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European Journal of Cancer 36 (2000) 2164–2170

European Journal of Cancer

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Gelatinolytic activity of matrix metalloproteinase-2 and -9 in oesophageal carcinoma; a study using *in situ* zymography

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Received 20 March 2000; received in revised form 7 June 2000; accepted 9 June 2000

Abstract

In this study, we investigated the activity of matrix metalloproteinase (MMP)-2 and -9 by gelatine zymography, immunostaining and *in situ* gelatine zymography in 30 oesophageal squamous-cell carcinomas. The gelatinolytic activity *in situ* was detected in all cases with different patterns of localisation. Significant gelatinolysis by stromal cells adjacent to tumour nests was found in 12 cases. Strong gelatinolytic activity appeared within the tumour nest itself in 13 cases. In the other 5 cases, both stromal cells and tumour cells showed the gelatinolytic activity. Gelatine zymography demonstrated a correlation between vascular invasion and activation of MMP-9. It also demonstrated a correlation between lymph node metastasis, lymphatic or vascular invasion and activation of MMP-2. These results suggest that MMPs play an important role in the invasion of oesophageal carcinoma.

Keywords: Matrix metalloproteinase-2; Matrix metalloproteinase-9; *In situ* zymography; Gelatinolytic activity; Oesophageal cancer

1. Introduction

Patients with oesophageal cancers have a poor prognosis due to extensive local tumour invasion and frequent spread to lymph nodes or other metastatic sites at the time of the diagnosis [1,2]. The invasion of the tumour requires destruction of the extracellular matrix and connective tissue surrounding the tumour cells and the basement membrane [3–5]. Matrix metalloproteinases (MMPs) degrade components of the extracellular matrix [6]. MMPs are classified as gelatinases, collagenases, stromelysins, membrane-type matrix metalloprotease (MT-MMP), based mainly on the *in vivo* substrate specificity of the individual MMP [7–11]. Type IV collagen is an important protein of the basement membrane. Type IV collagenases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have been reported to be especially important in the process of tumour invasion and metastasis [12–15]. These enzymes are secreted as inactivated proMMPs [16], and activated by proteolytic cleavage [17,18]. A MT-MMP that catalyses proteolytic cleavage of MMP-2 has been identified [19]. MMP-3 has been reported to cleave proMMP-9 and activate it [20].

Using gelatine zymography, it has been reported that there is a relationship between the production of Type IV collagenases and malignant disease [21–24]. Our previous studies have shown that activation of proMMP-2 may be an indicator of lymph node metastasis of breast cancer [25]. The localisation of active gelatinase *in situ*, however, has been difficult to analyse. The biggest problem has been the poor resolution of *in situ* zymography [26–28].

We have developed a gelatine film *in situ* zymography with high resolution and reproducibility. We applied this method to study the role of MMPs in malignant tumours. In combination with conventional zymography and densitometry, we have investigated whether MMPs play an important role in the invasion of oesophageal carcinoma.

2. Materials and methods

Thirty human oesophageal cancers were resected at the Department of Surgery II, Nagoya City University Medical School Hospital, Nagoya, Japan. The samples

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0959-8049/00/$ - see front matter
PII: S0959-8049(00)00297-5

were snap-frozen in Tissue-Tek O.C.T. compound (Miles Inc., Elkhart, IN, USA) by dipping in liquid nitrogen. Blocks of embedded tissue were stored at −80°C until used for immunostaining and *in situ* zymography. Frozen sections were made using a cryostat. Paraffin sections of the surgical specimens fixed in formalin were stained with haematoxylin and eosin.

2.1. Tissue extract

Tissue samples (200 mg) were rinsed in sterile phosphate-buffered saline (PBS), homogenised in 2 ml TNC buffer (50 mM Tris–HCl, 0.15 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3·, 0.05% Brij35), and then were centrifuged at 15 000 rpm for 15 min. Supernatants were stored at −80°C until used for gelatine zymography. The protein concentration of the supernatant was measured by Lowry's method using a commercial kit (DC protein assay kit, Bio-Rad, Hercules, CA, USA).

