Prognostic Significance of Matrix Metalloproteinase 2 and Tissue Inhibitor of Metalloproteinase 2 Expression in Prostate Cancer

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Matrix metalloproteinases (MMPs) are proteolytic enzymes capable of degrading the structural support network for normal and malignant cells, promoting neoplastic cell invasion and metastasis. Tissue inhibitors of metalloproteinases (TIMPs) maintain connective tissue integrity by modulating MMP activity. Formalin-fixed paraffin-embedded tissue sections from 138 prostatic adenocarcinomas (PACs) were immunostained by a combined automated/manual method using monoclonal antibodies against MMP2 and TIMP2. Immunoreactivity was semiquantitatively scored based on stain intensity and distribution, and results were correlated with Gleason grade, pathologic stage, ploidy status, and disease recurrence. One hundred five of 138 (76%) and 113/138 (82%) PACs expressed MMP2 and TIMP2, respectively. Co-expression was observed in 94/138 (68%) of PACs ($P = .01$), correlated with advanced tumor stage ($P = .05$), and tended to be associated with disease recurrent cases ($P = .07$). TIMP2 expression individually correlated with advanced tumor stage ($P = .04$) and reached near significance with disease recurrence ($P = .06$). MMP2 expression was also more frequent in recurrent PACs, although this value did not reach statistical significance ($P = .07$). However, on multivariate analysis, only pathologic stage ($P = .009$) and ploidy status ($P = .03$) independently predicted disease recurrence. In conclusion, MMP2 and TIMP2 are co-expressed in a majority of PACs and correlate with prognostic variables. Interestingly, contrary to

the previously documented anti-tumor effects of TIMPs, TIMP2 expression appears to have a tumor-promoting role in PACs and warrants further investigation.

KEY WORDS: Immunohistochemistry, MMP, Prognosis, Prostate cancer, TIMP.

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Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, degrade the basement membrane and extracellular matrix, facilitating cell migration, tumor invasion, and metastasis (1–5). There are at least 20 human MMPs, divided into the collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs; 1–5). Tissue inhibitors of metalloproteinases (TIMPs) are the major endogenous regulators of MMPs and consist of four homologous members (TIMP1–4; 6–8). TIMPs are multifunctional proteins that inhibit cell invasion *in vitro* and tumorigenesis and metastasis *in vivo* (6). Although each TIMP appears capable of inhibiting several MMPs, these proteins exhibit preferential inhibitory capacity; for example, TIMPs1 and 2 selectively inhibit MMP9 and 2, respectively (9).

Increased expression of MMPs has been associated with poor prognosis and shortened patient survival in a variety of malignancies including carcinomas of the esophagus (10), stomach (11), colon (12), breast (13), pancreas (14), lung (15), kidney (16), and ovary (17). TIMP expression has been associated with both tumor suppressor or anti-metastatic effects and tumor-promoting effects in selected cancers (18–20). MMP and TIMP expression in prostate cancer has been recently reviewed (21). Both MMPs and TIMPs have been characterized in prostate cancer cell lines (22–25) and clinical samples from prostate cancer patients (26–33), with conflicting results. Similarly, serum levels of circulating MMPs and TIMPs have shown variable

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capability of predicting disease progression (34–37). The aim of the current study was to evaluate the immunohistochemical expression of MMP2 and TIMP2 in prostate cancer and determine whether the expression of these markers correlates with prognostic variables, including patient survival.

MATERIALS AND METHODS

Patients and Specimens

One hundred thirty-eight randomly selected prostatic adenocarcinomas (PACs) treated by radical retropubic prostatectomy obtained from the files of the Albany Medical Center Hospital between 1987 and 1997 were included in this study. All hematoxylin and eosin-stained slides from each case were reviewed, and tumors were graded according to the Gleason system (38) and staged according to TNM criteria (39). Multiple blocks were identified based on the presence of adequate tumor and the representative nature of the overall grade. For statistical evaluations, tumors with Gleason scores of 6 or lower were considered as low grade, and tumors with Gleason scores of 7 or higher were considered as high grade. Statistical analysis was also performed using a three-tier scheme isolating tumors with Gleason score of 7. Serum PSA levels as measured by the Hybritech Tandem method (Hybritech) were obtained from review of the patients' medical records. Postoperative PSA of >0.4 ng/mL on two consecutive occasions after prostatectomy was considered as biochemical evidence of disease recurrence.

