# Immunochemical Staining of MT2-MMP Correlates Positively to Angiogenesis of Human Esophageal Cancer

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**Abstract.** *Matrix metalloproteinases (MMPs) play an important role in the pathological processes of degradation of extracellular matrix and destruction of basement membrane, which leads to tumor invasion and metastasis. In the present study, we investigated membrane-type 2 MMP (MT2-MMP) expression pattern in esophageal cancer tissues collected from 103 patients, and explored MT2-MMP expression pattern in correlation to patients' clinicopathological features, intratumoral angiogenesis and postoperative prognoses. The intensity of immunochemical staining of MT2-MMP was significantly positively correlated to the intratumoral angiogenesis of esophageal cancer tissues. Positive MT2-MMP immunoreactions were found in 85.4% of total tumor sections, whereas none or very weak MT2-MMP staining occurred in normal esophageal tissues. In addition, MT2-MMP immunochemical intensities were significantly correlated to tumor size, but not to patient's gender, age, invasion depth, lymph node metastasis and distant metastasis. Moreover, MT2-MMP levels could not be applied for predicting patients' survival rate although the H-score cut-off value showed the overall survival rate of patients with low MT2-MMP protein level to be better than those with high MT2-MMP protein level.*

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Esophageal cancer is a quite aggressive malignancy of the upper gastrointestinal tract that ranks as the sixth cause of cancer deaths (1). Based on its distinct histopathological characteristics, esophageal cancer can be divided into two major types, namely adenocarcinoma and squamous cell carcinoma (SCC). The latter accounts for 90% of all esophageal cancer cases worldwide (2). Surgical resection of the tumor at the primary site is a standard treatment for esophageal cancer, and shows favorable trends for postoperative prognosis (3). Moreover, numerous alternative therapies have been available for the treatment of esophageal cancer, including chemotherapy, radiotherapy and immunotherapy, or a combination of these therapies. Due to the aggressive nature of this disease, patients suffering from esophageal cancer commonly undergo systemic and local recurrences even after curative operation, and the 5-year survival rate remains dismal (4).

It has been demonstrated that matrix metalloproteinases (MMPs) are related to tumor invasion and metastasis in most tumors (5). MMPs consist of more than 25 well-characterized members of secreted or transmembrane proteins that degrade the extracellular matrix (ECM) and basement membrane macromolecules (6). According to their structures and substrate specificities, MMPs are predominantly divided into five subgroups: collagenases, gelatinases, stromelysins, membrane-type MMPs (MT-MMPs) and other MMPs (7). MMPs regulate the tumor microenvironment, and their expression levels and/or activation might be altered in many human cancer tissues (5). Moreover, many studies have shown significant associations between MMP expression and the patient's clinicopathological features, as well as postoperative prognosis, which suggested that MMP levels might be used as biomarkers and therapeutic targets in human cancer (8). As yet, however, the expression patterns and physiopathological functions of MT-MMPs, a minority of the MMP family, have not been well documented in tumors. MT2-MMP was first characterized by Takino *et al.* (9) in 1995, and was

subsequently identified as being involved in the activation of MMP-2 and may act as an anti-apoptotic factor in cancer cells (10, 11).

In the present study, we examined MT2-MMP expression in esophageal cancer tissues and further investigated MT2-MMP expression in relation to patients' clinicopathological features, the intratumoral microvessel density (MVD) and postoperative prognosis.

## Patients and Methods

*Samples*. Esophageal cancer samples were collected from 103 patients who underwent surgical resection between January 2001 and March 2005 in the hospital (75 men and 28 women; median age at diagnosis was 58 years). No patients received pre-operative chemotherapy or radiotherapy. All cases were confirmed as being esophageal SCC, and the tumor-node-metastasis (TNM) stages were assigned according to the American Joint Committee on Cancer Criteria (12). Patients' clinical parameters are shown in Table I and patients' survival intervals were dated to the end of March 2010. Ten normal esophageal tissues from the non-malignant portion were used as controls. The present study was approved by the Ethics Committee of the hospital.

