Increased Expression of Matrix Metalloproteinase-2: A Diagnostic Marker but Not Prognostic Marker of Papillary Thyroid Carcinoma

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Abstract

Background: Matrix metalloproteinases are proteolytic enzymes that degrade extracellular matrix components. Numerous studies have demonstrated that individual MMPs play a crucial role in tumor invasion and metastasis.

Objective: To examine the expression of MMPs and their inhibitor TIMP-2 in neoplastic and normal thyroid tissues.

Methods: We examined 33 cases of thyroid tumor (papillary, follicular and medullary carcinoma, follicular adenoma and multinodular goiter). MMP protein content and activity were measured by enzyme-linked immunosorbent assay and gel zymography. Immunohistochemistry was also performed.

Results: The thyroid tissues examined secreted MMP-2 and 9 as well as TIMP-2, but only MMP-2 was significantly higher in papillary carcinoma cases compared to the adjacent normal tissue or to the other tumor entities. Increased MMP-2 immunohistochemical staining was demonstrated in the neoplastic papillary epithelial component. No significant difference was seen between papillary carcinomas with lymph node metastases and those without.

Conclusions: Increased MMP-2 expression may be useful as a diagnostic marker to differentiate papillary carcinoma from other thyroid neoplasms, but it cannot serve as a useful prognostic marker.

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Tumor invasion is a cascade of sequential events that involves detachment of malignant cells from their site of origin and invasion through the surrounding stroma into lymphovascular channels. All these steps are associated with extracellular matrix degradation, and the proteolytic breakdown of major ECM components requires specific proteases. Matrix metalloproteinases are proteolytic enzymes that degrade ECM components at neutral pH [1, 2]. Numerous studies have demonstrated that individual MMPs play crucial roles in tumor invasion and metastasis [reviewed in Refs. 3–5]. Expression of MMP-2 (72 kd type IV collagenase or gelatinase A) is considered especially important as an indicator of tumor aggressiveness in a variety of neoplasms [6,7].

Few studies have examined the association between MMP activity and thyroid neoplasia in clinical samples [7–11] or *in vitro* cell lines [12–14]. MMP activity was found to be greater in thyroid carcinoma cell lines with highly metastatic potential as compared to less aggressive cell lines [7]. Immunohistochemical studies have demonstrated that neoplastic epithelium from thyroid carcinoma tissue expresses higher levels of MMP-2 protein than thyroid epithelium from non-neoplastic tissue [8,10,11].

In this study, we examined the expression of MMPs and the tissue inhibitor of metalloproteinase-2 (TIMP-2) in a variety of neoplastic and non-neoplastic thyroid tissues by gel zymography and ELISA and correlated these findings with immunohistochemistry.

Materials and Methods

Clinical samples and histology

Fresh tissue samples were obtained at thyroidectomy from 33 patients: 12 patients with papillary carcinoma, 7 with multinodular goiter, 6 with follicular adenoma, and 4 each with follicular carcinoma and medullary carcinoma. Five of the patients with papillary carcinoma had lymph node metastases at presentation. Surgical specimens were fixed in 10% buffered formalin and paraffin sections were stained with hematoxylin and eosin. The specimens were evaluated according to the WHO Classification of Thyroid Tumors criteria [15].

Tissue sampling and homogenization

Samples of tumor and normal thyroid tissue taken from an adjacent site (approximately 0.1 g each) were rapidly frozen and stored in liquid nitrogen until homogenization. The samples were homogenized in 300 l of PBS followed by centrifugation at 3,000 rpm for 5 minutes. The supernatant was separated and the protein concentration determined by the Bradford reagent (Bio-Rad Hercules, California, USA). The samples were used both for zymography and ELISA experiments.

MMPs = matrix metalloproteinases
ECM = extracellular matrix

ELISA = enzyme-linked immunosorbent assay
PBS = phosphate-buffered saline

ELISA

The concentration of MMP-2 and tissue inhibitor of metalloproteinase-2 in the tissue homogenates was measured using human MMP-2 and TIMP-2 ELISA kits (Amersham Life Sciences, Braun Schweig, Germany). Values are expressed as ng/mg protein.

