Virchows Arch (2001) 438:121–128
DOI 10.1007/s004280000286

ORIGINAL ARTICLE

Hiroyuki Maeta · Shigetsugu Ohgi · Tadashi Terada

Protein expression of matrix metalloproteinases 2 and 9 and tissue inhibitors of metalloproteinase 1 and 2 in papillary thyroid carcinomas

Received: 26 May 2000 / Accepted: 27 June 2000 / Published online: 4 November 2000

Abstract Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play an important role in tumor invasion and metastasis. There have been only a few studies on the protein expression of MMPs and TIMPs in thyroid carcinomas. Therefore, we investigated the protein expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in 86 papillary thyroid carcinomas using immunohistochemistry, semiquantitative scoring morphometry of immunohistochemistry, gelatin zymography, and western blotting. We also examined the correlations between the immunohistochemical scores and several clinicopathological parameters. The immunoreactivities of MMP-2, MMP-9, TIMP-1, and TIMP-2 were largely located in the tumor cells or non-tumor follicular cells and to a much lesser extent in the fibroblasts and endothelial cells in the tumor and non-tumor regions. Compared with non-tumor regions, these four proteins tended to be overexpressed in the tumor cells; the overexpression was found in 64 of 86 (74%), 80 of 86 (93%), 79 of 86 (92%), and 64 of 86 (74%) cases for MMP-2, MMP-9, TIMP-1, and TIMP-2, respectively. Gelatin zymography showed distinct bands of MMP-2 and MMP-9 in tumor extracts but vague bands in non-tumor extracts. Western blotting revealed the specific bands of MMP-2 and MMP-9 in both tumor and non-tumor extracts. Morphometric scoring revealed that high expression of these proteins significantly correlated with large tumor size, presence of lymph node metastasis, high clinical stage, high intrathyroidal invasion, and high vascular invasion. These data suggest that MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins and activities are increased in tumors cells of papillary thyroid carcinomas and that they play an important role in the invasion and metastasis of papillary thyroid carcinomas.

Keywords Thyroid cancer · Matrix metalloproteinase · Immunohistochemistry · Gelatin zymography · Western blot

H. Maeta · T. Terada (✉)
Second Department of Pathology, Tottori University, Faculty of Medicine, 86 Nishi-cho, Yonago 683–8503, Tottori, Japan
e-mail: terada@grape.med.tottori-u.ac.jp
Tel.: +81-859-348018, Fax: +81-859-348348

S. Ohgi
Departments of Surgery, Tottori University, Faculty of Medicine, Yonago, Japan

Introduction

Matrix metalloproteinases (MMPs) are secreted proteinases required for extracelluler matrix degradation in tumor invasion and are generally classified into some different families of closely related members, such as collagenases, gelatinases, and stromelysins [24]. MMP-2 (72 kDa type IV collagenases, gelatinase A) and MMP-9 (92 kDa type IV collagenases, gelatinase B) are critical factors in basement membrane degradation [22]. Therefore, these MMPs may participate in the various steps of tumor invasion, metastasis, and progression in several carcinomas [5, 27]. Their activities are controlled through distinct mechanisms, and their expressions are regulated at the level of gene expression during proenzyme activation by proteolysis and during binding of the proenzymes or active enzymes to specific inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs) [2, 30]. The latent forms of MMP-2 (proMMP-2) and MMP-9 (proMMP-9) bind to TIMP-2 and TIMP-1, respectively. Membrane type of MMPs (MT-MMPs) present on tumor cell surfaces is thought to play a crucial role in the invasive phenotype of carcinomas. It has been reported that activation of MMP-2 correlates with MT1-MMP expression [39]. It has been shown in vitro that MT1-MMP might be associated with TIMP-2, both acting together as receptors for proMMP-2 and leading to cleavage of the zymogens [41]. MMP-9 is not activated by MT-MMPs, but is activated by plasmin, stromelysin-1 (MMP-3), and MMP-2 [15].

Papillary carcinoma of the thyroid are generally associated with slow growth and good prognosis. However, some cases show a relatively early recurrence, severe in-

vasion, or multiple lymph node metastasis, though their tumor size is small. It would be important to identify the characteristics of thyroid carcinoma which have such a high risk for invasion and metastasis. Only a few studies are available about the expression and activation of MMP-2 and MMP-9 in normal and malignant thyroid tissues. The first report by Campo et al. [8], who used immunohistochemistry, found that the amount of MMP-2 correlates with the transformation to a malignant phenotype. Some studies showed that mRNA levels of MMPs and TIMPs were present mainly in normal thyrocytes and thyroid carcinoma cell lines, while others reported that mRNA levels were determined in thyroid-derived fibroblasts [4, 20, 42]. Nakamura et al. [26] reported that overexpression of proMMP-2 mRNA and MT-1MMP mRNA in the thyroid carcinoma cells but not in the stromal cells were important in the malignant behavior using in situ zymography. However, to the best of our knowledge, there have been no comprehensive studies on the protein expression using a large number of cases in thyroid carcinoma.

The aim of this study was to investigate the expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 immunoreactive proteins in thyroid papillary carcinoma and to examine their clinicopathological correlations, such as invasive propensity and metastatic potential. We examined the existence and localization of these MMPs and TIMPs at the protein level and also observed the production and activation level of these enzymes in thyroid papillary carcinoma. The results suggest that overexpression and activation of these proteinases and their inhibitors in the thyroid tumor tissue play an important role in invasive and metastatic behavior in thyroid carcinomas.

