

## The thymidylate synthase tandem repeat promoter polymorphism: A predictor for tumor-related survival in neoadjuvant treated locally advanced gastric cancer

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We evaluated DNA polymorphisms in the thymidylate synthase (TS) and 5,10-methylene-tetrahydrofolate reductase (MTHFR) genes for an association with response and survival in locally advanced gastric cancer treated with 5-FU based preoperative chemotherapy (CTx). DNA of 238 patients (CTx-group: total  $n = 135$ , completely resected (R0)  $n = 102$ ; without CTx: R0  $n = 103$ ) was isolated from blood or from nontumorous tissues. In the CTx-group, genotyping of the tandem repeat and the G/C polymorphism in the triple repeat in the promoter region of the TS gene and of the C677T polymorphism of the MTHFR gene was performed. None of the TS or MTHFR genotypes were associated with histopathological response and only the TS tandem repeat polymorphism was significantly related to survival (all patients  $n = 135$ ,  $p = 0.002$ ; R0 resected patients  $n = 102$ ,  $p = 0.007$ ; log-rank test). Multivariate analysis revealed ypN ( $p < 0.001$ ) and the TS tandem repeat polymorphism as independent prognostic factors in the CTx-R0-group ( $p = 0.003$ ). Analyzing the prognostic significance of the TS polymorphisms in the R0-group without CTx, TS genotypes were not significantly associated with survival. Comparing survival between R0 patients with and without CTx in the respective TS genotype groups of the tandem repeat polymorphism, a significant survival benefit for the patients with CTx was found for the 2rpt/2rpt ( $n = 49$ ;  $p = 0.002$ ) and 2rpt/3rpt genotypes ( $n = 99$ ;  $p = 0.004$ ), but not for the 3rpt/3rpt genotype ( $n = 57$ ;  $p = 0.93$ ). Patients' survival after CTx was associated with the TS tandem repeat polymorphism. CTx did not improve survival of patients with the 3rpt/3rpt genotype. Thus, a different therapy might be more appropriate for these patients.

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**Key words:** thymidylate synthase; MTHFR; DNA polymorphism; preoperative chemotherapy; 5-fluorouracil; cisplatin; gastric carcinoma

Neoadjuvant chemotherapy for advanced gastric cancer has been used in several clinical trials. However, only 30–40% of patients respond and the majority undergoes several months of toxic, expensive therapy without a survival benefit.<sup>1–3</sup> Response to chemotherapy is considered to be highly complex and may be influenced by specific genetic alterations in the tumors as well as by inherited interindividual variability in genes involved in drug metabolism. Presently, there is no reliable assay that can be used to predict chemotherapy response in gastric carcinoma in clinical practice. Thus, the identification of molecular genetic parameters that are associated with response and prognosis is of utmost interest.

Most chemotherapeutic regimens used in the neoadjuvant treatment of advanced gastric carcinoma contain 5-FU. 5-FU is an inhibitor of thymidylate synthase (TS), a key enzyme in nucleotide metabolism. The promoter enhancer region of the TS gene contains polymorphic 28 base pair tandem repeats, and the presence of the triple repeat (3rpt) has been shown to be associated with a 2–4 fold increase in protein expression in comparison to the double repeat (2rpt).<sup>4,5</sup> In addition, a G > C polymorphism in the second repeat of the 3R allele has been demonstrated to alter the tran-

scriptional activity of the TS gene.<sup>6,7</sup> Because of this functional relevance, these polymorphisms have been studied for response prediction in various 5-FU based chemotherapeutic settings in different tumor types, but the results have been inconsistent.<sup>8–15</sup>

5,10-Methylenetetrahydrofolate reductase (MTHFR) is a key regulatory enzyme in folate metabolism, which converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. As 5,10-methylenetetrahydrofolate also serves as a substrate for the conversion of dUMP to dTMP, which is catalyzed by TS, MTHFR and TS activity are interconnected and dependent on one another. A common C677T polymorphism in the MTHFR gene leads to a substitution of alanine by valine at codon 226 (Ala226Val). The enzyme encoded by the 677TT genotype has been demonstrated to have only 30% activity compared to the protein encoded by the 677CC genotype.<sup>16</sup> In metastatic colorectal cancer treated with fluoropyrimidines, a significant correlation has been reported between the presence of at least one T allele and response.<sup>17</sup> To the best of our knowledge, no data are available concerning a possible association between TS or MTHFR polymorphisms with clinical outcome in preoperative chemotherapy of gastric carcinoma.

The goal of our study was to analyze the polymorphisms of the promoter region of the TS gene and the C677T polymorphism in the MTHFR gene in order to test the hypothesis that they might be used as predictive and prognostic markers for neoadjuvant chemotherapy of advanced gastric carcinoma. As the tandem repeat polymorphism of the TS gene revealed a prognostic relevance, we compared the results regarding this gene to a group of patients with locally advanced completely resected gastric cancer without neoadjuvant treatment.

### Material and methods

#### Patient characteristics

In the group of patients, who received preoperative chemotherapy, the retrospective polymorphism analysis was done as a part of consecutive phase II studies evaluating preoperative chemother-

**Abbreviations:** BSA, body surface area; CI, confidence interval; CTx, preoperative chemotherapy; 5-FU, 5-fluorouracil; MTHFR, 5,10-methylenetetrahydrofolate reductase; PLF, cisplatin/leucovorin/5FU; R-category, resection category; rpt, repeat; RFLP, restriction fragment length polymorphism; TS, thymidylate synthase; ypN, pathological nodal status after chemotherapy; ypT, pathological tumor category after chemotherapy. Grant sponsor: KKF; Grant number: 8744168.

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apy in 192 patients with locally advanced gastric carcinomas (cT3-4, any N, M0) from October 1991 to January 2003.<sup>2,18</sup> DNA of 135 patients (70%) receiving more than 50% of the projected dose of chemotherapy was available. The clinical and histopathological data of the 135 patients that were included did not differ from those of the entire group of 192 patients. Patients older than 70 years and patients with bleeding of the primary tumor or gastric outlet syndrome were excluded from neoadjuvant chemotherapy. Eligibility requirements and staging procedures were as previously described.<sup>19</sup> As control group, 103 completely resected patients staged cT3-4, any N, cM0, by endoscopy, endoluminal ultrasound and CT scan undergoing primary surgery after the exclusion of peritoneal carcinomatosis by a diagnostic laparoscopy from February 1995 to October 2002, were included. All patients had to be fit for extended surgery. The clinical and histopathological data are included in Table I.

