Thymidylate Synthase Messenger RNA Expression in Plasma from Patients with Colon Cancer: Prognostic Potential

Vanessa Garcia, José M. García, Cristina Peña, Javier Silva, Gemma Domínguez, Alicia Hurtado, Isabel Alonso, Rufo Rodríguez, Mariano Provencio, and Félix Bonilla

Abstract

Purpose: Thymidylate synthase (TS), a critical target in fluorouracil-based chemotherapy, is a prognostic marker in colon carcinomas and a predictor of response to treatment. Tumor RNA has been detected in plasma from cancer patients and is associated with poor prognosis. This is the first study to examine extracellular TS mRNA in plasma from patients with colon carcinoma, and its possible relation with TS promoter enhancer region (TSER) polymorphism.

Experimental Design: TS expression was measured in plasma from 88 patients and 26 controls, and in a tumor subgroup of this series by quantitative PCR. Genotyping for TSER polymorphism was done in 60 patients. Clinicopathologic variables were correlated with these molecular changes.

Results: TS mRNA was detected in plasma in 47% of patients, showing significant differences from healthy controls. Patients with TS mRNA in plasma had higher levels of TS in tumor tissue than patients without. The presence of TS mRNA was associated with lymph node metastases and more advanced stages. Polymorphism TSER 3/3 was found in 38% of cases, and was significantly correlated with high amounts of TS mRNA in plasma.

Conclusions: Our results suggest that TS mRNA in plasma originated from tumors, it may indicate poor prognosis and might help to classify tumors in Dukes’ stages B and C. The TSER genotype may influence TS mRNA expression in plasma.

Colon carcinoma is the second most commonly diagnosed cancer and the second cause of death from cancer in developed countries. For many years, 5-fluorouracil (5-FU) has been used to treat colon carcinoma, in combination with oxaliplatin, and others. Several studies have examined the role of chemotherapeutic target enzymes such as thymidylate synthase (TS; refs. 1–3). TS converts 2’-deoxyuridine 5’-monophosphate to thymidine 5’-monophosphate, which is required for DNA synthesis and repair. It is an important target for fluoropyrimidines such as 5-FU and folate-based inhibitors. However, their mechanisms of inhibition are different. The inhibition of DNA synthesis by 5-FU is based on its conversion to 5-fluoro-2’-deoxyuridine 5’-monophosphate, which binds covalently to TS with a folate cofactor, forming a ternary complex that blocks the 2’-deoxyuridine 5’-monophosphate binding site and inhibits thymidine 5’-monophosphate synthesis (4–7), whereas antifolates block the folate-binding site in TS (8–10).

Multiple studies have shown the importance of the relative levels of TS as a determinant of cellular sensitivity to 5-FU (11–15). In addition, patients with higher levels of TS in tumors have significantly worse clinical outcome, irrespective of their response to treatment (16–19). However, there have been some studies which failed to show these associations (18, 20).

In conclusion, TS may be a predictor of response to 5-FU-based therapies, as well as being a prognostic marker (1).

A tandem repeat sequence in the TS promoter enhancer region (TSER) is associated with alterations in TS mRNA and protein levels (21). TS gene expression is significantly higher in tumors with three/three-repeat sequence (TSER 3/3) than in those with TSER 2/2 and TSER 2/3 (22–24), and this higher expression might affect the response to treatment and clinical outcome (25, 26).

Early detection of colon carcinoma could improve survival (27), and molecular techniques and new methods to detect metastatic or recurrent disease in preclinical or presymptomatic phases of the disease may contribute to this strategy. However, many methods have limited sensitivity or specificity, or entail invasive procedures (28). Several studies have detected increased concentrations of circulating nucleic acids in the plasma or serum of cancer patients, and higher levels in patients with metastases than in those with localized disease (29, 30). DNA alterations in the tumors such as mutations of oncogenes or tumor suppressor genes, deletions, microsatellite alterations, and hypermethylation of promoters, have been found in genetic material from the plasma of patients with various types of cancer (31–34). Several studies have reported an association between tumor-related plasma DNA and poor
prognosis, suggesting their use as prognostic marker (35–38). Moreover, several types of tumor-associated mRNA in plasma, such as tyrosinase and telomerase, have also been detected in cancers such as melanoma, breast, colon, and nasopharyngeal cancers (39–45). mRNA-based amplification methods could offer greater specificity and sensitivity. In a previous study, we reported a correlation between circulating tumor cells and circulating tumor mRNA in colon cancer (43), and found that mRNA is more sensitive than DNA in the plasma of patients with breast cancer (46). Additionally, the association between tumor mRNA in plasma and pathologic variables compatible with more aggressive tumors, was also observed (41, 43, 44). Therefore, the presence of tumor RNA in plasma could allow the development of noninvasive diagnostic and prognostic tools for use during the follow-up of patients who have undergone surgery.