Immunohistochemistry

To analyze for the expression of MMP2 and TIMP2 proteins, contiguous 4- μ m sections were cut from a single block of formalin-fixed, paraffin-embedded tissue randomly chosen from those initially identified; sections were placed on charged slides. After deparaffinization, primary antibody incubation was performed by an automated system (Ventana Medical Systems, Tucson, AZ) for MMP2 and manually for TIMP2. Pertinent details regarding antibodies and staining procedure are summarized in Table 1. The remainder of the staining procedure included incubation with a biotinylated anti-mouse secondary antibody, diaminobenzidine substrate, and hematoxylin counterstain and was

performed on the Ventana ES automated immunohistochemistry system. Negative-control slides were incubated with isotype-matched immunoglobulin in parallel with each batch of staining to confirm the specificity of the antibodies.

Staining Interpretation

Staining results were interpreted without prior knowledge of clinical and pathologic parameters by two observers using a consensus method. For all markers, both the intensity of staining and approximate percentage of positive tumor cells were considered in the semiquantitative assessment, as previously published (40, 41). Briefly, the distribution of positive staining in the tumors was graded as focal ($\leq 10\%$), regional (11–50%), and diffuse ($>50\%$). The staining intensity was subjectively scored as weak, moderate, or intense. Staining patterns of moderate diffuse, moderate regional, intense regional, and intense diffuse were considered as increased expression of each protein.

Quantitative DNA Analysis

Quantitative analysis of DNA content was determined for each case using 5- μ m tissue sections stained by the Feulgen reaction and evaluated by the CAS 200 image analyzer (Tripath Corp., Burlington, NC), as previously described (42, 43).

Statistical Analysis

Statistical comparisons were performed using Stata software (Stata Corp, College Station, TX). Correlation between protein expression and pathologic variables was performed using the χ^2 univariate analysis. Survival curves for all univariate analyses were assessed using the Kaplan-Meier method. Overall survival was defined as the interval between surgery and postsurgical biochemical disease recurrence. Multivariate analyses of clinicopathologic parameters, including survival, were performed using the Cox proportional hazards model. The level of significance was set at .05.

RESULTS

Of the 138 PACs, there were 75 (54%) low-grade and 63 (46%) high-grade tumors. At prostatectomy, there were 78 (57%) organ-confined tumors (Stages I and II) and 60 (43%) advanced-stage (Stages III

TABLE 1. Antibodies and Immunohistochemical Procedure

Antibody	Manufacturer	Clone	Citrate Antigen Retrieval (min)	Antibody Dilution	Primary Antibody Incubation	Positive Controls
MMP2	Novocastra	4D3	60	1:10	32 min at 41°C	Colon carcinoma
TIMP2	Neomarkers	2TMP04	60	1:10	Overnight at 4°C	Breast carcinoma

and IV) cancers. Of the 77 cases tested for total DNA content, 52 (68%) were diploid, and 25 (32%) were nondiploid. A total of 131/138 (95%) had sequential serum PSA follow-up information available. Of these 131 patients, 50(38%) had biochemical post-surgical disease recurrence.

Immunohistochemistry and Statistical Analysis

Immunostaining pattern for both proteins was cytoplasmic, with tumor cells showing moderate to intense positivity, as opposed to relatively weaker expression in the benign elements, which served as internal control in each case. One hundred five of 138 (76%) PACs expressed MMP2, and 113/138 (82%) expressed TIMP2. There was an overall significant coexpression of MMP2 and TIMP2 in 94/138 (68%) PACs ($P = .01$; Fig. 1). The co-expression of MMP2 and TIMP2 correlated with advanced tumor stage ($P = .05$) and reached near-significance as a univariate predictor of disease recurrence ($P = .07$). TIMP2 expression individually correlated with advanced tumor stage ($P = .04$; Fig. 2) and reached near significance with disease recurrence ($P = .06$; Fig. 3). MMP2 expression was also more frequent in the PACs that recurred, although this value did not reach statistical significance ($P = .07$). On univariate analysis, neither MMP2 nor TIMP2 expression correlated with tumor grade (using either the two- or three-tier scheme) or DNA ploidy.