Materials and methods

Tissue specimens and clinical data

Surgically resected cases (86) of papillary thyroid carcinoma were used. After the surgical resection, the tumor specimen was immediately divided into two parts in each case. One part was fixed in neutral buffered 10% formalin and embedded in paraffin, and another part was snap frozen in liquid nitrogen and stored at –80°C until use. As a control, three non-tumor thyroid tissues were obtained from the three thyroid tumor cases.

The age of the subjects ranged from 25 years to 83 years, with an average of 54 years. The male to female ratio was 14 to 72. The histology of the thyroid carcinomas was of ordinary type in 78 (91%) cases, of follicular variant in 6 (7%) cases, of oxyphilic variant in 1 (1%) case, and of encapsuled variant in 1 (1%) case. There were no cases of tall cell variant. The number of tumors per case is one in 55 (62%) cases, two in 30 (35%) cases, and three in 3 (3%) cases. Multiple serial sections of 3-μm thickness were obtained from each specimen. Two of them were stained with hematoxylin and eosin and with elastic van Gieson for assessment of vascular invasion. The rest were subjected to immunohistochemical staining.

Immunohistochemistry

Sections were immunohistochemically stained for MMP-2, MMP-9, TIMP-1, and TIMP-2 using the standard avidin-biotin-peroxidase complex (ABC) method. In brief, the sections were processed to unmask antigens by means of microwave oven heating in 10 nm/l citric acid buffer (pH 6.0) and subsequent detergent treatment using polyoxyethylene sorbitan monolaurate (Tween 20) for 30 min. The sections were then treated with normal serum for 20 min, followed by the application (4 C overnight) of primary monoclonal antibodies against MMP-2 [clone 42–5D11, class immunoglobulin (Ig) G/κ, dilution 1:1000], MMP-9 (clone 56–2A4, class IgGl/κ, diluton 1:800), TIMP-1 (clone 147–6DI1, class IgGl/κ, dilution 1:100), or TIMP-2 (clone 67–4Hl1, class IgGl/κ, dilution 1:800; Fuji Chemicals, Toyama, Japan). The sections were then treated with biotinylated secondary antibodies (Vector Lab, Burlingame, Calif.), and then with the ABC (Vectastein ABC kit, Vector Lab) for 1 h each. The reaction products were developed in 3.3′-diaminobenzidine tetrahydrochloride solution containing 0.03% H_2O_2. Nuclei were lightly counterstained with hematoxylin. No staining was obtained when nonimmune serum or phosphate-buffered saline (PBS) was used instead of the primary antibodies.

Semiquantitative scoring of immunohistochemistry

We used a semiquantitative scoring system to evaluate the results, as previously reported [13, 14, 37]. As shown in Table 1, immunoreactivity was assessed by the percentage of positive cells and intensity of staining, each being scored from 0 to +3. The scoring was done in thyroid tumor tissue and in the surrounding non-tumor thyroid. The former was categorized as “tumor score” and the latter as “non-tumor score”. The final score was “tumor score” minus “non-tumor score”.

This scoring system was adopted because the immunoreactivities were heterogeneous in distribution and intensity in both the tumor and non-tumor region in a given case. Therefore, we evaluated, simultaneously, the percentage and intensity of immunoreactivities in a given tumor and non-tumor region. In addition, the immunoreactivities in non-tumor regions varied from case to case; they were heterogeneous in the distribution and intensity among different cases. This may be because all samples did not have equal status of antigenic expression, probably due to different fixation time and/or different prefixation intervals. Therefore, we examined the percentage and intensity of immunoreactivities in the non-tumor regions, and used them in non-tumor regions as internal controls, so that we utilized the final score (tumor score minus

Table 1 Semiquantitative scoring of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) used in the present study

Extent score	Intensity score
1. Evaluation of immunohistochemistry	
0: 0%	0: no immunoreaction
+1: 1–33% positive	+1: mild immunoreaction
+2: 34–66% positive	+2: moderate immunoreaction
+3: 67–100% positive	+3: severe immunoreaction
2. Tumor score = extent score + intensity score in tumor	
Non-tumor score = extent score + intensity score in non-tumor	
3. Final score = tumor score–non-tumor score	

non-tumor score) as described above in order to avoid these artifacts. We believe that this scoring system reflects more exactly the antigenic expression than do other simple methods. In fact, we used this scoring system in previous papers [13, 14, 37].

Gelatin zymography for MMP-2 and MMP-9

The proenzyme and activated forms of MMP-2 and MMP-9 were detected using gelatin zymography in 15 fresh samples of papillary carcinoma and three controls. Tissue samples were cut into small pieces, homogenized at 4 C with a homogenizer in Tris buffer (50 mM tris HCl, pH 7.5, 75 mM NaCl), and centifuged at 5000×*g* for 20 min. The supernatant was aliquoted, and the protein content was determined using the method of Bradford [6]; Bio-Rad reagents (Bio-Rad Laboratories, Hercules, Calif.) were used, and bovine serum albumin was used as the standard. Tissue extracts (containing 50 µg protein) were electrophoresed on 7% sodium dodecyl sulfate (SDS)-polyacrylamide gels containing gelatin (2 mg/ml) (DIFCO Lab., Detroit, Mich.) as described previously [29]. After electrophoresis, the gels were rinsed twice in 2.5% Triton X-100 and incubated at 37 C for 20 h in 0.2 M NaCl, 5 mM $CaCl_2$, and 50 mM Tris-HCl buffer (pH 7.6) containing 0.02% Briji-35 (Sigma Chemical Co., St.Louis, Mo.). The gels were stained with 0.25% Coomassie blue and destained in 25% methanol and 10% acetic acid in H_2O.

