Thymidylate synthase (TS) is an enzyme that plays an important role in cellular proliferation and growth (1), catalyzing the methylation of fluorodUMP to dTMP, an essential precursor for DNA synthesis (2). 5-Fluorouracil (5-FU) is an anticancer agent active in several types of cancer through TS inhibition and the block of DNA synthesis (3). Previously, several studies investigated the level of TS expression, mostly analyzing the methylation of fluorodUMP to dTMP, an essential role in cellular proliferation and growth (1), catalyzing the methylation of fluorodUMP to dTMP, an essential precursor for DNA synthesis (2).

5-Fluorouracil (5-FU) is an anticancer agent active in several types of cancer through TS inhibition and the block of DNA synthesis (3). Previously, several studies investigated the level of TS expression, mostly testing transcript quantification by real-time PCR, and higher TS levels were associated with poor prognosis and/or an adverse response to 5-FU treatment in esophageal (4), breast (5), head and neck (6), bladder (7), and non–small cell lung cancer (8), although conflicting results have been reported in colorectal carcinoma (9, 10).

Neuroendocrine tumors (NET) are a rare and heterogeneous group of tumors with specific biological, histopathologic, and clinical features (11–13). NETs of the gastroenteropancreatic (GEP) system are classified according to WHO (14) as follows: well-differentiated (WD) NETs with benign behavior, WD neuroendocrine carcinomas (NEC) with low-grade malignancy, and poorly differentiated (PD) NECs (PD-NEC) with a high grade of aggressiveness and poor prognosis. The classification of pulmonary NETs differentiates typical carcinoid (TC) and atypical carcinoid (AC) and PD-NECs of the small–cell lung cancer (SCLC) and large–cell NEC (LCNEC) types (15–17). Both classifications identify a benign group of tumors (WD-NEC and TC) and a highly malignant counterpart (SCLC, LCNEC, and PD-NEC), being low-grade malignant tumors (atypical carcinoid, WD-NEC) in an intermediate position. In WD-NEC, surgical resection is the preferred treatment, but in the case of a neoplastic spread outside the primary site or recurrence after surgery, no standard systemic treatment is currently recommended. Therapy with IFN-a or somatostatin analogues, such as octreotide, have been used as a first-line treatment, but they are generally associated to a low response rate (18–20). Treatment of NETs with 5-FU has been proposed with promising results (21–25), although no data are currently available on the expression of TS in NETs to support antifolate drug based strategies.

Thymidylate Synthase Expression in Gastroenteropancreatic and Pulmonary Neuroendocrine Tumors

Paolo Ceppi,1 Marco Volante,2 Anna Ferrero,3 Luisella Righi,2 Ida Rapa,2 Rosj Rosas,2 Alfredo Berruti,3 Luigi Dogliotti,3 Giorgio V. Scagliotti,1 and Mauro Papotti2

Abstract

Purpose: The predictive role of the quantification of thymidylate synthase (TS) in tumors treated with antifolate drugs, such as 5-fluorouracil (5-FU), has been extensively reported in a variety of human tumors. Neuroendocrine tumors (NET) represent potential targets of antifolate agents, but no data on TS expression level in these tumors are currently available.

Experimental Design: A series of 116 NETs were collected, including 58 gastroenteropancreatic (GEP) and 58 lung NETs. In 24 well-differentiated GEP neuroendocrine carcinomas (WD-NEC), a 5-FU – based treatment was given. Total RNA was extracted from microdissected paraffin blocks. TS mRNA quantification was done by real-time PCR, whereas protein expression was evaluated by immunohistochemistry.

Results: By means of both quantification by real-time PCR and immunohistochemistry, a higher TS expression in pulmonary small cell lung cancer and large cell NEC compared with typical and atypical carcinoids was observed ($P < 0.01$). Similarly, in GEP tumors, a higher TS expression in poorly differentiated carcinomas than both WD-NEC and benign tumors ($P < 0.01$) was found. In patients with WD-NEC treated with 5-FU, high TS mRNA levels were associated with shorter time to progression ($P = 0.002$) and overall survival ($P = 0.04$). This negative prognostic role was confirmed in multivariate analysis adjusting for major prognostic variables ($P = 0.01$). No association between TS mRNA and survival was observed in WD-NEC patients not receiving 5-FU.

