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Immunohistochemical detection of metallothionein in carcinomatous and normal human gastric mucosa

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Summary. Utilising a specific monoclonal mouse antibody (E9), metallothionein (MT) expression has been immunohistochemically investigated in 112 formalin-fixed paraffin-embedded surgical gastric samples, 38 of which were early carcinomas (EGC) and 74 advanced ones (AGC); clinico-pathological details and follow-up data (ranging from 3 to 197 months, mean 60.5 months) were available. Eighty-nine portions of gastric mucosa adjacent to examined carcinomas (transitional mucosa) were also analysed; in addition, 22 biopsies of normal gastric mucosa were studied as tissue control. The MT immunoreactivity was evaluated by staining and intensity-distribution scores. A various MT positivity was appreciable in the cytoplasm and nucleus of antrum or body gastric epithelial cells in 100% of normal control biopsies. 75/112 (67%) gastric carcinomas showed MT immunoreactivity with a significant lower expression in AGC. No relationships were encountered between MT immunostaining and clinico-pathological data; in addition, no difference in the Kaplan-Meier survival curves of patients with various MT expression was achieved. When the transitional mucosa was examined, 84/89 (94%) samples were stained although the immunoreaction was not always concordant with that encountered in adjacent carcinomatous elements. The significant statistical decrease of MT scores observed by us moving from normal to neoplastic gastric mucosa allows us to exclude the hypothesis of an overexpression of MT in gastric carcinomas.

Key words: Metallothionein, Gastric mucosa, Gastric carcinoma, Immunohistochemistry, Prognosis

Introduction

Metallothionein (MT) is a low molecular weight protein (6-7 kD) with a high content of cysteinyl residues and strong affinity for divalent heavy metal ions such as zinc, copper, cadmium, mercury, silver and platinum (Kagi and Nordberg, 1979; Nishimura et al., 1989). MT is considered to be involved in various processes such as storage of essential metals (Webb and Cain, 1982; Panemangalore et al., 1983; Schmid et al., 1993), binding of large amounts of potentially toxic metal ions with a sequestration function (Nomiyama et al., 1982; Goering and Klaassen, 1984; Nishimura et al., 1989; Schmid et al., 1993) and scavenging of free radicals in tissues and cells (Sato and Bremner, 1993). By immunohistochemistry, MT has been demonstrated in both the nucleus and the cytoplasm of cells in various organs of humans (Savino et al., 1984; Clarkson et al., 1985; Nartey et al., 1987) and animals (Banerjee et al., 1982; Danielson et al., 1982; Bataineh et al., 1986; Umeyama et al., 1987; Hazelhoff Roelfzema et al., 1989; Nishimura et al., 1989, 1991; Jasani et al., 1998; Fuentealba and Mullins, 1999).

In human neoplastic pathology (Jasani and Schmid, 1997), the presence of MT has been immunocytochemically revealed in testicular embryonal carcinomas (Kontozoglou et al., 1987), thyroid tumours (Nartey et al., 1987), transitional cell carcinomas of the bladder (Bahnson et al., 1991; Siu et al., 1998), in situ and invasive breast carcinomas (Fresno et al., 1993; Schmid et al., 1993; Bier et al., 1994; Haerslev et al., 1994; Douglas-Jones et al., 1995), cervical intraepithelial and invasive squamous carcinomas (McCluggage et al., 1998) as well as in malignant melanomas (Zelger et al., 1993), salivary gland tumours (Sunardhi-Widyaputra et al., 1995) and hepatocellular (Deng et al., 1998) and pancreatic (Ohshio et al., 1996) carcinomas. In addition, immunohistochemical evidence of MT has been reported in colorectal adenocarcinomas (Öfner et al., 1994; Giuffrè et al., 1996), with a variable, not always concordant, immunoreactivity in corresponding lymph node and distant metastases (Giuffrè et al., 1996). On the light of these grounds, the aim of the present study was to systematically investigate the immunohistochemical distribution pattern of MT in the normal gastric mucosa, in mucosa adjacent to cancerous tissues as well as in gastric carcinomas, either early or advanced. The possible correlation between immunohistochemical data and morphological characteristics of gastric carcinomas as well as the prognostic significance of MT detection

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*Dedicated to my father on the occasion of his 80th birthday

have also been investigated.

