PERGAMON

European Journal of Cancer 38 (2002) 1879–1887

European Journal of Cancer

www.ejconline.com

Variation in MT expression in early-stage depressed-type and polypoid-type colorectal tumours

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Received 18 December 2001; received in revised form 9 April 2002; accepted 13 June 2002

Abstract

Metallothionein (MT) expression is observed in various carcinomas, but its role is not fully understood. To clarify the clinicopathological significance of MT, 87 colorectal adenomas and 128 early-stage carcinomas were immunohistochemically analysed for MT expression. The degree of MT immunostaining of a specimen was graded according to the proportion of MT-positive cells; negative ($<5\%$) and positive (focally 5–50%, diffusely $>50\%$). MT expression significantly decreased with tumour development. For carcinomas, MT-positivity was significantly associated with depth of invasion (T1 60% versus T2 33%; $P<0.01$), vascular involvement (positive 35% versus negative 61%; $P<0.01$) and morphology (polypoid 62% versus depressed 26%; $P<0.01$). Regarding MT-positive distribution, the diffuse-positive rate in MT-positive polypoid lesions was 28%, while MT-positive depressed lesions were all diffusely stained ($P<0.01$). In conclusion, our results suggested that decreasing MT expression is an early event in colorectal carcinogenesis and may reflect local invasion. Furthermore, MT-positive distribution may reflect genetic differences between the polypoid and depressed-type.

Keywords: Metallothionein; Colorectal carcinoma; Depressed-type; Proliferating cell nuclear antigen; Immunohistochemistry

1. Introduction

Metallothionein (MT) is a low molecular weight cysteine-rich protein (6–7 kD), which can bind to sequester divalent heavy metal ions such as zinc, copper and cadmium. By immunohistochemistry, MT expression is frequently demonstrated in both the nucleus and cytoplasm of cells in various organs of humans and experimental animals [1]. MT appears to play an important role in the detoxification of toxic metals, for example cadmium, and probably in cellular protection against ionising radiation and alkylating agent cytotoxicity [1–3]. In addition, this protein may play an important role in the homeostasis of zinc, a metal that is important for tumour growth and progression [1].

Some direct evidence indicates that the presence of MT has been observed immunocytochemically in several kinds of carcinomas, so it is possible that MT plays a role in some carcinogenic processes [4]. MT expression seems to be significantly associated with progression and poor prognosis in breast carcinoma [5], malignant melanoma [6] and pancreatic carcinoma [7]. However, in colorectal adenocarcinomas, MT-positivity is inversely related to the tumour stage and lymph node involvement [8]. These results suggest that MT expression decreases with colorectal tumour development, and that MT-positive colorectal carcinoma has a favourable clinical outcome.

Of interest is the phase at which the alteration of MT protein expression occurs during the development of colorectal cancer. There have been a few reports

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0959-8049/02/$ - see front matter
PII: S0959-8049(02)00233-2

concerning the expression of MT protein in colorectal cancers, including early-stage lesions [8–10], but this issue has yet not been fully investigated.

During colon carcinogenesis, adenocarcinomas are thought to arise from adenomas through a stepwise series of changes (the adenoma–carcinoma sequence) [11]. Morphological, epidemiological and molecular biological evidence is available to support this theory. An important aspect of this model is that the risk of an adenoma containing carcinoma increases with increasing size. However, depressed-type colorectal cancers, which may have different characteristics from polypoid types, have been increasingly reported with recent advances in endoscopic instruments and techniques [12–14]. These lesions are often less than 1 cm in diameter (Fig. 1a and b). Histologically, a sharp transition from normal-appearing colonic mucosa to carcinoma is seen without an adenomatous component in the vicinity (Fig. 1c and d). Such lesions appear not to fit the adenoma-carcinoma sequence and have been given the name '*de novo* carcinoma' [15,16]. Previous reports have shown an aggressive nature for the depressed-type carcinoma [16,17], but the biological differences between the depressed-type and polypoid-type carcinomas still remain obscure.

