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Original Paper

Immunohistochemical Determination of Thymidylate Synthase in Colorectal Cancer—Methodological Studies

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With the aid of specific monoclonal antibodies, an immunohistochemical technique has recently been developed for the detection of intratumoral thymidylate synthase (TS). This technique can be applied to paraffin-embedded material suitable for retrospective studies. In order to examine this technique further, the TS enzyme activity of lysates from frozen-stored colorectal cancer (CRC) specimens were compared with their immunohistochemical TS staining intensity (arbitrarily graded from 0 to 3). A statistically significant correlation between these two methods on a total of 25 tumour specimens ($P < 0.001$) was observed. The staining intensity in different areas of 48 paraffin-embedded CRCs was examined. Sixty-seven per cent of the tumours were homogeneously stained (either grades 0–1 or 2–3), 33% showed a heterogeneity in TS staining. Increased TS expression correlated with more advanced Dukes' stage ($P < 0.001$). It is concluded that TS immunostaining intensity reflects TS enzyme activity in colorectal tumours and is well suited for paraffin-embedded material. The TS immunostaining pattern is heterogeneous in up to one-third of the tumours. © **1997 Elsevier Science Ltd.**

Key words: thymidylate synthase, colorectal cancer, immunohistochemical determination, heterogeneity, enzyme activity, Dukes' stage
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INTRODUCTION

NEW HOPE for an improved prognosis of patients with colorectal cancer (CRC) has recently appeared after decades of fruitless efforts. Pre-operative radiation therapy and a modified surgical technique have been reported to reduce the local recurrence rate and possibly also prolong the survival of patients with rectal cancer [1–4]. Postoperative adjuvant treatment of CRC patients with specific monoclonal antibodies seems promising [5] and prolonged 5-fluorouracil (5-FU)-based chemotherapy has been shown not only to reduce the recurrence rate but also to significantly prolong survival [6]. Candidates for adjuvant 5-FU-based chemotherapy, which causes severe side-effects in some patients, are those who are at high risk of developing disseminated disease. Invariably, the stage of the tumour is taken into consideration when deciding whether chemotherapy is advisable. The classification of Dukes', or modifications thereof, has been widely used since its publication in 1932 [7]. At present, considerable efforts are being made to determine whether factors other than tumour stage, such as altered gene expressions of cancer cells, may predict prognosis.

The enzyme thymidylate synthase (TS) which catalyses the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) can now be immunohistochemically detected. Recently, it was demonstrated that immunohistochemically detected intracellular levels of this enzyme in primary rectal cancer correlates with both disease-free and overall survival independent of Dukes' stage [8]. Its possible relationship to the sensitivity of 5-FU-based chemotherapy is still unclear. However, one of the cytotoxic metabolites of 5-FU, fluorodeoxyuridine monophosphate (FdUMP), functionally inactivates TS thereby inhibiting DNA synthesis (for reviews, see refs [9] and [10]).

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Thus, in patients with CRC, the clinical value of intratumoral TS levels as a determinant of prognosis and as a predictor of 5-FU sensitivity needs to be urgently defined.

Several such studies have been performed using different methods for TS determinations. The FdUMP binding assay can measure total protein [11], whereas the dUMP assay is a catalytic assay [12]. Measurement of TSmRNA uses the PCR technique [13, 14] and immunohistochemical TS detection utilises specific monoclonal antibodies [15, 16]. The aims of the present study were: to compare the TS enzyme activity of tumour lysates with a recently developed immunohistochemical staining technique using the monoclonal antibody TS 106 [15, 16]; to examine TS expression in different parts of a primary CRC; and to determine any association between TS expression of primary CRC and Dukes' stage.

MATERIALS AND METHODS

Patients

All patients of this study had undergone surgery at Södersjukhuset, Stockholm. 25 consecutive patients who had been operated on for primary CRC during the years 1993–1994 were included in the study comparing two techniques for TS determination (see below). The mean age of the patients was 70 years (range 41–90 years) and there were 13 women and 12 men. 21 had carcinoma of the colon and 4 carcinoma of rectum. Tumours from 48 patients who had been operated on for primary CRC during the years 1994–1995 were studied immunohistochemically for TS expression within different sections of the tumour, and TS expression was compared to Dukes' stage. The mean age of the patients was 69 years (range 30–90 years) and there were 21 women and 27 men. 29 had carcinoma of the colon and 19 carcinoma of the rectum.