Materials and methods

Surgical samples from 112 patients (60 male, 52 female; age range 21-84 years, mean age 61.2 yrs) resected for gastric carcinoma were included in this study: 38 were diagnosed as early gastric carcinoma (EGC), while 74 were advanced gastric carcinoma (AGC). In 89 samples, a portion of gastric mucosa adjacent to carcinomas (transitional mucosa) was available for evaluation. The carcinomas were classified and staged according to WHO criteria (Watanabe et al., 1990) and the UICC recommendations (Hermanek and Sobin, 1992), respectively; the main clinico-pathological data being summarized in Table 1. Preoperative radiation and/or chemotherapy were not performed in all cases. Data concerning follow-up and cause of death were obtained from city registry offices: four patients who died during surgery or in the first month of follow-up were excluded from the survival study.

In addition, 22 biopsies of normal gastric mucosa (16 from the body and 6 from the antrum) of 16 non-neoplastic patients (10 male, 6 female; age range 45-74 years, mean age 56.4 yrs.) were also analyzed as tissue control.

All samples were fixed in 10% neutral formalin for 12-24 hrs at room temperature (RT) and then embedded in paraffin at 56 °C. From each tissue block, two consecutive 3 μm-thick sections were cut and mounted on silane-coated glass, then dewaxed in xylene, rehydrated in graded ethanols and submitted respectively to haematoxylin-eosin stain and to immunohistochemistry. The immunoreaction was performed utilising a monoclonal mouse anti-MT reactive against a single and highly conserved epitope shared by the isoforms I and II (clone E9, commercially obtained from Dako, Denmark, w.d. 1:100) applied overnight at 4 °C; successively, swine anti-mouse globulin antiserum (1:20, purchased from Dako, Denmark) and ABC complex for 30 min at RT and 3,3'-diaminobenzidine tetrahydrochloride-H_2O_2 substrate solution for 10 min at RT in darkness were applied. Finally, slides were lightly counterstained with Mayer's haematoxylin.

To test the specificity of MT staining, the specific MT-E9 antiserum was omitted or replaced by either phosphate-buffered saline or normal rabbit serum and negative results were obtained. Positive controls were represented by normal human kidney. Immunostained sections were estimated by light microscopy using a x20 and x40 objective lens and x10 eyepiece. The assessment of immunostained sections was performed on a consensus basis by two pathologists (G.T. and G.G.) using a double-headed microscope. The percentage of stained cells (staining score) was graded for semiquantitative purposes as follows: 0 (no staining); 1 (>0 to 5%); 2 (>5 to 50%); 3 (>50%), as previously performed (Giuffrè et al., 1996). In addition, an intensity-distribution (ID) score was calculated by multiplying, for each case, the staining score by the staining intensity (weak=1; moderate=2; strong=3), similarly to that elsewhere reported (Douglas-Jones et al., 1995; Anstey et al., 1996).

The possible correlation between immunohistochemical data and morphological characteristics of carcinomas was investigated using non-parametric methods (chi-square test, Mann-Whitney U-test, Kruskal-Wallis H-test). Survival analysis was performed by the Kaplan-Meier method utilising the type of tumour as a strata variable and grouping the patients in two different manners: the first according to staining score values, the second according to ID-score. For the comparison of the survival curves, the Mantel-Cox long-rank test was used. A P value less than 0.5 was considered statistically significant.

Results

All samples of normal gastric mucosa showed MT positivity variously expressed in the cytoplasm and nucleus of antrum and body elements (Fig. 1). In the antrum, the staining was localized in the glandular mucous cells (6 of 6 cases) as well as in the surface mucous cells (4 of 6 cases); in the body, the immunoreactivity was mainly encountered in the lower half of the oxyntic glands (15 of 16 cases) and also in the cells of the neck zone (15 of 16 cases), while surface mucous cells were more rarely stained (5 of 16 cases) (Fig. 1). The staining and ID-scores relative to normal mucosa are reported in Table 2.

