Increased Expression of Matrix Metalloproteinases 2 and 9 and Tissue Inhibitors of Metalloproteinases 1 and 2 Correlate with Poor Prognostic Variables in Renal Cell Carcinoma

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ABSTRACT

Purpose: Matrix metalloproteinases (MMPs) degrade components of the extracellular matrix and are implicated in tissue remodeling and tumor infiltration. Tissue inhibitor of metalloproteinases (TIMPs) inhibit enzymes of the MMP family and preserve stromal integrity, thus inhibiting tumor migration. Although numerous studies on several human carcinomas have demonstrated a role for MMPs in tumor metastasis and patient survival, their prognostic role in patients with renal cell carcinoma (RCC) has not been well defined. More importantly, the recently documented paradoxical functions of TIMPs have not been characterized in these neoplasms.

Experimental Design: Five-μm, formalin-fixed, paraffin-embedded tissue sections from 153 RCCs were immunostained using specific antibodies against MMP2, MMP9, (Novocastra, Burlingame, CA) TIMP1, and TIMP2 (Neo-Markers, Fremont, CA) proteins. Immunostaining was semiquantitatively scored based on intensity and distribution, and results were correlated with histological and prognostic variables.

Results: The rates of increased expression of MMPs and TIMPs in RCC were as follows: MMP2, 67%; MMP9, 43%; TIMP1, 46%; and TIMP2, 73%. Each of these four markers individually correlated with histological tumor type with a vast majority of papillary and sarcomatoid RCCs expressing these proteins as compared with clear cell tumors (P range, 0.0001–0.003). Significant coexpression of MMPs and TIMPs was observed (P = 0.0001). Increased immunoreactivity for each of these proteins correlated with high tumor grade (P range, 0.0001–0.01). On univariate analysis, expression of each of these markers correlated with shortened survival (P range, 0.004–0.05). On multivariate analysis, including tumor grade, stage, and all four markers, only advanced stage (P = 0.047) and increased TIMP1 expression (P = 0.007) independently predicted shortened survival.

Conclusion: Increased expression of MMP2, MMP9, TIMP1, and TIMP2 proteins in RCCs correlate with poor prognostic variables including shortened patient survival. The paradoxical poor prognostic implication of TIMP overexpression complements the recently documented dual function of TIMPs and warrants further investigation.

INTRODUCTION

Proteolytic degradation of the ECM is a fundamental aspect of cancer development and a key event in the regulation of tumor proliferation and metastasis. MMPs are a family of zinc-dependent endopeptidases that are collectively capable of degrading most components of the basement membrane and ECM, facilitating cell migration (1). Given their ubiquitous presence, MMPs play an important role in several physiological and benign pathological processes such as in embryogenesis and inflammatory diseases (2, 3). MMPs are secreted as inactive proenzymes and are transformed into active forms after cleavage of a propeptide domain of the molecule (4). On the basis of their structure, cell localization, and substrate specificity, the >20 human MMPs are divided into several groups such as collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs; Ref. 5). TIMPs are the major endogenous regulators of MMPs and consist of four homologous members (TIMP1–4) identified to date (6). Although each TIMP appears capable of inhibiting cell invasion in vitro and tumorigenesis and metastasis in vivo (6), 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; e.g., TIMP1 and -2 selectively inhibit MMP9 and -2, respectively (7).

Numerous investigators used various techniques and reported the prognostic significance of increased MMP expression in several human malignancies. A significant correlation between increased MMPs and poor prognosis, including shortened patient survival, has been documented in carcinomas of the esophagus (8), stomach (9), colon (10), breast (11), pancreas (12), prostate (13), lung (14), and ovary (15). Although early studies have shown TIMPs to have antitumor or antimetastatic effects, more recent reports indicate a dual function with positive correlation between increased TIMP levels and poor out-

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The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; DAB, 3,3′-diaminobenzidine.
come in some human malignancies (16–18). With regard to MMP expression in RCC, there are only a few studies in the literature, all of which used small numbers of cases and reported variable findings. Kugler et al. (19) isolated RNA from 17 fresh tumor specimens and, using a reverse transcription-PCR technique, demonstrated a correlation between increased MMP:TIMP ratio and aggressive tumor behavior. Subsequently, Lein et al. (20) reported increased MMP9 levels in both tumor tissue and sera of patients with RCC but found no correlation with prognostic variables such as tumor grade and stage.

The aim of the current study was to evaluate the immunohistochemical expression of MMP2, MMP9, TIMP1, and TIMP2 proteins in RCC and to determine whether the expression of these markers correlates with prognostic variables including survival.

