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Advances in Brief

Levels of Matrix Metalloproteases in Bladder Cancer Correlate with Tumor Grade and Invasion

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Abstract

We have used quantitative zymography to measure levels of the type IV collagenases matrix metalloproteinase (MMP)-9 and MMP-2 in 42 biopsies of transitional cell carcinoma and in 7 biopsies of normal bladder. Mean levels of MMP-9 were significantly higher in tumor compared with normal samples ($P = 0.08$). Levels of MMP-9 and active MMP-2 increased with tumor grade (test for trend, $P = 0.002$ and $P = 0.05$, respectively). Levels of MMP-9 and activated MMP-2 were also higher in invasive tumors than in superficial ones ($P = 0.001$ and $P = 0.008$, respectively). *In situ* hybridization studies showed that the mRNAs for both MMP-2 and MMP-9 were located chiefly in the stroma rather than epithelial tumor cells and were concentrated at the interface between the two tissues.

Introduction

Normal epithelial cells are separated from underlying mesenchyme by a specialized layer of extracellular matrix termed the basement membrane. This consists of laminin and fibronectin with type IV collagen forming the main structural element. Degradation of this protective structure is essential if tumor cells are to invade into surrounding mesenchyme and metastasize to distant sites (1, 2). MMPs[1] are a family of degradative enzymes involved in remodeling extracellular components in health and disease. Two MMPs have type IV collagenolytic activity, namely M_r 72,000 type IV collagenase (MMP-2) and M_r 92,000 type IV collagenase (MMP-9). These enzymes are expressed in a variety of tumor types including skin (3), colon (4), breast (5), and prostate (6). However, few studies have quantified and compared levels of type IV collagenases in normal and malignant tissue. Here we have used quantitative gel substrate analysis, zymography, to compare levels of MMP-2 and MMP-9 in biopsies of normal bladder and TCCs of the bladder, and *in situ* hybridization to identify the localization of these enzymes. We have found striking association between levels of the MMPs and tumor grade and invasiveness.

Materials and Methods

Tissue Samples. All tissue samples were cryopreserved in liquid nitrogen following their removal from the patient. Seven bladder specimens were obtained from patients undergoing nephrectomy, where the ureter had been removed with a cuff of normal bladder, in the process of renal transplantation surgery. Bladder tumor samples were obtained by cystodiathermy. Five-μm-thick sections were cut from the surface area of tumors and homogenized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer containing glycerol (10% v/v), sodium dodecyl sulfate (1% w/v), and bromophenol blue using Treff microhomogenizers (Scott Lab) for gel substrate analysis. Adjacent sections were cut for protein estimation, *in situ* hybridization, and histopathological examination. Tumor grade was assessed by three histopathologists without knowledge of the results of quantitative zymography. Sections of paraffin-embedded tumors were also examined to assess tumor grade. The independent grading was scored and a consensus grade was determined. Sections of paraffin-embedded tumors were examined to determine whether muscle invasion was evident. Ethical committee approval was obtained for the use of these biopsies.

Protein Estimation. Single 5-μm-thick cryostat sections from each tumor were homogenized in 1% (w/v) sodium dodecyl sulfate and diluted 10-fold in water before measuring protein content against bovine serum albumin using the Bradford method as described by Davies *et al.* (5).

Gelatin Zymography. Gelatinolytic zymography was performed as described by Brown *et al.* (7). Because separation according to size occurs, this method can distinguish between MMP-2 and MMP-9. Separate quantification of the inactive and activated forms of MMP-2 was also possible, although the resolution of the gels was insufficient to allow distinction between the two forms of MMP-9. Homogenized tissue samples were applied directly, without heating or reduction, to the zymogram. MMP activity affects not only the intensity of the resulting band but also its size. Conventional linear densitometric analysis was therefore inadequate for assessment of gelatinolytic activity. We have used computer-assisted image analysis in order to overcome this problem as described in Ref. 5. Conditioned media from human melanoma RPMI 7951 cells and from 12-*O*-tetradecanoylphorbol-13-acetate-stimulated HT1080 fibrosarcoma cells were used as MMP standards (5). RPMI 7951 constitutively secretes the M_r 72,000 proform of MMP-2 (Fig. 1, *Lane 1*), and the activity contained in 20 μl of conditioned media was defined as 100 arbitrary units of MMP-2. 12-*O*-tetradecanoylphorbol-13-acetate-stimulated HT1080 cells also secreted small amounts of the proform of MMP-2, larger amounts of its M_r 59,000/62,000 activated forms, and MMP-9 (Fig. 1, *Lane 2*). The amount of MMP-9 in 20 μl of this conditioned media was defined as 100 arbitrary units.

