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Immunohistochemical detection of membrane-type-1-matrix metalloproteinase in colorectal carcinoma

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Summary We investigated whether the expression of membrane-type-1 matrix metalloproteinase (MT1-MMP), matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2) was consistent with the proposed roles of these proteins in promoting metastasis in colorectal cancer. The expression of MT1-MMP was significantly more frequent in deeply invasive carcinomas ($P = 0.007$) and in cases of vascular invasion ($P = 0.02$). The frequency of detection of MMP-2 in the stroma was much greater in vascular invasion-positive cases (42%) than in negative cases (20%; $P = 0.02$). The rate of detection of TIMP-2 in tumour cell cytoplasm increased with the depth of invasion ($P = 0.03$). TIMP-2 in the stroma was found more frequently in tumours with lymphatic invasion and lymph node metastasis ($P < 0.05$). Significant correlations were found between detection of MT1-MMP and MMP-2 in tumour cell cytoplasm ($P < 0.05$), of MT1-MMP and TIMP-2 in tumour cell cytoplasm ($P < 0.01$), and of MMP-2 and TIMP-2 in tumour cell cytoplasm ($P < 0.01$). Immunohistochemical detection of MT1-MMP and TIMP-2 might be useful for monitoring infiltration in colorectal carcinoma but is not correlated with distant metastases. © 2000 Cancer Research Campaign

Keywords: membrane-type-1-matrix metalloproteinase; matrix metalloproteinase-2; tissue inhibitor of metalloproteinase-2; immunohistochemistry; distant metastases; colorectal carcinoma

Distant metastasis is one of the most important determining factors in the prognosis of colorectal cancer. Degradation of the extracellular matrix that surrounds tumour cells is an essential step in the processes of tumour invasion and metastasis. Matrix metalloproteinases (MMPs) degrade various components of the extracellular matrix. In particular, MMP-2 (gelatinase A; also called type-IV collagenase and 72-kDa gelatinase) degrades type IV collagen of the basement membrane, as well as gelatin, and type V, VII and X collagens (Collier et al, 1997). MMP-2 is expressed in different types of human epithelial cancer, such as breast (Davies et al, 1993), ovarian (Naylor et al, 1994), pancreatic (Bramhall et al, 1996), and gastric (Sier et al, 1996) cancer, and its level seems to be related to malignancy and invasion. The activity of MMP-2 is modulated by a tissue inhibitor of metalloproteinase-2 (TIMP-2) (Hayakawa, 1994; Stetler-Stevenson et al, 1993*a*). Moreover, there have been several reports that the level of MMP-2 is not related to malignancy and invasion (Visscher et al, 1994; Ring et al, 1997) and that it is the level of TIMP-2 that is related to these processes (Visscher et al, 1994; Ring et al, 1997; Murashige et al, 1996). These results contradict the original proposed functions of MMP-2 and TIMP-2. In addition, membrane-type-1 matrix metalloproteinase (MT1-MMP) was recently identified (Sato et al, 1994). Conflicts among the above results might be resolved by studies of the functional interplay between MT1-MMP, MMP-2 and TIMP-2. The primary aim of the present study was to investigate whether the results of immunohistochemical detection of MT1-MMP, MMP-2 and TIMP-2 in colorectal cancers might be consistent with the proposed roles of these proteins in the promotion of the metastatic behavior of tumours.

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MATERIALS AND METHODS

Patients and tumour samples

A total of 92 adenocarcinomas of the colon and rectum were studied. Tumours were obtained surgically between 1988 and 1993 at the Department of Surgery II, Oita Medical University. There were 11 cases of simultaneous distant metastasis and nine cases of allochronic distant metastasis. All specimens were fixed in 10% buffered formalin and embedded in paraffin. The tumours were staged according to the standard TNM classification (Sobin and Wittekind, 1997).

Immunohistochemistry

For immunohistochemical analysis, 4 μm-thick sections were cut from formalin-fixed, paraffin-embedded blocks and placed on silan-coated slides. After deparaffinization, the sections were incubated in 3% hydrogen peroxide for 20 min in order for devitalization of the peroxidase. Deparaffinized and rehydrated specimens were heated in 10 mM citrate buffer, pH 6.0, for 10 min in an autoclave at 121°C. After cooling to room temperature (RT) for 30 min, the specimens were incubated with normal rabbit serum for 15 min at RT. Then they were incubated with various primary antibodies, namely, monoclonal antibody against MT1-MMP (114–6G6, 1:25; Fuji Chemical Industries, Japan), monoclonal antibody against MMP-2 (42-5D11, 1:100; Fuji Chemical Industries) or monoclonal antibody against TIMP-2 (67-4H11, 1:100; Fuji Chemical Industries) for 16 h at 4°C. After incubation, immunohistochemical staining was performed by the standard

