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Predictive value of thymidylate synthase expression in resected metastases of colorectal cancer

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Abstract

Recent investigations have focused on the prognostic value of thymidylate synthase (TS) assessment in metastases of colorectal carcinoma (CRC). In order to evaluate the prognostic impact of TS expression after resection of metastases of colorectal cancer followed by systemic adjuvant chemotherapy, we performed an immunohistochemical characterisation of TS in the primary tumours and in the corresponding radically resected hepatic and pulmonary metastases. An additional objective was to compare the levels of TS in primary and metastatic disease. TS expression was assessed by immunohistochemistry using the monoclonal antibody TS 106. The study population consisted of 60 patients: 48 underwent liver and 12 lung resection. All of them received adjuvant chemotherapy after metastasectomy according to the Mayo Clinic schedule. In the 49 evaluable primary tumours, TS score was high in 53% and low in 47% of patients, while in the 60 metastatic samples TS immunostaining was high in 33% and low in 67%. There was a significantly smaller number of high TS expressors in metastatic than in primary tumours ($P<0.04$). No correlation was observed between TS expression and the site of the metastasis. TS status did not significantly correlate with the median disease-free interval (DFI) after metastasectomy, although this parameter was longer for patients with low TS immunoreactivity in the resected metastases than for those with high TS lesions (19.6 versus 13.8 months). Patients with high TS levels, however, had a significantly shorter median overall survival (OS) (27.6 months) than those with low TS expression (36.3 months) ($P<0.008$). TS status in the resected metastases confirmed its independent prognostic value in the multivariate analysis and was the only prognostic marker of OS in the subgroup of patients with resected liver metastases. These results suggest that high TS levels in resected metastases of colorectal cancer are associated with a poor outcome after surgery and 5-FU adjuvant therapy; therefore, a prospective assessment of TS levels in resected colorectal metastases could be useful to define which patients will most likely benefit from 5-FU adjuvant therapy after metastasectomy. Chemotherapeutic agents that target TS may not be the appropriate adjuvant treatment after metastasectomy for patients with a high TS expression in the resected metastases of colorectal cancer.

Keywords: Thymidylate synthase; Immunohistochemistry; Metastases; Colorectal cancer

1. Introduction

Colorectal cancer (CRC) is the second cause of death from cancer in Western countries. Conventional management of stage IV CRC in most patients is palliative, but in selected patients with metastatic disease confined to the liver or to the lung, metastasectomy may offer a survival advantage [1,2]. Surgery alone, however, does not offer a 5-year survival rate in more than 20–30% of cases.

In a few non-randomised trials of patients with colorectal cancer, it has been possible to discern a trend towards an improvement in the disease-free interval (DFI) due to adjuvant therapy after liver metastasectomy compared with historical or matched pair control groups [3,4]; however, the published randomised trials comparing intraarterial chemotherapy with observation alone failed to demonstrate an improvement in overall survival (OS) [5,6]. At present, no data are

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available on the use of adjuvant chemotherapy after pulmonary metastasectomy. Substantial variations are observed in the long-term survival of patients with similar conventional prognostic factors, suggesting that biological factors may play an important role in determining survival after resection of metastases. Recent studies focused on the prognostic value of thymidylate synthase (TS) assessment in metastases of CRC [7–13]. TS is the rate-limiting enzyme in the synthesis of pyrimidine nucleotides required for DNA synthesis and is also a critical target for fluoropyrimidines, which are widely used in the treatment of gastrointestinal and other epithelial tumours [14]. Increased expression of the TS protein may be a major mechanism of resistance to fluoropyrimidines in both clinical and preclinical studies [15–17]. The intrinsic cell TS content may be a predictor of response to 5-fluorouracil (5-FU) and floxuridine (FUdR) chemotherapy [7–10,13,18]. TS enzyme expression is a powerful prognostic marker of disease recurrence and survival in epithelial malignancies. Patients with colorectal cancer and high TS expression had a significantly worse prognosis regardless of disease stage [19,20]; moreover, high TS expression in primary breast samples predicted for a poor clinical outcome in patients with node-positive disease [21].

