Urinary gelatinase activities (matrix metalloproteinases 2 and 9) in human bladder tumors

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Abstracts. The ability to degrade type IV collagen, the major component of the basement membrane, is unique to gelatinases A and B. These two matrix metalloproteinases (MMPs) are most often linked to the malignant phenotype of tumor cells, and their expression is elevated in several cases of human tumor aggressiveness and overall survival. By gelatin zymography, we verified MMP activity in the urine of patients with bladder cancer. Of these patients, 10 had well-, 8 had moderately and 7 had poorly differentiated bladder cancer. The urine of healthy volunteers with no evidence of disease was used for controls. Zymography showed five dominant gelatinolytic bands of 240, 220, 130, 92 and 72 kDa in tumor samples, whereas only traces of MMP were detected in the urine of healthy subjects. The majority of cancerous urine samples showed MMP-9 lytic activity but only a few contained MMP-2. Moreover, MMP-9 content is enhanced in the urine from patients with high-grade and advanced-stage bladder tumors. Finally, we determined the urinary levels of urinary bladder cancer (UBC), tissue polypeptide-specific antigen (TPS) and protein 22 of nuclear matrix (NMP22). The levels of TPS and NMP-22 were higher in G3 bladder cancer than in G1 and G2 neoplasias. The urinary values of these two biomarkers correlated with the increase in MMP-9 lytic activity in high-grade and advanced-stage bladder cancer.

Introduction

The diagnosis of bladder cancer is primarily based on combined information provided by urinary cytology and cystoscopy (1,2). Cytological examination of urine involves the assessment of morphological changes in tumor-derived cells among the exfoliated cells in urine. This morphology-based examination is specific for the diagnosis of bladder carcinoma but inevitably demonstrates low sensitivity in patients with low-grade tumors because the cytological abnormality is slight (3). To date, cystoscopy remains the gold standard when diagnosing malignancies of this organ. This examination is required not only for diagnosis but is repeated at 3-month intervals in follow-up because no other method available is adequately sensitive and specific. Cystoscopy is an invasive, relatively costly and uncomfortable diagnostic method, which may also be inconclusive at times, particularly in the case of cystitis (4). Therefore, there is a pressing need for non-invasive methods to diagnose carcinoma of the bladder, as well as for follow-up surveillance because of its high progression and recurrence rates. Urinary tumor markers represent an option of interest for early detection and surveillance of the disease because of sample accessibility and the direct contact of urine with the tumor. Several classes of proteins have been investigated with variable results (5,6 and refs. therein). In particular, nuclear matrix proteins (NMPs) and cytokeratins (CKs) have gained the widest acceptance.

Continuous changes in gene products are associated with the transition from the normal urothelium layering on a stable basement membrane to benign hyperplasia and then to transitional cell carcinoma of the bladder and basement membrane rupture. Increased expression of two basement membrane collagen IV-degrading matrix metalloproteinases (MMPs), i.e. 72- and 92-kDa gelatinases known as gelatinases A and B respectively, appears to be associated with invasiveness and disease progression (7-9). The ability to degrade type IV collagen, the major component of the basement membrane, is unique to gelatinases A and B. These two MMPs are most often linked to the malignant phenotype of tumor cells, and their expression is elevated in several cases of human tumor aggressiveness and overall survivall (10,11). Studies have reported that these two MMPs are enhanced in the sera and urine of patients with cancer (12,13).

In the present preliminary study, we determined MMP-2 and MMP-9 activity levels in urine from patients with bladder carcinoma and healthy volunteers using gelatin zymography. These levels were compared with traditional urinary cytology and the urinary levels of urinary bladder cancer (UBC), tissue polypeptide-specific antigen (TPS) and protein 22 of nuclear matrix (NMP22) with the following objectives: a) to

