Increased Expression of Matrix Metalloproteinase-2 and Tissue Inhibitor of Metalloproteinase-2 Is Correlated with Poor Prognostic Variables in Patients with Thymic Epithelial Tumors

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BACKGROUND. A distinction between noninvasive, invasive, and metastatic thymoma on the basis of the cytologic features is difficult. The current study investigated whether the expression of MMP and TIMP was correlated with tumor invasiveness and prognosis in patients with thymoma.

METHODS. Tumor tissue samples were obtained from 42 patients with thymic epithelial tumors between 1974 and 2001 at Tokushima University Hospital. Three-micrometer-thick, formalin-fixed, paraffin-embedded tissue sections were immunostained using specific antibodies against MMP-2, MMP-9, TIMP-1, and TIMP-2.

RESULTS. MMP-2 expression was detected in 30 tumors (71%), and TIMP-2 expression was detected in 31 tumors (74%). MMP-9 expression was detected in 22 of 36 tumors (61%), and TIMP-1 expression was detected in only 7 tumors (19%). MMP-2 and TIMP-2 expression levels were very low (10% and 0%, respectively) in noninvasive tumors but were very high (91% and 97%, respectively) in invasive tumors. In thymic epithelial tumors, the more progressive the clinical stage of tumor, the higher the strongly positive rate of MMP-2 and TIMP-2 expression. There was no correlation between positivity for MMP-9 and stage. Twenty-five percent of Type AB thymomas and 50% of Type B1 thymomas expressed MMP-2 and TIMP-2. Most of Type A, Type B2, Type B3, and Type C thymomas expressed MMP-2 and TIMP-2. There were significant differences in disease-free survival at 5 years between patients without and with MMP-2 expression (91% vs. 55%, respectively) and patients without and with TIMP-2 expression (100% vs. 53%, respectively).

CONCLUSIONS. MMP-2 and TIMP-2 are key enzymes for invasiveness of thymic epithelial tumors. The expression of these proteins can predict a poor outcome in patients with thymoma. *Cancer* 2003;98:1822–9.
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KEYWORDS: thymoma, matrix metalloproteinase, tissue inhibitor of metalloproteinase, invasiveness, immunohistochemistry.

Thymic epithelial tumors mainly consist of thymoma, thymic carcinoma, and thymic carcinoid.[1] Thymoma was defined as a benign tumor or a low-grade, malignant tumor of the thymic epithelium with some interesting features: association with myasthenia gravis (MG) or other autoimmune disease, histologic variability, and heterogeneity of malignant behavior.[1, 2] A distinction between noninvasive, invasive, and metastatic thymomas on the basis of the cytologic features is difficult. However, thymic carcinoma and thymoma can be distinguished histologically in most cases.[2] Several studies have assessed

factors associated with the invasiveness of thymoma, such as the nuclear areas of epithelial cells, the nuclear DNA content, proliferative activity (proliferating cell nuclear antigen [PCNA], Ki-67, mitotic figures), the argyrophilic nucleolar organizer region (AgNOR), and p53 expression.[3–7] However, there are no indicators that distinguish invasive thymoma from noninvasive thymoma.

In our previous study, we used gelatinolytic zymography to demonstrate that the gelatinolytic activity of active matrix metalloproteinase (MMP)-2 was correlated significantly with invasiveness in thymic epithelial tumors.[8] The MMPs are a family of zinc-dependent endopeptidases capable of degrading most components of the basement membrane and extracellular matrix (ECM).[9] MMPs are secreted as inactive preenzymes and are transformed into active forms after cleavage of a propeptide domain of the molecule.[10] In particular, MMP-2 and MMP-9, which are capable of degrading Type IV collagen (the major component of the basement membrane), are involved in the spread of various carcinomas. Tissue inhibitors of metalloproteinase (TIMPs) are the major endogenous regulators of MMPs and consist of four homologous members.[11] Each TIMP exhibits preferential inhibitory capacity for MMPs; e.g., TIMP-1 and TIMP-2 selectively inhibit MMP-9 and MMP-2, respectively.[12] The objective of the current study was to evaluate the immunohistochemical expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins in thymic epithelial tumors and to determine whether their expression is correlated with the invasiveness and recurrence of the tumor.

