

# Enhanced Production and Activation of Progelatinase A Mediated by Membrane-Type 1 Matrix Metalloproteinase in Human Papillary Thyroid Carcinomas<sup>1</sup>

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## ABSTRACT

Matrix metalloproteinases (MMPs) are believed to be involved in the invasion and metastasis of various human carcinomas. In the present study, the production levels of seven different MMPs (MMP-1, -2, -3, -7, -8, -9, and -13), the activation of the zymogen of MMP-2 (proMMP-2), the expression of membrane-type MMPs (MT1-, MT2-, and MT3-MMPs), and the tissue localization of the activated enzyme were examined in human invasive papillary thyroid carcinomas. Sandwich enzyme immunoassays revealed that among the MMPs examined, only the MMP-2 production level is significantly enhanced in the carcinoma tissues compared with the follicular adenoma and normal control thyroid tissues. Gelatin zymography indicated that the proMMP-2 activation ratio is considerably higher in carcinomas with lymph node metastasis than it is in those without metastasis, follicular adenomas, or normal controls ( $P < 0.01$ ). Northern blot analysis of the expression of MT1-, MT2-, and MT3-MMPs, which are known to activate proMMP-2 *in vitro*, demonstrated the predominant expression of MT1-MMP mRNA in the carcinoma tissues (15 of 15 cases), whereas MT2-MMP expression was confined to 26% of the cases (4 of 15 cases), and no consistent expression of MT3-MMP was observed. MT1-MMP mRNA expression levels correlated with the proMMP-2 activation ratio ( $r = 0.692$ ;  $P < 0.01$ ), but such a correlation was not obtained with MT2-MMP. There was also a direct correlation between MT1-MMP expression and lymph node metastasis ( $P < 0.05$ ). *In situ* hybridization indicated that both carcinoma and stromal cells express MT1-MMP transcripts (five of six cases). MT1-MMP was also immunolocalized to carcinoma and stromal cells in all of the carcinoma samples (26 of 26 cases), which were positive for MMP-2. *In situ* zymography indicated definite gelatinolytic activity in the carcinoma cell nests, which was abolished by incubation of the carcinoma samples with a synthetic MMP inhibitor before the reaction. These results suggest for the first time that among seven different MMPs, the production of proMMP-2 and its MT1-MMP-mediated activation in the carcinoma cell nests play an important role in the lymph node metastasis of human invasive papillary thyroid carcinomas.

## INTRODUCTION

A characteristic of malignant tumor cells is their ability to invade the surrounding stroma and metastasize. The high frequency at which MMPs<sup>3</sup> are detected at mRNA and/or protein levels in invasive tumor cell lines and various human carcinomas suggests that MMPs are implicated in invasion and metastasis (1, 2). Among the MMPs, MMP-2 (gelatinase A) is considered to be especially important in the degradation of the ECM that is associated with the malignant behavior

of the tumor cells (3, 4). Similar to most MMPs, MMP-2 is secreted as an inactive zymogen (proMMP-2); therefore, overexpression is not sufficient for the *in vivo* action of MMP-2, and activation must be a prerequisite to its functioning in the specific tissue (5). ProMMPs can be activated by various factors such as organomercurials, serine proteinases, hypochlorous acid, and acid exposure (5), and serine proteinases including trypsin, plasmin, plasma kallikrein, and neutrophil elastase may be generally important as *in vivo* activators for proMMPs (3, 5). However, proMMP-2 is unique in that it is not activated by the serine proteinases (6) but rather by MT-MMPs with a transmembrane domain (7–10). Although four different MT-MMPs (MT1-, MT2-, MT3-, and MT4-MMP) have been identified (7–10), MT4-MMP is distinguished from the others because of its low homology to other MT-MMPs (less than 30% homology to MT1-MMP at the amino acid level; Ref. 10) and lack of activator function.<sup>4</sup>

Human thyroid carcinoma tissues have been reported to express MMP-1 (tissue collagenase; Ref. 11) and MMP-2 (12, 13), and these MMPs are localized in the fibroblasts of the stroma adjacent to or close to the invading tumor cells (11–13). On the other hand, thyroid carcinoma cell lines with a highly metastatic potential are known to exhibit higher MMP-2 activity than the less metastatic cell lines (14). However, these reports did not study the expression of other MMPs including MT-MMPs or the activation of proMMP-2 in human thyroid carcinomas. In addition, tissue localization of activated MMP-2 has not been studied in any carcinoma tissues.

In the present study, we examined the production levels of MMP-1, MMP-2, MMP-3 (stromelysin 1), MMP-7 (matrilysin), MMP-8 (neutrophil collagenase), MMP-9 (gelatinase B), and MMP-13 (collagenase 3); the expression and localization of MT1-, MT2-, and MT3-MMPs; correlations between the expression of MT-MMPs and proMMP-2 activation; and the tissue localization of gelatinolytic activity in human invasive papillary thyroid carcinomas. The results suggest that overexpression of proMMP-2 and its MT1-MMP-mediated activation in the carcinoma cell nests are important in the malignant behavior of thyroid carcinomas.

## MATERIALS AND METHODS

**Clinical Samples and Histology.** Fresh tissue samples of thyroid were obtained from patients with primary papillary carcinoma (26 cases) and follicular adenoma (9 cases) who underwent surgery in the University Hospital, School of Medicine, Kanazawa University and used for histology and immunostaining. The patients with carcinoma ranged in age from 19–85 years (mean  $\pm$  SD, 51.1  $\pm$  13.6 years), and those with adenoma ranged in age from 27–64 years (mean  $\pm$  SD, 48.9  $\pm$  9.5 years). Surgical specimens were fixed with 10% buffered formalin, and paraffin sections were stained with H&E. The specimens were assessed according to the WHO International Classification of Thyroid Tumors standard criteria. There were stage I (5 cases), stage II (8 cases), and stage III (13 cases) carcinomas. The stages were determined according to the tumor-node-metastasis (TNM) classification of papillary thyroid carcinoma. Control normal thyroid tissues (26 cases) were also obtained from sites remote from the carcinomas.