On multivariate analysis, only tumor stage ($P = .009$) and DNA ploidy status ($P = .03$) independently predicted disease recurrence.

DISCUSSION

MMP expression has been reported to be low or undetectable in most benign elements but is substantially increased in a majority of human malignancies (10–21). Analysis of both primary and metastatic tumors has shown increased relative MMP expression at the metastatic site, supporting a role in tumor migration and spread (44). Additionally, increased MMP levels have been reported in the plasma and urine of patients with a variety of advanced malignancies (45). Cancer outcome studies have also shown that increased expression of MMPs is associated with shortened patient survival (10, 12, 46). Aberrant expression of MMPs in prostate cancer was first described using *in situ* hybridization in 1991 (31). MMP7 expression has been linked to prostate cancer pathologic stage and incidence of metastasis (33). Using both Northern analysis and *in situ* hybridization, Still and co-workers (27) linked increased MMP2 and TIMP2 to high tumor grade and advanced tumor stage of the disease. Increased MMP2 expression has also been associated with high tumor Gleason score (28). Finally,

increased MMP expression also has been implicated in the development of prostate cancer, as evidenced by increased levels found in carcinomas *versus* benign prostatic hypertrophy and prostatic intraepithelial neoplasia (47, 48).

Serum measurements of MMPs in prostate cancer have yielded conflicting correlations with disease outcome. Several studies have found a correlation between circulating MMPs (MMP1,2,3) and circulating TIMPs (TIMP1,3) and advanced or progressive disease (34, 36); others have failed to confirm this association (35).

In the present study, increased immunohistochemical co-expression of MMP2 and TIMP2 was associated with advanced tumor stage and reached near-significance as a predictor of disease recurrence. TIMP2 expression correlated with tumor grade and predicted disease recurrence on univariate analysis but was not an independent predictor when tumor stage and DNA ploidy status were included in the multivariate analysis model.

At the time of their discovery, TIMPs were considered to be tumor suppressor proteins. Recombinant TIMP2 was shown to inhibit invasion of HT 1080 fibrosarcoma cells *in vitro* (49). Increased TIMP expression has been associated with decreased tumor growth, invasiveness, and metastasis in a variety of prostate cancer and non-prostate cancer cell lines (22–25, 49–52). However, the results of the current study, demonstrating a poor prognostic significance in prostate cancer for increased TIMP2 expression, are contrary to the original tumor suppressor role hypothesized for TIMPs and more in line with recent evidence documenting a multifunctional complex role for TIMPs. Nemith *et al.* (53) described the growth-promoting abilities of TIMP2 in several human cell types, including fibroblasts, keratocytes, lymphocytes, and stem cells. Increased TIMP1 and TIMP2 mRNA levels have been correlated with tumor stage, lymph node metastasis, and shortened survival in patients with carcinomas of colon (18), breast (19), and bladder (54). Our findings of the poor prognostic role of increased TIMP2 expression in prostate cancer also concur with the data of Kugler *et al.* (16), which demonstrated a correlation of increased TIMP2 levels with aggressive phenotype in renal cell carcinoma.

Although the paradoxical positive effect of TIMP in tumor progression is not completely understood, the tumor-promoting activity may be due either to proteolytic degradation of ECM or direct influence on cell survival and growth. TIMP2 is reported to regulate matrix degradation, acting through a membrane type MMP (MT1-MMP; 55, 56). MT1-MMP is a key enzyme in tumor angiogenesis and metastasis, hydrolyzes a variety of ECM components, and is a physiologic activator of pro-MMP2

FIGURE 1. An example of the same case of prostatic adenocarcinoma showing co-expression of MMP2 (A) and TIMP2 (B) proteins. (3,3'-diaminobenzidine; hematoxylin, 200 $\times$).