Western blotting

The 15 tumor samples and three control samples were used. Protein loads of 20 µg were applied onto a 10% polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane (Amersham, San Diego, Calif.). The membrane was blocked with blocking solution (2.5% nonfat milk in PBS) for 20 min at room temperature and then incubated with 5 µg/ml of antibody in the blocking solution. The antibodies to MMPs were the same as those used in the immunohistochemistry. After extensive washing with T-PBS (0.05% Tween20-PBS), the membrane was reprobed with antimouse IgG (class IgG/Fab′, dilution 1:1000, MBL, Nagoya, Japan) conjugated with horseradish peroxidase in blocking solution for 2 h. Detection of signals was performed using an enhanced chemiluminescent technique using an ECL kit (Amersham, Bucks, UK).

Correlations between immunohistological scores and clinicopathological parameters

Immunohistochemical results were correlated with tumor size, lymph node metastasis, distant metastasis, clinical stages, intrathyroidal invasion, vascular invasion, and presence of satellite tumors. The tumor size was categorized into four groups according to the pTNM system [32]. Clinical stage was determined with the use of the International Union Against Cancer (UICC) system [32]. Intrathyroidal invasion was subjectively classified into none, mild, moderate, and severe, depending on the morphologic invasive features. Vascular invasion means both lymphatic and venous invasion and was subjectively categorized as none, mild, moderate, and severe, according to the density of the vascular lumina-containing tumor cells. Satellite tumors imply daughter tumors remote from main tumors and are considered to be due to lymphatic spreads.

Statistical analysis

Scores were expressed as mean±SD. Spearman rank correlation test, analysis of variance (ANOVA) test, and Mann-Whitney's U test were employed with a significant level of $P<0.05$.

Results

Immunohistochemistry

In the tumor regions, the number of positive immunoreactivities (tumor score >0) was 81 of 86 (94%), 71 of 86 (83%), 81 of 86 (94%), and 81 of 86 (94%) cases for MMP-2, MMP-9, TIMP-1, and TIMP-2, respectively (Table 2). MMP-2 (Fig. 1a, b), MMP-9 (Fig. 1c), TIMP-1 (Fig. 1d), and TIMP-2 (Fig. 1e) were expressed in the tumor cell cytoplasm with a homogeneous or granular pattern. Endothelial cells and fibroblasts in the tumor stroma were occasionally positive for the four proteins, although the stromal expressions were much lower than those in the tumor cells.

In the non-tumor regions, the number of positive immunoreactivities (non-tumor score >0) was 78 of 86 (91%), 53 of 86 (42%), 75 of 86 (87%), and 78 of 86 (91%) cases for MMP-2, MMP-9, TIMP-1, and TIMP-2, respectively (Table 2). The four proteins were expressed mainly in the follicular cells and to a much lesser extent in the fibroblasts and endothelial cells in the stroma. The expressions in the tumor regions tended to be higher than in non-tumor regions (Fig. 1a and Table 2). That is, the final scores were 0 or more in 64 of 86 (74%), 80 of 86 (93%), 79 of 86 (92%), and 64 of 86 (74%) cases for MMP-2, MMP-9, TIMP-1, and TIMP-2, respectively (Table 2).

Gelatin zymography

The MMP-2 active form, proMMP-2, MMP-9 active form, and proMMP-9 showed four major and clear proteolytic bands at 62 kDa, 72 kDa, 82 kDa, and 92 kDa, respectively, in the gelatin gels from the tissues homogenates from tumor tissues, and two unclear bands from the

Table 2 The number (%) of cases. *MMP* matrix metalloproteinase; *TIMP* tissue inhibitors of metalloproteinase

Scores	MMP-2	MMP-9	TIMP-1	TIMP-2
Tumor score				
0	5 (6%)	15 (17%)	5 (6%)	5 (6%)
1–2	22 (26%)	22 (26%)	13 (15%)	14 (16%)
3–4	27 (31%)	22 (26%)	30 (35%)	39 (45%)
5–6	32 (37%)	27 (31%)	38 (44%)	28 (33%)
Non tumor score				
0	8 (9%)	33 (38%)	11 (13%)	8 (9%)
1–2	28 (33%)	42 (49%)	50 (58%)	29 (34%)
3–4	30 (35%)	9 (10%)	18 (21%)	33 (38%)
5–6	20 (23%)	2 (2%)	7 (8%)	16 (19%)
Final score				
<0	22 (26%)	6 (7%)	7 (8%)	22 (26%)
0	24 (28%)	21 (24%)	16 (19%)	17 (20%)
1–2	28 (33%)	28 (33%)	33 (38%)	32 (37%)
3–4	11 (13%)	27 (31%)	28 (33%)	13 (15%)
5–6	1 (1%)	4 (47%)	2 (2%)	2 (2%)