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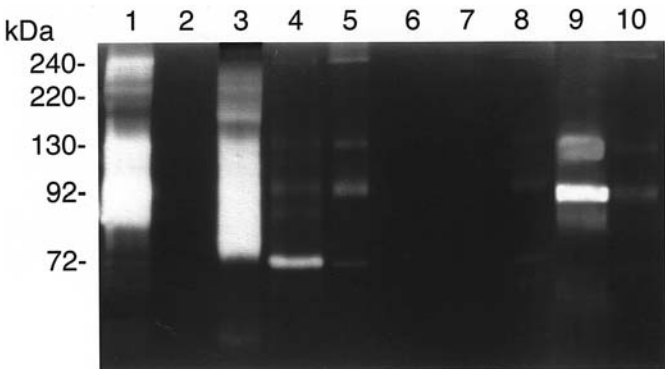

Figure 1. Gelatin zymography of bladder cancer urine specimens. Molecular weight standards are shown on the left. Lane 1, T4 G3 bladder cancer (patient MA); lane 2, T4 G3 (patient MA) in presence of EDTA 20 mM; lane 3, T1 G3 (patient DG); lane 4, Ta G1 (patient FE); lane 5, Ta G2 (patient DM); lane 6, T4 G3 (patient MA) in presence of 1.10 phenathroline 10 mM; lane 7, T1 G3 (patient DG) in presence of 1.10 phenathroline 10 mM; lane 8, T1 G2 (patient DP); lane 9, T3 G3 (patient SS); lane 10, Ta G1 (patient ZZ).

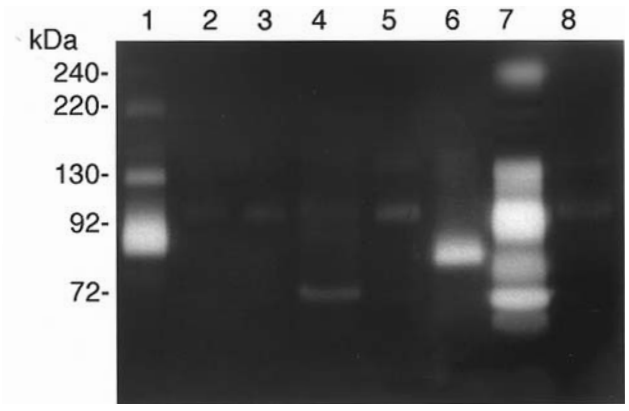

Figure 2. Gelatin zymography of bladder cancer urine specimens. Molecular weight standards are shown on the left. Lane 1, T4 G3 (patient PC); lane 2, Ta G2 (patient PP); lane 3, Ta G1 (patient VL); lane 4, Ta G1 (patient EE); lane 5, Ta G2 (patient DD); lane 6, T3 G3 (patient TT); lane 7, T4 G3 (patient RL); lane 8, Ta G1 (patient ZM).

support the diagnostic and prognostic value of urinary MMP-2 and MMP-9 in bladder cancer; and b) to determine the possible association of activity levels of urinary forms of gelatinases with the current clinical parameters and urine markers in use for bladder cancer disease management.

Materials and methods

Urine collection. Patients were chosen for the study, their first morning urine samples were collected, and the clinical status was verified by a urologist. Urine was collected from healthy volunteers (112 samples to determine tumor marker cut-off values, and 20 samples tested for MMP activity) with no evidence of disease (NED) and patients who underwent cystoscopy or surgical procedures at the Department of Urology of the Faculty of Medicine of the 2nd University of Naples. The diagnosis of tumors was made using the usual clinical laboratory criteria and confirmed by histopathological findings. The age of patients was between 46 and 86 years, and there were 23 males and 2 females. The tumors were histologically classified for grade and stage according to the pTNM classification (2). Prior to analysis, urine samples were tested using the Multistix Combur test (Roche Diagnostic GmbH Mannheim). Urine samples positive for leukocytes were excluded because of confounding leukocytic gelatinases. Microscopic hematuria present in most cancer samples was not quantified but grossly hematuric samples were excluded.

Urine sample preparation. Samples were frozen immediately after collection and stored frozen (-20˚C) until assay. The samples were thawed and an aliquot of each sample (15 ml) was centrifuged at 3200 rpm for 10 min at 4˚C, and the supernatant creatinine concentrations were determined using a commercial kit according to the manufacturer's instructions. An aliquot of the supernatant from each sample (2 ml) was dialyzed against double-distilled water in 16-mm dialysis tubing with a molecular weight cut-off (MWCO) of M_r 3500 overnight. Following dialysis, urine samples were centrifuged at 3200 rpm for 10 min at 4˚C, the supernatant was collected and aliquots were stored at -20˚C until used to detect tumor markers (UBC, TPS and NMP22) and matrix metalloproteinases (MMP-2 and MMP-9).