Gelatin zymography

Essentially, the same method as that described by Kleiner et al. was used [16]. Samples for analysis (50 μg protein from each stored supernatant sample) were prepared by dilution into a buffer (4x) consisting of 0.4M Tris (pH 6.8), 5% SDS, 20% glycerol and 0.02% bromophenol blue. The samples were applied onto an 8% polyacrylamide gel (PAGE) containing 0.5% gelatin. After 90 minutes of electrophoresis, the gel was incubated for 30 min at room temperature in 30 ml of 2.5% Triton X-100 in a rotary shaker. The Triton X-100 solution was decanted, replaced with 30 ml of enzyme buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij 35) and incubated again for 30 min at room temperature in a rotary shaker. The solution was decanted, replaced with fresh enzyme buffer and incubated overnight at 37°C. The gel was then stained with 0.5% Coomassie Blue G in 30% methanol and 10% acetic acid for 10 min at room temperature in a rotary shaker, then washed with water until clear bands were visualized. Finally, the gel was incubated for 30 min in 45% methanol 5% glycerol prior to drying overnight between sheets of cellophane. Areas of proteolytic activity were visualized by the absence of staining. Each sample was run twice. The bands were quantitated by densitometric analysis of the zymograms using the Bio Imaging gel documentation system (Dinco & Renium, Jerusalem, Israel) and TINA software (Raytest, Staubenhardt, Germany).

Cell culture

The following thyroid carcinoma cell lines were kindly provided by Dr. G. Juillard (University of California, Los Angeles): MRO-87-1 (follicular), ARO-81-1 (anaplastic), and NPA (papillary). For each experiment, cells were plated onto 24-well plates in RPMI-1640 medium and 10% fetal calf serum at 37°C in 5% CO_2. The following day, the medium was replaced with serum-free medium (RPMI-1640 and 0.3% bovine serum albumin) and the cells were cultured for an additional 2 days. After incubation, the cells were washed and fresh serum-free medium added. After 24 hours the medium was collected and stored at -70°C. The supernatants were examined by gel zymography.

Immunohistochemistry

Representative sections from formalin-fixed paraffin-embedded tissue blocks containing tumor and normal tissue were cut at 5 μ, deparaffinized and dehydrated with xylene and graded alcohol. Sections were blocked with 10% goat serum for 30 min and then incubated with monoclonal antibodies toward MMP-2 (clone 42-5D11, Oncogene Research Products, Cambridge, MA, USA) at a concentration of 1/10 for 90 min at 37°C. The labeled streptavidin-biotin-alkaline phosphatase method using the Histostain-Plus kit and AP red substrate from Zymed Laboratories (South San Francisco, CA) was employed. Positive controls consisted of sections from a case of invasive breast carcinoma. Negative control slides did not exhibit staining when the primary antibody was replaced by a matched isotype mouse monoclonal antibody to a non-related antigen.

The degree of immunoreactivity was assessed similarly to the system described by Campo et al. [8]. The intensity of the staining was graded as 1+, 2+ or 3+. In cases with variable staining intensities, the most common pattern was recorded. The extent of staining was considered as 1+ if the number of reactive cells was less than 25%, 2+ (25–75% reactive cells), and 3+ (more than 75%). The final score was obtained in each case by adding the intensity to the extent score. Immunoreactivity was then classified according to the combined score as weak (scores 1 and 2), moderate (3 and 4), or strong (5 and higher). All slides were scored independently by two observers (M.B.R. and O.Y.). Six cases with discordant results were reevaluated to obtain agreement.

Statistical analysis

Statistical analysis of the data was performed using the Student *t*-test. A *P* value of less than 0.05 was considered significant.

