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Thymidylate synthase genotyping is more predictive for therapy response than immunohistochemistry in patients with colon cancer

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Thymidylate synthase (TS) is a potentially valuable marker for therapy response since it is the molecular target of 5-fluorouracil (5-FU). TS can be analyzed at the DNA (gene polymorphisms and amplification) and protein level (immunohistochemistry). This study investigated the predictive role of TS at the DNA and protein levels in patients with N^+ colon cancer ($n = 38$). Tumor and normal tissues were genotyped using PCR for variable number of tandem repeats (VNTR), a single nucleotide polymorphism (SNP) in the 3R allele and a 6 bp deletion (1494del6) in the *TS* gene. Tumor tissues were additionally analyzed for loss of heterozygosity (VNTR polymorphism). A newly developed real time PCR assay was used to detect the presence of *TS* gene amplifications in tumor tissues. VNTR analysis in normal tissue was significantly associated with distant tumor recurrence (8% for 2R/2R *vs.* 52% for patients carrying a 3R allele, $p = 0.038$) and cancer-specific survival ($p = 0.021$). IHC was not found to be significantly associated with patients' outcome. No correlations between *TS* gene polymorphisms and IHC were found. However, *TS* gene amplification was correlated with a strong IHC staining intensity. In conclusion, this study indicates that DNA based analysis is more predictive for patients' outcome than TS IHC.
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Key words: thymidylate synthase; polymorphism; 5-fluorouracil; colon cancer; gene amplification

A substantial number of patients with colon cancer who received adjuvant 5-fluorouracil (5-FU)-based therapy will not benefit from it. Therefore, predictive markers are needed in order to discriminate between responsive and nonresponsive patients. Thymidylate synthase (TS) is a central enzyme in DNA synthesis and is a potentially valuable marker since it is the molecular target of 5-FU. TS protein expression is affected by 3 different functional polymorphisms in the untranslated regions (UTRs) of the gene. Sensitivity to 5-FU based therapy might be largely influenced by the intracellular levels of the TS protein.

TS protein levels can be studied directly by western blotting, enzyme activity assays,[1,2] ELISA[3,4] and immunohistochemistry (IHC).[5,6] The mainstream method is IHC because it is a relatively cheap, widely implemented technique that enables studying protein expression *in situ*. At the DNA level, TS protein expression is affected by different underlying functional polymorphisms as shown by several functional studies.[7–12] These polymorphisms are the following: a variable number of tandem repeats (VNTR) containing 2 (2R) or 3 (3R) repeats of 28 bp,[7] a single nucleotide polymorphism (SNP) of a G to C substitution in the 3R allele[9] in the 5′UTR and a 6 bp deletion at nucleotide 1494 in the 3′UTR (1494del6).[12] Recently, a SNP of a G to C substitution in the first repeat of the 2R allele has also been found (hereafter referred to as the 2RC allele).[13,14] TS mRNA with 3 repeats has greater translation efficiency than mRNA with 2 repeats.[7,11] Individuals with a 3R/3R genotype will, in theory, have higher TS protein levels than individuals homozygous for the 2R allele. Furthermore, the SNP has been described to decrease translation efficiency of the 3R allele to the level of the 2R allele.[8–10] The 1494del6 polymorphism was found to be associated with decreased mRNA stability and lower TS protein levels.[12]

Although *TS* expression can be studied using either IHC or genotyping, it is still unclear which methodology is clinically most valuable, as recently indicated in the ASCO 2006 update for recommendations for the use of tumor markers in gastrointestinal cancer.[15] In addition, the experimental procedures for both methods remain unclear. In case of IHC it is undecided which antibody should be used and on which tissue type (tumor, normal or metastatic) it should be performed.[16,17] These differences in technical approach, the semiquantitative nature of IHC analysis, observer dependence and incomplete standardization for IHC assessment could account for the significant heterogeneity between studies investigating the predictive role of TS with IHC. In the adjuvant and advanced settings, a predictive role of TS IHC was found by some studies[2,18] but enfeebled by others,[19–23] which was also confirmed by the meta-analysis conducted by Popat *et al.*[24] With respect to TS genotyping, it is still unclear which polymorphism(s) should be analyzed and whether the germline or somatic genotype should be investigated. That is, *TS* genotype in tumor tissue can deviate due to chromosomal aberrations such as loss of heterozygosity (LOH) and gene amplification.[25–27]

This study aimed to perform a detailed analysis of the predictive values of TS at both the protein level (IHC) and DNA level (genotyping) in patients with colon cancer. In order to achieve this, TS was thoroughly evaluated with these 2 methodologies in both tumor and normal tissue in a homogenous patient population of patients with N^+ colon adenocarcinoma who received post operative 5-FU based chemotherapy.

Materials and methods

Patient population

Thirty-eight patients treated in the Catharina hospital in Eindhoven, The Netherlands, were investigated in this study. Patients diagnosed with an adenocarcinoma in the colon between 1995 and 2002 and staged as any T, N^+, M0 (stage III) were selected for this retrospective study. Patient and tumor characteristics are depicted in Table I. This specific patient population was studied because all patients received adjuvant chemotherapy and the absence of metastasis at time of diagnosis enabled us to study the distant recurrence free interval as a clinical parameter. All patients received postoperative 5-FU based chemotherapy according to the National treatment guidelines. Chemotherapy consisted of leucovorin (20 mg/m^2) iv. bolus followed by 5-FU (370–425 mg/m^2) iv bolus. Both drugs were administered on days 1 to 5 of each cycle (28 days), patients received 6 cycles. For 5 patients metastatic tissue from liver ($n = 2$, obtained 13 and 27 months after primary surgery), ovarium ($n = 1$, collected 48 months after primary

Abbreviations: bp, base pair; CSS, cancer-specific survival; DR, distant recurrence; ELISA, enzyme-linked immuno sorbent assay; IHC, immunohistochemistry; LOH, loss of heterozygosity; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; TS, thymidylate synthase; UTR, untranslated region; VNTR, variable number of tandem repeats; 1494del6, a 6-bp deletion at nucleotide 1494 in the 3′UTR region of the *TS* gene; 5-FU, 5-fluorouracil.