MATERIALS AND METHODS

Patients and Specimens. One hundred fifty-three randomly selected surgically resected RCC specimens obtained from the files of the pathology departments at Albany Medical Center Hospital and Georgetown University Medical Center between 1991 and 1999 were included. All of the H&E-stained slides from each case were reviewed and tumors were histologically classified as clear cell, papillary, chromophobe, and sarcomatoid RCC. Tumors were graded according to Fuhrman’s system (21) and staged according to TNM criteria (22). For statistical evaluations, grade 1 and 2 tumors were considered as low grade and grade 3 and 4 as high grade (23). Similarly, stage 3 and 4 tumors were considered in the advanced stage category. Follow-up information was obtained from review of the patients’ medical records.

Immunohistochemistry. To analyze for the expression of MMP2, MMP9, TIMP1, and TIMP2 proteins, contiguous 5-µm sections were cut from a representative block of formalin-fixed, paraffin-embedded (FFPE) tissue and placed on charged slides. After deparaffinization, primary antibody incubation was performed manually for MMP9 and TIMP2 and by an automated system (Ventana Medical Systems, Tucson, AZ) for MMP2 and TIMP1. Pertinent details regarding antibodies and staining procedure are summarized in Table 1. The remainder of the staining procedure included incubation with a biotintylated antimouse secondary, DAB substrate and hematoxylin counterstain and was performed on the Ventana ES automated immunohistochemistry system (Ventana Medical Systems, Tucson, AZ). 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 pathological parameters by two observers (B. V. S. K., S. K.) using a consensus method. For all markers, both the intensity of staining and the approximate percentage of positive tumor cells were considered in the semiquantitative assessment as published previously (24–25). Briefly, the distribution of positive staining in the tumors was graded as focal (≤10%), regional (11–50%), or diffuse (>50%). The staining intensity was subjectively scored as weak, moderate, or intense. Staining patterns of moderate diffuse, intense regional, and intense diffuse were considered as increased expression of each protein.

Statistical Analysis. Statistical comparisons were performed using Stata software (Stata Corp, College Station, TX). Correlation between protein expression and pathological variables was performed using the χ2 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 death, the end point being either death or the closing date of this study. Multivariate analyses of clinicopathological parameters including survival were performed using the Cox proportional hazards model. The level of significance was set at 0.05.

RESULTS

Of the 153 RCCs, there were 118 clear cell, 22 papillary, 8 chromophobe, and 5 sarcomatoid carcinomas. The tumors were categorized as grade 1 and 2 in 114 (75%) cases and grade 3 and 4 in 39 (25%) cases; and stage 1 and 2 in 104 (68%) cases and stage 3 and 4 in 49 (32%) cases. Follow-up information was available for 139 (91%) patients. Whereas 34 (24%) patients died of disease, the remaining 105 (76%) were alive at the time of the last follow-up (mean, 40 months; range, 2–95 months).

Immunohistochemistry. The frequency of increased expression of each protein and correlation with histological tumor type, grade, and survival are summarized in Table 2. Immunostaining pattern for each protein was cytoplasmic, with tumor cells showing moderate-to-intense positivity as opposed to relatively weaker expression in the benign elements. There was an overall significant coexpression of MMPs and TIMPs in all of the RCCs (P = 0.0001). Of particular interest was the similarity in the frequency of expression of MMP2 and TIMP2, and MMP9 and TIMP1, supporting their preferential association. The non-clear cell RCCs (Fig. 1, A–D) showed a significant predilection to overexpress each of these proteins as compared with clear cell tumors.

Increased expression of each protein correlated with high tumor grade (Figs. 2, A–D and 3, A–D). However, no correlation was found between increased expression of these proteins and advanced tumor stage.