The MT expression observed in 75 of 112 (67%) cases of gastric carcinoma is shown in Table 2, with the corresponding staining and ID-scores. The positivity was localized in the cytoplasm (Fig. 2a), although a combined nuclear/cytoplasmic reactive pattern was also seen in neoplastic elements (Fig. 2b,c). In some cases, immunoreactive neoplastic cells were found in direct contact with negative ones (Fig. 2b-d). The stroma, intermingled with neoplastic glands, sometimes showed stained fibroblasts. Analysing staining score values (Table 1), a significantly less frequent MT immunoexpression was encountered in AGC than EGC (χ^2=8.794; df=3; P=0.033), while a trend towards this correlation (χ^2=2.881; df=1; P=0.089) was encountered utilising the ID-score. No relationships were found between other clinico-pathological data and MT expression in gastric carcinomas utilising either the staining score or the ID-score values. Moreover, the same results were obtained in EGC or AGC groups separately.

At the time of the present study, 59 (54.6%) patients (7 EGC and 52 AGC) had died of disease, and 49 (45.4%) (31 EGC and 18 AGC) were alive. The mean follow-up time of all patients was 60.5 months (ranging from 3 to 197), while for alive patients it was 101.4 months (ranging from 36 to 197); in detail, for this latter group, the mean follow-up time was 100.5 months (ranging from 36 to 197) for EGC and 102.8 months

Table 1. Clinico-pathological data in 112 gastric carcinomas investigated and their correlation with MT score.

		Nr.	STAINING SCORE				INSTENSITY-DISTRIBUTION SCORE
			0	1	2	3	Mean (±SD)
Sex	Male	60	21	20	14	5	2.38 (2.80)
	Female	52	16	13	13	10	3.25 (3.48)
Age	≤ 50	24	11	7	4	2	1.96 (2.91)
	> 50	88	26	26	23	13	3.01 (3.19)
Tumour location	Cardia	8	4	1	1	2	3.13 (4.16)
	Body	27	8	11	4	4	2.56 (3.14)
	Body-Antrum	9	3	4	1	1	2.22 (3.15)
	Antrum	68	22	17	21	8	2.91 (3.08)
Type of tumour	EGC	38	12	6	11	9	3.74 (3.60)
	AGC	74	25	27	16	6	2.30 (2.79)
Histological type	Tubular	59	19	16	13	11	3.19 (3.46)
	Papillary	10	1	5	3	1	3.00 (2.83)
	Mucinous	12	3	5	4	0	2.08 (2.02)
	Signet-ring cell	31	14	7	7	3	2.23 (2.97)
Laurén class	Intestinal	80	23	25	20	12	3.03 (3.22)
	Diffuse	32	14	8	7	3	2.19 (2.93)
Histological grading	Low grade	65	24	19	14	8	2.52 (3.10)
	High grade	47	13	14	13	7	3.15 (3.21)
TNM class	pT1	38	12	6	11	9	3.74 (3.60)
	pT2	31	10	13	7	1	1.90 (2.24)
	pT3	33	12	11	7	3	2.42 (2.99)
	pT4	10	3	3	2	2	3.10 (3.67)
	pN0	47	17	9	12	9	3.21 (3.48)
	pN1	35	9	12	11	3	2.74 (2.83)
	pN2	30	11	12	4	3	2.17 (2.95)
Stage	Ia	28	11	4	7	6	3.43 (3.66)
	Ib	23	5	6	8	4	3.39 (3.22)
	II	19	6	7	5	1	2.26 (2.60)
	IIIa	16	5	7	3	1	2.19 (2.69)
	IIIb	14	5	4	4	1	2.50 (3.06)
	IV	12	5	5	0	2	2.08 (3.34)
Clinical course	Alive	49	18	10	13	8	3.00 (3.38)
	Death for cancer	59	19	20	13	7	2.64 (3.06)

Table 2. Detection of MT in normal gastric mucosa, carcinomas and transitional mucosa.

		STAINING SCORE				INSTENSITY-DISTRIBUTION SCORE
	Positive rate	0	1	2	3	Mean (±SD)
Normal gastric mucosa	100% (22/22)	0	6	8	8	5.14 (3.00)
Gastric Carcinoma	67% (75/112)	37	33	27	15	2.79 (3.15)
Transitional Mucosa	94% (84/89)	5	30	49	5	3.17 (2.21)

(ranging from 48 to 144) for AGC.

A univariate analysis of survival data of MT expression, evaluated either by staining- or ID-scores, failed to be of prognostic significance, even when the type of tumour (EGC or AGC) was used to define the strata.