In this study, we performed an immunohistochemical characterisation of TS in the primary CRC and in the corresponding radically resected hepatic and pulmonary metastases in order both to evaluate its relationship with other clinical and pathological features and to compare its expression in primary and metastatic disease. Using univariate and multivariate analyses, we also investigated the prognostic value of TS expression after resection of metastases and systemic adjuvant chemotherapy.

2. Patients and methods

2.1. Patients

The study included 60 patients with liver or pulmonary metastases from CRC who underwent liver or lung resection with curative intention, between December 1988 and December 1998 at Università Cattolica del S. Cuore, Rome, Italy.

Eligibility requirements were the following: (1) R0 resection of hepatic or pulmonary metastases, (2) no evidence of other sites of disease, (3) less than three metastases resected, (4) tumour-free resection margins, (5) systemic adjuvant chemotherapy after metastasectomy with a regimen containing 5-FU (according to the Mayo Clinic schedule, 5-FU 425 mg/m^2 plus folinic acid 20 mg/m^2 for 5 days, every 28 days for six cycles), (6) availability of paraffin-embedded material and clinical data.

Patients' characteristics are listed in Table 1. The median age of the patients was 59.5 years (range 32–77 years); 37 were male and 23 female; 48 patients underwent liver resection (20 for synchronous and 28 for metachronous metastases) and 12 patients underwent lung resection. In 49 patients, the paraffin-embedded block of the primary tumour was available and a comparison of the expression of TS with the corresponding metastases was possible. 25 patients (8 Dukes' stage B and 17 Dukes' stage C) had received a regimen containing bolus 5-FU as adjuvant chemotherapy after surgery for the primary tumour: 23 received the Mayo Clinic regimen therapy as previously reported and 2 received Machover's regimen therapy with 5-FU 370 mg/m^2 plus folinic acid 200 mg/m^2 for 5 days, every 28 days for six cycles. Metastases that appeared less than 6 months after primary tumour resection were considered synchronous. Clinical follow-up was available for all patients, with a median follow-up of 32.5 months (range 2–85 months).

2.2. Immunohistochemistry

TS protein expression was evaluated with the avidin–biotin–complex (ABC) immunohistochemical technique, using the TS 106 monoclonal antibody, kindly provided by Prof. P.G. Johnston, The Queens University of Belfast, UK [22]. Positive controls, which are cases known to show high TS expression; negative controls, in which the primary antibody was omitted, and duplicate samples were included in each batch of TS immunostaining.

The slides were independently examined under a light microscope and scored by three investigators who were blinded to both the clinical and pathological data, and used standard criteria [19,23] to define both the TS 106 staining intensity based on a visual grading scale (0–3) and heterogeneity of staining (focal, $\leqslant 25\%$ of tumour staining positive; diffuse, $>25\%$ of tumour staining positive). Intensity levels 0–1 were grouped together as low intensity staining, while levels 2–3 were grouped as high intensity staining. The area with the highest staining intensity was used for the grading of staining and all fields of the tissue section were evaluated.

There was close agreement ($>85\%$) in the evaluation of the TS intensity. The tumour samples with conflicting scores were re-evaluated and agreed on by two investigators. TS staining in duplicate tissue samples was similar from one batch to the next.

2.3. Definitions and statistical analysis

All survival data were updated in March 2001. DFI was calculated from the date of lung or liver resection to the date of relapse or death in the case of patients who died without relapse. OS was defined as the interval