*Immunohistochemistry*. Formalin-fixed and paraffin-embedded tissues were cut into 3-μm-thick consecutive sections, and were dewaxed in xylene and rehydrated in graded ethanol solutions. Polyclonal rabbit against human MT2-MMP antibody (diluted in 1:600; Chemicon, USA) and monoclonal mouse against human CD34 antibody (ready to use; Maixin Biotechnology Limited Corporation, Fuzhou, P.R. China) were used in the present study. The CD34 antigen was retrieved by heating the slides in a citrate solution (10 mmol/l, pH 6.0) for 30 min. In brief, sections were immersed in a 0.3% hydrogen peroxide solution for 30 min to block endogenous peroxidase activity, rinsed in phosphate buffered saline (PBS) for 5 min, and then incubated with primary antibodies at 4˚C overnight. A negative control was performed by omitting the primary antibodies. The sections were then incubated with horseradish peroxidase-labeled goat against mouse/rabbit secondary antibody (ready to use; Maixin Biotechnology Limited Corporation). Diaminobenzene was used as the chromogen and hematoxylin as the nuclear counterstain.

*Evaluation of MT2-MMP expression and intratumoral MVD*. All slides were examined independently by two pathologists who were not informed of the patients' clinical data. The MT2-MMP immunostaining intensities were assessed according to the H-score method described by Hammes *et al*. (13):

H-score=(% unstained tumor cells ×0) + (% tumor cells stained weakly ×1) + (% tumor cells stained moderately ×2) + (% tumor cells stained strongly ×3).

The H-scores ranged from 0 (100% negative tumor cells) to 300 (100% strong staining tumor cells). In the present study, we ranked the intensity of the immunochemical staining as negative, low intensity (0<H-score≤105) and high intensity (H-score >105).

Immunopositive staining of endothelial cell marker, CD34, was chosen for the evaluation of intratumoral MVD as described previously (14). In brief, tumor sections were first examined at low magnification (×40), and five intratumoral areas with the most intense neovascularization were selected. The positive CD34 immunostaining microvessels, as well as single endothelial cells, were counted per high power field (HPF, ×200 magnification).

Table I. *Correlation between MT2-MMP expression and clinical parameters*.

| Clinical parameters | MT2-MMP immunochemical staining | | *P*-Value |
|---|---|---|---|
| | Low (%) | High (%) | |
| Gender | | | |
| Male | 46 (61.3) | 29 (38.7) | 0.2994 |
| Female | 14 (50.0) | 14 (50.0) | |
| Age (years) | | | |
| <60 | 35 (55.6) | 28 (44.4) | 0.4861 |
| ≥60 | 25 (62.5) | 15 (37.5) | |
| Tumor size (cm) | | | |
| <3.5 | 27 (71.1) | 11 (28.9) | 0.0440 |
| ≥3.5 | 33 (50.8) | 32 (49.2) | |
| Depth of tumor (T) invasion | | | |
| $pT_1$ | 8 (61.5) | 5 (38.5) | 0.7277† |
| $pT_2$ | 27 (58.7) | 19 (41.3) | |
| $pT_3$ | 19 (57.6) | 14 (42.4) | |
| $pT_4$ | 6 (54.5) | 5 (45.5) | |
| Lymph node (N) metastasis | | | |
| $N_0$ | 35 (64.8) | 19 (35.2) | 0.1563 |
| $N_1$ | 25 (51.0) | 24 (49.0) | |
| Distant metastasis (M) | | | |
| $M_0$ | 54 (62.1) | 33 (37.9) | 0.0670 |
| $M_1$ | 6 (37.5) | 10 (62.5) | |
| TNM Stage | | | |
| I | 8 (61.5) | 5 (38.5) | 0.2128† |
| II | 34 (60.7) | 22 (39.3) | |
| III | 12 (66.7) | 6 (33.3) | |
| IV | 6 (37.5) | 10 (62.5) | |

$pT_1$, Invasion of lamina propria or submucosa; $pT_2$, invasion of muscularis propria; $pT_3$, invasion of adventitia; and $pT_4$, invasion of adjacent structures. $N_0$, No regional lymph-node metastasis; $N_1$, regional lymph-node metastasis. $M_0$, No distant metastasis; $M_1$, metastasis to cervical nodes, celiac nodes and other distant metastases. †Chi-square test for trend.

