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Thymidylate synthase expression and genotype have no major impact on the clinical outcome of colorectal cancer patients treated with 5-fluorouracil

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ABSTRACT

Background and objectives: Thymidylate synthase (TS) expression levels appear to be related to response to 5-fluorouracil-(5-FU)-based chemotherapy in colorectal cancer (CRC) patients. Three polymorphisms have been proposed as modulators of TS expression: a tandemly repeated sequence (2R/3R) in the 5′ UTR, a SNP (G > C) within the 3R allele and a 6 bp deletion in the 3′ UTR.

To evaluate the influence of *TS* expression and polymorphisms on clinical outcome of 5-FU-treated patients we performed a comprehensive genetic analysis on 63 CRC patients.

Methods: TS expression levels were analyzed in normal and tumor tissues. *TS* coding sequence and UTR polymorphisms were investigated on DNA from normal tissue. LOH analysis was performed to determine tumor genotype.

Results: A difference in disease-free survival (DFS), although not statistically significant, was observed between high and low mRNA expression levels: patients with low levels showed longer DFS. The 2R2R genotype showed significantly lower expression than the 3R3R and 2R3R genotypes in normal tissue. No other *TS* polymorphism was associated with mRNA expression or clinical outcome.

Conclusions: The results obtained in this pilot study indicate that the number of 5′ UTR repeats is the major genetic determinant of TS expression. The lack of association with other polymorphisms might be partially explained by the existence of linkage disequilibrium in the *TS* gene. Our data support the growing evidence that TS control may require multiple mechanisms acting in close coordination with one another and suggest that *TS* genotyping alone in tumor samples is not sufficient to accurately predict response to 5-FU.

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1. Introduction

More than 50 years after its introduction into clinical practice, 5-fluorouracil (5-FU) is still a fundamental drug in the treatment of colorectal cancer (CRC) and many other tumors, either alone or in combination with other drugs [1,2]. Several variables associated with genes involved in the 5-FU metabolic pathway have been studied for their potential relationship with clinical outcome and response to chemotherapy. To date the most widely studied molecular marker is thymidylate synthase (TS), the biological target of 5-FU and related drugs. TS is the key enzyme of the *de novo* synthesis of deoxythymidine monophosphate (dTMP), that catalyzes the methylation of deoxyuridine monophosphate to dTMP [3,4], an essential step in DNA synthesis.

The expression level of the *TS* gene appears to be related to clinical outcome and response to 5-FU chemotherapy and has been suggested as a potential prognostic and/or predictive marker. Johnston et al. [5] first demonstrated a correlation between low *TS* levels and improved 5-year disease-free survival (DFS) and overall survival (OS) in rectal cancer patients receiving 5-FU adjuvant chemotherapy. A meta-analysis by Popat et al. [6] showed that CRC patients with advanced disease treated with TS inhibitors had a significantly better OS if they had low *TS* expression in primary tumors

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or metastases, whereas a predictive role of *TS* expression was not established for the adjuvant setting.

Three different polymorphisms in the *TS* untranslated regions (UTRs) have been proposed as modulators of *TS* mRNA transcriptional and translational efficiency. The 5′ UTR contains a variable number of 28 bp tandem repeats (VNTR) [7,8]. Although up to nine repeats have been described, the vast majority of *TS* alleles harbor either 2 or 3 repeats, creating genotypes defined as 2R/2R, 2R/3R and 3R/3R, respectively. The 3R alleles present a G > C single nucleotide polymorphism (SNP) at the 12th position of the second repeat [9]. The two alleles of this SNP are defined as 3RG and 3RC, respectively. The third polymorphism is a 6 bp insertion/deletion at nucleotide 1494 within the 3′ UTR [10]. In addition, a further SNP in the VNTR region, consisting of a C > G substitution in the first repeat of the 2R allele, has recently been described [11].

Many studies have been conducted to investigate whether *TS* genotypes might explain differences in mRNA expression levels, but the results are heterogeneous and even controversial.