The protocol was reviewed and approved by the local ethic committee, and informed consent was obtained according to institutional regulations.

TABLE I - PATIENT CHARACTERISTICS

	Study group: neoadjuvant treated patients		Control: R0 with primary resection	
	No. of patients	%	No. of patients	%
<b>Preoperative characteristics</b>				
Total	135	100	103	100
Age $\pm$ standard deviation	56 $\pm$ 10		69 $\pm$ 14	
Range	23-70		31-94	
Sex				
Female	38	28	43	42
Male	97	72	60	58
Localization				
Proximal third	87	64	43	42
Middle third	37	28	26	26
Distal third	11	8	33	32
Lauren classification				
Intestinal	60	44	52	51
Nonintestinal	75	56	51	49
Grading				
G1/2	23	17	26	25
G3/4	112	83	77	75
Response				
Responder	33	24		
Nonresponder	102	76		
Chemotherapy regimens				
PLF <sup>1</sup>	121	90		-
E-PLF <sup>2</sup>	10	7	-	
Paclitaxel-PLF <sup>3</sup>	4	3		
<b>Postoperative characteristics</b>				
Resection	129/135	96	103/103	100
R-category				
R0	102/129	79	103/103	100
R1/2	27/129	21	-	
ypT-category				
ypT0	7/129	5	-	
ypT1	8/129	6	-	
ypT2	81/129	63	54/103 (pT2)	52
ypT3	28/129	22	44/103 (pT3)	43
ypT4	5/129	4	5/103 (pT4)	5
ypN-category				
ypN0	50/129	38	36/103 (pN0)	35
ypN1	44/129	34	32/103 (pN1)	31
ypN2	24/129	19	26/103 (pN2)	25
ypN3	11/129	9	9/103 (pN3)	9

<sup>1</sup>PLF protocol: 50 mg/m<sup>2</sup> body surface area (BSA) cisplatin over 1 hr at weeks 1, 3 and 5, 500 mg/m<sup>2</sup> BSA leucovorin for 2 hours, followed by 2000 mg/m<sup>2</sup> BSA 5-FU given as continuous infusion over 24 hours at weeks 1, 2, 3, 4, 5 and 6. <sup>2</sup>E-PLF protocol: In addition, epirubicin 30 mg/m<sup>2</sup> BSA at weeks 2, 4, 6 was given. <sup>3</sup>Paclitaxel-PLF: In addition, paclitaxel 85 mg/m<sup>2</sup> BSA at weeks 1,3,5 was given.

### Preoperative chemotherapy

The preoperative chemotherapy protocol consisted of 50 mg/m<sup>2</sup> body surface area (BSA) cisplatin over 1 hr at weeks 1, 3 and 5, 500 mg/m<sup>2</sup> BSA leucovorin for 2 hr, followed by 2000 mg/m<sup>2</sup> BSA 5-FU given as continuous infusion over 24 hr at weeks 1, 2, 3, 4, 5 and 6 in 121 patients. Additionally, paclitaxel 85 mg/m<sup>2</sup> BSA at weeks 1,3,5 was given in 4 patients, epirubicin 30 mg/m<sup>2</sup> BSA at weeks 2,4,6 was given in 10 patients.

### Surgery, histopathology and response evaluation

In patients with proximal gastric cancer, a transhiatal extended gastrectomy and an extended D2-lymphadenectomy (resection of the lymph node groups 1 and 2 according to the Japanese Research Society for Gastric Cancer), including a left retroperitoneal lymphadenectomy were performed; for patients with the tumor localization in the middle or distal third, a total gastrectomy with D2-lymphadenectomy was performed.

Histopathological workup was done by standardized protocols including the pTNM-categories, resection margins (no microscopically residual tumor: R0; microscopically residual tumor: R1; macroscopically residual tumor: R2), grading, tumor localization and Lauren subtype. Complete tumor resection (R0 resection) in this study was defined according to the histopathological work up and not according to the surgeon.

Response evaluation was performed histopathologically and clinically as previously described.<sup>19-22</sup> For histopathological response evaluation, the macroscopically identifiable tumor bed of the resected tumors was completely examined histologically. For the purpose of this study, all patients with less than 10% residual tumor cells were classified as responders. All other patients were classified as nonresponders.<sup>20</sup> Patients were also classified as nonresponding when tumor progression occurred during chemotherapy.<sup>19</sup>

### Patient followup

After resection, patients were followed for a period of 3-month interval, by abdominal and chest computed tomography (CT) and endoscopy. Tumor-related survival was calculated from the first day of chemotherapy or from the day of resection in patients with primary operation. No patient was lost to follow up.

### DNA isolation

DNA isolation from blood lymphocytes was performed after cell lysis, proteinase K digestion followed by phenol and chloroform extraction according to standard procedures or by using a DNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturers' instructions. DNA from formalin-fixed, paraffin-embedded nontumorous tissues was isolated as previously described.<sup>23</sup>

### Genotyping

PCR of the polymorphic region in the promoter of the *TS* gene was performed using published primer sequences.<sup>9</sup> The PCR reactions were run in 25  $\mu$ l of a reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.0 mM MgCl<sub>2</sub>, 0.01% gelatin, 200  $\mu$ M dNTP and 0.4  $\mu$ M of each primer. After an initial denaturation step at 94°C for 4 min., 35-40 cycles were performed consisting of 30 sec at 64°C and 30 sec at 72°C, followed by a final extension of 7 min at 72°C. The triple repeat (3R) of the promoter region resulted in a 144 bp and the double repeat (2R) in a 116 bp fragment, which were separated and visualized by electrophoresis on a 2% agarose gel. The G > C polymorphism within the triple repeat was determined by RLFP analysis, using *HaeIII* digestion as published.<sup>24</sup> Patients with the *TS* 2rpt/2rpt, 2rpt/3C, 3C/3C genotypes were classified as low *TS* producers and patients with the 3G/3G, 3G/3C, 2rpt/3G genotypes were classified as high *TS* producers as described.<sup>13,24</sup>

Genotyping of the C677T *MTHFR* polymorphism was performed by allelic discrimination using the 5' nuclease PCR assay

(TaqMan). Primers and probes for the TaqMan assay were designed using Primer Express software (PE Applied Biosystems). Primer sequences were as follows: forward, ggctgacctgaagcacttgaa; reverse, aaagaaagctgctgatgatg. Probes for the C or T allele were 5' labelled with either FAM or VIC fluorogenic dyes. The sequences were C-allele, tgcgggagccgatt; T-allele, tgcgggagtcgatt. PCR amplification was performed in 15 µl containing 1.8 µl DNA, 1× TaqMan Universal Master Mix (PE Applied Biosystems), 200 nM for the probe of the C or T allele, respectively, and 900nM each primer. Cycle conditions were 15 sec at 92°C and 1 min at 60°C for 40 cycles after an initial incubation of 2 min at 50°C and 10 min at 95°C. Assays were run in duplicates.