In the present study, we reconfirmed the relationship between decreasing MT expression and tumour development, and investigated the stage at which alteration of MT expression occurs in colorectal carcinogenesis. Next, we tried to indicate the difference in biological behaviour between the polypoid-type and the depressed-type of early-stage colorectal carcinoma through the pattern of MT immunohistochemical expression. We excluded advanced-stage colorectal carcinomas in this study because of their low MT-positive rate [8] and the difficulties encountered in deciding

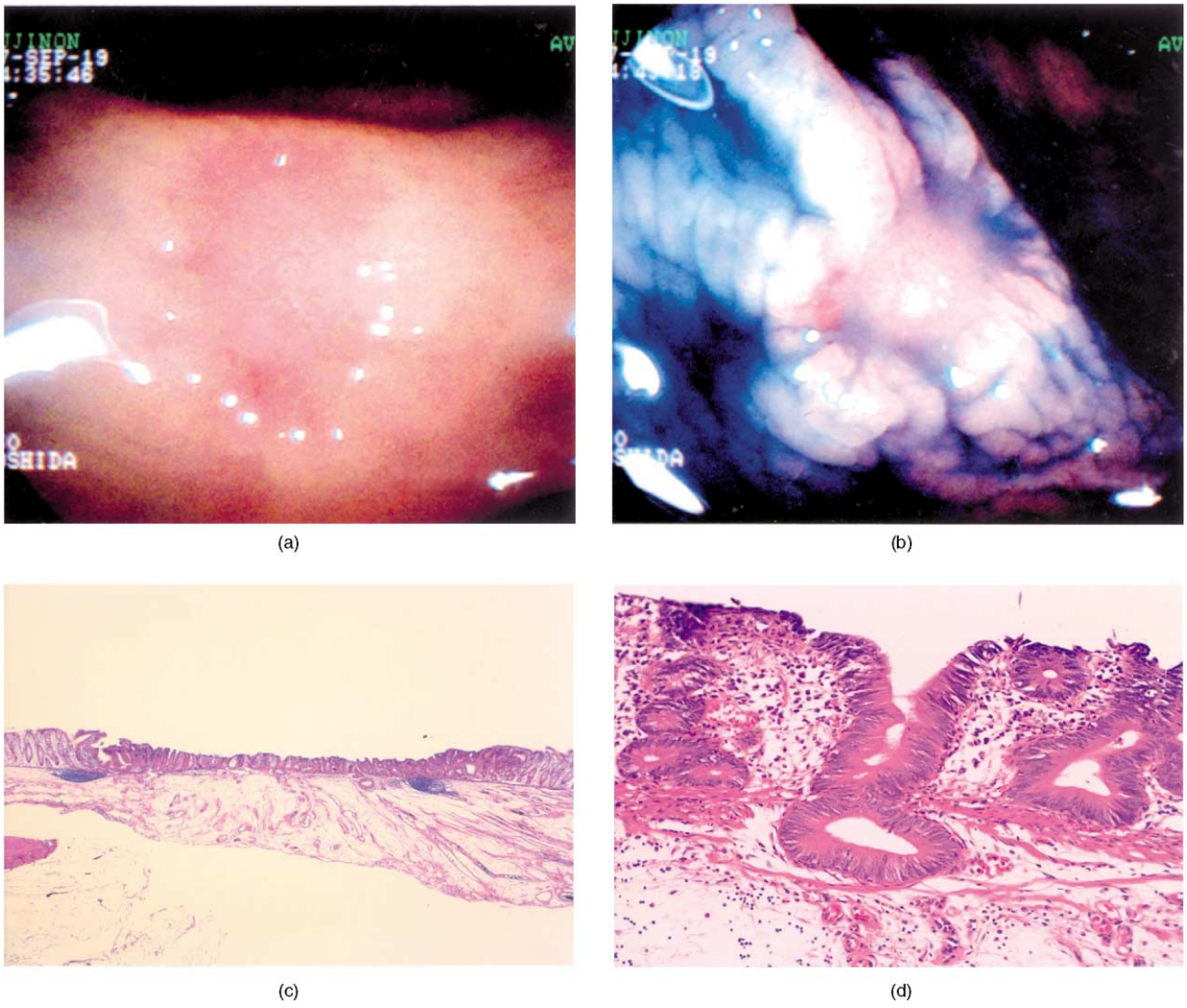

Fig. 1. An example of a depressed-type carcinoma. (a) Endoscopic view of a reddished depressed-type carcinoma (8 mm in diameter) in the descending colon. (b) After spraying with indigo carmine solution the lesion appears to have a depression. (c) Cross-sectional view of histological specimen shows endophytic growth. Haematoxylin-eosin (H-E), original magnification ×12.5. (d) Well differentiated tubular adenocarcinoma showing minimal submucosal invasion. H-E, original magnification ×200.