***In Situ* Hybridization.** Antisense MMP-2 and MMP-9 were generated from the pGEM3-72K and pGEM3-92K (provided by British Bio-technology, Ltd., Oxford, United Kingdom) using the relevant RNA polymerase (Promega Biotech, Madison, WI). The negative control was sense RNA generated from the same vector linearized in the opposite direction. *In vitro* transcriptions were performed using Promega Biotech transcription kits to incorporate [^{35}S]UTP (Amersham International; SJ 1303). Restriction enzymes were all obtained from Pharmacia (Milton Keynes, United Kingdom). *In situ* hybridization was carried out on cryostat sections as in Ref. 8.

Statistical Analysis. *P* values were calculated using the Mann-Whitney test. A nonparametric test for trend over ordered groups was used to make comparisons across different histopathological grades (9).

Results

MMP-2 and MMP-9 activities were measured by quantitative zymography in 7 biopsies of normal bladder and 42 biopsies of TCC of the bladder of which 4 were grade I, 24 were grade II, and 14 were grade III. The activities of MMP-2 and MMP-9 were calculated in arbitrary units/10 μg of protein. The activity of MMP-2 was resolved into its inactive proform (M_r 72,000) and the M_r 59,000 and M_r 62,000 activated species which appeared as a doublet in the gels.

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[1] The abbreviations used are: MMP, matrix metalloproteinase; TCC, transitional cell carcinoma.

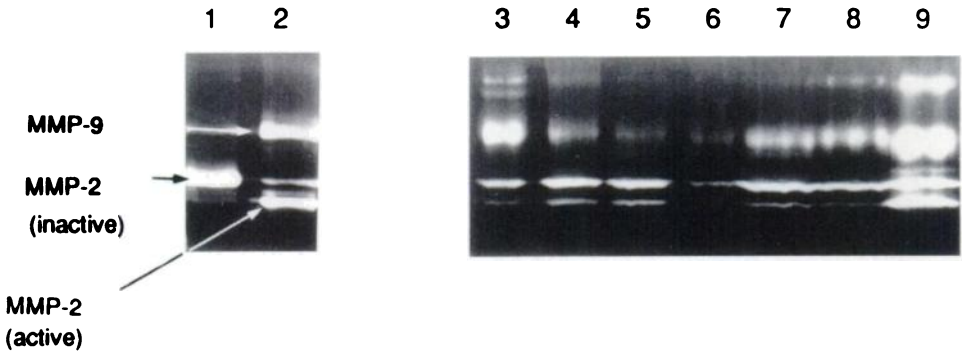

Fig. 1. Zymogram of bladder biopsies. *Lane 1*, MMP-2 (inactive) standard; *Lane 2*, MMP-9 and MMP-2 (activated) standards; *Lanes 3–9*, biopsies of bladder.

Fig. 1 shows a representative zymogram of bladder biopsies. *Lanes 1* and *2* contain MMP standards as described in "Materials and Methods." The single band in *Lane 1* corresponds to the inactive proform of MMP-2. This band is faintly visible in *Lane 2*, and below this a M_r 59,000/62,000 doublet corresponds to the active forms of MMP-2. Above these in *Lane 2*, a single band of M_r 92,000 corresponds to all forms of MMP-9. *Lanes 3–9* contain bladder biopsies. Samples 3, 4, 5, 7, 8, and 9 are of TCC, while sample 6 is of normal bladder. All