*Statistical analyses*. Correlations between MT2-MMP expression and patients' clinical parameters as well as intratumoral MVD were analyzed by the Chi-square test or Mann-Whitney test, respectively. MT2-MMP expression in relation to patients' postoperative prognoses was examined by the log-rank survival analysis. All statistical analyses were performed by using the GraphPad Prism 4.0 software package (GraphPad Software, Inc., San Diego, USA). A *p*-value less than 0.05 was considered as being statistically significant.

## Results

*Immunochemical staining of MT2-MMP in esophageal tissues*. As shown in Figure 1, positive MT2-MMP immunochemical staining was predominantly observed on the membrane and in cytoplasm of tumor cells (Panel B),

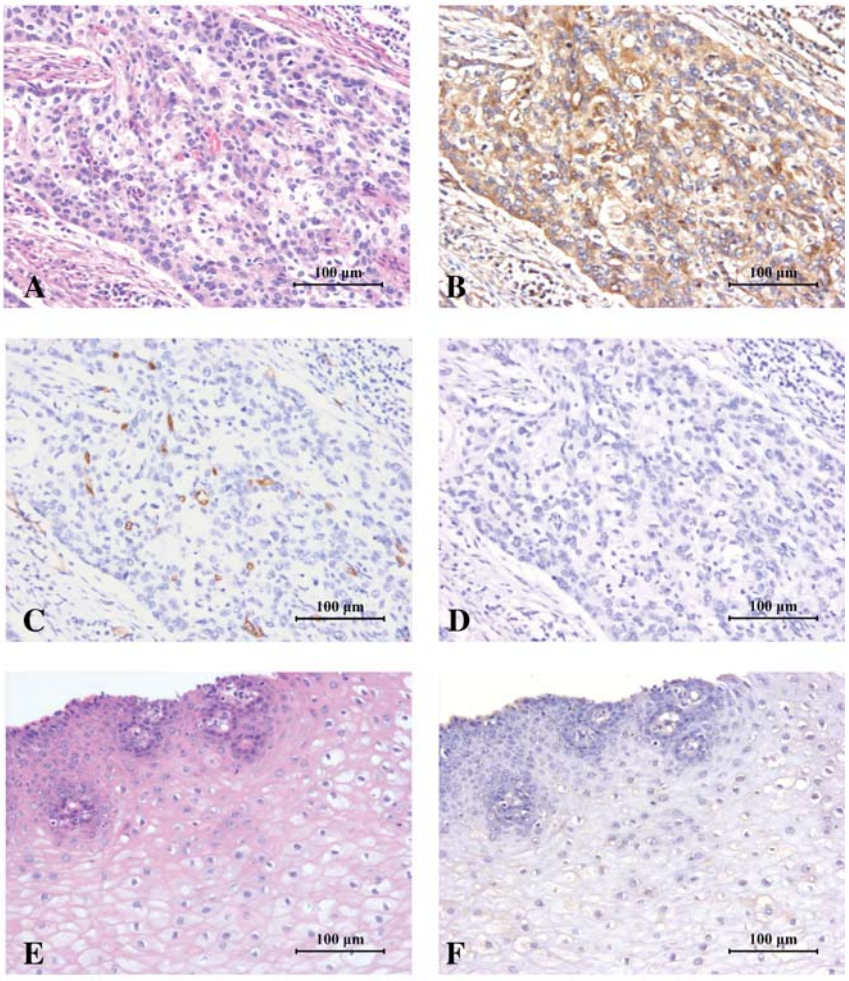


Figure 1. *Immunochemical staining of MT2-MMP and intratumoral microvessels. The figure represents one of the consecutive sections of an esophageal cancer specimen with high MT2-MMP immunoreactivity. A: H&E staining. B: MT2-MMP-positive immunochemical staining. C: Intratumoral microvessels estimated by the immunoreaction staining of anti-CD34 monoclonal antibody. D: Negative control. E: H&E staining. F: Negative MT2-MMP immunoreaction staining of normal esophageal tissue.*