In the gastric peritumour mucosa, 84 of 89 (94%) samples were positively stained (Table 2); this immunoreaction was not always concordant with that encountered in adjacent carcinomatous elements, as shown in Table 3.

When staining- and ID-scores relative to gastric carcinomas as well as normal and transitional mucosa groups were compared by the chi-square test (χ^2=50.813; df=6; P=0.0001) and the Kruskal-Wallis H-test (χ^2=17.073; df=2; P=0.0002), highly significant differences were found.

Discussion

In the present study, we have performed a systematic investigation on the immunohistochemical MT expression in normal, neoplastic and transitional gastric mucosa. In particular, a clear cytoplasmic and/or nuclear

localization of MT has been documented in glandular and surface mucous cells of the antrum as well as in the lower half of the oxyntic glands and in cells of the neck zone of the body in all normal gastric control specimens; these findings have not been reported until now in the stomach, although MT immunoexpression has been previously described in unaffected mucosae of other portions of human gastrointestinal tract such as ileum and right or left colon (Clarkson et al., 1985; Giuffrè et al., 1996). The immunohistochemical presence of MT in normal gastric epithelium may be attributable to its scavenging capability; in fact, it has been claimed that MT is of importance in cellular defense mechanisms against hydroxyl free radicals (Sato and Bremner, 1993) as well as in the homeostatic control of Zn and Cu (Bremner, 1987).

Table 3. MT expression in 89 gastric carcinomas samples and corresponding transitional mucosa.

GASTRIC CARCINOMA	TRANSITIONAL MUCOSA	
	MT +	MT -
MT +	63% (56/89)	3% (3/89)
MT -	32% (28/89)	2% (2/89)

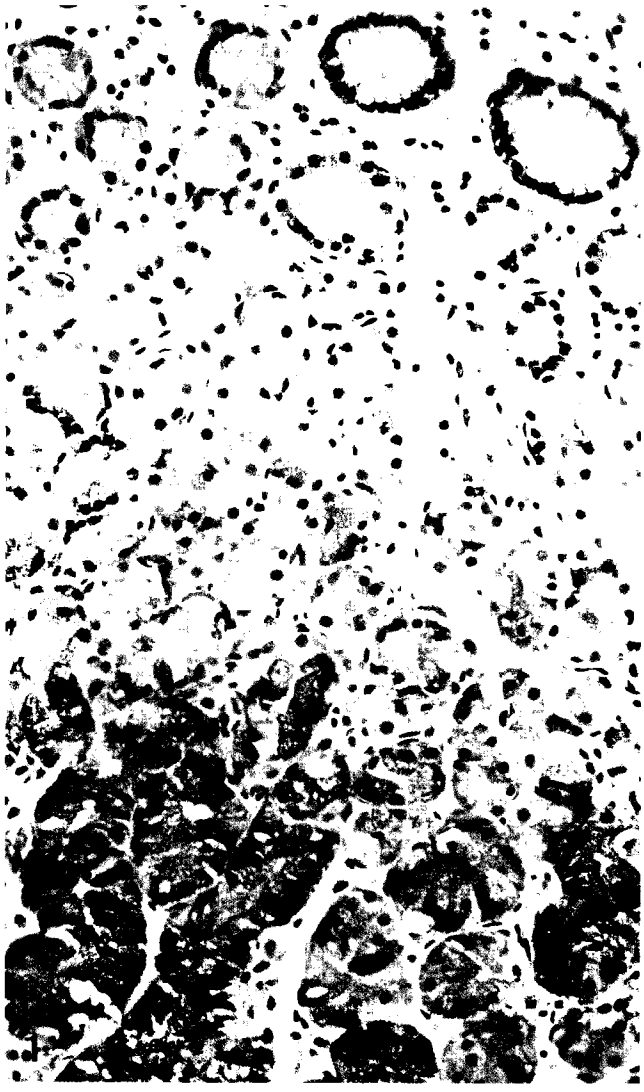

Fig. 1. Normal gastric mucosa. An evident MT immunoreactivity is mainly encountered in epithelial cells of the lower half of the oxyntic glands. MT-ABC complex, Mayer's haematoxylin nuclear counterstain. x 50

Moreover, exchange reactions between Zn-MT and the apoform of Zn-enzymes MT have been reported in vitro (Udom and Brady, 1980), suggesting MT is involved in metal-transfer reactions in vivo; therefore, the immunohistochemical expression of MT in non-neoplastic mucous and zymogenic gastric lineages should also suggest a possible role of MT as a donor of Zn to these apoenzymes, since Zn is required for synthesis of DNA and DNA-repair enzymes (Sato and Bremner, 1993).

