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An immunohistochemical study of TIMP-3 expression in oesophageal squamous cell carcinoma

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Tissue inhibitor of metalloproteinase-3 (TIMP-3) inhibits the activity of matrix metalloproteinase, which may play an important role in carcinoma invasion and metastasis. We have investigated the relationship between TIMP-3 reduction and clinicopathological factors in oesophageal squamous cell carcinoma (ESCC). We examined tissue specimens that had been removed from 90 patients with thoracic oesophageal cancer who had undergone surgery between 1983 and 2001. Immunohistochemical staining was performed by the standard streptavidin–biotin method. Immunostaining of TIMP-3 was seen in the cytoplasm of cancer cells and normal oesophageal epithelial cells, particularly in cells located in shallow areas of the tumour. TIMP-3 preserved (+), moderate (±), and reduced (−) cases accounted for 30, 27, and 33 of the 90 patients, respectively (33, 30, 37%). Significant correlations were observed between TIMP-3 expression and depth of tumour invasion ($P=0.001$), number of lymph node metastases ($P=0.003$), infiltrative growth pattern ($P=0.003$), and disease stage ($P=0.005$). The survival rates of patients with TIMP-3 (−) cancer were significantly lower than those of patients with TIMP-3 (+) and TIMP-3 (±) cancer ($P=0.0003$). The mean 5-year survival rates of patients with TIMP-3 (+), (±), and (−) were 50, 58, and 21%, respectively. In conclusion, decreased expression of TIMP-3 protein correlates with invasive activity and metastasis. This makes the prognosis for patients with cancer that has lost TIMP-3 significantly less favourable than that for patients with cancer that has maintained TIMP-3.

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The extracellular matrix is an important structure in maintenance of tissue organisation and in the suppression of cellular proliferation and migration. Excess destruction of the extracellular matrix is associated with many pathologies, including atherosclerosis, rheumatoid arthritis, and cancer progression (Liotta *et al*, 1980; Tryggvason *et al*, 1987; Palletier *et al*, 1990). The intricate balance between net extracellular matrix deposition and degradation is controlled by a complex system of tightly regulated protease enzymes and their endogenous inhibitors. Matrix destruction is thought to be a key event in both the local invasion and distant metastasis associated with tumour progression, and genes that inhibit these processes may be useful for cancer therapy. Matrix metalloproteinases (MMPs), a family of Zn^{2+} metalloproteinases involved in the degradation of extracellular matrix macromolecules, are associated with tissue destruction under various pathological conditions. Previous experimental and clinicopathological studies have revealed a good correlation between expression of MMPs and invasive phenotype of tumour cells or frequency of metastasis (Stetler-Stevenson *et al*, 1993; Sato *et al*, 1994). Expression of MMP-1, -2, -3, -7, and -9 in oesophageal cancer has already been reported (Shima *et al*, 1992; Ohashi *et al*, 2000). In addition, Ohashi *et al* (2000) have reported the expression of tissue inhibitor of MMP (TIMP)-1 and -2, MT1-MMP, and MT2-MMP by immunohistochemical staining.

The TIMPs are a family of molecules that inhibit the proteolytic activity of MMPs, which also play an important role in tumour invasion and metastasis (Sato *et al*, 1992; Miyagi *et al*, 1995; Wang *et al*, 1997).

TIMP-3 is a secreted 24-kDa protein that, unlike other TIMP family members, binds to the extracellular matrix. Its functions have been reported as inducing apoptosis of cancer cells (Ahonen *et al*, 1998; Baker *et al*, 1999), and suppressing tumour growth and angiogenesis (Anand-Apte *et al*, 1996, 1997; Bian *et al*, 1996). Furthermore, TIMP-3 expression decreases at the invasive edge of poorly differentiated adenocarcinoma of the human colon, which suggests that a regional loss of TIMP-3 may contribute to their increased invasiveness (Powe *et al*, 1997). However, the relationship between TIMP-3 expression and clinical features (tumour invasiveness, metastasis and prognosis) in patients with ESCC is not known.

In this study, an immunohistochemical analysis of TIMP-3 protein expression was performed to determine the relationship between TIMP-3 reduction and clinicopathological factors in ESCC.