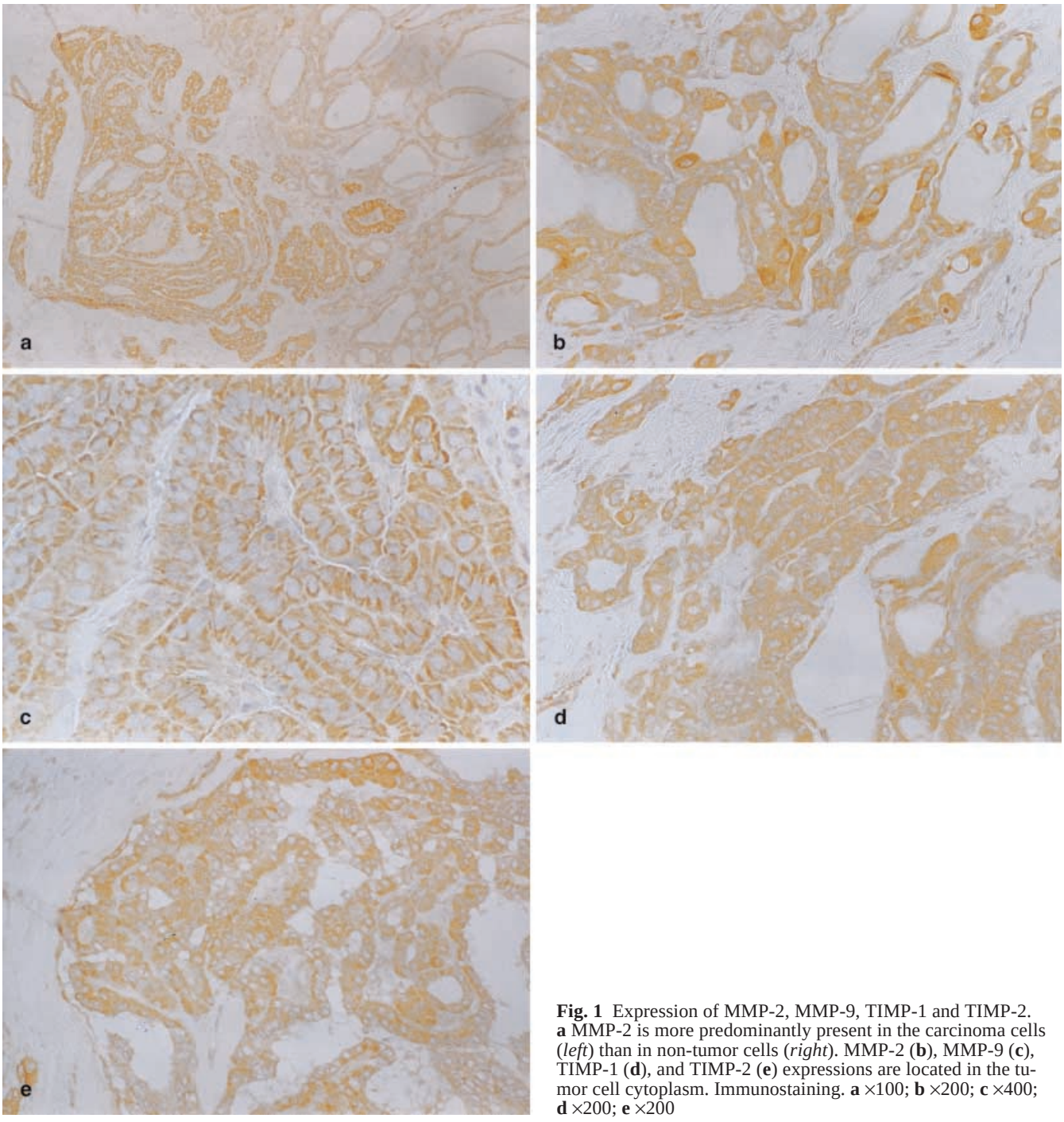

Fig. 1 Expression of MMP-2, MMP-9, TIMP-1 and TIMP-2. **a** MMP-2 is more predominantly present in the carcinoma cells (*left*) than in non-tumor cells (*right*). MMP-2 (**b**), MMP-9 (**c**), TIMP-1 (**d**), and TIMP-2 (**e**) expressions are located in the tumor cell cytoplasm. Immunostaining. **a** ×100; **b** ×200; **c** ×400; **d** ×200; **e** ×200

tissue homogenates from non-tumor tissues (Fig. 2). The number of samples with enzymatic active bands for MMP-2 and MMP-9 was 15 of 15 (100%) in tumor tissues and 2 of 3 (66%) in non-tumor tissues. Moreover, the activity, which was judged from the size of band area, was significantly higher in the tumor tissues than in the non-tumor tissues ($P<0.05$), and the cases with strong enzymatic activities of MMP-2 and MMP-9 tended to show high immunohistochemical scores (data not shown).

Western blotting

Single bands were found at 72 kDa for MMP-2 and at 92 kDa for MMP-9 in each sample (Fig. 3a, b). Although MMP-2 and MMP-9 were demonstrated in both tumor and non-tumor extracts, there was a tendency for the bands to be larger in tumor extracts than in non-tumor extracts. The cases positive for MMP-2 and MMP-9 with western blotting were also positive for them when using immunohistochemistry.