In vitro experiments have shown that the 3R/3R genotype is associated with higher levels of *TS* gene expression than the 2R/2R genotype [8]. Subsequent experiments on DNA samples from CRC patients provided support to these results, indicating that the 3R sequence has greater transcriptional efficiency than the 2R allele [12,13]. The influence of the VNTR on *TS* expression has been ascribed to the presence of a USF family E-box consensus element in repeat units containing the G nucleotide at position 12. The G > C substitution eliminates the USF-1 binding site, thus abolishing the translation enhancer effect of the 3R allele [9]. Each of the first two repeats of the 3RG allele has a G at this position; therefore, 3RG alleles contain two potential USF-1 binding sites, whereas the G is present only in the first repeat of 2R and 3RC alleles, that consequently have a single USF-1 site.

The ins/del polymorphism within the 3′ UTR seems to modulate *TS* expression by affecting mRNA stability; the 6bpdel allele has been associated with decreased mRNA stability *in vitro* and lower intratumoral *TS* expression *in vivo* [14,15].

By contrast, other studies did not detect any correlation between *TS* polymorphisms and mRNA or protein expression levels [16–18], or even a significantly decreased *TS* mRNA expression in samples from patients with the 3R/3R genotype [19].

Discrepancies in results among different studies may be due to methodological differences or incomplete analysis leading to partial results. Some groups analyzed *TS* expression levels by immunohistochemistry (IHC) [14,15,20] while others used real time quantitative PCR [12,21,22]. In addition, loss of heterozygosity (LOH), that modifies *TS* genotype in tumor cells, has been reported to affect tumor response and survival and TS expression [21,23].

In order to take into account all major intrinsic factors potentially involved in the relationship between *TS* genotype, expression and clinical response to 5-FU, we performed a comprehensive pilot study; this involved investigation of the whole *TS* coding sequence, 5′ and 3′ UTR polymorphisms and mRNA expression in normal and matching tumor tissues of a series of CRC patients receiving treatment with 5-FU only. We analyzed possible associations between *TS* genotype and expression as well as those between these experimental data and survival parameters (DFS and OS). Additional associations between *TS* genotype, expression and clinical/pathological characteristics were explored.

2. Materials and methods

2.1. Tissue samples

Primary tumor and corresponding colonic mucosa explants obtained from 63 CRC patients at surgery were frozen in liquid nitrogen until molecular analysis. Normal colonic mucosa was taken at a distance of ~10 cm from the tumors. Immediately after resection, the tumor sample was divided into equal portions after washing and removal of necrotic tissues. Some specimens were fresh frozen in liquid nitrogen, and one portion was embedded in paraffin to confirm histologically that it was not significantly contaminated by normal or necrotic tissue, or lymphocytes.

All samples were collected before combination chemotherapy became standard practice both in the adjuvant setting and for advanced disease in CRC patients. Patients treated in the adjuvant setting received folinic acid and 5-FU according to the schedule described in the pooled analysis by the IMPACT investigators [24]. Patients treated for advanced disease received chemotherapy according to the Machover regimen [25]. In both cases high-dose folinic acid was administered.

Informed consent was obtained from all patients for the use of specimens and clinical/pathological data for research purposes according to the guidelines established by the local ethical committee.

2.2. Gene expression analysis

Total RNA was isolated from tumor and normal tissues using a Trizol RNA isolation kit with glass-fiber filter purification methodology (RiboPure kit, Ambion Inc., Austin, TX, USA). RNA concentration and purity were verified by a Gene Quant II spectrophometer (Pharmacia Biotech, Cambridge, UK).

cDNA was generated from 10 μg of total RNA using random primers and the M-MLV reverse transcriptase RNase H minus (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol.

Real-time polymerase chain reaction (RT-PCR) analysis was performed with the ABI PRISM 7900HT Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

A predesigned and validated gene-specific probe-based TaqMan Gene Expression Assay from Applied Biosystems (Foster City, CA, USA) was used for the *TS* gene. Reactions were performed using Taqman Fast Universal PCR Master Mix No AmpErase UNG; two to three replicates for each reaction were plated onto 96-well plates. The PCR program was 95 °C for 20 s and 40 cycles of 95 °C for 1 s and 60 °C for 20 s.

The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as endogenous reference for standardization (TaqMan Endogenous Control concentration-limited primer, Applied Biosystems, Foster City, CA, USA), and the Human Reference Total RNA (Stratagene, La Jolla, CA, USA) was used as a calibrator sample. The expression levels of *TS* mRNA were normalized to the endogenous reference and expressed relative to the calibrator as $2^{-\Delta\Delta CT}$, according to the comparative CT method [26]. A validation experiment was performed to demonstrate that the efficiencies of *TS* and reference gene amplifications were approximately equal, using a standard curve method with several dilutions of the cDNA calibrator sample.