<sup>4</sup> M. Seiki and H. Sato, unpublished data.

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<sup>3</sup> The abbreviations used are: MMP, matrix metalloproteinase; proMMP, corresponding zymogen; MT-MMP, membrane-type MMP; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EIA, enzyme immunoassay; TIMP, tissue inhibitor of metalloproteinase.

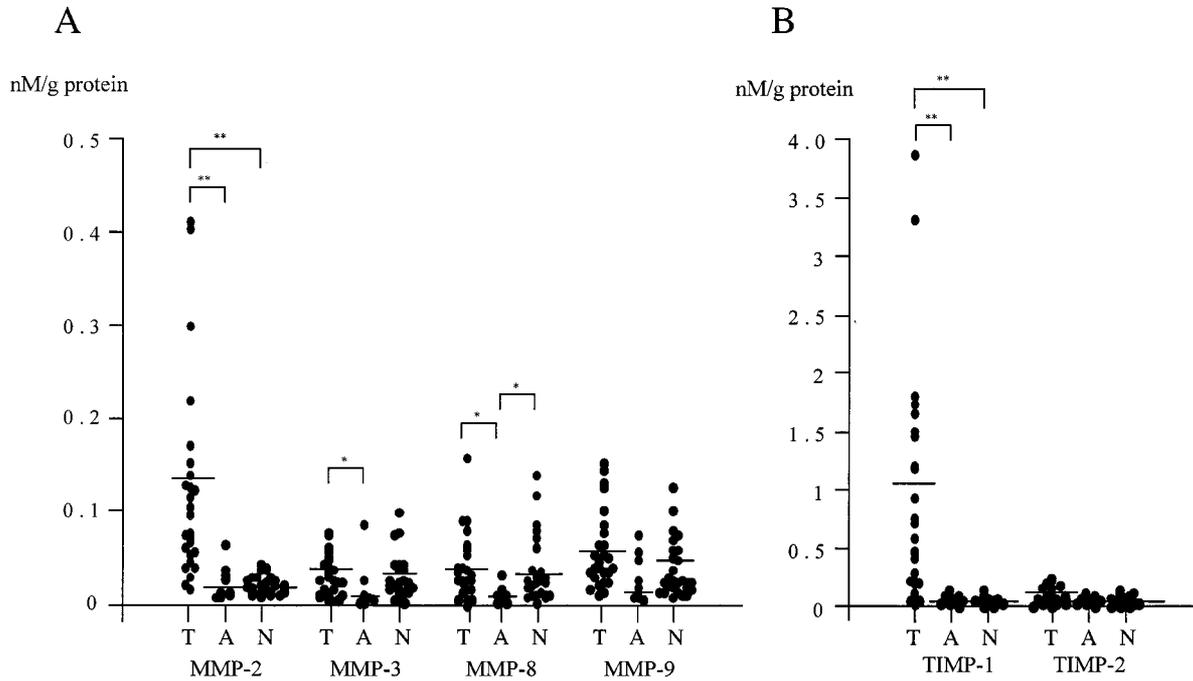


Fig. 1. The amount of MMPs (A) and TIMPs (B) in the supernatants of homogenates from carcinoma, follicular adenoma, and normal control tissues. Tissue homogenates were prepared from thyroid carcinoma (T; n = 26), follicular adenoma (A; n = 9), and normal control tissues (N; n = 26). MMP-2, -3, -8, and -9 and TIMP-1 and -2 were measured by corresponding EIA systems, and values (nM/g protein) were calculated as described in "Materials and Methods." Bars, mean. \*, P < 0.05; \*\*, P < 0.01 (Mann-Whitney U test).

**Tissue Homogenates and Sandwich EIAs.** A portion of fresh thyroid tissue samples (26 carcinomas, 26 normal controls, and 9 adenomas) obtained for immunostaining was homogenized without fixation in 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 10 mM CaCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, and 0.05% Brij 35. The supernatants were prepared by centrifugation, and protein concentrations were determined by the dye binding method according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

The concentrations of MMP-1, -2, -3, -7, -8, -9, and -13 and TIMP-1 and -2 in the supernatants were measured by the corresponding sandwich EIA systems for these MMPs and TIMPs as described previously (15–23). The EIA systems for MMP-1, -3, -8, and -13 measure both the precursor and active forms of the MMPs, but those for MMP-2, -7, and -9 detect only their latent forms. The EIA for TIMP-1 determines the whole amount of TIMP-1 including free TIMP-1 and the complexed forms with active MMPs and proMMP-9 (22). However, the EIA for TIMP-2 detects free TIMP-2 and TIMP-2 complexed with active MMPs, but not the complex with proMMP-2 (23). Values were expressed as nM/g protein. The following molecular weights derived from the amino acid sequence were used for calculation: MMP-1, M<sub>r</sub> 51,896; MMP-2, M<sub>r</sub> 70,930; MMP-3, M<sub>r</sub> 52,188; MMP-7, M<sub>r</sub> 27,916; MMP-8, M<sub>r</sub> 51,098; MMP-9, M<sub>r</sub> 76,291; MMP-13, M<sub>r</sub> 51,647; TIMP-1, M<sub>r</sub> 20,695; and TIMP-2, M<sub>r</sub> 21,740. The molar ratios of the total amounts of MMP-1, -2, -3, -7, -8, -9, and -13 to those of TIMP-1 and TIMP-2 were calculated by dividing the values of the MMPs with those of TIMP-1, TIMP-2, and MMP-2 in each case, assuming that all of the MMP-2 detected by the assay was the proMMP-2-TIMP-2 complex (4).