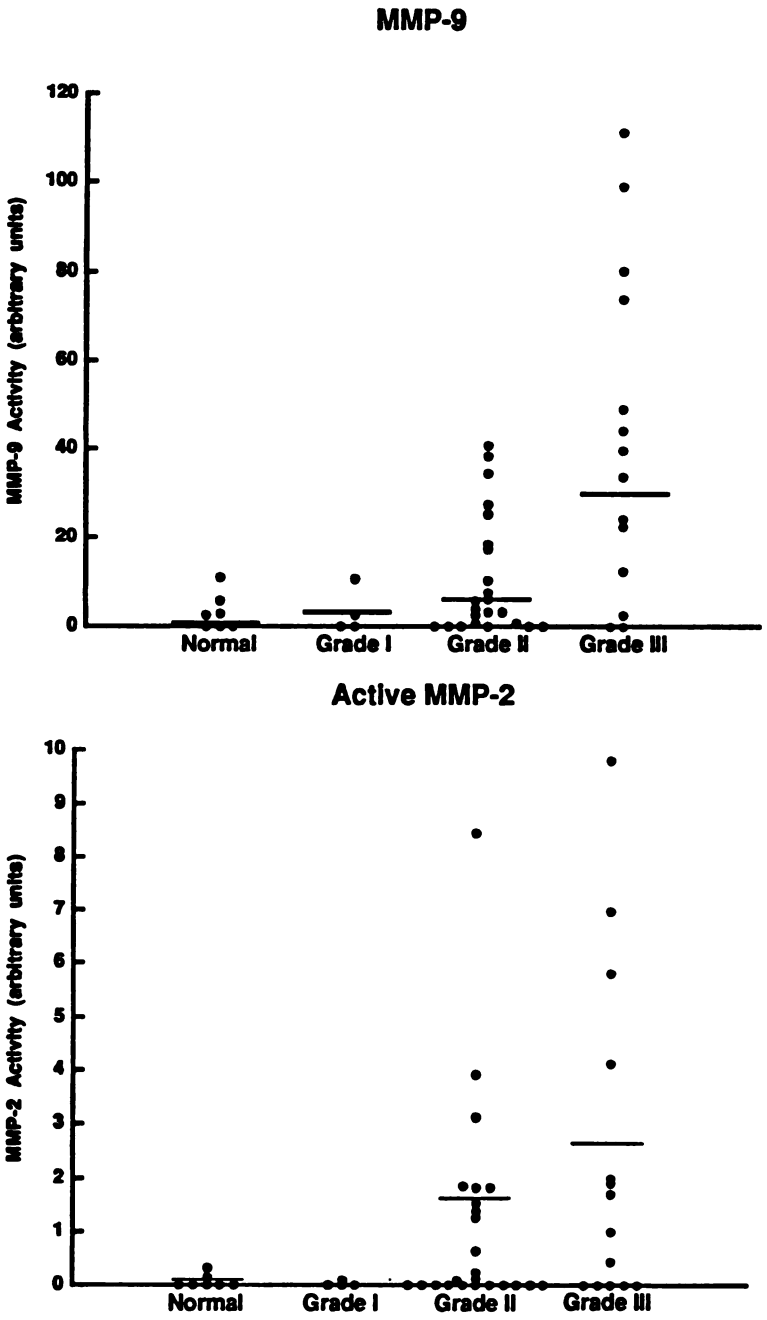

Fig. 2. Levels of MMP-2 and MMP-9 in biopsies of normal and malignant bladder were determined by quantitative zymography and compared to tumor grade. *a*, MMP-9; *b*, MMP-2 (active). *Bars*, SEM.