Results

Gelatin zymography

Gel zymography of thyroid tissue homogenates (tumor and normal tissue) revealed two main bands: around 92 kd, corresponding to MMP-9, and a band around 72 kd, corresponding to MMP-2 [Figure 1 shows a representative experiment]. The MMP activity of all samples examined was determined as the ratio between each band in the tumor sample as compared to the band in the normal adjacent tissue [Figure 2]. MMP-2 levels were significantly higher in the samples of tumor from papillary carcinoma cases as compared to the control ($P<0.001$). Furthermore, the mean MMP-2 level was significantly higher ($P<0.05$) in the papillary carcinoma subgroup compared to all other subgroups. No significant difference was seen between the mean level of MMP-2 in the five cases of papillary carcinoma with lymph node metastases compared to the six cases of papillary carcinoma with localized disease. No significant difference in MMP-2 activity was seen when comparing tissue from follicular carcinoma, adenoma, multinodular goiter or medullary carcinoma to their adjacent normal tissues [Figure 2].

Bands at 92 kd corresponding to MMP-9 were seen in all tissue homogenates [Figure 1]. However, no significant difference in MMP-9 activity was seen among any of the subgroups examined and the corresponding normal tissues, or among the specific subgroups (data not shown).

ELISA

MMP-2 was measured by ELISA in tissue homogenates from both benign and malignant thyroid tissue from 17 patients: 8 papillary carcinomas, 6 follicular adenomas and 3 multinodular goiters [Table 1]. The mean level of MMP-2 in papillary carcinomas was significantly higher ($P<0.005$) than that in the adjacent normal tissue (44.6 11.4 and 7.7 2.1 ng/mg protein, respectively) and higher than that detected in follicular adenomas or multinodular goiters (18.5 5.1 and 10.5 4.4 ng/mg protein, respectively) ($P<0.05$). No

TIMP-2 = tissue inhibitor of metalloproteinases

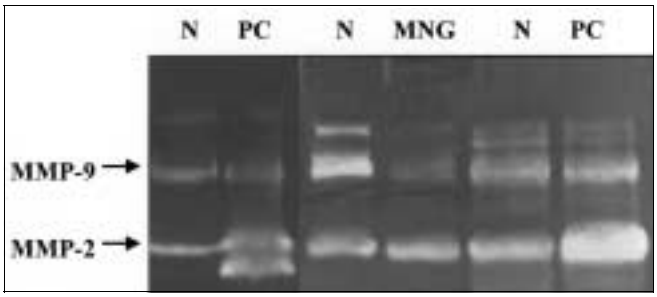

Figure 1. A representative figure of gelatin zymography. Supernatants of thyroid tissue homogenates (50 g protein/lane) from papillary carcinoma (PC), multinodular goiter (MNG), and the adjacent normal tissue (N) were subjected to gelatin zymography as described in Material and Methods.

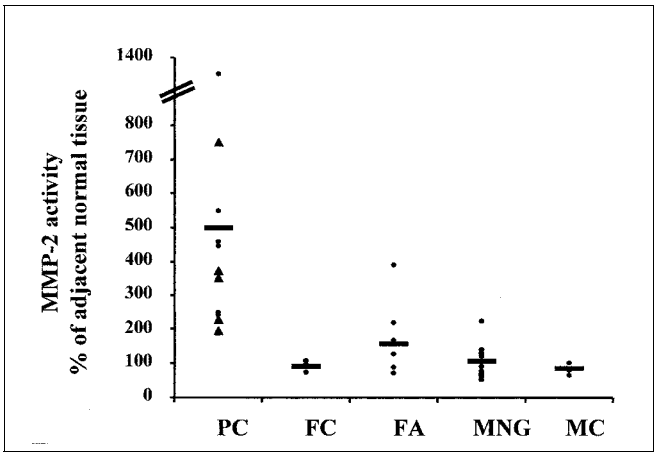

Figure 2. Densitometric analysis of gelatin zymography. On each lane 100 μg of protein was loaded. The bands were quantitated by computer densitometric analysis and were calculated as % of the adjacent normal tissue. PC = papillary carcinoma (n = 1), MNG = multinodular goiter (n = 11), FA = follicular adenoma (n = 6), FC = follicular carcinoma (n = 3), MC = medullary carcinoma (n = 3), N = normal adjacent tissue. The bars represent the mean value. Triangles represent cases with lymph node metastasis, while circles represent those without.

significant difference was seen between the mean level of MMP-2 in the five cases of papillary carcinoma with lymph node metastases compared to the seven cases of papillary carcinoma with localized disease.