2.2. Immunohistochemistry

Monoclonal antibodies against human MMP-2 (75-7F7) and MMP-9 (56-2A4) have been characterised [20]. For immunohistochemistry, the frozen sections (6 μm) were stained with primary antibodies or non-immune mouse IgG for 15–18 h at 4°C after blocking endogenous peroxidase with 1% H_2O_2. Non-specific binding was blocked with 10% normal rabbit serum. Concentration of the primary antibody used for immunostaining was 2.5 μg/ml for anti-MMP-2, 2 μg/ml for anti-MMP-9 and 10 μg/ml for non-immune mouse IgG. After rinsing in PBS, the sections were incubated with a biotinylated rabbit antibody to mouse IgG (SAB-PO Kit. Nichirei Co., Inc., Tokyo, Japan) for 10 min, washed and then incubated with streptavidin–peroxidase complex (SAB-PO Kit) for 5 min at room temperature. Colour was developed with 0.03% 3,3′-diaminobenzidine tetrahydrochloride in 50 mM Tris–

Table 1
Clinicopathological characteristics of patients and gelatinase activity

Patient	Age/sex	Stage[a]	Depth of invasion[a]	Lymph node metastasis	Lymphatic invasion	Vascular invasion	Immunohistochemistry		*In situ* zymography	Zymography (activation ratio)	
							MMP-9	MMP-2		MMP-9	MMP-2
1	69/M	IV	a1	–	+	–	–	–	T	0[b]	0[b]
2	73/M	III	a2	+	+	–	–	+	T	0	0
3	71/M	I	mp	–	–	+	–	–	I	0.13	0
4	62/M	IV	a2	–	–	–	–	+	I	0.26	0
5	64/M	III	mp	–	+	–	–	+	I	0	0.15
6	76/M	IV	a3	–	–	–	–	+	I	0.03	0.19
7	68/F	III	a2	–	+	+	+	+	T	0	0.22
8	68/M	III	a2	+	+	+	–	+	I	0.37	0.24
9	78/F	III	mp	+	+	+	–	–	T	0.24	0.24
10	68/F	III	a2	–	+	–	+	–	I	0	0.27
11	65/M	III	a2	+	+	+	–	+	T and I	0.11	0.27
12	71/M	I	mp	–	–	–	–	+	T	0	0.3
13	69/M	II	a1	+	+	+	+	–	T	0	0.36
14	64/M	IV	a1	+	+	–	+	+	T and I	0	0.37
15	73/M	IV	a1	+	+	+	+	–	I	0.19	0.38
16	68/M	II	a1	+	+	–	+	–	T	0	0.38
17	63/M	II	mp	–	–	–	+	+	T and I	0.11	0.39
18	74/F	IV	a2	+	+	–	+	–	I	0.08	0.42
19	71/M	III	a2	+	+	+	+	+	T and I	0.12	0.42
20	59/M	IV	a2	+	+	–	+	+	T	0	0.43
21	71/M	III	a2	+	+	+	–	–	I	0.12	0.44
22	71/M	III	a1	+	+	+	+	–	T and I	0.05	0.44
23	63/M	III	a2	+	+	+	+	–	I	0.2	0.46
24	69/M	III	a2	+	+	+	+	+	T	0.33	0.46
25	72/M	III	a2	+	+	+	+	+	T	0.17	0.49
26	69/M	IV	a1	+	+	+	+	+	I	0.12	0.50
27	64/M	IV	a1	–	+	+	+	+	I	0.28	0.55
28	64/M	IV	a3	+	+	+	+	–	T	0.22	0.56
29	58/F	IV	a2	+	+	+	–	+	T	0.09	0.67
30	59/M	IV	a1	+	+	+	+	+	T	0.10	0.80

T, gelatinolytic activity positive in tumour cells; I, gelatinolytic activity positive in stromal cells.

[a] Guidelines for the clinical and pathologic studies on carcinoma of the oesophagus, 8th edn Japanese Society for Esophageal Disease.

[b] Activation ratio; density of active band/sum of densities of inactive and active band.

HCl buffer, pH 7.6, containing 0.006% H_2O_2. Counterstaining was performed with 1% methyl green. We scored the staining as positive when more than 30% of the tumour cells stained for MMP.

2.3. In situ *zymography*

Gelatinolytic activity in the tissue specimen was analysed using a newly developed gelatine-coated film. This 6 μm thick gelatine-coated film contains calcium and was developed in collaboration with Fuji Photo Film Co., Ltd, Tokyo, Japan. Frozen sections (6 μm) were placed on this film. Films were incubated in a humidified chamber at 37°C for various lengths of time. After incubation, the film was stained with 1% Amido Black 10B (Wako Inc., Tokyo, Japan) in 70% (v/v) methanol and 10% (v/v) acetic acid for 15 min. The film was destained with distilled water and a 70% (v/v) methanol, 10% (v/v) acetic acid solution. Lysis of the substrate was assessed by examination under a light microscope.