On the basis of such evidence, we designed the present study to investigate: (a) the presence at diagnosis of detectable TS mRNA in plasma from patients with colon carcinoma, (b) whether the levels of TS expression in plasma are associated with the TSER genotype, (c) the possible correlation between the presence of this mRNA in plasma and specific pathologic variables of the primary tumors, and (d) possible association between high amounts of TS mRNA in plasma and poor prognosis. We used real-time PCR to measure TS because it is a sensitive, precise, and rapid technique (47, 48), and could enhance the sensitivity of the detection of tumor-derived RNA in patients’ plasma (49).

**Materials and Methods**

**Plasma samples and clinicopathologic variables.** Informed consent was obtained from all participants following an explanation of the nature of the study, as approved by the research ethics board of our hospital. All patients were considered sporadic cases on the basis that no clinical antecedents of familial adenomatous polyposis were reported, and those that met the clinical criteria for hereditary nonpolyposis colon cancer (Amsterdam criteria) were excluded. Between March 2002 and September 2004, blood samples (20 mL) were taken from 90 patients with colorectal carcinoma by venipuncture before intervention on the day of surgery. Blood samples from 27 healthy blood donors were also obtained at the hematology unit of our hospital. Plasma was prepared by centrifugation of peripheral blood at 2,500 rpm for 25 minutes and divided into aliquots, which were snap-frozen at ~80°C until processing. Samples of tumor and normal tissue from 60 patients of our series were obtained sequentially immediately after surgery, snap-frozen in liquid nitrogen, and stored at –80°C until processing (38 patients) or fixed in 10% formalin and processed by paraffin embedding (22 patients). All tumors were examined histologically to confirm the diagnosis of adenocarcinoma, establish the pathologic stage, and select the areas that contained at least 75% tumor cells.

The following variables were obtained from the medical records of the 90 patients: age, sex, tumor site, lymph node metastases, pathologic stage, histologic grade, vascular invasion, and evidence of polyps (defined by the presence of polyps in the surgical sample). Pathologic stage was assessed using Dukes’ classification.

**Patient characteristics.** We examined 90 patients with colorectal carcinomas, consisting of 37 women (41%) and 53 men (59%), in which 94% of patients were >50 years old. Most of the tumors were intermediate stages: 6 cases were classified as Dukes’ A, 50 were Dukes’ B, 32 were Dukes’ C, and 2 were Dukes’ D. Forty-one specimens were classified as well-differentiated, 41 as moderately differentiated, and 8 as poorly differentiated. The follow-up of our series had a median of 24 months (range, 9–39 months).

**Nucleic acids isolation.** Plasma mRNA was obtained from 1 mL of sample by Dynabeads mRNA DIRECT Kit. Plasma was incubated with 100 μL of Dynabeads Oligo (dT) for 10 minutes at room temperature. mRNA was eluted in 10 mmol/L Tris-HCl. For isolation of nucleic acids from tissues, RNA was extracted from ~30 mg of tumor and corresponding normal samples by RNeasy mini kit (Qiagen Inc., Hilden, Germany), and DNA was extracted from snap-frozen tissue using a nonorganic method (S-4520 kit; Oncor Inc., Gaithersburg, MD) and from paraffin-embedded tissues using chelating resin. The DNA and RNA extracted were quantified spectrophotometrically.