For quality control, at least 20 samples were analyzed twice or analyzed in parallel by direct sequencing giving consistent results in all cases. DNA sequencing was performed by cycle sequencing with fluorescent labeled dye terminators and separation with an automated sequencing system (ABI 377, Perkin-Elmer, Branchburg, New Jersey).

*Statistical analysis*

Association between the different genotypes and clinical and histopathological features were analyzed by  $\chi^2$ -, Kruskal-Wallis or Fisher's exact test where appropriate. Survival rates were estimated according to Kaplan-Meier. Comparisons between different groups of patients were performed with the log-rank test. Relative risks were estimated by calculating hazard ratios (HR) from Cox proportional hazard models. Prognostic significance of potential parameters was determined by univariate and multivariate analysis. All statistical testing was two-sided and conducted at the 0.05 significance level. SPSS software (SPSS Inc., Chicago, IL 11.5) was used.

**Results**

*Patients, treatment and followup*

Hundred and thirty-five patients who had received preoperative chemotherapy were included in the study. 129 (96%) patients could be resected, 102 (79%) patients received complete (R0) resections. There were 33 responders and 102 nonresponders. The mean followup for the surviving patients was 38.1 months (range 4.8–112.3). The median overall survival was 77.8 months.

In the control group of 103 completely resected patients without preoperative chemotherapy, the mean follow up for the surviving patients was 45.1 months (range 0.4–112.1) and the median survival was 33.8 months.

There was no significant differences with respect to histopathological classification and tumor grading between the patients treated with and without neoadjuvant chemotherapy. With respect to tumor localization and age of the patients, a significant preponderance of distally located tumors ( $p < 0.001$ ) and of patients with older age ( $p < 0.001$ ) were observed in the group without neoadjuvant chemotherapy. Therefore, we tested the *TS* polymorphisms with respect to age and tumor localization without any significant association (all  $p$ -values  $> 0.05$ ).

The patient characteristics are summarized in Table I.

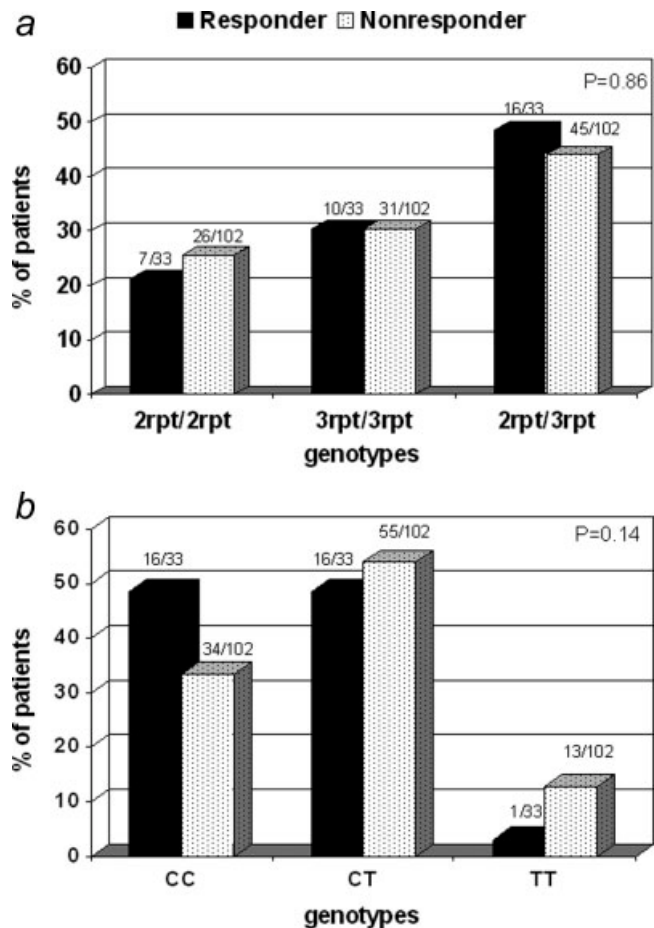
*Allele frequencies*

In the group of patients with preoperative chemotherapy, 33 (25%) patients showed the 2rpt/2rpt genotype, 61 (45%) the 2rpt/3rpt genotype and 41 (30%) the 3rpt/3rpt genotype of the *TS* tandem repeat polymorphism. Inclusion of the G/C polymorphism in the triple repeat in our analysis and classification of the patients in *TS* high producers (3G/3G, 3G/3C, 2rpt/3G) and *TS* low producers (2rpt/2rpt, 2rpt/3C, 3C/3C), 83 (62%) were low and 51 (38%) were high producers. One patient could not be evaluated for the G/C polymorphism. With respect to the *MTHFR* gene, 50 (37%) of the patients had the CC genotype, 71 (53%) the CT genotype and 14 (10%) the TT genotype.

