Thymidylate Synthase Expression in Advanced Colorectal Cancer Predicts for Response to Raltitrexed

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ABSTRACT

Purpose: The purpose of this research was to evaluate the predictive value of expression of thymidylate synthase (TS) and other genes for response to raltitrexed (RTX).

Experimental Design: Twenty-five patients with metastatic colorectal cancer received RTX 3 mg/m2 3-weekly. Pretreatment tumor biopsies were analyzed for TS, dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP), folypolyglutamate synthetase, and reduced folate carrier mRNA expression by real-time reverse transcription-PCR. TS protein expression was evaluated by immunohistochemistry using a polyclonal TS antibody.

Results: Twenty patients were evaluable for response and gene expression. Six of 20 (30%) achieved a partial response. Median TS/β-actin was 5.7 x 10^3 (range, 2.2–42 x 10^3). Median TS/β-actin was 3.7 x 10^3 in responding patients and 6.1 x 10^3 in nonresponders (P = 0.048). Five of 6 patients with TS/β-actin ≤ 4.1 x 10^3 and 1 of 14 with higher values responded (P = 0.002). Overall survival was 21.7 months in patients with TS/β-actin ≤ 4.1 x 10^3 and 5.7 months in patients with higher values (P = 0.013). No correlations were seen between expression of TP, DPD, reduced folate carrier, or folypolyglutamate synthetase mRNA and response or survival. Weak TS staining was seen in 10 of 21 tumors evaluable for immunohistochemistry, including 5 responders All 4 of the patients with both weak staining and TS/β-actin ≤ 4.1 x 10^3 responded.

Conclusions: High TS mRNA expression predicts nonresponse to RTX. By contrast with 5-fluorouracil, high levels of TP and DPD mRNA are not associated with RTX resistance. Limited genomic fingerprinting could optimize single-agent therapy, allowing combinations to be reserved for high TS-expressing patients or for treatment failures, with potential reductions in toxicity and cost.

INTRODUCTION

Colorectal cancer is a leading cause of cancer-related morbidity and mortality in the Western world, second only to lung cancer as a cause of cancer death in Western populations. Despite advances in the surgical and adjuvant management of early disease, ~50% of patients who have potentially curative surgery relapse and eventually die from their disease. The mainstay of treatment for advanced disease is chemotherapy, with LV-modulated5 5-FU being the agent of choice and producing objective tumor response rates of around 20–25% (1). 5-FU, via its active metabolite 5-fluoro-2′-deoxyuridine-5′-monophosphate is an inhibitor of TS, the rate-limiting enzyme in the de novo synthesis of 2′-deoxythymidine-5′-monophosphate (2). It is also converted to other active metabolites such as 5-fluorouridine-5′-triphosphate, which are incorporated into RNA and inhibit protein synthesis (3).

The mode of action of 5-FU is thought to be schedule dependent (4, 5), and clinical studies have demonstrated improvement in response rates over bolus 5-FU when the drug is administered by protracted infusion (6). Biomodulation of 5-FU by agents such as folic acid and methotrexate has also improved response rates over single agent bolus 5-FU by the same magnitude as infusional therapy (7). However, the overall response rate for these treatments remained of the order of 25%. Recently, combination studies of 5-FU with new agents, such as the capecitabine analogue topoisomerase 1 inhibitor irinotecan (8, 9) and the platinum derivative oxaliplatin (10), have shown response rates rise to ~50% and have even shown a modest survival advantage for 5-FU and irinotecan. However, this is not without cost, and these regimens are potentially associated with greater toxicity and expense. This was highlighted in recent results showing an increased rate of treatment-related mortality in two large studies of the combination of irinotecan and 5-FU (11).

A novel approach to direct TS inhibition was the development of the specific antifolate TS inhibitor RTX (12). RTX is taken up into the cell by an active carrier-mediated transport