**Real-time PCR.** TS mRNA levels was normalized using two reference housekeeping genes, succinate dehydrogenase complex subunit A (SDHA) and ubiquitin C (UBC), in each sample, and calculating the geometric average as described by Vandesompele et al. (50). The relative concentrations of the target and the reference genes were calculated by interpolation using a standard curve of each gene plotted from the same serial dilution of cDNA from tumor tissue. For tissues, we calculated the expression level of target gene in patients as the ratio: target in normal tissue / target in tumor tissue [R (N / T)]. For the synthesis of first-strand cDNA, mRNA was reverse-transcribed using the Gold RNA PCR core kit (PE Biosystems), according to the manufacturer’s instructions. Random hexamers were used as primers for cDNA synthesis. Although DNA does not bind to the beads, we designed specific intron-spanning primers for real-time PCR to avoid possible contamination.

Real-time PCR was done in a Light-Cycler apparatus (Roche Diagnostics, Mannheim, Germany) using the LightCycler-FastStart DNA MasterPLUS SYBR Green 1 kit (Roche Diagnostics) according to the manufacturer’s instructions. The primers and annealing temperatures are shown in Table 1. At the end of the PCR cycles, melting curve analyses, and electrophoresis of the products on nondenaturing 8% polyacrylamide gels together with a molecular weight marker were done to confirm the generation of the specific PCR product expected. The

<table>
<thead>
<tr>
<th>Primmers</th>
<th>Sequence</th>
<th>Annealing temperature (°C)</th>
</tr>
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<tbody>
<tr>
<td>TS</td>
<td>5’CCAGTGGAGGCTTTTGGG3’F</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>5’CGTCAAGGTTGTTGTTATGAG3’R</td>
<td></td>
</tr>
<tr>
<td>UBC</td>
<td>5’ATTGCGTGCGGTTTCTG3’F</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>5’TGCCTTGGACATTCTCGATG3’R</td>
<td></td>
</tr>
<tr>
<td>SDHA</td>
<td>5’TGGGAAACAGGGAGCTCG3’F</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>5’CCACCACTGACATCAAATCAG3’R</td>
<td></td>
</tr>
<tr>
<td>TS-TSER</td>
<td>5’AAAAGGCGGCGGGAAGGGG3’T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’TCCGAGCGGCGCACAGGCG3’R</td>
<td>65</td>
</tr>
</tbody>
</table>
Allelic band on the gels was detected by nonradioisotopic means using a commercially available silver staining method (51). The bands were sequenced in an ABI Prism 377 DNA sequencer apparatus (PE Applied Biosystems, Foster City, CA).

**Polymerase chain reaction.** PCR was done in a final volume of 25 μL using 0.2 units of Taq Gold DNA polymerase and 1× PCR buffer (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ), 200 μmol/L deoxynucleotide triphosphates, 30 pmol of each primer (Table 1), 2 mmol/L MgCl2, and 10% DMSO. PCR was carried out in a thermocycler (Perkin-Elmer, Cetus, Foster City, CA); each sample was denatured at 94°C for 11 minutes and subjected to 30 cycles (denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and elongation at 72°C for 30 seconds) followed by a final 12-minute extension at 72°C. The amplified products were mixed with 6 μL of loading buffer (0.02% xylene cyanol and 0.02% bromophenol blue), and run on nondenaturing 8% polyacrylamide gels for 2 hours at 500 V. After gel electrophoresis, the allelic band was detected by a nonradioisotopic technique using a commercially available silver staining method (51). The number of repeats in representative bands was determined by DNA sequencing in an ABI Prism 377 DNA sequencer apparatus (PE Applied Biosystems).

**Data analysis.** The TS expression data in plasma were not normally distributed (Kolmogorov-Smirnov test), hence, the geometric average, instead of the arithmetic, was used to describe the gene expression, and the Kruskal-Wallis test was applied. Data distribution of TS expression in tumors was normalized using a log10 transformation. The association between the presence of TS mRNA in plasma and the levels of expression in tissues was analyzed using ANOVA test. The correlation between two qualitative variables was analyzed using the χ2 test. Two-tailed P ≤ 0.05 were considered significant. Statistical analysis was done using the SPSS package (version 11.0).