the original morphological types from which these advanced-stage lesions are derived. In addition, we immunohistochemically analysed the cellular proliferative activity in early-stage colorectal carcinomas, and considered its relationship with MT expression. In this study, we evaluated proliferating cell nuclear antigen labelling index (PCNA-LI) as an indicator of cellular proliferative activity since. Previous reports have suggested the usefulness of PCNA-LI for prognostic information in many premalignant and malignant tumours, especially colorectal carcinoma [18,19].

2. Patients and methods

2.1. Subjects

The study material consisted of colorectal neoplastic lesions with adjacent normal mucosa resected either endoscopically or surgically at the Kobe University Hospital from 1991 to 2000. Late stage cancers (T3 and T4 stage in the TNM classification system [20]) were not included. There were 215 lesions from 175 patients, none of whom had a familial history of polyposis. The main clinical data of subjects are summarised in Table 1. There were 87 adenomatous lesions and 128 carcinoma specimens; 86 (86/128; 67%) T1 stage (invading the submucosal layer) tumours and 42 (42/128; 33%) T2 stage (invading the muscularis propria) tumours. The degree of histological differentiation was well differentiated in 78 (78/128; 61%) lesions and moderately to poorly differentiated in 50 (50/128; 39%) lesions. Among the surgically resected cases, nodal metastases were negative in 87 cases (87/100; 87%), and positive in 13 cases (13/100; 13%). Early-stage colorectal carcinomas can be morphologically classified into three types; polypoid, flat-elevated and depressed types [21]. The polypoid-type was defined as tumours in which the height of the neoplastic mucosa was more than twice the thickness of the adjacent non-neoplastic mucosa. The flat-elevated type was defined as tumours with a neoplastic mucosa not greater than twice the thickness of the adjacent non-neoplastic mucosa. The depressed-type was defined as tumours in which the height of the neoplastic mucosa was less than that of the adjacent non-neoplastic mucosa. Out of 128 carcinoma cases, there were 52 (41%) polypoid lesions and 46 (36%) depressed lesions. 30 cases (23%) consisted of flat-elevated lesions and unclassified types in which the intramucosal lesions were absent.

2.2. Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were deparaffinised in xylene and rehydrated in a graded ethanol series, washed with phosphate-buffered saline (PBS, pH 7.6), and microwaved in 10 mM citrate buffer (0.01 mol/l, pH 6.0) for 15 min. Deparaffinised sections were incubated with 0.3% hydrogen peroxide for 20 min to block the intrinsic peroxidase, and then washed in PBS. The sections were then incubated with bovine

Table 1
Frequency of variables investigated in 215 colorectal adenomas and carcinomas

	Adenoma ($n=87$)	T1 carcinoma ($n=86$)	T2 carcinoma ($n=42$)
Age range (mean±S.D.) (years)	39–85 (63.8±10.6)	44–87 (65.1±9.5)	42–86 (65.0±10.5)
Sex (no of patients)			
Male	67 (77%)	54 (63%)	26 (62%)
Female	20 (23%)	32 (37%)	16 (38%)
Tumour location (no. of patients)			
Proximal colon	27 (31%)	23 (27%)	9 (21%)
Distal colon and rectum	60 (69%)	63 (73%)	33 (79%)
Tumour size range (mean ±S.D.) (mm)	13–70 (11.9±9.7)	6–100 (18.5±13.1)	12–65 (32.3±12.0)
Histological differentiation (no. of patients)			
Well		53 (62%)	25 (60%)
Moderately to poorly		33 (38%)	17 (40%)
Vascular involvement (no. of patients)			
Positive		24 (28%)	33 (79%)
Negative		62 (72%)	9 (21%)
Lymph node metastasis (no. of patients)[a]			
Positive		4 (7%)	9 (21%)
Negative		54 (93%)	33 (79%)

S.D., standard deviation.
[a] Data is given for surgically resected patients.