Determinations of TS enzyme activity

Tumour specimens (less than 0.5 cm^3) were collected, one from each patient taken during surgery. The specimens had, at the time of surgery, been freed of adjacent tissues, and placed on an ice-bed for a maximum of 3 h before being stored at −70°C. Half the frozen tumour was fixed in formalin and embedded in paraffin for immunohistochemical analysis (see below) and the other half was used for determination of TS enzymatic activity as described by Roberts [12]. Tumour specimens were minced with scissors, washed in Tris–NaCl buffer and pressed through a nylon net. These tumour-derived cells were sonicated for 20 s at +4°C followed by centrifugation at 14 000 rpm for 30 min. The protein concentration of the lysates was determined by the BioRad Protein assay [17]. Different dilutions of the lysates in 0.6 ml, corresponding to protein concentrations ranging from 10 to 125 μg/ml, were added to 100 μl of a reaction mixture containing 0.15 M Tris–HCl (pH 7.5), 10 mM NaF, 9 mM DL-dithiothreitol, 0.052% formaldehyde, 100 μM tetrahydrofolate, 67 μM dUMP (Sigma), 49 kBq of (5-^{3}H) dUMP (0.57 TBq/mmol, Amersham) and 0.1% bovine serum albumin. The mixture was incubated for 30 min at 37°C. The reaction was terminated by adding 40 μl ice-cold 20% trichloroacetic acid and 10 μl of unlabelled dUMP (5 mg/ml). 200 μl of a charcoal suspension (100 mg/ml) was then added to adsorb unreacted (^{3}H)dUMP, incubated for 1 h and then pelleted by centrifugation. 100 μl of the supernatant was mixed with 4.5 ml of Gold-TM scintillation fluid (Canberra Packard) and the radioactivity was determined by a Packard scintillation counter and expressed as counts per minute (cpm).

Immunohistochemical determination of TS

A slight modification of the method described by Johnston and associates [13, 16] was used. Paraffin-embedded tumour specimens were sliced into 6 μm sections, deparaffinised in xylene, rehydrated by incubation in decreasing concentrations of ethanol and then washed with phosphate-buffered saline (PBS) with Tris. Endogenous peroxidase activity was quenched with 3% hydrogen peroxidase in PBS for 10 min followed by rinsing for 5 min in tap water and 5 min in PBS. The sections were then covered with 20% horse serum and then incubated for 90 min at room temperature with TS106 monoclonal antibodies. After rinsing with PBS–Tris, the sections were incubated with biotin conjugated anti-mouse secondary antibodies (Vectastain ABC, Vector Lab) for 30 min, rinsed and incubated with a peroxidase-conjugated biotin–avidin complex for 30 min. Finally, they were incubated in PBS–Tris buffer containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.005% hydrogen peroxide and counterstained with haematoxylin. The intensity of TS staining of the tumour cells was arbitrarily graded from 0 (no staining) to 3 (highest-intensity staining) as described [8]. Each time a set of tumour samples were stained we included sections from tumours which were previously classified as 0 and 3 as references. The agreement of TS intensity reached by two independent observers was more than 90%. In some cases, grading was determined by consensus.

Data processing and statistical evaluations

Mean values were calculated on an arithmetic basis. The statistical correlations of the data presented in Figure 1 and Table 3 were calculated using the Spearman correlation test and the data in Table 1 with the chi-square exact trend test (two-tailed).

RESULTS

Comparison between TS enzyme activity and intensity of TS staining in CRC specimens

In serial dilutions of tumour specimens, the TS enzyme activity increase was linear up to a plateau. The maximum activity in most tumour specimens was noted at a protein concentration of 75 μg/ml. The tumour specimens which were examined for TS enzyme activity were also graded immunohistochemically for TS expression. The highest enzyme activity on the linear part of the curve of each patient was plotted against its staining intensity (Figure 1). Using

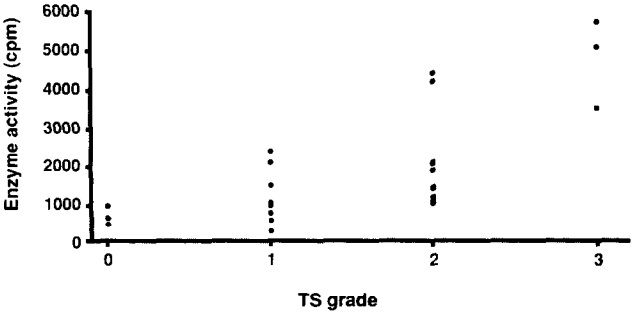

Figure 1. TS staining intensity (expressed in TS grades) and maximum enzyme activity of 25 individual primary CRCs.

Table 1. Intensity of TS staining (low/high) in different parts of CRCs

Dukes' stage	Homogeneous (%)	Heterogeneous (%)
A–C ($n=48$)	32 (67)	16 (33)
A ($n=12$)	9 (75)	3 (25)
B ($n=21$)	14 (67)	7 (33)
C ($n=15$)	9 (60)	6 (40)

Spearman's correlation test, we found a statistically significant correlation between the two methods ($r_s=0.70$, $P<0.001$).