MATERIALS AND METHODS
Patients
Tumor tissue samples were obtained from 42 patients with thymic epithelial tumor who underwent surgery ($n = 37$) or biopsy ($n = 5$) between 1974 and 2001 at Tokushima University Hospital. Twenty-six patients were female, and 16 patients were male. The median patient age was 55.7 years $\pm$ 15.1 years (range, 23–81 years). MG was related to disease in 11 patients (26%). All 42 thymic epithelial tumors were classified according to the World Health Organization (WHO) histologic classification system.[13] All tissues were referred to one of the authors (K.M.) for histologic consultation, and that author was one of the collaborators on *Histological Typing of Tumours of the Thymus* by J. Rosai.[13] There were 3 patients with Type A tumors, 8 patients with Type AB tumors, 9 patients with Type B1 tumors, 4 patients with Type B2 tumors, 6 patients with Type B3 tumors, and 12 patients with Type C tumors. The clinical stage of each thymic epithelial

tumor was determined according to the criteria of Masaoka et al.[14] There were 10 patients with noninvasive tumors (Stage I), 12 patients with Stage II tumors, 10 patients with Stage III tumors, 4 patients with Stage IVa tumors, and 6 patients with Stage IVb tumors. Thirty-six patients underwent total resection of the tumor, 1 patient underwent subtotal resection, and 5 patients underwent biopsy only. Radiochemotherapy was administered to 12 patients, radiotherapy was administered to 10 patients, and chemotherapy was administered to 5 patients. Follow-up information with respect to survival was available for 41 patients.

Gelatin Zymography
To evaluate the association between gelatinolytic activity and immunoreactivity, we examined the gelatinolytic activity of MMP-2 in 16 thymic epithelial tumors using quantitative gelatinolytic zymography, which has been described previously.[8] For gelatinolytic zymography, some 5-μm cryostat sections were homogenized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (10 μL buffer per mg of tissue) containing 50 mM Tris-HCl, pH 6.8; 10% glycerol; 1% SDS; and 0.01% bromophenol blue. Lysate was clarified by centrifugation. Samples were separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS and 1% gelatin as a substrate. Thereafter, gels were incubated in reaction buffer containing 50 mM Tris/HCl, pH 8.0; 0.5 mM CaCl$_2$; and 10^{-6} M ZnCl$_2$ for 16 hours at 37 °C. Gels were stained for 10 minutes in 10% methanol, 5% glacial acetic acid, and 1% Coomassie Brilliant Blue G-250 (Wako; Osaka, Japan) and then destained in the same solution without dye. The gelatinolytic activity of each collagenase was evident as a clear band against the blue background of stained gelatin. We quantified the intensity of bands (92 kilodaltons [kD], 72 kD, and 62 kD) by gelatinolytic zymography using National Institutes of Health image analysis software (Version 1.56; National Institutes of Health, Bethesda, MD).

Immunohistochemical staining for MMP-2, MMP-9, TIMP-1, and TIMP-2
All tissues were fixed in formalin and embedded in paraffin wax, sectioned at 3 μm, and stained with hematoxylin and eosin. Adjacent sections were immunostained using the monoclonal antibodies to MMP-2 (500 μg/mL; clone 42-5D11; 1:150 dilution; Fuji Chemical Industries, Takaoka, Japan), MMP-9 (500 μg/mL; clone 56-2A4; 1:150 dilution; Fuji Chemical Industries), TIMP-1 (500 μg/mL; clone 147-6D11; 1:150 dilution; Fuji Chemical Industries), and TIMP-2 (500 μg/mL; clone 67-4H11; 1:150 dilution; Fuji Chemical Industries) or nonimmune mouse immunoglobulin G

