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Dietmar Öfner · Hans Maier · Bernhard Riedmann
Tanja Bammer · Astrid Rumer · Günther Winde
Werner Böcker · Bharat Jasani · Kurt W. Schmid

Immunohistochemical metallothionein expression in colorectal adenocarcinoma: correlation with tumour stage and patient survival

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Abstract Metallothioneins (MTs), a set of ubiquitous low-molecular-weight proteins essential for the protection of cells against heavy metal ion toxicity, were demonstrated immunohistochemically using a monoclonal antibody (E9) against a conserved epitope of I and II isoforms in a series of 109 colorectal adenocarcinomas. In a semiquantitative analysis strong MT expression in the majority of tumour cells was observed in 34 (31%) cases, 24 (22%) tumours showed a focal MT positivity, and 51 (47%) almost completely lacked MT expression. These differences in MT expression were statistically significantly ($P<0.05$) associated with the tumour stage (Dukes classification) and the lymph node involvement at the time of operation (pN stages). However, in contrast to previous findings obtained on a variety of tumours, MT positivity was associated with a favourable clinical outcome in colonic carcinoma, which may indicate their different biological behaviour. Survival curves of cases with MT-positive and MT-negative status differed from each other in a univariate analysis (Mantel-Haenszel: 8.9, $P<0.05$) but lost significance when a multivariate analysis was carried out by means of the Cox proportional regression model with Dukes' stages as a stratification factor. It is concluded that immunohistochemically demonstrated MT expression is significantly associated with tumour stages but does not represent an independent prognostic variable in colorectal cancer. However, it may provide important information about some of the biological mechanisms underlying progression in colorectal cancer.

Key words Metallothionein · Immunohistochemistry
Colorectal cancer · Prognosis

D. Öfner · W. Böcker · K.W. Schmid (✉)
Gerhard-Domagk-Institute of Pathology, University of Münster, Domagkstrasse 17, D-48149 Münster, Germany

D. Öfner · B. Riedmann · T. Bammer · A. Rumer
Department of Surgery I, University Hospital, Innsbruck, Austria

H. Maier
Department of Pathology, University of Innsbruck, Innsbruck, Austria

G. Winde
Department of Surgery, University of Münster, Münster, Germany

B. Jasani
Immunocytochemical and Molecular Pathology Unit, University of Wales College of Medicine, Cardiff, UK

Introduction

Metallothioneins (MTs) are a set of ubiquitously expressed low-molecular-weight proteins with a high content of cysteins exhibiting a selective binding affinity for heavy metal ions [19]. MTs appear to play a homeostatic role in the control of extracellular zinc and detoxification of copper and particularly cadmium. Furthermore, MTs are implicated in transient response to any form of stress or injury (inflammation, ischaemia, ionising radiation, alkylating agent cytotoxicity) providing a cytoprotective mechanism against potential damaging effects of oxygen-derived free radicals (for review, see [19]). Immunohistochemically detectable over-expression of MTs has been demonstrated in a variety of human tumours. Recently, a statistically significant correlation of MT expression and poor clinical outcome was found in invasive ductal carcinomas of the breast [14, 16, 31, 33, 35] and malignant melanomas [34, 38, 43]. In the present study, 109 colorectal carcinomas were investigated immunohistochemically with a monoclonal anti-MT antibody reactive against a conserved epitope shared by I and II isoforms of human MTs. The present study was designed to assess MT expression and its distribution patterns as a possible prognostic variable in colorectal cancer with regard to histological grading and staging.

Patients and methods

Tumour tissues from 109 consecutive cases of colorectal adenocarcinoma (43 rectal carcinomas, 45 carcinomas of the left colon, and 21 of the right colon; 56 male, 53 female patients; mean age 67.8 years, range 35–90 years) were investigated in this study. All patients had been operated upon between 1984 and 1986 at the

Table 1 Frequency of prognostic variables investigated in 109 colorectal adenocarcinomas

Parameter	No	%
Tumour type		
intestinal	87	80
mucinous	17	16
signet ring cell	5	4
Histological grading		
well	15	13
moderate	69	64
poor	25	23
Lymphocytic infiltration		
mild	43	39
moderate	49	45
significant	17	16
Dukes stage		
A	11	10
B	48	44
C	32	30
D	17	16
pT stage		
pT1	12	11
pT2	34	31
pT3	51	47
pT4	11	10
pN stage		
pN0	56	51
pN1	13	12
pN2	20	18
pN3	7	6
pNx	13	12
Tumour site		
right hemicolon	21	19
left hemicolon	45	41
rectum	43	40
Age		
≤65 years	38	35
>65 years	71	65
Sex		
male	56	51
female	53	49