serum (BSA) albumin for 10 min at room temperature to reduce the non-specific immunoreactivity, and washed in PBS. We immunostained tissue sections with anti-MT antibody E9, a mouse monoclonal antibody (DAKO, Carpinteria, CA, USA) and with anti-PCNA antibody PC10, a mouse monoclonal antibody (DAKO). Treated tissue sections were incubated at room temperature for 60 min with E9 antibody or PC10 antibody diluted 1:200, respectively, in Tris–HCL buffer containing carrier protein and 15 mM sodium azide. After rinsing with PBS, the sections were incubated with peroxidase labelled anti-mouse immunogloblin (DAKO Envision System, DAKO) for 30 min. Sections were washed with PBS and coloured with 3,3′-diaminobenzidine (DAB). Finally, the sections were counterstained lightly with haematoxylin, dehydrated and mounted. To test the specificity of MT staining or PCNA staining, the specific MT-E9 or PCNA-PC10 antiserum was omitted or replaced with normal mouse serum: negative results were obtained. The immunohistochemical localisation of MT was either cytoplasmic or nuclear in the tumour and normal gland cells (Fig 2a and b). Peripheral nerves and smooth muscle cells present in each section served as positive controls for MT.

2.3. The evaluation of MT expression and PCNA-LI

The evaluation of MT expression followed the method previously described in Refs. [8–10]. The degree of MT immunostaining in a specimen was graded according to the proportion of MT-positive cells; negative (none detectable or less than 5% MT-positive cells) and positive (positive cells are 5% or more more). PCNA-LI was defined as the mean percentage of tumour cells showing PCNA-positive staining per 1000 cancer cells counted in five randomly chosen microscopic fields (×200). All assessments were performed without knowledge of the pathological, surgical or endoscopic data.

2.4. Statistical analysis

Statistical analyses were performed using the Chi-squared test and Mann–Whitney U test. A P value of less than 0.05 was considered significant.

3. Results

3.1. MT expression in colorectal tumours and adjacent normal mucosa to the tumour

To clarify the relationship between colorectal tumour development and MT expression, we immunohistochemically compared colorectal tumours with normal mucosa adjacent to those tumours. In all portions of normal mucosa adjacent to tumours, MT expression was positive. In adenomas, MT expression was negative in 29 cases (33%) and positive in 58 cases (67%) (Fig. 3a). In carcinomas, MT expression was negative in 65 cases (51%) and positive in 63 cases (49%) (Fig. 3b–e). There was a significant decrease of MT expression in the colorectum with tumour progression (normal mucosa versus adenomas or carcinomas $P<0.01$, and adenomas versus carcinomas $P<0.05$).

3.2. Relationship between clinicopathological findings and MT expression, PCNA-LI in carcinomas

The relationship between clinicopathological findings and MT expression and PCNA-LI in the carcinoma cases is shown in Table 2. The positive rate of MT expression was significantly associated with the depth of invasion and vascular involvement ($P<0.01$, respectively). PCNA-LI was significantly associated with the depth of invasion, vascular involvement, lymph node metastasis ($P<0.01$, respectively) and histological differentiation ($P<0.05$).

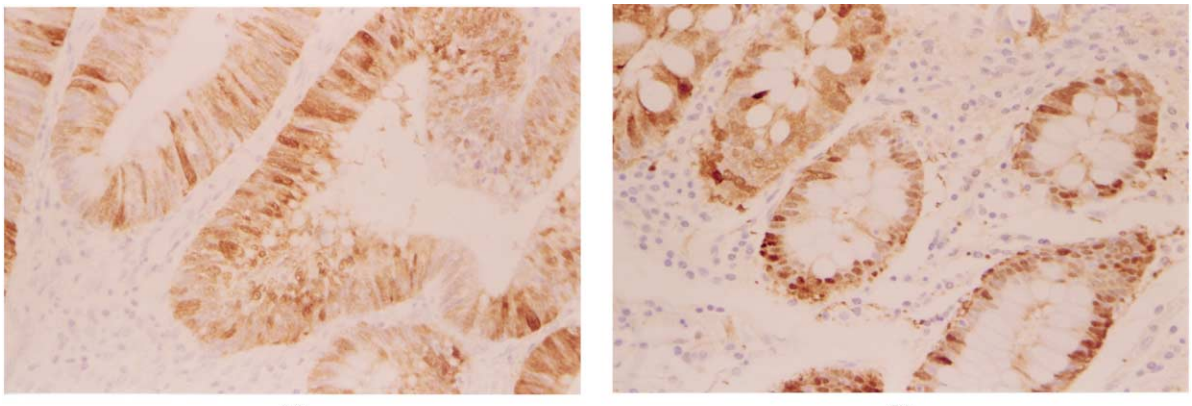

Fig. 2. MT immunostaining in tumour cells and normal gland cells. (a) MT-positive tumour cells (haematoxylin counterstaining, original magnification ×200). (b) Normal gland cells (haematoxylin counterstaining, original magnification ×200).