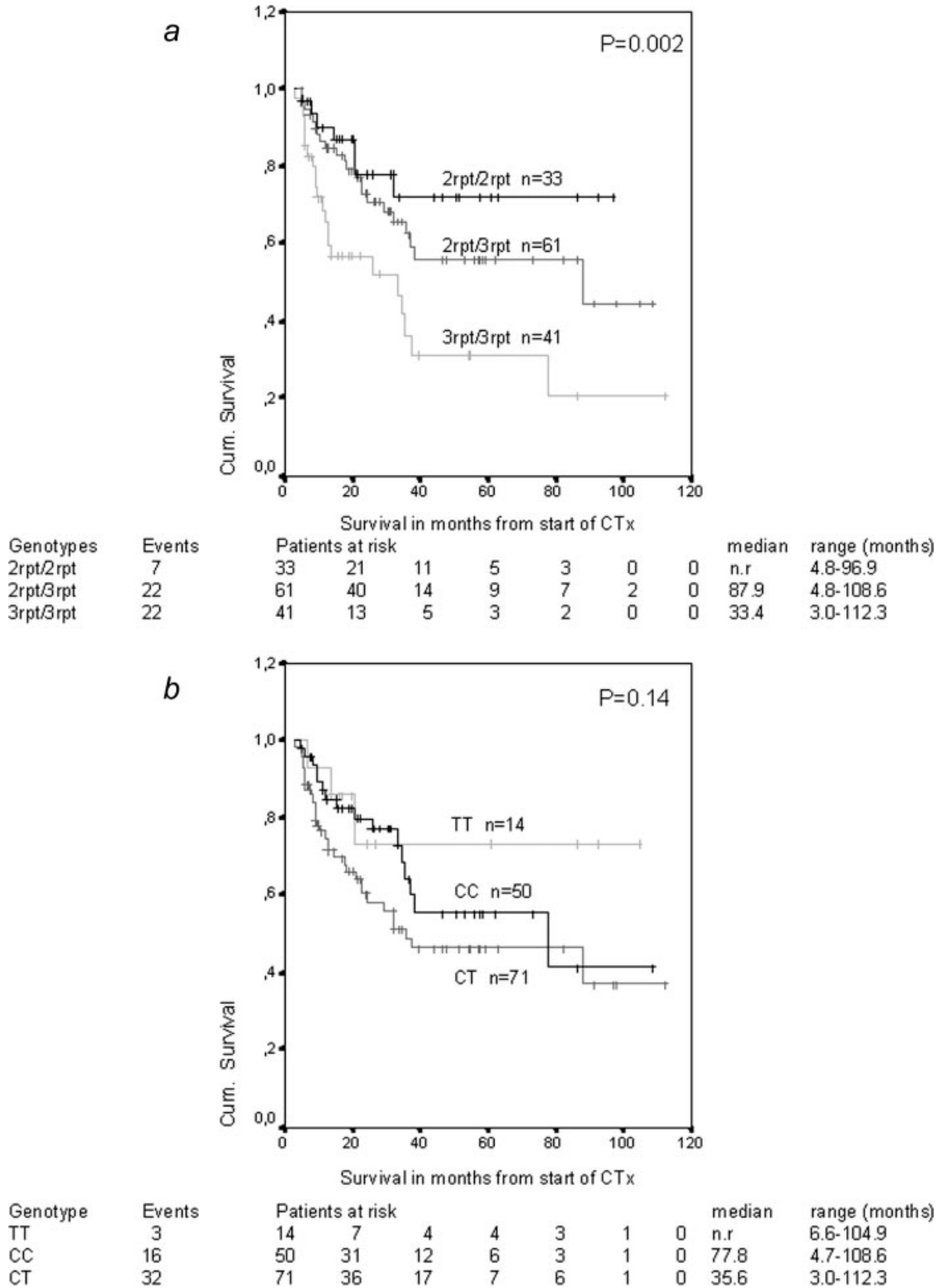
In the control group of patients without preoperative chemotherapy ( $n = 103$ ), 22 (21%) patients showed the 2rpt/2rpt genotype, 53 (52%) the 2rpt/3rpt genotype and 28 (27%) the 3rpt/3rpt genotype of the *TS* length polymorphism. For 101 patients, genotyping of the G/C polymorphism in the triple repeat was successful: 63 (62%) patients were low and 38 (38%) were high *TS* producers. Regarding the *TS* genotypes, either considering the tandem repeat polymorphism alone or using the classification of a low and high *TS* producing genotype, no statistical difference for the *TS* genotype distributions could be found between R0 resected patients with and without neoadjuvant treatment ( $p = 0.60$  and  $p = 0.77$ ).

*Genotypes and response*

For the *TS* tandem repeat polymorphism, there was no association with response. Among the 33 responding patients, 7 (21%) demonstrated the 2rpt/2rpt genotype, 16 (49%) the 2rpt/3rpt genotype and 10 (30%) the 3rpt/3rpt genotype; and among the 102 nonresponding patients, 26 (26%) had the 2rpt/2rpt, 45 (44%) had the 2rpt/3rpt and 31 (30%) the 3rpt/3rpt genotype ( $p = 0.86$ ) (Fig. 1a). The classification in high and low *TS* producing genotypes showed no association with response as well. Among the 33 responding patients, 14 (42%) demonstrated the high and 19 (58%) the low *TS* producing genotype and among the 101 evaluable nonresponding patients, 37 (37%) demonstrated the high and 64 (63%) the low *TS* producing genotype ( $p = 0.68$ ).