2.4. *Gelatine zymography*

Zymography was performed using sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) containing 0.2% gelatine according to the method of Hibbs and colleagues [29]. The tissue extract was incubated at 37°C for 20 min in SDS sample buffer without reducing agent and then electrophoresed on 8% polyacrylamide gels at 4°C. After electrophoresis, the gels were washed in 2.5% Triton-X 100 and incubated for 16 h at 37°C in 50 mM Tris–HCl, pH 7.4, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% NaN_3 and then stained with 0.1% coomassie brilliant blue R250. Computer-assisted image analysis of the gels was performed [14]. Relative activity of an MMP was calculated by dividing the density of the active band by the sum of the densities of the bands for the active and proMMP. The effect of various inhibitors such as ethylene diamine tetraacetic acid (EDTA), 1,10-phenanthroline and phenylmethylsulphonyl fluoride (PMSF) on gelatinolytic activity was also tested.

2.5. *Statistical analyses*

The correlation between clinicopathological factors and immunostaining for MMP-2 and MMP-9 was examined by the χ^2-test and the Kruskal–Wallis test for categorical and quantitative data respectively. The correlations between the activation of MMP-2, -9 and lymph node metastasis, local invasion, lymphatic and vascular invasion were examined by Student *t*-test. The correlations between activation of the MMP-2, -9 and gelatinolytic activity were examined by Student *t*-test. *P* values below 0.05 were considered significant.

3. Results

3.1. *Clinicopathological characteristics of the patient*

The clinicopathological characteristics of each patient are presented in Table 1.

3.2. *Immunohistochemistry*

Localisation of MMP-2 and MMP-9 was examined in carcinoma samples from 30 cases. Antibodies to MMP-2 and MMP-9 stained the carcinoma cells intracytoplasmically (Fig. 1a) in approximately 60.0% of cases. In most positive cases, cell membrane of some carcinoma cells was also stained with the antibody against MMP-2 (Fig. 1a). In many cases keratinising lesion (arrow) within carcinoma tissue and vascular endothelium was also immunostained with the antibodies against MMP-2 and -9 (Fig. 1b). Stromal fibroblasts and vascular endothelial cells were also positive for

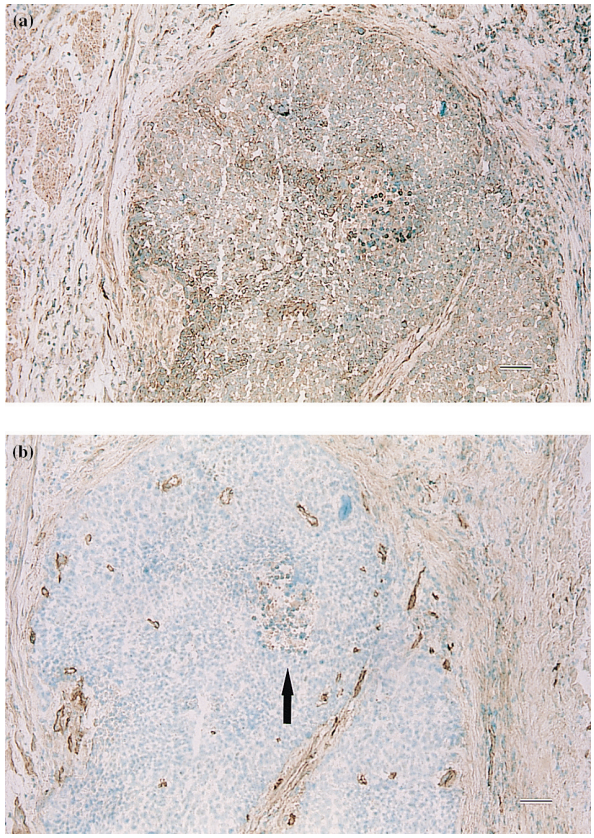

Fig. 1. Immunohistochemistry for MMP-2 (a) and MMP-9 (b) of a representative oesophageal cancer. Counterstained with methyl green. In this sample, cancer cells are positive for MMP-2 (a). For MMP-9, the keratinising lesion (arrow) within carcinoma tissue and vascular endothelium are positive (b). MMP-9 staining of tumour cells is faint in this sample. This specimen was the same as the one used in Fig. 2(a) and (b) for *in situ* gelatine zymography. Staining was mainly cytoplasmic with significant staining of the plasma membrane for MMP-2. Stromal cells also stained for both MMP-2 and -9. Bar = 50 μm.