In epithelial compartments of gastric mucosa adjacent to carcinomas, a distinct MT immunostaining was present in 94% of samples, with a corresponding decrease of ID-score in comparison to that observed in normal control cases. Moreover, in gastric carcinomas the MT-positivity was encountered in only 67% of cases, with further reduction of ID-score. In addition, a non concordant MT expression between gastric carcinoma and its transitional mucosa was found in 31/89 cases, 28 of which showed MT positivity only in non-carcinomatous mucosa. Therefore, in the light of these observations, we are unable to demonstrate an overexpression of MT in gastric carcinomas since a significant statistical decrease of MT scores has been observed moving from the normal to neoplastic gastric mucosa. However, in our cases of gastric carcinomas, the immunohistochemical evidence of MT did not show any correlation with sex, age, tumour location, histological type or grade, TNM classes, stage and clinical course, while a significant decrease in MT rate was found in AGC in comparison to EGC, either with staining- or ID-scores. The possible explanations of the reduced MT immunoexpression in neoplastic samples in comparison to normal and peritumour gastric mucosa may be related to different mechanisms. Firstly, neoplastic elements may utilize MT faster in order to have a greater availability of Zn for their turnover, even if a reduced MT storage cannot be excluded. Additionally, it may be hypothesized that during carcinogenesis other MT isoforms are produced as a consequence of different gene-expression; in fact, it is well known that in humans 10 functional MT isoforms and 7 non functional ones have been demonstrated as a result of a family of genes (Friedline et al., 1998). However, the commercially available antiserum used by us is reactive only against a conserved N-terminal epitope shared by MT-I and MT-II isoforms and therefore it is unable to distinguish between either MT-I

