Quantitative Immunohistochemical and *In Situ* Hybridization Analysis of Metalloproteinases in Prostate Cancer

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Abstract. *The role of matrix metalloproteinases (MMPs) as markers of tumor progression in prostate cancer (CaP) is complex and poorly understood. Using computerized image analysis, the differential expression of interstitial collagenase (MMP-1), gelatinase B (MMP-9), matrilysin-1 (MMP-7) and the membrane-type 1-MMP (MT1-MMP) in the epithelium and stroma of human prostate neoplastic tissues were investigated. Using immunohistochemistry and in situ hybridization techniques, 38 paraffin-embedded prostatic samples were analyzed and CaP was compared with prostate intraepithelial neoplasia (PIN) and its normal adjacent prostate (NAP) counterpart. The association of MMP protein and mRNA expression with Gleason histological tumor grade and TNM clinical stage was also determined. In most prostatectomy specimens examined, detectable amounts of MMP-1, MT1-MMP, MMP-7 and MMP-9 proteins and MT1-MMP and MMP-9 mRNA were found in the epithelial and stromal components of CaP, PIN and NAP. MMP expression was significantly stronger in the epithelium than in the stroma ($p<0.01$). In the epithelium of normal and preneoplastic prostate tissue, MMP-1, MMP-9 and MT1-MMP were preferentially expressed in secretory luminal cells; conversely, MMP-7 was concentrated in basal cells. Epithelial and stromal expressions of MMPs differed in normal, preneoplastic and CaP tissues. Whereas MMP-1 was overexpressed in NAP epithelial glands and progressively decreased from PIN to CaP, MMP-7, MMP-9 and MT1-MMP were more strongly expressed in CaP than in PIN and NAP tissue. The MMPs investigated reached their highest levels in prostate tumors with high Gleason scores. The differential MMP expression in epithelial and stromal prostate tissue supports the previous hypothesis that MMPs may be autocrine and paracrine mediators of the stroma-epithelial interaction, an event that plays a critical role in regulating normal and abnormal prostate growth. MMP gene regulation changes during the early stage of prostate cancer. Differential expression of MMP components in CaP may reflect the malignant phenotype and more aggressive tumor behavior.*

As proteolytic enzymes that mediate local extracellular matrix (ECM) and basement membrane degradation, matrix metalloproteinases (MMPs) participate in tumor invasion, metastasis and progression (1). MMPs belong to a family of more than 20 zinc endopeptidases capable of degrading numerous components of the ECM (2). MMPs are classified on the basis of their substrate specificity and protein structure. According to the chronological order in which they were identified, MMPs have been categorized into 4 groups: collagenases, degrade interstitial collagens (type I, type II and type III collagens); gelatinases, degrade basement membrane collagen (type IV collagen); stromelysins, degrade proteoglycans, fibronectin and laminin; matrilysins, have proteolytic activities against a wide range of substrates (proteoglycans, fibronectin, laminin and type IV collagen) and membrane-type (MT)-MMPs, directly digest some ECM components (including type I, II and III collagens, fibronectin, vitronectin and laminin) and are key enzymes in initiating ECM breakdown (3-6). The MT-MMPs contain a transmembrane domain anchoring the enzyme to the cell surface. MT-MMPs are involved in the activation of latent MMPs and also exhibit proteolytic activity against components of the ECM, potentially facilitating egress of cancer cells from a tumor deposit (6).

Current research, reappraising the role of MMPs in tumor invasion and metastasis, shows that, in addition to

Abbreviations: NAP, normal adjacent prostate; BPH, benign prostate hyperplasia; PIN, prostate intraepithelial neoplasia; CaP, prostate carcinoma; NISH, nonisotopic *in situ* hybridization; IHC, immunohistochemistry; MMP, metalloproteinase.

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ECM dissolution, MMPs play crucial roles in a variety of biological and pathological processes, including apoptosis, cell proliferation and cell differentiation (6, 7). Many factors (including growth factors and their receptors) interact with MMPs, as substrates of individual MMPs and mediators of apoptosis and cell adhesion molecules (8, 9), contributing to tumor development and progression (6). Hence, MMPs are not only responsible for the proteolytic dissolution of the basement membrane, an important step in metastasis, but also regulate tumor growth by maintaining growth factor access to the ECM and by regulating angiogenesis (10).

A positive correlation between tumor progression and increased MMP expression has been described in various human cancers, including prostate tumors (11). In prostate cancer, MMP mRNA and protein expression correlate with advanced or metastatic disease (12). Despite extensive research on MMPs in animal prostate tumors (13), malignant prostatic cells (14-18) and cell cultures of human prostatic tissue (19), few immunohistochemical and *in situ* studies have reported the expression profile of epithelial and stromal MMPs in human benign and malignant prostatic tissue. Information is therefore limited regarding MMP localization within prostate cancer tissue and their potential usefulness as prognostic markers in prostate cancer.

Our aim in this *in vivo* study was to characterize the epithelial and stromal expression of interstitial collagenase (MMP-1), gelatinase B (MMP-9) and matrilysin-1 (MMP-7) MMPs, and a membrane-type 1 (MT1)-MMP (as representatives of the 4 different groups of MMPs) in NAP, PIN and CaP tissues from 38 patients who had undergone radical prostatectomy and to assess immunohistochemical and *in situ* MMP levels by quantitative computer-assisted image analysis.

Materials and Methods

Tumor specimens. In a retrospective study, 38 formalin-fixed, paraffin-embedded prostatic specimens (all containing NAP, PIN and CaP areas), obtained from 38 patients (age range 51 to 88 years, mean age 68.11 years) with a diagnosis of CaP, who had undergone radical prostatectomy in the Department of Urology, University "La Sapienza", Rome, Italy, were examined. For pathological classification, tumors were graded using the Gleason system for histological grading of CaP (20): 1 tumor was Gleason score 5; 5 were score 6; 24 score 7; 7 score 8; and 1 was score 9.