Table 1 Antibodies and immunohistochemical procedure

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Clone</th>
<th>Citrate antigen retrieval (min)</th>
<th>Antibody dilution</th>
<th>Primary antibody incubation</th>
<th>Positive controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP2</td>
<td>Novocastra</td>
<td>4D3</td>
<td>60</td>
<td>1:10</td>
<td>32 min at 41°C</td>
<td>Colon carcinoma</td>
</tr>
<tr>
<td>MMP9</td>
<td>Novocastra</td>
<td>2C3</td>
<td>None</td>
<td>1:15</td>
<td>Overnight at 4°C</td>
<td>Colon carcinoma</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Neomarkers</td>
<td>102D1</td>
<td>None</td>
<td>1:10</td>
<td>32 min at 41°C</td>
<td>Breast carcinoma</td>
</tr>
<tr>
<td>TIMP2</td>
<td>Neomarkers</td>
<td>2TMP04</td>
<td>60</td>
<td>1:10</td>
<td>Overnight at 4°C</td>
<td>Breast carcinoma</td>
</tr>
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</table>
Survival Analysis. On both \( \chi^2 \) and Cox univariate analysis, high tumor grade \((P = 0.02\) and \(P = 0.01\), respectively) and advanced stage \((P = 0.003\) and \(P = 0.04\), respectively) correlated with death (Fig. 4, A and B). On \( \chi^2 \) analysis, increased expression of each of the four proteins correlated with survival (Table 2). However, on Cox univariate analysis, which takes into account the duration of survival, only the increased expression of MMP9 \((P = 0.01)\) and TIMP1 \((P = 0.006)\) correlated with a shortened patient survival (Fig. 5, A and B). By the latter analysis, increased expression of MMP2 \((P = 0.07)\) and TIMP2 \((P = 0.1)\) showed a trend toward shortened survival that did not reach statistical significance. On multivariate analysis of tumor grade, stage, and all four proteins using Cox proportional hazards model (Table 3), only tumor stage \((P = 0.047)\) and increased TIMP1 expression \((P = 0.007)\) independently predicted shortened survival. Although the remaining variables were not significant indicators of patient survival, this model (Table 3) suggests a relatively higher magnitude of death hazard for MMP9 and TIMP2 expression over tumor grade, which supports the role of these proteins in regulating matrix degradation. The relatively lower death hazard for nuclear grade in this model may reflect the proliferative capacity of tumor cells rather than their ability to spread.

DISCUSSION

Tumor growth, invasion, and metastasis is a complex, multistep process that is facilitated by the proteolytic degradation of components of the ECM and basement membrane. The role of MMPs in this process has been firmly established based on numerous previously published experimental and clinical studies. Increased expression of various MMPs has correlated with enhanced metastatic properties in rat embryo fibroblasts (26) and cancer cell lines of rat bladder, mouse lung, and human prostate (27–29). In human tumors, MMP expression has been reported to be low or undetectable in most benign elements and substantially increased in a majority of malignancies, including carcinomas of colon, breast, lung, pancreas, and prostate (10–14). Analysis of both primary and metastatic tumors demonstrated increased MMPs at the metastatic site, which supports their role in tumor migration and spread (30). Additionally, increased MMP levels have been reported in the plasma and

Table 2  Increased expression of MMPs and TIMPs by tumor type, grade, and survival

<table>
<thead>
<tr>
<th>Protein</th>
<th>Increased expression (%)</th>
<th>Tumor type (%)</th>
<th>Grade (%)</th>
<th>Survival (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Clear cell</td>
<td>Non-clear cell</td>
<td>High</td>
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<tr>
<td>MMP2</td>
<td>67</td>
<td>59</td>
<td>91</td>
<td>85</td>
</tr>
<tr>
<td>MMP9</td>
<td>43</td>
<td>38</td>
<td>63</td>
<td>79</td>
</tr>
<tr>
<td>TIMP1</td>
<td>46</td>
<td>38</td>
<td>77</td>
<td>74</td>
</tr>
<tr>
<td>TIMP2</td>
<td>73</td>
<td>67</td>
<td>91</td>
<td>89</td>
</tr>
</tbody>
</table>

Fig. 1 An example of the same case of papillary RCC showing moderate-to-intense, diffuse, cytoplasmic immunoreactivity within tumor cells for MMP2 (A), MMP9 (B), TIMP1 (C), and TIMP2 (D) proteins; ×200; staining: DAB and hematoxylin.
urine of patients with cancers of colon, breast, prostate, and bladder compared with levels in healthy subjects (31). Furthermore, there is ample evidence correlating increased expression with poor prognosis (8, 10, 32). With regard to genitourinary tumors, Gohji et al. (33) described increased serum levels of MMP2 and MMP3 in patients with urothelial cancer that correlated with disease progression and poor outcome. Studies on MMP/TIMP expression in RCCs have been limited and have reported variable findings. Kugler et al. (19) analyzed MMP2, MMP9, TIMP1, and TIMP2 in 17 RCCs by PCR and demonstrated a strong correlation between increased gene expression and tumor stage. Additionally, these authors also reported that the MMP:TIMP ratio significantly increased in a graded fashion from normal tissues to organ-confined tumors and metastatic carcinomas. In contrast, Lein et al. (20) reported that only MMP9 was increased in RCCs and found no correlation with tumor type, grade, or stage. Our results are more in keeping with Kugler’s data, in that an increased expression of MMP2, MMP9,
TIMP1, and TIMP2 portend a poor prognosis in patients with RCC. Furthermore, we also demonstrated that increased TIMP1 protein expression independently predicted shortened patient survival.