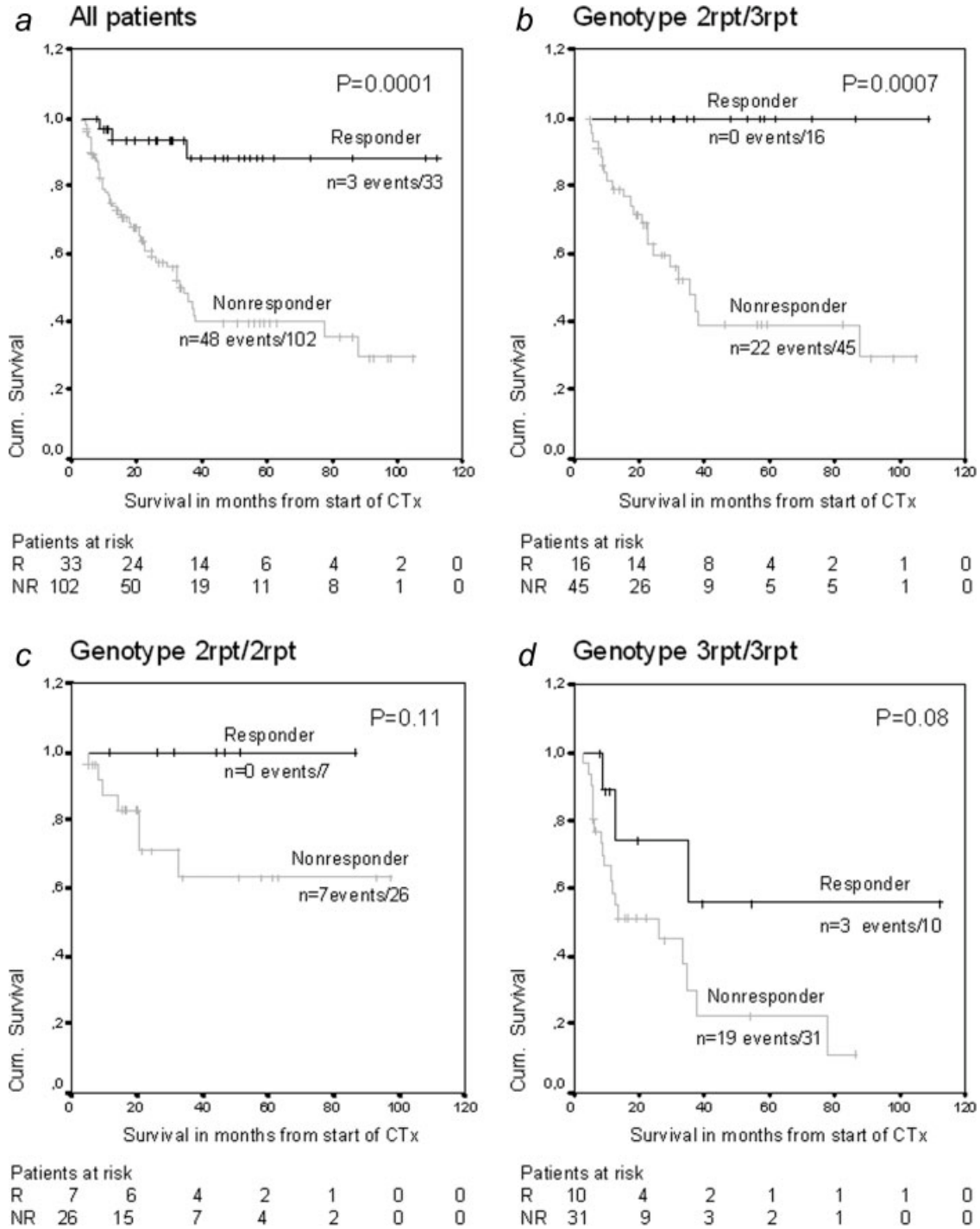
**FIGURE 1** – (a) Association of *TS* tandem repeat genotype with response.  $p$ -value was calculated by  $\chi^2$ -test. (b) Association of *MTHFR* genotype with response.  $p$ -value was calculated by  $\chi^2$ -test.



**FIGURE 2** – Survival curves of the neoadjuvant treated patient in relation to the genotypes. The numbers of events during the period and the number of patients at risk after each 20-month interval are indicated. *p*-values were calculated by the log-rank test (n.r: not reached). (a) *TS* tandem repeat genotype and (b) *MTHFR* genotype.

For the *MTHFR* genotypes, there was no statistically significant association between the respective genotypes and response, although the TT genotype was more frequently found in nonresponding patients. Among the 33 responding patients, 16 (48%)

showed the CC genotype, 16 (48%) the CT and 1 (4%) the TT genotype. In the 102 nonresponding patients, 34 (33%) were homozygous CC, 55 (54%) were heterozygous CT and 13 (13%) were homozygous TT (*p* = 0.14) (Fig. 1b). There was also no associa-



**FIGURE 3** – Survival curves of responding and nonresponding patients in relation to the *TS* tandem repeat genotype. The number of events during the period and the number of patients at risk after each 20-month interval are indicated. *p* values were calculated by the log-rank test. R (responder), NR (nonresponder). (a) All patients; the median survival for the responders was not reached, for the nonresponder, it was 35.0 months. (b) Heterozygous (2rpt/3rpt) patients; the median survival for the responders was not reached, for the nonresponders, it was 35.6 months. (c) Homozygous 2rpt/2rpt patients; the median survival for the responders and for the nonresponders was not reached. (d) Homozygous 3rpt/3rpt patients; the median survival for responders was not reached, for the nonresponders, it was 26.0 months.

tion between response and the presence of at least one T allele ( $p = 0.18$ ).

*Genotypes and prognosis*

*All patients of the CTx group.* In the neoadjuvant treated group, considering the *TS* tandem repeat polymorphism, the three

different *TS* genotypes demonstrated a statistically significant association with tumor related survival ( $p = 0.002$ ) (Fig. 2). In this group, a strong statistically significant survival difference was found between responding and nonresponding patients ( $p = 0.0001$ ) (Fig. 3a). Considering the survival of the responding and nonresponding patients in the respective *TS* genotype groups,

there was a strong, statistically significant survival difference for the patients with 2rpt/3rpt genotype ( $p = 0.0007$ ). All of the 16 responding patients with the 2rpt/3rpt genotype were still alive (Fig. 3b). In contrast, the responding and nonresponding patients with the 2rpt/2rpt genotype showed no statistically significant survival difference ( $p = 0.11$ ) (Fig. 3c). Similarly, patients homozygous for the triple repeat (3rpt/3rpt) showed only a trend for an improved survival in responders ( $p = 0.08$ ) (Fig. 3d). Of note, the survival rate of the responders with the 3rpt/3rpt genotype was almost identical to the survival rate of the nonresponders with the 2rpt/2rpt genotype (Figs. 3c and 3d).

No association with survival was found using the classification of the *TS* genotypes in high and low *TS* producers ( $p = 0.70$ ).

The *MTHFR* polymorphism was not significantly related to survival ( $p = 0.14$ ) (Fig. 2b). The relative risks for the different genotypes of the *TS* and *MTHFR* genes are shown in Table II.

Cox regression analysis for the neoadjuvant treated patients ( $n = 135$ ) including response to chemotherapy, genotype of the *TS* tandem repeat polymorphism, ypT category, ypN category and R category revealed ypN and the *TS* polymorphism and R category as prognostic factors in the stepwise analysis (Table III).