**Northern Blot Analysis.** Total RNA was extracted by the acid guanidinium-phenol chloroform method (7). Because some samples were not sufficient for RNA isolation, 15 specimens of carcinoma and control normal thyroid tissues obtained from sites remote from the carcinoma and 9 follicular adenomas were used for RNA preparation. RNA samples (25 μg/lane) were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde and transferred onto Hybond N<sup>+</sup> membranes (Amersham, Tokyo, Japan). The membranes were hybridized with <sup>32</sup>P-labeled probes for MT1-MMP (1.2-kb cDNA fragment corresponding to nucleotides 1647–2889; Ref. 7), MT2-MMP (1.2-kb fragment corresponding to nucleotides 273–1526; Ref. 8), MT3-MMP (2.1-kb fragment corresponding to nucleotides 1–2107; Ref. 9); and GAPDH as described previously (7, 9). As a control, total RNA was extracted from OSC-19 cells (a highly metastatic oral squamous cell carcinoma cell line), which are known to express the MT-MMPs (9), and the samples were pro-

cessed in a similar way. The blotted membranes were scanned by a Bioimage Analyzer BAS 1000 (Fuji Photo Film, Co., Ltd., Tokyo, Japan). For a quantitative analysis of the expression levels of the MT-MMPs, the radioactivity of the bands of MT-MMPs was corrected for the RNA loading data obtained from GAPDH signals (24).

**In Situ Hybridization.** To verify the origin of cells expressing MT1-MMP mRNA, the samples (six carcinomas and six normal cases) that showed MT1-MMP expression in carcinoma tissues by Northern blotting were used for *in situ* hybridization according to the methods described previously (25). Briefly, the cDNA (4.5-kbp) encoding MT1-MMP was subcloned into Bluescript SK ± (Stratagene, La Jolla, CA), and radiolabeled single-stranded RNA probes were prepared using <sup>35</sup>S-labeled UTP. The probes were hydrolyzed in 0.1 M sodium carbonate buffer (pH 10.2) containing 10 mM DTT to an average size of 300 bases, and paraffin sections of the tissues that were treated with 20 μg/ml proteinase K (Promega Biotec, Oakland, CA) and 0.0025% acetic anhydride (Eastman Kodak, Rochester, NY) in 0.1 M triethanolamine (pH 8.0; Eastman Kodak) were hybridized with antisense RNA or sense RNA for ~12 h at 50°C. The slides were then treated with 20 μg/ml RNase A, washed under stringent conditions (washed with 2× SSC, 0.5× SSC, and 0.1× SSC two times each for 30 min at 50°C), and air-dried. They were dipped in NR-M2 emulsion (Konica, Tokyo, Japan) and subjected to autoradiography by a 3–4-week exposure.

**Immunohistochemistry.** Tissue samples of the carcinoma (26 cases), control normal thyroid remote from the tumor (26 cases), and follicular adenoma (9 cases) were fixed with periodate-lysine-paraformaldehyde fixative (4) for 18–24 h at 4°C, and the paraffin sections were reacted with the monoclonal antibodies to MT1-MMP (30 μg/ml; clone 114-6G6), MT2-MMP (30 μg/ml; clone 162-22G5), and MMP-2 (10 μg/ml; clone 75-7F7) or nonimmune mouse IgG (30 μg/ml). After reactions with biotinylated horse IgG to mouse IgG (Vector Laboratories, Burlingame, CA) and an avidin-biotin-peroxidase complex (DAKO, Glostrup, Denmark), color was developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) as described previously (4). The ratio (percentage) of immunostained carcinoma cells:total carcinoma cells was measured by observing five different fields at a magnification of ×200 without knowledge of the EIA or clinicopathological data.

**Gelatin Zymography.** Gelatinolytic activity in thyroid tissue homogenates (26 carcinomas, 26 normal controls, and 9 adenomas) was examined by gelatin zymography. The supernatants (20 μg protein/lane) were subjected to SDS-

PAGE using gelatin-containing gels as described previously (4). The ratios of proMMP-2 activation were estimated by computer-assisted densitometric analysis of the  $M_r$  62,000 and  $M_r$  68,000 proteolytic bands, which correspond to the active and latent species of MMP-2, respectively (4).

**In Situ Zymography.** Fresh specimens of carcinoma with normal thyroid tissues (five cases) were embedded without fixation in Tissue-Tek OCT compound (Miles, Inc., Elkhart, IN). Frozen sections were made by a cryostat (Miles, Inc.) and mounted onto gelatin films that were coated with 7% gelatin solution (Fuji Photo Film Co., Ltd.). The films with sections were incubated for 12 h at 37°C and stained with 1.0% Amido Black 10B. The expression of MT1-MMP and MMP-2 in the carcinoma tissues was ascertained by immunohistochemistry. As a control, carcinoma tissues were incubated in DMEM containing 0.2% lactalbumin hydrolysate with or without 50  $\mu$ M BB94 (British Biochem, Oxford, United Kingdom) for 3 h at 37°C, and the frozen sections were treated in a similar way as described above.

**Statistics.** Statistical analyses were performed using the  $\chi^2$  test and the two-tailed Mann-Whitney *U* test. *P*s less than 0.05 were considered significant.