avidin-biotin-peroxidase complex (ABC) technique with an LSAB kit (Nichirei, Tokyo, Japan) and 3,3′-diaminobenzidine as the chromogen. Nuclei were counterstained with haematoxylin. For evaluation of immunohistochemical staining, specimens were divided into two groups as follows. The immunopositive cell area was used for the evaluation of the immunohistochemical staining of MT1-MMP, MMP-2 and TIMP-2 monoclonal antibody: negative, 0–10%; positive, > 10%. Expression of MMP-2 and TIMP-2 was also evaluated in terms of immunostaining of the tumour cell cytoplasm and the stroma. A clinicopathologic study was performed by reference to the depth of invasion, lymphatic invasion, venous invasion, lymph node metastasis, and distant metastasis.

Statistical analysis

Correlations between the expression of each antigen and the various clinicopathologic factors were examined by the χ-squared test, Fisher's exact probability test and Mann-Whitney's U-test. Furthermore, correlations between the expression of pairs of antigens were studied by the χ-squared test.

RESULTS

Immunohistochemical staining of MT1-MMP, MMP-2 and TIMP-2

Immunohistochemical staining indicated that MT1-MMP was localized predominantly in the tumour cell cytoplasm and it was weakly or not expressed in normal tissue (Figure 1A). The frequency of samples positive for MT1-MMP was 36% (33/92). MMP-2 (Figure 1B) and TIMP-2 (Figure 1C) were localized by immunostaining in the tumour cell cytoplasm and the stroma. The frequency of samples positive for MMP-2 in the tumour cell cytoplasm was 20% (18/92) and in the stroma it was 30% (27/92). Thirteen samples (14%) in 92 were positive for MMP-2 in both the tumour cell cytoplasm and the stroma. The frequency for samples positive for TIMP-2 in the tumour cell cytoplasm was 32% (29/92) and in the stroma it was 47% (43/92). Eighteen samples (20%) in 92 were positive for TIMP-2 in both the tumour cell cytoplasm and the stroma.

Correlations between the expression of each antigen and the various clinicopathologic factors

Table 1 shows the correlations between the expression of each antigen and the various clinicopathologic factors. The frequency of immunodetection of MT1-MMP increased with increases in the depth of invasion (P = 0.007; Mann-Whitney U-Test). The percentage of MT1-MMP-positive cases was significantly higher in vascular invasion-positive cases (54%) than in invasion-negative cases (29%; P = 0.02; χ-squared test). The percentage of cases positive for MMP-2 in the stroma was significantly higher in vascular invasion-positive cases (42%) than in invasion-negative cases (20%; P = 0.02; χ-squared test). The frequency of detection of TIMP-2 in the tumour cell cytoplasm increased with increases in the depth of invasion (P = 0.03; Mann-Whitney U-test). TIMP-2 was detected in the stroma more frequently in tumours with lymphatic invasion and lymph node metastasis (P < 0.05; χ-squared test) than in tumours without such features.