4 The abbreviations used are: LV, leucovorin; 5-FU, 5-fluouracil; TS, thymidylate synthase; RTX, raltitrexed; RFC, reduced folate carrier; FPGS, folypolyglutamate synthetase; IHC, immunohistochemistry; RT-PCR, reverse transcription-PCR; DPD, dihydropyrimidine dehydrogenase; TP, thymidine phosphorylase; CEA, carcino-embryonic antigen; CT, computed tomography; TAMRA, 6-carboxytetramethylrhodamine; 6FAM, 6-carboxyfluorescein.
system, via the RFC. Once within the cell it undergoes rapid metabolism by the enzyme FPGS into polyglutamated species, which are up to 70-fold more potent inhibitors of TS than the parent compound. RTX is associated with similar response rates to biomodulated and infusional 5-FU while causing less neutropenia and mucositis (13, 14). Of four large Phase III studies carried out with RTX, two have shown a small benefit in failure-free survival for 5-FU-based therapy, with one showing an overall survival benefit (15, 16), which has resulted in some muting of the initial enthusiasm for RTX. Nevertheless three of four studies have shown no difference in overall survival between 5-FU-based chemotherapy and RTX, and it is licensed in many countries as a single agent for the treatment of metastatic colorectal cancer. The ease of delivery and generally favorable toxicity profile make RTX an ideal agent for use in combination, and several clinical studies are ongoing in which RTX is being combined with other agents such as irinotecan (17) and oxaliplatin (18, 19), with highly promising results. Other schedules using single-agent RTX are also under investigation such as a 14-day schedule (20).

The inability to achieve higher response rates without the cost of added toxicity from drug combinations, and the failure to impact significantly on survival, have stimulated considerable research into mechanisms of resistance in tumors to these agents. Retrospective studies of primary rectal tumor sections with IHC showed that the degree of TS protein expression predicted for survival, with higher expression having an adverse effect (21). This was also demonstrated in primary breast cancer (22). However, TS expression in primary tumors failed to predict for outcome of chemotherapy in advanced disease (23). Studies in advanced disease have shown that both high TS mRNA expression quantified by RT-PCR and high TS protein expression predicted for a poor response to fluoropyrimidine-based therapy in colorectal, gastric, and head and neck cancer (24–27). Additional studies have shown that high levels of expression of TP (28) and DPD, the rate-limiting enzyme in the catabolic degradation of 5-FU (29), are also predictors of 5-FU treatment failure. TP is also the angiogenic factor platelet-derived endothelial cell growth factor, and it is thought that it is this which may account for this clinical effect, as in vitro studies show that 5-FU cytotoxicity may actually be enhanced by high TP levels (30).

The aims of this study were to investigate the relationship between TS mRNA and protein expression in pretreatment biopsies of colorectal cancer metastases, and to correlate these with response to RTX. In addition it was proposed to study the influence of levels of expression of TP and DPD on response to RTX, and to compare the results obtained with those seen in the previous studies with 5-FU-based chemotherapy. Furthermore, it was planned to examine the levels of expression of FPGS and RFC, two proteins known to be involved with cellular uptake and retention of RTX, and to assess whether there was any relationship with response.

**PATIENTS AND METHODS**

**Eligibility**

Patients were eligible for this study if they had a histological diagnosis of colorectal cancer with either locally advanced or metastatic disease for which no curative therapy was available. Disease had to be amenable to repeat biopsy. In addition, patients were required to have normal platelet counts and blood clotting, and a life expectancy of at least 12 weeks. Patients were not suitable if they had received adjuvant systemic 5-FU treatment for primary disease within 12 months from study entry or any systemic treatment for advanced disease.

**Pretreatment Evaluation**

Patients were asked to discontinue any dietary folic acid supplements on study entry. Baseline full blood count, urea and electrolytes, liver function tests, coagulation studies, and CEA. A staging abdomino-pelvic CT scan was performed, which confirmed metastatic disease and also allowed assessment as to the suitability for biopsy. If suitable, patients gave informed written consent and were entered in the study, which was approved by the Research and Ethics Committees of the Royal Marsden Hospital.

**Sample Collection**

Study participants underwent pretreatment (day −1) and post-treatment (day 5) tumor biopsies with the first course of RTX (day 0). This consisted of CT-guided needle biopsy of liver or other accessible metastatic disease. Two to three tissue cores each weighing ~10 mg and ~10 mm long were obtained. Each core was placed on a labeled glass slide, and 1–2 mm from both ends were cut and were placed in formalin. The rest of the core was immediately frozen in liquid nitrogen before storage at −70°C. The interval between biopsy and freezing was <1 min. In addition, blood was taken 24 h after the first injection of RTX and again on day 5. Blood samples were collected onto ice, and were centrifuged at 10,000 rpm for 10 min. After this, the plasma was pipetted into 1.8-ml cryotubes and stored at −20°C pending analysis.

**Treatment**

RTX (Tomudex) was supplied by Astrazeneca Pharmaceuticals and given by a 15-min i.v. infusion via a peripheral vein at a dose of 3 mg/m² once every 21 days.

**Assessment of Response**

Radiological assessments of response by CT scanning were performed at 12 and 24 weeks unless disease progression was suspected clinically, in which case treatment was withheld until a scan had been performed. Tumor response was objectively assessed using WHO criteria (31).