Conclusions: This study, for the first time, (a) reports the differential TS expression in the spectrum of NETs and (b) indicates TS as a possible predictive marker of treatment efficacy in WD-NEC patients treated with 5-FU.

Thymidylate synthase (TS) is an enzyme that plays an important role in cellular proliferation and growth (1), catalyzing the methylation of fluorodUMP to dTMP, an essential precursor for DNA synthesis (2). 5-Fluorouracil (5-FU) is an anticancer agent active in several types of cancer through TS inhibition and the block of DNA synthesis (3). Previously, several studies investigated the level of TS expression, mostly testing transcript quantification by real-time PCR, and higher TS levels were associated with poor prognosis and/or an adverse response to 5-FU treatment in esophageal (4), breast (5), head and neck (6), bladder (7), and non–small cell lung cancer (8), although conflicting results have been reported in colorectal carcinoma (9, 10).

Neuroendocrine tumors (NET) are a rare and heterogeneous group of tumors with specific biological, histopathologic, and clinical features (11–13). NETs of the gastroenteropancreatic (GEP) system are classified according to WHO (14) as follows: well-differentiated (WD) NETs with benign behavior, WD neuroendocrine carcinomas (NEC) with low-grade malignancy, and poorly differentiated (PD) NECs (PD-NEC) with a high grade of aggressiveness and poor prognosis. The classification of pulmonary NETs differentiates typical carcinoid (TC) and atypical carcinoid (AC) and PD-NECs of the small–cell lung cancer (SCLC) and large–cell NEC (LCNEC) types (15–17). Both classifications identify a benign group of tumors (WD-NEC and TC) and a highly malignant counterpart (SCLC, LCNEC, and PD-NEC), being low-grade malignant tumors (atypical carcinoid, WD-NEC) in an intermediate position. In WD-NEC, surgical resection is the preferred treatment, but in the case of a neoplastic spread outside the primary site or recurrence after surgery, no standard systemic treatment is currently recommended. Therapy with IFN-a or somatostatin analogues, such as octreotide, have been used as a first-line treatment, but they are generally associated to a low response rate (18–20). Treatment of NETs with 5-FU has been proposed with promising results (21–25), although no data are currently available on the expression of TS in NETs to support antifolate drug based strategies.
This study aimed at testing TS expression levels in a large series of GEP and pulmonary NETs by means of quantification by real-time PCR and immunohistochemistry on paraffin-embedded specimens. In addition, the potential prognostic or predictive role of TS expression in NETs was investigated.

**Materials and Methods**

**Patients and samples.** One hundred and sixteen paraffin-embedded surgical or biopsy specimens were collected (from the pathology files of the University of Turin Medical School) from patients affected by NETs of GEP (n = 58) or lung (n = 58) origin from 1992 to 2006. These included 50 WD (6 benign and 44 low grade malignant) and 8 PD GEp tumors, as well as 5 pulmonary TCs, 17 ACs, 16 LCNECs, and 20 SCLCs. Patients characteristics are summarized in Table 1. Overall, 32 of 116 (28%) patient samples were obtained from metastatic sites. Tumors of the GEP tract were from pancreas (n = 18), intestine (n = 29), liver (n = 2), stomach (n = 2), and of occult origin (n = 7). Among the malignant cases (n = 105), 24 patients (20%) with advanced GEp WD-NEC were prospectively enrolled in a trial which investigated the administration of octreotide and 5-FU protracted continuous infusion (25). In this subset of patients, two had unresectable locally advanced disease and 22 had metastatic disease, of which 18 showed the presence of liver metastases (82%).

In the remaining 81 patients, 11 (13%; four GEp WD-NECs, three ACs, and four LCNECs of the lung) had no available outcome information and were excluded from the survival analysis. The patients considered for survival analysis (n = 70) were grouped as WD-NEC (n = 30) and PD-NEC (n = 40) independently of tumor site and were treated with surgery, somatostatin analogues, or chemotherapy (not considered for survival analysis (24)).