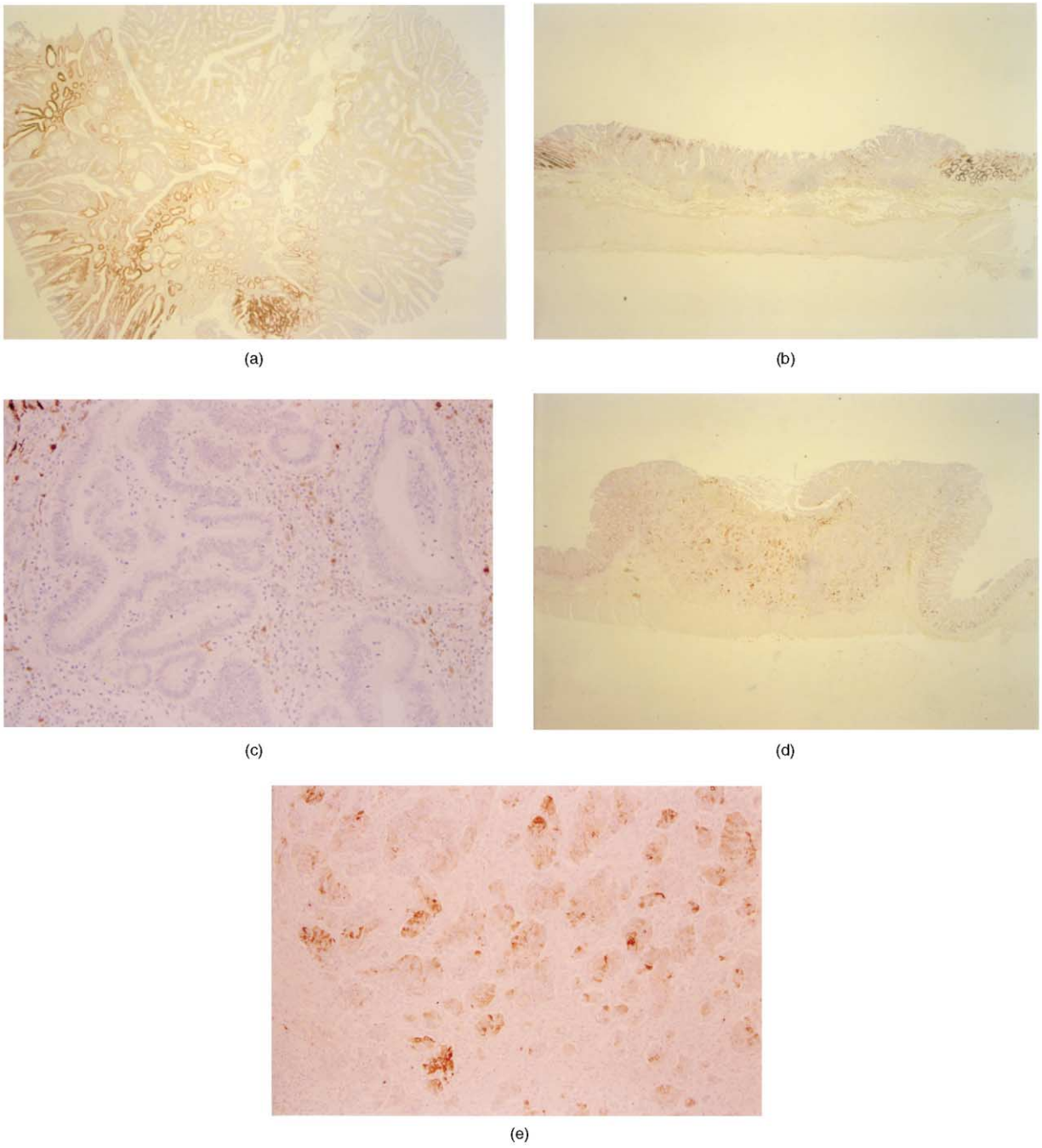

Fig. 3. MT immunostaining in colorectal tumours and normal mucosa adjacent to the tumour. (a) MT-positive adenoma (haematoxylin counterstaining, original magnification ×12.5). (b) MT-negative depressed-type T1 carcinoma with MT-positive normal mucosa (haematoxylin counterstaining, original magnification ×12.5). (c) Higher magnification of the invasive area in the same lesion as shown in (b) (haematoxylin counterstaining, original magnification ×100). (d) MT-positive depressed-type T2 carcinoma (haematoxylin counterstaining, original magnification ×12.5). (e) Higher magnification of the invasive area in the same lesion as shown in (d) (haematoxylin counterstaining, original magnification ×100).

3.3. Relationship between morphological type and MT expression and PCNA-LI in carcinomas

In order to clarify the characteristics of the depressed-type carcinomas, a comparison between the polypoid-type and the depressed-type was made (Table 3). Flat-elevated-type lesions were excluded from this comparison. The mean sizes of the polypoid lesions and depressed lesions were 22.2 ± 10.3 and 18.6 ± 12.4 mm, respectively. The depth of invasion, histological differentiation, vascular involvement and lymph node metastases in both groups were not significantly different. The positive rate of MT expression in the depressed lesions (12/46; 26%) was significantly lower than that in the polypoid lesions (32/

Table 2
Relationship between clinicopathological findings and MT expression, PCNA-LI in T1 and T2 carcinomas

	Number of cases	Number of MT-positive cases	*P* value	PCNA-LI ((%)±S.D.)	*P* value
Depth of invasion					
T1	86	52 (60%)	<0.01	38.1±7.7	<0.01
T2	42	14 (33%)		43.0±6.7	
Histological differentiation					
Well	78	39 (50%)	0.83	38.5±7.9	<0.05
Moderately to poorly	50	24 (48%)		41.7±7.1	
Vascular involvement					
Positive	57	20 (35%)	<0.01	42.5±7.5	<0.01
Negative	71	43 (61%)		37.5±7.2	