2.3. TS genotyping

Genomic DNA extraction was performed using BIOROBOT EZ1 (Qiagen, Italy) and EZ1 DNA Tissue Kit (Qiagen, Italy) according to the manufacturer's protocol.

In order to detect rare coding sequence variations, genomic DNA samples were screened by denaturing high-performance liquid chromatography (dHPLC) on a Transgenomic Wave System (Transgenomic Co., Omaha, NE, USA). Primers and conditions used for PCR amplification and dHLPC analysis are available upon request.

Amplification of the 5′ UTR tract containing the VNTR and G > C SNP was performed as previously described [22]. PCR products were electrophoresed onto a 2.5% agarose gel. Amplification products of 213 and 241 bp were observed for the 2R and 3R alleles, respectively. Ten microliters of the amplification products from all samples were subsequently digested with the restriction enzyme *HaeIII*, which allows recognition of the G/C SNP in the 3R allele. *HaeIII* digestion also allows to investigate the C/G SNP located in the proximal repeat of the 2R allele. Digested PCR products were electrophoresed onto non-denaturing polyacrylamide gels.

For 1494del6 bp analysis, genomic DNA was amplified by PCR using the following primers: forward 5′-CAAATCTGAGG-GAGCTGAGT, reverse 5′-TGAGCAGATAAGTGGCAGTACA in a reaction containing 100 ng DNA, 1.5 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphates, 30 pmol of each primer and 1.25 U of Taq polymerase. Cycling conditions were: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s, for 35 cycles. The amplified fragments were digested with *DraI* and the products separated on a 2.5% agarose gel. The expected fragment sizes were 92 bp + 60 bp for the common allele (6bpins) and 146 bp for the rare one (6bpdel).

LOH was investigated by comparing genotypes of matched tumor and normal tissue from the same patient. Patients homozygous for the VNTR, G > C and 6 bp deletion polymorphisms were analyzed for other microsatellite markers located upstream and downstream of the *TS* gene region (*D18S170*, *D18S1140*, *D18S1372*, *D18S498*, *D18S59*). Primers were labelled with 6-FAM to allow detection of the amplified products by an ABI Prism 310 Genetic Analyser. LOH was determined by assessment of peak height ratios between tumor and constitutional alleles using the following formula:

$$\frac{\text{constitutional allele 2/constitutional allele 1}}{\text{tumor allele 2/tumor allele 1}}.$$

A ratio >1.5 indicated loss of allele 2, a ratio <0.5 indicated loss of allele 1, and a ratio between 0.51 and 1.49 indicated retention of both alleles.

LOH analysis could be performed on 60 out of 63 patients because of unavailability of two tumor DNA samples and due to the presence of microsatellite instability, that prevented LOH assessment, in a third sample. Samples showing LOH were defined as 2R/loss and 3R/loss to indicate the allele that was retained in tumor DNA.

Table 1
Number of USF-1 binding sites in *TS* 5′ UTR alleles and genotypes.

Genotype	No. of USF-1 binding sites[a]
2R/loss	1 (1/0)
2R/2R	2 (1/1)
2R/3RG	3 (1/2)
2R/3RC	2 (1/1)
3RC/loss	1 (1/0)
3RG/loss	2 (2/0)
3RG/3RC	3 (2/1)
3RG/3RG	4 (2/2)

[a] In brackets: numbers of USF-1 binding sites for each allele comprised in the genotype combination.

2.4. Statistical analysis

The correlations between *TS* mRNA expression in tumor and normal tissue and clinical/pathological characteristics (sex, histotype, tumor site, stage and grading) and genotypes were analyzed using ANOVA and *t*-test. The association between genotypes and clinical/pathological features was analyzed by the χ^2 test.

In order to evaluate the relationships between response to therapy and genotypes or pathological characteristics, patients were categorized as responders or non responders on the basis of their disease-free and disease recurrence status, respectively, and the χ^2 test was used.

The paired Student's *t*-test was used to analyze the correlation of *TS* gene expression between normal and tumor tissues.