**Tumors of the GEP tract were from pancreas (n = 116 (28%) patient samples were obtained from metastatic sites. **

**Immunohistochemistry in paraffin-embedded tissues.** From each paraffin block, 5-µm-thick sections were obtained and stained with H&E for conventional histologic examination. In addition, serial sections collected on charged slides were processed for immunohistochemical staining. Briefly, after deparaffinization and rehydration through graded alcohols and phosphate-buffer saline at pH 7.5, endogenous peroxidase activity was blocked by methanol and 0.3% hydrogen peroxide for 15 min. For antigen retrieval, the sections were treated in a microwave oven at 92°C for 30 min. For antigen retrieval, the sections were treated in a microwave oven at 92°C for 30 min. The slides were then incubated for 40 min at room temperature with the primary mouse anti-TS antibody (clone TS106, dilution 1:100; Zymed). The immune reaction was revealed with a biotin-free detection system based on a dextran chain-linked to the secondary antibody and peroxidase (En Vision, Dako). Slides were counterstained with hematoxylin, dehydrated, and mounted. A colorectal carcinoma specimen was included as a positive control, whereas negative controls were obtained omitting the primary antibody.

For statistical analyses, the immune reaction was considered as positive when present in >5% of the tumor cell population.

**Microdissection, RNA isolation, and cDNA synthesis.** From each paraffin block of representative tumor areas, serial sections with a thickness of 10 µm were prepared and stained with nuclear Fast Red (Sigma-Aldrich). Malignant cells were selected under microscope magnification (50× to 100×) and dissected from the slide using a scalpel. RNA isolation was done as previously described (26). In brief, tissue samples were heated at 92°C for 30 min in 4 mol/L DTT–GTC/sarcosine [4 mol/L guanidinium isothiocyanate, 50 mmol/L Tris–HCl (pH 7.5), 25 mmol/L EDTA; Invitrogen]. Fifty microliters of 2 mol/L sodium phosphate buffer (pH 4.0) followed by 600 µL of freshly prepared phenol/ chloroform/isoamyl alcohol (250:50:1) were added to the tissue suspensions. The suspension was centrifuged at 13,000 rpm for 8 min in a chilled (8°C) centrifuge. The upper aqueous phase was removed and combined with glycogen (10 µL) and 300 to 400 µL of isopropanol. The tubes were placed at -20°C for 30 to 45 min to precipitate the RNA. After centrifugation at 13,000 rpm for 7 min in a chilled (8°C) centrifuge, the supernatant was carefully poured off, the pellet was resuspended in 50 µL of 5 mmol/L Tris, and the cDNA synthesis was done as previously described (27).

**Real-time PCR analysis.** Relative cDNA quantification of TS and β-actin (internal reference gene) was done using a fluorescence-based real-time detection method, as previously described (28). Each measurement was done in duplicate, and the comparative C_t method was used. To further normalize across samples, the highest DC_t value was subtracted from each DC_t, to give the ∆DC_t values. These values were converted to relative expression levels by the following formula: 2^(-∆DC_t) (29). The sequences of the primers and probe used were as follows (30): TS forward 5’-GGCCTCGGTGTGCCTTT-3’; reverse 5’-GATGTGCGCAATCATGTACGT-3’; probe (FAM)-5’-ACCACGCGCCAGCTACACCGCCG-3’ (TAMRA); β-actin forward 5’-TGACCCGCCACTACACCTGT-3’; reverse 5’-TCTTAATGTCCGAGCGAGGTG-3’; probe (FAM)-5’-ACACAGCGCGCCAGCCGCGCCG-3’ (TAMRA). The PCR reaction mixture consisted of 1.200 nmol/L of each primer, 200 mmol/L probe, 200 mmol/L each of dATP, dCTP, dGTP, dTTP, 3.5 mmol/L MgCl2, and 1× Taqman Universal PCR Master Mix to a final volume of 20 µL (all reagents were from PE Applied Biosystems). Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min.