Lymph node metastasis					
Positive	13	6 (46%)	0.95	53.5±4.6	<0.01
Negative	87	41 (47%)		37.9±6.5	

MT, metallothionein; PCNA-LI, proliferating cell nuclear antigen labelling index.

Table 3
Relationship between morphological type and MT expression, PCNA-LI

	Polypoid type ($n=52$)	Depressed type ($n=46$)	*P* value
Depth of invasion			
T1	36 (69%)	29 (63%)	0.52
T2	16 (31%)	17 (37%)	
Histological differentiation			
Well	36 (69%)	23 (50%)	0.05
Moderately-poorly	16 (31%)	23 (50%)	
Vascular involvement			
Positive	19 (37%)	24 (52%)	0.09
Negative	33 (63%)	22 (48%)	
Lymph node metastasis			
Positive	5 (17%)	5 (12%)	0.54
Negative	25 (83%)	38 (88%)	
MT expression			
Positive	32 (62%)	12 (26%)	<0.01
Negative	20 (38%)	34 (74%)	
PCNA-LI ((%)±S.D.)	38.6±7.1	41.9±7.5	<0.05

MT, metallothionein; S.D., standard deviation.

52; 62%; $P<0.01$). The PCNA-LI in the depressed lesions (41.9±7.5%) was significantly higher than that in polypoid lesions (38.6±7.1%; $P<0.05$).

3.4. Relationship between morphological type and the pattern of MT-positive distribution

MT-positivity was divided into a focally positive pattern (positive cells are 5–50%) and diffusely positive pattern (more than 50%) according to a method previous described in Ref. [8]. Due to the observed MT-positive distribution on the basis of this definition, we noticed that MT staining patterns were clearly divided into two types. In all focal MT-positive lesions, MT staining had a 'scattered' pattern, while in all diffuse MT-positive lesions, MT staining had a 'uniform' pattern. In the 32 MT-positive polypoid-type carcinomas, MT expression was diffuse in 9 cases (28%) and focal in 23 cases (72%, Fig. 4a). In the 12 MT-positive depressed-type carcinomas, MT expression was diffuse in all cases (100%, Fig. 4b). The rate of diffuse MT expression in the MT-positive depressed lesions was significantly higher than that in the MT-positive polypoid lesions ($P<0.01$).