Table 1
Patients' characteristics

Variable	No.
All patients	60
Sex	
Male	37
Female	23
Age (years)	
⩽60	28
>60	32
Primary tumour	
Tumour site	
Rectum	17
Colon	43
Grading[a]	
G1	3
G2	28
G3	22
Extension into serosa	
−	54
+	6
Lymphatic spread	
−	25
+	35
Dukes' stage	
B	20
C	20
D	20
Adjuvant chemotherapy	
Dukes' B	8
Dukes' C	17
Liver metastases	48
Delay from primary tumour	
Synchronous	20
Metachronous	
<2 years	22
>2 years	6
No. of resected metastases	
1	39
2–3	9
Size of the larger deposit	
<5 cm	38
⩾5 cm	10
Pre-operative CEA (μg/l)	
⩽5	15
>5	33
Lung metastases	12
Delay from primary tumour	
<2 years	8
>2 years	4
No. of resected metastases	
1	11
2–3	1
Size of the larger deposit	
<5 cm	11
⩾5 cm	1
Pre-operative CEA (μg/l)	
⩽5	6
>5	6

G, grade; CEA, carcinoembryonic antigen.
[a] Evaluated in 53 of the 60 primary tumours.

between the resection of lung or liver metastases and death or the date of the last follow-up evaluation. Both DFI and OS were calculated according to the Kaplan–Meier method [24]. All reported P values are two-sided.

TS status in the resected metastases (high, low) was compared with other factors of proven prognostic value (1, 2): age (⩽60 years, >60 years), lymphatic spread of the primary tumour (present, absent), extension into the serosa of the primary tumour (present, absent), time of metastases (synchronous, metachronous), number of metastases resected (single, multiple), preoperative carcinomembryonic antigen (CEA) level (⩽5 μg/l, >5 μg/l).

The contribution of prognostic variables to survival was analysed using the log-rank test [25] for univariate analysis and the proportional hazards model of Cox [26] for the multivariate analysis. Variables found to be significant prognostic factors in the univariate analysis were tested in the multivariate analysis.

3. Results

In the 49 evaluable primary tumours, TS score was high in 26 (53%) and low in 23 patients (47%). All samples showed diffuse TS staining. There was no significant association between TS expression in the primary tumours and the other clinical–pathological features (sex and age of the patients, histological grade, site, stage, extension into the serosa and lymphatic spread of the primary tumour).

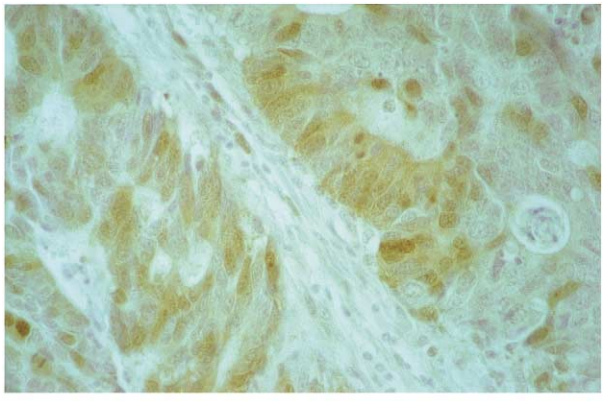
TS immunostaining was high in 20 (33%) and low in 40 (67%) of the 60 metastatic samples. There was a significantly smaller number of high TS expressors in metastatic than in primary tumours ($P<0.04$).

The comparison between TS expression levels in the metastases and primary tumours was as follows: 31 cases did not show any difference, 14 had a lower TS level and 4 cases had a higher TS expression. The distribution of TS-positive cells was diffuse, as for the staining pattern observed in the primary tumours. Representative metastatic samples of high and low TS levels are shown in Fig. 1a and b, respectively. There was no correlation between TS expression and the site of the secondary lesions: a high level of TS immunoreactivity was observed in 16 (33%) of the 48 liver metastases and in four (33%) of the 12 lung metastases.