In the context of tumor invasion, the original concept of TIMPs was that of an inhibition of MMPs, thus serving as anti-invasive/anti-metastatic agents (as evidenced by data from both experimental and clinical studies). Recombinant TIMP2 was shown to inhibit the invasion of HT 1080 fibrosarcoma cells in vitro (34). Increased TIMP expression has been associated with decreased tumor growth, invasiveness, and metastasis in cancer cell lines of the stomach (35), pancreas (36), lung (14), and breast (37). However, the results of the current study, which demonstrate a poor prognostic significance for increased TIMP1 and TIMP2 expression, are contrary to the previous notion and more in line with recent evidence documenting a multifunctional complex role for TIMPs. Nemeth et al. (38) described the growth-promoting abilities of TIMP2 in several human cell types including fibroblasts, keratocytes, lymphocytes, and stem cells. With regard to human cancers, increased TIMP1/TIMP2 mRNA levels correlated with tumor stage, lymph node metastasis, and shortened survival in patients with carcinomas of the colon (16), breast (17), and bladder (39). Our findings of the poor prognostic role of increased TIMP expression concur with the data of Kugler (2), in which the author demonstrated a correlation of increased TIMP2 levels with aggressive phenotype in RCC.

The mechanisms supporting the paradoxical positive effect of TIMP in tumor progression are not completely understood and are the subject of intense investigation. This tumor-promoting activity may be attributable to either proteolytic degradation of ECM or direct influence on cell survival and growth. TIMP2, acting through a membrane-type MMP (MT1-MMP; 40, 41), is reported to regulate matrix degradation. MT1-MMP is a key enzyme in tumor angiogenesis and metastasis, hydrolyzes a variety of ECM components, and is a physiological activator of pro-MMP2 (42). 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, which results in increased levels of its active form. It has also been reported that some TIMPs can directly affect cell growth/survival independent of their actions on MMPs. Stimulation of cell growth by TIMPs is thought to be

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
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<tr>
<td>MMP2</td>
<td>-0.0959459</td>
<td>0.6312752</td>
<td>0.879</td>
</tr>
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<td>Grade</td>
<td>0.2526737</td>
<td>0.4672274</td>
<td>0.589</td>
</tr>
<tr>
<td>TIMP2</td>
<td>-0.3059951</td>
<td>0.5548827</td>
<td>0.581</td>
</tr>
<tr>
<td>MMP9</td>
<td>0.5454763</td>
<td>0.4471249</td>
<td>0.222</td>
</tr>
<tr>
<td>Stage</td>
<td>0.7109947</td>
<td>0.3576072</td>
<td>0.047</td>
</tr>
<tr>
<td>TIMP1</td>
<td>1.000858</td>
<td>0.3685423</td>
<td>0.007</td>
</tr>
</tbody>
</table>
mediated by c-AMP-dependent activation of protein kinase A (43) and increased tyrosine phosphorylation (44). Cell survival is prolonged by the TIMP1-mediated up-regulation of antiapoptotic protein bcl-x<sub>L</sub> and decreased nuclear factor κB activity (45). What are the factors that regulate TIMP activity toward tumor suppression or tumor promotion? Several factors that are likely to play a key role include local TIMP concentration, cellular distribution, association with pro-MMPs, and presence of a putative “TIMP receptor” (46, 47).

In view of the important role in tumor invasion and metastasis, inhibitors of MMP activity have been investigated as a method of preventing/decreasing tumor spread. Several pharmaceutical companies are currently developing low-molecular-weight MMP inhibitors for clinical use. Clinical trials involving batimastat (British Biotech), a potent broad-based inhibitor of MMPs 1, 2, 3, and 9 (48) and marimastat (British Biotech), a second-generation water-soluble synthetic MMP inhibitor, have been evaluated in patients with pancreatic, pulmonary, ovarian, and mammary carcinomas.

In conclusion, MMPs and their inhibitors are key enzymes for tumor progression, and increased expression of these proteins is associated with poor outcome in patients with RCC, which supports a potential role for synthetic MMP inhibitors in the management of this disease. The paradoxical poor prognostic significance of TIMP overexpression, as demonstrated in this study and in recent literature, warrants further investigation to fully understand the complex MMP/TIMP interactions and to evaluate the potential efficacy of TIMP modulation in cancer therapy.

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25. Kawamura, H., Kameyama, S., Kawai, K., Tenaka, Y., Nan, L., Barch, D., Stetler-Stevenson, W. G., and Oyasu, R. Marked acceleration...


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