## RESULTS

**Sandwich EIAs.** MMP-2, -3, -8, and -9 and TIMP-1 and -2 were measurable by the EIA systems in approximately 90% of the carcinomas, but MMP-1, -7, and -13 were detectable in only 10% of the cases. The levels of MMP-2, -3, -8, and -9 in the carcinomas were  $0.139 \pm 0.168$ ,  $0.042 \pm 0.03$ ,  $0.048 \pm 0.053$ , and  $0.063 \pm 0.057$  nm/g protein (mean  $\pm$  SD), respectively, and only the MMP-2 level was significantly higher than that found in the control normal tissue ( $0.023 \pm 0.014$  nm/g protein) or follicular adenoma ( $0.023 \pm 0.019$  nm/g protein; Fig. 1A;  $P < 0.01$ ). Although the production levels of MMP-3 and -8 in the carcinomas were also significantly higher than those in the adenomas (MMP-3,  $0.018 \pm 0.028$ ; MMP-8,  $0.013 \pm 0.009$  nm/g protein;  $P < 0.05$ ), there was no difference in the levels between the carcinoma and normal control samples (Fig. 1A). The TIMP-1 level ( $1.072 \pm 1.376$  nm/g protein) was significantly higher in the carcinomas than it was in the normal ( $0.058 \pm 0.054$  nm/g protein) and adenoma ( $0.046 \pm 0.044$  nm/g protein) samples ( $P < 0.01$ ; Fig. 1B). However, there was no difference in the TIMP-2 levels between the carcinoma ( $0.132 \pm 0.155$  nm/g protein), normal ( $0.07 \pm 0.04$  nm/g protein), and adenoma ( $0.043 \pm 0.037$  nm/g protein) samples (Fig. 1B). When the production levels of these MMPs and TIMPs were compared with clinicopathological factors such as lymph node metastasis, no correlations were obtained. The molar ratio of the total amounts of MMPs:TIMPs was significantly higher in the carcinoma samples (mean  $\pm$  SD,  $0.45 \pm 0.25$ ) than in the adenoma ( $0.18 \pm 0.10$ ;  $P < 0.01$ ) and normal samples ( $0.24 \pm 0.08$ ;  $P < 0.01$ ).

**Activation of ProMMP-2.** ProMMP-2 activation in the supernatants of thyroid tissue homogenates was analyzed by gelatin zymography (Fig. 2A). Computer-assisted image analyses of the intensity of the proteolytic bands showed that the activation ratio (the ratio of the  $M_r$  62,000 active form:proMMP-2 and active forms) is significantly higher in the carcinoma samples ( $0.26 \pm 0.15$ ;  $n = 26$ ) than in the normal control ( $0.08 \pm 0.05$ ;  $n = 26$ ) or follicular adenoma samples ( $0.04 \pm 0.04$ ;  $n = 9$ ;  $P < 0.01$ ; Fig. 2B). In addition, the activation ratio was significantly higher in the carcinoma samples with lymph node metastasis ( $0.31 \pm 0.16$ ;  $n = 14$ ) than in those without metastasis ( $0.19 \pm 0.12$ ;  $n = 12$ ;  $P < 0.05$ ; Fig. 2B). The activation ratio was also remarkably higher in the stage III carcinomas ( $0.33 \pm 0.16$ ;  $n = 13$ ) than in the stage I and II carcinomas ( $0.19 \pm 0.12$ ;  $n = 13$ ;  $P < 0.05$ ; data not shown).

**mRNA Expression of MT1-, MT2-, and MT3-MMPs and Its Correlation with ProMMP-2 Activation.** The MT1-MMP transcript was detected by Northern blot analyses in all of the carcinoma samples (15 of 15 cases), whereas negligible or no hybridization was

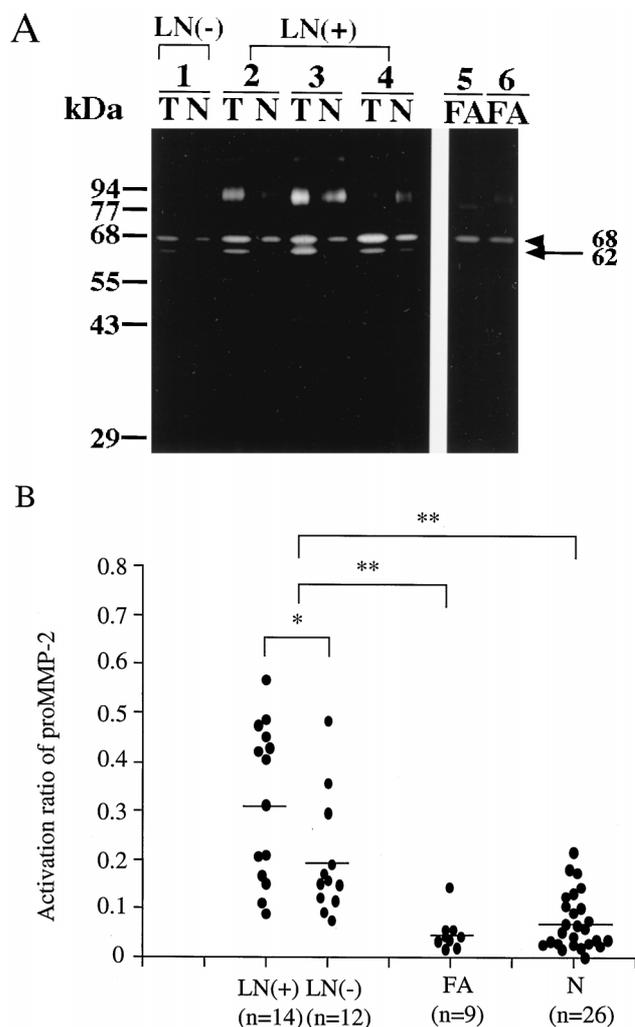


Fig. 2. Analysis of the activation ratios of proMMP-2 by gelatin zymography. A, gelatin zymography. The supernatants of the homogenates from carcinoma (T), normal thyroid (N), and follicular adenoma tissues (FA) were subjected to gelatin zymography as described in "Materials and Methods." Four representative carcinoma samples with or without lymph node metastasis [LN(+)] and LN(-), respectively] and two follicular adenoma samples are shown. Major gelatinolytic activities with  $M_r$  68,000 (arrowhead) and  $M_r$  62,000 (arrow) corresponding to proMMP-2 and MMP-2, respectively, are indicated. The molecular weights of the protein standards are as follows: phosphorylase b,  $M_r$  94,000; transferrin,  $M_r$  77,000; BSA,  $M_r$  68,000; heavy chain IgG,  $M_r$  55,000; ovalbumin,  $M_r$  43,000; and carbonic anhydrase,  $M_r$  29,000. B, activation ratios of proMMP-2. The ratios were measured by a computer-assisted densitometric analysis of the gels as described in "Materials and Methods." LN(+), carcinoma with lymph node metastasis; LN(-), carcinoma without metastasis; FA, follicular adenoma; N, normal samples. Bars, mean. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