3.5. Relationship between MT expression and PCNA-LI in colorectal tumours

In the 121 MT-positive lesions, the PCNA-LI was 35.2±8.0%. In 94 MT-negative lesions, the PCNA-LI was 37.2±9.1%. MT expression was not associated with the PCNA-LI. Furthermore, although we analysed the adenoma cases and carcinoma cases separately, there was no significant difference in the PCNA-LI between the two groups.

4. Discussion

The expression of MT is associated with a poor prognosis in most carcinomas [5–7], but in colorectal carcinomas low MT expression has been reported to be related to poor prognosis [8]. These results suggested that there is variability in the biological behaviour of MT according to the tumour origin, and that in colorectal carcinoma low MT expression could be a marker for malignant potential. In the present study, MT staining was observed in all portions of normal colorectal mucosa adjacent to the tumour, and there was a significant decrease in MT expression with tumour progression. Previous studies also showed a significant decrease in MT content in both adenomas and carcinomas compared with normal colonic mucosa [9,22].

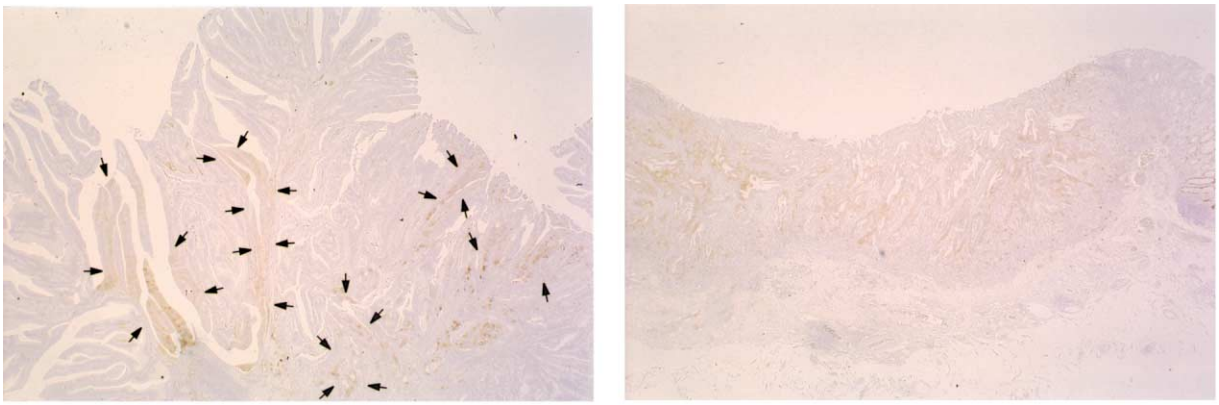

Fig. 4. The pattern of MT-positive distribution in colorectal carcinoma. (a) MT-focally positive polypoid-type T1 carcinoma (haematoxylin counterstaining, original magnification ×20). Scattered MT-positive areas of both adenoma and carcinoma (the deepest invasive portion) are shown by arrows. (b) MT-diffusely positive depressed-type T1 carcinoma (haematoxylin counterstaining, original magnification ×20).

Our findings show that in carcinoma cases decreasing MT expression is significantly related to the depth of invasion and vascular involvement, but not to histological differentiation and lymph node metastasis. These results suggest that decreasing MT expression may be an early event in colorectal tumour progression, and that it may affect local invasion, but not cell differentiation or lymph node metastasis. However, a previous report showed that MT expression was related to lymph node involvement in colorectal carcinomas [8]. The discrepancy between that study and ours could have been caused by differences in the clinical characteristics of the subjects studied. The previous study included only 11 Dukes' A cases (10%) and all other cases were more advanced (97 cases; 90%), while our study included only T1 and T2 stage cases. In our analysis, decreasing MT expression seems to be an early event in colorectal carcinogenesis. Therefore, we think that it is important to analyse MT expression in early phase colorectal tumour development, such as adenomas and early-stage carcinomas.

Recently, flat- or depressed-type colorectal tumours have been increasingly detected not only in Japan, but also in other countries [12–14]. Kudo and colleagues argued that depressed cancers have an invasive tendency despite their small size and should be regarded as *de novo* carcinomas [16,17]. We have also reported that the depressed-type carcinoma must be distinguished from the polypoid-type carcinoma because of its different biological behaviour, and that depressed-type carcinoma may have a more malignant potential than the polypoid-type carcinoma [21,23].

