ANTICANCER RESEARCH *24*: 3875-3880 (2004)

Thymidylate Synthase Gene Promoter Polymorphisms are Associated with TSmRNA Expressions but not with Microsatellite Instability in Colorectal Cancer

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Abstract. *Background: Microsatellite instability (MSI) is a biological characteristic of most tumours, being involved in 85% of hereditary non-polyposis colorectal cancer (HNPCC). It also occurs in 10-15% of sporadic colorectal cancers (CRC). HNPCC appears to be caused by germline mutations in mismatch repair (MMR) genes, which are responsible for repairing single base-pair mismatches. MSI is also associated with a better response of CRC to adjuvant chemotherapy with fluoropyrimidines. We investigated any relationship between the MSI status and the TSmRNA expression, the polymorphisms of 5-Fluorouracil (5-FU cellular target, the enzyme thymidylate synthase (TS) and TS expression evaluated by means of immunohistochemistry. Materials and Methods: A series of 80 colorectal cancers was evaluated for MSI and polymorphisms in the 3'UTR and the 5'UTR of the TS gene by a PCR assay. TSmRNA was quantified by real-time PCR and the TS expression by immunohistochemical assay. Results: There was no significant association between the polymorphisms in the TS gene and the MSI or between the TSmRNA expression and the MSI status. CRC with a 3R/3R or 2R/3R genotype showed a significantly higher TSmRNA expression than those with the 2R/2R genotype ($p=0.001$ and $p=0.028$, respectively). Another significant association was found between the TSmRNA expression and the TS immunohistochemical determination ($p=<0.05$). No association was found between the polymorphism of the 3'UTR and the TSmRNA expression. Conclusion: Our data show that there is no association between MSI status and the polymorphisms in the 3' and 5' UTRs and the TS expression. Tumour samples displaying the 3R/3R or 2R/3R genotype of TS have higher TSmRNA levels than the 2R/2R genotype. Polymorphic variant of the 3'UTR does not influence the TSmRNA level. We found a relationship between the TSmRNA expression, evaluated by real-time PCR, and with the TS level determined by immunohistochemical assay. Thus, genotyping of the 5'UTR and quantification of the TSmRNA expression in human CRC could be considered as predictors for response to 5FU-based chemotherapy. The evaluation of the TS expression by means of immunohistochemistry assay remains a safe and reliable assay in CRC.*

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Key Words: Colorectal cancer, thymidylate synthase, pharmacogenomic, microsatellite instability, polymorphism, molecular therapeutic.

Major advances in the treatment of cancer have resulted from the recent revolution in medical interventions. Significant heterogeneity in the efficacy and toxicity of chemotherapeutic agents is consistently observed across the human population (1).

Administration of the same dose of a given anticancer drug to a population of patients results in a range of toxicity, from unaffected to lethal events. While many clinical variables have been associated with drug response (age, gender, diet, organ function, tumour biology), genetic differences in drug disposition and drug targets can have a great impact on treatment outcome (1-3).

The cellular targets for the majority of chemotherapy agents contain genetic polymorphisms (4), but prospective identification of patients likely to benefit from (or be harmed by) chemotherapy is not currently possible for most treatments.

This is particularly important in the current health care environment, where cost containment and evidence-based initiatives are having a significant influence on patient care.

Much research is being concentrated on isolating and studying new genetic markers able to identify patients

bearing tumours at high risk of progression or drug resistance, one of them (MSI) with a possible role in the therapeutic strategy of colorectal cancers (CRC).

Microsatellites are small DNA sequences repeated in tandem that show length polymorphisms and are found throughout the human genome. Because they are repetitive, microsatellites are prone to strand slippage and replication errors (5).

MSI occurs when there is disruption of the DNA mismatch-repair system. Failure to repair mismatches can generate mutations characterised by small insertions or deletions. Such mutations alter microsatellite length and are visualised as bandshifts on gel electrophoresis (6). MSI was first seen in HNPCC (7) and is now known to occur in more than 90% of colorectal cancers in individuals with this inherited predisposition (5). About 15% of sporadic colorectal cancers (those occurring without a familial predisposition) are MSI (8). The MSI status of colorectal cancer has been assessed for its prognostic value. Some studies found no survival difference between tumours positive and negative for MSI (9), but they were based on small samples and predated the routine use of adjuvant chemotherapy. More recent studies report a substantial survival advantage in MSI cancers compared with microsatellite stability (MSS) cancers (8, 10), when they are treated with 5-FU-based chemotherapy. The main antitumour activity and cytotoxicity of this compound has been ascribed to a potent inhibition of thymidylate synthase (TS).