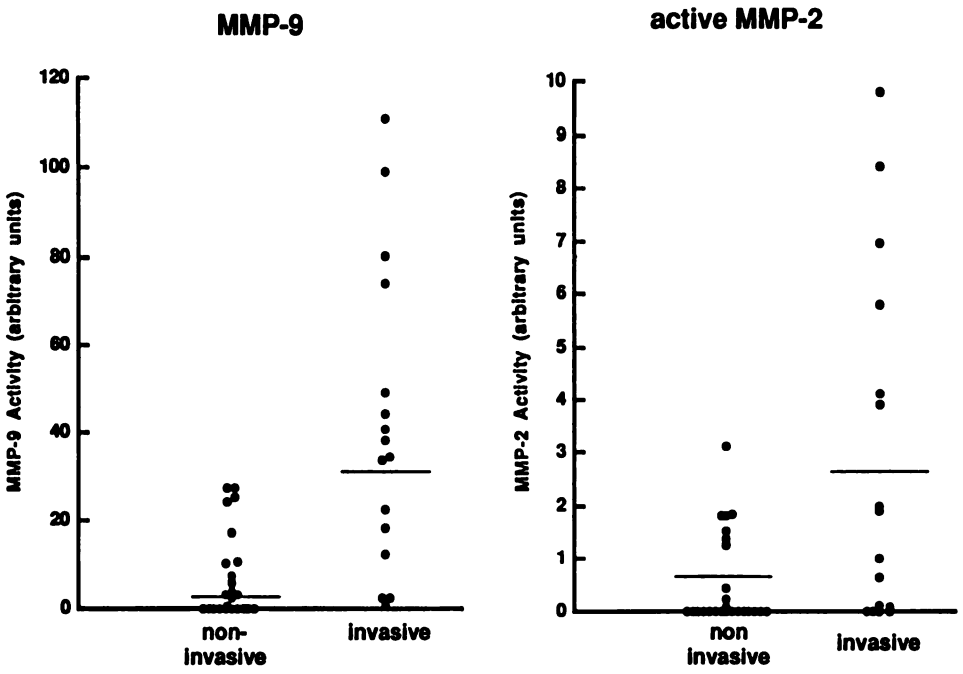

Fig. 3. Levels of MMP-2 and MMP-9 in bladder biopsies were determined by quantitative zymography and compared between invasive and noninvasive tumors. *a*, MMP-9; *b*, MMP-2 (active). *Bars*, SEM.