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TABLE I – PATIENT AND TUMOR CHARACTERISTICS

Factor	*N* (%)[1]
Age (median), [range]	(64.5), [39–74]
Sex	
Male	28 (74%)
Female	10 (26%)
Localization	
Cecum	8 (21%)
Appendix	0 (0%)
Colon ascendens	3 (8%)
Hepatic flexure	4 (10.5%)
Colon transverse	2 (5.3%)
Splenic flexure	3 (7.9%)
Colon descendens	2 (5.3%)
Sigmoid colon	16 (42.1%)
Invasion depth	
T2	4 (10.5%)
T3	30 (79%)
T4	4 (10.5%)
Lymph node status	
N1	28 (74%)
N2	10 (26%)
Tumor differentiation grade	
Well differentiated	2 (5.3%)
Moderately	24 (62.3%)
Poorly or undifferentiated	11 (28.9%)
Unknown	1 (2.6%)
Comorbidity	
No comorbidity	21 (55%)
One additional disease	9 (24%)
Two or more additional diseases	7 (18%)
Unknown	1 (3%)

[1]In case of scale variables, the median and range values are depicted.

surgery) and peritoneum ($n = 2$, collected 17 and 7 months after primary surgery) was also available.

Patient data were obtained from the Comprehensive Cancer Centre South (Eindhoven, Cancer Registry). Trained registrars recorded the following tumor characteristics: tumor grade (well/moderately differentiated *vs.* poorly or undifferentiated tumors), postoperative tumor depth (T1/T2, T3, T4) and lymph node involvement (N1, N2). Clinical factors such as adjuvant chemotherapy, year of diagnosis and, if applicable, dates of death were also recorded. Additional data on recurrence dates were retrieved from patient records.

TS *genetic analysis*

DNA isolation. Genomic DNA was obtained from archival formalin-fixed paraffin-embedded normal and tumor tissues. In case of tumor specimens, enrichment was performed by macro dissection of tumor areas with more than 50% tumor cells.[28] DNA was extracted by overnight incubation with proteinase K (Merck, Darmstadt, Germany) at 56°C followed by boiling for 5 min.[28] Subsequently, DNA was purified using the high pure PCR template preparation kit (HPPTP kit, Roche Diagnostics, Mannheim, Germany).

VNTR analysis. The VNTR region was amplified by a PCR. The primers and PCR conditions used were previously described by Kawakami *et al.*[29] and were optimized for our laboratory settings. Briefly, primers: 6-FAM 5′-GCGGAAGGGGTCCTGCCA-3′ and 5′-TCCGAGCCGGCCACAGGCAT-3′, 1 unit of AmpliTaq Gold Polymerase, PCR Gold buffer (Applied Biosystems, Foster City, CA), 1.5 mM $MgCl_2$ and 5% DMSO (Merck, Darmstadt, Germany). Primers were labeled to facilitate, if necessary, more detailed fragment analysis using the ABI Prism 310 Genetic Analyzer. PCR conditions were as follows: preheating: 5 min at 95°C; 40 cycles: 45 sec at 95°C, 45 sec at 58°C, 45 sec at 72°C and 5 min at 72°C. The presence of the 3R (135 bp) or 2R (107 bp) repeats was evidenced after electrophoresis on a 2% agarose gel and ethidium bromide staining. In the case of heterozygous patients, both PCR products were observed.

SNP analysis. Digestion of the VNTR PCR products with *HaeIII* during 1 hr at 37°C (New England Biolabs, Ipswich, United Kingdom) was used to detect the SNP of a G to a C substitution in the 3R allele.[8] In case of a SNP, an additional 94 bp fragment was observed after gel electrophoresis on a 3% agarose gel and ethidium bromide staining. In order to study the SNP in the first repeat of the 2R allele which has been recently described,[13,14] fragments after digestion were additionally analyzed with the ABI Prism 310 Genetic Analyzer.

1494del6 analysis. The *TS* 6 bp deletion polymorphism at nucleotide 1494 was assessed using PCR and primers 6-FAM 5′-CAAATCTGAGGGAGCTGAGT-3′ and 5′-CAGATAAGTG GCAGTACAGA-3′,[30] 1 unit of AmpliTaq Gold Polymerase, PCR Gold buffer (Applied Biosystems) and 2.5 mM $MgCl_2$ for amplification. PCR conditions were similar to the ones described for the VNTR analysis. PCR products were analyzed with the ABI Prism 310 Genetic Analyzer in order to distinguish between the +6 bp and −6 bp alleles.

Allelic imbalance

Loss of heterozygosity. Loss of heterozygosity (LOH) was analyzed in patients heterozygous for the VNTR polymorphism (2R/3RG, 2R/3RC) ($n = 16$). LOH was determined by assessment of the peak surfaces ratios acquired by ABI Prism 310 Genetic Analyzer. After analysis in normal tissue the peak surface of the 2R allele was divided by the surface of the 3R allele (ratio in normal tissue). This number was divided by the ratio between the 2R and 3R allele found in tumor tissue. An obtained LOH ratio of 0.76–1.00 or 1.00–1.30 indicated retention of both alleles. An LOH ratio of $\leq$0.59 (loss 3R) or $\geq$1.70 (loss 2R) indicated LOH. Intermediate ratios (0.60–0.75 and 1.3–1.69) were considered as inconclusive.

TS gene amplification. TS gene amplification was determined by a newly developed multiplex quantitative real time PCR using a LightCycler 2.0 apparatus (Roche Molecular Biochemicals, Mannheim, Germany) and Taqman probes. *TS* gene copy number was normalized to the reference β-*globin* gene. For both genes, specific Taqman probes (Applied Biosystems) were newly designed to anneal to region to be amplified as a duplex PCR assay by using the LightCycler Probe Design software 2.0 to fit proper primer/probe criteria for optimal multiplex analysis. Two differently labeled Taqman probes enabled simultaneous quantification of the *TS* and reference gene; respectively FAM-5′-AGGCCAT TACTTTGCCATAATTGTACGACC-3′ and VIC-5′-AGTCTG CCGTTACTGCCCTGTGG-3′. In case of the *TS* gene, PCR primers with the following sequences were used: 5′-GCTTT GGGAAAGGTCTGG-3′ and 5′-CGGACATGAGGAGCAAT TAC-3′. These primers amplified a portion of the TS gene resulting in a fragment of 99 bp.[27] PCR primers used for the reference gene (β-*globin*), 5′-ACACAACTGTGTTCACTAGC-3′ and 5′-CAACTTCATCCACGTTCACC-3′ resulting in a fragment of 110 bp were previously described.[31] The PCR reaction for both genes was performed using a LC FastStart DNA Mastermix Plus Hybridization Probes kit (Roche). PCR conditions were 10 min 95°C; 45 cycles of 10 sec at 95°C, 10 sec at 55°C, 10 sec at 72°C. The normalized amplification rate (relative quantification) was calculated with the Light Cycler Relative Quantification Software (Roche). A *TS*/β-*globin* ratio of > 1.5 was considered positive for *TS* gene amplification. This amplification ratio was calculated by dividing the ΔC_p of the target gene by the ΔC_p of the reference gene.