(Southern Biotechnology Associates Inc., Birmingham, AL). We used the Dako Envision kit (peroxidase/3,3′diaminobenzidine tetrahydrochloride; Dako, Glostrup, Denmark) and the Dako Automatic slide stainer (TechMate Horizon; Dako). Sections were counterstained slightly with hematoxylin to identify the nucleus under light microscopy. Cells that were not stained completely by the primary antibody were regarded as antigen-negative cells. Immunohistochemical staining of tumor sections was examined independently by two observers who were unaware of the clinical data. The numbers of tumor cells that stained positive with the primary antibody were counted in at least 100 tumor cells in randomly, nonrepetitive distributed, microscopic fields (×400 magnification), and the percentages of atypical cells that stained positive were scored. A *positive* score was defined as < 50% of tumor cells positively stained, and *strongly positive* was > 50% of tumor cells positively stained. All samples ($n = 42$) were immunostained by anti-MMP-2, and TIMP-2 antibodies, and 36 of 42 tumors were stained by anti-MMP-9 and TIMP-1 antibodies.

Statistical Analysis

Statistical analysis was performed using the Fisher exact test and the Spearman rank correlation procedure (nonparametric method). Survival analysis was performed by the method of Kaplan–Meier with respect to disease-free survival (DFS) (recurrence-free survival), and comparisons of survival with the log-rank test were made using SPSS for Windows (Version 11.0.1). P values < 0.05 were considered significant.

RESULTS

Gelatinolytic Activity and Immunoreactivity of MMP-2

The gelatinolytic activity and the immunoreactivity of MMP-2 in 16 thymic epithelial tumors are shown in Figure 1. The gelatinolytic activities of strongly positive, positive, and negative staining groups were 0.459 ± 0.392, 0.232 ± 0.301, and 0.118 ± 0.037, respectively. Immunoreactivity for MMP-2 was correlated with gelatinolytic activity (Spearman rank correlation: $r = 0.451$; $P = 0.079$).

Immunostaining for MMP-2, MMP-9, TIMP-1, and TIM-2

In specimens from 42 patients with thymoma, the expression levels of MMPs and TIMPs were variable and involved intracytoplasm of tumor cells but did not involve lymphocytes, which were admixed in the tumors. MMP-2, MMP-9, and TIMP-2 were expressed strongly in the periphery of the tumor nest, indicating an invasive lesion. Their expression was weak in the center of the tumor nest (Fig. 2A–C). MMP-2 expres-
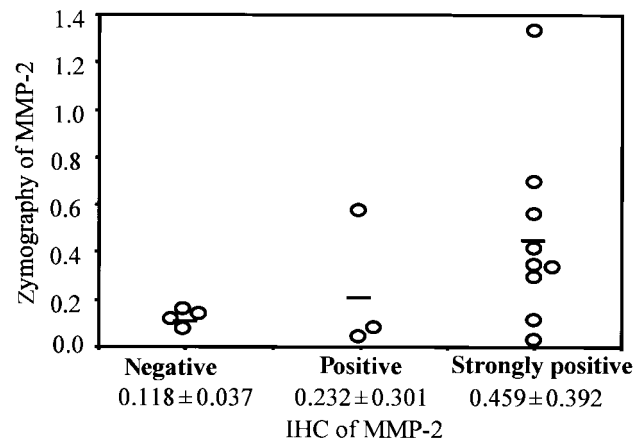