**Table 1. Characteristics of the 116 patients**

<table>
<thead>
<tr>
<th>n</th>
<th>M/F</th>
<th>Mean age (range)</th>
<th>TS protein</th>
<th>TS mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IHC positive</td>
<td>IHC negative</td>
</tr>
<tr>
<td>GEP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WD-NET</td>
<td>6</td>
<td>3/3</td>
<td>63 (45-82)</td>
<td>0</td>
</tr>
<tr>
<td>WD-NEC</td>
<td>44</td>
<td>23/21</td>
<td>59 (21-81)</td>
<td>5</td>
</tr>
<tr>
<td>PD-NEC</td>
<td>8</td>
<td>4/4</td>
<td>46 (30-85)</td>
<td>6</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>5</td>
<td>3/2</td>
<td>54 (28-70)</td>
<td>0</td>
</tr>
<tr>
<td>AC</td>
<td>17</td>
<td>10/7</td>
<td>59 (27-72)</td>
<td>6</td>
</tr>
<tr>
<td>LCNEC</td>
<td>16</td>
<td>14/2</td>
<td>59 (46-77)</td>
<td>13</td>
</tr>
<tr>
<td>SCLC</td>
<td>20</td>
<td>15/5</td>
<td>61 (44-80)</td>
<td>20</td>
</tr>
</tbody>
</table>

Note: The number of immunohistochemistry-positive and immunohistochemistry-negative patients and the median levels of TS mRNA expression are reported for each group. Abbreviations: M, male; F, female; IHC, immunohistochemistry.
Data analysis. To test differential TS protein expression among different histopathologic groups, the $\chi^2$ for trend test was used. The Mann-Whitney $U$ and Kruskal-Wallis tests were used to verify differential TS mRNA levels between groups. The Ki67 score of the 5-FU–treated patients was previously assessed in a clinical study recently presented (25). For the purpose of a correlation analysis with TS expression, a Spearman’s test was used. In the survival analysis, tumors were divided into groups according to tertiles of mRNA expression levels. First and second tertiles of TS distribution were grouped as low TS and compared with the third tertile (high TS). Survival analysis was done by Kaplan-Meier curves, and the significance was verified by log-rank test. Univariate and multivariate analysis of time to progression were carried out by means of the Cox proportional hazard model. All analyses were done using the statistical PC software package. The level of significance was set at $P < 0.05$.

Results

TS protein expression in NETs. TS immunohistochemistry staining in paraffin-embedded tumor sections was found positive in 50 of the 116 samples (43%), using 5% of positive cells as a cutoff value. According to different histopathologic categories, TS protein expression progressively increased from WD-NET to WD-NEC and PD-NEC ($P < 0.0002$). TS immunohistochemistry was present in only 5 of 44 WD-NEC (11%). In lung NETs, a significantly higher rate of TS expression in SCLC and LCNEC compared with TC and AC ($P < 0.0001$, all $m^2$ tests for trend) was observed. As a source of potential pitfall, no differences in the TS immunohistochemical distribution were observed comparing tumor samples obtained from small biopsies or surgical specimens. The results are summarized in Table 1. Figure 1 shows TS immunostaining in WD-NEC and PD-NEC. In the subgroup of 24 patients treated with 5-FU + long acting octreotide, we also investigated the correlation between Ki67 score, which was assessed previously to enroll patients in the clinical trial (25), and TS expression, and the results showed that the two markers were independently expressed ($R_s = 0.04$, $P = 0.85$).

Quantification of TS mRNA levels in NETs. TS expression level (ranging from 1 to 52.5, unit less ratios) was measured with the relative $\Delta C_t$ method from all the investigated samples. Table 1 shows the relative TS mRNA levels according to different histologic tumor types. Consistently with immunohistochemical data, TS mRNA transcripts were significantly higher among PD-NEC of the GEP system compared with WD-NEC and WD-NET ($P = 0.002$) and, among lung tumors, in SCLC and LCNEC compared with pulmonary TC and AC ($P < 0.001$, all Kruskall-Wallis tests). No significant differences were found comparing samples obtained from primary tumors (84 cases) and from metastases (32 cases), as well as among GEP tumors of different locations, in terms of TS expression. TS mRNA expression levels in GEP and pulmonary NETs are shown in Fig. 2A and B, respectively.

Prognostic and predictive significance of TS mRNA levels in NETs. Because of the lower rate of positive cases detected by immunohistochemistry in the group of WD tumors, TS transcript quantification was selected for the survival analysis.
Patients were split by adopting cutoff values according to tertiles of distribution of TS mRNA expression, and patients in first and second tertile (low TS) were compared with those in the third one (high TS).