while none or very weak staining was found in normal esophageal tissues (Panel F). A total of 88 out of 103 (85.4%) specimens of esophageal cancer tissues showed positive MT2-MMP immunochemical staining. In order to determine the importance of MT2-MMP expression level in esophageal cancer, we further sub-grouped 103 patients into three groups according to the intensity of MT2-MMP immunochemical staining with the following results: H-score=0, 15 cases; 0<H-score≤105, 45 cases and H-score>105, 43 cases.

*Intensity of MT2-MMP immunochemical staining and intratumoral MVD*. The median intratumoral MVD was 34.0/HPF in esophageal cancer tissues, and the number of intratumoral MVD was significantly higher in MT2-MMP-positive tissues than in MT2-MMP-negative tissues ($p$=0.0220, U=201.5) (Figure 2A). Moreover, Figure 2B shows that the intratumoral MVD was higher in the subgroup of 0<H-score≤105 ($p$=0.0207, U=201.5) and in the subgroup of H-score>105 ($p$=0.0530, U=213.0) than in the MT2-MMP-negative group, which indicates that intratumoral MVD is related to MT2-MMP expression.

*MT2-MMP immunochemical staining in relation to patients' clinical data and prognoses*. As shown in Table I, MT2-MMP immunoreactivity was only significantly correlated to the tumor size ($p$=0.0440), whereas it was not correlated to patient's gender, age, invasion depth and lymph node metastasis. Concerning distant metastasis, although no significant statistical correlation was found there were more cases of $M_1$ (62.5%) than $M_0$ (35.2%) in the higher MT2-MMP group. When the H-score cut-off value of 105 was