FIGURE 1. Gelatinolytic activity and immunoreactivity of matrix metalloproteinase (MMP)-2. IHC: immunohistochemistry.
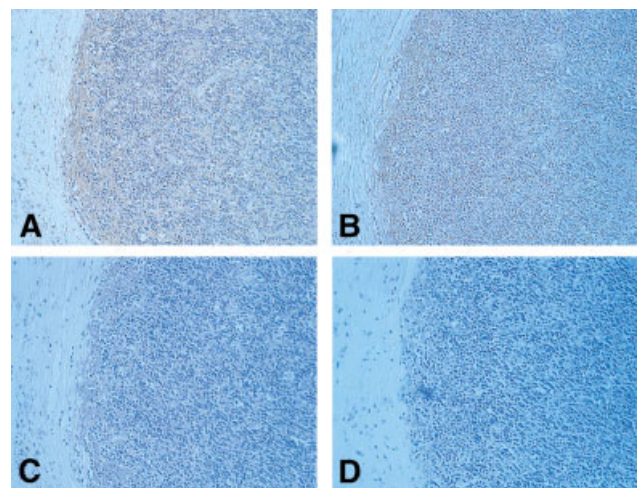

FIGURE 2. Immunostaining of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1, and TIMP-2. (A) Tumor sample from a woman age 43 years with Stage IVb, Type B1 thymoma exhibits strong staining for MMP-2 in tumor cells. (B) Strong staining for TIMP-2 in tumor cells. (C) Staining for MMP-9 in tumor cells. (D) No staining for MMP-1 in tumor cells. Original magnification ×200 (A–D).

sion was detected in 30 specimens (71%), with 20 specimens (48%) showing strongly stained tumor cells. TIMP-2 expression was evident in 31 specimens (74%), with 23 specimens (55%) exhibiting strong staining. Twenty-eight tumors coexpressed MMP-2 and TIMP-2, whereas 10 tumors did not express either MMP-2 or TIMP-2. Immunoreactivity for MMP-2 was similar to immunoreactivity for TIMP-2 in positive specimens. MMP-9 expression was detected in 22 of 36 specimens (61%), with 7 specimens (19%) exhibiting strong staining. TIMP-1 expression was detected in only 7 of 26 specimens (19%), with no specimens exhibiting strong staining.

FIGURE 3. Expression of each matrix metalloproteinase (MMP) and World Health Organization histologic classification. MMP-2 and tissue inhibitor of metalloproteinase (TIMP)-2: Type A, $n = 3$; Type AB, $n = 8$; Type B1, $n = 9$; Type B2, $n = 4$; Type B3, $n = 6$; Type C, $n = 12$. MMP-9 and TIMP-1: Type A, $n = 3$; Type AB, $n = 8$; Type B1, $n = 8$; Type B2, $n = 4$; Type B3, $n = 5$; Type C, $n = 8$.
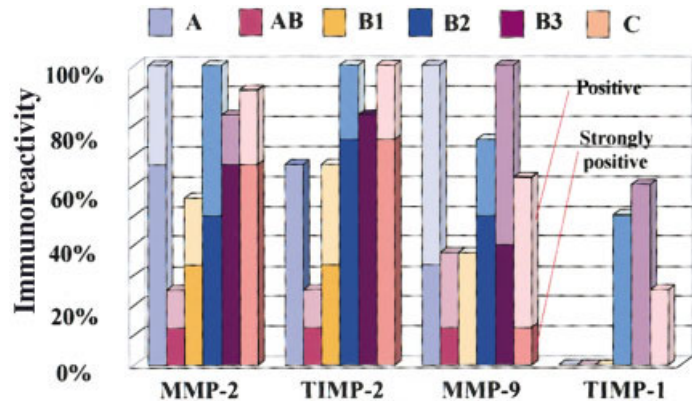
Expression of Each MMP and WHO Histologic Classification

All Type A thymomas expressed MMP-2, 2 thymomas (67%) expressed TIMP-2, and approximately 66% of thymomas strongly expressed both (Fig. 3). Twenty-five percent of Type AB thymomas and about 60% of Type B1 thymomas expressed MMP-2 and TIMP-2, and 50% strongly expressed both. Most of Type B2, B3, and C thymomas (83–100%) expressed MMP-2 and TIMP-2. Fifty percent of Type B2 thymomas and approximately 66% of Type B3 or C thymomas strongly expressed MMP-2; and about 80% of Type B2, B3, and C tumors strongly expressed TIMP-2. Although the pattern of MMP-9 expression in thymomas (except Type C) was similar to the pattern of MMP-2 and TIMP-2 expression, the frequency of tumors with strong expression was less than for MMP-2 and TIMP-2. However, TIMP-1 was expressed in some of Type B2, B3, and C thymomas.