Immunohistochemistry

Immunohistochemical staining of TS was performed on 4 μm sections obtained from paraffin-embedded normal and tumor tissues using the TS-106 antibody (1:1000, DakoCytomation, Glostrup, Denmark, kindly provided by DakoCytomation, The Netherlands). This clone is commonly used to investigate TS with

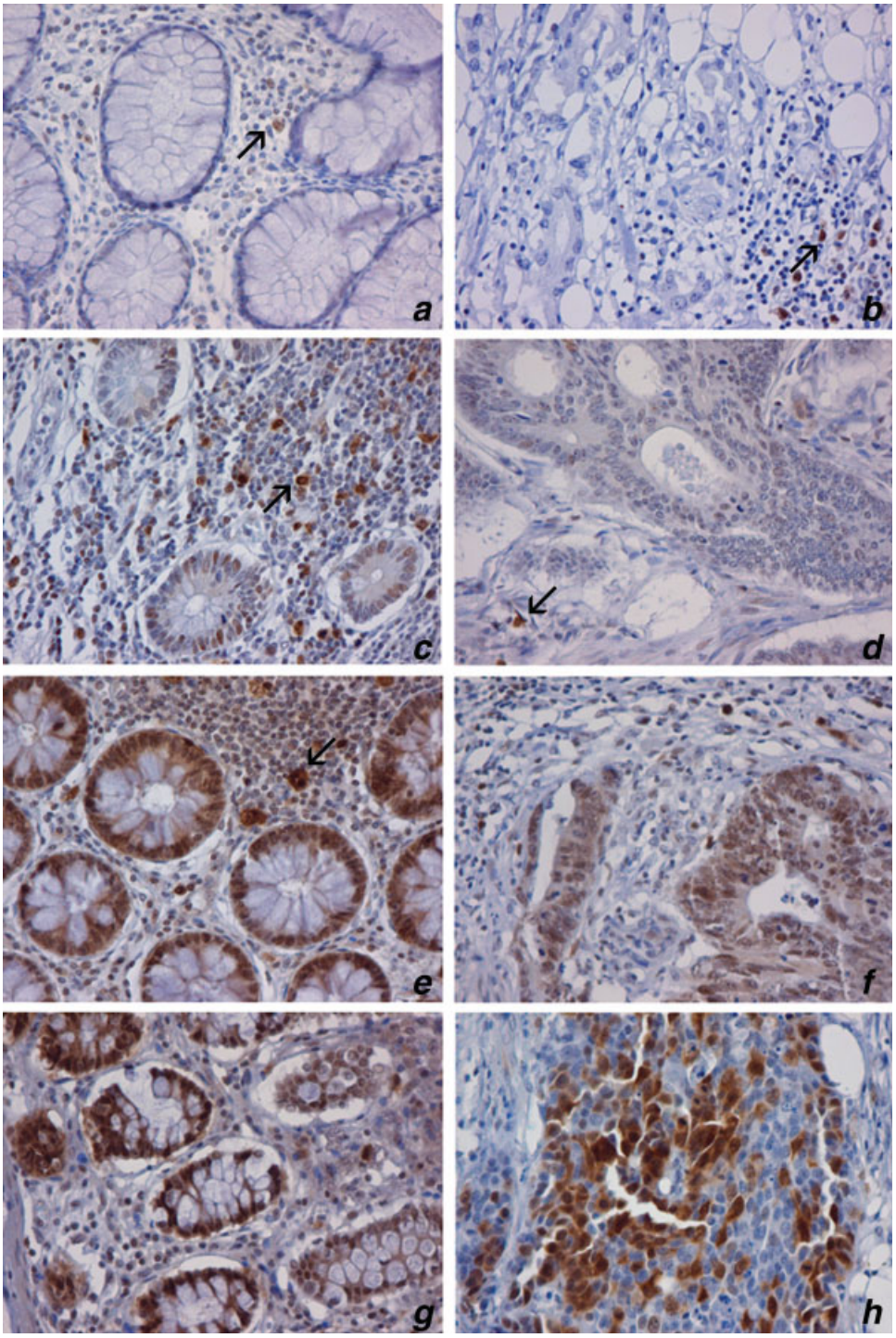


FIGURE 1 – Different staining intensities observed after TS IHC with monoclonal antibody TS-106 (DakoCytomation) in tumor tissue (*b*, *d*, *f* and *h*) and normal tissue (*a*, *c*, *e* and *g*), original magnifications: 200X. Arrows depict intensely stained immature lymphocytes. Staining intensities of 0, 1, 2 and 3 are respectively indicated by panels *a* and *b*, *c* and *d*, *e* and *f* and *g* and *h*.

IHC, as reviewed by Popat *et al.*[24] Staining was performed using the PowerVision plus method (ImmunoVision, Brisbane, CA) and visualization occurred with 3,3′-diaminobenzide hydrochloride solution (DAB). Briefly, paraffin sections were dewaxed and rehydrated. Endogenous peroxidase activity was blocked by incubation for 30 min. in aquadest containing 3% H_2O_2 (Fisher Chemicals, Fair Lawn, NJ) and 0.1% sodium azide (Fisher Chemicals). Antigen retrieval consisted of boiling in an EDTA buffer (pH 9.0). Subsequently, slides were allowed to cool down for at least 30 min. Before applying the antiserum, slides were pretreated with 0.5% casein (Sigma-Aldrich, St. Louis, MO) and 0.1% sodium azide in phosphate-buffered saline (PBS) for at least 10 min. To reduce nonspecific staining, antibodies were dissolved in PBS with 2% goat serum (Gibco, Invitrogen Corporation Carlsbad, CA) and 0.1% sodium azide. Next, slides were incubated in a humidity chamber with the primary antibody for 20 hr (overnight) at

4°C. Staining was developed with PowerDAB (ImmunoVision) during 5 min. Slides were counterstained with hematoxylin solution, dehydrated and enclosed with Permount (Fisher Chemicals). All reactions were performed at room temperature, unless stated otherwise. All slides were stained simultaneously in order to reduce intersample variability. Test slides of normal and cancer tissue revealed that (immature) lymphocytes were intensely stained and that normal epithelial cells were primarily stained in the basal area of the colonic crypts. These observations were in agreement with the performance characteristics of the antibody as mentioned in the DakoCytomation datasheet.