5′ UTR *TS* genotypes were grouped according to the number of USF-1 binding sites (Table 1). Comparisons were performed between patients with 1–2 USF-1 *TS* binding sites and with 3–4 USF-1 *TS* binding sites for both normal and tumor tissue.

Genotypes of the 3′UTR were also considered, alone and in combination with 5′ genotypes. The presence of linkage disequilibrium (LD) between 5′ and 3′ UTR *TS* polymorphisms was investigated

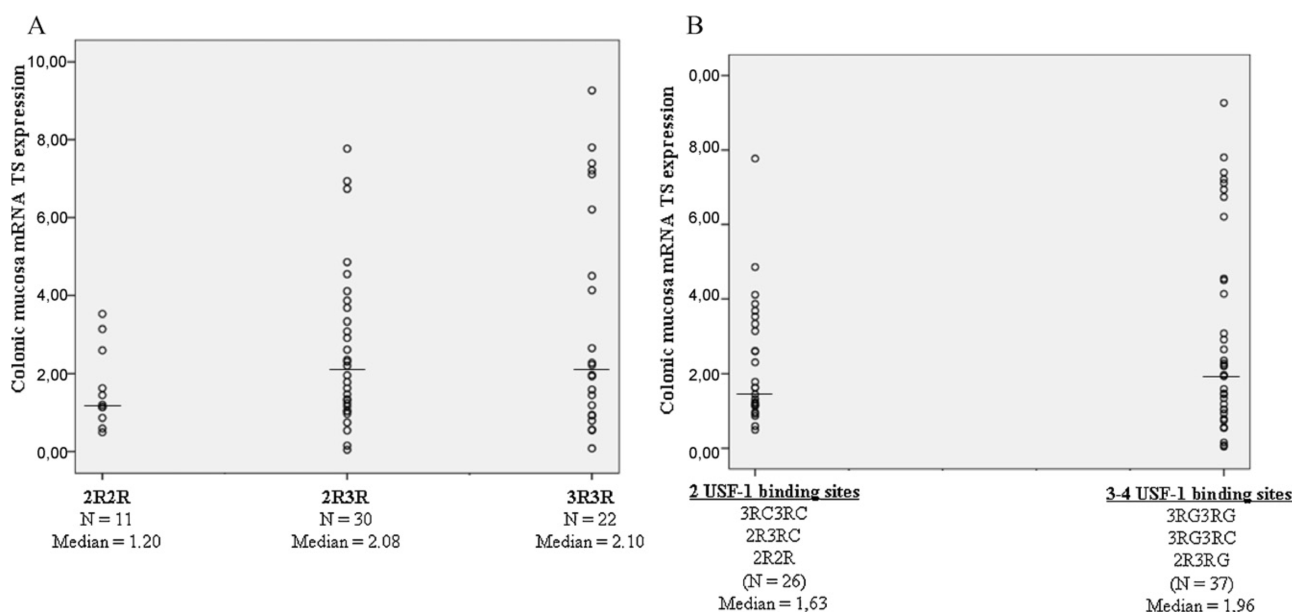

Fig. 1. Relationships between *TS* expression and 5′ UTR polymorphisms in normal tissue. (A) Relationship with VNTR number (2R2R vs 3R3R, $p = 0.020$; 2R2R vs 2R3R, $p = 0.049$; 2R3R vs 3R3R, $p = 0.322$); (B) Relationship with complete 5′ UTR haplotype (VNTR number and G > C SNP; 2 USF-1 binding site vs 3–4 USF-1 binding sites, $p = 0.258$); —, median value.

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Table 2
Main clinical/pathological features of colorectal cancer patients.

No. of patients	63
Age	
Median value	61
Range	23–76
Sex	
M	32
F	31
Stage (AJCC)	
II	27
III	25
IV	11
Grading	
G2	52
G3	11
Histotype	
Adenocarcinoma	58
Colloid	5
Site of primary tumors	
Left colon	23
Transverse colon	6
Right colon	9
Rectum	25
Type of 5-FU chemotherapy	
Adjuvant	52
Palliative	11

Table 3
Constitutional and tumor *TS* genotypes in colorectal cancer patients.