The tumors were then evaluated clinicopathologically using the tumor-node metastatic (TNM) staging system (21): 28 tumors were organ-confined diseases (pT2) and 10 extraprostatic diseases (pT3). The samples analyzed were then arbitrarily divided into three histological groups: low (scores 5 and 6); intermediate (score 7); and high (scores 8 and 9) Gleason score tumors, and into two pathological stages: pT2 and pT3. PIN areas were graded as low-grade and high-grade. The reported values refer only to high-grade PIN.

Immunohistochemical (IHC) analysis. Ten serial sections (3 μm thick) from prostatic blocks were cut onto slides coated with 3 aminopropyl-ethyl-xylene (Sigma-Aldrich, S.R.L, Milan, Italy) and stained using the avidin-biotin-peroxidase complex (ABC) method (reagents from Dako S.p.A., Milan, Italy) as previously reported (22). The following primary mouse monoclonal antibodies (supplied by Chemicon International, Inc.) were used at 1:100 dilution and 4˚C overnight incubation: anti-MMP-1 (clone 41-1E5), IgG2a/k fraction; anti-MT1-MMP (human membrane type 1-MMP (clone 114-6G6) IgG1/k fraction; anti-MMP-9 (clone 56-2A4) IgG/K fraction; and anti-MMP-7 (Matilysin/PUMP-1)(clone ID-2) IgG_{2b} fraction. Sections stained with the anti-MMP-7 antibody were previously microwaved for 5 min at 750 W (two cycles). All reactions included appropriate positive controls (breast tissue) and negative controls (the primary antibody was replaced by normal swine serum), using biotinylated secondary antibodies and avidin-peroxidase reagents (Dako, S.p.A.) and chromogen diaminobenzidine-hydrogen peroxidase (DAB) (Sigma-Alderich, S.R.L).

Non-isotopic in situ hybridization (NISH) analysis. The specimens were *in situ* hybridized according to a previously described technique (22) using double fluorescein-isothio-cyanate (FITC) staining. The following hybriprobes™ human MMP-9, 29 bases (2006-2034) (23) and MT-MMP 28 bases (1144-1171)(24) of the coding region were used at a concentration of 1 pmol/ml 20 units (60 μl) per ml of hybridization buffer. To verify the specificity of the detection signal, positive and negative controls were included in each batch of sections. As positive controls, the probes Poly-d (T) and β-actin 29 bases (1092-1120) hybriprobe™ (supplied with the kit) were used in the same experimental protocol and processed in parallel with the main hybriprobes™, to validate the technique, the quality of the tissue, the integrity of mRNA and to permit semi-quantitative assessment of the signal. In the negative controls, the probes were replaced with hybridization buffer. For indirect immunogenic detection of the signal, the sections were incubated for 2 h in a humid chamber with anti-FITC antibody linked to alkaline phosphatase (Chemicon, Boehringer Mannheim) diluted 1/500 in block buffer, followed by two 10 min rinses in washing buffer at pH 9.5 (100 mM Tris HCl, 100 mM NaCl and 50 mM $MgCl_2$). Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) stock solution were used as chromogen substrate for visualization of the signal. The slides were counterstained with Mayer's hematoxylin and mounted with aqueous mounting medium.

Computer-assisted image analysis. Three representative areas of each sample (assessed by IHC and *in situ* analysis) were randomly selected for each tumor tissue sample from the three areas examined (NAP, PIN, CaP) in the light microscope Olympus Uplan FI (20x objective) and were captured with a digital camera (Nikon). Quantification of areas of interest was done with IMAGE-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA). With the use of the color-recognition software, the specific brownish color (immuno-histochemistry) or blue-black (*in situ* hybridization) for positive areas was selected. After selection, these areas were quantified (pixels/camp) using the histogram function of the software, as previously described (25, 26). For each field, the number of positive areas was expressed as a fraction of the epithelium-stroma area (positive areas divided by the overall field area). The integrated optical density (IOD) of the blue or the red staining was measured after standard OD calibration. Parallel serial negative control sections were measured and their optical densities were subtracted from those of the positively-stained sections, in each sample. The results are expressed in pixels and represent an average of the three visual fields. The error bars represent standard

error (SE). Data are given as optical density per unit surface area (1 μm^2). For each slide, the means+SE of ColorPercent area (ColorPercent=ColorArea/(tissueArea) 2x100) were calculated.

Statistical analysis. The results are reported as mean ± standard error (SEM) and are expressed in arbitrary densitometric units. The data were analyzed by the Multistat program (Biosoft, Cambridge, 1988). Chi-square analysis was used to test differences in the percentage of tumors expressing MMP-1, MT1-MMP, MMP-7 and MMP-9 proteins and MT1-MMP and MMP-9 mRNA in the epithelium and stroma. The paired Student's *t*-test and Chi-square test were used to analyze differential expression of MMP ligands in the epithelial and stromal compartments of the three areas examined. A possible correlation between the various MMP proteins and mRNA in the three neoplastic and non-neoplastic areas was determined by linear regression analysis using rank correlation. The Chi-square test was used to determine the association of MMP ligand levels with Gleason score histological grade and TNM clinical stages. *P* values less than 0.05 were considered to indicate statistical significance.

Results

MMP-1 protein expression. The MMP-1 protein detected in the cytoplasm of epithelial cells was diffusely localized in the luminal cells of prostatic neoplastic acini and in the ducts of NAP and PIN (Figure 1 A, B). MMP-1 immunoreactivity was also expressed in stromal tissue and blood vessels around NAP, PIN and in single stromal cells adjacent to CaP areas (Figure 1 C). MMP-1 expressions were significantly higher in the epithelium than in the stroma of benign and malignant areas (NAP, $p=0.0006$); (PIN, $p=0.0003$; and CaP, $p=0.0004$, by Chi-square test) (Table I) (Figure 2 A, B and C). NAP epithelial and stromal cells showed higher, though not significantly higher, MMP-1 immunoreactivity than PIN and CaP (Figure 3 A and B). In normal prostatic epithelium, the MMP-1 levels were significantly higher than MMP-7 ($p=0.0283$ by Student's *t*-test) (Figure 3 A).

Epithelial and stromal MMP-1 protein levels were significantly higher in high- (Gleason scores 8, 9) and intermediate- (Gleason score 7) grade than in low-grade (Gleason scores 4, 5, 6) CaP ($p=0.01$ by Chi-square test). Conversely, no correlation was found between MMP-1 expression and TNM stage.