TS staining intensity and localization were evaluated in matched tumor and normal tissues by 2 observers, one being a pathologist (ITG), who were not acquainted with patients' clinical outcome. Slides were evaluated with respect to several IHC parameters by both observers at the same time in order to achieve a consensus score immediately. Staining intensity was categorized as follows: (0) no staining, (1) weak staining, (2) moderate staining and (3) intense staining (Fig. 1). Immature lymphocytes displayed a consistently high TS staining intensity (3) and were therefore used as an internal reference point. In addition to the overall staining intensity (the intensity that was observed in more than 50% of tumor cells), the strongest staining intensity and percentage of cells stained with this intensity were also assessed. Furthermore, the staining localization was scored as nuclear (N) or nuclear and cytoplasmatic (N+C). Finally, the ratio between the overall intensity observed in the tumor and normal epithelia was assessed. A ratio of 0 indicated equal staining intensities. When the normal tissue showed a stronger intensity than the tumor tissue, the ratio was −. In case of reverse observations the ratio was scored as +.

Assignment to TS categories

Patients were categorized into low or high TS producers based on their genotypes (Table II) and immunohistochemically assessed protein levels. Dichotomization into low and high TS producers was performed for both tumor and normal tissue. Heterozygous individuals were categorized as high producers following a dominant model. The 3RC allele was considered to encode for amounts of TS proteins equal to a 2R allele. Therefore, the 3RC/3RC and 2R/3RC genotypes were categorized as low TS producers. In case of IHC, TS levels were dichotomized based on the chromogen intensity as follows: low TS producers: an overall staining intensity of 0–1, high TS producers: an overall staining intensity of 2–3 as described by several other studies.[19,22,32,33]

Statistical analysis

Relations between various parameters were analyzed using the chi-square and one-way ANOVA method. The correlation of the *TS* amplification assay which was performed in duplicate was determined using the Pearson correlation. Survival analyses of time to cancer related death or the occurrence of distant metastasis were performed using the Kaplan-Meier method, with the time of surgery as entry date. Patients who experienced a metastasis during treatment with chemotherapy, *i.e.*, 6 months, were excluded from survival analysis ($n = 4$) since these patients probably had metastasis undetected at time of diagnosis. These patients had 2R/2R ($n = 2$) and 2R/3R ($n = 2$) genotypes and will be categorized as low (2) and high (2) TS producers, respectively, after dichotomization. The patient population, excluding patients with metastasis at time of diagnosis, was used to investigate the proof of principle. Investigations on the total population were additionally performed in order to test the predictive value of TS in the setting of the intention to treat.

Differences in observed survival between groups were tested for statistical significance using log-rank tests. A p value of < 0.05 was considered statistically significant.

TABLE II – DISTRIBUTION OF THE *TS* POLYMORPHISMS

Polymorphism	Genotype	Frequency (%)	Predicted level of protein expression
VNTR[1]	2R/2R	13 (34%)	Low
	2R/3R	16 (42%)	High
	3R/3R	9 (24%)	High
VNTR+SNP[1]	2R/2R	12 (32%)	Low
	2RC/2R	1 (3%)	Low
	2R/3RG	8 (21%)	High
	2R/3RC	8 (21%)	Low
	3RG/3RG	4 (10%)	High
	3RC/3RG	1 (3%)	High
	3RC/3RC	4 (10%)	Low
VNTR+SNP+LOH[2]	2R/loss	6 (37%)	Low
	3RC/loss	0	Low
	3RG/loss	2 (13%)	High
	2R/3RC R	3 (19%)	Low
	2R/3RG R	1 (6%)	High
	2R/3RC IR	1 (6%)	Low
	2R/3RG IR	3 (19%)	High
1494del6[1]	+6bp/+6bp (wt)	22 (58%)	High
	+6bp/−6bp	15 (39%)	High
	−6bp/−6bp	1 (3%)	Low

R, retention; IR, intermediate ratio; wt, wild type.
[1]Dominant model.–[2]Only analyzed in tumor tissue of patients heterozygous for the VNTR polymorphism ($n = 16$).

Results

Distribution of TS *polymorphisms*

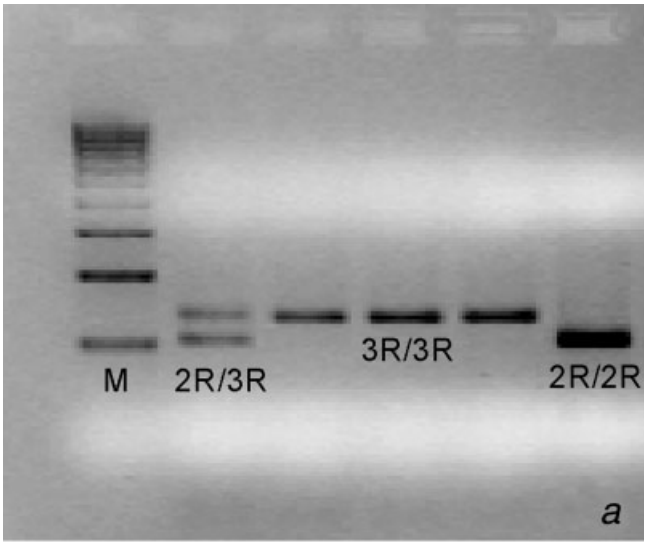
VNTR analysis and subsequent digestion resulted in clear visible bands after gel electrophoresis (Figs. 2*a* and 2*b*). The presence of a 6 bp deletion in the 3′UTR region could be easily analyzed after detailed fragment analysis with the ABI Prism Genetic Analyzer (Fig. 2*c*).