	Constitutional genotype (%)[a]	Tumor genotype (%)[b]
5′ UTR		
2R/LOH	–	9 (*15*)
2R2R	11 (*17*)	9 (*15*)
2R3R	30 (*48*)	12(*20*)
2R3RC	14	7
2R3RG	16	5
3R/LOH	–	20 (*33*)
3RC	–	8
3RG	–	12
3R3R	22 (*35*)	10 (*17*)
3RG3RG	13	5
3RG3RC	8	5
3RC3RC	1	
3′ UTR		
6bpdel/LOH	–	12 (*20*)
6bpins/LOH	–	17 (*28*)
6bpdel/6bpdel	15 (*24*)	7 (*12*)
6bpins/6bpins	22 (*35*)	12 (*20*)
6bpdel/6bpins	26 (*41*)	12 (*20*)

[a] $n = 63$.
[b] $n = 60$ (tumor genotype was not analyzed in 3 patients due to unavailability of DNA from tumor tissue).

using MIDAS software [27]; D′ values >0 indicate the presence of LD.

Patients were divided into high and low *TS* mRNA level groups using the median value as a cut-off. Patients who died due to causes unrelated to colorectal cancer were excluded. OS was calculated from surgery to the date of last follow-up or death, DFS from surgery to the first evidence of disease. Median follow-up time was computed for all patients alive at the time of analysis. Survival curves were estimated by the Kaplan Meier method and compared with the log-rank test.

Analyses were carried out using the SPSS version 15 Software. P values <0.05 were considered significant.

3. Results

Clinical/pathological characteristics of the patients investigated are reported in Table 2. The series was comprised of 32 males and 31 females, with a median age at diagnosis of 61 years (range 23–76). All patients received 5-FU chemotherapy: 52 as adjuvant and 11 as palliation.

Interindividual variation in *TS* mRNA expression was 25.5 (*TS*/*GAPDH* ratios: 0.35–8.93; median value 1.53) and 231.5 fold (*TS*/*GAPDH* ratios: 0.04–9.26; median value 1.93) in tumor and normal tissue, respectively (data not shown). However, overall, mean *TS* mRNA expression was not significantly different between tumor and normal tissue ($p = 0.076$, mean ± SD: 1.96 ± 1.82 vs 2.67 ± 2.27, respectively).

Genotype distributions of the VNTR, G > C SNP, ins/del 6 bp polymorphisms and their combinations in tumor or normal tissue are shown in Table 3. LOH was found in 29/60 (48%) samples. The recently described C > G SNP in the first repeat of the 2R allele was absent in this series. Genotype frequencies of the 5′ VNTR and 3′ 6 bp deletion polymorphisms were within Hardy–Weinberg equilibrium (data not shown). Analysis of allelic combinations at these sites showed the presence of LD: the 3RG allele was associated with the 6bpdel allele in the 3′ UTR (D′ = 0.68) and, on the other hand, the 2R allele was associated with absence of 6bpdel (D′ = 0.67).

No sequence variation was found within the *TS* coding sequence by extensive dHPLC screening.

The VNTR genotype was associated with *TS* expression levels in normal tissues, with the 2R2R genotype showing significantly lower mRNA expression than the 3R3R and 2R3R genotypes ($p = 0.020$ and 0.049, respectively; Fig. 1a). However, there was no significant association in normal colonic mucosa between *TS* expression and the complete 5′UTR genotypes (VNTR combined with the G > C SNP), when these were divided into 2 groups corresponding to the presence of 2 and 3–4 USF-1 binding sites, respectively (Fig. 1b).

No other relationship was observed between genotype and *TS* expression levels either in normal or tumor tissue. In particular, *TS* expression levels were similar between tumor samples with 1–2 and 3–4 USF-1 binding sites (Fig. 2).

Although not significant, a difference in DFS was observed between high and low tumor *TS* mRNA expression levels in the group of patients with completely resected tumors (Fig. 3): patients with low *TS* mRNA levels had a longer DFS ($p = 0.122$). On the other