In the subset of malignant cases not treated with 5-FU (n = 70), survival analysis revealed that TS levels were not correlated with survival once the tumors were grouped by histologic subtypes (P > 0.05) or grade of differentiation. In the latter case, Kaplan Meier curves did not show significant statistical differences neither in WD (n = 30, P = 0.55; Fig. 3A) nor in PD carcinomas (n = 40, P = 0.86; Fig. 3B) irrespective of their GEP or pulmonary origin.

In WD-NECs (n = 24) homogeneously treated with somatostatin analogue and continuous 5-FU infusion, the median time to progression was 20.4 months, whereas the median overall survival was not reached. Univariate analysis showed that 5-FU–treated patients with higher TS levels (third tertile, n = 8) had worse outcome in terms of time to progression (P = 0.002; Fig. 4A) and overall survival (P = 0.04; Fig. 4B) than those with lower levels (first and second tertiles, n = 16). None of the other prognostic considered variables (age, gender, performance status, site of primary disease, and response to therapy) were significantly correlated with survival, except for the Ki67 score (5% cutoff value), as reported elsewhere (25). In multivariate analysis, both TS (hazard ratio, 4.81; 95% confidence interval, 1.45-7.67; P = 0.01) and Ki67 (hazard ratio, 4.80; 95% confidence interval, 1.14-7.97; P = 0.03) were found to be independent prognostic markers (Table 2).

**Discussion**

This is the first report in which TS expression level and its prognostic role were analyzed in a large series of NETs. Both quantification by real-time PCR and immunohistochemistry detected a significantly higher expression of TS in PD-NECs compared with WD-NETs or carcinomas both of GEP and pulmonary origin (all P < 0.01). According to these results, TS expression at mRNA and protein level seems to be strictly correlated with tumor differentiation, this being higher in PD tumors of each group. Because the efficacy of TS-inhibiting drugs, such as 5-FU, was shown to be inversely correlated with TS
expression (31), the results of this study could provide further insights on the limited activity of 5-FU in PD-NEC (32, 33). In conclusion, this work for the first time reported a differential TS expression in the spectrum of NETs and (b) indicates the possible predictive role of TS expression levels in NEC patients treated with 5-FU–based therapy. This study adds novel insights which might open the line of future prospective trials based on 5-FU administration in patients with WD-NETs.

Table 2. Cox univariate and multivariate analysis of time to progression of 5-FU–treated patients

<table>
<thead>
<tr>
<th></th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TTP (median)</td>
<td>HR (range)</td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;58</td>
<td>25.8</td>
<td>1.19 (0.55-6.64)</td>
</tr>
<tr>
<td>&gt;58</td>
<td>22.3</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>35.2</td>
<td>1.27 (0.37-4.29)</td>
</tr>
<tr>
<td>F</td>
<td>22.3</td>
<td></td>
</tr>
<tr>
<td>PS (ECOG)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>21.8</td>
<td>0.39 (0.13-1.21)</td>
</tr>
<tr>
<td>1 + 2</td>
<td>21.6</td>
<td></td>
</tr>
<tr>
<td>Site of disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>22.9</td>
<td>0.97 (0.46-2.02)</td>
</tr>
<tr>
<td>Intestine</td>
<td>22.9</td>
<td></td>
</tr>
<tr>
<td>Occult</td>
<td>16.6</td>
<td></td>
</tr>
<tr>
<td>Response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>32.1</td>
<td>0.29 (0.06-1.39)</td>
</tr>
<tr>
<td>SD</td>
<td>22.2</td>
<td></td>
</tr>
<tr>
<td>Ki67 (median)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>26.2</td>
<td>3.67 (1.04-12.09)</td>
</tr>
<tr>
<td>High</td>
<td>14.8</td>
<td></td>
</tr>
<tr>
<td>TS (tertiles)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st + 2nd</td>
<td>28.4</td>
<td>4.25 (1.4-12.35)</td>
</tr>
<tr>
<td>3rd</td>
<td>14.9</td>
<td></td>
</tr>
</tbody>
</table>

Note: Patients were divided into groups according to tertiles of mRNA expression levels. First and second tertiles of TS distribution were grouped as low TS and compared with the third tertile (high TS).

Abbreviations: TTP, time to progression; HR, hazard ratio; PS (ECOG), performance status according to Eastern Cooperative Oncology Group; PR, partial responder; SD, stable disease.

References


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