**Statistical Considerations**

At the time of initiation of this study, data from similar studies was very limited. Estimates as to the probable range of tumor TS expression were based on those reported by Johnston et al. (25). Assuming a similar population distribution, a minimum of 4 patients in each arm gave at least 80% power, at the two-sided α significance level of 0.05, of detecting a similar difference between means. On the basis of a response rate to RTX of ~25% (32), this meant a minimum total of 16 patients, provided all of the required samples were collected from each
patient. To allow for incomplete sampling, 20 patients were initially recruited, this number being expanded to 25 after interim review. Statistical comparisons between groups were made using the Mann-Whitney U test. All of the data were collected prospectively.

The cutoff value for TS mRNA expression between low and high TS expressers first quoted by Leichman et al. (27) was the median TS:β-actin ratio in their cohort of 46 patients i.e., 3.5 × 10^{-3}. In their study, patients with TS:β-actin ratios less than or equal to the median had a response rate of 52% and a median survival of 13.6 months, compared with 5% and 8.2 months for patients with TS:β-actin ratios greater than the median. They also noted that they were no responses among 19 patients with a TS:β-actin ratio >4.1 × 10^{-3}. Subsequently, the same group (29) proposed an algorithm based on levels of TS, TP, and DPD, which was able to predict nonresponse to 5-FU in their cohort of 46 patients (27) was 4.1, DPD

### Table 1  Predictive values for expression of TS, DPD, and TP mRNA based on 33 patients (29)

<table>
<thead>
<tr>
<th>Relative gene expression</th>
<th>Response rate</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>TS &lt; 4.1</td>
<td>57%</td>
<td>&lt;0.001</td>
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<tr>
<td>TS &lt; 4.1 and DPD &lt; 2.5</td>
<td>92%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TS &lt; 4.1, DPD &lt; 2.5 and TP &lt; 18</td>
<td>100%</td>
<td>&lt;0.001</td>
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</table>

### Laboratory Analyses

#### RT-PCR

**RNA Extraction and cDNA Synthesis.** Total RNA was isolated by a single-step guanidinium isothiocyanate method using the QuickPrep Micro mRNA Purification kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) according to the manufacturer’s instructions (33).

Isolated mRNA was dissolved in 50 μl of 5 mmol/liter Tris-HCl (pH 7.5). For cDNA synthesis, 20 μl 5 × Moloney murine leukemia virus buffer [250 mM Tris-HCl (pH 8.3); 375 mM KCl; 15 mM MgCl_2; [Life Technologies, Inc., Gaithersburg, MD]; 10 μl DTT [100 mM; Life Technologies, Inc.]; 10 μl deoxynucleotide triphosphate [each 10 μM; Amersham Pharmacia Biotech]; 0.5 μl random hexamers [A_{50 nm} dissolved in 550 μl of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA; Amersham Pharmacia Biotech]; 2.5 μl BSA [3 mg/ml in 10 mM Tris-HCl (pH 7.5) Amersham Pharmacia Biotech]; 2.5 μl RNase inhibitor [5 × 1000 units; Amersham Pharmacia Biotech]; and 5 μl Moloney murine leukemia virus reverse transcriptase [200 units/μl; Life Technologies, Inc.], added to a total volume of 50.5 μl.

**Real Time RT-PCR Quantification.** Only pretreatment biopsies were assessed for mRNA expression. Quantitation of cDNAs of the genes of interest and an internal reference gene (β-actin) was done using a fluorescence based real-time detection method [ABI PRISM 7700 Sequence Detection System (Taqman); Perkin-Elmer Applied Biosystems, Foster City, CA] as described previously (34, 35). In brief, this method uses a dual labeled fluorogenic oligonucleotide probe that anneals specifically within the forward and reverse primers. Laser stimulation within the capped wells containing the reaction mixture causes emission of a 3’ quencher dye (TAMRA) until the probe is cleaved by the 5’ to 3’ nuclease activity of the DNA polymerase during PCR extension, causing release of a 5’ reporter dye (6FAM). Thus, production of an amplicon causes emission of a fluorescent signal that is detected by the TaqMan charge-coupled device detection camera. The amount of signal produced at a threshold cycle within the purely exponential phase of the PCR reaction, in relation to the internal standard, provides a relative gene expression level.

The PCR reaction mixture consisted of 600 nM of each primer, 200 nM probe, 2.5 units AmpliTaq Gold Polymerase, 200 μM each dATP, dCTP, dGTP, 400 μM dUTP, 5.5 mM MgCl_2, and 1 × Taqman Buffer A containing a reference dye, to a final volume of 25 μl [all reagents Perkin-Elmer (PE) Applied Biosystems]. Cycling conditions were 50°C for 10 s, 95°C for 10 min, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min.