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MATERIALS AND METHODS

Patients and tissue samples

The tissue specimens used had been removed from 90 patients with thoracic ESCC who had undergone surgery at Gunma University Hospital between 1983 and 2001. Written informed consent to participate in the study was obtained from each patient before surgery, according to the ethical guidelines of our university. All patients underwent potentially curative surgery without preoperative therapy. There were 76 men and 14 women, aged 40–78 years (mean age: 60.9 years). Tumour stages were classified according to the fifth edition of the TNM classification of the International Union against Cancer (UICC). Evaluation of tumour differentiation was based on histological criteria of the guidelines of the Japanese Society for Esophageal Diseases (1999). The mean postoperative follow-up period was 33.9 months (range: 6.2–192.2 months).

Specimens were fixed in 10% formaldehyde solution and embedded in paraffin. We examined sections that contained both a tumour-invasive portion and normal oesophageal epithelium.

Antibodies

The monoclonal antibody (Mab) specific for TIMP-3 (clone 136-13H4) was purchased from Daiichi Fine Chemical Co. Ltd., Toyama, Japan.

Immunohistochemistry

Immunohistochemical staining was performed by the standard streptavidin–biotin (SAB) method. Briefly, each 4-μm tissue section was deparaffinised, then rehydrated and incubated with fresh 0.3% H_2O_2 in methanol for 30 min at room temperature. After rehydration through a graded ethanol series, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 120°C for 5 min, then cooled to 30°C. After incubation with normal rabbit serum for 30 min, the tissue sections were removed by blotting. The sections were then incubated at 4°C overnight with anti-TIMP-3 Mab at a dilution of 1 : 800 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, then washed in PBS and incubated with secondary antibody for 30 min at room temperature. Immunohistochemistry was performed using a Histofine SAB-PO(M) kit (Nichirei, Tokyo, Japan). The chromogen was 3,3′-diaminobenzidine tetrahydrochloride, applied as a 0.02% solution containing 0.0055% H_2O_2 in 50 mM ammonium acetate – citric acid buffer (pH 6.0). The sections were lightly counterstained with haematoxylin. Negative controls were prepared by substituting normal mouse serum for primary antibody, and no detectable staining was evident.

Evaluation of immunostaining for TIMP-3 expression

We evaluated immunostaining of cancer cells in all layers of the oesophageal epithelium. When more than 80% of carcinoma cells in a given specimen were positively stained as well as normal epithelium in the same section, the sample was classified as TIMP-3 preserved (TIMP-3 (+)); when less than 30% were stained, as TIMP-3 reduced (TIMP-3 (−)); and when 30–80% of cells were stained, as TIMP-3 moderate (TIMP-3 (±)).

Statistical analysis

Statistical analysis was performed using unpaired two-group *t* test for age and number of lymph node metastases. The χ^2 test was used to analyse the effects of sex, differentiation, location, and TNM clinical classification. Survival curves were calculated by the Kaplan–Meier method, and analysis was carried out by the log-rank test.