TABLE II - GENOTYPE AND RELATIVE RISKS IN NEOADJUVANT TREATED PATIENTS<sup>1</sup>

Gene	Genotype	Number (%)	Death (%)	Rel. risk	95% CI	p-value	
<b>Chemotherapy</b>							
<i>TS</i>		135					
	2rpt/2rpt	33 (25)	7 (21)	1			
	2rpt/3rpt	61 (45)	22 (36)	1.63	0.70–3.82	0.26	
<i>TS</i>	3rpt/3rpt	41 (30)	22 (54)	3.60	1.53–8.44	0.003	
		134				0.70	
	Low <sup>2</sup>	83 (62)	30 (36)	1			
<i>TS</i>	High <sup>3</sup>	51 (38)	20 (39)	1.12	0.63–1.97	0.70	
	<b><i>MTHFR</i></b>						
<i>MTHFR</i>		135					
	CC	50 (37)	16 (32)	1			
	TT	14 (10)	3 (21)	0.64	0.18–2.19	0.47	
<i>MTHFR</i>	CT	71 (53)	32 (45)	1.57	0.86–2.87	0.14	
	<b>Control group</b>						
	<i>TS</i>		103				
2rpt/2rpt		22 (21)	12 (55)	1			
2rpt/3rpt		53 (52)	29 (55)	1.04	0.53–2.4	0.91	
<i>TS</i>	3rpt/3rpt	28 (27)	11 (39)	0.84	0.37–1.90	0.67	
		101					
	Low <sup>2</sup>	63 (62)	30 (48)	1			
<i>TS</i>	High <sup>3</sup>	38 (38)	21 (55)	1.63	0.93–2.86	0.09	

<sup>1</sup>Cox regression analysis. –<sup>2</sup>*TS* high producing genotype: 3G/3G, 3G/3C, 2rpt/3G. –<sup>3</sup>*TS* low producing genotype: 3C/3C, 2rpt/3C, 2rpt/2rpt.

**Completely resected (R0) patients of the CTx group.** Among the 102 neoadjuvant treated completely resected patients, the association of the *TS* tandem repeat polymorphism genotype and patient survival remained significant ( $p = 0.007$ ). The median survival for patients with the 3rpt/3rpt genotype was 31.3 months (range: 2.2–106.7), whereas the median survival for the two other genotypes was not reached yet. Classification of the patients in high and low *TS* producers revealed a trend for an association of patients with the low producing genotypes and better survival ( $p = 0.08$ ). The median survival of the patients with high producing genotypes was 36.0 months, the median survival of the patients with the low producing genotypes was not reached yet.

The *MTHFR* genotype had no prognostic impact in neoadjuvant treated completely resected patients ( $p = 0.23$ ).

The stepwise Cox regression analysis including the all significant prognostic factors of the univariate analysis (response, ypT, ypN and *TS* tandem repeat polymorphism) revealed ypN and *TS* length polymorphism as independent prognostic factors for the completely resected patients (Table III).

**Completely resected (R0) patients without CTx.** In the control group of the completely resected patients without chemotherapy, there was no significant survival difference between the patients in relation to the *TS* genotypes regarding the tandem repeat polymorphism ( $p = 0.83$ ) (Fig. 4). Grouping the patients into high and low *TS* producers demonstrated a trend for an association of patients with the low producing genotypes and better survival ( $p = 0.09$ ). The median survival of the patients with high producing genotypes was 25.8 months; the median survival of the patients with the low producing genotypes was 61.9 months. The relative risks of the various *TS* genotypes are included in Table II. Multivariate analysis identified pN-category ( $p = 0.001$ ) and pT-category ( $p = 0.047$ ) as the only significant prognostic factors.

Analyzing the survival difference for completely resected patients with respect to neoadjuvant treatment versus primary resection, a significant survival benefit for those patients receiving chemotherapy and demonstrating the 2rpt/2rpt genotype ( $p = 0.002$ ) or the 2rpt/3rpt genotype ( $p = 0.004$ ) was found, but not for patients demonstrating the 3rpt/3rpt genotype ( $p = 0.93$ ) (Fig. 5).

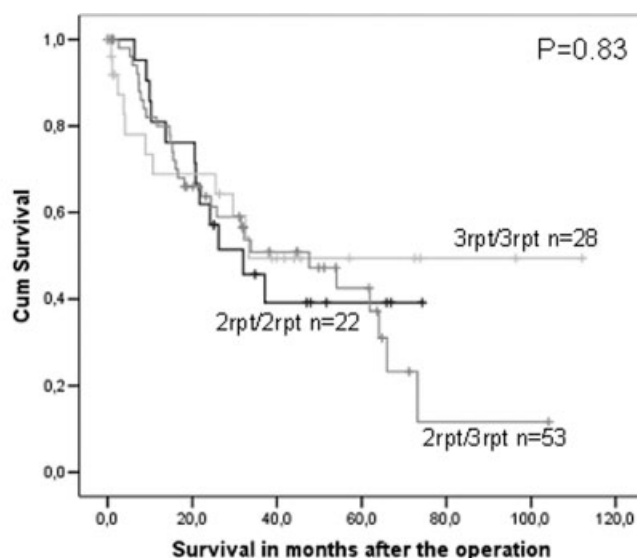
**Discussion**

This study is, to the best of our knowledge, the first and largest study analyzing the influence of polymorphisms in the promoter enhancer region of the *TS* gene and of the C677T polymorphism in the *MTHFR* gene, on the responsiveness to continuous 5-FU based chemotherapy and patient survival in neoadjuvant treated gastric cancer. The most important observation in this study indicates a major impact of the genotype of the *TS* tandem repeat polymorphism on patient survival only in the setting of neoadju-

TABLE III - MULTIVARIATE COX REGRESSION ANALYSIS FOR TUMOR-RELATED SURVIVAL

Factor	All patients ( $n = 135$ ) <sup>1</sup>			Completely resected patients ( $n = 102$ ) <sup>2</sup>		
	p	Rel. risk	95% CI	p	Rel. risk	95% CI
ypN						
ypN0		1			1	
ypN1	0.0009	5.47	2.08–14.94	0.002	7.61	2.09–27.73
ypN2	<0.0001	8.70	3.07–24.62	0.0002	12.01	3.20–45.07
ypN3	<0.0001	11.67	3.59–37.96	<0.0001	40.80	7.30–228.20
<i>TS</i> genotype						
2rpt/2rpt		1			1	
2rpt/3rpt	0.90	1.06	0.44–2.58	0.13	3.22	0.70–14.79
3rpt/3rpt	0.0008	4.57	1.88–11.14	0.003	9.70	2.14–43.91
R-category						
R0		1				
R1/2	0.0001	4.16	2.08–8.33			

CI, confidence interval; ypT, pathological tumor category after chemotherapy; ypN, pathological nodal status after chemotherapy; R-category, resection category. –<sup>1</sup>Included factors: ypT, ypN, response R-category, *TS* genotype. –<sup>2</sup>Included factors: ypT, ypN, response *TS* genotype.