The primers and probe sequences used were as follows: TS primers: GGCTTCGGTGTGCCTTT and GATGTCGCAAT-TGATGTACGT; probe 6FAM (carboxyfluorescein)-5’-AA-CATGCGCAGCTACGCCCTG-3’ TAMRA (N,N,N’,N’-tetramethyl-6-carboxyrhodamine); TP primers: CCTGGCGAGCAGGATCTCC and TCCAGAAGTTCCTATCAGGAACTG; probe 6FAM5’-CAGGCGAGATGTCACGCCACC-3’ TAMRA; DPD primers: TCACGTGGACGCTGAGCTGT and TGCGGCAAGTGGAACACCA; probe 6FAM5’-CCCGCGACTC-CTACTGAGCACACAGG3’ TAMRA; FPGS primers: CCTCGTCCTAGCCTGATTT and GGAGCAGTGGTCTGGAAGAT, probe 6FAM5’-CATGCGCTGCAATTGATCAGG-3’ TAMRA; RF primers: CTTCTGCTGTCTAGCAGT and GCCCGAGAGCTACGTTCA; probe 6FAM5’-ATTGCGAAACGCCGCTGTTGGAGAAGACCT-3’ TAMRA; and β-actin primers: TGGAGCGGCTGACATCC and TCTT-GTAATGTCAAGCAGATT, probe 6FAM5’-ACCCACAC- GGCCAGCGC3’ TAMRA.

**Statistical Analysis of RT-PCR Results.** TaqMan analysis yield values that are expressed as ratios between two absolute measurements (gene of interest:internal reference gene).

### TS IHC

The avidin-biotin complex immunohistochemical technique was used to detect TS in tissue specimens, using the Vectastain kit (Vector Laboratories). Paraffin-embedded tissue sections 4-μm thick were deparaffinized in Histoclear (National Diagnostics) and rehydrated through graded alcohols. Endogenous peroxidase was quenched with 3% hydrogen peroxide/methanol mixture (1:4) for 10 min. Sections were rinsed and preincubated with 2% blocking serum for 30 min, followed by incubation with TS polyclonal antibody (Obtained from Dr. G. Wynne Aherne) for 1 h. After rinsing the tissue sections in phosphate buffered saline-Tween for 10 min, 100 μl of secondary antibody (biotinylated horse antirabbit) was added for 30
min. Tissue sections were again rinsed in PBST for 10 min and incubated with avidin-biotin complex for 30 min. Sections were rinsed in PBST and incubated with diaminobenzene substrate (Sigma) for 15 min. Tissues were finally rinsed in PBST for 5 min and tap water for 5 min, and counterstained with Mayer’s hematoxylin (Sigma) for 1 min. The tissue sections were subsequently dehydrated in graded ethanol, cleared in Histoclear, and mounted with glass coverslips using DPX. Each run included positive and negative controls.

**Scoring of IHC.** Tissue sections were examined and scored by two independent observers without knowledge of clinical outcome or RT-PCR results. TS expression was quantitated using a visual grading system based on the intensity of grading (0–3). The highest degree of staining seen in a tumor was used as its score. The mean of the two scores was calculated, and each section was assigned as strong (mean score ≥1.5) or weak (mean <1.5) staining.

**Quantitation of Plasma and Tumor RTX Concentration**

Day 1 and 5 plasma samples, and post-treatment biopsies were assayed for RTX concentrations by radioimmunoassay using the method described previously by Aherne et al. (36).

**RESULTS**

Twenty-five patients were recruited into this study between February 1996 and February 2000. These included 2 patients who received reduced doses of RTX from the start of treatment because of impaired renal function, and who were therefore ineligible for the study, and have been excluded from the analysis. In addition, 2 patients had no tumor in their biopsy and were therefore not evaluable for either IHC or for gene expression profiling. One sample provided inadequate mRNA for analysis: this patient was only evaluable for IHC. The procedures were well tolerated, with no significant morbidity from the liver biopsies other than pain, which responded to simple analgesia. In particular there was no significant hemorrhage. Nevertheless, 8 patients declined the post-treatment biopsy.

A median number of 4 cycles of RTX were given per patient (range, 1–16). The median event-free survival of the entire cohort after RTX therapy was 4.7 months, whereas the median overall survival was 13.9 months. Nineteen patients had died at the time of analysis.