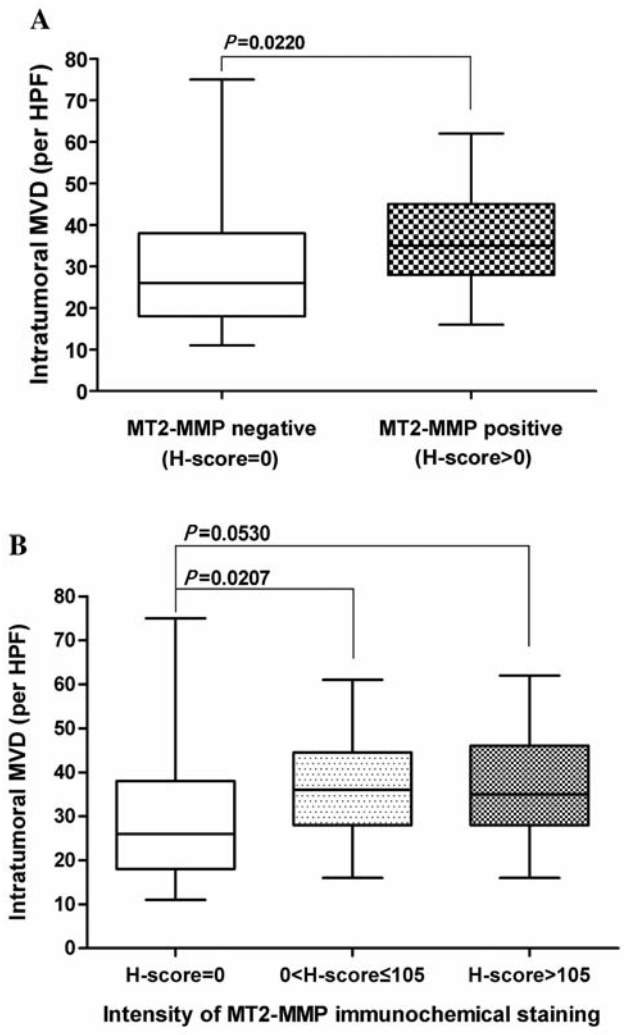


Figure 2. *Correlation between MT2-MMP expression and intratumoral microvessel density (MVD). A: Intratumoral MVD in the MT2-MMP-positive group. B: Intratumoral MVD in the MT2-MMP-positive subgroups according to the H-score cut-off.*

selected (Figure 3A), the overall survival rate of patients with low MT2-MMP expression was better than those with high MT2-MMP expression ($p$=0.1851, hazard ratio=1.368, 95% CI: 0.8512-2.303, Figure 3B), although it was not statistically significant, which suggested that MT2-MMP expression level cannot directly predict survival of esophageal cancer patients.

## Discussion

It has been demonstrated that degradation of the ECM is an essential step of tumor invasion and metastasis, and MMPs contribute to this pathological process in human malignancies (15). Studies on MMP expression patterns in human esophageal cancer demonstrated there was a significant correlation between MMP expression and patients' clinicopathological features as well as postoperative prognosis (16-18). Yamamoto *et al*. (16) reported that MMP-7 was detected in 49% esophageal cancer tissues and was positively correlated to tumor invasion depth, stages and recurrence, and negatively correlated to patients' disease-free/overall survival, which suggests an important role of MMP-7 in the progression of esophageal cancer and a potential biological marker for the targeted therapy and the prediction of recurrence and poor prognosis. Gu *et al*. (17) reported that MMP-9 was an independent prognostic factor for patients suffering from esophageal cancer, and MMP-9 levels positively correlated to tumor differentiation, vessel permeation and lymph node metastasis.

Six MT-MMPs have been described: four type-I transmembrane proteins (MT1-, MT2-, MT3- and MT5-MMP) and two glycosylphosphatidylinositol (GPI)-anchored proteins (MT4-MMP and MT6-MMP). The transmembrane domain is particularly important for the functioning of pericellular proteolysis (19). It has been demonstrated that MT-MMPs could have an important role in tumor angiogenesis (20, 21). MT1-MMP promoted tumor growth and angiogenesis accompanied by up-regulation of vascular endothelial growth factor expression (22), *via* the activation of Src-tyrosine kinases (23). In addition, *in vitro* studies have demonstrated that MT1-, MT2- and MT3-MMP were able to induce capillary-like tube formation (21, 24). Moreover, several studies explored whether MT2-MMP might also be involved in the progression of certain types of human cancer, such as that of the breast (25), cervix (26), ovary (27) and colorectum (28). Lyall *et al*. reported that higher MT2-MMP mRNA levels had longer disease-free survival intervals on stage III colorectal cancer patients (29).

In the present study, we further examined MT2-MMP protein levels in esophageal cancer tissues and found that the intensity of the MT2-MMP immunochemical staining was positively correlated to the intratumoral MVD, which suggests that MT2-MMP may be a potential regulator of intratumoral angiogenesis in the progression of human esophageal cancer. MT2-MMP immunoreactivity was also significantly correlated to the tumor size, and potentially correlated to distant metastasis, whereas it was not correlated to patient's gender, age, invasion depth and lymph node metastasis and postoperative prognosis. However, there was no statistically significant difference in patients' survival intervals after surgical resection under 1 to 5 years between the high and low MT2-MMP expression when categorized by the following variables: gender, age, tumor size, invasion depth, nodal metastasis, distant metastasis and TNM stage (data not shown). Thus, it is concluded that although MT2-MMP is positively correlated to intratumoral MVD in esophageal cancer and to the tumor size, it is not correlated to patients' survival intervals.