Genotypes	Events	Patients at risk							median	range (month)
2rpt/2rpt	12	22	16	6	3	-	-	-	32.0	0.1-74.4
2rpt/3rpt	29	53	31	16	9	1	1	-	47.6	0.9-104.2
3rpt/3rpt	11	28	15	8	4	2	1	-	33.3	0.2-112.1

**FIGURE 4** – Survival curves of completely resected patients not treated with neoadjuvant chemotherapy in relation to the *TS* tandem repeat genotype. The number of events during the period and the number of patients at risk after each 20-month interval are indicated. *p*-values were calculated by the log-rank test.

vant chemotherapy. In addition, comparison of survival between completely resected patients with or without neoadjuvant treatment indicated that neoadjuvant chemotherapy did not improve survival in patients with the 3rpt/3rpt genotype.

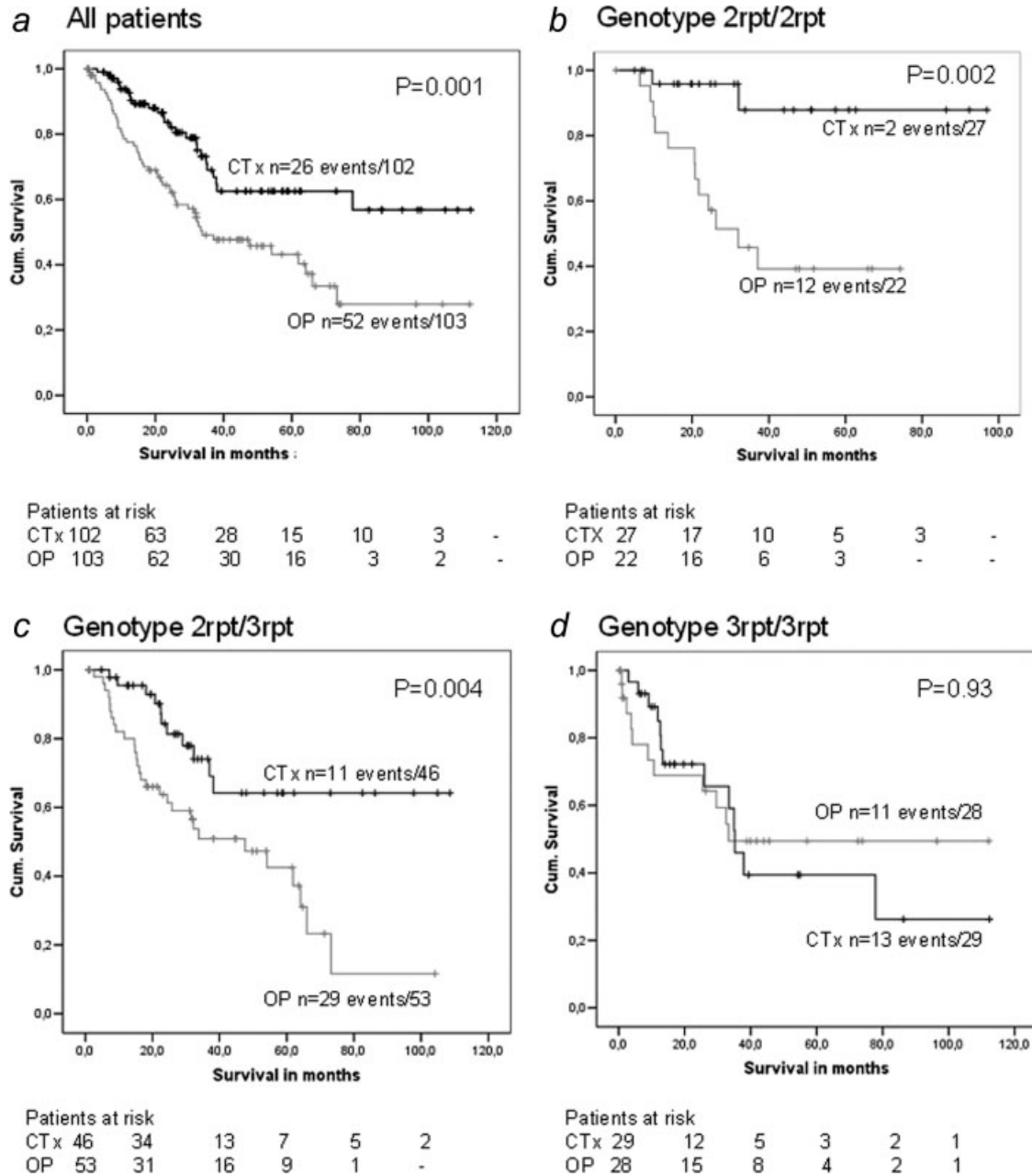
The distribution of the *TS* genotypes of the tandem repeat and the G/C polymorphism in the triple repeat corresponds well to the data of gastric and colorectal cancer patients in Western countries, to an Australian and to a white control population.<sup>7-10,14,25</sup> In the neoadjuvant setting, patients homozygous for the triple repeat of the *TS* polymorphism showed a statistically significant worse prognosis in our study. Noticeably, however, the proportion of responders and nonresponders was similar among the different genotypes, indicating that the *TS* genotype is not related to response at least in terms of the established criteria for response evaluation, which were used in our study. Patients who were heterozygous for the *TS* tandem repeat polymorphism showed the strongest survival difference between responding and nonresponding patients. On the other hand, responding patients homozygous for the double or triple repeat had a tendency toward better survival, but the differences were not statistically significant. This indicates that in the heterozygote group, response to chemotherapy may exert the strongest ability to improve survival. Although 135 patients with chemotherapy have been included in this study, the respective subgroups with the various genotypes are different in size, which can affect statistical power. Interestingly, however, there was nearly no difference between survival rates between responders with the 3rpt/3rpt genotype and nonresponders with the 2rpt/2rpt genotype.