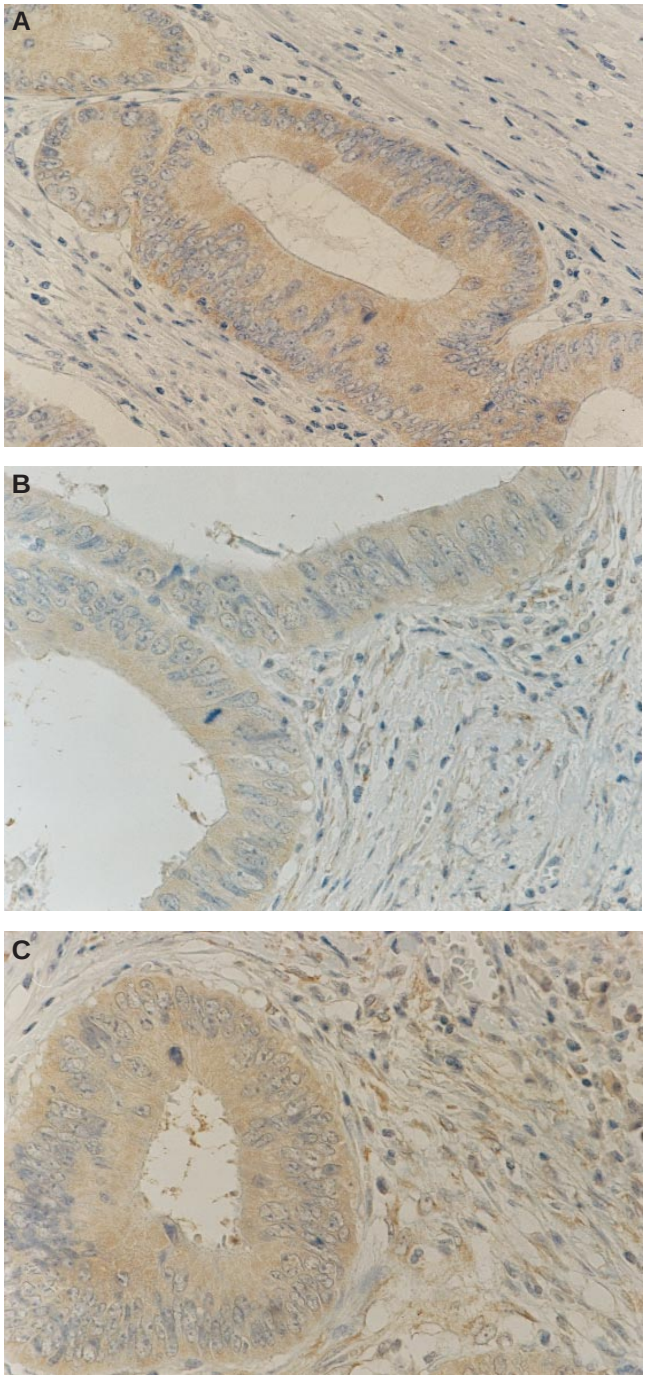

Figure 1 Immunolocalization of MT1-MMP (**A**), MMP-2 (**B**) and TIMP-2 (**C**) in samples of human colorectal carcinomas. Immunostaining was performed with monoclonal antibodies raised against MT1-MMP, MMP-2 and TIMP-2 as described in Materials and methods. Immunostaining revealed MT1-MMP is in the cytoplasm of tumour cells (**A**), while MMP-2 (**B**) and TIMP-2 (**C**) were immunostained in the tumour cell cytoplasm and the stroma, respectively (original magnification × 400).

Correlations among antigens

The percentage of cases positive for MT1-MMP was significantly higher in cases positive (61%, 11/18) for MMP-2 in the tumour cell cytoplasm than in negative cases (30%, 22/74; P = 0.013; χ-squared test). The percentage of cases positive for MT1-MMP was

Table 1 Correlations between the expression of MT1-MMP, MMP-2 and TIMP-2 and clinicopathologic factors

	MT1-MMP		MMP-2				TIMP-2			
			Tumour cell cytoplasm		Stroma		Tumour cell cytoplasm		Stroma	
Variable	**Positive rate (%)**	***P* value**	**Positive rate (%)**	***P* value**	**Positive rate (%)**	***P* value**	**Positive rate (%)**	***P* value**	**Positive rate (%)**	***P* value**
Depth of invasion[a]										
T1	6	0.007	28	NS	28	NS	28	0.03	39	NS
T2	36		7		25		11		46	
T3 and T4	48		24		33		46		50	
Lymphatic invasion[b]										
Positive	44	NS	19	NS	44	NS	33	NS	58	0.04
Negative	29		20		29		31		37	
Venous invasion[b]										
Positive	54	0.02	15	NS	42	0.02	42	NS	62	NS
Negative	29		21		20		27		41	
Lymph node metastasis[b]										
Positive	34	NS	14	NS	37	NS	34	NS	60	0.046
Negative	37		23		25		30		39	
Distant metastasis[b]										
Positive	45	NS	15	NS	20	NS	40	NS	35	NS
Negative	33		21		32		29		50	

NS, Not significant; [a]Mann-Whitney's U-test; [b]chi-squared test or Fisher's exact probability test

significantly higher in cases positive (55%, 16/29) for TIMP-2 in the tumour cell cytoplasm than in negative cases (27%, 17/63; $P < 0.01$; χ-squared test). The percentage of cases positive for MMP-2 in the tumour cell cytoplasm was significantly higher in cases positive for TIMP-2 in the tumour cell cytoplasm (41%, 12/29) than in negative cases (10%, 6/63; $P < 0.01$; χ-squared test). The frequency of samples positive for all three (MT1-MMP, MMP-2 and TIMP-2) was 12% (11/92), and the frequency of samples negative for all three was 23% (21/92).