MMP-7 protein expression. The immunolocalization of MMP-7 showed cytoplasmic staining in the epithelium and stroma of NAP, PIN and CaP tissues (Figure 1 D, E and F). Although some MMP-7 immunoreactivity was detected in luminal cells, it appeared concentrated mainly in the basal cells of NAP and PIN areas. In all prostatic tissues examined (NAP, PIN and CaP) the levels were significantly higher in the epithelium than in the stroma (NAP, $p=0.0028$; PIN, $p=0.0001$; CaP, $p=0.0001$ by Chi-square test) (Table I; Figure 2 A, B and C). MMP-7 expression significantly increased from NAP to PIN ($p=0.0032$) and from NAP to CaP, in the epithelium and stroma ($p=0.0013$; $p=0.0001$) (Figure 3 A and B). Epithelial MMP-7 expression differed in the three areas. In NAP, the MMP-7 levels were lower than those of MMP-1 ($p=0.0283$); in PIN, MMP-7 was more strongly expressed than MMP-9; and in CaP, MMP-7 was significantly more strongly expressed than MT1-MMP ($p=0.0381$ by Student's *t*-test) (Figure 3 A and B).

The epithelium and stroma of high-grade CaP contained significantly higher levels of MMP-7 protein than intermediate-grade (Gleason scores 8, 9 *vs* Gleason score 7, $p=0.002$; $p=0.0037$) and than low-grade tumors (Gleason scores 8, 9 *vs.* Gleason scores 5, 6, $p=0.0006$; $p=0.0182$ by Chi-square test). No correlation was found between TNM stages and MMP-7 immunoreactivity in the stroma. In contrast, epithelial MMP-7 protein levels were higher in more advanced TNM stage tumors (pT3 *vs.* pT2, $p=0.001$ by Chi-square analysis).

MMP-9 protein and mRNA expression. MMP-9 protein staining was identified in the cytoplasm of epithelial and stromal fibroblastic cells. In the prostatic acini and ducts of NAP and PIN tissue, MMP-9 proteins were expressed in secretory luminal cells rather than basal cells. In PIN, MMP-9 showed discontinuous staining in the basal cell layer. In CaP, MMP-9 immunostaining concentrated in the cytoplasm of neoplastic acini and the intensity increased in the lumen (Figure 1 G, H and I). Stromal tissue showed focal MMP-9 immunoreactivity. In all three areas examined, the MMP-9 levels were significantly higher in the epithelium than in the stroma (NAP, $p=0.0041$; PIN, $p=0.0203$; CaP, $p=0.0038$) (Table I) (Figure 2 A, B and C). In the epithelium of PIN, the MMP-9 protein was significantly less strongly expressed than MMP-7 ($p=0.0142$, Student's *t*-test) (Figure 3 A). As prostate tissue progressed from a normal to a premalignant state (high-grade PIN) into the malignant state (CaP), MMP-9 immunoreactivity increased in the epithelium, reaching significance only in the stroma ($p=0.0001$, by Chi-square test) (Figure 3 B).

MMP-9 mRNA hybridization signals were detected in the epithelium and stroma of all three tissues examined and were localized in the same sites as MMP-9 proteins (Figure 4 A, B and C). Epithelial and stromal MMP-9 mRNA hybridization signals increased non significantly as the prostate tissue progressed from NAP, to PIN and CaP (Figure 3 C and D). No significant difference was found in stromal MMP-9 protein and mRNA expression in the three areas examined. As the histological grade of the tumor progressed, epithelial and stromal MMP-9 protein and mRNA expressions increased: high-grade tumors (Gleason scores 8, 9) expressed MMP-9 mRNA and protein more strongly than lower-grade tumors (Gleason scores 5, 6) ($p<0.01$). While epithelial and stromal MMP-9 protein levels were higher in more advanced TNM stage tumors (pT3 *vs.* pT2, $p=0.0001$ and 0.006), the MMP-9 mRNA levels showed no relationship with TNM stage.