In the follicular adenoma and multinodular goiter subgroups, no significant difference was seen between the level of MMP-2 in the normal and pathologic tissues [Table 1]. The mean levels of TIMP-2 did not differ significantly between the normal and pathologic tissues or between any of the specific subgroups.

We correlated the zymogram and ELISA MMP-2 data and the correlation was highly significant (r =0.73, P<0.001, n = 16).

Immunohistochemistry

As shown in Table 2, normal follicular cells from the vast majority of multinodular goiters exhibited either weak or no staining for MMP-2. In the neoplastic cases, the normal follicular epithelium adjacent to the neoplastic tissue was either negative for MMP-2 or exhibited weak/moderate staining. Positive staining was also seen in smooth muscle or blood vessels, stromal cells in fibrotic areas and at the pericapsular interface. Of all samples tested, only papillary carcinoma exhibited strong cytoplasmic MMP-2 immunostaining. The degree of MMP-2 staining of the neoplastic epithelium in cases of papillary carcinoma was strong in eight cases and moderate in four. In contrast to papillary carcinoma, the neoplastic epithelium of follicular neoplasms and medullary carcinomas exhibited either weak or moderate staining.

MMP activity in thyroid carcinoma cell lines

Supernatants from papillary, follicular and anaplastic cell lines were analyzed by gel zymography. A distinct band at 72 kd, corresponding to MMP-2, was seen in the supernatant taken from the papillary cell line and was not detectable in the other two lines (data not shown)

Table 1. MMP-2 and TIMP-2 levels (ng/mg protein) in benign and malignant thyroid tissue

	Papillary carcinoma		Follicular adenoma		Multinodular goiter	
	N	T	N	T	N	T
MMP-2	7.7 ± 2.1	44.6 ± 11.4*	11.1 ± 2.8	18.5 ± 5.1	12.3 ± 2.4	10.5 ± 4.4
TIMP-2	298 ± 36	463 ± 108	247 ± 28	292 ± 40	331 ± 39	492 ± 170

Supernatants of tissue homogenates of three cases of multinodular goiter, six cases of follicular adenoma and eight cases of papillary carcinoma were evaluated by ELISA. Results are presented as ng/mg protein of the mean ± SE.
T = tumor, N = adjacent normal tissue
* P<0.001 compared to normal tissue and other subgroups.

Table 2. Immunohistochemical staining of MMP-2 in benign and malignant thyroid tissue

	No. of cases	Negative	Weak	Moderate	Strong
Multinodular goiter	7	4	2	1	–
Follicular adenoma	6	1	4	1	–
Follicular carcinoma	4	–	3	1	–
Papillary carcinoma	12	–	–	4	8
Medullary carcinoma	4	–	2	2	–

Immunoreactivity of the benign and neoplastic thyroid epithelium was scored as described in the Materials and Methods section.

Discussion

This study demonstrates that only papillary thyroid carcinomas in contrast to normal thyroid, multinodular goiter, follicular or medullary neoplasms, express elevated levels of MMP-2. Increased MMP-2 protein levels and activity were detected in papillary carcinomas both by gel zymography and ELISA, and MMP-2 expression was immunolocalized to the tumor epithelium. Expression of MMP-9 and TIMP-2 did not differ significantly between papillary carcinomas and adjacent normal tissue or the other thyroid pathologic tissues.

Observations from other non-thyroid experimental models and clinical studies illustrate the critical role of MMP-2 in tumor progression [5]. Indeed, high levels of MMP-2 expression are associated with aggressive tumor behavior and phenotype in carcinomas of the esophagus [17], pancreas [18], prostate [19], lung [20] and ovary [21]. A recent study by Nakamura et al. [10] demonstrated that of seven MMPs tested, only MMP-2 expression levels were elevated in papillary carcinomas. In contrast to the Nakamura study, which compared MMP levels in papillary carcinomas to follicular adenomas and adjacent normal tissues, we also measured MMP-2 in four follicular and four medullary carcinomas. Surprisingly, increased expression of MMP-2 was highly specific only for papillary carcinoma. All papillary carcinomas demonstrated at least a twofold increase in MMP-2 activity by gel zymography compared to normal thyroid tissue and in contrast to other thyroid neoplasms. Lastly, of three thyroid carcinoma cell lines tested for MMP-2 activity, only the one derived from a papillary carcinoma exhibited MMP-2 expression.