TS catalyses the conversion of dUMP to dTMP, the source of thymidine nucleotides for DNA synthesis. 5-FU is metabolised into the analogue FdUMP that inhibits, in a competitive manner, the conversion of dUMP to dTMP, which leads to the inhibition of DNA synthesis (11).TS levels vary considerably among tumours and the response to 5-FU is influenced by the intratumoural activity of the enzyme, with high levels generally being associated with a poor response (12, 13). TS expression has been shown to be influenced both *in vitro* and *in vivo* by a polymorphic tandem repeat sequence in the 5'-untranslated region (5'UTR). Increasing the number of repeats leads to an increase in TS expression (14, 15). Whether the polymorphism affects TS expression on a transcriptional or translational level is discussed controversially. Whereas Kawakami *et al.* (16) concluded from their results that the three-repeat-sequence confers a greater translational efficiency, Pullarkat *et al.* (17) found a higher TS mRNA expression together with an increasing number of tandem repeats. A recently detected 6-bp deletion polymorphism in the 3'-untranslated region of the TS gene might also influence transcriptional and/or translational efficiency or mRNA stability (18).

Recently, studies were conducted to investigate whether genotyping for the TS polymorphism might identify patients with advanced CRC responding well to 5-FU-based chemotherapy. The data suggested that patients homozygous for the double tandem repeat compared to those with a triple repeat had a survival benefit from 5-FU therapy or therapy with the precursor molecule capecitabine (19-21).

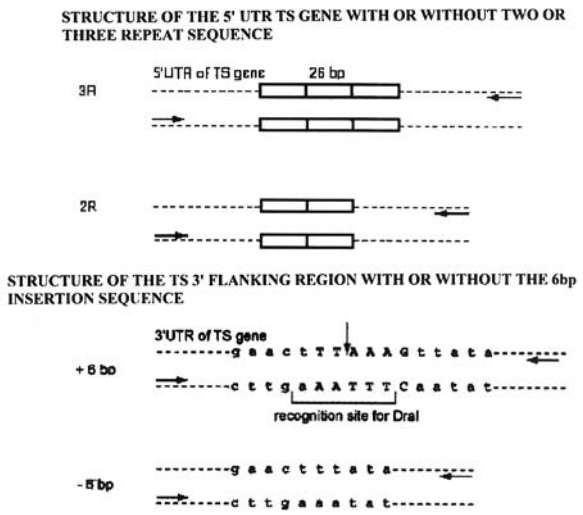

Figure 1. *Schematic view of the TS gene polymorphic regions. A structure of the TS 5' UTR with the 3-or-2 repeat sequence. Horizontal arrows, location of PCR primers. B structure of the TS 3' flanking region with and without the 6 bp insertion sequence. Capital letters, insertion sequence; horizontal arrows, location of PCR primers; vertical arrow, restriction site for DraI.*

The aim of this study was to investigate whether there was a correlation between the MSI phenotype and the TS mRNA levels and with the presence of the 5' and 3' UTR polymorphisms in CRC. We analyzed the TS mRNA expression by means of real-time PCR and the TS genotype concerning the 3'UTR and 5'UTR which has been implicated in influencing TS expression levels. As we performed the TS mRNA levels in paraffin-embedded tumour samples using a LightCycler–TS mRNA quantification Kit (Roche), we also compared the TS mRNA levels obtained in this way with the protein TS levels obtained by immunohistochemical method using the monoclonal antibody 106, to show any difference between the two assays.

Materials and Methods

Cases. Formalin-fixed, paraffin-embedded samples retrieved from archival material of 80 (previously untreated) patients affected by primary colorectal cancer, were reviewed. Fifty patients were males and 30 females. The age ranged from 40 to 90 years, with a mean age of 65 years.