observed in those of the normal thyroid and follicular adenoma tissues (Fig. 3A). MT2-MMP mRNA was expressed selectively in the carcinomas, but the expression was confined to 26% of the cases (4 of 15 cases; Fig. 3A). In contrast, MT3-MMP mRNA was undetectable in all of the specimens examined (Fig. 3A). When the expression levels of MT1-MMP and MT2-MMP (MT-MMP/GAPDH) in the carcinoma samples were compared with clinicopathological factors, only MT1-MMP expression was significantly higher in the carcinoma samples from the patients with lymph node metastasis ( $0.203 \pm 0.043$ ;  $n = 9$ ) as compared to those without metastasis ( $0.148 \pm 0.039$ ;  $n = 6$ ;  $P < 0.05$ ; Fig. 3B). However, such a correlation was not seen between MT1-MMP expression and other factors such as tumor size of the carcinomas. There was no correlation between MT2-MMP expression and the clinicopathological factors (data not shown). When the activation ratio of proMMP-2 was plotted against the MT1-MMP or

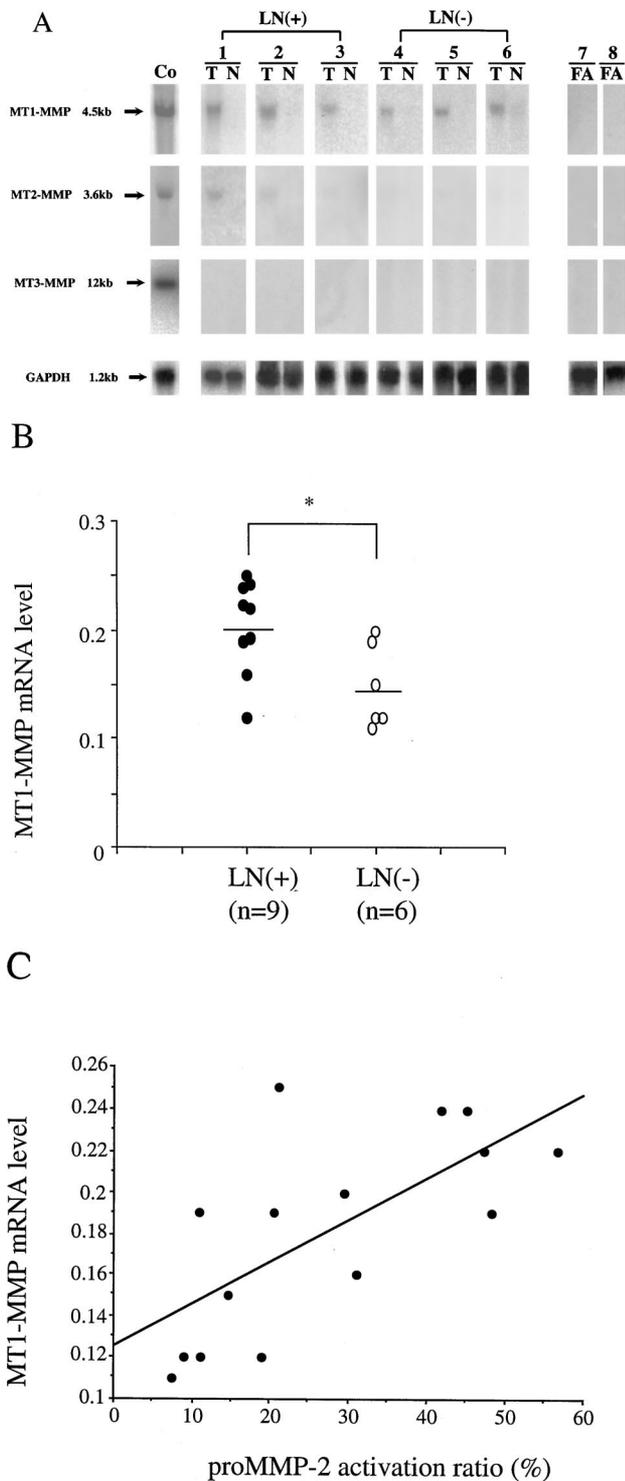


Fig. 3. mRNA expression of MT1-, MT2-, and MT3-MMPs (A); correlation of MT1-MMP expression with lymph node metastasis (B); and correlation of MT1-MMP expression with the proMMP-2 activation ratio (C) in human thyroid carcinomas. A, total RNA was extracted from the carcinoma (T), normal control (N), and follicular adenoma tissues (FA), and Northern blotting for MT1-, MT2-, and MT3-MMPs was carried out as described in "Materials and Methods." Six representative carcinoma samples (three cases with and three cases without lymph node metastasis) and two follicular adenomas are shown. Molecular sizes of the transcripts are as follows: 4.5 kb, MT1-MMP; 3.6 kb, MT2-MMP; 12 kb, MT3-MMP; and 1.2 kb, GAPDH. LN(+) and LN(-) indicate carcinoma cases with or without lymph node metastasis, respectively. RNA from OSC-19 cells (Co) was used as a positive control for the Northern blotting. B, the MT1-MMP mRNA levels in carcinomas with lymph node metastasis [LN(+)] or without lymph node metastasis [LN(-)] were compared after standardization with those of GAPDH as described in "Materials and Methods." Bars, mean. \*,  $P < 0.05$ . C, the activation ratios of proMMP-2 were plotted against the MT1-MMP mRNA levels in the carcinoma cases ( $n = 15$ ). A direct correlation is observed ( $r = 0.692$ ;  $P < 0.01$ ).

MT2-MMP mRNA expression level in each case, the ratio had a direct correlation with MT1-MMP expression ( $r = 0.692$ ;  $P < 0.01$ ; Fig. 3C), but not with MT2-MMP (data not shown).