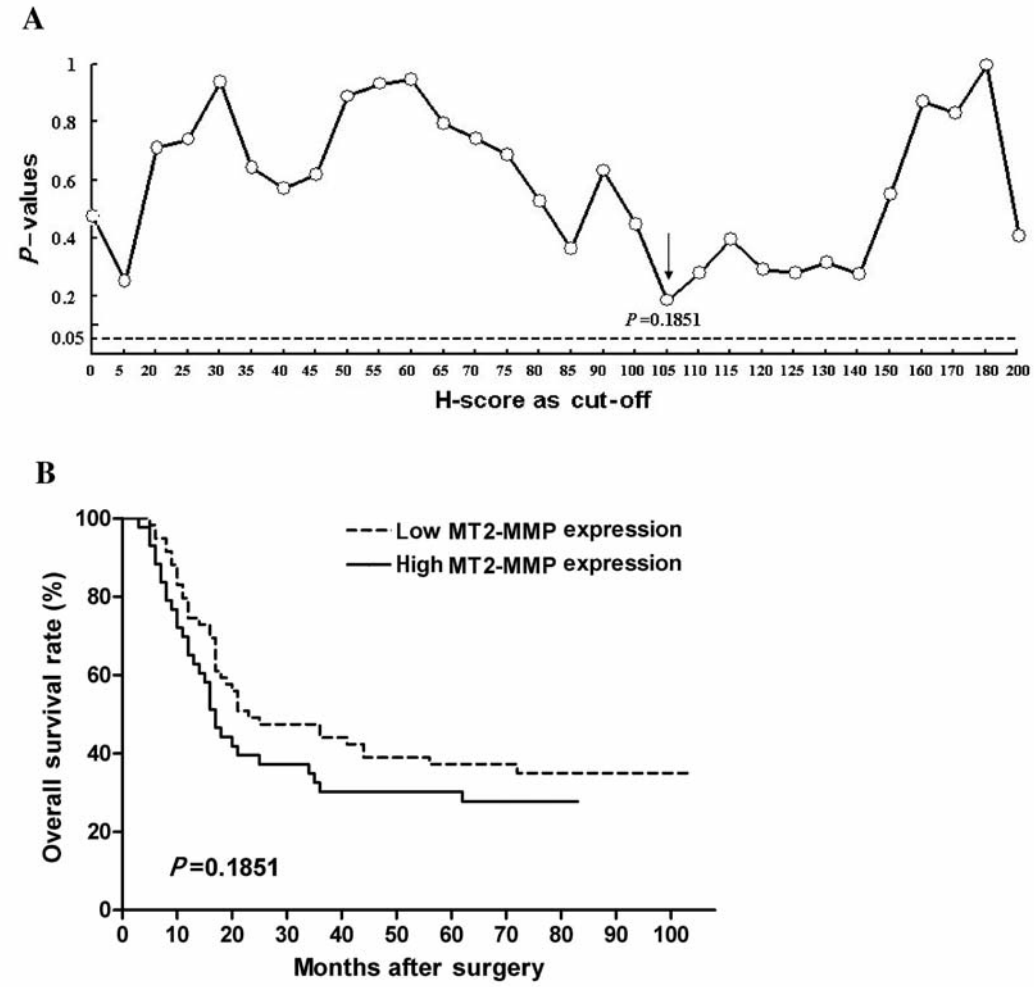


Figure 3. *Relationship between MT2-MMP expression and patients' survival intervals. A: Minimum P-value seek was conducted according to the literature (30, 31) in log-rank survival analysis when using different MT2-MMP immunostaining H-score values as cut-offs, and when H-score=105 was set as the cut-off value, the minimum p-value=0.1851 was found and selected to divide patients into low/high MT2-MMP expression groups. B: Log-rank survival analysis performed when the H-score=105.*

## Competing Interests

The Authors declare that they have no competing interests.

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