Papers

Prognostic evaluation of metallothionein expression in human colorectal neoplasms

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Abstract

Aim—To investigate the role of metallothionein in colorectal tumours and the possible relation with other factors associated with tumour progression: expression of cathepsin D (CD), CD44, p53, Rb, bcl-2, c-erbB-2, epidermal growth factor receptor (EGFR), proliferation indices (Ki-67, proliferating cell nuclear antigen (PCNA)), and conventional clinicopathological variables.

Methods—The immunohistochemical expression of metallothionein was investigated in 23 cases of colorectal adenoma and 94 adenocarcinomas. Metallothionein expression was examined by the avidin-biotin peroxidase immunoperoxidase (ABC) using the monoclonal mouse antibody E9, on formalin fixed, paraffin embedded tissue.

Results—Positive metallothionein expression (> 5% of neoplastic cells) was observed in 30.4% of adenomas and 25.5% of adenocarcinomas, while 8.7% of adenomas and 14.9% carcinomas showed focal metallothionein positivity. In contrast, 60.9% of adenomas and 59.6% of carcinomas almost completely lacked metallothionein expression. In the series of adenocarcinomas, metallothionein expression was inversely correlated with CD44 in neoplastic cells ($p = 0.01$). There was no statistically significant difference of metallothionein expression, or the other variables examined, between adenocarcinomas and adenomas.

Conclusions—Metallothionein expression does not seem to indicate aggressive biological behaviour in colorectal adenocarcinomas, in comparison with the other types of carcinoma. The inverse correlation with CD44 could suggest that the decreased metallothionein expression may contribute to the metastatic spread of the lymph node involvement in colorectal cancer. Metallothionein expression does not seem to represent an independent prognostic marker in colorectal cancer.

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Metallothionein is a low molecular weight cysteine-rich protein, which has the ability to bind and sequester heavy metal ions such as zinc, copper, cadmium, and mercury.¹ Synthesis of metallothionein is induced in various tissues by these metal ions as well as by endogenous factors such as glucocorticoids, interferon, interleukin-1, and vitamin D.^{2,3} Metallothioneins have the ability to bind to large quantities of metal ions, thus causing an intracellular reservoir of sequestration function for essential or potentially toxic ions such as zinc and copper. Furthermore, metallothioneins play an important role in the detoxification of toxic metals, for example cadmium,⁴ and probably in the cellular protection against ionising radiation and alkylating agent cytotoxicity.^{1,5,6} Metallothionein overexpression has been found in various carcinoma cell lines resistant to certain anticancer drugs.^{5,7,8} This protein may play a role in the development of drug resistance as well as in tissue resistance to metal carcinogens. Metallothionein also plays an important role in the homeostasis of zinc, a metal important to tumour growth and progression.⁹ Furthermore, metallothioneins are often associated with rapidly proliferating tissues, such as fetal and neonatal liver.¹⁰⁻¹² There are mechanisms whereby metallothionein and p53 may interact in the control of cell division.¹³ In addition, in vitro studies have shown that exposure to a metal chelating agent induces a reversible conformation change in wild type p53 to the mutant form. It was thought that binding of zinc ions to cysteinyl residues stabilises the tertiary structure of p53.¹⁴ Some direct evidence indicates that certain tumour types have highly concentrated metallothionein. The mechanism of tumour metallothionein expression is not fully understood. There is evidence that metallothionein may play a role in various carcinogenic processes. Metallothionein expression seems to be significantly associated with progressive disease and with poor prognosis in invasive ductal carcinoma of the breast,¹⁵⁻¹⁹ malignant melanoma,²⁰ and pancreatic carcinomas.²¹ In contrast, in colorectal carcinomas, metallothionein positivity is associated with a favourable clinical outcome, which may indicate variability of the biological significance

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Table 1 Antibodies used

Antibodies	Supplier	Dilution	Incubation time
Metallothionein, E9	Dako	1: 50	1 hour
Cathepsin D (O13A)	Dako	1: 300	30 minutes
CD44 (Clone DF 1485)	Dako	1:40	Overnight*
DO-7 (IgG2b)†	Ylem	1: 200	Overnight*
Anti-Rb (AB-5)†	Oncogene	1: 80	Overnight*
Anti-bcl-2(M0887)†	Dako	1: 40	Overnight*
C-erbB-2 (OM-11-925)	ICI, Cambridge	1: 500	1 hour
EGFR (AB-4)	Oncogene	1: 500	1 hour
PC-10	Dako	1: 50	1 hour
Ki-67	Dako	1: 10	30 minutes

*With microwave oven antigen retrieval.

†Citrate buffer incubation.

of metallothionein according to the tumour type.²² To elucidate the role of metallothionein in colorectal tumours we studied the immunohistochemical expression of metallothionein in conjunction with other potential prognostic variables such as cathepsin D, CD44, p53, Rb, bcl-2, c-erbB-2, epidermal growth factor receptor (EGFR), and proliferating activity estimated by the MIB-1 and proliferating cell nuclear antigen (PCNA). We also assessed the conventional clinicopathological indices (age, sex, tumour size, tumour grade, and Duke's stage).