**In Situ Hybridization for MT1-MMP.** Cells expressing MT1-MMP mRNA in thyroid carcinomas were identified by *in situ* hybridization. The signals for MT1-MMP were observed with the antisense RNA probe in both carcinoma and stromal cells surrounding the invasive tumor cell nests (five of six cases; Fig. 4A), but stromal cells in the normal thyroid tissue showed no hybridization signals (data not shown). The sense probe gave only a background signal in the carcinoma and normal tissues (Fig. 4B).

**Immunohistochemistry.** MT1-MMP was immunolocalized to the carcinoma cells and stromal cells in all of the carcinoma cases (26 of 26 cases; Fig. 5A). On average, approximately 40% of the total carcinoma cells ( $40.3 \pm 19.4\%$ ) were positively immunostained in each case. In follicular adenomas, neither tumor cells nor stromal fibroblasts were immunostained in any of the cases (data not shown). Also, no staining was seen in the control normal samples (data not shown). MT2-MMP was immunolocalized mainly to the carcinoma cells (Fig. 5B), but staining was observed in only 35% of the cases (9 of 26 cases). Immunoreactive carcinoma cells for MT2-MMP were  $18.5 \pm 19.1\%$  of the total carcinoma cells in positive cases. In addition, MMP-2 was immunolocalized in the carcinoma cells and stromal fibroblasts in all of the carcinoma cases (26 of 26 cases; Fig. 5C). In most cases, fibroblasts and vascular endothelial cells in the carcinoma tissue were also immunostained for MMP-2. The staining for these MMPs in the stromal cells was definitely positive in the carcinoma tissues, but it was weak or negative in the normal tissue remote from the carcinoma. No staining was obtained with nonimmune mouse IgG (Fig. 5D).

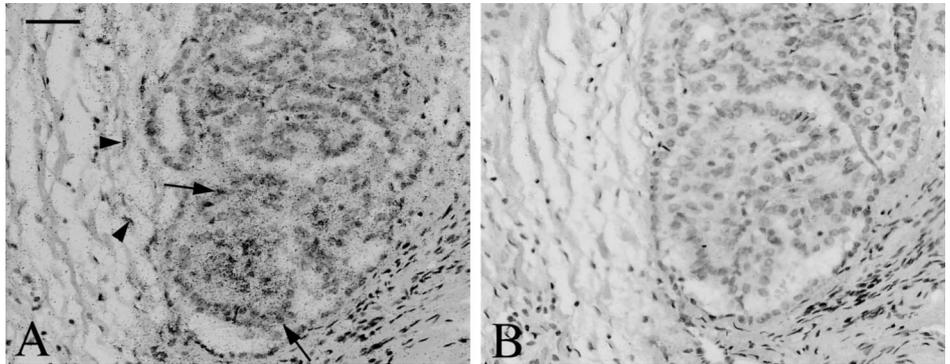
**Detection of Gelatinolytic Activity by in Situ Zymography.** *In situ* zymography using gelatin films revealed that strong gelatinolytic activity is present mainly in the carcinoma cell nests (Fig. 6, A and B). Although weak activity was also detected in the stromal components adjacent to the neoplastic islands, no activity was recognized in the normal thyroid tissue remote from the carcinoma (Fig. 6, E and F). The activity in the carcinoma cell nests was almost completely blocked in carcinoma samples that had been incubated with BB94 (Fig. 6, C and D). Distributions of the activity were consistent with the immunolocalization of MT1-MMP and MMP-2.

**DISCUSSION**

The present studies have demonstrated for the first time that among seven different MMPs (MMP-1, -2, -3, -7, -8, -9, and -13), only the production level of MMP-2 is remarkably higher in the carcinomas than in the noncarcinoma tissues. The enhanced production of MMP-2 protein and/or MMP-2 activity have been reported in many human carcinomas, including stomach (4), breast (26), and lung (27) carcinomas. Using our EIA systems, we have examined the production levels of the seven MMPs in several human carcinomas and found that the production of proMMP-2 is usually enhanced to a similar level in these carcinomas. However, thyroid carcinomas are unique in that the production levels of other MMPs in the carcinoma tissue are negligible, whereas other carcinomas including carcinomas of endometrium,<sup>5</sup> breast,<sup>6</sup> and head and neck<sup>7</sup> commonly show an increased production of MMP-1, -3, -7, -8, and -9 as well as MMP-2. Among the two TIMPs (TIMP-1 and -2), the TIMP-1 level was also remarkably higher in the thyroid carcinoma than in the noncarcinoma tissues, and a similar finding was obtained for the other carcinomas. Thus, overproduction of TIMP-1 in the carcinoma

<sup>5</sup> H. Ueno, K. Yamashita, I. Azumano, M. Inoue, and Y. Okada, unpublished data.  
<sup>6</sup> H. Nakamura, K. Yamashita, H. Veno, and I. Okada, unpublished data.  
<sup>7</sup> T. Shimada, H. Nakamura, K. Yamashita, Y. Marakami, N. Fujimoto, and Y. Okada, unpublished data.

Fig. 4. *In situ* hybridization for MT1-MMP in the thyroid carcinoma tissues. *In situ* hybridization was performed with the RNA probes as described in "Materials and Methods." Note that signals of MT1-MMP mRNA are detectable in both carcinoma (arrows) and stromal cells (arrowheads) with the antisense probe (A). The sense probe gives only a background signal in the carcinoma tissue (B). H&E stain. Bar, 50  $\mu$ m.