All twenty-three of the eligible patients had completed treatment with RTX at the time of this analysis, and all were evaluable for response. There were 7 of 23 responders (30%). Twenty patients were evaluable for both response and gene expression (see above), and in these, there were 6 partial responses, giving an overall response rate of 30% in evaluable patients. All 6 of the responses were based on the biopsied lesion. One of the 6 responders had a differential response with reduction in size of liver metastases but small volume progression of lung metastases. This patient has been classified as a responder based on the outcome in the biopsied site. Three patients died on the study without follow-up radiological assessment. Of these, 2 had clear clinical and biochemical evidence of disease progression, with increasing levels of CEA. The third had clinical disease progression with continued weight loss and deteriorating performance status, although there was no increase in CEA and no symptoms suggesting toxicity from RTX. All 3 of the patients have been classified as nonresponders.

**Tumor TS mRNA Expression**

Tumor TS:β-actin mRNA ratios were available for 20 patients. The median TS:β-actin in tumor biopsies was $5.3 \times 10^3$ (range, $2.2–42 \times 10^3$). For responding patients, median TS:β-actin was $3.7 \times 10^3$ compared with $6.1 \times 10^3$ for nonresponding patients ($P = 0.048$, Mann-Whitney U test). Five of 6 responding patients had TS:β-actin $\leq 4.1 \times 10^3$ compared with 1 of 14 nonresponders. This difference was statistically significant ($P = 0.002$, two-tailed Fisher’s exact test). Fig. 1 shows the tumor TS mRNA expression according to tumor response. Tumor TS mRNA expression also correlated with survival: patients with TS:β-actin ratios $>4.1 \times 10^3$ had a median survival time of 5.7 months, whereas patients with TS:β-actin ratios $\leq 4.1 \times 10^3$ had a median survival of 21.7 months ($p = 0.013$). Fig. 2 shows the survival curves for these two groups.

**Expression of Other Genes Linked to Failure of 5-FU/LV Therapy**

**TP.** Median TP:β-actin was $15.1 \times 10^3$ (range, $1.9–194 \times 10^3$), and there was considerable overlap in expression between responding and nonresponding patients. Interestingly there was a trend for median TP to be higher in responders ($30.5 \times 10^3$) than nonresponders ($15.1 \times 10^3$), although this was not statistically significant ($P = 0.54$, Mann-Whitney U test). Of particular note, 3 of the 6 responding patients had
TP-β-actin \( \geq 18 \times 10^3 \), the cutoff level for nonresponse to 5-FU. These results are illustrated in Fig. 3.

DPD. As would be expected, there was no significant difference in DPD mRNA expression between responders and nonresponders, although there was a trend for higher DPD expression in nonresponders. Overall median DPD-β-actin was \( 1.3 \times 10^3 \) (range, 0.13–71 \( \times 10^3 \)), with medians 0.93 \( \times 10^3 \) and 1.83 \( \times 10^3 \) in responders and nonresponders, respectively (\( p = 0.1 \), Mann-Whitney \( t \) test). One patient with DPD \( > 2.5 \times 10^3 \) responded to treatment, suggesting that high DPD does not in itself prevent response to RTX (Fig. 4).

Expression of Genes Specifically Relevant to RTX

Metabolism

RFC. Although the RFC is thought to be the main membrane carrier for RTX (12), relative RFC mRNA expression was up to 1000-fold less than that of the other genes assessed (population median level: 31.7; range, 0–272). RFC mRNA was not detected in 5 patients, including 2 whose tumors responded to RTX. There was no difference in the median RFC-β-actin expression between responders (2.22) and nonresponders (52.0; \( p = 0.30 \), Mann-Whitney \( U \) test).

FPGS. No differences were noted between responders and nonresponders in terms of FPGS mRNA expression (32.6 \( \times 10^3 \) \( \text{versus} \) 24.5 \( \times 10^3 \) respectively; \( P = 0.97 \), Mann-Whitney \( U \) test). Median FPGS-β-actin for the entire cohort was 28.4 \( \times 10^3 \) (range, 2.86–101 \( \times 10^3 \)), whereas 2 of 6 responding patients had very low tumor FPGS (<4.0 \( \times 10^3 \)), suggesting that even low levels of expression of this enzyme are sufficient for polyglutamation and drug activity.