Expression of Each MMP and Masaoka Clinical Stage

The positive rates of staining for MMP-2 and TIMP-2 were very low (10% and 0%, respectively) in noninvasive tumors (Stage I) (Fig. 4). In contrast, the positive rates of staining for MMP-2 and TIMP-2 were very high (91% and 97%, respectively) in invasive tumors (Stage II–IV). There was a significant difference in the levels of MMP-2 and TIMP-2 expression between noninvasive thymomas and invasive thymomas (Fisher exact test; $P < 0.0001$). Furthermore, the strongly positive rate for MMP-2 was zero in Stage I tumors, 50–55% in Stage II and III tumors, and 73% in Stage IV tumors. The strongly positive rate for TIMP-2 was zero in Stage I tumors, 55% in Stage II tumors, and 73–90% in Stage III and IV tumors. The more progressive the clinical stage of the tumor, the higher the strongly positive rates for MMP-2 and TIMP-2 were in thymic epithelial tumors (Spearman rank correlation: MMP-2: $r = 0.596$; TIMP-2: $r = 0.654$, respectively; $P < 0.0001$).

There was no correlation between the positive rate for MMP-9 and stage (Fig. 4A).

Figure 4B shows the correlation between clinical stage and thymomas, except Type C thymoma (thymic carcinoma). The positive rates of staining for MMP-2 and TIMP-2 were very low (10% and 0%, respectively) in noninvasive tumors. Conversely, the positive rates for MMP-2 and TIMP-2 were very high (90% and 95%) in invasive tumors. There was a significant difference in the levels of MMP-2 and TIMP-2 expression between noninvasive tumors and invasive tumors (Fisher exact test; $P < 0.0001$). Furthermore, the strongly positive rate for MMP-2 was zero in Stage I tumors, 50–55% in Stage II and III tumors, and 80% in Stage IV tumors. The strongly positive rate for TIMP-2 was zero in Stage I tumors, 55% in Stage II tumors, and 80–100% in Stage III and IV tumors. The more progressive the clinical stage of tumor, the higher the strongly positive rates for MMP-2 and TIMP-2 in thymic epithelial tumors (Spearman rank correlation: MMP-2: $r = 0.706$; TIMP-2: $r = 0.805$, respectively; $P < 0.0001$). There was no correlation between the positive rate for MMP-9 and disease stage.