### Results

**TS mRNA expression levels.** This study was done by real-time PCR in plasma from 90 colon carcinoma patients and 27 healthy controls. mRNA of housekeeping genes was detected in the plasma of 88 (98%) patients and 26 (96%) controls, confirming both the presence and integrity of RNA in plasma. The cases in which housekeeping mRNA was not found were eliminated from the study. TS mRNA in plasma was identified in 47% of patients and in 23% of controls (P = 0.030, χ2). Among the plasma samples with the presence of TS mRNA, the geometric average of TS expression levels was eight times higher in patients than in controls (P = 0.002, Kruskal-Wallis; Fig. 1A).

Analysis of TS expression of 24 tumor and normal counterpart tissues of our series of 88 patients was done. Seven tumors (29%) had high levels of TS mRNA, at least 4-fold above the normal ratio, and four tumors (17%) showed underexpression. Patients with TS mRNA in the plasma had higher levels of TS expression in tumor tissue (geometric average, 13) compared to patients without TS mRNA in plasma (geometric average, 0.78; P = 0.033, ANOVA; Fig. 1B).

**Correlation of TS mRNA in plasma with clinicopathologic characteristics.** We analyzed the possible relations between the presence of TS mRNA in plasma and a series of clinicopathologic variables of the tumors (Table 2). We found a significant correlation with lymph node metastasis (P = 0.024, χ2), and a trend for the stage (P = 0.056, χ2) was also observed. However, for this last variable, there were few cases of stages A and D (five and two, respectively), which could modify the statistical analysis. When we eliminated these two stages and analyzed stages B and C, a significant association was observed between the presence of TS mRNA in plasma and Dukes’ C (P = 0.006, χ2).

We assessed whether the relative amount of TS expression was associated with the clinicopathologic variables of the patients, but no association was found.

**Analysis of TSER polymorphism.** We studied the TSER genotype in 60 cases of our series. Due to the difference in size of the repetitions (28 bp) the TSER polymorphism was studied by PCR (Fig. 2). The frequencies of TSER genotypes were as follows: 7 tumors had TSER 2/2 (12%), 30 tumors had TSER 2/3 (50%), and 23 tumors had TSER 3/3 (38%). No significant correlations were observed between TSER genotype and presence of TS mRNA in plasma. Among the cases with the presence of TS...
mRNA, Kruskal-Wallis analysis showed a significant increase in TS expression in plasma from patients with TSER 3/3 \( (P = 0.027) \). Thus, the geometric average was 3.1 and 3.5 times higher in TSER 3/3 than in TSER 2/2 and 2/3, respectively (Fig. 1C). We observed a significant correlation between TSER 2/2 and the presence of polyps \( (P = 0.018, \chi^2) \). There was no correlation between clinicopathologic features and any TSER genotype.

**Discussion**

The role of proteins involved in the action of chemotherapeutic agents is an exciting area. TS has received considerable attention because it is a critical target for fluoropyrimidine treatment. All studies of the significance of TS in colorectal cancer have been done in primary tumors, lymph node, and distant metastases (1). We attempted to detect TS mRNA in plasma from patients with colon carcinoma and to assess its possible value as a prognostic marker because it could be a noninvasive tool for prognosis.

We detected TS mRNA in the plasma of 47% of the patients at the time of diagnosis. There was a significant difference with normal controls both in the number of cases in which TS mRNA was present, and in the levels of this nucleic acid among cases in which it was detected. These results suggest that the most plausible origin of TS mRNA observed in plasma of colon cancer patients is the primary tumor.

Additionally, we analyzed tumor and normal tissues from 24 patients in our series to confirm the presence of TS mRNA in tumors, and to compare our rates with those reported previously. The proportion of patients with high levels of TS reported in the literature ranges from 14% to 80%, with a median of 51%, most of these results having been obtained by immunohistochemistry (1). The number of cases showing overexpression in the present study was within this range, although it was lower than the median. This discrepancy could be due to the small number of cases analyzed and the different techniques used.

The percentage of cases with the presence of TS mRNA in plasma (47%) was higher than the percentage of tumors with high levels of expression (29%). These differences could be due to the arbitrary definition of high levels of TS in tumors, which we defined as 4-fold above the normal ratio. However, when we studied TS in tumors as a quantitative variable, we found a significant correlation between high levels of TS in tumors and the presence of TS mRNA in plasma. This association suggests that the presence of TS in tumors could also be reflected in plasma. Several studies have reported a small number of alterations present only in plasma (35, 52).