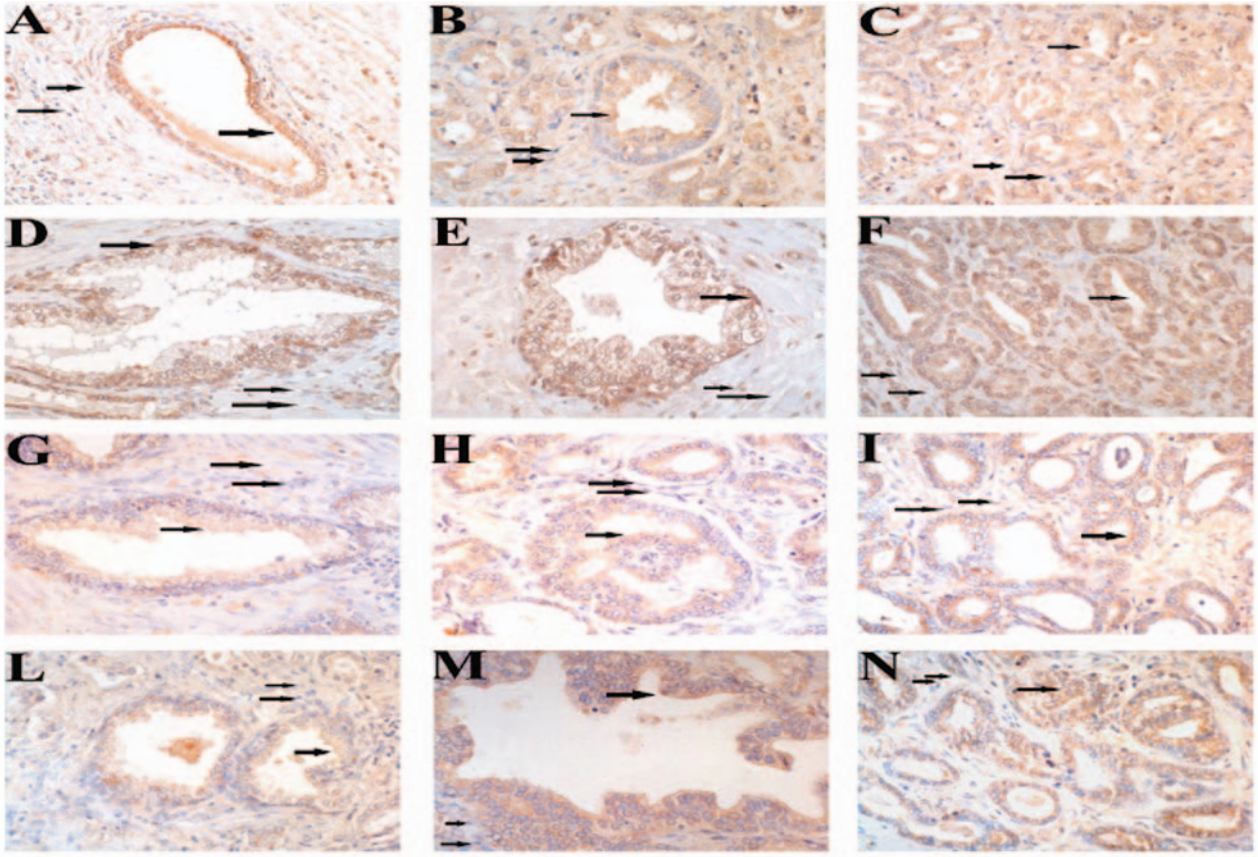

Figure 1. *Immunoreactivity for matrix metalloproteinase (MMP), MMP-1, MMP-7, MMP-9 and MT1-MMP proteins: A) Area of normal prostate (NAP) showing MMP-1 immunostaining localized mainly and diffusely in the secretory luminal cells (arrow). Stromal cells stained diffusely for MMP-1 (double arrows); B) Area of prostatic intraepithelial neoplasia (PIN) showing stronger MMP-1 immunoreactivity in luminal cells than in basal cells (arrow). Epithelial cells PIN stained more strongly than stromal cells (double arrows); C) Area of prostatic carcinoma (CaP) showing MMP-1 immunostaining in neoplastic acini (arrow) and in surrounding stroma (double arrows); the stroma contained less MMP-1 than the epithelial cells; D) Area of NAP showing MMP-7 immunostaining localized in basal cells (arrow) rather than in luminal cells. Epithelial cells stained more diffusely for MMP-7 than stromal cells (double arrows); E) Area of PIN showing a discontinuous MMP-7 staining pattern in the basal cell layer (arrow). Stromal cells showed diffuse MMP-7 staining (double arrows), but MMP-7 levels were lower in the stroma than in the epithelium; F) Area of CaP showing MMP-7 luminal staining (arrow) concentrated in the cytoplasm of neoplastic acini: the intensity of staining was higher in malignant epithelium than in the stroma (double arrows); G) Area of NAP showing MMP-9 immunostaining mainly in luminal cells (arrow). MMP-9 immunostaining was weaker in stromal than in epithelial cells; H) Area of PIN showing MMP-9 cytoplasmic membrane staining, localized on luminal cells (arrow). The surrounding stroma (double arrows) contained lower MMP-9 levels than the epithelium; I) Area of CaP. Note the cytoplasmic MMP-9 immunoreactivity in neoplastic acini and weaker staining in the surrounding stroma; L) Area of NAP showing MT1-MMP immunostaining mainly in luminal cells (arrow). MT1-MMP immunostaining was weaker in stromal (double arrows) than in epithelial cells; M) Area of PIN showing MT1-MMP cytoplasmic membrane staining, localized on luminal cells (arrow). The surrounding stroma (double arrows) contained lower MT1-MMP levels than the epithelium; N) Area of CaP. Note the cytoplasmic membrane staining for MT1-MMP in neoplastic acini (arrow) and weaker staining in the surrounding stroma (double arrows); (DAB X 400).*

MT1-MMP protein and mRNA expressions. Immunolocalization of the MT1-MMP showed cytoplasmic staining in the epithelium and stroma of NAP, PIN and CaP tissues (Figure 1 L, M and N). In most specimens (36/38) from NAP tissue, the MT-1 immunoreactivity appeared diffusely distributed, mainly in luminal secretory cells: the levels were significantly higher in the epithelium than in the stroma ($p=0.0128$ by Chi-square test) (Table I) (Figure 2 A, B and C). MT1-MMP immunoreactivity was also detected in PIN areas, luminal cells staining more intensely than basal cells. Stromal cells around PIN areas stained significantly less diffusely and intensely than epithelial cells ($p=0.0069$) (Table I). In CaP, epithelial and stromal cells exhibited MT1-MMP immunoreactivity; the epithelium stained more intensely than the stroma ($p=0.0203$) (Table I). In the epithelial and stromal compartments, the MT1-MMP expression increased, but not significantly, from NAP tissue to PIN, to CaP (Figure 3 A and B).

MT1-MMP mRNA hybridization signals were invariably cytoplasmic. In the epithelium of NAP and PIN, MT1-MMP

Table I. *Evaluation of metalloproteinase (MMP) protein and mRNA expressions in the epithelium and stroma of normal prostate tissue (NAP), intraepithelial prostatic neoplasia (PIN) and prostatic carcinoma (CaP) by computer-assisted quantitative image analysis.*