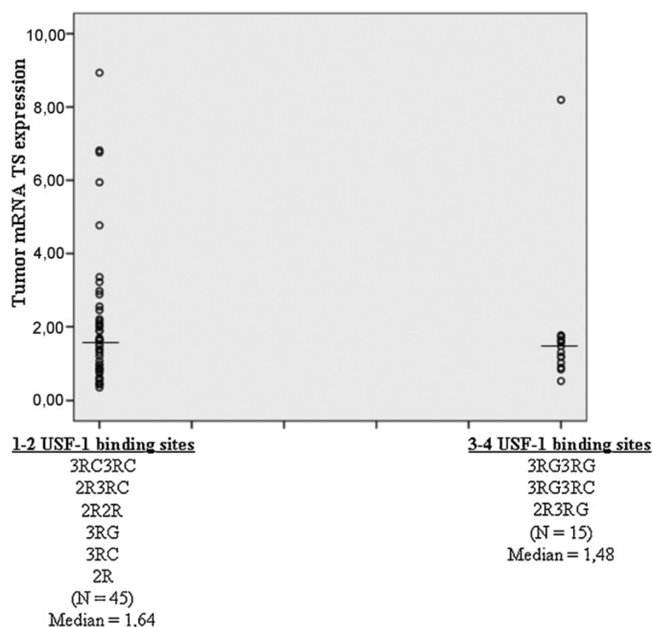

Fig. 2. Relationship between *TS* expression and 5′ UTR genotype (VNTR and G > C SNP) in tumor tissues (1–2 USF-1 binding site vs 3–4 USF-1 binding sites, $p = 0.626$); —, median value.

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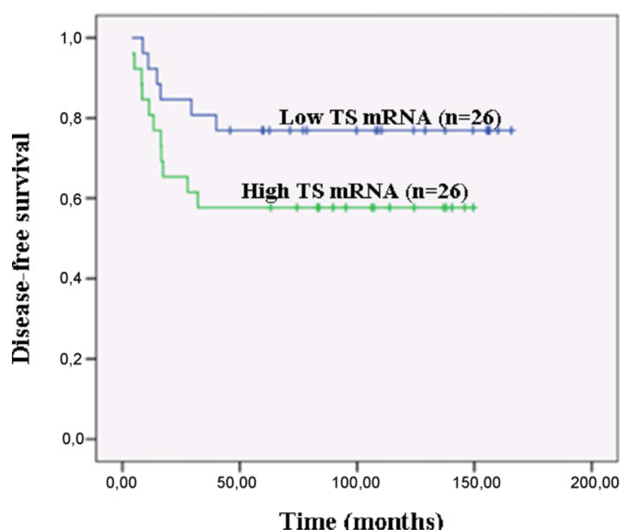

Fig. 3. Survival parameters of patients who received 5-FU chemotherapy according to *TS* gene expression levels. Low *TS* mRNA expression <1.53 (median value); high *TS* mRNA expression >1.53 (median value); $n = 52$, $p = 0.122$.

hand, no difference was observed between survival parameters (OS and DFS) and *TS* expression in normal tissue (data not shown).

Combined analysis of *TS* VNTR and G > C genotypes both in normal and tumor tissue showed that completely resected patients ($n = 52$) with 1–2 USF-1 binding sites had a prolonged survival compared to patients with 3–4 USF-1 binding sites. However this difference was not statistically significant (Fig. 4). Similarly, no significant difference was observed when OS was evaluated in the entire case series ($n = 63$) (data not shown).

Evaluation of *TS* 3′ UTR polymorphism alone or in combination with the 5′ UTR genotype did not show any significant difference in relation to survival parameters (data not shown).

No relationship was observed between clinical/pathological features and *TS* expression levels either in normal or tumor tissues. Likewise, no correlation between allele and genotype frequencies in the 5′ or 3′ UTR, including repeat number in the VNTR, G > C SNP and 6 bp deletion polymorphisms, and clinical/pathological features were observed (data not shown).

Finally, no association between clinical features/*TS* genotypes/*TS* expression and response to therapy was observed, with the exception of a statistically significant value ($p = 0.007$) between Duke's stage and response: patients with Duke's stage B had a lower incidence of disease recurrence (47.8%) compared to stage C patients (69.3%).

4. Discussion

In the present study we have extended our previous analysis of the relationship between *TS* genotype, *TS* mRNA levels, and response to 5-FU treatment in CRC patients [22]. To this purpose, we investigated expression and genotypes in both normal colonic mucosa and tumor tissue and we analyzed further genetic variables, including the whole *TS* coding sequence in constitutional DNA, LOH in the *TS* region, 3′ UTR polymorphisms, and estimate of LD.