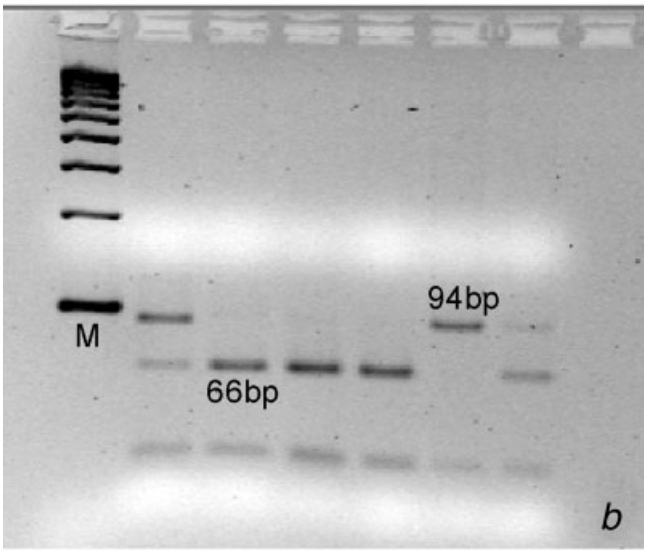
The distribution and categorization of the 3 different polymorphisms is depicted in Table II. The majority (42%) of the patients was found to be heterozygous for the VNTR polymorphism. Although 4 up to 9 repeats in the VNTR region have been described,[34,35] no alleles with more than 3 tandem repeats were observed. Seventeen of the 33 3R alleles (52%) revealed to have a G to C substitution. Combining the VNTR polymorphism with the SNP analysis resulted in the following genotype distribution: 32% 2R/2R, 3% 2RC/2R, 21% 2R/3RC, 21% 2R/3RG, 10% 3RC/3RC, 10% 3RG/3RG and 3% 3RG/3RC (Table II). The majority (58%) of the patients had no 6 bp deletion in the 3′UTR region. VNTR with subsequent SNP analysis were performed in both tumor and normal tissue. Genotypes were similar in both groups except for heterozygous individuals exhibiting LOH.

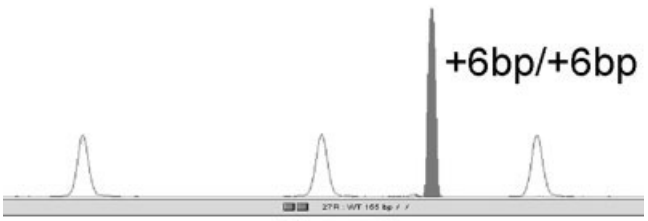
Allelic imbalance

Loss of heterozygosity. LOH was found in tumor specimens in 8 of 16 (50 %) patients heterozygous for the VNTR polymorphism. Of the remaining 8 patients, retention and intermediate ratios were observed in 4 patients each (Table II). Additional LOH analysis affected the tumor genotype in 21% of the total patient population. Loss of the 3R allele was most frequently observed (75%). Heterozygous tumors with loss of the 3R allele were categorized as low TS producers and tumors with loss of the 2R allele remained high TS producers.

TS gene amplification. In this study a new real time PCR was developed to determine *TS* gene amplification. This was done by comparing the amount of PCR products of the β-*globin* reference gene with the *TS* gene. For this it is crucial that the efficiency, as measured by the curve slopes, of both PCR reactions is comparable. Our data showed that in control DNA (15 ng/μg human genomic DNA, LightCycler control kit DNA, Roche Applied







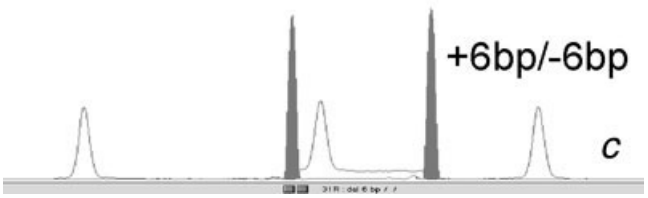
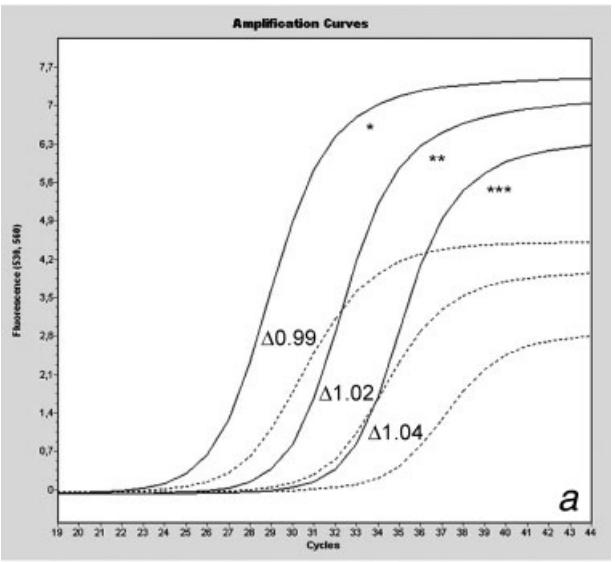


FIGURE 2 – Representative images of *TS* PCR products after gel electrophoresis. (*a*) Variable number of tandem repeat (VNTR) analysis; (*b*) subsequent digestion of the amplified VNTR region. The appearance of a fragment of 94 bp indicates a base pair substitution of a C to a G. In panel 1C, PCR products after analysis with the ABI Prism 310 Genetic Analyzer are depicted. M, marker.



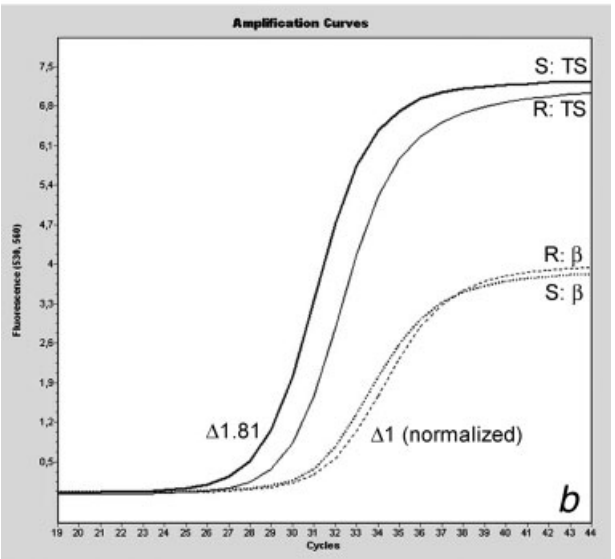


FIGURE 3 – Amplification curves observed after the newly developed real time PCR assay, performed on DNA obtained from formalin-fixed paraffin-embedded tissue. A dilution series (panel *a*) and an example of *TS* gene amplification (panel *b*) are depicted. Panel *a*: amplification curves of the *TS* gene (solid line) and β-*globin* gene (dotted line), *: undiluted, **: 10X diluted, ***: 100X diluted. The Δ indicated the difference of C_p (crossing point) values between the *TS* and reference gene for each dilution. The C_p value is defined as the cycle at which the fluorescent signal exceeds the threshold value as determined by the automatic fit point module. Panel *b*: S: sample (tumor DNA), R: reference (normal DNA), Δ: difference in C_p values between the *TS* and β-*globin* gene. The amplification ratio was calculated by dividing the ΔC_p of the target gene by the ΔC_p of the reference gene.