Methods

Tissue from surgical specimens resected from 94 patients with primary colorectal carcinoma and from 23 patients with colorectal adenomas was collected and processed by standard techniques to paraffin wax embedding, after fixation in neutral buffered formal saline for 24–28 hours. In 53 cases one additional sample was taken shortly after surgical removal and snap frozen in isopentane liquid nitrogen, using an embedding medium for frozen tissue specimens, OCT compound (Tissue Tek-Miles). The frozen specimens were stored at -80°C until processing. Consecutive 4 μm sections were stained with haematoxylin and eosin for histological diagnosis. Slides from all tumours were reviewed and divided according to WHO classification with regard of the grade of dysplasia; there were 13 adenomas with low

dysplasia and 10 with high dysplasia. Histologically, all colorectal carcinomas were adenocarcinomas. The age of patients at the time of surgery ranged from 26 to 86 years. According to WHO criteria, the grade of differentiation was high in 21 cases, moderate in 65, and low in seven. According to Dukes' stage there were 12 cases of stage A, 42 cases of stage B, and 40 cases of stage C disease. In most cases the adjacent normal mucosa was also examined.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry on one or two selected blocks from each case was performed on 4 μm tissue sections using the avidin-biotin and immunoperoxidase method as previously described.²³ To unmask the epitopes of bcl-2, p53, and Rb, we used treatment with microwaves and the sections were placed in 10 mM citrate buffer, pH 6.0. Metallothionein, cathepsin D, CD44, c-erbB-2, EGFR, and PCNA staining was achieved without using the heat mediated antigen retrieval method. Subsequently, the sections were treated with 0.3% hydrogen peroxide (H_2O_2 in methanol) for 30 minutes to block endogenous peroxidase. The sources and dilution of the antibodies are shown in table 1. Tumour sections subjected to the whole procedure except for incubation with the primary antibody were used as substitute "negative" controls. For Ki-67 staining cryostat sections 4 μm thick were air dried and fixed in absolute acetone. They were stained with monoclonal antibodies against Ki-67 (Dako) using a sensitive two step indirect immunoperoxidase technique.

Immunohistochemical evaluation

Immunostaining (nuclear, cytoplasmic, or membrane cytoplasmic) was calculated as the percentage of positive tumour cells in relation to the total cell number in representative fields. Every stained nucleus was considered positive, irrespective of intensity. Only intense membrane immunostaining was considered to represent the overexpression of c-erbB-2 protein, as it has been shown to yield the best prognostic associations.²⁴

STATISTICAL ANALYSIS

The association of continuous variables was confirmed using a non-parametric test for two or several independent samples, or the Spearman bivariable correlation. Probability (p) values under 0.05 were considered statistically significant.

Results

The immunohistochemical localisation of metallothionein was both cytoplasmic and nuclear in the carcinoma cells (fig 1). Normal colorectal epithelium adjacent to carcinoma was negative or showed strong to weak cytoplasmic positivity for metallothionein, and the staining was confined to the proliferative zone, that is the lower crypts. Some peripheral nerves and smooth muscle cells were also stained. In 25% of carcinomas and 30% of adenomas, strong metallothionein expression was observed in the majority of tumour cells, while focal metal-

Figure 1 Strong metallothionein expression in a case of colorectal cancer. The reactivity is confined to the nuclei and the cytoplasm of the majority of neoplastic cells. Note the low metallothionein nuclear expression in the "normal" adjacent mucosa at the right. (Avidin-biotin-peroxidase complex stain, $\times 138$.)

Table 2 Expression of metallothionein in correlation with clinicopathological features of colorectal cancer

	Metallothionein expression			p Value
	(-)	<10%	>10%	
Age (years)				NS
<50	17	4	6	
>50	36	10	16	
Sex				NS
Female	29	5	13	
Male	25	9	10	
Tumour size (cm)				NS
<5	25	3	12	
>5	29	8	8	
Grade				NS
G1	16	3	2	
G2	37	10	18	
G3	3	—	4	
Duke's stage				NS
A	9	2	1	
B	25	7	10	
C	22	5	13	

Table 3 Metallothionein expression in correlation with potential prognostic variables in colorectal cancer

Prognostic variable	Metallothionein expression			p Value
	(-)	<5%	>5%	
Cathepsin D				NS
<10	18	2	6	
>10	26	11	15	
CD44				p=0.01
(-)	2	—	6	
<10	4	—	—	
>10	48	14	18	
p53				NS
<5	23	5	10	
>5	32	9	14	
Rb				NS
<10	25	5	7	
>10	31	9	16	
bcl-2				NS
<5	44	9	19	
>5	9	5	2	
c-erbB-2				NS
<25	27	2	7	
>25	5	1	4	
EGFR				NS
<25	47	11	18	
>25	9	3	6	
Ki-67				NS
<50	15	1	2	
>50	11	1	7	
PCNA				NS
<50	8	6	3	
>50	46	8	19	

EGFR, epidermal growth factor receptor; PCNA, proliferating cell nuclear antigen.

lothionein positivity occurred in 15% of the carcinomas and 9% of the adenomas; 60% of the carcinomas and 60.9% of the adenomas almost completely lacked metallothionein expression. Results of metallothionein expression with CD, collagen type IV, p53, pRb, bcl-2, c-erbB-2, EGFR proliferating indices, and the clinicopathological features are shown in tables 2 and 3. Metallothionein expression did not correlate statistically with any of the variables examined except for CD44: carcinomas with a low metallothionein expression showed high CD44 expression ($p = 0.01$). There was no significant difference of metallothionein expression between adenocarcinomas and adenomas.