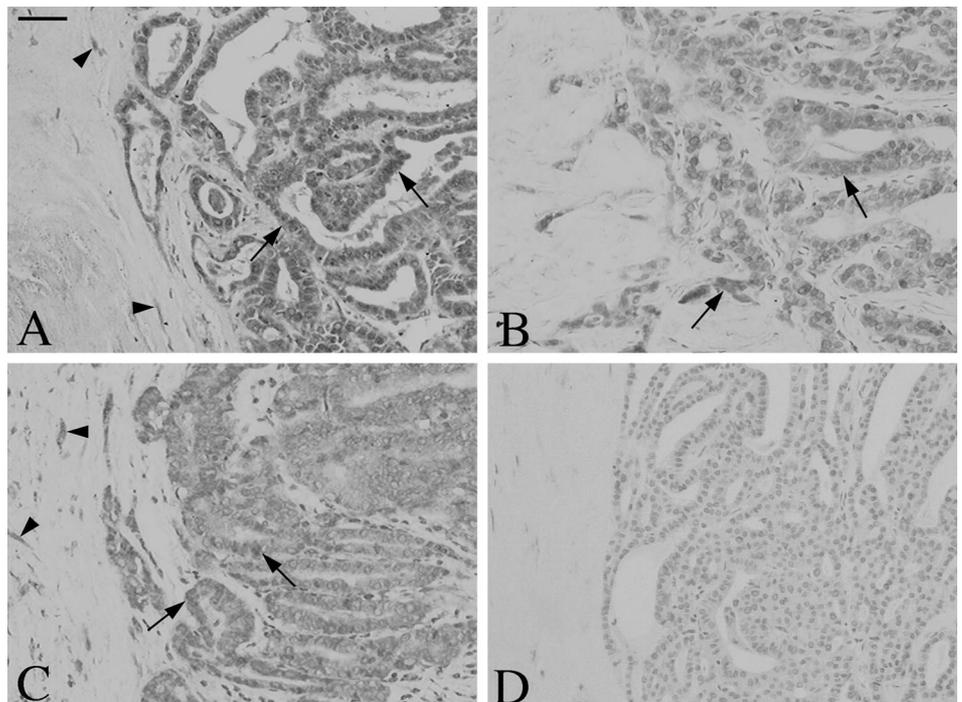
tissue may be a common feature in human carcinomas. However, the TIMP-2 level did not differ between the carcinoma and noncarcinoma tissues, suggesting a different regulatory mechanism for TIMPs in human carcinoma tissues. Although the molar ratios of MMPs:TIMPs are in favor of the proteinases in carcinomas of the head and neck,<sup>7</sup> breast,<sup>6</sup> and endometrium,<sup>5</sup> the thyroid carcinomas had an overwhelming production of TIMP-1 over the MMPs. Papillary thyroid carcinomas have the potential for invasion and metastasis, but they are usually localized in the thyroid tissues, surrounded by a fibrous capsule, and metastasis is confined to the local neck lymph nodes. Thus, the profile of MMP and TIMP production might be related to the pathology of the carcinoma.

The activation of proMMP-2 was almost selectively observed in the thyroid carcinomas, and the activation ratio was significantly higher in carcinomas with lymph node metastasis than it was in those without metastasis, although the production level itself had no correlation with lymph node metastasis. Similar correlations of proMMP-2 activation with metastasis have been reported in human breast (26), lung (27), and stomach carcinomas (4). It is therefore suggested that the activation of proMMP-2 in the carcinoma tissues (but not its production level) is a determinant of metastasis. When the activation rate of proMMP-2 was compared among the human carcinomas, the rate in papillary thyroid carcinomas ( $0.26 \pm 0.15$ ) appears to be much lower than that in carcinomas of the head and neck ( $0.59 \pm 0.18$ ),<sup>7</sup> lung

( $0.54 \pm 0.09$ ; Ref. 27), and breast ( $0.52 \pm 0.13$ ; Ref. 26). The data might be related to the preferable prognosis of the papillary thyroid carcinomas (5-year survival, 96%; Ref. 28). Additional studies on a large number of cases with a long follow-up are needed to clarify the correlations between proMMP-2 activation and the prognosis of papillary thyroid carcinomas.

Previous studies have demonstrated that MT1-, MT2-, and MT3-MMPs can activate proMMP-2 *in vitro* (7, 29, 9), and our recent studies indicated that MT1-MMP is responsible for the activation of proMMP-2 in human stomach (30), lung (27), and breast (26) carcinomas. In the present studies, we have demonstrated for the first time that among MT1-, MT2-, MT3-MMPs, the expression of MT1-MMP is predominant in human thyroid carcinomas. Because the mRNA expression level of MT1-MMP but not MT2-MMP or MT3-MMP directly correlates with proMMP-2 activation, MT1-MMP is considered to be responsible for activation in the carcinoma tissues. A discrepancy between the mRNA and protein expression of MT1-MMP has been reported in human breast carcinomas; MT1-MMP was immunostained mainly in the carcinoma cells (26), but mRNA localized predominantly to the stromal cells by *in situ* hybridization (31). However, such a finding was not observed in the thyroid carcinomas, because both the carcinoma and stromal cells were labeled by immunohistochemistry and *in situ* hybridization in the present studies.

Fig. 5. Immunolocalization of MT1-MMP, MT2-MMP, and MMP-2 in the thyroid carcinoma tissues. Thyroid carcinoma tissues were immunostained with monoclonal antibodies against MT1-MMP (A), MT2-MMP (B), and MMP-2 (C) or nonimmune mouse IgG (D) as described in "Materials and Methods." Note that MT1-MMP, MT2-MMP, and MMP-2 are immunostained within the carcinoma (arrows) and stromal cells (arrowheads), whereas no staining is observed with nonimmune IgG. Hematoxylin counterstain. Bar, 50  $\mu$ m.