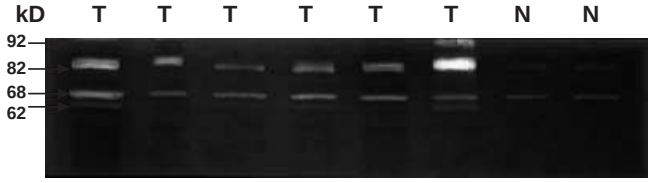

Fig. 2 Enzymic activity analysis of MMP-2 and MMP-9. Sample of the homogenates from tumor tissues (*T*) and non-tumor tissues (*N*) were subjected to gelatin zymography. MMP-2 active form, proMMP-2, MMP-9 active form, and proMMP-9 show four major clear proteolytic bands at 62 kDa, 68 kDa, 82 kDa and 92 kDa, respectively, in the gelatin gels corresponding to the molecular weight of MMP-2 and MMP-9. Low levels of both MMP-2 and MMP-9 activity are found in the non-tumor tissues specimens

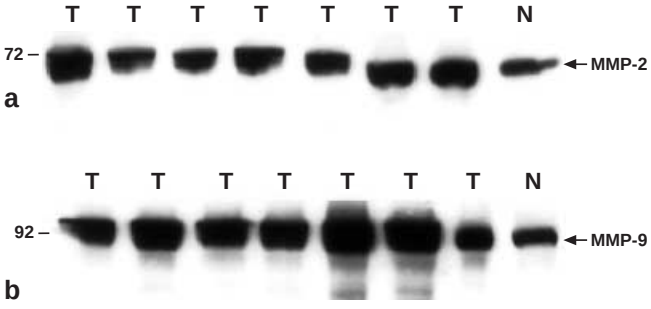

Fig. 3 In every tumor sample, there was a band of MMP-2 (**a**), MMP-9 (**b**). Sample of the homogenates from tumor tissues (*T*) and non-tumor tissues (*N*) were subjected to western blotting. The amount of MMP-2 (**a**) and MMP-9 (**b**) is rather high in *T* and rather low in *N*. Immunohistochemistry of the same cases showed expression of these immunoreactive proteins in tumor tissues and non-tumor tissue

Correlations between imunohistochemical scores and clinicopathological parameters

The results for the correlation between MMP-2, MMP-9, TIMP-1, and TIMP-2 scores and clinicopathological data are shown in Table 3. The MMP-2 high score significantly correlated with large tumor size, presence of lymph node metastasis, high clinical stage, high intrathyroidal invasion, and high vascular invasion. MMP-9 high score significantly correlated with the presence of lymph node metastasis and high intrathyroidal invasion. TIMP-1 and TIMP-2 high scores significantly correlated with large tumor size, high clinical stage, high intrathyroidal invasion, and high vascular invasion.

Discussion

The present study is the second report of the protein expression of MMP and TIMP in thyroid carcinoma. The first report was that of Campo et al. [8], who demonstrated that MMP-2 protein expression is associated with tumor invasion and metastasis in thyroid carcinoma. In the present study, immunolocalization of MMP-2, MMP-9, TIMP-1, and TIMP-2 was found largely in the tumor cells and much less frequently in the fibroblasts and endothelial cells in the tumor stroma. There has been controversy about the immunolocalization of TIMPs in non-thyroid carcinomas. Some reports suggested that tumor cells were the main source of MMPs and TIMPs, while others stressed that MMPs and TIMPs were localized in mesenchymal cells in the tumor stroma [19, 21, 30, 36, 38]. The present study suggests that, in thyroid papillary carcinomas, the main source of MMP-2, MMP-9, TIMP-1, and TIMP-2 is the tumor cells rather than stromal mesenchymal cells. Gelatin zymography and western blotting in this study also support these immunohistochemical findings.

The present study showed that follicular cells and stromal cells in the non-tumor thyroid are positive for MMP-2, MMP-9, TIMP-1, and TIMP-2. However, their expressions were much stronger in follicular cells than in stromal cells. These findings suggest that follicular cells in non-tumor thyroid contain MMP-2, MMP-9, TIMP-1, and TIMP-2 under normal conditions. In the present study, the immunoreactivities of MMP-2, MMP-9, TIMP-1, and TIMP-2 were higher in tumor cells than in follicular cells in non-tumor regions in a majority of cases, suggesting that follicular cells produce or accumulate much more MMP-2, MMP-9, TIMP-1, and TIMP-2 during the carcinogenesis and progression of papillary thyroid carcinomas. The findings that MMPs and TIMPs correlated with large tumor size and invasiveness may support this suggestion.

MMP-2 and MMP-9 are secreted from cells as latent zymogen forms, and their activities in the extracellular environment are controlled by various activators and TIMPs [2]. In carcinoma tissues, it has been suggested that the disruption of the balance between MMPs and TIMPs may be a factor in the progression of tumors to more malignant phenotypes [2, 11, 17, 30, 33]. TIMP-1 preferentially affects MMP-9, and TIMP-2 affects MMP-2. Moreover, MMP-2 activation involves interactions among proMMP-2, MT1-MMP, and TIMP-2, and MMP-2 is activated by forming a complex of them [9, 26]. In the present study, gelatin zymography of tumor tissues showed four distinct bands of proMMP-2, proMMP-9 and active forms of MMP-2 and MMP-9, suggesting that pro-MMP converts to active forms in thyroid papillary carcinomas. The present study did not investigate the molecular mechanism of MMP, TIMP, and MT-MMP. Further study on the molecular pathology remains to be clarified in thyroid tumors.