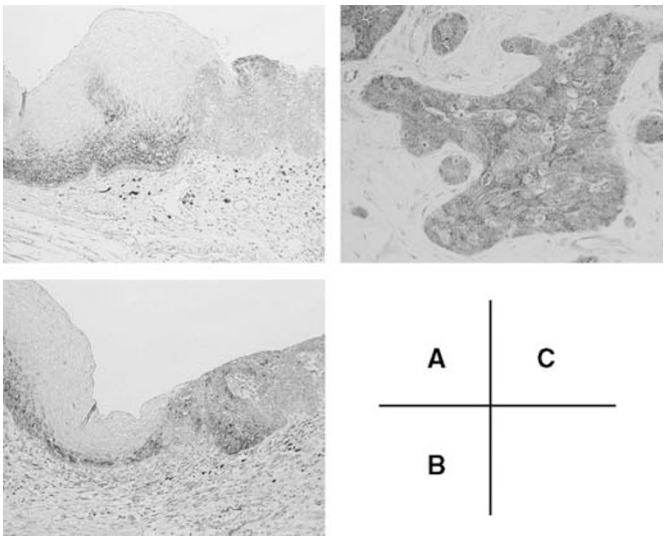

Figure 1 Representative photomicrographs of tissue sections immunostained for TIMP-3. (**A**) TIMP-3 was detected in the cytoplasm of the basal cells, parabasal cells, and leucocytes in normal oesophageal epithelium (left side). In this case, TIMP-3 expression in the cancer cells (right side) was weaker than that in the normal epithelium. This case was regarded as TIMP-3-reduced (× 100). (**B**) The arrowhead indicates primary oesophageal cancer with TIMP-3 protein expression (× 100). This case was regarded as TIMP-3 preserved. (**C**) High-power view of the immunohistochemistry. TIMP-3 was detected in the cytoplasm of cancer cells (× 200).

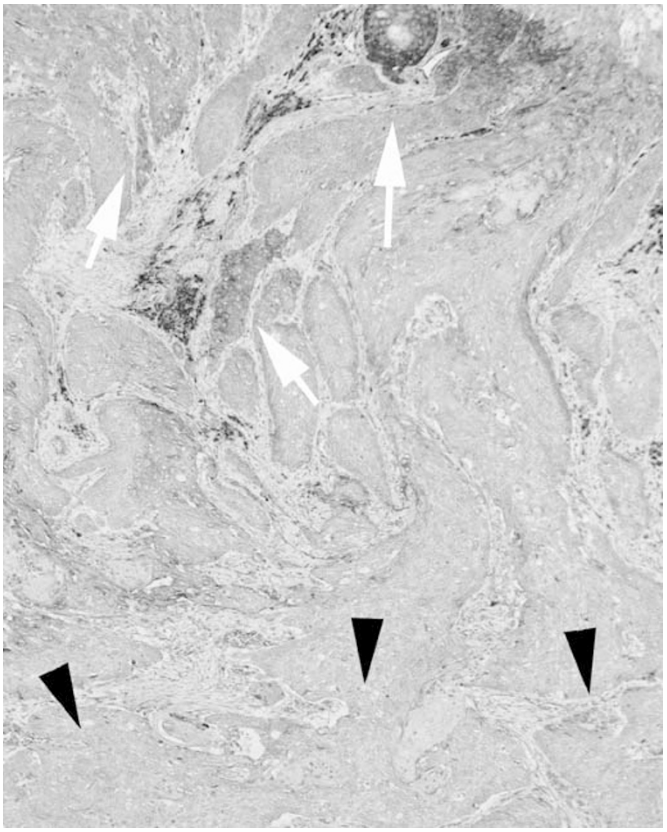

Figure 2 Representative photomicrographs of tissue sections immunostained for TIMP-3 (× 50). Black arrowheads indicate deep areas of tumour invasion. Cancer cells from these areas did not express TIMP-3 protein. White arrowheads indicate shallow areas of tumour, where cancer cells expressed TIMP-3 protein.

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RESULTS

Relationship between TIMP-3 expression and clinicopathological features

Tissue inhibitor of metalloproteinase-3 expression in ESCC was investigated by immunohistochemical analysis of formalin-fixed, paraffin-embedded specimens using a TIMP-3 specific Mab. In normal oesophageal tissue, immunostaining of TIMP-3 was detected in the cytoplasm of the basal cells, parabasal cells and stromal cells (Figure 1A, B). Immunostaining of TIMP-3 was seen in the cytoplasm of all cancer cells (Figure 1B, C). Expression of TIMP-3 was preserved in shallow areas of the tumour, but it was

Table 1 The correlationship between clinicopathological characteristics and TIMP-3 expression