No differences in the levels of TS expression in the metastatic samples were found in relation to the above-mentioned clinical–pathological features or to the delay of metastases from primary tumour, to the number and size of resected metastases, or to the pre-operative CEA levels. No differences in TS staining intensity were observed between metastatic tumour samples from patients who had received prior 5-FU as adjuvant chemotherapy after resection of the primary tumour and those who had not. However, in the subgroup of

patients with resected liver metastases, a high TS score was found in 10 of the 20 patients who previously received 5-FU in the adjuvant setting and in only 6 of the 28 patients who did not receive adjuvant chemotherapy after primary tumour resection ($P=0.038$) (Table 2). Median DFI after metastasectomy and adjuvant systemic chemotherapy was 19.4 months (range 2+ to 75+ months); 19.3 months (range 2+ to 75+ months) in patients who underwent liver resection and 37.7 months (range 8+ to 61+ months) in patients who underwent lung resection ($P=$ non significant (NS)). Univariate analysis showed a significant predictive relevance of the extension into serosa and of preoperative CEA on the DFI (Table 3). TS status did not significantly affect the DFI, although the median DFI after resection of metastases was longer for patients with low TS immunoreactivity than for those with high TS metastatic lesions (19.6 versus 13.8 months). The predictive value of preoperative CEA was retained in the multivariate model for DFI (Table 3).

Median OS after metastasectomy and adjuvant systemic chemotherapy was 29.5 months (range 2+ to 85+ months); 27.6 (range 2+ to 75+ months) in patients who underwent liver resection and 44.4 months (range 8+ to 85+ months) in patients who underwent lung resection ($P=$NS). Univariate analysis showed that extension into the serosa of the primary tumour and the TS status in secondary lesions appeared to significantly influence OS after metastasectomy and adjuvant chemotherapy (Table 3). Patients who underwent surgery for metastatic disease, and had high TS levels in the metastases had a significantly shorter median OS (27.6 months) than those with low TS expression (36.3 months) ($P<0.008$) (Fig. 2). The extension into the serosa of the primary tumour and the TS status in the resected metastases confirmed their independent prognostic value in the multivariate analysis (Table 3). TS status was the only prognostic marker of OS in the subgroup of patients with resected liver metastases (Table 3).



(a)

(b)

Fig. 1. Immunohistochemical staining of colorectal liver metastasis using thymidylate synthase (TS) 106 monoclonal antibody demonstrating high (a) and low (b) intensity tumour cell staining for TS (magnification 250×).

4. Discussion

In our study, no association was found between TS immunoreactivity in the primary tumours and the other

Table 2
Relationship between clinical–pathological characteristics and TS expression in the resected metastases

Variable	No.	High TS	*P* value
Site			
Liver	48	16	1.000
Lung	12	4	
Delay from primary tumour			
Synchronous	20	6	0.860
Metachronous			
<2 years	30	11	
>2 years	10	3	
Number of resected metastases			
1	50	16	0.624
2–3	10	4	
Size of the largest deposit			
<5 cm	49	18	0.238
⩾5 cm	11	2	
Preoperative CEA (μg/l)			
⩽5	21	10	0.059
>5	39	10	
Previous adjuvant chemotherapy			
Yes	25	11	0.138
No	35	9	
Liver metastases			
Adjuvant chemotherapy			
Yes	20	10	0.038
No	28	6	
Lung metastases			
Adjuvant chemotherapy			
Yes	5	1	0.400
No	7	3	

TS, thymidylate synthase; CEA, carcinoembryonic antigen.

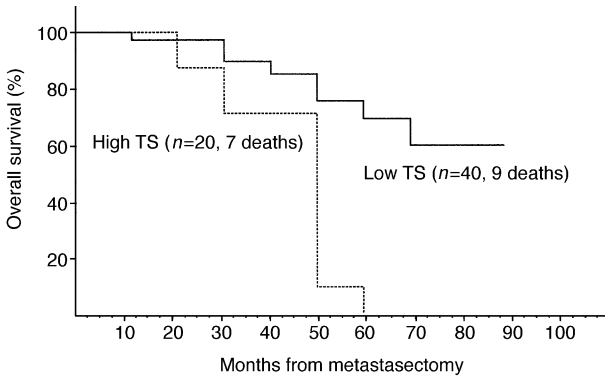


Fig. 2. Thymidylate synthase (TS) expression and overall survival after metastasectomy and systemic adjuvant therapy. Kaplan–Meier curves for the probability of survival of the 60 patients after resection of metastases, that were evaluated for TS expression, are shown. The probability of surviving for patients with high TS was significantly lower than the probability for patients with low TS expression ($P < 0.008$).

clinical–pathological features of the primary tumours, including Duke's stage, which is considered by other authors [19,20] to correlate with TS levels. In agreement with Findlay and colleagues [27], we believe that this finding is consistent with the evidence that all patients in our series, regardless of their initial Dukes' stage, ultimately relapsed, and the TS levels found may therefore be different from those in a population of relapse-free survivors of a similar stage.