Science) the efficiency of the *TS* and β-*globin* amplification gene was equal; also after analyzing a 10-fold dilution-series. The curve shape and slope as observed after analysis of the control DNA were confirmed in formalin-fixed paraffin-embedded tissue. Serial dilutions of DNA isolated from tumor samples showed similar amplification curves for the *TS* and β-*globin* gene (Fig. 3*a*). *TS* gene amplification could be determined by this newly developed assay in 37 of the 38 patients (Table III). Amplification was performed in duplicate. The correlation between these 2 measure-

TABLE III – COMPARISON BETWEEN *TS* GENOTYPES AND IMMUNOHISTOCHEMISTRY

TS nr	G T VNTR and SNP	G N VNTR and SNP	1494del6	IHC, N	IHC, T[1]	Mean amp ratio PT[1]	IH M	Mean amp ratio, M
TS01	3C/3C	3C/3C	+6bp/+6bp	2	2	0.6		
TS02	2/2	2/2	+6bp/+6bp	2	3	2.3		
TS03	3G/3G	3G/3G	−6pb/+6bp	2	2	1.0	3	1.71
TS04	2/_	2/3C	+6bp/+6bp	·	2	1.0		
TS05	3G/_	2/3G	−6pb/+6bp	0	2	0.8		
TS06	2/_	2/3C	+6bp/+6bp	2	1	1.3		
TS07	2/2	2/2C	+6bp/+6bp	2	3	1.2		
TS08	3C/3C	3C/3C	−6pb/+6bp	2	1	1.0		
TS09	2/2	2/2	+6bp/+6bp	2	1	0.8		
TS10	2/2	2/2	+6bp/+6bp	1	0	0.7		
TS11	3C/3C	3C/3C	+6bp/+6bp		1		1	0.76
TS12	3G/3G	3G/3G	+6bp/+6bp	0	0	0.8	2	0.87
TS13	2/3G	2/3G	−6pb/+6bp	2	1	0.9		
TS14	2/3C	2/3C	−6pb/+6bp	2	1	0.9	2	0.56
TS15	2/_	2/3G	−6pb/+6bp	2	2	1.0		
TS16	2/2	2/2	−6pb/+6bp	1	1	0.5		
TS17	2/_	2/3G	−6pb/+6bp	1	2	1.8		
TS18	2/2	2/2	−6pb/+6bp	3	2	1.2		
TS19	3G/3G	3G/3G	+6bp/+6bp	2	2	1.2		
TS20	2/_	2/3C	−6pb/+6bp	3	1	1.4		
TS21	2/2	2/2	+6bp/+6bp	2	2	1.2		
TS22	2/2	2/2	+6bp/+6bp	2	2	1.1		
TS23	2/2	2/2	−6pb/+6bp	2	1	0.8		
TS24	2/3C	2/3C	+6bp/+6bp	1	1	0.7		
TS25	2/2	2/2	−6pb/+6bp	2	1	0.5		
TS26	2/2	2/2	+6bp/+6bp	2	1	0.9		
TS27	3G/3C	3G/3C	−6pb/+6bp	2	1	0.8		
TS28	2/_	2/3C	−6pb/+6bp	1	1	0.8		
TS29	3G/3G	3G/3G	−6pb/−6pb	1	1	0.8		
TS30	2/3G	2/3G	+6bp/+6bp	3	2	0.9		
TS31	2/3C	2/3C	+6bp/+6bp	2	2	1.0		
TS32	2/2	2/2	+6bp/+6bp	2	2	0.9		
TS33	2/3G	2/3G	+6bp/+6bp	2	1	0.9		
TS34	2/3C	2/3C	+6bp/+6bp	1	0	1.0		
TS35	2/3G	2/3G	−6pb/+6bp	2	1	1.0		
TS36	3C/3C	3C/3C	+6bp/+6bp	1	0	1.1	2	0.88
TS37	3G/_	2/3G	+6bp/+6bp	2	2	1.1		
TS38	2/2	2/2	+6bp/+6bp	2	1	0.7		

G, genotype; T, tumor; N, normal; PT, primary tumor; M, metastasis; IHC, immunohistochemical staining intensity observed in the majority of normal/tumor cells; amp ratio, amplification ratio of the *TS* gene.

[1]The mean amplification ratio was found to be correlated to the IHC intensity as observed in the majority of the tumor cells ($p = 0.001$). This correlation is also depicted in Figure 5.

ments was highly significant ($p < 0.001$, correlation coefficient = 0.927, Pearson correlation) indicating that the amplification assay was highly reproducible. A mean amplification ratio of >1.5 was found in 2 out of 37 tumors (5%) (Fig. 3*b*). In addition, 1 out of 5 (20%) metastases had a mean ratio of >1.5. The primary tumor (TS03) of the metastasis with *TS* gene amplification did not demonstrate amplification (mean ratio 1.0).

Immunohistochemical assessment of TS

Respectively 11%, 47%, 37% and 5% of the tumors displayed an overall staining intensity of 0, 1, 2 and 3 (Table IV). Overall TS staining intensity in the normal mucosa could be evaluated in 36 out of 38 patients; normal tissue could not be found in 1 patient, in case of another patient IHC of normal tissue could not be assessed due to an artefact. Staining was predominately localized in the nucleus (tumor tissue: 71%, normal tissue 89%).

TABLE IV – DISTRIBUTION OF THE DIFFERENT STAINING INTENSITIES AND STAINING LOCALIZATION OBSERVED IN THE MAJORITY OF THE TUMOR/EPITHELIAL CELLS

IHC score	Tissue	
	Normal	Tumor
0	2 (6%)	4 (11%)
1	8 (22%)	18 (47%)
2	23 (64%)	14 (37%)
3	3 (8%)	2 (5%)
Localization	n: 32 (89%); n + c: 4 (11%)	n: 27 (71%); n + c: 11 (29%)

IHC, immunohistochemistry; n, nucleus; c, cytoplasm.

Prognostic significance

The different IHC parameters (overall and strongest staining intensities, staining localization and the ratio of staining between normal and tumor cells) failed to demonstrate a significant association with distant recurrence (DR) and cancer-specific survival (CSS). TS dichotomization based on the VNTR analysis in normal tissue (as summarized in Table II) was found to be of prognostic significance for DR ($p = 0.038$) and CSS (5-year survival proportions for TS low: 88 % and TS high: 37%, $p = 0.021$) (Figs. 4*a* and 4*b*). As illustrated in Figure 4*c*, additional SNP analysis of DNA extracted from normal tissue did not add prognostic information. This was also observed for DR.