Parameters	Total	TOMP-3 preserve ($n=30$)	TOMP-3 moderate ($n=27$)	TOMP-3 reduce ($n=33$)	*P*–value
Age (mean ± s.d., years)	60.9 ± 8.3	60.9 ± 7.5	59.9 ± 8.2	61.8 ± 9.1	NS
Gender					
Male	76	23	23	30	
Female	14	7	4	3	0.295
Differentiation					
Well	22	7	6	9	
Moderate	45	15	15	15	
Poor	23	8	6	9	0.957
Location					
Upper	12	1	6	5	
Mid-thora	55	21	18	16	
Lower	23	8	3	12	0.060
TNM clinical classification					
T					
T1	34	20	9	5	
T2	13	2	6	5	
T3	37	8	11	18	
T4	6	0	1	5	0.001
N					
N0	36	16	11	9	
N1	54	14	16	24	0.108
M					
M0	74	28	21	25	
M1	16	2	6	8	0.147
Stage					
I	23	15	4	4	
II	28	7	12	9	
III	23	6	5	12	
IV	16	2	6	8	0.005
No. of lymph node metastases (mean ± s.d.)	—	1.3 ± 1.8	3.1 ± 5.9	2.5 ± 3.4	0.003
Lymphatic invasion					
ly (−)	28	13	7	8	
ly (+)	62	17	20	25	0.206
Blood vessel invasion					
v (−)	49	18	14	17	0.756
v (+)	41	12	13	16	
Infiltrative growth pattern					
inf α	22	14	2	6	
inf β	60	12	23	25	
inf γ	8	4	2	2	0.003
Intraepithelial spread					
ie (−)	45	14	10	21	
ie (+)	45	16	17	12	0.111
Intramural metastasis					
IM0	80	27	25	28	
IM1	10	3	2	5	0.619

s.d. = standard deviation.

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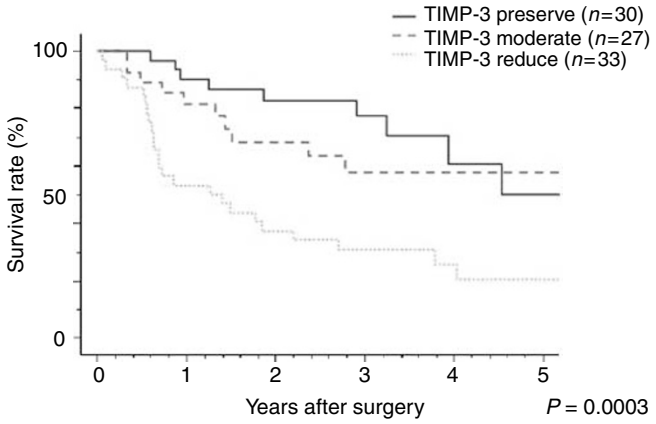

Figure 3 Relationship between overall postoperative survival and TIMP-3 expression. Patients with TIMP-3-reduced cancer had a significantly poorer prognosis than those with TIMP-3 preserved or moderate expression (5-year survival rates: TIMP-3 reduced, 21%; TIMP-3 moderate, 58%; TIMP-3 preserved, 50%; $P = 0.0003$).

reduced in deep areas of the same tumour (Figure 2). TIMP-3 preserved, moderate, and reduced cases accounted for 30, 27, and 33 of the 90 patients, respectively (33, 30, 37%). The relationship between the clinicopathological characteristics of patients with ESCC and TIMP-3 expression is summarised in Table 1. A significant correlation was observed between TIMP-3 expression and depth of tumour invasion ($P = 0.001$), number of lymph node metastases ($P = 0.003$), infiltrative growth pattern ($P = 0.003$), and disease stage ($P = 0.005$). However, there was no significant association with age, sex, tumour location, presence of regional lymph node metastasis, lymphatic invasion, blood vessel invasion, intraepithelial spread, intramural metastasis, or presence of distant metastasis.

The survival rates of patients with TIMP-3 (−) cancer were significantly lower than those of patients with TIMP-3 (+) and TIMP-3 (±) cancer ($P = 0.0003$; Figure 3). The mean 5-year survival rates of patients with TIMP-3 (+), (±), and (−) were 50, 58, and 21%, respectively. Multivariate analysis showed that TIMP-3 overexpression was not a prognostic factor by itself, in contrast to depth of tumour invasion, lymph node metastasis, or disease stage (data not shown).