DNA extraction and MSI analysis. TS-positive cells were, through microdissection, isolated from paraffin-embedded, formalin-fixed specimens and DNA was extracted using the Dneasy tissue kit purchased from Qiagen. The technique of microdissection allowed molecular analysis of microsatellites instability only on tumour cells

Table I. *Polymorphism in the 5'UTR of the TS gene in microsatellite stable (MSS) and unstable (MSI) colorectal cancer.*

	2R/2R	2R/3R	3R/3R
MSI	15 (31.3%)	13 (28.1%)	20 (40.6%)
MSS	3 (9.5%)	15 (47.6%)	14 (42.9%)
Total	18	28	34

Table II. *Polymorphism in the 3'UTR of the TS gene in MSS and MSI colorectal cancer.*

	TS genotype		
	+6bp/+6bp	+6bp/- 6bp	- 6bp/- 6bp
MSI	19 (56.5%)	11 (34.8%)	3 (8.7%)
MSS	14 (52.6%)	9 (31.6%)	4 (15.8%)
Total	33	20	7

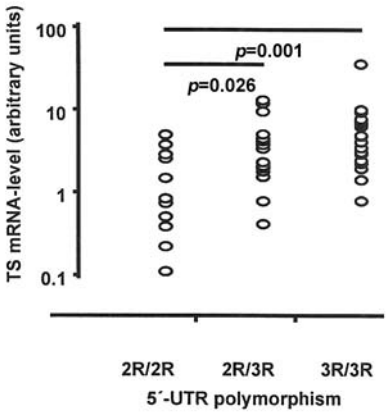

Figure 2. *Colorectal cancers showed different TS mRNA levels according to their 5'UTR polymorphism (p<0.004). Levels were significantly higher in tumours harbouring triple repeats (3R) compared with those homozygous for 2R.*

Table III. *Immunohistochemical evaluation of the TS levels compared with those obtained by RT PCR assay (p<0.05).*

% of cells positivity	Patients n.	Median TS/RT Ratio	Min	Max
Gr.1: 0 – 1+	11	0.78	0.11	11.74
Gr.2: 2+	15	2.82	0.41	7.64
Gr.3: 3+ – 4+	17	4.93	2.06	13.00
Intensity of staining	Patients n.	Median TS/RT Ratio	Min	Max
Gr.1: 0 – 1+	12	0.80	0.11	6.24
Gr.2: 2+	16	3.93	0.41	11.74
Gr.3: 3+ – 4+	15	4.93	2.06	13.00

positive for TS and avoided contamination of normal tissue. The following loci were examined: Bat-25, Bat-26, Bat-40, APC, D2S 123 and Mfd 15, according to international guidelines (22). DNA was amplified through PCR techniques and PCR products were carried out on polyacrilamide gel.

Polymerase chain reaction of the 5' UTR polymorphism. Polymerase chain reaction analysis was performed amplifying 200 ng template DNA in 20 μl of reaction mix including 10% of DMSO 1x reaction buffer, $MgCl_2$ at a final concentration of 3mM, 0.4 mM of each primer, 0.2 mM dNTP and 1 U Taq polymerase (platinum Taq, Invitrogen, Groningen, Netherlands).

Primers: Forward: 5'-AAA AGG CGC GCG GAA GGG GTC CT-3'; reverse 5'- TCC GAG CCG GCC ACA GGC AT-3' were used. After 35 cycles of amplification (94˚C 40 sec, 62˚C 40 sec, 72˚C 40 sec), amplification products were electrophoresed in 3% agarose gel. Products of 220 bp (2R/2R), and 248 bp (3R/3R), or both of these products (2R/3R), depending on the TS genotype, were obtained.

Polymerase chain reaction of the 3' UTR polymorphism. The 3' UTR analysis was carried out by polymerase chain restriction amplification/RFLP analysis. Briefly, the fragment containing the polymorphism was amplified by PCR as previously described by Urlich *et al.* (23). As the presence of a 6bp insertion creates a Dral restriction site, the amplified fragments were digested with this enzyme and separated on 5% agarose gel.

LyghtCycler PCR for the quantification of TS expression. Total RNA was extracted from five 5-mm paraffin sections. The tumour area was scraped manually from the slide. Extraction was done using the High Pure RNA Paraffin Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. cDNA synthesis and subsequent PCR steps were performed using the LightCycler–TS mRNA quantification Kit (Roche). Briefly, cDNA was reverse transcribed from total RNA using AMV reverse transcriptase and specific primers for the TS gene as well as for the reference gene glucose-6-phosphate dehydrogenase (G6PDH). Afterwards, fragments of the TS cDNA and the G6PDH cDNA were amplified by PCR. The reaction mix with the solutions from the kit was prepared and the appropriate amount of template DNA, as well as the calibrator DNA provided with the kit, or PCR grade water as negative control, were added. The amplicons were detected in separate capillaries by using two specific pairs of fluorescence-labelled hybridization probes. The fluorescence signals were measured after each primer annealing step.