	NAP		PIN		CaP	
	Total area	Percentage (%) area stained mean±SE	Total area	Percentage (%) area stained mean±SE	Total area	Percentage (%) area stained mean±SE
MMP-1						
epithelium	12393.21±2058.39	30.31±2.59	15909.65±2489.35	27.36±2.81	12709.43±1914.32	26.36±2.81
stroma	12947.63±2204.47	9.96±1.95	9162.83±1164.89	6.85±1.71	10104.34±2028.29	6.88±2.89
MMP-7						
epithelium	15061.15±2514.37	17.12±2.18	18619.59±3101.67	35.44±2.49	15300.89±2024.02	37.42±4.13
stroma	10395.96±983.71	3.32±0.60	9510.36±980.91	5.91±0.67	5827.68±744.19	10.83±1.55
MMP-9						
epithelium	9716.98±15759.95	18.98±1.6	13083.48±1665.71	19.92±2.20	19268.82±2427.17	28.54±3.53
stroma	12955.44±2135.11	4.83±0.80	7260.20±306.05	7.61±1.35	9121.78±1367.95	12.09±2.52
mRNA MMP-9						
epithelium	15555.48±1871.52	20.61±2.34	12007.64±1673.53	22.99±2.00	17407.73±2645.04	30.09±2.50
stroma	11141.33±1682.87	13.08±1.46	10308.92±1705.74	13.95±1.74	5667.69±749.77	22.48±3.18
MT1						
epithelium	13806.60±1903.19	19.74±2.31	16118±2251.53	23.30±2.90	13343.66±2154.03	23.90±2.53
stroma	11698.65±1632.93	7.48±1.86	12826.98±2517.40	8.36±1.75	9335.27±1659.05	11.90±2.07
mRNA MT1						
epithelium	13317.54±1864.99	24.71±2.21	14203.63±2056.44	26.64±2.04	15266.54±2672.31	30.01±3.74
stroma	17043.72±3261.33	13.16±1.81	13202.78±1983.11	14.35±2.34	8414.22±1236.98	18.19±3.21

by Chi-square test
*$p<0.05$
*$p<0.01$

mRNA tended to localize in the luminal rather than basal cells. In NAP, PIN and CaP, the epithelium gave a higher signal than the stroma ($p=0.0385$; $p=0.0313$; $p=0.0507$ by Chi-square tests) (Figure 2 D, E and F). In the epithelium and stroma, the MT1-MMP mRNA signals increased, but not significantly, from NAP tissue to PIN to CaP. In the epithelium of PIN and CaP, the MT1-MMP protein levels were lower than those of MMP-7 ($p=0.0595$; $p=0.0381$, by Student's *t*-test). No significant differences were found between epithelial and stromal MT-1 protein levels and mRNA expression.

The epithelium of high-grade tumors (Gleason scores 8, 9) expressed MT1-MMP mRNA and protein more strongly than that of low-grade tumors (Gleason scores 5, 6) ($p=0.032$; $p=0.0143$). Stromal MT1-MMP mRNA levels were significantly higher in high-grade and more advanced stage tumors (pT3) than in low-grade and less advanced stage tumors (pT2) ($p=0.00$; $p=0.031$).

Linear regression analysis disclosed a positive correlation between MMP-1 and MMP-9 levels ($p=0.0189$; r=018) in the epithelium and between MMP-1 and MMP-7 levels in the stroma of NAP ($p=0.020$; r=0.037); but a negative correlation between MMP-1 and MMP-7 in the epithelium of PIN ($p=0.055$ r =–0.030). MT-1 MMP levels correlated weakly with those of MMP-1 and MMP-9 in the epithelium of CaP ($p=0.030$, r=0.034; $p=0.04$, r =0.032, respectively).

Discussion

The findings obtained by immunohistochemistry and *in situ* hybridization and assessed by quantitative computer-assisted image analysis clearly show differential epithelial and stromal MMP gene expression in the three areas of radical prostatectomy samples examined, NAP, PIN and CaP. Our *in vivo* study therefore further underlines the involvement of the MMP axis in human prostate cancer development and progression and its correlation with advanced prostatic disease.

Quantitative image analysis of our immunohistochemical and *in situ* prostatic samples showed that the four MMPs studied – MMP-1, MMP-7, MMP-9 and MT1-MMP – were expressed mainly in the epithelial cells of the three prostate areas analyzed. Evidence of stronger epithelial than stromal