IHC

Strong staining for TS protein was seen in 11 of 21 tumor samples taken before treatment. Of the 10 patients with low TS, there were 5 responses (50%), compared with 2 of 11 responses (18%) seen in high TS tumors. The relationship between TS mRNA and TS protein expression was not clear-cut. There was no direct correlation between mean TS IHC score and mRNA expression, although there was a trend for the higher IHC scores of 2–3 to be associated with higher mRNA expression, as is shown in Fig. 5. Paired data were available for 20 patients. Eight of 13 patients with TS-β-actin \( > 4.1 \times 10^3 \) also expressed high levels of protein, whereas lower levels of TS mRNA expression were associated with weak staining for TS protein in only 4 of 7 patients. Interestingly, the combination of low TS mRNA and low protein expression was associated with response to RTX in 4 of 4 cases. Of the 8 cases where the TS mRNA and protein scores were discordant there was 1 response (in a patient with high mRNA expression and weak staining), and there was also 1 responder in the group of 7 patients with both high mRNA and protein expression. Thus, the combination of low TS mRNA and protein expression may be a better predictor of outcome than either parameter alone.

Post-treatment biopsies were available for 15 patients. Paired (pre- and post-treatment) samples were available for only 13 patients. There was only limited evidence of TS up-regulation in these samples. Although the absolute TS score was increased in 10 of 13 paired samples, an increase from weak to strong staining was only recorded in 3 of 13 patients, with no change in 8 patients and a reduction from strong to weak staining in 2 patients. No firm conclusions may be drawn from these data.

Tumor and Plasma RTX Concentrations

Mean plasma RTX concentration was 8.5 ± 3.5 nm on day 1 (24 h after treatment), and 5.3 ± 2.2 nm on day 5. Day 5 tumor biopsies were available for analysis in 15 patients. RTX was below the limit of detection in 6 of 15 biopsies (including 2 who responded to treatment), and these have been assigned the value of 0.04 mmol/g (the detection limit) for the purposes of analysis. The detectable values ranged from 0.074 to 0.68 mmol/g (mean 0.2 ± 0.2 mmol/g). No apparent correlation was seen between tumor or plasma RTX concentration and response to RTX.

DISCUSSION

Studies of paired tissues show that tumors in general express 2–10-fold higher TS levels than normal tissues (23, 24, 37, 38), whereas ~50% of tumor specimens have high levels of TS message and/or protein. A considerable number of retrospective clinical studies have shown that high levels of TS mRNA and/or protein expression are associated with a low probability of response to 5-FU/LV and also poorer survival. Conversely there are a few studies that have failed to show such a correlation, although these are often limited by small numbers (Refs. 21–23, 25–27, 37–46; Table 2). One factor encountered when reviewing the literature is that different assays were used to quantify TS expression. Whereas activity assays would appear to be the most indicative of TS function, it would be difficult to apply
such assays to large-scale clinical use, as they require larger amounts of fresh tissue. Quantification of TS protein may be the next best technique with the advantage that it is possible to perform IHC on archive material. However, the scoring of tissue sections using visual scales based on intensity and extent of staining are considerably observer-dependent; scores can be influenced by the degree of tissue heterogeneity within the sections, and immuno-positivity gives no indication of the biological activity on the protein. Despite this, several studies have found correlation between TS protein expression and outcome using this method (Table 2).

Quantification of TS mRNA using techniques such as RT-PCR is attractive in that it provides an objective and quantitative method of studying gene expression. This method also requires fresh tissue, although there are ongoing developments that might allow its use on archive material (47). TS protein is subject to translational regulation in that the free protein interacts with TS message in a negative feedback loop (48). It has also been suggested recently that polymorphisms of the TS gene, rather than the number of gene copies, may influence the degree of protein expression (49). Thirdly, TS is known to be amplified after exposure to chemotherapy, possibly by interruption of the above negative feedback loop (50), so that the correlation between pretreatment levels of the enzyme and those subsequent to drug exposure has not been studied. While these factors would appear to militate against a correlation between the degree of mRNA and protein expression, investigators have demonstrated reasonable correlation between the two (25), hence justifying the use of mRNA expression. Different groups have developed their own variations of this technique, each quoting different cutoff values between high and low TS gene expression, thus complicating the comparison of results from different studies. In our study, this problem was partly overcome by analyzing tissue samples using a well-established technique in the same laboratory, which has reported extensively on this method (Table 2).