Calculation of the relative amount of TS mRNA compared to the reference gene was done using the LightCycler Relative Quantification Software (Roche). The final results were expressed as the ratio of TS/reference mRNA copies (T:R) in the samples relative to the T:R ratio in the calibrator DNA. The T:R ratio in the calibrator DNA has a value of one.

Immunohistochemical determination of TS. Three micron sections from formalin-fixed tissue blocks, dewaxed and rehydrated, were used. Antigen retrieval was carried out using Dako antigen retrieval fluid and microwaving at full power for a total of 15 minutes, in three 5-minute sessions, adding distilled water during the break between each microwaving. Sections were then treated with 3% hydrogen peroxidase for 5 minutes, followed by TS 106 (purchased from NeoMarkers) antibody to TS at a dilution 1:10 overnight at 4˚C. Visualization was obtained by incubation with anti-mouse rabbit and goat antibody (Dako) for 15 minutes, followed by a streptavidin-biotin peroxidase complex (Dako) for 15 minutes and, finally, Dako chromogen for 5 minutes. Mayer's haematoxylin was used to counterstain the sections. As a positive control, lymphoid germinal centre cells and normal mucous glands were used for TS.

The TS staining positivity was semi-quantitatively evaluated as follows:

Intensity of staining. A semi-quantitative score was adapted from 1+ to 4+ for positive cases: lack of staining was classified as 0.

Percentage of staining. A semi-quantitative score was adopted: 1 (less than 10% tumour cells stained) to 4+ (all cells stained). Lack of staining was classified as 0. The immunohistochemical assay was performed in 43 patients.

Statistical analysis. The relationship between the TS genotype regarding the 5'UTR polymorphism or the 3'UTR polymorphism and the MSI status was analysed using a contingency table analysis with Chi-squared test. To show any relationship between the quantification of the TS mRNA levels by LightCycler assay with the immunohistochemical one, the Mann-Whitney test was adopted. To investigate the relationships between TS genotype or MSI status and the TS expression as measured by LightCycler PCR, a Kruskal-Wallis test was performed followed by a Wilcoxon-Mann-Whitney test as appropriate. Calculations were performed using a commercially available computer program (JMP 5.0, SAS Institute Inc, Cary, NC, USA). p values < 0.05 were considered to be statistically significant.

Results

Polymorphism in the number of the TS repeat sequences in its 5' UTR and MSI status in human colorectal cancer. The TS genotype in 80 samples of DNA isolated from CRC cancer tissues using the PCR assay was analysed. Of those 80 cancer samples, 48 were MSI and 32 MSS. In the 5' UTR of the TS gene (Figure 1A) we found a PCR product of 144bp in those cancer samples homozygous for the triple repeat (3R/3R), a PCR product of 116bp in those homozygous for the double repeat (2R/2R), while both PCR products (144 and 116bp) were present in heterozygous cancer samples (2R/3R). In Table I is shown the rate of each genotype and its distribution into MSI and MSS cases. We did not find any relationship between the MSI status and the number of repeats in the 5'UTR ($p=0.22$).

Polymorphism in the number of TS repeat sequences in its 3' UTR and MSI status in human colorectal cancer. We evaluated the presence of the 6bp fragment in the 3'UTR region of the TS gene (Figure 1B) in 60 tumour cancer samples from patients bearing CRC (33 MSI and 27 MSS). The tumour samples homozygous for the 6bp insertion (+6bp/+6bp) displayed two fragments of 70 and 88 bp, tumour samples homozygous for the 6 bp deletion, displayed a 152 bp PCR product, while cancer heterozygous samples displayed a combination of the three fragments (70, 88 and 152 bp). In Table II is shown the rate of each genotype and its distribution to MSI and MSS cases. In the 3'UTR region we also did not find any association between the presence or absence of 6bp-insert and the MSI status ($p=0.55$).

Evaluation of the TS mRNA levels. We performed the TS mRNA levels from 80 CRC samples by means of real-time PCR on a LightCycler. We found different significant TS mRNA expression ($p<0.004$) in tumours with increasing copy-number of repeats (2R/2R *versus* 2R/3R *versus* 3R/3R). Moreover, we found significantly higher TS mRNA levels in the 2R/3R group ($p<0.026$) and in the 3R/3R group ($p<0.001$) compared with the 2R/2R group (Figure 2).

We did not find any relationship between the polymorphism of the 3' UTR and the TS mRNA levels ($p=0.65$) or between the TS mRNA expression with the MSS and MSI status ($p=0.56$).

