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.

Keywords: oesophageal cancer; tissue inhibitor of matrix metalloproteinase 3 (TIMP-3); invasion; prognosis; immunohistochemistry

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 Zn2⁺- 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.
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% H₂O₂ 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% H₂O₂ 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 for TIMP-3 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 χ² 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.
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 correlation between clinicopathological characteristics and TIMP-3 expression

| Parameters                          | Total   | TIMP-3 preserve (n = 30) | TIMP-3 moderate (n = 27) | TIMP-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                     | 0.957   |
| Poor                                | 23      | 8                        | 6                        | 9                      |         |
| Location                            |         |                          |                          |                        |         |
| Upper                               | 12      | 1                        | 6                        | 5                      |         |
| Mid-thorae                          | 55      | 21                       | 18                       | 16                     |         |
| Lower                               | 23      | 8                        | 3                        | 12                     | 0.060   |
| TNM clinical classification         |         |                          |                          |                        |         |
| T                                   |         |                          |                          |                        |         |
| T1                                  | 34      | 20                       | 9                        | 5                      | 0.001   |
| T2                                  | 13      | 2                        | 6                        | 5                      |         |
| T3                                  | 37      | 8                        | 11                       | 18                     |         |
| T4                                  | 6       | 0                        | 1                        | 5                      |         |
| N                                   |         |                          |                          |                        |         |
| N0                                  | 36      | 16                       | 11                       | 9                      | 0.108   |
| N1                                  | 54      | 14                       | 16                       | 24                     | 0.147   |
| M                                   |         |                          |                          |                        |         |
| M0                                  | 74      | 28                       | 21                       | 25                     |         |
| M1                                  | 16      | 2                        | 6                        | 8                      |         |
| Stage                               |         |                          |                          |                        |         |
| I                                   | 23      | 15                       | 4                        | 4                      | 0.005   |
| II                                  | 28      | 7                        | 12                       | 9                      |         |
| III                                 | 23      | 6                        | 5                        | 12                     |         |
| IV                                  | 16      | 2                        | 6                        | 8                      |         |
| 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                      | 0.206   |
| Ly (+)                              | 62      | 17                       | 20                       | 25                     |         |
| Blood vessel invasion               |         |                          |                          |                        |         |
| v (−)                               | 49      | 18                       | 14                       | 17                     | 0.756   |
| v (+)                               | 41      | 12                       | 13                       | 16                     |         |
| Infiltrative growth pattern         |         |                          |                          |                        |         |
| inf α                               | 22      | 14                       | 2                        | 6                      | 0.003   |
| inf β                               | 60      | 12                       | 23                       | 25                     |         |
| inf γ                               | 8       | 4                        | 2                        | 2                      |         |
| Intraepithelial spread              |         |                          |                          |                        |         |
| ie (−)                              | 45      | 14                       | 10                       | 21                     | 0.111   |
| ie (+)                              | 45      | 16                       | 17                       | 12                     |         |
| Intramural metastasis               |         |                          |                          |                        |         |
| IM0                                 | 80      | 27                       | 25                       | 28                     | 0.619   |
| IM1                                 | 10      | 3                        | 2                        | 5                      |         |

s.d. = standard deviation.
TIMP-3 expression in oesophageal cancer

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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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