There have been many studies on the role played by MMP and TIMP [3, 12, 22, 23, 30, 34, 35]. These studies have shown that high expression of MMP is associated with high tumor invasion and metastasis and shortened survival in non-thyroidal carcinomas [7, 10, 16, 18, 21, 28, 31, 40]. Our findings that high expression of MMP-2 and MMP-9 in tumor cells was associated with large tumor size, high stage, high intrathyroidal invasion, capsular invasion, high vascular invasion, and higher lymph node metastasis, may indicate that MMP-2 and MMP-9 play an important role in tumor invasion and metastasis in papillary thyroid carcinomas.

Table 3 Correlations between MMP-2, MMP-9, TIMP-1 and TIMP-2 scores and clinicopathological data. *NS* not significant; *UICC* International Union Against Cancer

	n	MMP-2 score	*P* value	MMP-9 score	*P* value	TIMP-1 score	*P* value	TIMP-2 score	*P* value
Tumor size									
T1 (1 cm)	10 (12%)	–1.20±0.71		0.50±0.70		0.10±0.66		–0.80±0.63	
T2 (1 cm<<4 cm)	57 (66%)	0.58±0.23	*P*=0.006[a]	1.88±0.23	NS	1.77±0.20	*P*=0.008[a]	0.67±0.2	*P*=0.007[a]
T3 (4 cm)	2 (2%)	2.00±0.00		2.50±0.50		2.50±1.50		1.00±1.00	
T4 (beyond the capsule)	17 (20%)	1.059±0.41		1.59±0.44		2.47±0.42		1.65±0.58	
Lymph node metastasis									
Absent	30 (35%)	–0.10-1.97		1.00±1.76		1.27±1.95		0.23±2.05	
Present	56 (65%)	0.82±1.81	*P*=0.033[c]	2.04±1.80	*P*=0.014[c]	1.98±1.60	NS	0.95±2.05	NS
Distant metastasis									
Absent	85 (99%)	0.51±0.21		1.68±0.20		1.73±0.19		0.67±0.224	
Present	1(1%)	0.00±0.00	NS	1.00±0.00	NS	2.00±0.00	NS	2.00±0.00	NS
UICC stage									
I	31 (36%)	–0.31 (36%)		0.13±0.31	1.23±0.34	1.29±0.31		–0.10±0.32	
II	16 (19%)	–0.06±0.53		1.69±0.43	1.31±0.51	0.25±0.55			
III	38 (44%)	1.26±0.28	*P*=0.002[a]	2.05±0.29	NS	2.26±0.25	*P*=0.043[a]	1.47±0.32	P<0.001[a]
IV	1 (1%)	0.00±0.00		1.00±0.00		2.00±0.00		2.00±0.00	
Intrathyroidal invasion									
None	23 (27%)	–0.65±1.58		0.87±1.18		0.48±1.81		–0.39±1.70	
Mild	15 (17%)	0.53±2.10		1.07±1.79		1.67±1.50		0.80±1.86	
Moderate	38 (44%)	0.87±1.77	*P*=0.002[a]	2.24±1.92	*P*=0.011[a]	2.13±1.38	*P*<0.001[b]	0.82±2.03	*P*=0.001[b]
Severe	10 (12%)	1.70±1.70		2.30±2.11		3.20±1.62		2.60±1.95	
Vascular invasion									
None	23 (27%)	–0.52±1.37		0.87±1.63		0.61±1.43		–0.30±1.43	
Mild	21 (24%)	0.24±2.59		1.76±2.10		1.05±1.66		0.24±2.36	
Moderate	28 (33%)	0.89±1.34	*P*=0.002[c]	2.18±1.61	NS	2.39±1.37	*P*<0.001[b]	1.07±1.78	*P*=0.001[b]
Severe	14 (16%)	1.79±1.58		1.86±1.96		3.29±1.38		2.29±2.02	
Satellite tumors									
Absent	53 (62%)	0.30±2.05		1.42±1.66		1.59±1.92		0.59±2.17	
Present	33 (38%)	0.81±1.63	NS	2.09±2.07	NS	1.97±1.45	NS	0.88±1.90	NS

[a] Spearman rank correlation test
[b] Analysis of variance (ANOVA test)
[c] Mann-Whitney's U test

The present study is the first report of the protein expression of TIMP in thyroid carcinomas. The present study demonstrated that TIMP-1 and TIMP-2 were overexpressed in tumor cells relative to non-tumor thyroid and that the expressions of TIMP-1 and TIMP2 were associated with high tumor size and high invasiveness. In other carcinomas, TIMPs were previously reported to be associated with low invasiveness and low metastatic potential [1, 25]. However, recent studies have suggested that TIMPs and active MMPs are associated with the malignant potential of cancers [2, 19]. Our findings also suggest that high expression of TIMP-1 and TIMP-2 is associated with high malignant potential in papillary thyroid carcinomas.

Finally, the present study is a kind of immunohistochemical study. However, the present study also employed the gelatin zymography and western blot analysis, both of which showed increased activity and amount of MMP-2 and MMP-9 in tumors relative to non-tumor thyroid. Therefore, the immunohistochemical data of the present study seem to be valid and strengthened by these techniques. In summary, the present study of papillary thyroid carcinoma suggests that MMP-2, MMP-9, TIMP-1, and TIMP-2 are mainly produced in tumor cells, that these proteins are increased during carcinogenesis, and that these four proteins are associated with high malignant potential.