Department of Surgery I, Innsbruck University Hospital, Austria, either with curative (n = 92) or palliative intent (n = 17). Patients who died within 30 days of surgery, with adjuvant chemo- and/or radiotherapy, or members of families with familial adenomatosis coli or hereditary nonpolyposis colorectal cancer were excluded from this study.

Representative cross-sections of the tumours (in 68 cases one tumour block, in 41 cases two tumour blocks) were routinely processed (formalin-fixed and paraffin-embedded) and were classified according to the Dukes classification [8], the TNM staging system [39], and the WHO grading system [27]. Lymphocytic infiltration at the advancing edge of the tumour was determined according to the criteria of Jass et al. [18]. Table 1 gives a detailed description of staging and grading results. The mean follow-up period of the 55 patients still alive (April 1994) or lost during the follow-up period (n = 3) was 79 months.

In 78 of the 109 cases, normal and/or transitional mucosa adjacent to the carcinoma was present on the sections investigated. All 11 Dukes A and 14 Dukes B cases showed a residual adenomatous tumour component. Liver metastases were investigated in two Dukes D cases.

Antibody

The preparative details of the monoclonal anti-MT antibody (designated as E9) used in this study have been described elsewhere [35]. The antibody was a generous gift from Professors J.M. Stark, A. Cryer, and J. Kay, University of Wales, Cardiff, UK. This antibody has been used in its unfractionated ascites form to demonstrate immunoreactive MT in a variety of formalin-fixed and paraffin-embedded tissues of rat and human origin [9, 10, 15, 17]. The antibody is now commercially available in its purified form from Dako (Copenhagen, Denmark).

Staining procedures

The monoclonal MT antibody was applied overnight at 4°C in a humidified chamber [dilution in phosphate-buffered saline (PBS) containing 0.6% bovine serum albumin 1:30,000], followed by a goat anti-mouse bridging antibody (1:30 in PBS; 30 min at room temperature; Dako) and a polyclonal mouse APAAP complex (1:100 in PBS; 60 min at room temperature; Dianova, Hamburg,

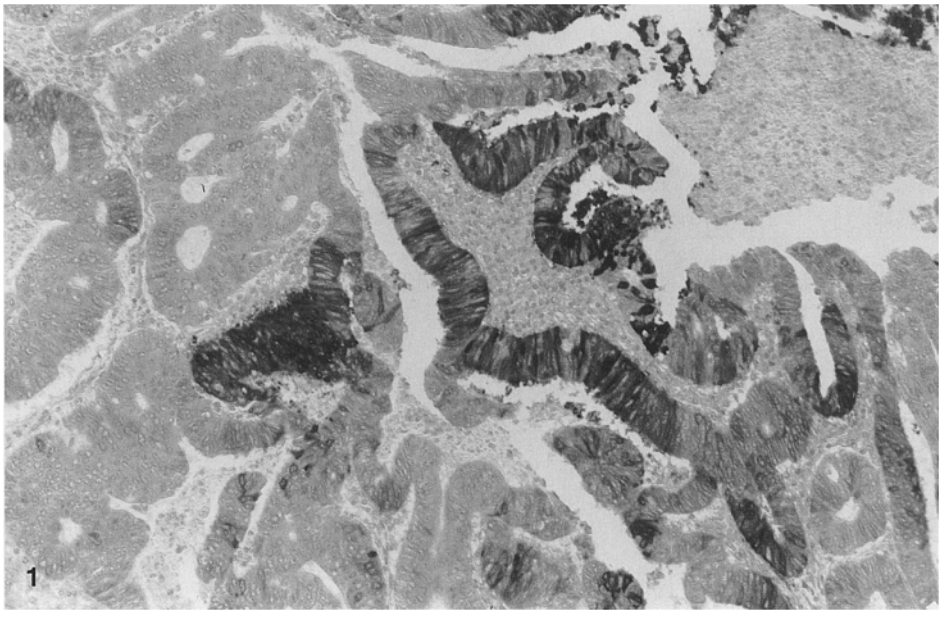

Fig. 1 Focally accentuated immunhistochemical expression of MT in a Dukes B colorectal carcinoma. APAAP-technique, ×40