The data from this study show a similar correlation between TS expression and response to the specific TS inhibitor RTX as has been reported for 5-FU-based therapy. We used the same cutoff level for TS expression, which had been derived retrospectively in previous studies, and we have demonstrated in this prospective study that it is also valid for our own population treated with a different agent. Our data confirm that high TS mRNA expression in biopsies predominantly from hepatic me-
tastases of colorectal cancer predicts for treatment failure. This presents an exciting opportunity for the pretreatment classification of patients based on likelihood of response to 5-FU or RTX therapy. Patients with low TS gene expression could be treated with folinic acid modulated 5-FU or RTX with anticipated response rates of >50% and without the added toxicity of combined therapy with newer agents. Patients with high TS gene expression have a much smaller chance of response and in this group, treatment with combinations or non-TS targeting agents such as irinotecan would be warranted. However, there are several observations that influence the interpretation of such data. Firstly, it has been demonstrated that TS expression in primary tumor samples may not be predictive of response of metastases to treatment (23) and, therefore, patients require biopsies of recurrent disease. Secondly, TS expression in different metastatic sites, e.g., local recurrence versus liver versus lung, may vary and could lead to differential responses to treatment at different disease sites (51). Thirdly, this method is influenced by the degree of tissue heterogeneity, with more fibrotic and necrotic biopsies probably expressing less TS mRNA. This issue can only be overcome by incorporating microdissection techniques into the method, at the risk of making it more unwieldy and less applicable to general high-throughput use.

High TS expression does not only adversely influence response to specific TS inhibitors, but also correlates with response to non-TS targeting therapy such as chemotherapeutic agents/combinations with diverse cellular targets (21, 39), and also survival after hepatic resection (40). Secondly, several studies including our own have shown high TS expression to be an adverse prognostic factor for survival irrespective of response to chemotherapy (Table 2). Paradoxically, in the adjuvant setting two studies (21, 52) showed no benefit from adjuvant chemotherapy in low TS-expressing tumors but an improvement in survival after treatment in high TS-expressing tumors. It is significant that such a strong correlation between TS expression and survival was demonstrated in our study, because most of the patients were selected on the basis of having bulky metastatic disease to facilitate biopsy, and greater disease bulk is a well-recognized adverse prognostic feature (53). These observations suggest that TS expression is indicative of other differences in tumor biology, which in turn influence the natural history of the disease. Indeed, data show that TS protein can interact with the proto-oncogene c-myc (54) as well as the tumor suppressor gene p53 (55). Additionally, in one study, TS influenced response to therapy only in tumors with wild-type p53 and not in those with mutated p53 (41). All of the above point to the possibility that TS may have a more fundamental role in cellular homeostasis and that differences in its expression may be indicative of underlying variations in tumor cell survival and apoptosis.

It is not surprising that DPD gene expression appeared to bear no relationship to response to RTX, but it is significant because of the marked association between elevated tumor DPD and nonresponse to 5-FU/LV (29, 56). This finding highlights the potential for using RTX in tumors with high DPD, thereby circumventing 5-FU resistance through increased catabolism while preserving TS as the main focus of antitumor action.
Interestingly, in contrast to the 5-FU data, there appeared to be no association between high TP gene expression and resistance to RTX. Indeed, 3 of 6 responding patients in this study had tumors that expressed very high levels of TP. These data do not support the hypothesis that the angiogenic properties of TP are responsible for its role in 5-FU failure, because this effect of TP would also be expected to confer resistance to RTX. The alternative hypothesis of high TP expression enhancing the bioconversion of 5-FU to 5-fluoro-2'-deoxyuridine would seem more plausible. Enhanced TP activity may also favor RTX cytotoxicity by reducing the amount of thymidine available for rescue of thymidylate-depleted cells after TS inhibition (57).

The lack of correlation between TS mRNA levels below the response threshold and TS protein is disappointing, whereas this correlation was stronger between higher mRNA expression and increased protein, confirming the data from previous studies. IHC has obvious advantages in terms of its wider application as a predictive tool, but is hampered by several factors