References

1. Albini A, Melchiori A, Santi L, Liotta LA, Brown PD, Stetler-Stevenson WG (1991) Tumor cell invasion inhibited by TIMP-2. J Natl Cancer Inst 83:775–779
2. Anina EY, Robert E, Hewitt RE, Kleiner DE, Stetler-Stevenson WG (1996) Molecular regulation of cellular invasion-role of gelatinase A and TIMP-2. Biochem Cell Biol 74:823–831
3. Apte SS, Olsen BR, Murphy G (1995) The gene structure of tissue inhibitor of metalloproteinases (TIMP)-3 and its inhibitory activities define the distinct TIMP gene family. J Biol Chem 270:14313–14318
4. Aust G, Hofmann A, Laue S, Rost AK, Köhler T, Scherbaum WA (1997) Human thyroid carcinoma cell lines and normal thyrocytes: expression and regulation of matrix metalloproteinase-1 and tissue matrix metalloproteinase inhibitor-1 messenger-RNA and protein. Thyroid 7:713–724
5. Azzam HS, Arand G, Lippman ME, Thompson WE (1993) Association of MMP-2 activation potential with metastatic progression in human breast cancer cell lines independent of MMP-2 production. J Natl Cancer Inst 85:1758–1764
6. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
7. Brown PD, Bloxidge RE, Stuart NS, Gatter KC, Carmichael J (1993) Association between expression of activated 72-kilodalton gelatinase and tumor spread in non-small-cell lung carcinoma. J Natl Cancer Inst 85:574–578
8. Campo E, Maria J, Merino J, Liotta L, Neumann R, Stetler-Stevenson WG(1992) Distribution of the 72-kD type IV collagenase in nonneoplastic and neoplastic thyroid tissue. Hum Pathol 23:1395–1401
9. Corcoran ML, Hewitt RE, Kleiner DE, Stetler-Stevenson WG (1996) MMP-2: expression, activation and inhibition. Enzyme Protein 49:7–19
10. Davies B, Wasan H, Abel P, Williams G, Krausz T, Neal D, Thomas D, Hanby A, Balkwill F (1993) Levels of matrix metalloproteinases in bladder cancer correlate with tumor grade and invasion. Cancer Res 53:5365–5369
11. Davies B, Miles DW, Happerfield LC, Naylor MS, Babrow LG, Rubens RD, Balkwill FR(1993) Activity of type IV collagenases in benign and malignant breast disease. Br J Cancer 67:1126–1131
12. Declerck YA, Perez N, Shimada H, Bonne TC, Langley KE, Taylor SM (1992) Inhibition of invasion and metastasis in cell transfected with an inhibitor of metalloproteinases. Cancer Res 52:701–708
13. Endo K, Terada T (1999) Protein expression of CD44 (standard and variant isoforms) in hepatocellular carcinoma: relationship with tumor grade, clinocopathologic parameters, p53 expression, and patient's survival. J Hepatol 32:78–84
14. Endo K, Ueda T, Ueyama J, Ohta T, Terada T (1999) Immunoreactive E-cadherin, alpha-catenin, beta-catenin and gammma-catenin proteins in hepatocellular carcinoma: relationships with tumor grade, clinicopathologic parameters, and patient's survival. Hum Pathol 31:558–565
15. Friedman R, Toth M, Pena D, Mobashery S (1995) Activation of progelatinase B (MMP-9) by gelatinase A(MMP-2). Cancer Res 55:2548–2555
16. Gohji K, Fujimnoto N, Fujii A, Komiyama T, Okawa J, Nakajima M (1996) Prognostic significance of circulating matrix metalloproteinase-2 to tissue inhibitor of metalloproteinase-2 ratio in recurrence of urotherial cancer after complete resection. Cancer Res 56:3196–3198
17. Gress TM, Müller-Pillasch F, Lerch MM, Friess H, Büchler M, Adler G (1995) Expression and in-situ localization of genes coding for extracellular matrix degrading proteases in pancreatic cancer. Int J Cancer 62:407–413
18. Grigioni WF, D'Errico A, Fortunato C, Fiorentino M, Mancini AM, Stetler-Stenvenson WG, Sobel ME, Liotta LA, Onisto M, Garbisa S (1994) Prognosis of gastric carcinoma revealed by interactions between tumor cells and basement membrane. Mod Pathol 7:220–225
19. Grignon DJ, Sakr W, Toth M, Ravery V, Angulo J, Shamsa F, Pontes JE, Crissman JC, Fridman R (1996) High levels of tissue inhibitor of metalloproteinase-2 (TIMP-2) expression are associated with poor outcome in invasive bladder cancer. Cancer Res 56:1654–1659
20. Hofmann A, Laue S, Rost A, Scherbaum WA, Aust G (1998) mRNA levels of membrane type 1 matrix metallopoteinase (MT1-MMP), MMP-2 and MMP-9 and of their inhibitors TIMP-2 and TIMP-3 in normal thyrocytes and thyroid carcinoma cell lines. Thyroid 8:203–214
21. Höyhtyä M, Fridman R, Komarek D, Porter-Jordan K, Stetler-Stevenson WG, Liotta LA, Liang CM (1994) Immunohistochemical localization of matrix metalloproteinase 2 and its specific inhibitor TIMP-2 in neoplastic tissues with monoclonal antibodies. Int J Cancer 56:500–505
22. Liotta LA (1984) Tumor invasion and metastasis – role of the basement membrane. Am J Pathol 117:339–348
23. Liotta LA, Stevenson WG (1990) Metalloproteinases and cancer invasion. Semin Cancer Biol 1:99–106
24. Matrisian LM (1992) The matrix-degrading metalloproteinases. Bio Essays 14:455–463
25. Montgomery AM, Mueller BM, Reisfeld RA, Taylor SM, DeClerck YA (1994) Effect of tissue inhibitor of metalloproteinase-2 expression on the growth and spontaneous metastasis of a human melanoma cell line. Cancer Res 54:5467–5473
26. Nakamura H, Ueno H, Yamashita K, Shimada T, Yamamoto E, Noguchi M, Fujimoto N, Sato H, Seiki M, Okada Y (1993) Enhanced production and activation of progelatinase A mediated by membrane type 1 matrix metallopreoteinase in human papillary thyroid carcinomas. Cancer Res 59:467–473
27. Nomura H, Fujimoto N, Seiki M, Mai M, Okada Y (1996) Enhanced production of matrix metalloproteinases and activation of matrix metalloproteinase 2 (gelatinase A) in human gastric carcinomas. Int J Cancer 69:9–16