Overall, no significant correlations between *TS* alleles, genotypes or expression, and clinical parameters, including response to 5-FU, were observed. Although the difference was not statistically significant, results on the relation between survival and *TS* expression are in keeping with those obtained in our previous study [22] as well as by other authors [5,12,28,29]: patients with low *TS* expression levels tend to have a prolonged DFS compared to those with high *TS* expression levels in tumor tissue.

Since both *in vitro* and *in vivo* data indicate that intragenic polymorphisms may influence *TS* expression levels [8,9,12,30], we investigated potential genotype/mRNA correlations in this series. Among the different intragenic *TS* variants analyzed, a significant association was only found between 5′ UTR 28 bp repeat number and *TS* mRNA expression in normal, but not in tumor, tissue. The intrarepeat G > C SNP apparently did not have a major influence on the effects of repeat numbers, since no difference was observed when complete 5′ UTR genotypes were assessed against expression, clinical characteristics, survival and response to 5-FU treatment.

Since the influence of the VNTR on *TS* expression has been attributed to the presence of one USF family E-box consensus element in repeat units containing the G nucleotide, we classified *TS* 5′ UTR genotypes according to the number of USF-1 binding sites. The absence of a significant correlation between complete 5′ UTR genotype and clinical parameters indicates that the number of USF-1 sites does not have a major influence on survival.

These findings suggest that the number of repeats could be more important than their sequence differences for the regulation of *TS* mRNA expression *in vivo*. Other authors reported similar results, confirmed both at the RNA and protein level, on CRC tissue samples [31]. On the other hand, a positive correlation between TS protein expression/activity and the 3RG allele in normal mucosa has also been observed [32]. This latter observation is consistent with the experimental results that provided original evidence for a role of the G > C SNP on *TS* transcriptional efficiency [9]. It has also recently been suggested that the position of the G nucleotide in the repeat

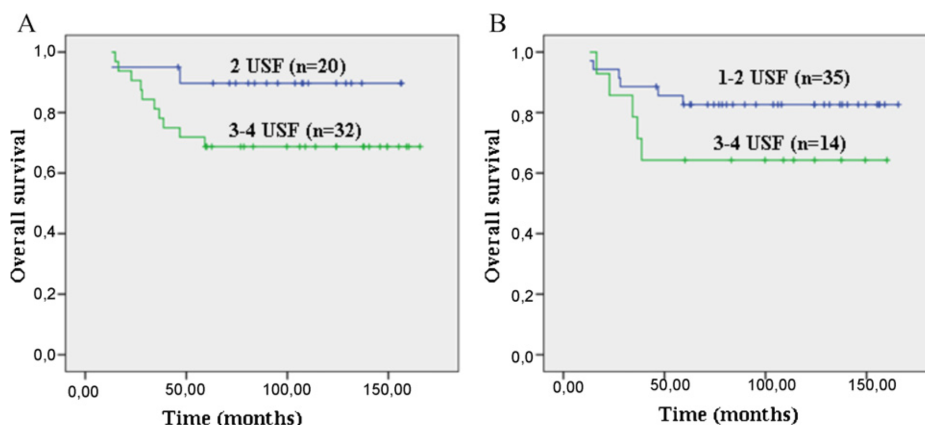

Fig. 4. Overall survival according to the number of USF binding sites in the *TS* 5′UTR observed in constitutional ($n = 52$, $p = 0.090$) (A) and tumor genotypes (including LOH data; $n = 49$, $p = 0.162$) (B) of CRC patients.

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cluster may be important for transcriptional efficiency: its location in the most 5′ repeat has been found to be associated with high expression levels, regardless of the presence of additional G-containing repeats [33]. However, while *in vitro* studies are very important to understand the pathophysiological mechanisms of *TS* regulation, they may not reflect more complex *in vivo* conditions, since the regulation of *TS* expression is likely dependent on multiple *cis* and *trans* factors.

The apparently contrasting results on the effects of VNTR repeat number and *TS* mRNA expression obtained in mucosa and tumor samples could be related to the occurrence of somatic mutations and epigenetic alterations involving *TS* as well as additional loci implicated in the control of *TS* expression in tumor cells. Deletions of chromosome 18 are a frequent event in colorectal carcinogenesis and, when they involve the *TS* locus, they can cause a reduction in its expression levels. So far, most studies analyzing *TS* prognostic and predictive role have not considered the possible occurrence of LOH in tumor cells. In addition, while most previous studies limited LOH analysis to cases that were heterozygous for one of the three *TS* intragenic polymorphisms [17,21,23,34], we investigated flanking extragenic polymorphisms in order to increase the number of informative samples. Overall, the results obtained indicate that LOH alone cannot account for the different correlations between genotype and RNA expression observed in normal and tumor tissue, and that other factors are implicated in the control of *TS* expression in CRC cells.