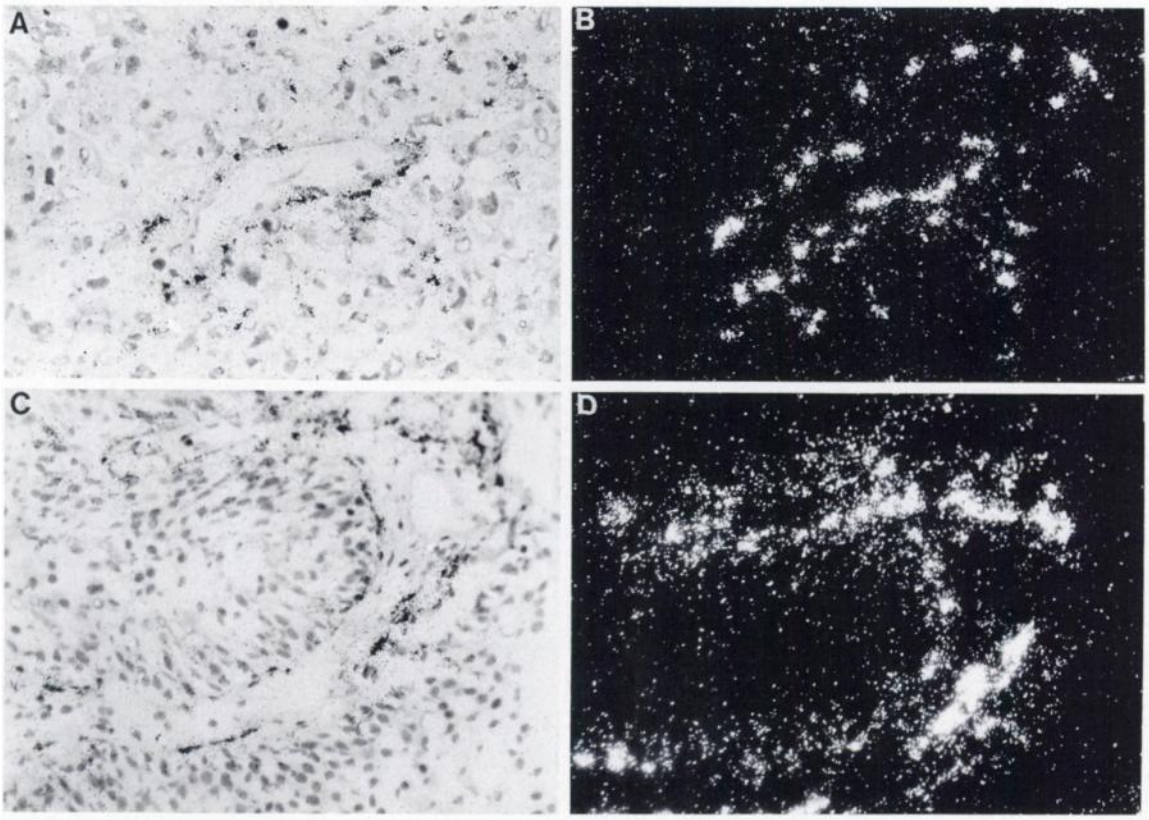

Fig. 4. *In situ* hybridization studies on biopsies of transitional cell carcinomas of the bladder. *a* and *b*, light and dark field views of MMP-9 expression; *c* and *d*, light and dark field views of MMP-2 expression. × ~1000.

samples in Fig.1 expressed the inactive form of MMP-2, ranging from 6.3 units (sample 6) to 43.5 units (sample 9). All biopsies of TCC expressed activated forms of MMP-2 ranging from 5.1 units (sample 3) to 47.7 units (sample 9), but no activated MMP-2 was detectable in the sample of normal bladder (sample 6). Variation in the levels of MMP-9 between the samples was greater, ranging from 0 (below limits of detection; samples 4, 5, and 6) to 103.0 units (sample 9).

Four of the seven normal bladder biopsies expressed MMP-9 (57%), six expressed the proform of MMP-2 (86%), and two expressed its active form (29%). Thirty-two of the 42 bladder tumor biopsies expressed MMP-9 (76%), 25 expressed the proform of MMP-2 (60%), and 24 expressed its active form (57%). Fig. 2 shows the variation in levels of MMP-9 (Fig. 2*a*) and activated MMP-2 (Fig. 2*b*) with tumor grade. Variation in gelatinolytic activity between dif-

ferent sections of the same tumor has been shown by us (5) and others (10, 11) to be negligible. Due to variations in histopathological grading, we used the independent opinions of three histopathologists given after examination of frozen and paraffin sections. All three gave the same grading for 27 of the 42 samples and 2 of the 3 agreed for the remaining 22 cases. The majority decision was taken in these cases.

Levels of MMP-9 in normal bladder [1.7 ± 0.8 (SEM) units; $n = 7$] were significantly lower than mean levels in all samples from malignant tumors (13.6 u ± 4.0; $P = 0.02$). The levels of MMP-9 increased significantly through tumor grade (test for trend, $P = 0.006$). Levels of the inactive proform of MMP-2 did not differ between normal and malignant bladders (2.7 ± 0.6 units *versus* 3.0 ± 0.5 units) and did not increase through tumor grade (test for trend, $P = 0.7$). Levels of activated MMP-2 were higher in malignant tissues than in normal bladders (1.9 ± 0.4 units *versus* 0.07 ± 0.05 units), but this did not reach statistical significance because of the number of negative values in all groups ($P = 0.1$). However, levels of activated MMP-2 increased with increasing histological grade (test for trend, $P = 0.05$).

Paraffin sections of the cases of TCC under investigation were examined to determine if the tumors were invasive or superficial. From a total of 41 malignant cases where the information was available, 16 were muscle invasive and 25 were superficial (pT1/pTa). Invasive tumors were generally of higher histopathological grade (6 of 16, grade 2; 10 of 16, grade 3). Quantitative zymography showed that invasive tumors contained significantly higher quantities of MMP-9 ($P = 0.001$) and activated MMP-2 ($P = 0.008$) than superficial tumors (Fig. 3). The survival data available on the patients studied were insufficiently mature to determine if there were any relationships between MMP levels and survival or occurrence of distant metastasis.

***In Situ* Hybridization Studies.** We performed *in situ* hybridization studies on 20 biopsies of TCC to determine which cells expressed mRNA for MMP-2 and MMP-9. MMP-9 expression was located almost exclusively in stromal elements of the tumor rather than in epithelial areas. Fig. 4, *a* and *b*, shows a blood vessel surrounded by a narrow band of stroma and epithelial tumor areas. Nonepithelial tumor elements positive for MMP-9 surround the blood vessel.