28. Nuovo GJ, Mac Connell PB, Simsir A, Valea F, French DL (1995) Correlation of the in situ detection of polymerase chain reaction-amplified metalloproteinase complementary DNAs and their inhibitors with prognosis in cervical carcinoma. Cancer Res 55:267–275
29. Rao JMP, Steck PA, Mohanam S, Stetler-Stevenson WG, Liotta LA, Sawaya R (1993) Elevated levels of Mr92,000 type IV collagenase in human brain tumors. Cancer Res 53:2208–2211
30. Ring P, Johansson K, Höyhtyä M, Rubin K, Lindmark G (1997) Expression of tissue inhibitor of metalloproteinases TIMP-2 in human colorectal cancer – a predictor of tumor stage. Br J Cancer 76:805–811
31. Sier C, Kubben F, Ganesh S, Heerding MM, Griffioen G, Hanemaaijer R, Krieken J, Lamers C, Verspaget H (1996) Tissue levels of matrix metalloproteinases MMP-2 and MMP-9 are related to overall survival of patients with gastric carcinoma. Br J Cancer 74:413–417
32. Sobin LH, Wittekind CH (1997) TNM classification of malignant tumours, 5th edn. International Union Against Cancer. Wiley, New York, pp 47–50
33. Stetler-Stevenson WG, Krutzsch HC, Wachner MP, Marguiles IMK, Liotta LA (1989) The activation of human type IV collagenase proenzyme. Sequence identification of the major conversion product following organomercurial activation. J Biol Chem 264:1353–1356
34. Stetler-Stevenson WG, Krutzsch HC, Liotta LA (1989) Tissue inhibitor of metalloproteinase (TIMP-2). A new member of the metalloproteinase inhibitor family. J Biol Chem 264:17347–17378
35. Strongin AY, Marmer BL, Grant GA, Goldberg GI (1993) Plasma membrane-dependent activation of the 72 kDa type IV collagenase is prevented by complex formation with TIMP-2. J Biol Chem 268:14033–14039
36. Talvensaari-Mattila A, Pääkkö P, Höyhtyä M, Blanco-Sequeiros G, Turpeenniemi-Hujanen T (1998) Matrix metalloproteinase-2 immunoreactive protein – a marker of aggressiveness in breast carcinoma. Cancer 83:1153–1162
37. Terada T, Ashida K, Kitamura Y, Matsunaga Y, Takashima K, Kato M, Ohta T (1998) Expression of epithelial-cadherin, alpha-catenin and beta-catenin during human intrahepatic bile duct development: a possible role in bile duct morphogenesis. J Hepatol 28:263–269
38. Tryggvarson K, Hoyhtya M, Pyke C (1993) Type IV collagenases in invasive tumors. Breast Cancer Res Treat 24:209–218
39. Ueno H, Nakamura H, Inoue M, Imai K, Noguchi M, Sato H, Seiki M, Okada Y (1997) Expression and tissue localization of membrane-type 1, 2, and 3 matrix metalloproteinase in human invasive breast carcinomas. Cancer Res 57:2055–2060
40. Urbanski SJ, Edwards DR, Maitland A, Leco KJ, Watson A, Kossakowska AE (1992) Expression of metalloproteinases and their inhibitors in primary pulmonary carcinomas. Br J Cancer 66:1188–1194
41. Will H, Atkinson SJ, Butler GS, Smith B, Murphy G (1996) The soluble catalytic domain of membrane type 1 matrix metalloproteinase cleaves the propeptide of progelatinase A and initiates autoproteolytic activation. Regulation by TIMP-2 and TIMP-3. J Biol Chem 271:17119–17213
42. Zedenius J, Ståhle-BäckDahl M, Enberg U, Grimelius L, Larsson C, Wallin G, BäckDahl M (1996) Stromal fibroblasts adjacent to invasive thyroid tumors: expression of gelatinase A but not stromelysin 3 mRNA. World J Surg 20:101–106