The lack of any association between individual and combined *TS* polymorphisms and clinical outcome following 5-FU treatment in this series of CRC patients is in accordance with the results of recent studies [35,36]. Other studies have reported contrasting results on the clinical significance of the three common UTR *TS* polymorphisms [16,17,37,38]. It should be considered that some authors assign high and low expression levels to the 3R3R and 2R2R genotype, respectively, on the basis of previously published data [35–38]. However, these assignments may not be correct, since we, as well as other authors [16,17], have observed that *TS* expression levels do not correlate with *TS* genotype.

In general, expression and survival analyses in relation to genotype are complicated by the presence of multiple *TS* polymorphisms that occur in different haplotype combinations containing alleles with either synergic or opposite consequences on *TS* mRNA levels. The existence of LD, documented by us and other authors [14,35], may partially explain the discrepancies between results obtained *in vitro* and on clinical samples. Since the 3RG allele is associated with the 3′ 6 bp deletion, that is thought to reduce mRNA stability, the overall effect should depend on the interaction between these variants. The same applies to other *TS* haplotype combinations.

Rare DNA variants in the *TS* coding sequence may also influence the effects of the UTR polymorphisms [39]. However, none of the patients included in this study, that is the first one to screen the whole *TS* coding sequence for prognostic purposes, showed alterations.

Furthermore, *TS* transcription and translation are likely influenced by other genes, whose sequence (e.g. *p53*) [40] and expression (e.g. *AEG-1*) [41] can be altered in tumor tissues. It has been suggested that the *p53* status could play a role in *TS* expression in tumor cells, by altering transcription and/or translation levels [40]. It has also been shown that astrocyte elevated gene-1 (*AEG-1*), known to augment invasion, metastasis and angiogenesis, directly contributes to 5-FU resistance, since it induces the expression of *LSF* (late SV40 factor), a transcription factor that regulates the expression of *TS* [41].

The TS protein has also been shown to inhibit the translation of *TS* mRNA in an autoregulatory manner [42], suggesting that *TS* genotyping alone is not sufficient to accurately predict response to 5-FU. It is also likely that other genes coding for enzymes or proteins involved in the mechanism of action and in the inactivating or activating metabolism of 5-FU are involved in the outcome of colorectal cancer patients treated with 5-FU [43].

It has also been observed that an increase in the intracellular pool of reduced folates following exposure to folinic acid enhances 5-FU antitumor activity [44,45]. This is due to an increased inhibition of TS enzyme activity via the formation of a covalent ternary complex among the active 5-FU metabolite 5-fluoro-2'-deoxyuridine-5'-monophosphate, TS and the 5,10-methylene tetrahydrofolate cofactor [3,4,46]. This ultimately leads to greater sensitivity to 5-FU [47] and improves response rate and overall survival in colorectal cancer patients compared with 5-FU alone [48]. Different size and composition of cellular folate cofactor pools may thus be another important factor of variability to 5-FU treatment. The use of high-dose folinic acid in our case series [24,25] potentially allowed maximal enhancement of clinical 5-FU antitumor activity. However, interindividual variability in the size and composition of cellular folate pools, possibly mediated by polymorphisms of folate transporters or metabolic enzymes (e.g. *MTHFR*) [49] may have occurred in our series. This aspect was not studied and warrants further investigation on larger series.

In conclusion, we did not find significant correlations for most parameters evaluated in this study, despite comprehensive analysis of *TS* gene variants and RNA expression in both normal colonic and tumor tissue. However, due to the relatively limited sample size, weak effects of additional genotypic variations cannot be completely excluded. Further investigations on larger sample series are needed to clarify whether TS has a relevant role in determining response to FU treatment and can be used in the clinical setting. Methodological issues related to quantitation of TS RNA, protein expression and enzyme activity, as well as the potential involvement of additional constitutional and acquired genetic factors will also need to be addressed.

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