MMP-2 showed a distribution similar to that of MMP-9. Positive cells were located in the stroma and not in the epithelial areas (Fig. 4, *c* and *d*). The precise cell types which were positive for MMP-9 and MMP-2 could not be identified unambiguously upon morphological grounds alone. The expression of mRNA for both MMPs was most marked in stromal cells closest to the advancing tumor front and declined with increasing distance from the tumor.

Discussion

There is accumulating evidence that type IV collagenases play an important role in cancer cell invasion and metastasis (1, 12). Earlier studies showed the potential importance of these enzymes in tissue culture and animal models of invasion and metastasis (13, 14). More recently immunohistochemical and *in situ* hybridization studies have demonstrated the presence of these enzymes in a wide variety of human tumors (3, 4, 6, 15). However, the physiological significance of type IV collagenases to invasive progression of these tumors remains unclear partly because there have been relatively few quantitative studies. In this study we have used quantitative zymography to demonstrate that biopsies of bladder tumors of higher histological grade (which are associated with a poorer prognosis) express increased levels of both MMP-9 and the activated form of MMP-2. These present findings extend results we have obtained in an earlier study in breast cancer. In 43 breast cancer biopsies, mean levels of MMP-9 were higher in grade III tumors compared with lower grades, benign, and normal tissues. There was also a significant trend towards expression of active MMP-2 in more poorly differentiated tumors (5). In a study of 22 non-small cell lung cancer biopsies, there was a strong association between levels of activated MMP-2 and tumor spread (11). Taken together, these three studies provide compelling evidence of an association between tumor spread and expression of MMP-2 and MMP-9.

mRNA for MMP-9 and MMP-2 was expressed chiefly by the stromal cells and not the tumor cells themselves. Occasionally, endothelial cells in the fibrovascular cores of the tumors expressed mRNA for both type IV collagenases, but cells of macrophage/fibroblast morphology accounted for most of the message. *In vitro* studies have shown that cells of the mononuclear phagocyte lineage (16, 17) are the major producers of MMP-9, while MMP-2 is secreted by resting fibroblasts in culture (18). Occasional tumor cells in a minority of the biopsies also contained mRNA for MMP-2 and MMP-9. However, whether stroma or tumor cell derived, mRNA was invariably concentrated at the interface between these two components of tumor, suggesting that cell-cell contact or paracrine factors such as cytokines are responsible for the induction of the gene. Similar results have been reported in other tumor types including squamous cell carcinoma of the skin (3), breast carcinoma (5), and colorectal tumors (4). The expression of the enzymes was often localized around the tumor blood vessels. This raises the possibility that these enzymes may facilitate angiogenesis. This observation could, however, be fortuitous; stromal cores typically contain blood vessels and these are likely to be close to the tumor islands and under the same MMP-regulating influences as other stromal elements. The conditions present in high grade tumors which cause activation of MMP-2 need to be identified. In tissue culture, activation is mediated by a cell surface-bound protease sensitive to MMP-specific inhibitors. This is not on the surface of resting fibroblasts but is present following stimulation with concanavalin A (18, 19).

Patients with bladder TCC may present with superficial or invasive tumors. One major clinical problem in superficial bladder cancer is identification of those patients who will progress to advanced tumors or a metastatic state (20). At present, there are no biological markers available to enable an accurate prognosis for these patients. Our results show that fundamental biochemical differences exist between low- and high-grade bladder tumors which cause different patterns of expression of type IV collagenases. Different patterns of MMP expression may explain the differences in invasive and metastatic behavior between tumors. Recently, MMP inhibitors have been developed and are under laboratory investigation. Our present study suggests that these inhibitors may have a clinical role in the treatment of bladder carcinomas.