Our finding of an association of the *TS* tandem repeat polymorphism with survival, but not with histopathological response in the neoadjuvant treated group, may point to response mechanisms on the molecular or cellular basis, which do not necessarily translate into a measurable tumor shrinkage and are not revealed by standardized response evaluation methods. Response evaluation in our study was essentially based on histopathological methods. Analyzing the *TS* length polymorphism with respect to clinical response rates, determined by endoscopy, CT and endoscopic ultrasound,

gave essentially the same results (data not shown), which confirms our overall findings. Although histopathological and clinical response evaluation methods are highly associated with survival in our as well as in previous studies,<sup>1,2</sup> it has to be emphasized that the accuracy of response evaluation is not perfect. It has been suggested, that despite being accepted as a valid measure of antitumor activity, response rates do not capture the full effect of treatment benefit and that patients with nonresponding tumors, but demonstrating an increase in survival may benefit from delay in tumor progression.<sup>26</sup> The exact mechanism by which the *TS* genotypes affect survival and response in neoadjuvant treated gastric cancer must be highly complex and is not known in detail. As we did not find any association of the *TS* tandem repeat polymorphism with survival in the untreated group of patients, the prognostic value of this *TS* polymorphism in the neoadjuvant treated patients seems to be chemotherapy dependent without being measurable by the response evaluation methods used in our study.

In the untreated control group of patients, the preponderance of distally located tumors and of patients with older age may be related to the inclusion criteria used for neoadjuvant treatment, which encompasses age younger than 70 years and exclusion of patients with gastric outlet stenosis. Because of the older age of patients in the control group, direct comparison of the results might be hampered by age related comorbidity. Indeed, we found an improved survival for younger patients (< 70 years) compared to older patients in this group (*p* = 0.02) (data not shown). However, analysing the *TS* tandem repeat polymorphism with survival separately in both age groups, we found essentially the same results, revealing no association of this polymorphism with survival (*p* = 0.44, < 70 years; *p* = 0.99, > 70 years). In addition, our results that the *TS* genotypes demonstrated no association with either age or tumor localization makes a bias of the overall results because of this differences unlikely.

As patients with the 3rpt/3rpt genotype treated with neoadjuvant chemotherapy did not demonstrate a survival benefit in comparison to untreated patients, a different chemotherapy or primary resection should be discussed for this group of patients.



**FIGURE 5** – Survival curves of completely resected patients with and without neoadjuvant chemotherapy in relation to the *TS* tandem repeat genotype. The number of events during the period and the number of patients at risk after each 20-month interval are indicated. *p* values were calculated by the log-rank test. OP (untreated patients with primary operation), CTx (patients with neoadjuvant chemotherapy). (a) All patients; the median survival for the neoadjuvant treated patients was 33.8 months, for the untreated patients, it was not reached. (b) Heterozygous (2rpt/3rpt) patients; the median survival for the neoadjuvant treated patients was not reached, for the untreated patients it was 47.6 months. (c) Homozygous 2rpt/2rpt patients; the median survival for the neoadjuvant treated patients was not reached, for the untreated patients it was 32.0 months. (d) Homozygous 3rpt/3rpt patients; the median survival for neoadjuvant treated patients was 35.2, for the untreated patients, it was 33.3 months.

Other studies in gastric cancer revealed only a weak association for the combined 2rpt/2rpt and 2rpt/3rpt genotypes with prolonged survival in a study of 51 patients treated with a 5-FU based postoperative chemotherapy.<sup>27</sup> However, it has to be emphasized that in this study, genotyping was performed from tumor DNA, which may lead to a false positive classification of homozygosity in the case of constitutional heterozygosity and concomitant loss of one allele in the tumor. Kawakami analyzed the *TS* genotypes including a G/C single nucleotide polymorphism within the repeat polymorphism in the promoter region and a 6-bp deletion polymorphism in the 3' untranslated region of the gene, for an association with dis-

ease free survival in gastric cancer patients treated with fluorouracil-based adjuvant chemotherapy and found a significant better outcome for carriers of low *TS* expression genotypes.<sup>13</sup> In another study, in gastric cancer treated with 5-FU/cisplatin in the palliative setting, patients with a favorable *TS* genotype demonstrated a trend for an increased survival, but did not show an association with response.<sup>14</sup> The findings of these studies are essentially in line with our results in the neoadjuvant setting. However, in our study, the division in only two groups with high and low *TS* producing patients did not lead to a better prediction of response or prognosis. In contrast, no statistically significant difference in survival could

be found any more. If only the patients with the 2rpt/3rpt genotype were divided into low and high producers and the 2rpt/2rpt were defined as low and the 3rpt/3rpt as high producers, the results remained statistically significant ( $p = 0.009$ ). Thus, the effect of the *TS* polymorphisms on survival seems to be primarily associated with the *TS* tandem repeat polymorphism in our study.

Regarding the *MTHFR* polymorphism, there was neither a statistically significant association with response nor with prognosis in our study. However, in contrast, to what was expected, the TT genotype showed a trend for an association with nonresponse, with a similar genotype distribution in the whole study, as reported by others.<sup>28,29</sup> Patients with this genotype showed a better survival rate. However, in light of the relatively small number of patients with the TT genotype, interpretation of this finding has to be taken with care and needs to be analyzed in a higher number of patients. For colorectal cancer, a statistically significant correlation of the T allele with response was found,<sup>17</sup> but no survival difference was shown between patients with the wild type CC genotype and patients with at least one T allele by others.<sup>30</sup>

Because all patients received more than 50% of the projected dose of chemotherapy and none of the included patients developed severe toxicity during the second cycle of chemotherapy, this study was not suitable to analyze an association between the tested polymorphisms and toxicity.

Despite the relatively homogenous group of 135 patients with locally advanced gastric cancer in our study, the study has to be

considered as exploratory and its retrospective nature clearly precludes immediate use of *TS* and *MTHFR* genotyping in clinical practice. Further, large-scale, prospective studies will be needed to confirm and extend our findings.

The fact that DNA of only 70% of all patients included in phase II studies was available for analysis in our study, and the long median survival time of 77.8 months must also be taken into consideration. The long median survival might be due to a preferential collection of DNA from blood samples from surviving patients from the early years of the phase II studies. To exclude a bias, the correlation of the genotypes with survival was repeated for 87 patients treated from the beginning of 1999 and having a median survival of 37 months, which corresponds to a total of 97% patients treated during that time. Essentially, the same results for an association of the *TS* genotype of the tandem repeat polymorphism with survival were obtained for all patients ( $p = 0.01$ ) and for the 70 completely resected patients ( $p = 0.003$ ). This indicates that a bias due to a preferential inclusion of long surviving patients is unlikely.

In conclusion, our study demonstrates for the first time in locally advanced gastric cancer that the constitutional *TS* tandem repeat polymorphism has a strong influence on the prognosis of patients treated preoperatively with continuous infusional 5-FU. This observation and other genetic variants influencing patient's prognosis and response could substantially contribute to the future design of individualized cancer treatment.

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