(57). TIMP2 forms a complex with MT1-MMP and pro-MMP2 on the cell surface, promoting hydrolysis of pro-MMP2 to its active form (MMP2) and resulting in degradation of ECM. Also, formation of this complex decreases the autocatalysis of MT1-MMP, resulting in increased levels of its active

form. It also has been reported that some TIMPs can directly affect cell growth and survival, independent of their actions on MMPs. Stimulation of cell growth by TIMPs is thought to be mediated by c-AMP-dependent activation of protein kinase A (58) and increased tyrosine phosphorylation (59).

FIGURE 2. A Stage 3 prostatic adenocarcinoma with perineural invasion expressing TIMP2 protein (A) in comparison to its absence in tumor glands of a Stage 2 cancer (B). (3,3'-diaminobenzidine; hematoxylin, 200 $\times$).

Cell survival is prolonged by the TIMP1-mediated upregulation of anti-apoptotic protein bcl-XL and by decreased NF Kappa B activity (60). Several additional factors that may play key roles in the TIMP promotion of cancer progression include the following: local TIMP concentration, cellular distribu-

tion, association with pro-MMPs, and presence of TIMP receptors (61, 62).

In view of their important role in tumor invasion and metastasis, inhibitors of MMP activity have been investigated as a method of preventing or decreasing tumor spread. Clinical trials involving

FIGURE 3. Kaplan-Meier estimates showing correlation between TIMP2 protein expression and postsurgical disease recurrence ($P = .05$).

batimastat (British Biotech), a potent, broad-based inhibitor of MMPs 1, 2, 3, and 9 (63), and marimastat (British Biotech), a second-generation, water-soluble synthetic MMP inhibitor, have been associated with clinical responses in pancreatic, pulmonary, ovarian, and mammary carcinomas (63). In preclinical studies of prostate cancer, anti-tumor effects of MMP inhibitors (doxycycline and chemically modified tetracyclines), both *in vitro* and *in vivo*, have been reported (21).

In conclusion, co-expression of MMP2 and TIMP2 proteins bears prognostic significance in patients with prostate cancer and supports a potential therapeutic role for synthetic MMP inhibitors. The paradoxical poor prognostic significance of TIMP2 expression warrants further investigation into the complex MMP-TIMP interactions and into the role of TIMPs in tumor evolution and spread.

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Book Review

Miettinen MM: *Diagnostic Soft Tissue Pathology, 1st Edition, 800 pp, London, Churchill Livingstone, 2002 (\$199.00).*

With his book on soft tissue pathology, Dr. Markku Miettinen continues the tradition of the Chairs of the Department of Soft Tissue Pathology in publishing superb textbooks in this area of diagnostic pathology. This time it is a 800-page, superbly illustrated (all microphotographs are in full color) review of traditional microscopic and up-to-date immunohistochemical and molecular genetic characteristics of lesions of the organ system commonly referred to as "soft tissues." Reading this outstanding book, one may wonder what constitutes soft tissues. The book goes into a great detail describing gastrointestinal stromal tumors, melanomas, and distribution of cytokeratins' expression; all this could be considered a bit of an unorthodox approach in writing of "soft tissues" textbook. However, these chapters and paragraphs are excellently incorporated into a textbook that provides the most recent views on the diagnosis and pathogenesis of entities found under the umbrella of soft tissues.

There are 21 chapters. A separate chapter on immunohistochemistry of soft tissue tumors is a

marvel and a reflection of the author's extensive personal involvement in research and diagnostic applications of this technique. This is followed by a comprehensive chapter on the genetics of soft tissue tumors by Dr. Jerzy Lasota, again reflecting the great personal research experience of the author. Each following chapter deals with a traditional histogenetic group of tumors and consistently lists the most important clinical features, followed by the review of the most relevant histologic features, immunophenotype, and genetic alterations. The references are as recent as 2002, which is highly impressive.

This new book, although approximately one-third the size of the now classic *Enzinger and Weiss's Soft Tissue Tumors*, manages to cover the same territory without compromising the quality. Future research on molecular mechanisms will undoubtedly shed more light on understanding of the diseases of soft tissues, which will hopefully lead to new editions of this thoroughly modern book.

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