DISCUSSION

MMP-2, which is a type IV collagen-degrading enzyme, is a very important factor in the infiltration and metastasis of several carcinomas. TIMPs are intrinsic inhibitors of MMPs and have been studied clinically as potential carcinostatic agents (Watson et al, 1995; 1996; An et al, 1997). Theoretically, MMP-2 and TIMP-2 should be immunolocalized only in fibroblasts and monocytes at the sites that produce MMP-2 and TIMP-2 (Poulsom et al, 1992; Liabakk et al, 1996; Pyke et al, 1993; Ito et al, 1995). However, they have also been immunolocalized in the cytoplasm and cell membranes of cancer cells (Tomita and Iwata, 1996; Nomura et al, 1996; Höyhtyä et al. 1994). In our study, we found that MMP-2 and TIMP-2 were immunostained not only in the stroma of tumours but also in the cytoplasm of cancer cells, as well as there being elevated frequencies of expression of MT1-MMP, MMP-2, and TIMP-2 in the cytoplasm of cancer cells. One reason for this is that TIMP-2 and proMMP-2 might have been anchored to the cell membrane by MT1-MMP (Sato et al, 1996; Imai et al, 1996).

Expression of MMP-2 was not strongly correlated with factors related to infiltration apart from vascular invasion in our study. One explanation of our results is that the immunostaining with the MMP-2-specific antibody used in this study did not reflect the activity of MMP-2 since the antibody recognized both MMP-2 and proMMP-2 (Fujimoto et al, 1993). Furthermore, Liabakk et al (1996) reported that less-advanced tumours at Dukes' stage A have higher levels of active MMP-2 than do more invasive tumours at Dukes' stage B. These results raise the possibility that MMP-2 might be necessary while the tumour is in the process of penetrating the bowel wall in tumours at Dukes' stage A but might be less important when tumours have spread beyond muscle into the surrounding adipose tissue, as at stage B. Consequently, the immunohistochemical detection of MMP-2 appears not to be an appropriate indicator of infiltration and metastasis in a clinical setting.

The expression of TIMP-2 has been reported to be closely correlated with the progression of human colorectal cancer, a proposal that conflicts with the original function of TIMP-2 as an inhibitor of MMPs (Murashige et al, 1996; Tomita and Iwata, 1996). In our study, expression of TIMP-2 in the tumour cell cytoplasm was correlated with depth of invasion and expression of TIMP-2 in the stroma was correlated with lymphatic invasion and lymph node metastasis. These results indicate that TIMP-2 is a reliable indicator of the progression of human colorectal carcinoma. These results are in conflict with the original proposed functions of inhibitors of MMPs for the following reasons. TIMPs counteract the proteolytic functions of MMPs in a stoichiometric manner, at a ratio of 1:1 (Stetler-Stevenson et al, 1993*b*). However, the presence of excess TIMPs, as compared to MMPs, in tumour tissue might indicate that an abnormal ratio in tumour tissue was associated with growth and metastasis, and the binding and biological actions of MMPs and TIMPs might be altered in the presence of excess TIMPs (Tomita and Iwata, 1996; Kossakowska et al, 1996). In addition, the TIMP-2-specific antibody used in our study also recognized the proMMP-2/TIMP-2 complex (Höyhtyä et al, 1994). TIMP-2 in this complex functions as an activator of proMMP-2. Accordingly, under difficult circumstances, such as when quantifying TIMP-2 and MMP-2, immunohistochemical detection of TIMP-2 might indicate that TIMP-2 is essential for the activation of proMMP-2.

Recently, MT1-MMP was identified in the cell membranes of transfected cells that expressed MT-MMP and the expression of

MT1-MMP induced the activation of the precursor to MMP-2, proMMP-2 (Sato et al, 1994). There were few previous reports of immunohistochemical detection of MT1-MMP in colorectal carcinoma. In tumour cells of invasive lung carcinomas, MT1-MMP was immunolocalized in the carcinoma cells but not in the parenchymal or stromal cells of the surrounding normal tissue (Sato et al, 1994). In gastric carcinoma, MT1-MMP was predominantly immunolocalized in the carcinoma cells, and some carcinoma cells showed immunostaining on the cell membrane (Nomura et al, 1995). We found that MT1-MMP was immunolocalized predominantly in colorectal carcinoma cells, while its levels were low or it was absent in normal tissue. Moreover, expression of MT1-MMP was correlated with vascular invasion in cases of gastric carcinoma (Nomura et al, 1995) and with lymph node metastases in cases of lung carcinoma (Tokuraku et al, 1995) in previous studies. The present study showed that expression of MT1-MMP was correlated with vascular invasion and the depth of invasion. These results suggest that the expression of MT1-MMP might reflect infiltration in colorectal carcinoma.

Thus, immunohistochemical detection of MT1-MMP and TIMP-2 provides appropriate indices of infiltration but expression is not correlated with distant metastases in colorectal carcinoma. Our results indicate that not only MMPs but also other factors are required for the development of distant metastases.

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