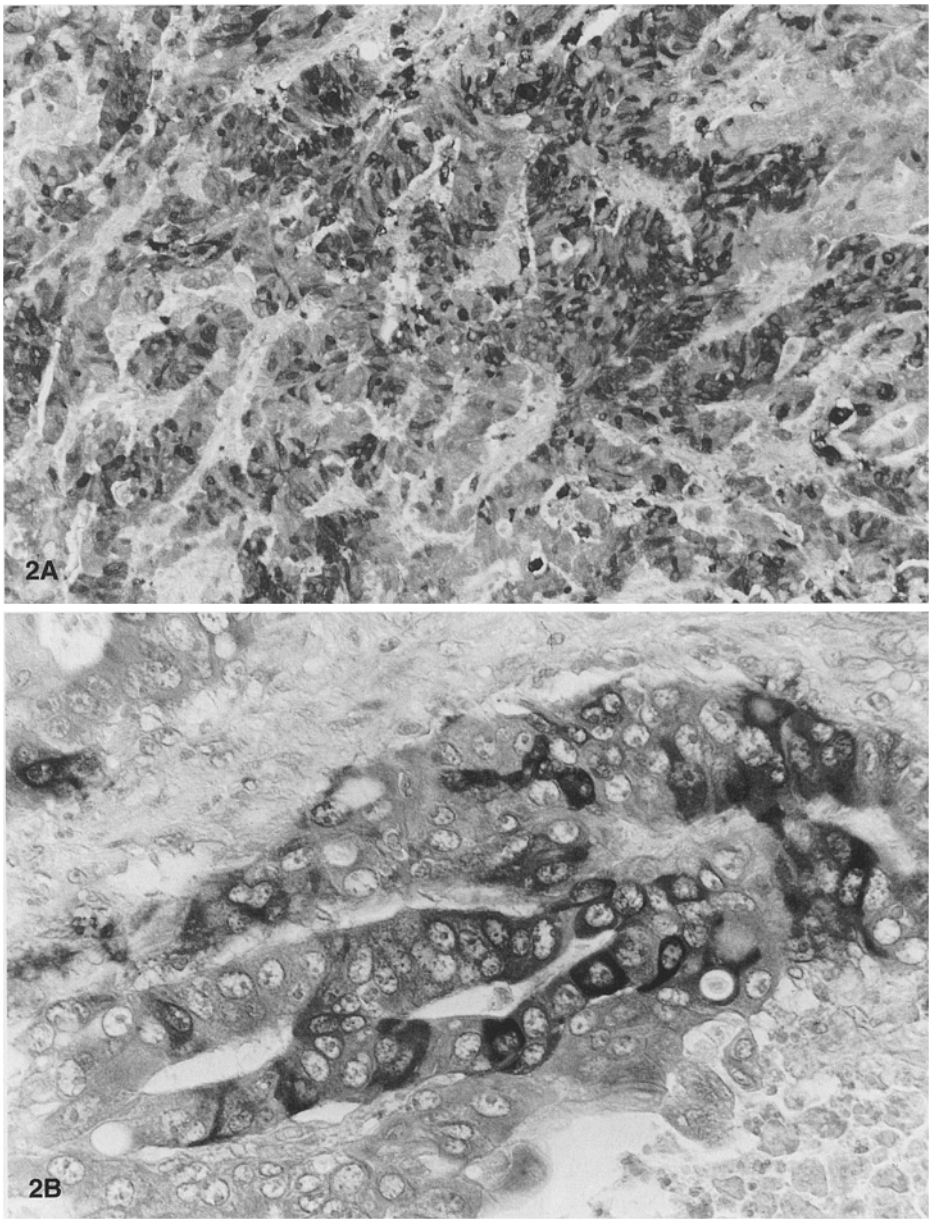

Fig. 2A Moderate to strong metallothionein (MT) immunoreactivity in the majority of tumour cells in a Dukes B colorectal carcinoma. APAAP-technique, ×40. **B** Higher magnification of a Dukes C colorectal carcinoma demonstrating moderate to strong MT staining mainly located in the cytoplasm of tumour cells. Some tumour cells show both nuclear and cytoplasmic MT positivity. APAAP-technique, ×250

FRG). The bridging antibody and the APAAP complex were applied on a semi-automatic immunostaining device ("Omnibus"; Quartett, Berlin, Germany). Subsequently the enzyme reaction was developed for 25 min at room temperature in a freshly prepared new fuchsin solution containing naphthol-bi-as-phosphate. Finally the sections were counterstained with haematoxylin and mounted in Kayser's glycerine gelatine. Omission of the primary antibody was used as a negative control, and normal human kidney as a positive control.