To our knowledge, the present study is the first report of MT expression in depressed-type early cancers. We showed that the positive rate of MT expression in the depressed type was significantly lower than that in the polypoid-type, and that PCNA-LI in the depressed-type was significantly higher than that in the polypoid type. In the two groups that we analysed, the depth of invasion, histological differentiation, vascular involvement and lymph node metastases were not significantly different. Therefore, the significant differences in MT expression and PCNA-LI may reflect the biological differences in early-stage colorectal carcinomas. In addition, considering that decreasing MT expression is associated with local invasion and poor prognosis as mentioned above, this may be a predictor of the malignant potential of the early-stage depressed type.

However, the relationship between biological characteristics and location in colorectal tumours is interesting. Concerning this, consensus has not yet been reached. In this study, in both adenoma and early-stage carcinoma cases, MT expression was not associated with tumour location, and in carcinoma cases morphological type was not associated with tumour location (data not shown). To clarify the relationship between the biological characteristics and location in colorectal tumours, further study will be necessary.

Generally, protruding tumours are considered to develop through the adenoma-carcinoma sequence based on the multistep genetic model for colorectal tumorigenesis [11]. Heterogeneous subpopulations identified within colorectal carcinomas [24,25] may be due to this multistep carcinogenesis. In contrast, depressed-type tumours are thought to arise through another developmental mechanism without some of the intervening steps.

In this study, we evaluated MT expression using the 5% cut-off value according to previous reports on MT expression in colorectal tumours [8–10]. In these reports, MT-positivity was classified according to the proportion of MT-positive cells only. However, owing to our careful observation of the MT-positive distribution, we noticed that classification of MT-positivity on the basis of the proportion of MT-positive cells was related to the pattern of MT-positive distribution.

Moreover, we noticed a significant relationship between the pattern of MT-positive distribution and morphological type. Our study showed that the rate of diffuse MT expression in MT-positive depressed lesions (100%) was significantly higher than that in MT-positive polypoid lesions (28%). We speculate that, in polypoid lesions, a decrease of MT expression occurs sporadically within the whole lesion because of its development through multistep carcinogenesis. As the result, the rate of focal MT expression becomes higher and may reflect heterogeneity within the lesion. However, in depressed lesions a decrease in MT expression occurs simultaneously through *'de novo*' carcinogenesis without some of the intervening steps. Therefore, in depressed lesions, the rate of diffuse MT expression becomes higher and focal MT expression is rare. Thus, there is a possibility that the pattern of decreasing MT expression reflects the differences in genetic characteristics between these two groups.

The details of the genetic characteristics with regard to the depressed-type cancer have not yet been elucidated. Infrequent detection of *K-ras* gene mutation is one of the major characteristics demonstrated by molecular analysis of flat- or depressed-type tumours [21,26,27]. There have been various reports about p53 immunostaining of these tumours, but the relationship between the morphology of colorectal tumours and p53 immunoexpression still remains unclear [12,28–30]. Although the number of studies and reports about flat- or depressed-type carcinomas (*de novo* carcinomas) is increasing [12–14], few studies have reported the molecular biological characteristics of this type of carcinoma. Thus, it is necessary to accumulate more data, especially from molecular biological analyses.

Our results showed that there was no significant difference in the PCNA-LI between MT-positive and MT-negative lesions. These results suggest that decreasing MT expression is not reflected in the tumour-proliferative activity. These factors may independently affect the malignant potential in colorectal carcinogenesis. One possible reason why there was no relationship between tumour-proliferative activity and MT expression is that, in addition to cell division, many other factors contribute to the progression, growth and invasion of tumours. MT plays a role in drug resistance or the detoxification of toxic metals. PCNA-LI is one of the markers of tumour proliferation, which reflects cell division, and it is known to be a marker of malignant potential. MT expression may reflect different factors from proliferative activity, for example an abnormality of metabolism in tumour cells, but the role of MT expression in tumour cells has not been clarified and will require further study.

In conclusion, our study suggests that decreasing MT expression is an early event in colorectal tumour progression and may affect local invasion. Furthermore, MT expression may reflect biological differences in carcinogenesis between the depressed-type and polypoid-type carcinomas.

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