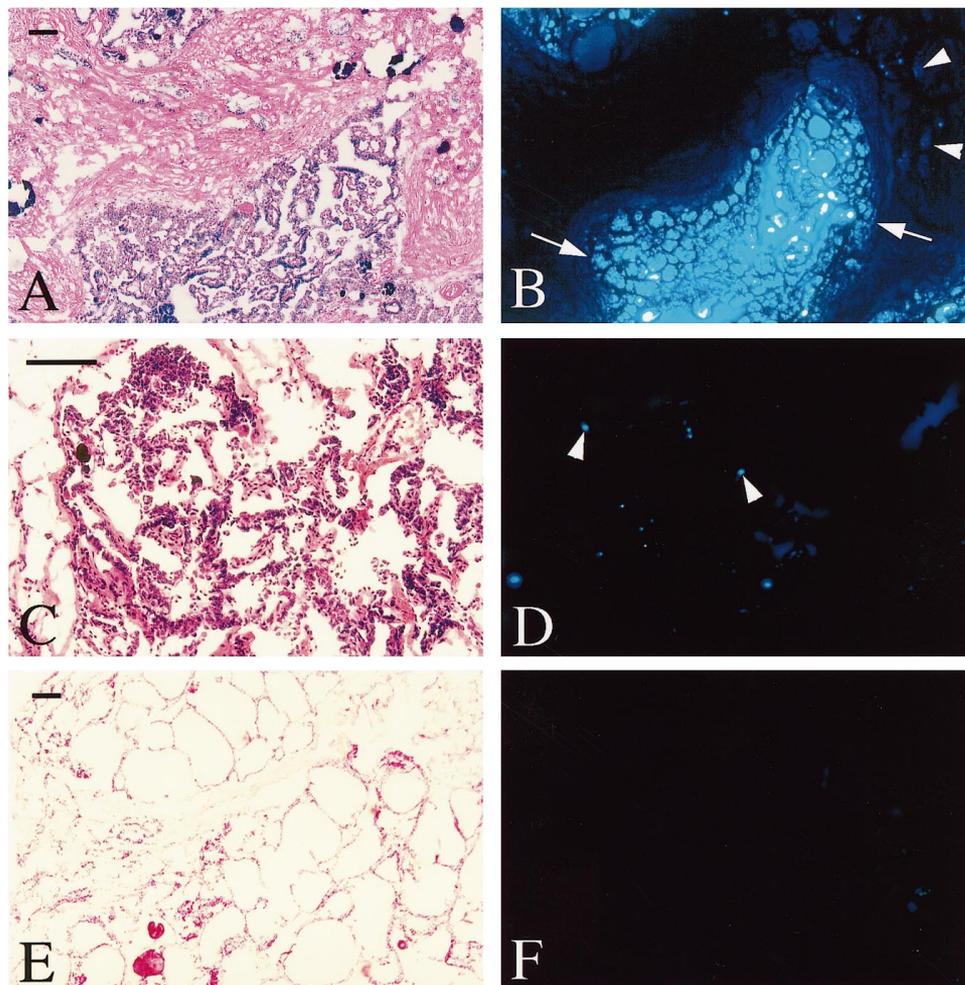


Fig. 6. *In situ* zymography of the carcinoma and normal thyroid tissues. Serial frozen sections were made and stained with H&E (A, C, and E) or subjected to *in situ* zymography (B, D, and F) as described in "Materials and Methods." A and B, carcinoma tissues without treatment. C and D, carcinoma tissues preincubated with BB94. E and F, normal thyroid tissue without treatment. Note the strong gelatinolytic activity in the carcinoma cell nests (B, arrows) and the abolishment of activity in the BB94-treated carcinoma tissue (D). Patchy focal foci of calcification (B and D, arrowheads) in the carcinoma tissues probably show unstained spots in the *in situ* zymography because the dye cannot permeate through the gelatin films in the calcified foci. Bar, 100  $\mu$ m.

MT2-MMP was also expressed in approximately one-third of the analyzed thyroid carcinoma tissues and immunostained in the carcinoma cells. However, there was no correlation between MT2-MMP expression and proMMP-2 activation, which suggests a minor role for MT2-MMP in the activation in thyroid carcinomas. Recent biochemical studies have shown that MT2-MMP has proteolytic activity to ECM macromolecules such as fibronectin, nidogen, perlecan, and laminin (32). Thus, it is conceivable that like MT1-MMP (33), MT2-MMP is also involved in the direct pericellular ECM degradation by the carcinoma cells, which express this enzyme.

One of the interesting findings in the present study is that the gelatinolytic activity in thyroid carcinoma tissues is detected predominantly in the carcinoma cell nests by *in situ* zymography. The activity was abolished in the carcinoma samples incubated with BB94 before the sectioning. Thus, the gelatinolytic activity can be ascribed to MMPs. Gelatin is a nonspecific substrate susceptible to many MMPs (5, 6). However, because the major MMPs produced in thyroid carcinomas are MMP-2 and MT1-MMP, it is reasonable to believe that these MMPs are responsible for the activity. MT1-MMP was originally cloned as an activator of proMMP-2 (7), and its implications for proMMP-2 activation *in vivo* have been demonstrated in various human carcinomas (26, 27, 30). Although these studies indicated the colocalization of MT1-MMP and MMP-2 proteins in the carcinoma cells as shown in the present study, this study is the first to demonstrate that gelatinolytic activity of MMP-2 activated by MT1-MMP is expressed in the carcinoma cell nests. MMP-2 and MT1-MMP were also expressed in the stromal cells, but the present data suggest that

major gelatinolytic activity is present in the carcinoma cell nests but not in the stromal cells. This may be because of the low cellularity of the stromal fibroblasts compared with that of the carcinoma cells. Because MT1-MMP also has ECM-degrading activities including weak gelatinolytic activity (33), the data do not exclude the possibility that MT1-MMP itself, in addition to MMP-2, is partly involved in the activity in the carcinoma cell nests. Another important point is that the activity was detected in carcinoma cell nests where the TIMP-1 level was highly elevated as demonstrated by the EIA system. Because TIMP-1 does not inhibit the MT1-MMP activity *in vitro* (34), this suggests the possibility that TIMP-1 may not function as an inhibitor of gelatinolytic activity in the carcinoma cell nests. Additional studies are needed to demonstrate this hypothesis.

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## Enhanced Production and Activation of Progelatinase A Mediated by Membrane-Type 1 Matrix Metalloproteinase in Human Papillary Thyroid Carcinomas

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