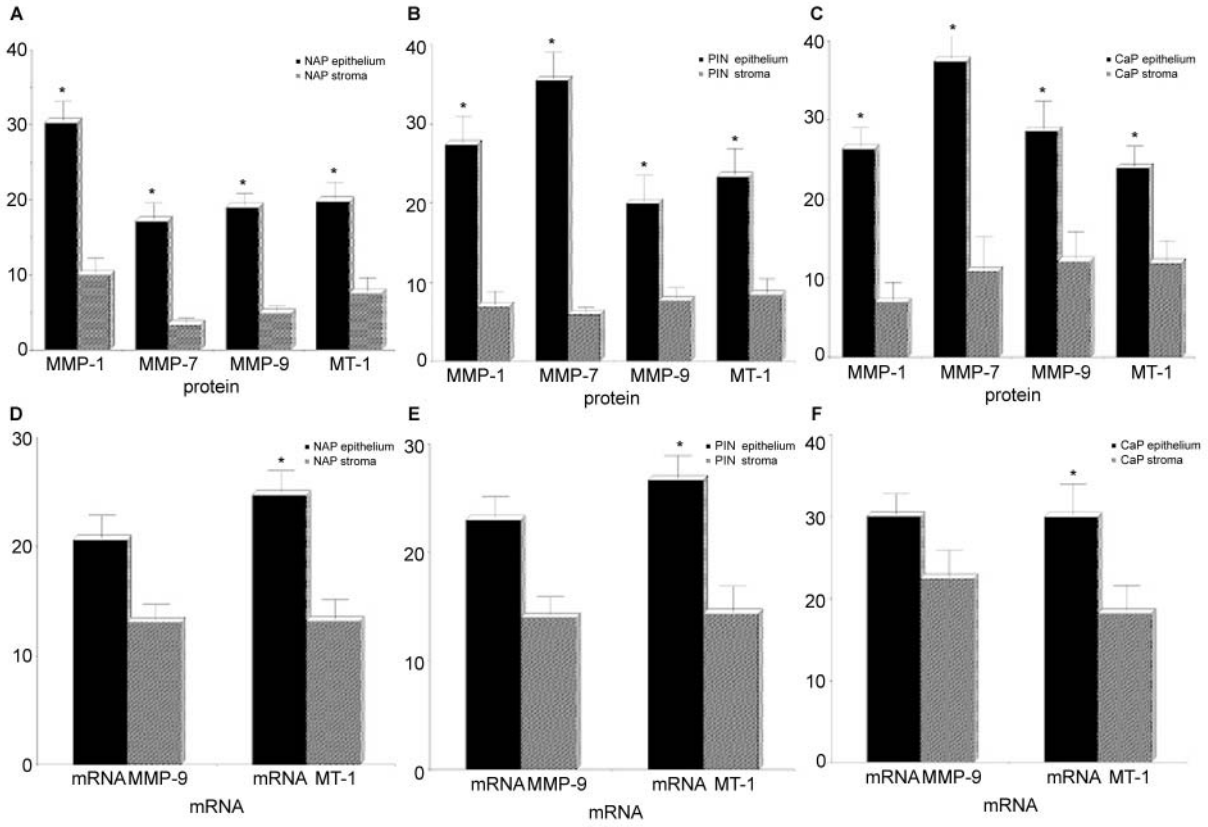

Figure 2. *MMP-1, MMP-7, MMP-9 and MT1-MMP protein and mRNA levels in 38 prostatectomies: comparison between the epithelium and stroma of normal prostate (NAP), prostatic intraepithelial neoplasia (PIN) and prostatic carcinoma (CaP) tissues: A) NAP: normal prostatic epithelial tissue yielded significantly higher MMP-1, MMP-7, MMP-9 and MT1-MMP expressions than in the stroma ($p<0.01$); B) PIN tissue: intraepithelial prostatic tissue gave significantly higher MMP-1, MMP-7, MMP-9 and MT1-MMP levels than in the stroma around PIN ($p<0.01$; $p<0.05$) C) CaP areas: the intensity of MMP-1, MMP-7 and the MMP-9 immunostaining and MT1-MMP protein were significantly greater in the epithelium than in the stroma ($p<0.01$; $p<0.05$) D) NAP showed a significantly higher MT1-MMP mRNA signal in the epithelium than in the stroma ($p<0.05$); E) PIN tissue: note the significantly higher MT1-MMP mRNA signal in the epithelial than in the stromal cells ($p<0.05$) F) CaP tissue: the MT1-MMP mRNA signal was significantly higher in epithelial neoplastic acini than in the stroma ($p<0.05$ by Chi-square test).*

MMP expression in prostate tissues indicated that MMPs are synthesized and secreted by carcinoma as well by stromal cells (12, 27-28).

Whereas some studies, in agreement with our findings, reported significantly higher MMP-1, MMP-7, MMP-9 and MT1-MMP levels in the epithelium than in the stroma of CaP (12), others, on a wide variety of cancer tissues, reported that MMP-1 expression was concentrated mainly in the stromal cells (fibroblasts, monocytes, macrophages) (29-33). Increased MMP-9 expression has been reported in studies on human prostatic cell cultures. MMP-9 is up-regulated at the transcriptional level in murine prostate carcinoma cells (14). Whether the tumor cells themselves or the stroma surrounding the tumor cause change is controversial. Co-cultures of prostate cancer cells with stromal cells derived from various sources could induce MMP-9 expression in prostate cancer cells at the transcriptional level, resulting in higher levels of pro-MMP-9 protein in the culture media (14). The discrepancies presumably reflect the various quantitative methods used (ELISA, immunoblotting and quantitative real-time PCR) and samples examined and reflect the neoplastic heterogeneity of prostate tissue. In our immunohistochemical and *in situ* hybridization study, computer-assisted image analysis enabled us to document and quantify MMP protein in the epithelium and stroma of the benign and malignant human prostate cancer tissues studied.

All the prostate tissues examined also expressed higher epithelial than stromal levels of matrilysin (MMP-7). Matrilysin is produced by human prostate cancer, is catalytically active and, therefore, plays a role in tumor progression (34). Matrilysin is capable of degrading a diverse set of ECM proteins (35) and its expression enhances the invasiveness of prostate cancer cell lines (34). Why prostate cancer cells overexpress matrilysin is unknown. A plausible reason is that paracrine factors from cells within the surrounding stroma stimulate matrilysin secretion (15).