Discussion

The biological mechanisms underlying metallothionein overexpression in tumours are not currently understood. Although expression of

this protein has been associated with poor prognosis in a variety of tumours,¹⁵⁻²¹ in colorectal carcinomas it was shown that low expression was related to metastatic spread with lymph node involvement at the time of operation.²² This suggested that there was variability in the biological significance of metallothionein according to tumour type. However, in that study metallothionein expression was not considered as an independent prognostic variable in analysing the survival data. Mulder *et al* have reported a significantly decrease metallothionein content (determined by radioimmunoassay) in both adenomas and carcinomas compared with normal colonic mucosa.²⁵ In our current study, although there was a decreased metallothionein expression with higher Duke's stage, we found no significant association between metallothionein expression and tumour progression. Furthermore, the metallothionein expression in some cases in adjacent normal mucosa, in adenomas as well as in carcinomas, suggests that this is an early event in tumour progression. This is in line with the findings in breast cancer and melanomas.¹⁸⁻²⁰ However, the mechanism of tumour metallothionein expression is not fully understood. Hainaut and Milner¹⁴ found the altered conformation of the tumour suppressor protein p53 on the exposure to a metal chelating agent, thus suggested that binding of zinc ions to cysteinyl residues stabilises the tertiary structure of p53. Metallothioneins also have a high affinity for zinc ions and thus could act as intracellular sequestrators of zinc. Thus cells containing metallothionein in sufficient quantity to reduce intranuclear zinc ion levels and thus induce functional inactivation of p53 would acquire growth advantages and thus be able to proliferate and accumulate mutational events.

It has been hypothesised that mutation induced metallothionein overexpression may interfere with the function of zinc finger DNA binding transcription factors²⁶ involved in the controlling of the expression of a wide range of genes regulating cell proliferation and apoptosis, such as p53, and conferring a growth advantage on the mutated cells. It has been also shown that metallothionein specific antisense oligonucleotides cause growth arrest and induction of apoptosis in metallothionein overexpressing breast carcinoma cells.²⁷

In our study, although metallothionein and p53 were observed in both benign and malignant lesions, no correlation was detected between them in either group. These results are in line with reports¹⁸ concerning duct in situ carcinoma of the breast, which indicate that metallothionein and p53 overexpression may arise from independent mechanisms in early breast neoplasia.

We also found no correlation with proliferation indices, antiapoptotic bcl-2 protein, or the other potential markers of prognosis. An interesting finding was the inverse correlation between metallothionein and CD44 expression, though CD44 expression has been shown to be associated with metastasis and poor prognosis in colorectal cancer.²⁸⁻³⁰ This finding

supports that of a previous study in which decreased metallothionein expression in colorectal carcinomas was correlated with metastatic state.²² In our previous study we found that CD44 expression in colorectal carcinomas was positively correlated with tumour size, tumour and stromal cell cathepsin D expression, and pRb expression. We also found an inverse correlation between CD44 expression and the antiapoptotic protein bcl-2. Thus we assumed that CD44 expression may be involved in the process of invasion and metastasis, probably with the cooperation of cathepsin D, and its expression could be an indicator of poor prognosis in colorectal adenocarcinomas.³¹ In addition, the inverse correlation between metallothionein and CD44 expression would support the view that in colorectal cancer the metallothionein expression is associated with a favourable outcome.²² This would tend to confirm the different biological role of metallothionein expression in colorectal cancer, possibly because in colorectal tissue there is considerable inflammatory infiltrate and this could induce metallothionein synthesis through interferon production by activated T lymphocytes. However, the biological mechanisms underlying metallothionein overexpression in tumours are not currently understood. In a recent study, it was shown in normal colorectal mucosa that metallothionein overexpression is a result of somatic mutation in cells showing no obvious morphological evidence of a neoplastic phenotype.³² Thus it is still not clear whether the metallothionein expression in colorectal tumours represents an addition marker of the biological behaviour or whether it is an incidental finding.

CONCLUSIONS

Our results confirm that metallothionein expression does not indicate aggressive biological behaviour in colorectal adenocarcinomas compared with other types of carcinoma. The inverse correlation with CD44 could suggest that the decreased metallothionein expression may contribute to metastatic spread and lymph node involvement. In addition, metallothionein expression does not seem to be an independent marker of prognosis in colorectal cancer.

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