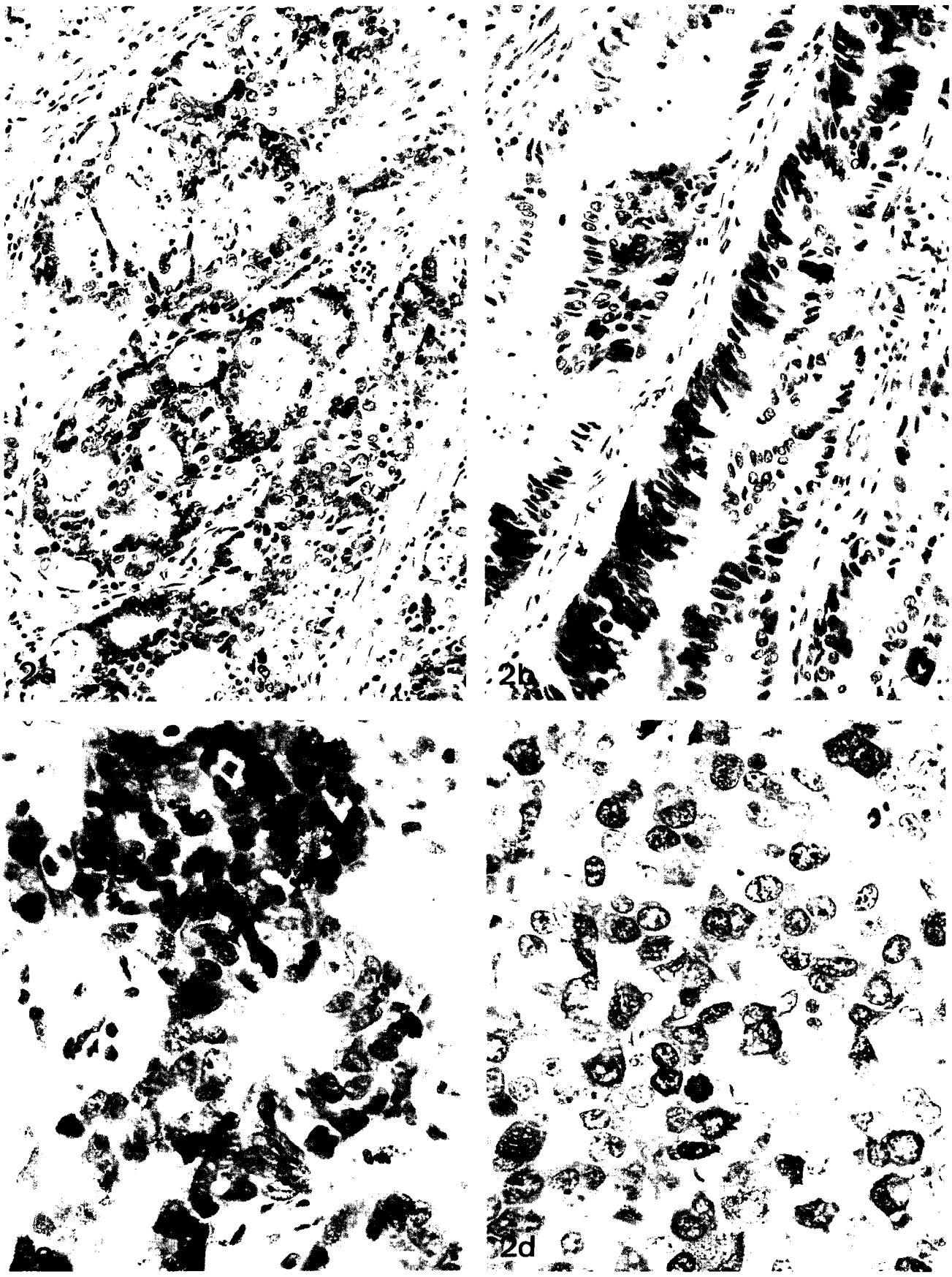

Fig. 2. Gastric carcinoma. MT staining mainly localized in the cytoplasm **(a)**; a combined nuclear/cytoplasmic reactive pattern is also seen **(b, c)**, with a strong immunostaining in neoplastic elements intermingled with negative ones **(b, c, d)**. MT-ABC complex, Mayer's haematoxylin nuclear counterstain. a, b, x 50; c, d, x 100

and MT-II isoforms or metal-bound and metal-free forms of MT or to detect other MT isoforms, as elsewhere suggested (Jasani and Schmid, 1997).

The biological significance of MT expression in human tumours as well as its association with prognosis are not fully understood and conflicting explanations have been reported (Fresno et al., 1993; Schmid et al., 1993; Zelger et al., 1993; Bier et al., 1994; Haerslev et al., 1994; Öfner et al., 1994; Douglas-Jones et al., 1995; Sunardhi-Widyaputra et al., 1995; Giuffrè et al., 1996; Ohshio et al., 1996; Deng et al., 1998; McCluggage et al., 1998; Siu et al., 1998). In detail, a direct correlation between MT expression and poor clinical outcome has been demonstrated in some malignancies, such as invasive breast ductal carcinomas (Fresno et al., 1993; Schmid et al., 1993; Haerslev et al., 1994) and malignant melanomas (Zelger et al., 1993). In contrast, no correlation has been made with tumour differentiation or tumour aggressiveness in MT-positive thyroid (Nartey et al., 1987) and testicular embryonal carcinomas (Kontozoglou et al., 1987). Finally, a favourable clinical outcome of MT-positive colonic carcinoma has been suggested (Öfner et al., 1994), even if we have shown MT immunoreactivity both in stage I and II colorectal carcinomas and in advanced ones as well as in corresponding metastases of the latter, excluding thus a relationship between MT staining and a better prognosis of the neoplasia (Giuffrè et al., 1996). Nevertheless, in the present study, no significant differences in the survival curves regarding patients affected by gastric carcinomas with different MT expression were achieved, neither when the type of tumour was utilized as a strata variable. Therefore, the hypothesis the MT immunopositivity in gastric carcinomas represents a prognostic parameter should be rejected. However, in node-positive (N1-N2) gastric carcinomas, it has been shown that MT staining did not correlate with any clinicopathological variables and did not affect disease-free survival (Monden et al., 1997), even if the method used for evaluating the MT immunoreactivity was over-simplistic and the study was only addressed to the drug resistance of cancer cells, without analysis of MT expression in the normal gastric mucosa taken from control non-neoplastic patients.

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