DFS in Patients with Thymoma and Expression of MMPs

The DFS rate was 91% for patients without MMP-2 expression and 55% for patients with MMP-2 expression at 5 years ($P = 0.0200$) (Fig. 5A). The DFS rate was 100% for patients without TIMP-2 expression and 53% for patients with TIMP-2 expression at 5 years ($P = 0.0053$) (Fig. 5B). There were significant differences in DFS between patients with and without MMP-2 expression and patients with and without TIMP-2 expression. The DFS rate was 75% for patients without MMP-9 expression and 75% for patients with MMP-9 expression at 5 years (Fig. 5C). The DFS rate was 81% for patients without TIMP-1 expression and 54% for patients with TIMP-1 expression at 5 years (Fig. 5D). There were no significant differences in DFS between
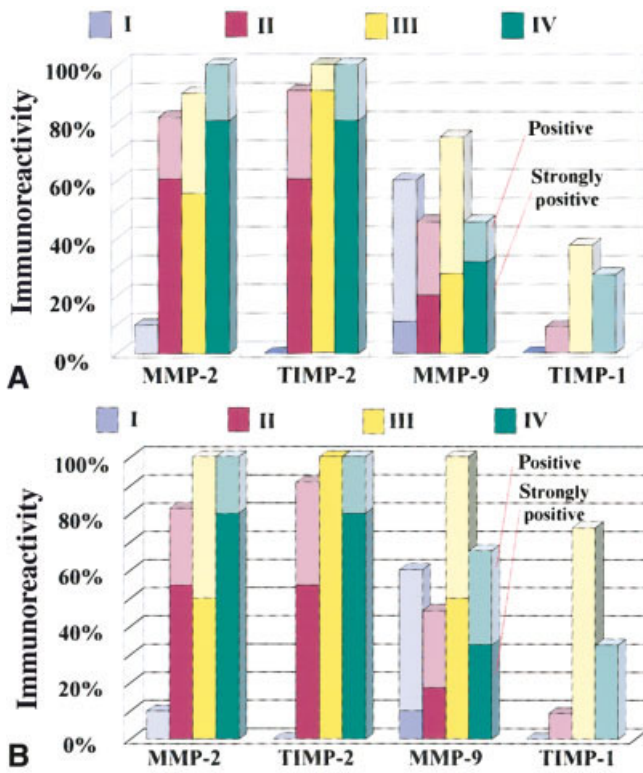
FIGURE 4. (A) Expression of each matrix metalloproteinase (MMP) and Masaoka clinical stage in thymomas. MMP-2 and tissue inhibitor of metalloproteinase (TIMP)-2: Stage I, $n = 10$; Stage II, $n = 12$; Stage III, $n = 10$; Stage IV, $n = 10$. MMP-9 and TIMP-1: Stage I, $n = 10$; Stage II, $n = 11$; Stage III, $n = 8$; Stage IV, $n = 7$. (B) Expression of each MMP and Masaoka clinical stage in thymomas except Type C thymoma (thymic carcinoma). MMP-2 and TIMP-2: Stage I, $n = 10$; Stage II, $n = 12$; Stage III, $n = 4$; Stage IV, $n = 4$. MMP-9 and TIMP-1: Stage I, $n = 10$; Stage II, $n = 11$; Stage III, $n = 4$; Stage IV, $n = 3$.

patients with and without MMP-9 expression or between patients with and without TIMP-1 expression.

DISCUSSION

It has been said that there is no histologic differences between noninvasive thymoma and invasive, disseminated, or metastatic thymoma.[2] Several studies have assessed factors associated with the invasiveness of thymoma, such as the nuclear areas of epithelial cells,[3] the nuclear DNA content,[4] proliferative activity (PCNA, Ki-67, mitotic figures),[5] the AgNOR,[6] and p53 expression.[7] The current study confirmed the existence of a significant correlation between MMP-2 expression and the invasiveness of thymic epithelial tumors using immunohistochemistry. First, we showed a tendency toward a positive relation between gelatinolytic activity and protein expression in MMP-2 (Fig. 1). Most invasive tumors (Stage II–IV; 91%) expressed MMP-2. In contrast, only 1 of 10 (10%) noninvasive tumors (Stage I) expressed MMP-2. Furthermore, immunoreactivity for MMP-2 was correlated significantly with Masaoka clinical stage. Except for Type C thymomas (thymic carcinoma), 18 of 30 invasive thymomas (90%) expressed MMP-2, whereas only 1 noninvasive thymoma (10%) expressed MMP-2. Immunoreactivity for MMP-2 was correlated significantly with Masaoka clinical stage, and MMP-2 expression was correlated with the degree of malignant behavior

of the thymoma and may be an indicator for distinguishing invasive thymoma from noninvasive thymoma. MMP-2 protein reportedly is correlated with poor prognostic variables in a variety of malignancies, such as breast,[15,16] lung,[17,18] stomach,[19] and head and neck carcinomas.[20]