Semiquantitative assessment of immunohistochemical staining patterns

Semiquantitative evaluation was performed by one of the authors (K.W.S.). The procedure was carried out twice by the same investigator with a 3-week interval. The staining pattern was assessed as follows. No MT detectable or less than 5% MT-positive cells, 5– <50% of tumour cells in a clustered focal distribution, and more than 50% diffusely distributed MT-positive cells.

Patient follow-up and statistical analysis

All data were entered into a Macintosh IIci microcomputer and statistical analysis was carried out using the SYSTAT statistical package [42] including the SURVIVAL supplementary module [40]. Patients were followed-up according to the oncological follow-up scheme of the Department of Surgery I, University of Innsbruck: clinical and laboratory examination (including tumour marker carcinoembryonic antigen) every 3 months within the first 3 years, every 6 months in years 4 and 5 after surgery, and once a year afterwards. Obligatory colonoscopy or barium enema and chest radiography was performed twice per year in years 1–3, and once a year thereafter until year 5 after operation. Additionally, the data concerning the date and cause of death were confirmed by the *Österreichisches Statistisches Zentralamt*, an institute of the Austrian government. The cumulative patient survival was estimated using the Kaplan-Meier method [21]; for comparison of the survival curves the log-rank test (method: Mantel-Haenszel) was used [20]. The Cox proportional hazards linear regression model [6], with Dukes stage as a stratification factor, was used to determine in a forward stepwise procedure which factors were associated simultaneously with survival. Esti-

mates of relative risks and 95% confidence intervals (CI) were generated from the estimates of regression coefficients and associated standard errors. Descriptive statistics for continuous measures are given as the mean with the respective standard deviation in parentheses; for discrete data frequency counts and percentages are tabulated and groups were compared using chi-square analysis with Yates' correction whenever appropriate.

Results

About half of the cases investigated (51 out of 109, 47%) almost completely lacked immunohistochemical detectable MT. In Dukes D cases 13 of 17 tumours (76%) were negative for MT; the remaining 4 cases showed only focal MT expression. Focal MT distribution (Fig. 1) was also found in 24 cases with increasing rates from Dukes A (9%) to Dukes B (18%) and Dukes C stages (31%). In contrast, pronounced staining of MTs (34 of all 109 cases, 31%, Fig. 2) was detected more frequently in Dukes A (45%) and B (43%) stages than in Dukes C cases (25%), whilst Dukes D stages lacked strong MT immunoreactivity (Table 2). This correlation was statistically significant (total chi-square: 14.9, $df = 6$, $P<0.05$). The distribution patterns of MTs were similarly associated with lymph node involvement at the time of operation. Tumours with advanced lymph node involvement (pN2 and pN3) were frequently MT negative or only focally positive (Table 3). The correlation between MT over-expression and pN stages was also statistically significant (total chi-square: 11.4, $df = 6$, $P = 0.05$). Correlations between MTs and other grading or staging parameters evaluated were not significant (Table 4).

Considerable MT staining was found in the normal and/or transitional mucosa in 63 of 78 cases (MT was focally or strongly positive in the respective tumours in 44 of 78 cases) which contained normal/transitional mucosa in the sections investigated. MT positivity of the normal/transitional mucosa was demonstrated in all 44 cases with concomitant MT-positive and 19 MT-negative tumours. Seventeen of 25 cases (8 Dukes A and 9 Dukes B cases) with residual adenomatous tumour components showed focal or diffuse MT positivity. Both liver metastases investigated were MT negative.