MMP-2 and MMP-9 (data not shown). There was no correlation between the immunostaining of MMP-2 or MMP-9 and lymph node metastasis, local invasion, lymphatic or vascular invasion (data not shown).

3.3. In situ *zymography*

The localisation of gelatinolytic activity was detected as a bright area due to gelatine digestion. To determine the optimum time of incubation, serial sections were incubated from 2 h to 7 days at 37°C. Visible lysis of gelatine began after approximately 4 h. Gelatinolytic activity could not be localised due to tissue disintegration after 24 h. The gelatinolytic activities were completely inhibited by EDTA or incubation at 4°C. Moreover, the lytic activity was completely inhibited at 4°C. We chose an incubation temperature of 37°C and an incubation time of 16 h for further experiments.

The localisation of gelatinolytic activity was analysed by *in situ* zymography in 30 oesophageal carcinomas. Gelatinolytic activity was detected in all cases and Fig. 2 demonstrates two different patterns of localisation of gelatinolytic activity (Fig. 2a and c). HE stained adjacent sections are also shown (Fig. 2b and d). In 13 cases, strong gelatinolytic activity appeared within the tumour nest itself, but not in the surrounding stromal cells (Fig. 2a and b). In 12 cases, significant gelatinolysis by stromal cells adjacent to tumour nests, but not by the tumour cells themselves was found (Fig. 2c and d). In 5 cases, both stromal cells and tumour nests showed gelatinolysis (data not shown). The sections used for Fig. 2(a) and (b) are adjacent to the ones used for Fig. 1(a) and (b). The area of gelatinolysis approximately coincided with that of MMP-2 and MMP-9 immunoreactivity; the tumour cells that were positive for MMP-2 immunoreactivity showed weak gelatinolysis and the area with MMP-9 immunoreactivity showed strong *in situ* gelatinolysis. However, there seemed to be many cells with positive MMP immunostaining that did not lyse gelatine in *in situ* zymography.

3.4. *Gelatine zymography*

The gelatinolytic activity of tissue extract was analysed by gelatine zymography in 30 oesophageal carcinomas. ProMMP-9 (92 kDa) and proMMP-2 (68 kDa) were found in all samples (representative 6 cases are shown in Fig. 3). The active band for MMP-2 (62 kDa)