Papillary carcinomas are not considered aggressive malignancies despite their invasive capacity. They are usually localized in the thyroid gland and, if they do metastasize, do not usually spread beyond the cervical lymph nodes. Thus, our finding that MMP-2 is only elevated in papillary carcinomas is intriguing considering that medullary and follicular carcinomas are both regarded as more aggressive tumors than papillary carcinomas, suggesting that MMPs, or matrix-degrading proteolytic enzymes other than MMP-2 and MMP-9, are probably involved in their progression. Furthermore, no significant differences were seen in the level of MMP-2 expression between follicular adenomas and follicular carcinomas. Thus, it appears that the measurement of MMP-2 (or MMP-9) activity is of no practical use in the often difficult differential diagnosis of follicular carcinoma from follicular adenoma neoplasms. Finally, and in contrast to other organ systems, no significant difference was seen in the degree of MMP-2 expression between papillary carcinomas with lymph node metastases and those without. Thus, MMP-2 expression cannot serve as a useful diagnostic marker, and provides further evidence that MMP-2 expression is not associated with tumor aggressiveness and suggests that other factors are active in papillary carcinoma progression. However, as MMP-2 expression was measured only in the primary tumor, the possibility exists that the level of MMP-2 expression associated with the metastatic deposit is higher than that present in the primary tumor.

In a recent study using morphometric immunohistochemistry, Maeta et al. [11] showed that increased levels of MMP-2 and MMP-9, TIMP1 and 2, correlated with large tumor size, presence of lymph node metastasis, high clinical stage, intrathyroidal invasion and vascular invasion. The correlation between MMP expression and parameters of tumor aggression described by Maeta's group differs from our findings [11]. Reasons for this difference may be technically related, for example the choice of the primary antibody, or based on a different immunohistochemical morphometric analysis scheme [11].

Similar to our results are those by Campo and associates [8], who demonstrated increased MMP-2 expression by immunohistochemistry in papillary carcinomas as compared to multinodular goiters and follicular adenomas. However, in contrast to our study, MMP-2 immuno-expression was also detected by Campo et al. [8] in medullary and follicular carcinomas. This discrepancy could be partially explained by methodology, such as choice of primary antibody and detection system. The fact that increased MMP-2 activity in follicular and medullary carcinomas was not detected by either gel zymography or ELISA in this report strengthens our observation. To the best of our knowledge, excluding the studies of Campo et al. [8], Nakamura et al. [10] and Maeta et al. [11], only one other study examined expression of MMP-2 in thyroid neoplasms [9]. Using *in situ* hybridization, Zedenius and co-workers detected increased expression of MMP-2 mRNA in invasive thyroid neoplasms [9]. In their study, and in contrast to this report and the two others above, MMP-2 expression was localized only in stromal fibroblasts and was not seen in neoplastic epithelial cells. However, our finding that the papillary carcinoma cell line expressed MMP-2 confirms that the source of MMP-2 protein in papillary carcinoma is the neoplastic epithelial cells.

No significant difference was seen in the levels of the MMP-2 inhibitor TIMP-2, as measured by ELISA, between the pathologic and normal tissue or between papillary carcinomas and benign thyroid tissue. The significance of this observation is confounded by the unclear role of TIMPs in tumor progression. TIMPs have a dual function in MMP regulation: they not only inhibit MMP activity but also play a key role in the cell surface-targeted activation of MMPs [22].

In conclusion, it seems that increased MMP-2 expression may be useful as a diagnostic marker to differentiate papillary carcinoma from other thyroid neoplasms, but it cannot serve as a useful prognostic marker.

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