Immunohistochemical determination of TS expression. We determined immunohistochemically the TS expression in 43 CRC samples (from the same patients in which the TS mRNA levels were evaluated by means of LightCycler (Table III).

Discussion

Genetic polymorphisms in the gene encoding TS have been shown to influence the response to 5-FU therapy. Multiple studies have demonstrated that both the TS mRNA and protein levels are inversely related to clinical antitumour response; indeed, survival of patients with advanced colorectal cancer is inferior if high TS expression is present (24, 25). The expression of TS is controlled, in part, by a polymorphism characterized by a multiple number of tandem repeats of a 28-bp sequence in the 5'-promoter enhancer region (TSER) of the gene.

There have been many studies which all suggest that patients homozygous for TSER*3 have a higher TS activity and a poorer response to 5-FU therapy than patients homozygous for TSER*2. In one study involving 65 rectal cancer patients, the probability of achieving tumour downstaging after radiation and 5-FU treatment was dependent on TSER genotype. TSER*2 carriers exhibited a 3.7-fold higher probability of achieving downstaging (a

measure of therapeutic success) when compared to TSER*3 homozygotes (26). Marsh *et al.* (20) also showed that TSER genotypes correlated with tumour response after 5-FU treatment in 24 metastasis colorectal cancer patients. This study showed that the TSER*2/TSER*2 genotype was nearly twice as common in the responders to chemotherapy compared to non-responders (40% *vs.* 22%, respectively), and also showed a decrease in median survival with increasing numbers of TSER repeats (median survival 16 months for TSER*2/TSER*2, 14 months for TSER*2/TSER*3, 12 months for TSER*3/TSER*3). This is confirmed by Pullarkat *et al.* (17) who showed that, in 50 patients receiving 5-FU for metastatic colorectal cancer, there was a higher response rate in patients with lower numbers of TSER repeats (50% for TSER*2/TSER*2, 15% for TSER*2/TSER*3 and 9% for TSER*3/TSER*3). These studies suggest that combined genotyping of TSER functional variants might be useful in selecting patients who are likely to tolerate and respond to 5-FU therapy. This is particularly important because of the availability of other active antitumour agents (*i.e.*, irinotecan, oxaliplatin), which can be used in combination with or in place of 5-FU if high TS is detected. In the present study, we therefore tested the hypothesis that there is an association between TS expression and MSI phenotype in colorectal carcinoma.

As described above, the TS expression depends on the genotype of the 5'UTR enhancer region of the corresponding gene. Recently, another polymorphism in the 3'UTR of the TS gene was described (18). Although the 3'end of the mRNA is non-coding, it may contain sequences determining mRNA stability or translational efficiency. Therefore, we investigated the frequency of both polymorphisms in 80 (60) samples of CRC of which 48 (33) showed microsatellite instability. The results show no significant correlation between the 5'UTR polymorphism and the MSI phenotype, nor between the 3'UTR polymorphism and the MSI phenotype.

To include unknown putative associations between MSI and TS, we investigated the correlation between TS mRNA level and MSI status by means of real-time quantitative PCR with the LightCycler System (Roche). Again, no significant correlation was seen.

Interestingly, we observed an overall higher mRNA level together with an increasing number of repeats in the 5'UTR. This finding was indepent of MSI status. Pullarkat *et al.* (17) also found the 28 bp tandem repeat to be associated with intratumoural TS mRNA levels. The mechanism of this regulatory effect is not yet clear. It has been suggested, by Marsh *et al.* (20), that the tandem repeats could form stem loop structures with an inverted repeat sequence located upstream from the enhancer region, thus allowing more efficient translation of TS mRNA. Our results suggest that an increased number of repeats in the enhancer region of the TS gene leads to enhanced cellular mRNA levels.

The major finding of our work was a lack of correlation between MSI and TS expression or TS polymorphisms. Our results suggest that the higher efficiency of 5-FU therapy in microsatellite unstable CRC is not associated with TS expression, but is due to other mechanisms which need further study for clarification, and that the evaluation of the TS expression by means of immunohistochemistry assay remains a safe and reliable method in CRC.

In accordance with others, we conclude that evaluating the TS genotype with regard to the 5'UTR or measuring intratumoural mRNA levels may become a useful tool to predict the response to 5-FU and to stratify patients for appropriate chemotherapy.

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Received March 24, 2004
Revised July 28, 2004
Accepted August 16, 2004