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References

1. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell, *64:* 327–336, 1991.
2. Matrisian, L. M., and Bowden, G. T. Stromelysin/transin and tumor progression. Semin. Cancer Biol., *1:* 107–116, 1990.
3. Pyke, C., Ralfliaer, E., Huhtala, P., Hurslainen, T., Dano, K., and Tryggvason, K. Localization of messenger RNA for M_r 72,000 and 92,000 type IV collagenases in human skin cancers by *in situ* hybridization. Cancer Res., *52:* 1336–1341, 1992.
4. Pyke, C., Ralfkiaer, E., Tryggvason, K., and Dano, K. Messenger RNA for two type IV collagenases is located in stromal cells in human colon cancer. Am. J. Pathol., *142:* 359–364, 1993.
5. Davies, B., Miles, D. W., Happerfield, L. C., Naylor, M. S., Bobrow, L. G., Rubens, R. D., and Balkwill, F. R.. Activity of type IV collagenases in benign and malignant

breast disease. Br. J. Cancer, *67:* 1126–1131, 1993.
6. Stearns, M. E., and Wang, M. Type IV collagenase (M_r 72,000) expression in human prostrate: benign and malignant tissue. Cancer Res., *53:* 878–883, 1993.
7. Brown, P. D., Levy, A. T., Margulies, I. M. K., Liotta, L. A., and Stetler-Stevenson, W. G.. Indepenedent expression and cellular processing of M_r 72,000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines. Cancer Res., *50:* 6184–6191, 1990.
8. Naylor, M. S., Stamp, G. W. H., and Balkwill, F. R. Investigation of cytokine gene expression in human colorectal cancer. Cancer Res., *50:* 4436–4440, 1990.
9. Cuzick, J. A Wilcoxon-type test for trend. Statistics Med., *4:* 87–90, 1985.
10. Brown, P. D., Bloxidge, R. E., Anderson, E., and Howell, A. Expression of activated gelatinase in human invasive breast carcinoma. Clin. Exp. Metastasis *11:* 183–189, 1993.
11. Brown, P. D., Bloxidge, R. E., Stuart, N. S. A., Gatter, K. C., and Carmichael, J. Association between expression of activated 72-kilodalton gelatinase and tumor spread in non-small-cell lung carcinoma. J. Natl. Cancer Inst., *85:* 574–578, 1993.
12. Waxman, J., and Wasan, H.. The architecture of cancer: tissue metalloproteinases amd their inhibitors. Br. Med. J., *305:* 1306–1307, 1993.
13. Garbisa, S., Pozzatti, R., Muschel, R. J., Saffiotti, U., Ballin, M., Goldfarb, R. H., Khoury, G., and Liotta, L. A. Secretion of type IV collagenolytic protease and metastatic phenotype: induction by transfection with c-Ha-*ras* but not c-Ha-*ras* plus Ad2-E1a. Cancer Res., *47:* 1523–1528, 1987.
14. Alvarez, O. A., Carmichael D. F., and De Clerck, Y. A. Inhibition of collagenolytic activity and metastasis of tumor cells by a recombinant human tissue inhibitor of metalloproteinases. J. Natl. Cancer Inst., *82:* 589–595, 1990.
15. Monteagudo, C., Merino, M. J., San-Juan, J., Liotta, L. A., and Stetler-Stevenson, W. G. Immunohistochemical distribution of type IV collagenase in normal, benign and malignant breast tissue. Am. J. Pathol., *136:* 585–592, 1990.
16. Hibbs, M. S., Hoidaland, J. R., and Kang, A. H. Expression of a metalloproteinase that degrades native type V collagen and denatured collagens by cultured human alveolar macrophages. J. Clin. Invest., *80:* 1644–1650, 1987.
17. Welgus, H. G., Campbell, E. J., Cury, J. D., Eisen, A. Z., Senoir, R. M., Wilhelm, S. M., and Goldberg, G. I. Neutral metalloproteinases produced by human mononuclear phagocytes. J. Clin. Invest., *86:* 1496–1502, 1990.
18. Overall, C. M., and Sodek, J.. Concanavalin A produces a matrix-degradative phenotype in human fibroblasts. J. Biol. Chem., *265:* 21141–21151, 1990.
19. Ward, R. V., Atkinson, S. J., Slocombe, P. M., Docherty, A. J. P., Reynolds, J. J., and Murphy, G. Tissue inhibitor of metalloproteinase-2 inhibits the activation of 72 kDa progelatinase by fibroblast membranes. Biochim. Biophys. Acta, *1079:* 242–246, 1991.
20. Able, P. D. Follow up of patients with "superficial" transitional cell carcinoma of the bladder. The case for a change in policy. Br. J. Urol., *72:* 135–142, 1993.

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