It was long been believed that TIMPs suppressed tumor invasion by inhibiting MMPs in experimental and clinical studies. Recombinant TIMP-2 inhibited the invasion of HT-1080 fibrosarcoma cells in vitro[21]; and increased TIMPs expression has been correlated with decreased tumor growth, invasiveness, and metastasis in tumor cell lines of the stomach,[22] pancreas,[23] and lung.[24] However, the current study demonstrated that TIMP-2 immunoreactivity was correlated positively with the degree of malignant behavior in thymomas, contrary to previous evidence. A recent biochemical analysis demonstrated that TIMP-2 contributed to the activation of MMP-2.[25–27] In the MMP-2 activation mechanism, pro-MMP-2 secreted by fibroblasts or tumor cells binds to TIMP-2 complexed with membrane Type 1 MMP (MT1-MMP) on the tumor cell surface, and the pro-MMP-2/TIMP-2/MT1-MMP ternary complex is formed. Pro-MMP-2 in the ternary complex is activated by MT1-MMP that is free from TIMP-2.[25–27] Recently, some studies reported that TIMP-2 plays a positive role in invasion
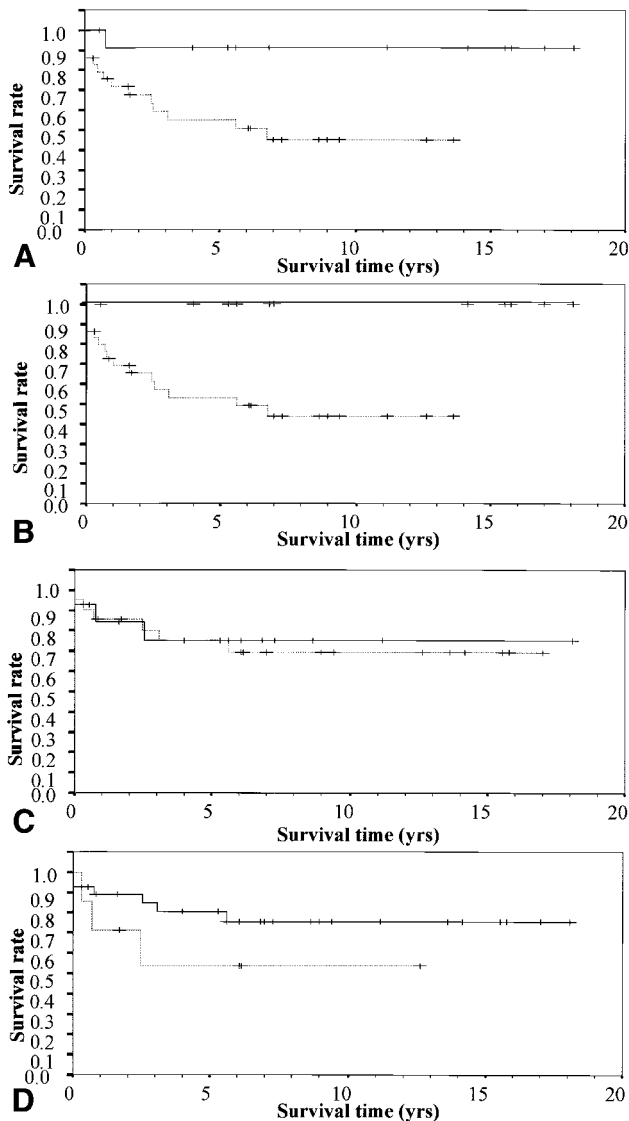
FIGURE 5. Disease-free survival of patients with thymoma according to expression of (A) matrix metalloproteinase (MMP)-2, (B) tissue inhibitor of metalloproteinase (TIMP)-2, (C) MMP-9, and (D) TIMP-1. Solid line: thymomas without expression of MMPs or TIMPs; dotted line: thymomas with expression of MMPs or TIMPs.