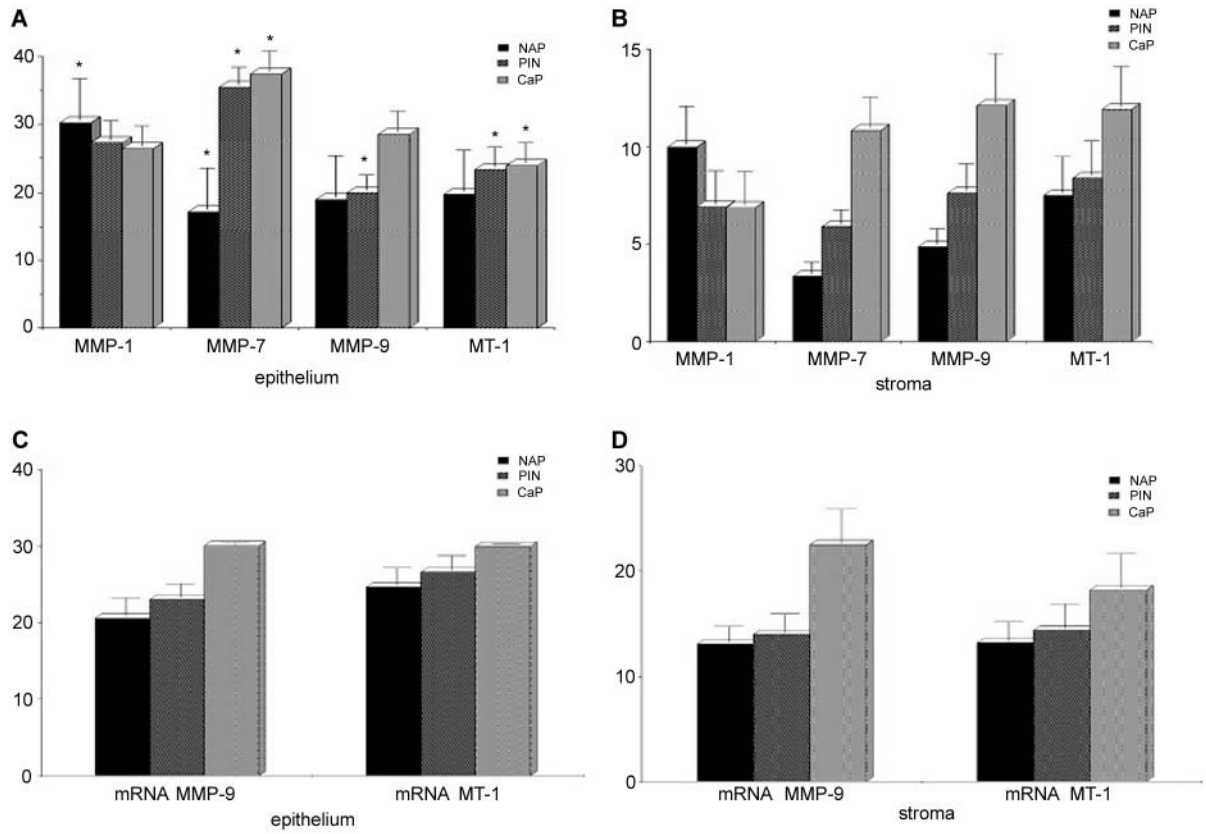

Figure 3. *Comparison of MMP-1, MMP-7, MMP-9 and MT1-MMP protein and mRNA expressions in the epithelial and stromal compartments of normal prostate (NAP), prostatic intraepithelial neoplasia (PIN) and prostatic carcinoma (CaP) tissues from 38 radical prostatectomies. In the epithelium (A) of NAP, MMP1 immunoreactivity was significantly higher than MMP-7 ($p=0.0283$ by Chi-square test) (asterisk); in PIN, MMP-7 immunoreactivity was significantly higher than MMP-9 and MT1-MMP ($p=0.0142$; $p=0.0595$); in CaP, MMP-7 immunoreactivity was higher than MT1-MMP ($p=0.0381$) (asterisk). The comparison between NAP, PIN and CaP areas by Chi-square test showed that in the epithelium: A) MMP-7 intensity scores increased significantly from NAP to PIN ($p=0.0032$) and from NAP to CaP ($p=0.0013$) (asterisk); in the stroma B) MMP-7 and MMP-9 increased significantly from NAP to PIN, and from NAP to CaP ($p<0.001$ by Chi-square test) (asterisk). C) MMP-9 and MT1-MMP mRNA increased, but not significantly, from NAP to PIN to CaP in the epithelium and D) in the stroma.*

Matrilysin, like the other members of the MMP family, is secreted in a latent pro-enzyme form. The range of substrates that matrilysin can directly degrade suggests that minimal matrilysin activity may greatly influence the invasive potential of the cells expressing this enzyme (15).

Scarce data are available on the gene expression of the other MMPs we studied, *i.e.* MT1-MMP, in prostate cancer (16, 19, 36). MT1-MMP appears important not only for destroying a variety of ECM molecules, but also for activating the latent form of secreted pro-MMP-2 (progelatinase A) and indirectly of pro-MMP-9 (pro-gelatinase B) (37). Finally, MT1-MMP alters cell properties and motility (38, 39). In our study, MMP-1 immunoreactivity was found in both the epithelium and stroma. This localization contrasts with previous studies reporting MT1-MMP and MMP-2 almost exclusively localized to the surrounding stroma and not to malignant tumor cells (19, 40). The stromal and epithelial cell MT1-MMP immunoreactivity found suggests that both the epithelium and stroma synthesize MT1-MMP and that paracrine and autocrine factors play an important role in regulating MT1-MMP in human prostate cancer cells. The restriction of matrilysin to the prostatic epithelium is characteristic of this MMP (34). The presence of MT1-MMP protein and mRNA in stromal and endothelial cells surrounding the neoplastic tissue may indicate that stromal and tumoral cells co-operate in facilitating invasion. In prostate cancer cells, as in other tumors (41), stromal cells can themselves produce MMPs or factors that induce MMP production, or both. *In vitro* studies on co-cultures with human fibroblasts demonstrated that cell-cell contact between carcinoma cells and fibroblasts enhances the production of MT1-MMP, induced by the fibroblast growth factor-1 (17).

Another finding relevant to the differential expression of MMPs in prostate cancer was that consistent changes in the