Intensity of TS staining in different parts of a CRC and relation to tumour stage

In order to examine the distribution of TS in different parts of a tumour, samples for staining were taken from around the periphery (usually from the upper, lower, left and right edges) and in the centre (3–6 samples from the same tumour). 21 of the 48 tumours (44%) had the same TS staining in all samples, 23 (48%) of the tumours displayed a difference of one TS grade and 4 tumours (8%) more than one TS grade. 12 of 48 tumours had a staining intensity range of 1–2, three had a range of 1–3 and one tumour ranged from 0 to 3. The central area of a tumour did not consistently express a lower or a higher staining intensity than the periphery. When grades 0 and 1 are defined as low-intensity staining and grades 2 and 3 as high-intensity staining, 67% of the CRCs were homogeneous with respect to TS expression and 33% heterogeneous (Table 1). As can be seen in Table 1, the frequency of tumours with a heterogeneous TS distribution tended to increase with increasing stage of tumour. This trend was not statistically significant. The data suggest that the probability of finding a representative area of the tumour for TS staining (low/high) with a single biopsy is 89% (Table 2). A second biopsy would raise the probability to 99%. The probability of finding an area of maximum TS staining (low/high) with a single biopsy is 81% and with two biopsies 96%. Moreover, there was a significant relationship between TS grade (highest value observed in a tumour) and Dukes' stage ($n=48$, $r_s=0.58$, $P<0.001$) (Table 3).

Table 2. Probability of finding a representative area for TS staining (low/high) and an area with maximum TS staining (low/high) with one biopsy

	Probability of finding a representative area of TS staining	Probability of finding the area of maximum TS staining
Dukes' stage A–C (236 samples)	89%	81%
Dukes' stage A (51 samples)	92%	88%
Dukes' stage B (109 samples)	88%	82%
Dukes' stage C (76 samples)	87%	74%

Table 3. Highest TS grade (grade 0–3) of 48 individual CRCs in relation to Dukes' stage

	TS grade			
	0	1	2	3
Dukes' stage				
A ($n=12$)	0	8	3	1
B ($n=21$)	0	5	10	6
C ($n=15$)	0	0	6	9

DISCUSSION

The ultimate aim of this ongoing study was to define the possible prognostic value of intratumoral TS concentration in primary CRC and to examine its possible linkage to different treatment modalities such as radiation therapy and 5-FU-based chemotherapy in both the adjuvant setting and advanced disease. The most convenient approach to answer these questions in a reasonably short time would be to analyse retrospectively paraffin-embedded material from CRC patients. As mentioned in the Introduction, there are several TS assays available. Most of them, however, require fresh or frozen tumour tissue and are thus not suitable for formalin-fixed paraffin-embedded material. The immunohistochemical determination of TS using the monoclonal antibody TS106 developed by Johnston and associates [15, 16] seems attractive for several reasons: (i) formalin-fixed paraffin-embedded tumours can be stained with this technique. This makes it possible to examine retrospectively the relationship between TS content and factors such as time to recurrent disease, survival and value of adjuvant treatments. (ii) A distinction can be made between the TS content of malignant and non-malignant cells in a tumour and it is possible to examine the staining pattern in relation to the histology in various parts of a tumour section. A possible disadvantage of the method is that the staining intensity of a tumour section is not, at present, based on an objective measurement but rather graded arbitrarily from 0 to 3 by one or more observers. The agreement between different observers, however, is high. Since this method and most other techniques simply reflect the amount of TS present and not the potential functional activity of this enzyme, we examined CRC specimens with respect to both enzyme activity and staining intensity. Because of the heterogeneity (see below), it is important to emphasise that both types of TS determinations were performed on one small specimen ($<0.5\,cm^3$ in size) and that the TS content, as determined by one or both of these methods, may have declined during storage of the tumour.

We observed a statistically significant correlation between the peak enzyme activity values and the TS staining intensity of the respective CRC samples (Figure 1). These results are analogous to those of other researchers, who observed a significant agreement between staining intensity and specific mRNA contents of tumour cells [13]. Thus, the TS immunohistochemical staining intensity reflects the potential catalytic activity of TS in a tumour.

Since different parts of a tumour may exhibit different TS activities, we examined different parts of CRCs. If most of the tumours are homogeneously stained, a very small sample would be representative of the whole tumour. The probability of finding the highest TS staining intensity (low/high) of a tumour is close to 100% when two biopsies are examined.

Since we have confirmed that there may be a significant correlation between TS grade and Dukes' stage [8], we are now examining the possible relationship between immunohistochemically determined TS levels and prognosis in primary CRC patients and comparing TS levels of metastasis with the primary CRC.

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