Although it was decided beforehand to exclude 4 patients who experienced a metastasis during the administration of chemotherapy (*i.e.*, within 6 months after surgery) because these patients would probably have stage IV disease at time of diagnosis instead of stage III disease, we also performed survival analysis on the total population based on clinical practice at time of diagnosis (Figs. 4*d* and 4*e*). Contrary to the IHC parameters, a strong trend towards a significant difference between TS low and high

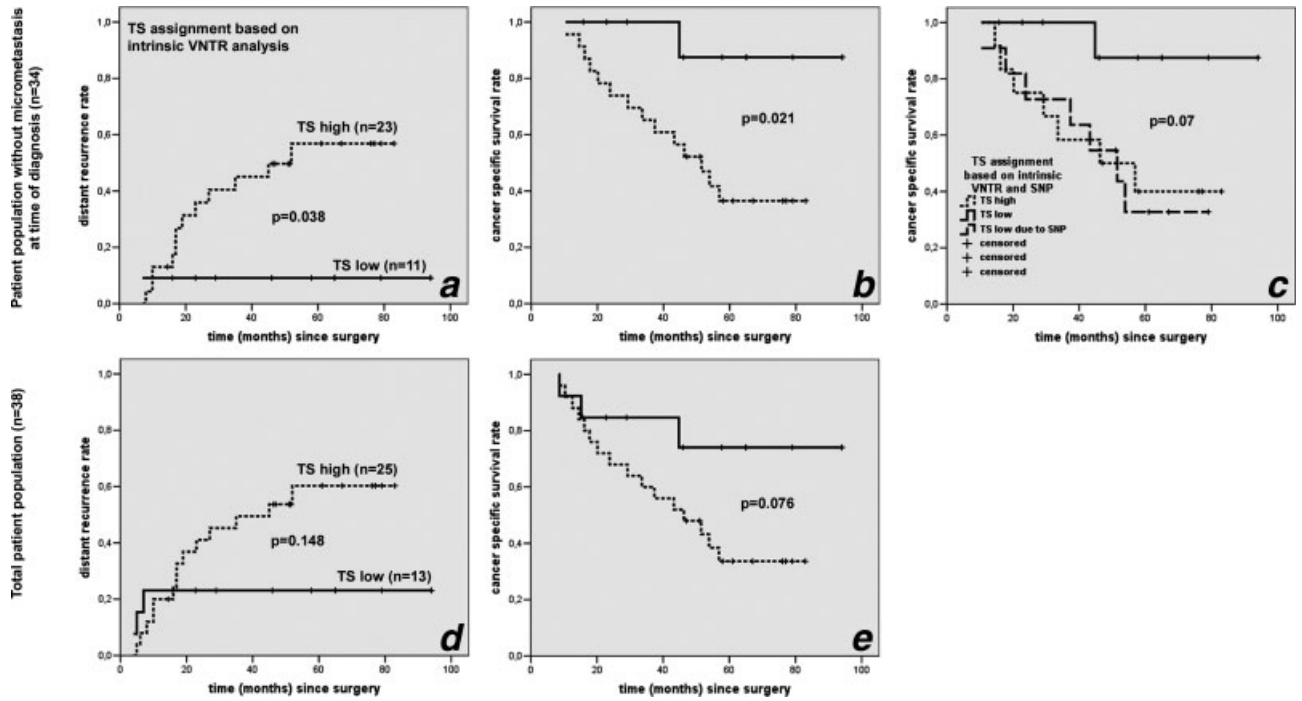


Figure 4 – Kaplan-Meier survival curves of distant recurrence (*a* and *d*) and cancer-specific survival (*b* and *e*). (*c*) Kaplan-Meier survival curve demonstrating the effect of SNP analysis in normal tissue. Patients with 3RC/3RC or 2R/3RC genotype behave differently from patients with the 2R/2R genotype. Patients developing a metastasis within 6 months were excluded from analysis, survival curves of this population are shown in *a–c*. Analysis performed on the total patient population ($n = 38$) are shown in panel *d* and *e*.

producers, especially with respect to CCS, could still be observed after analysis of the total population.

Correlation between TS *genotype and IHC*

No correlation was found between the predicted protein levels (high, low) based on *TS* polymorphisms and overall or strongest TS staining observed after IHC. This correlation was lacking for both tumor and normal tissues. Combining the strongest staining intensity with the percentage of maximal stained cells also did not result in a significant correlation with *TS* genotype. The other IHC parameters (staining localization, percentage of cells with strongest staining and staining ratio between normal and tumor tissue) also failed to show a correlation with *TS* genotype. However, the overall staining intensity was significantly correlated ($p = 0.001$, one-way ANOVA) with the mean amplification ratio (Fig. 5). This p value was still significant after correcting for multiple comparisons ($p < 0.0083$ in case of 4 groups). This relationship was mostly pronounced in tumors with gene amplification because these tumors were intensely stained in specific subpopulations of tumor cells as depicted in Figure 1*h*. The observed staining pattern of this tumor is very heterogeneous; some tumor cells are completely negative and others display an intensely stained nucleus.

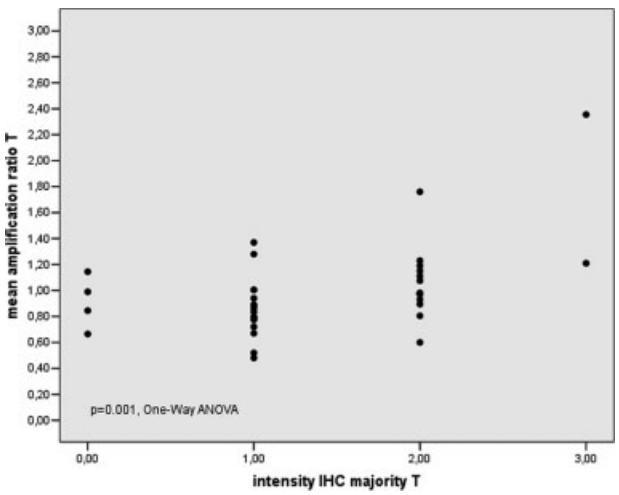


Figure 5 – Scatterplot depicting the correlation between *TS* gene amplification in the primary tumor and TS staining intensity as observed in the majority of the tumor cells. T, tumor.

Discussion

This study shows that the intrinsic variable number of tandem repeats (VNTR) was the only polymorphism of the TS gene demonstrating a significant association with distant recurrence (DR) and cancer-specific survival (CCS) (Figs. 4*a* and 4*b*). Assessment of the other 2 polymorphisms; the single nucleotide polymorphism (SNP) and the 3′UTR deletion (1494del6) did not add prognostic information. The different TS IHC parameters were also not predictive for patients' outcome.