and metastasis in carcinoma of the breast,[28] lung,[29] colon,[30] and bladder.[31]

The current study demonstrated that both MMP-2 and TIMP-2 were detected mostly on the tumor cell surface and cytoplasm of thymomas and that they stained more strongly in border areas between the tumor and the stroma, which was the invasive area, compared with the central area of the tumor. Furthermore, the immunoreactivity of TIMP-2 was the same as the immunoreactivity of MMP-2 in thymic epithelial tumors. These findings suggest that TIMP-2 com-

plexes with pro-MMP-2 and MT1-MMP and activates pro-MMP-2 to MMP-2. Some studies have indicated that the immunoreactivity and localization of these two proteins are similar.[32,33] MMP-2 and TIMP-2 were expressed in invasive thymomas but not in noninvasive thymomas, and their expression was correlated with the Masaoka clinical stage and with DFS. We speculate that the combined effect of these proteins destroyed the ECM around the tumor and enabled tumor cells to spread to distant sites from the invasion front of the tumor. These proteins may be indicators for invasiveness of thymic epithelial tumors and predictors for recurrence. Moreover, the presence of MMP-2 or TIMP-2 may influence the selection of therapeutic modality in patients with thymoma. Most authors do not recommend radiotherapy after patients undergo total resection for Stage I (noninvasive) thymoma, whereas the use of postoperative radiotherapy in patients with Stage II or III thymoma who undergo total resection is controversial.[34] The current study suggests that patients who have thymomas with expression of MMP-2 or TIMP-2 should be treated with postoperative radiotherapy. The expression of MMP-2 or TIMP-2 may be an indicator for radiotherapy in these patients.

In our previous study, we used gelatinolytic zymography to show that the gelatinolytic activity of MMP-9 was not correlated with invasiveness in thymic epithelial tumors.[8] The current study also confirmed this finding using immunohistochemistry. The expression of MMP-9 was not correlated with clinical stage, WHO histologic classification, or DFS. There were some discrepancies in the correlations between MMP-9 expression and tumor invasiveness in some tumor types.[33,35-38] We noted a small number of instances of TIMP-1 expression among thymic epithelial tumors. The current study demonstrated that neither MMP-9 nor TIMP-1 plays a large role in the invasiveness of thymic epithelial tumors.

The histologic classification of thymoma has remained a subject of controversy for many years.[39] In 1999, the WHO Consensus Committee published a histologic typing system for tumors of the thymus.[13] Thymoma has been stratified into six entities (Types A, AB, B1, B2, B3, and C) based on the morphology of epithelial cells and the lymphocyte-to–epithelial cell ratio. Some studies have demonstrated that the WHO histologic classification reflects the oncologic behavior of thymoma.[40,41] Type A and AB thymomas are benign, and Type B1 and B2 thymomas represent the borderline between benign and malignant thymoma. Conversely, Type B3 thymomas have malignant behavior, and Type C thymomas are more aggressive tumor and must be considered cancerous. Immuno-

reactivity for both MMP-2 and TIMP-2 was low in Type AB thymoma; moderate in Type B1 thymoma; and high in Type B2, B3, and C thymomas. This immunoreactivity reflects the oncologic behavior in each type of thymoma. Two of three Type A thymomas with strong expression of both proteins showed microinvasion to the capsule of the tumor (Stage II), whereas one Type A thymoma with weak expression of MMP-2 and no expression of TIMP-2 was noninvasive and showed benign behavior.

In conclusion, MMP-2 and TIMP-2 are key enzymes for the invasiveness of thymic epithelial tumors, and it is believed that TIMP-2 works as an activator of pro-MMP-2 in thymic epithelial tumors. Although no histologic differences exist between noninvasive thymoma and invasive, disseminated, or metastatic thymoma, expression of these proteins can be a predictor of poor outcome in patients with thymoma.

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