**Table 2** Clinical studies on the association of TS expression and outcome of chemotherapy

<table>
<thead>
<tr>
<th>Reference no.</th>
<th>Tumor type</th>
<th>n a</th>
<th>Technique</th>
<th>Outcome</th>
</tr>
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<td>21</td>
<td>Primary rectal</td>
<td>294</td>
<td>IHC</td>
<td>High TS associated with poorer DFS + OS Survival benefit of adjuvant chemo only in high TS tumors</td>
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<tr>
<td>25</td>
<td>Advanced colorectal and gastric</td>
<td>21</td>
<td>Western blotting (TS106) + RT-PCR</td>
<td>TS protein and mRNA expression correlated with response to 5-FU-based treatment in both tumor types</td>
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<tr>
<td>26</td>
<td>Primary gastric</td>
<td>65</td>
<td>RT-PCR</td>
<td>High TS associated with poorer OS Low TS correlated with response to 5-FU-based treatment</td>
</tr>
<tr>
<td>37</td>
<td>Primary colorectal</td>
<td>24</td>
<td>RT-PCR</td>
<td>High TS assoc. with poorer OS Low TS correlated with response to 5-FU</td>
</tr>
<tr>
<td>46</td>
<td>Head and neck</td>
<td>79</td>
<td>IHC TS 106 Moab</td>
<td>No correlation between TS expression and response to 5-FU</td>
</tr>
<tr>
<td>23</td>
<td>Primary colorectal</td>
<td>134</td>
<td>IHC TS Poab</td>
<td>TS expression in primary tumor did not correlate with response of metachronous metastases to 5-FU</td>
</tr>
<tr>
<td>27</td>
<td>Advanced colorectal</td>
<td>46</td>
<td>RT-PCR</td>
<td>High TS mRNA correlated with poor response to 5-FU. High TS associated with poorer OS</td>
</tr>
<tr>
<td>22</td>
<td>Primary breast</td>
<td>488</td>
<td>IHC TS 106 Moab</td>
<td>High TS associated with poorer DFS + OS in node +ve tumors. Survival benefit of adjuvant chemo only in high TS tumors</td>
</tr>
<tr>
<td>44</td>
<td>Primary colorectal</td>
<td>62</td>
<td>TS activity assay</td>
<td>Low TS activity associated with poor survival after surgery</td>
</tr>
<tr>
<td>40</td>
<td>Colorectal liver metastases</td>
<td>33</td>
<td>RT-PCR</td>
<td>High TS predicted for poor response to 5-FU. High TS associated with shorter DFS and OS after hepatic resection</td>
</tr>
<tr>
<td>42</td>
<td>Advanced colon</td>
<td>41</td>
<td>IHC TS Poab</td>
<td>High TS mRNA correlated with poor response to bolus 5-FU</td>
</tr>
<tr>
<td>58</td>
<td>Colorectal liver metastases</td>
<td>36</td>
<td>IHC TS 106 Moab</td>
<td>Low TS correlated with response to hepatic arterial fluorouridine. No correlation between TS expression and OS</td>
</tr>
<tr>
<td>43</td>
<td>Advanced breast</td>
<td>75</td>
<td>RT-PCR</td>
<td>No relation between TS gene expression and response to chemotherapy or OS</td>
</tr>
<tr>
<td>39</td>
<td>Head and neck</td>
<td>68</td>
<td>IHC TS 106 Moab</td>
<td>High TS associated with poorer OS; No correlation with response to 5-FU and non-5-FU containing chemo but few nonresponders</td>
</tr>
<tr>
<td>45</td>
<td>Advanced cervix</td>
<td>66</td>
<td>IHC TS Poab</td>
<td>Low TS association with improved OS</td>
</tr>
<tr>
<td>41</td>
<td>Primary colorectal</td>
<td>108</td>
<td>IHC TS 106 Moab</td>
<td>High TS in p53 +ve primary tumors predicted for poor response of metastases to MTX + 5-FU</td>
</tr>
</tbody>
</table>

a n = number of patients; Moab, monoclonal antibody; DFS, disease-free survival; OS, overall survival; Poab, polyclonal antibody; MTX, methotrexate.
contributing toward inaccuracy, such as small and often heterogeneous tumor samples, observer variation, and the inevitable overlap when trying to assign a continuous variable into definite categories. In addition, the IHC performed in this study was on very small tumor samples, which amplified the potential for error.

This study confirms the feasibility of treating patients with a drug regimen determined by the pattern of gene expression of their tumors. It is surprising, given the known histological heterogeneity of metastatic tumors, that such accurate predictions could be obtained from biopsy of a single lesion. Potential sampling errors and variations in TS mRNA expression between metastases to different sites will almost certainly preclude the achievement of 100% predictive value by this approach. However, the above studies have clearly shown that this method is able to distinguish between patient populations with up to a 10-fold difference in response rates, based on the analysis of a single variable. Inclusion of other factors such as DPD and TP may increase the predictive value even further and allow the prospective rationalization of therapy. This approach has the potential to achieve single-agent response rates equivalent to those of current combinations, without the added toxicity and financial cost, and with the additional benefit of reserving newer agents for second line therapy. At the cost of a single pre-treatment biopsy, it may be possible to acquire a limited genomic fingerprint of each tumor, enabling the individual tailoring of therapy. Although additional studies are required to assess whether or not this approach will prove superior to front-line combination therapy, the potential for improved therapeutic ratios and reduced cost make it an attractive proposition for future development, and a randomized study to confirm the benefits of a targeted approach to chemotherapy is warranted.

REFERENCES


Tumor TS mRNA Correlates with Response to Raltitrexed


Thymidylate Synthase Expression in Advanced Colorectal Cancer Predicts for Response to Raltitrexed


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