Figure 3 illustrates survival curves for all patients in the study in regard of Dukes stage and according to MT expression patterns. Univariate survival analysis of variables significantly associated with survival (Dukes stage, pT stage, pN stage, lymphocytic infiltration, MT scores, WHO grading) and variables which were not associated with prognosis (sex, age, tumour site) is shown in Table 5. Multivariate analysis by means of Cox regression model using Dukes stage classification as a stratification factor (relative hazard rates for patients with different Dukes stages were not constant over the follow-up intervals) revealed that only tumour grade according to WHO classification was associated with survival independently. The estimated increase in adjusted relative risk of death for patients with grade III tumours was 2.7 times higher (parameter estimate: 0.8; 95% CI: 0.2–1.4; $P = 0.006$) when compared with those with grade I and II tumours.

The semiquantitative assessment of MT staining pattern in the hands of an experienced investigator proved to be highly reproducible, since none of the cases were classified differently in the second evaluation performed several weeks after the first one.

Table 2 Immunohistochemical metallothionein (MT) expression in colorectal carcinoma with regard to Dukes stages (*neg* negative, *foc* focal, *pos* positive)

MT	Dukes	No.	%
neg		5	45.5
foc	A	1	9
pos		5	45.5
neg		19	39
foc	B	9	18
pos		21	43
neg		14	44
foc	C	10	31
pos		8	25
neg		13	76
foc	D	4	24
pos		0	0

Table 3 Immunohistochemical MT expression with regard to pN stages [pNx cases (n = 13, 4 Dukes A and 9 Dukes D cases) excluded]

MT	pN	No	%
neg		22	39
foc	pN0	10	18
pos		24	43
neg		4	31
foc	pN1	5	38
pos		4	31
neg		10	50
foc	pN2	6	30
pos		4	20
neg		6	86
foc	pN3	1	14
pos		0	0

Table 4 Correlation between MT scores and various prognostic variables investigated (*NS* not significant, *df* degrees of freedom)

Parameter	χ^2	*DF*	P
M stage	10.1	2	0.006
pN stage[a]	11.4	7	0.05
Dukes classification	14.9	6	0.02
pT stage	10.8	7	NS
Tumour site	6.3	4	NS
Lymphocytic infiltration	3.0	4	NS
Tumour type	1.7	4	NS
Histological tumour grade	1.6	4	NS
Age (≤65years vs>65years)	0.5	2	NS
Sex	2.1	2	NS

[a] pNx cases (n = 13, 4 Dukes A and 9 Dukes D cases) excluded

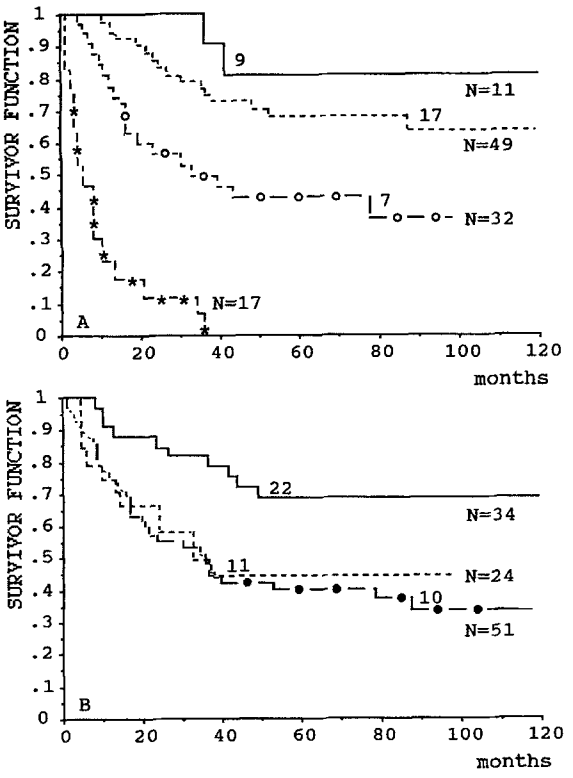

Fig. 3A,B Kaplan-Meier estimations. **A** Survival according to Dukes stages: – Dukes A, - - - Dukes B, -o-o- Dukes C, -*-*- Dukes D; Mantel-Haenszel: chi-square: 88.1, *df* = 3, *P* = 0.0001. **B** Survival in regard of MT scores: – positive, - - - focal positive, -●-●- negative; Mantel-Haenszel: chi-square: 8.9, *df* = 2, *P* = 0.01

Table 5 Prognostic factors examined in 109 colorectal carcinomas: a univariate approach to cancer-specific mortality (*NS* not significant, *df* degrees of freedom)