Different methods for quantitating the levels of TS protein and TS gene expression have been developed [28,29]. The development of antibodies to the TS protein has provided the opportunity to quantitate the levels of expression of this enzyme in routinely available, paraffin-embedded tumour samples, using low-cost immunohistochemical assays. This approach makes it possible to check for tumour heterogeneity, to avoid the contamination of tumour samples with normal tissues and to use archival material. Moreover, TS immunostaining, using the TS 106 antibody, has been shown to correlate with *TS* mRNA expression, assessed by the reverse transcriptase-polymerase chain reaction (RT-PCR) technique, in a series of gastrointestinal tumour samples [22].

Our data demonstrated a significantly higher TS expression in primary carcinomas than in metastases and no difference in the TS protein levels between liver and pulmonary metastases. 26 out of 49 patients (53%) showed high TS levels in primary disease compared with 20 out of 60 (33%) high expressors in the metastases ($P < 0.04$). Consistent with other findings, these data confirm that TS levels in the primary tumour were not able to predict TS expression in the corresponding metastatic sites. This variation may reflect a different regulation of TS expression in primary and in metastatic lesions [27,30]. Additional investigations are required to clarify the basis for the higher TS content of primary colorectal cancer compared with the corresponding metastases.

Two recent reports found a higher TS level in pulmonary than in liver metastases, using PCR and western blot analysis [11], and, using immunohistochemistry [13], a higher TS expression in abdominal recurrences than in liver metastases. In our study, comparison between TS protein levels in liver and lung metastases showed no difference. We think that the difference between our results and those reported by Gorlick and colleagues may be explained by the limited number of cases in both studies, consequently additional investigations are required to evaluate TS expression in the different metastatic sites.

There is evidence that treatment with 5-FU may induce increasing levels of TS [16]. We observed that the resected metastatic lesions of patients previously treated

Table 3
Predictive factors of recurrence and survival after metastasectomy. Univariate and multivariate analysis

Variable	Univariate analysis				Multivariate analysis			
	DFI		OS		DFI		OS	
	Median	*P* value	Median	*P* value	RR	*P* value	RR	*P* value
All pts					(95% confidence Intervals)		(95% confidence Intervals)	
Preoperative CEA								
⩽5 μg/l versus >5 μg/l	20.1 versus 8.6	0.023	–	–	1.9 (1.6–2.1)	0.024	–	–
Extension into serosa								
Absent versus present	19.6 versus 9	0.019	33 versus 19.5	0.012	–	–	2.4 (2.1–2.7)	0.040
TS status								
High versus low	–	–	27.6 versus 36.3	0.007	–	–	3.2 (3.0–3.4)	0.013
Liver metastases (48 patients)								
TS status								
High versus low	–	–	25.7 versus 35.3	0.024	–	–	4.3 (4.0–4.6)	0.038

DFI, disease-free interval; OS, overall survival; RR, relative risk; CEA, carcinoembryonic antigen. Non-predictive factors of recurrence and survival after metastasectomy were: age, lymphatic spread, delay from primary tumour, number of resected metastases.

with an adjuvant 5-FU chemotherapy after the resection of the primary tumour had higher mean TS protein levels than patients who did not receive adjuvant chemotherapy, although this difference was not statistically significant. In the subgroup of liver metastases, however, we found a positive correlation between prior adjuvant chemotherapy and high TS levels in metastases ($P = 0.038$). These results can be explained by the fact that, in our study, the delay between the primary tumour and liver metastases was shorter and the occurrence of these metastases was therefore closer in time to the administration of chemotherapy than that of the lung metastases.