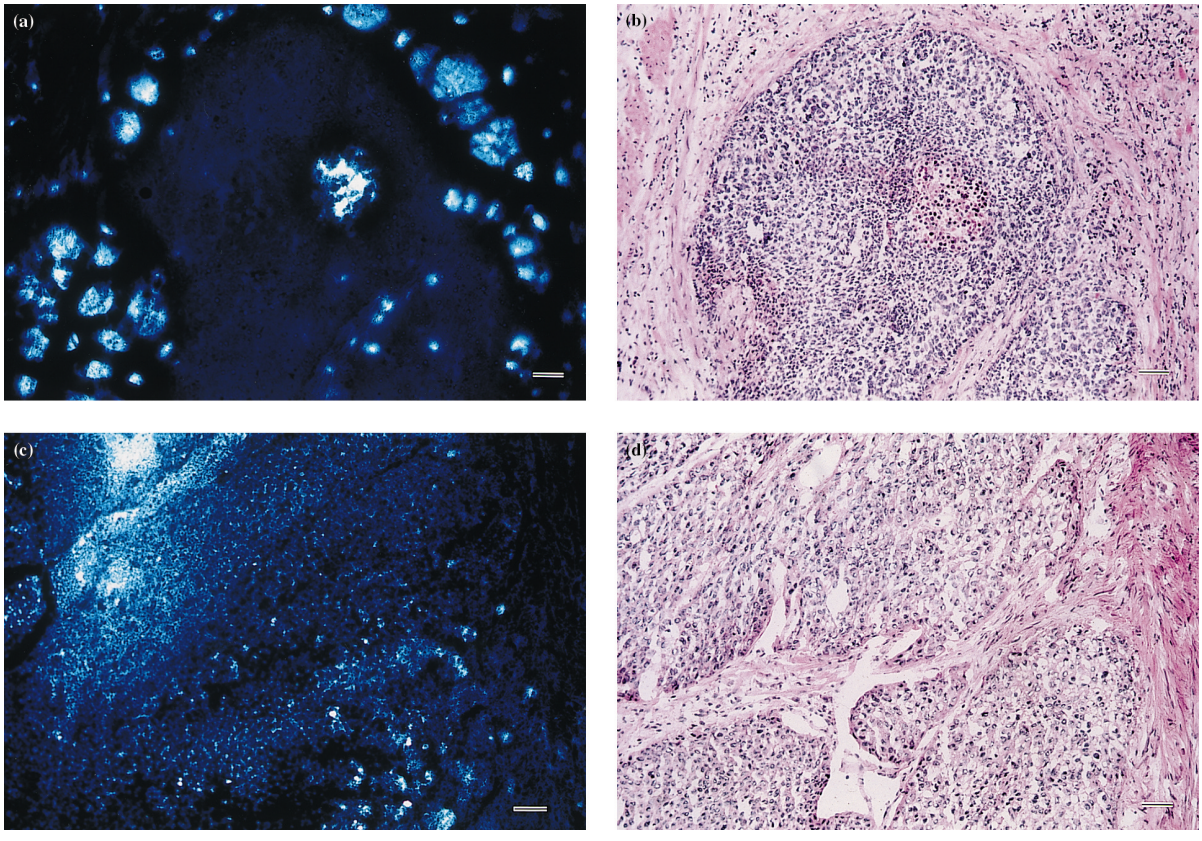

Fig. 2. *In situ* gelatine zymography (a and c) and matched haematoxylin and eosin (HE) staining (b and d) of representative oesophageal cancers. Tissue sections were incubated on a gelatine-coated film and the digested gelatine was visualised as a bright area. In some, gelatinolysis was noted in the tumour cells only (a), for HE staining see (b). In others, the stromal cells adjacent to tumour cells showed gelatinolysis (c), for HE staining see (d). Sections used for (a) and (b) were adjacent to the ones used for the immunostaining of MMP in Fig. 1. Bar = 50 μm.

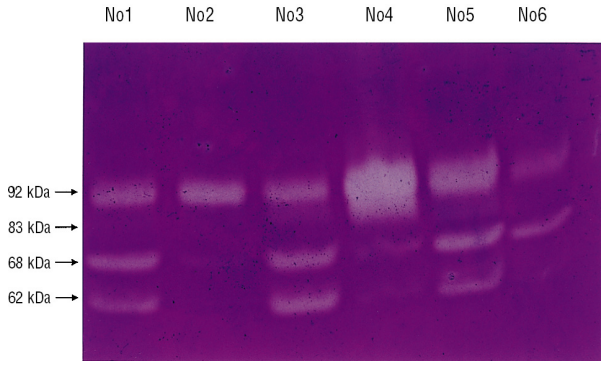

Fig. 3. Gelatine zymography of oesophageal cancers. 6 representative cases are shown. The positions of molecular weight markers are shown on the left. ProMMP-9 (92 kDa) are seen in all 6. ProMMP-2 (68 kDa) are seen in cases 1, 3, 4, 5 and 6 and faintly in case 2. Active MMP-2 (62 kDa) is seen in cases 1, 3 and 5 and faintly in cases 4 and 6. Active MMP-9 (83 kDa) is seen in case 4 and faintly in cases 3 and 5.

was detected in 26 cases (87%). The active band for MMP-9 (83 kDa) was present in 20 cases (67%).

Gelatinolytic activities of 92, 83, 68 and 62 kDa bands were almost completely inhibited by 10 mM EDTA and 1 mM 1,10-phenanthroline, but not by 2 mM-PMSF (data not shown).

Densitometric analysis showed that the relative activity of MMP-9 (the density of the active 83 kDa band relative to the sum of the densities of the bands of the proMMP-9 and active MMP-9) was significantly higher in the tumours that showed vascular invasion (v(+), 0.157±0.103) than in the tumours without vascular invasion (v(−), 0.039±0.078) ($P=0.0022$) (Fig. 4).

The relative activity of MMP-2 was significantly higher in the tumours with lymph node metastasis (n(+), 0.403±0.170) than in the tumours without lymph node metastasis (n(−), 0.213±0.190) ($P=0.011$). It was also higher in tumours with lymphatic invasion (ly(+), 0.381±0.183) than in tumours without lymphatic invasion (ly(−), 0.177±0.177) ($P=0.030$) and in tumours with vascular invasion (v(+), 0.415±0.181) than in tumours without vascular invasion (v(−), 0.242±0.17) ($P=0.014$) (Fig. 5).