	Univariate χ^2 for the log-rank test	*df*	*P*
Dukes stage	88.1	3	0.0001
pT stage	17.7	3	0.0001
pN stage[a]	25.7	3	0.0001
MT	8.9	2	0.01
Lymphocytic infiltration	8.6	2	0.01
Histological tumour grade	7.4	2	0.02
Type of tumour	4.0	2	NS
Tumour site	2.2	2	NS
Age (≤65years vs>65years)	0.1	2	NS
Sex	0.001	1	NS

[a] pNx cases (*n* = 13, 4 Dukes A and 9 Dukes D cases) excluded

Discussion

Adenocarcinoma of the colorectum remains the second leading cause of death from cancer in western countries [4]. Those who survive are diagnosed early and benefit from surgical resection. Recent advances in adjuvant chemotherapy in combination with biological response modifiers and/or radiation therapy of advanced colorectal cancer [11, 24, 26, 32] have promoted the need for better diagnostic and prognostic assessment to determine a group of patients that may benefit from these therapeutic strategies.

The results of the present study show that immunohistochemically detectable MTs are significantly correlated with variables associated with the development of lymph node or distant metastases (cf. Table 3). MT expression decreased with increasing metastatic spread. In none of our cases a strong MT expression of the primary tumour was associated with stage Dukes D or pN3. Our findings of a correlation between MT expression and metastatic tumour progression are in accordance with a previous biochemical study by Mulder and co-workers [28] reporting a significantly decreased MT content (determined by radioimmunoassay) both in adenomatous polyps and carcinomas when compared with normal colonic mucosa. However, they did not study any relationship of MT content to Dukes stage. The biological significance of these observations remains unclear. Our results suggest significant association of MT expression with tumour progression and it is tempting to postulate that MT-negative tumour subclones in advanced colorectal carcinomas may have a higher metastatic potential.

However, the mechanism(s) for tumour MT over-expression and its/their association with prognosis are not well understood and conflicting results have been reported. Induction of MT synthesis secondary to changes in tumour cell metabolism [41], increased cellular activity [25, 29, 30], activation of oncogenes [36], and as a protective response to DNA damage [1, 12, 13] have all been proposed to explain increased levels of MT associated with tumours. Raised levels may also result from reduced degradation of MT. The MT turnover in the cytoid fraction is strongly influenced by the specific metal ions bound to the protein. For example, apometallothionein (metal-free), zinc MT, and cadmium MT are apparently rapidly degraded by lysosomal extracts whilst copper-bound MT appears to have a prolonged half-life [5].

It is therefore of interest that a number of studies have indicated increased amounts of tissue copper associated with a variety of malignant tumour types including melanomas and breast cancers [2, 3, 7, 22, 23, 37]. Immunohistochemical MT over-expression was shown to be associated with poor clinical outcome in invasive ductal adenocarcinoma of the breast [14, 16, 35] and in malignant melanoma [34, 43]. The finding of the present study that immunohistochemical MT expression was related to better clinical outcome is most likely due to differing biological behaviour of colonic carcinomas. MT expression seems to be a common response in a variety of tumours. We observed MT expression in normal colonic mucosa adjacent to the tumour, in adenomas, and in the majority of tumours in Dukes stages A and B. These results suggest that expression is an earlier event in colonic carcinoma than in breast cancer and malignant melanoma [35, 43]. Further studies may elucidate a possible relationship with other factors associated with tumour progression [Ki-67; p53; silver-stained nucleolar organizer region associated proteins (AgNORs)]. Additionally the

results of the present study demonstrate distinct differences between different tumour types in terms of immunohistochemical MT-expression patterns.

Interestingly, in the study of Mulder and co-workers [28], raised levels of copper/zinc-superoxide dismutase (Cu/Zn-SOD) were found to be associated with an increasing degree of malignant change in colonic tumours. However, they did not assess whether the MT analysed in the same tumour tissue as Cu/Zn-SOD had an increased copper content. Copper is bound to MT with much higher affinity and stability than zinc or cadmium and it is therefore possible that increased MT content of tumours simply reflects increased uptake of copper in tumour cells. This question clearly needs to be resolved in order to understand better the possible involvement of MT in malignant tumour progression.

MT expression cannot be regarded as an independent prognostic variable in colorectal cancer; it may, however, provide new insight into biological mechanisms underlying the continued success of currently used staging systems.

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