DISCUSSION

Our immunohistochemical results suggest that expression of TIMP-3 protein is correlated with depth of tumour invasion, the number of lymph node metastases and disease stage. The expression of TIMP-3 protein was localised in shallow areas of the tumour, and was reduced in deep areas of the same tumour. In particular, no expression was observed at the invasive tumour front. This result indicated that invading cancer cells had lost their expression of TIMP-3. Furthermore, the prognosis for patients with cancer that had lost TIMP-3 was significantly less favourable than that for patients with TIMP-3 (+) and TIMP-3 (±) cancer.

Bachman *et al* (1999) showed that loss of TIMP-3 expression is associated with dense methylation of the 5′-CpG island in cell lines from many common human cancers. This methylation-associated silencing of TIMP-3 is tumour-specific, and associated with lack of TIMP-3 protein expression in primary cancers. Aberrant methylation of *TIMP-3* was demonstrated in primary cancers of the kidney, brain, colon, breast, and lung, but not in any of 41 normal tissue samples. Reduced TIMP-3 expression in ESCC may also have been caused by aberrant methylation in our study.

Baker *et al* (Ahonen *et al*, 1998; Baker *et al*, 1999) reported that TIMP-3 expression inhibits invasion and induces apoptosis in cancer cells. They used adenovirus-mediated gene delivery of TIMP-1, -2, and -3 to melanoma, cervical carcinoma, and fibrosarcoma cell lines. Their reports support ours. Tissue inhibitor of metalloproteinase-3 expression may be unfavourable for the survival of cancer cells, and cancer cells with a high grade of malignancy (more invasive and fewer apoptotic cells) may not express TIMP-3 for various reasons.

An imbalance between cell surface-associated proteinases and their inhibitors (higher concentrations of MMPs and lower concentrations of TIMPs) has been traditionally implicated in tumour expansion. However, it has been reported that some TIMPs function as growth factors (Hayakawa *et al*, 1992, 1994). For example, TIMP-1 and TIMP-2 have erythroid potentiating activity, and accelerate the growth of most cells (Hayakawa *et al*, 1992, 1994). Tissue inhibitor of metalloproteinase-3 has been reported to stimulate the proliferation of growth-retarded, nontransformed cells maintained under low-serum conditions (Yang and Hawkes, 1992). These reports do not coincide with our data, but growth activity is not always consistent with invasive activity.

Other functions of TIMP-3 have been reported, including suppression of tumour growth and angiogenesis (Anand-Apte *et al*, 1996, 1997; Bian *et al*, 1996; Spurbeck *et al*, 2002; Qi *et al*, 2003). Qi *et al* have demonstrated the ability of TIMP-3 to inhibit vascular endothelial growth factor (VEGF)-mediated angiogenesis and identified the possible mechanism involved. In ESCC, VEGF has been reported to be one of the most important angiogenesis factors (Shih *et al*, 2000; Kato *et al*, 2002). Therefore, TIMP-3 may suppress tumour angiogenesis indirectly by inhibiting the VEGF angiogenesis signal, and in turn, tumour growth.

Mori *et al* (2000)investigated TIMP-1 expression of mRNA and protein in ESCC, and reported that TIMP-1 expression is correlated with high-grade malignant behavior, and is an independent prognostic factor. The structure of the protein-coding exons is similar in the *Timp-1* and *Timp-3* genes (Apte *et al*, 1995). Moreover, TIMP-3 and TIMP-1 inhibition were quantitatively similar, implying that all TIMPs are equally efficient in MMP inhibition (Apte *et al*, 1995).

Decreasing expression of TIMP-3 in invasive oesophageal cancer cells may indicate that one of the functions of TIMP-3 is suppression of invasiveness. If cancers having invasive and metastatic ability do not express TIMP-3, preserving or increasing expression of TIMP-3 may lead to a novel cancer therapy. Matrix metalloproteinase inhibitor has recently been developed, and is undergoing trials as an investigational agent (Nozaki *et al*, 2003). Combination therapy with MMP inhibitor and TIMP-3 enhancement may be more effective than MMP inhibitor alone.

In conclusion, decreased expression of TIMP-3 protein correlates with invasive activity and metastasis. This makes the prognosis for patients with cancer that has lost TIMP-3 significantly less favourable than that for patients with cancer that has maintained TIMP-3.

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