The published data on systemic or intrahepatic chemotherapy after radical resection of liver metastases are inconclusive, due to the study design and drug schedules used [3–6]. No data at all have been published regarding the use of chemotherapy after lung resection.

In our study, we used one of the standard chemotherapy regimens employed in the adjuvant setting of CRC. The median DFI was 19.4 months (range 2+ to 75+) and the median OS was 29.5 months (range 2+ to 85+). We observed an advantage in median DFI and OS for patients who underwent lung resection compared with those who received a liver resection (37.7 months versus 19.3 months, and 44.4 months versus 27.6 months, respectively), but it was not significant.

The results of both the univariate and multivariate analyses demonstrate a significant inverse correlation between TS immunohistochemical staining in the resected metastases of colorectal cancers and OS after metastasectomy. In the univariate analysis, a different, but not significant, DFI was also observed in patients expressing low and high TS in metastases. Consistent with previous studies [1,2], other prognostic factors were detected in this cohort of patients. The extension into the serosa and preoperative CEA levels in the primary tumour have been shown to affect the DFI considered after the metastasectomy and the adjuvant therapy, but they are less predictive of OS than TS expression. Interestingly, the relationship between extension into the serosa and OS is independent of the chemotherapeutic agent used, whereas the predictive role of TS may be useful in selecting patients unlikely to benefit from TS inhibitors. Nevertheless, in the multivariate analysis TS immunostaining was the only predictor of clinical outcome in the subgroup of patients with liver metastases.

In agreement with our results, Bathe and colleagues found that increased *TS* gene expression (measured by the RT-PCR technique) is associated with a poor outcome in patients with liver metastases from colorectal carcinoma, whether resected or treated by chemotherapy only [12]. Taken together, these data confirm that TS expression reflects an inherently more aggressive behaviour of metastases.

Several explanations for this association have been proposed. The biological relevance of TS relates not only to the importance of this enzyme as a chemotherapeutic target, but also as a DNA synthetic enzyme associated with cell division and proliferation. Previous studies have shown an increase in TS of up to 40-fold in synchronous cells as they leave G0 and enter the S phase of the cell cycle [31,32]. More recently, a close association between TS expression and cell proliferation has been reported [33]; *in vitro* studies have showed that the TS protein binds to *c-myc* mRNA to form a ribonucleoprotein complex and to p53 mRNA, which results in decreased *p53* protein synthesis [34,35], suggesting that the prognostic effect of TS protein expression may be due to its involvement in the coordinated regulation of tumour suppressor or dominant oncogenes [36].

The results of our study show that TS levels in primary colorectal cancer do not reflect those observed in the corresponding metastases and fail to predict their response to 5-FU-based chemotherapy.

Our result also indicate that a prospective assessment of TS staining intensity in colorectal metastases could be useful to define which patients will most likely benefit from adjuvant therapy after metastasectomy. One possible explanation for failing to demonstrate a clinical benefit in patients treated with adjuvant chemotherapy after surgical resection of colorectal cancer is the lack of an individual treatment strategy. Our data suggest that TS assessment in resected metastases of colorectal cancer will render it possible to select appropriate drugs and to emphasise the benefit of the adjuvant treatment after metastasectomy.

Chemotherapeutic agents that target TS may not be the appropriate adjuvant treatment after metastasectomy for patients with a high TS expression in the resected metastases of CRC cancer, for whom other compounds, such as irinotecan or oxaliplatin, may be proposed as an initial therapy. Our data could also be useful in the neoadjuvant setting. In fact, a relevant number of patients with colorectal liver metastases have unresectable disease at presentation. Neoadjuvant therapy may convert a selected group of such patients into an operable state, as well it may be useful in resectable patients also. At present, there are many active agents that can be used singly or in combination. The assessment of the TS expression in metastases before surgery will render it possible to individualise the treatment according to the different TS levels and it will give the possibility of improving the results of chemotherapy in the neoadjuvant setting.

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