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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ARTICLE INFO

Article history:
Received 16 February 2011
Received in revised form 12 April 2011
Accepted 14 April 2011

Keywords:
Colorectal cancer
Thymidylate synthase
mRNA expression
Genotype
5-Fluorouracil

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 deoxynucleoside monophosphate (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 colorectal 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 may 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 folic 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 folic 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 spectrophotometer (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 Biosystem, 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 \(2^{-ΔΔCt}\), 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 dHPLC 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′-TGAGCAGATAAGTGGCAGTACA in a reaction containing 100 ng DNA, 1.5 mM MgCl2, 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 6bp deletion polymorphisms were analyzed for other microsatellite markers located upstream and downstream of the TS gene region (D18S170, D18S1140, D18S1372, D18S548, 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:

\[
\frac{\text{constitutional allele 2}}{\text{constitutional allele 1}} \quad \text{tumor allele 2} = \frac{\text{tumor allele 1}}{1}
\]

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.

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 \( \chi^2 \) 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 \( \chi^2 \) 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 5’TS binding sites and with 3–4 USF-1 5’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.

### Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of USF-1 binding sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>2R/loss</td>
<td>1 (1/0)</td>
</tr>
<tr>
<td>2R/2R</td>
<td>2 (1/1)</td>
</tr>
<tr>
<td>2R/3RC</td>
<td>3 (1/2)</td>
</tr>
<tr>
<td>2R/3RC</td>
<td>2 (1/1)</td>
</tr>
<tr>
<td>3RC/loss</td>
<td>1 (1/0)</td>
</tr>
<tr>
<td>3RC/3R</td>
<td>2 (2/0)</td>
</tr>
<tr>
<td>3RG/3RC</td>
<td>3 (2/1)</td>
</tr>
<tr>
<td>3RG/3RG</td>
<td>4 (2/2)</td>
</tr>
</tbody>
</table>

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

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 sites vs 3–4 USF-1 binding sites, \( p = 0.258 \)); --, median value.

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 palliative.

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 hand, the presence of 2 and 3–4 USF-1 binding sites in the tumor was associated with absence of 6bpdel ($D' = 0.67$).

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.

<table>
<thead>
<tr>
<th>Constitutional genotype (%)</th>
<th>Tumor genotype (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' UTR</td>
<td></td>
</tr>
<tr>
<td>2R/LOH –</td>
<td>9 (15)</td>
</tr>
<tr>
<td>2R2R 11 (17)</td>
<td>9 (15)</td>
</tr>
<tr>
<td>2R3R 30 (48)</td>
<td>12 (20)</td>
</tr>
<tr>
<td>2R3RC 14</td>
<td>7</td>
</tr>
<tr>
<td>2R3RG 16</td>
<td>5</td>
</tr>
<tr>
<td>3R/LOH –</td>
<td>20 (33)</td>
</tr>
<tr>
<td>3RC –</td>
<td>8</td>
</tr>
<tr>
<td>3RG –</td>
<td>12</td>
</tr>
<tr>
<td>3R3R 22 (35)</td>
<td>10 (17)</td>
</tr>
<tr>
<td>3R3RG/3R     13</td>
<td>5</td>
</tr>
<tr>
<td>3R3RC/3R     8</td>
<td>5</td>
</tr>
<tr>
<td>3R3RC/3R     1</td>
<td></td>
</tr>
<tr>
<td>3' UTR</td>
<td></td>
</tr>
<tr>
<td>6bpdel/LOH –</td>
<td>12 (20)</td>
</tr>
<tr>
<td>6bpins/LOH –</td>
<td>17 (28)</td>
</tr>
<tr>
<td>6bpdel/6bpdel –</td>
<td>12 (20)</td>
</tr>
<tr>
<td>6bpins/6bpins –</td>
<td>7 (12)</td>
</tr>
<tr>
<td>6bpdel/6bpins –</td>
<td>12 (20)</td>
</tr>
</tbody>
</table>

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

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.
features were observed (data not shown).

Overall survival according to the number of USF binding sites in the T5 gene (Fig. 3). The survival figures were plotted using the Kaplan Meier method. Comparison of survival was done by the log rank test, and a p value of 0.05 or less was considered significant. Survival was assessed by making comparisons among patients with different number of USF-1 binding sites. Similarly, no significant difference was observed when OS was evaluated in the entire case series (n = 63) (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/T5 genotypes/T5 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 T5 genotype, T5 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 T5 coding sequence in constitutional DNA, LOH in the T5 region, 3’ UTR polymorphisms, and estimate of LD.

Overall, no significant correlations between T5 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 T5 variants analyzed, a significant association was only found between 5’ UTR 28 bp repeat number and T5 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 were observed. 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 T5 3’ UTR genotypes according to the number of USF-1 binding sites. The absence of a significant correlation between complete 3’ 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 T5 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. 3](image-url) Survival parameters of patients who received 5-FU chemotherapy according to T5 gene expression levels. Low T5 mRNA expression <1.53 (median value); high T5 mRNA expression >1.53 (median value); n = 52, p = 0.122.

![Fig. 4](image-url) Overall survival according to the number of USF binding sites in the T5 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.

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 observed 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′ 6bp 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 folic 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 [3,46]. 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 folic 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 outcome of patients to 5-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.

Acknowledgements

Supported by a grant from the University of Florence (ex 60%) to MG, a grant from Ministero dell’Istruzione, dell’Università e della Ricerca, Rome (PRIN 2005) to TM, and by contributions of Ente Cassa di Risparmio di Firenze to Fiorgen, MG and EM, of Associazione Italiana per la Ricerca sul Cancro, Milan to EM and of Gruppo Oncologico Chirurgico Cooperativo Italiano, Florence to EM.

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