In a previous study done in tumors (different samples per tumor) and plasma, our results indicated the presence of heterogeneous tumor clones (53), which could explain some unmatched alterations between plasma and tumor DNA. Thus, the plasma of patients with cancer may be a global source of tumor nucleic acids, and therefore, TS mRNA in plasma could be a more representative marker of the disease than TS mRNA in tumor.

Studies of RNA in plasma of patients with different types of cancer have found a correlation between the presence of extracellular RNA and poor prognosis (41, 43, 44). Similarly, in this study, we observed that the presence of tumor-related TS mRNA in plasma is associated with lymph node metastasis and more advanced stages, suggesting a possible use as a prognostic factor. Moreover, due to the significant differences between B and C stages, the presence of TS mRNA in plasma may improve discrimination between stages in colon carcinoma. Studies in tumors have reported that high levels of TS expression were significantly associated with chemotherapy resistance, and poor disease-free survival and overall survival (11–19). However, the follow-up of our patients is too small to justify conclusions about the effect of adjuvant chemotherapy and overall survival.

**Table 2. Correlation between the presence of TS mRNA in plasma and clinicopathologic variables**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>N</th>
<th>Detection of TS mRNA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Presence</td>
<td>Absence</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>53</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>Female</td>
<td>35</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>Localization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>9</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Left</td>
<td>49</td>
<td>22</td>
<td>27</td>
</tr>
<tr>
<td>Right</td>
<td>30</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>25</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>No</td>
<td>63</td>
<td>27</td>
<td>36</td>
</tr>
<tr>
<td>Polyps</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Yes</td>
<td>30</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>No</td>
<td>58</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>32</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>No</td>
<td>56</td>
<td>21</td>
<td>35</td>
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<tr>
<td>Tumor differentiation</td>
<td></td>
<td></td>
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<tr>
<td>Well</td>
<td>40</td>
<td>18</td>
<td>22</td>
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<tr>
<td>Moderate</td>
<td>40</td>
<td>20</td>
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</tr>
<tr>
<td>Poor</td>
<td>8</td>
<td>3</td>
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<td>Stage</td>
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<td>A</td>
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<td>B</td>
<td>49</td>
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<td>C</td>
<td>32</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>D</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

NOTE: P value was calculated using the \( \chi^2 \) test. \( P \leq 0.05 \) was considered statistically significant.

*Statistical analysis without stages A and D.
Hypothetically, within stage C, patients with high TS mRNA in plasma could have resistance to fluoropyrimidines. Further studies are needed to address the potential of TS in plasma as a determinant of sensitivity to 5-FU.

Based on the association of TSER polymorphism and increase of TS mRNA in tumors (22–24), we examined the possible association of TSER polymorphism and TS mRNA in the plasma in 60 patients. The frequencies of TSER genotype were similar to those of the general Caucasian population and was lower than in the Asian population, as described previously (34, 55). No correlation between TSER 3/3 and the presence of mRNA in plasma was found, whereas within the group of patients with the presence of TS mRNA there was a relation between TSER 3/3 and high levels of extracellular plasma TS. Thus, the TSER genotype, which is a regulatory element for TS gene in tumors, may influence TS expression in plasma. The interaction between TSER genotype and other regulatory factors may be involved in the presence of extracellular tumor-related TS in plasma. There is controversy about the relation between TSER polymorphism and cancer. Previous studies have reported an association between TSER genotype and both response to 5-FU-based chemotherapy (25) and survival (26).

In conclusion, this is the first study of TS in plasma from patients with cancer. Our results indicate that the presence of TS mRNA in the plasma of patients with colon carcinoma is a possible prognostic marker which can be obtained by a noninvasive method. TSER polymorphisms may influence the levels of TS in plasma. Further studies of TS in plasma could offer a spectrum of results similar to those found in tumors, which might lead to possible applications as a serum marker of treatment response and prognosis in patients with colon carcinomas.

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References

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