The clinical implications of VNTR analysis with respect to the favorable outcome of patients with a 2R/2R genotype in the adjuvant setting was similar to that of several other studies.[11,36,37] In addition, the lack of prognostic information of VNTR combined with SNP analysis is in accordance with the findings of Dotor *et al*.[30] This is clearly illustrated in Figure 4*c*; patients who had low predicted protein levels due to a SNP in the 3R allele had a worse prognosis than patients with a 2R/2R genotype. However, others found that the 3R/3R genotype was associated with a better outcome which is in contrast to our findings.[30] This observation was confirmed by Jakobsen *et al*. investigating patients with disseminated disease.[38] However, nothing can be said about the differences in 5-FU sensitivity alone because the outcome is affected by both the intrinsic tumor biology and sensitivity to 5-FU. These effects of intrinsic tumor biology are probably reflected by our

findings that analysis of the total patient population, including patients with M+ disease at time of diagnosis, the significant difference between TS low and high producers disappeared (Figs. 4*d* and 4*e*). However, a clear trend towards a significant difference could still be observed. Since our study population is relatively small, it is difficult to study the effect of disseminated disease on the predictive role of TS and it would be interesting to study this in a larger study population which is currently under investigation.

In contrast to TS genotyping, this study demonstrated that IHC is not predictive for therapy response. A predictive role of TS IHC, in the adjuvant and advanced settings, was found in some studies[2,18] but enfeebled by others.[19–23]

This supports that TS IHC is a heavily operator-dependent method not easily reproducible in different laboratories. Differences in tissue fixation, observer dependent assessment of staining patterns, severe heterogeneity in staining intensities between cells and most importantly, antibody binding may all cause an inaccurate reflection of the protein content of the specimen.

In the present study, 52% of the 3R alleles revealed to have a G to C substitution. The frequency of this SNP varies between ethnic groups and has been described to be 56% for Caucasians[39] which is in agreement with our findings. Even though SNP analysis did not add prognostic information, it would have been desirable to study LOH in patients with 3 repeats heterozygous for the SNP because it can affect the predicted protein levels, *i.e*., 3RC/3RG individuals with loss of the 3RG allele would have low predicted protein levels. However, with current techniques, LOH analysis in 3RC/3RG individuals was found to be too challenging from a technical point of view. In the future, other techniques like primer extension might facilitate this analysis and is presently under investigation. In addition, we investigated the recently identified SNP in the fist tandem repeat of the 2R allele[13,14] in 1 patient who was homozygous for the VNTR but heterozygous for the SNP in the 2R allele (2R/2RC). The frequency of the 2RC allele has been described to be 4.2%[14] to 1.5%.[13] In our population, the frequency of the 2RC allele was found to be 1.3%, which is consistent with previous findings.

Although several in vivo and in vitro studies indicated that TS genotype and TS protein levels are correlated,[7–12] the present study indicated that this correlation could not be confirmed with IHC. Patients with a 3R/3R genotype did not show higher TS staining intensities than patients with a 2R/2R genotype. This finding might explain the discrepancies in literature concerning the predictive value of TS as indicated by the ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer.[15] Dotor and coworkers also observed that the 3 polymorphisms were not associated with IHC staining intensities (evaluated in 129 tumors).[30] Besides the technical considerations of IHC, several other mechanisms could account for the finding in the present study that IHC did not confirm the TS protein levels predicted by genotyping. Firstly, a variety of posttranslational mechanisms such as protein glycosylation, folding and protein–protein interactions can influence epitope-availability of monoclonal antibodies directed against TS. Secondly, the antibody used largely affects the staining pattern. Indeed, even using antibodies of the same clone (TS-106) but from different companies (Abcam *vs*. DakoCytomation) resulted in poor reproducibility (data not shown).

This is the first study reporting an association between *TS* gene amplification and IHC (Fig. 5). This observation makes it tempting to speculate that the variations in TS protein levels caused by the *TS* polymorphisms are very subtle and can therefore not be clearly visualized with IHC. *TS* gene amplification, on the other hand, can increase the concentration of TS proteins to such an extent that IHC staining patterns will be more pronounced in those specific tumor cells. Figure 1*h* depicts a staining pattern which can be best characterized as "dotted" with some tumor cells displaying an intense nuclear staining and others lacking staining. It was also observed that these intensely stained cells often displayed mitotic spindle figures indicating cell proliferation. Since amplification analysis was performed on DNA extracted from a selected tumor area containing >50% tumor cells and tumors are known to be very heterogeneous, the intensely stained cells would probably have an amplification ratio of for example 5 or 6 to compensate for those with no amplification. This hypothesis could be further investigated by using fluorescent *in situ* hybridization (FISH) since this technique can visualize differences in copy numbers between individual tumor cells.

TS gene amplification is not frequently observed in primary tumors in contrast to tissue exposed to 5-FU based therapy.[27] This is consistent with our results since we found that 5% (2/37) of the primary tumors demonstrated amplification *vs*. 20% (1/5) of the metastatic tissues. This percentage is in accordance with Wang *et al*. who reports *TS* gene amplification in 23% of liver metastases that have been exposed to 5-FU.[27] The single metastasis with *TS* gene amplification also demonstrated a strong (3) IHC staining intensity, which is in agreement with our finding that gene amplification is correlated with IHC staining intensity. However, due to small patient numbers firm conclusions cannot be drawn. Treatment with 5-FU might provoke selection of tumor cells with TS gene amplification.[40,41] Loss of heterozygosity (LOH), which can also result in allelic imbalance, was frequently reported at the site of the *TS* gene. LOH frequencies of 63% up to 73% were found in patients with a 2R/3R genotype.[25,42] These findings were confirmed by the present study which reported LOH in 8 of 16 (50 %) patients heterozygous for the VNTR polymorphism. Although we have performed macro dissection for purposes of tumor enrichment, the percentage of LOH reported could be underestimated if substantial numbers of normal cells are still present.

Although the number of patients in the present population is relatively small (38 patients), significant differences regarding CSS and DR based on VNTR analysis in germline DNA were obtained. Studying these germline DNA alternations has several advantages. Analysis can be performed on readily available tissue such as peripheral blood and does not require harvesting of tumor tissue. Moreover, genomic polymorphisms are stable providing certain robustness in favor of DNA based assays. To validate the data obtained by this study, *TS* genotyping will be further investigated in a large group of well defined patients with colorectal cancer to study the predictive value for response to 5-FU.

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