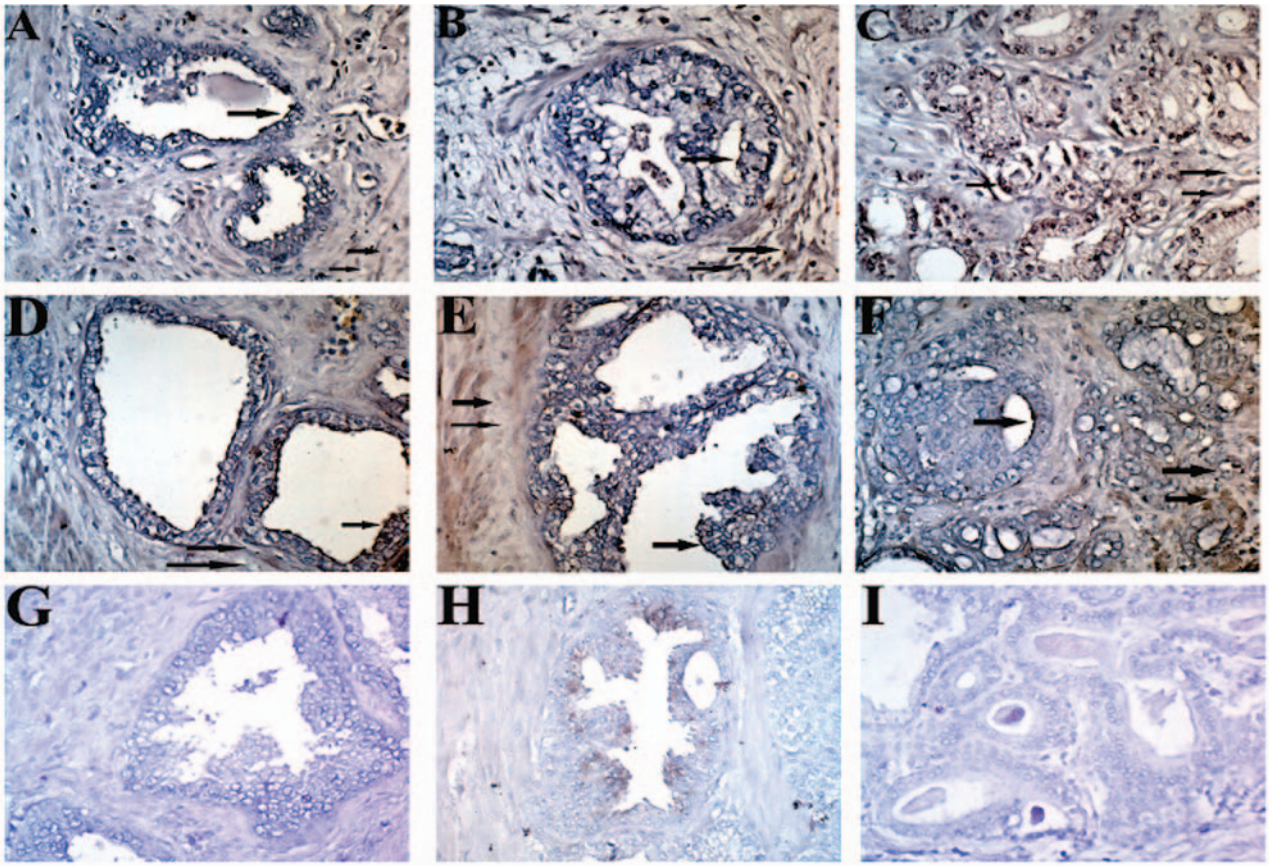

Figure 4. *In situ hybridization with non-isotopic MMP-9 and MT1 mRNA probes. A) Areas of normal prostate (NAP) showing MMP-9 mRNA localized in the luminal surface (arrow) of glands with a weaker signal than in the stroma (double arrows); B) Area of intraepithelial prostatic neoplasia (PIN) showing MMP-9 mRNA localized in luminal cells (arrow) and stroma containing larger amounts of MMP-9 mRNA than the epithelium; C) Area of prostatic carcinoma (CaP) showing a stronger MMP-9 mRNA signal in the epithelium (arrow) than in the surrounding stroma (double arrows); D) Area of NAP showing a stronger mRNA MT1-MMP signal in the luminal cells (arrow) of the glandular acini than in the surrounding stroma (double arrows); E) Area of PIN showing MT1-MMP mRNA localized in luminal cells (arrow), with a stronger signal in the epithelium than in the surrounding stroma (double arrows); F) Area of CaP showing a higher mRNA MT1-MMP signal in neoplastic acini (arrow) than in the supporting stroma (double arrows); G) Area of NAP; H) Area of PIN; and I) Areas of CaP (negative controls) (NTB/BCIPX400).*

localization and intracellular distribution of MMPs were associated with the transition from NAP to PIN to CaP. Whereas epithelial and stromal MMP-1 expressions decreased as prostate tissue progressed from a normal to a malignant state, MMP-7, MMP-9 and MT1-MMP expressions increased. Interestingly, epithelial MMP immunoreactivity distinctly differed in the three areas: MMP-1 was higher than MMP-7 in NAP, MMP-7 was higher than MMP-9 and MT1-MMP in PIN and MMP-7 was higher than MT1-MMP in CaP. These findings suggest that MMP-7 undergoes its functional change at an early stage of neoplastic transformation (in PIN tissue).

Changes in MMP-7 expression beginning early in neoplastic transformation also receive support from the differential cellular expression of MMPs which were found in normal prostate and PIN tissue compared with CaP. Whereas MMP-1, MMP-9 and MT1-MMP were preferentially expressed in the luminal secretory cells of NAP and PIN, MMP-7 was concentrated in the basal cell layers. Basal cell MMP-7 immunoreactivity indicates that in normal prostate and in the early stage of carcinogenesis the MMP signaling pathway remains intact. In NAP tissue, matrilysin expression was restricted to atrophic glands and was associated with inflammation (34, 42-43); in CaP, matrilysin was non-uniformly expressed by the tumor cells. Enhanced, but focal MMP-7 expression has also been observed by others in prostate cancer cell lines and other tumors (7, 44). The localization of MMP-1, MMP-9 and MT1-MMP observed in secretory luminal cells of NAP and PIN suggests that regulation of these enzymes is altered during the earliest stages of prostate cancer. Others, nevertheless, reported uniform MT1-MMP staining in basal cells of benign and in secretory cells of PIN glands (36), confirming that the transition from benign epithelium *via* PIN to cancer is associated with alterations in the localization and intracellular distribution of MT1-MMP in prostate epithelium.

The positive correlation between MMP-1 and MMP-9 in the epithelium of NAP, and between MMP-1 and MMP-7 in the stroma of NAP and the negative correlation between MMP-1 and MMP-7 in the epithelium of PIN suggest that MMPs closely interact with each other in all steps of carcinogenesis and CaP progression.

On investigation of the association of MMPs with tumor grade and clinical stage, a strong relationship was found between MMP-1, MMP-7, MMP-9 and MT1-MMP expressions and Gleason grade: the ratio significantly increased as the Gleason score increased. Whereas no differences were found in MMP-1 expression between organ- confined and advanced CaP, a strong association was found with the pathological stage for MMP-7 in the epithelium and for MMP-9 in the epithelium and stroma of MT1-MMP mRNA in the stroma. The stroma of advanced extraprostatic tumors (pT3) contained higher MT1-MMP mRNA signals than the stroma of organ-confined tumors (pT2). Some researchers have also reported a correlation of increased expression of MMP-1, MMP-7 and MMP-9 with the Gleason histological grade and pathological stage (6-7, 43, 45-46), while others reported a lack of correlation between MMP-1 and MT1-MMP expressions and these parameters (12, 36). Enhanced expressions of MMP-7 and MMP-9 have also been observed in many tumors (7, 33, 47-53), supporting the hypothesis that in prostate carcinomas, as in many other tumors, large quantities of MMP-7 and MMP-9 promote invasion and metastasis (10).

In conclusion, these findings provide evidence of differential MMP expression as prostate cancer progresses and suggest that MMPs may be autocrine and paracrine mediators of the stroma-epithelial interaction, an event that plays a critical role in regulating normal and abnormal prostate growth. The results also showed that MMP overexpression is associated with biologically more aggressive prostate cancer. Increased MMP production is an intrinsic property of invasive prostate cancer cells and could be used as a marker to differentiate between less aggressive and invasive prostate cancer.

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