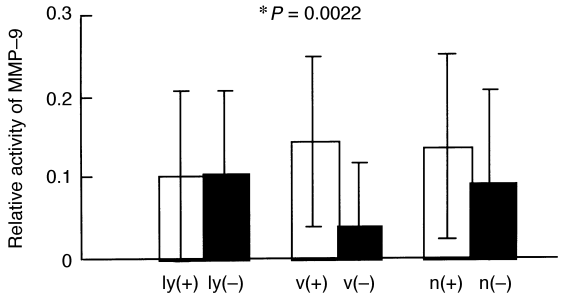

Fig. 4. Relative activity of MMP-9 according to the invasiveness of the oesophageal cancer. Those with vascular invasion (v(+)) ($n=18$) showed higher MMP-9 activity than those without vascular invasion (v(−)) ($n=12$). Lymphatic invasion (ly) or lymph node metastasis (n) did not show a correlation with MMP-9 activity. *Student t-test.

4. Discussion

Using a newly developed *in situ* zymography in combination with conventional gelatine zymography and immunohistochemistry, we have demonstrated an important role of MMPs in the invasion of oesophageal cancer. By immunohistochemistry, the percentage of cases with positive staining for MMP-2 and that for MMP-9 was approximately 60%. Murray and associates have reported that positive staining for MMP-2 and MMP-9 in oesophageal cancer was observed in 78% and 70% of samples, respectively [30]. We found no significant correlation between the presence of MMPs detected immunohistochemically and the invasion of oesophageal carcinoma. In addition, not all cells with positive MMP immunoreactivity showed gelatinolytic activity in our *in situ* zymography suggesting that the presence of MMP proteins may not directly lead to tumour cell invasion.

We have developed a new technique for *in situ* zymography using a gelatine-coated film. By optimising the incubation time and temperature we could obtain reproducible *in situ* zymography with a fine resolution of detail. However, there may be a difference in the amount of gelatinase and its inhibitors in different tissues and the optimal conditions may differ according to the tissue studied.

Using a similar method, Nakamura and colleagues have recently reported that *in situ* zymography indicated a gelatinolytic activity in the thyroid carcinoma cell nests, but not in the stromal cells [31]. Our data showed that there were gelatinolytic activities in the tumour nest and also in the stromal cells in some oesophageal carcinomas. This may suggest that oesophageal carcinoma produces more MMPs than thyroid carcinoma. Alternatively, since there is no fibrous capsule in oesophageal cancer, the activated MMPs may diffuse outside of the tumour nests.

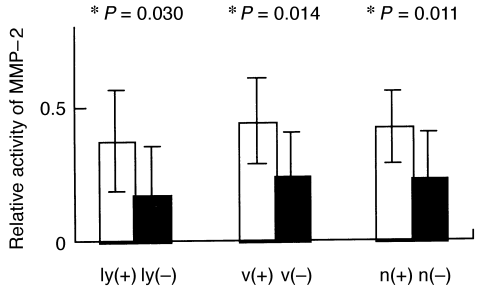

Fig. 5. Relative activity of MMP-2 according to the invasiveness of the oesophageal cancer. Those with vascular invasion (v(+)) ($n=18$) showed higher MMP-2 activity than those without vascular invasion (v(−)) ($n=12$). Those with lymphatic invasion (ly(+)) ($n=25$) showed higher MMP-2 activity than those without lymphatic invasion (ly(−)) ($n=5$). Those with lymph node metastasis (n(+)) ($n=21$) showed higher MMP-2 activity than those without lymph node metastasis (n(−)) ($n=9$). *Student t-test.

The activation of proMMP-2 was positively correlated with lymph node metastasis, lymphatic and vascular invasion (Fig. 5). Similar correlation of proMMP-2 activation with lymph node metastasis has been reported in human thyroid [31], breast [32], lung [33], stomach [34] and pancreatic carcinoma [35]. It is therefore possible that activation of proMMP-2 in carcinoma tissue is a determinant of metastasis in many cancers. In the present study, activation of proMMP-9 correlated positively with vascular invasion. This result suggests that not only MMP-2, but also MMP-9, may play an important role in the invasion of oesophageal carcinoma.

Using *in situ* and conventional zymography, the experiments described in this paper have added to the evidence that suggests MMP-2 and -9 play an important role in the invasion of oesophageal carcinoma and further studies are warranted to seek methods to control these enzymes in order to improve the prognosis of this intractable disease.

Acknowledgements

We are grateful to Yutaka Tamura (Fuji Photo film Co., Ltd, Japan) for providing us with gelatine films. We also thank Ms Shinobu Makino for technical assistance.

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