Materials. Gelatinases A and B were purchased from Hoffmann-La Roche Ltd. (Basel, Switzerland). Triton X-100, calcium chloride ($CaCl_2$) glycerol, gelatin, ethylene-diamine-tetraacetic (EDTA), and phenylmethylsulphonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO, USA). The creatinine assay reagent was from Sentinel Diagnostic CH. (Mi, Italy). All other reagents were available from commercial sources.

Gelatin zymography. Zymography was performed using 7.5% (w/v) polyacrylamide gels containing 0.1% (w/v) of gelatin. The amount of urine loaded on the gel was between 2 and 12 μl depending on the creatinine content. Urine samples were mixed with 5X sample buffer (10 mM Tris-HCl pH 6.8, 12.5% SDS, 5% sucrose, and 0.1% bromophenol blue) and applied directly without prior heating or reduction of the gel. After removing SDS from the gel by incubation in 2.5% (v/v) Triton X-100 for 1 h, the gels were incubated at 37˚C for 18 h in 50 mM Tris-HCl pH 7.6 containing 0.2 M NaCl, 5 mM $CaCl_2$, and 0.02% (w/v) Brij 35. Gels were stained for 1 h in 30% methanol, 10% glacial acetic acid containing 0.5% (w/v) Coomassie Brilliant Blue G250 and destained in the same solution without dye for several hours. Gelatinolytic activity of each collagenase was evident as a clear band against the blue background of stained gelatin. The molecular size of bands displaying enzymatic activity was identified by comparison with prestained standard protein, as well as with purified gelatinase A or gelatinase B. Following zymography, the degree of gelatin digestion was quantified using image analysis software (ImageQuant TL; Amersham, Chicago, IL, USA) according to the manufacturer's specifications. To normalize the possible difference between zymograms, an internal urine sample from a patient was incorporated in every gel. Urine gelatinase activity was expressed as the integrated density $x10^{-3}$ (volume) of all pixels above the background of each band.

Table I. Urinary MMP content related to UBC, TPS, and NMP22 in grade 1 human bladder cancer.

					Volume x10^{-3}							
Patient	Sex	Age (years)	Stage	Malignity	MMP 240 kDa	MMP 220 kDa	MMP 130 kDa	MMP-9 92 kDa	MMP-2 72 kDa	UBC ng/mg cr	TPS mU/mg cr	NMP22 U/mg cr
FE	M	69	Ta	Low	0	0	27	93	732	259.0	3292	2.1
ZZ	M	60	Ta	Low	23	0	32	109	0	8.0	303	4.5
DR	F	79	Ta	Low	0	0	0	0	0	13.2	627	7.1
NL	M	63	Ta	Low	0	0	0	0	0	23.0	362	8.1
MS	M	59	Ta	Low	0	0	0	0	0	7.9	82	1.8
MA	M	72	Ta	Low	0	0	0	0	0	5.4	219	2.6
DC	M	68	Ta	Low	0	0	0	0	0	20.0	350	9.1
VL	M	66	Ta	Low	0	0	0	41	0	34.0	146	4.7
EE	M	62	Ta	Low	0	0	0	50	142	210.0	760	3.3
ZM	F	68	Ta	Low	0	0	19	66	0	10.6	691	3.8

Measurement of tumor markers. UBC was detected using an immunoradiometric method with a commercial kit measuring CK8 and CK18, and values were expressed as ng/mg creatinine. TPS was detected by an immunoradiometic method with a commercial kit using a monoclonal antibody reacting with the epitope M3 of CK18. Commercial kits were obtained from IDL Biotech AB, Bromma, Sweden (UBC), and Byk Gulden, AB, Sangtec Medical Bromma, Sweden (TPS). NMP22 was determined using the Immulite analyzer with a commercial kit using antibodies that recognize the head domain of nuclear mitotic apparatus protein (NuMA) (Diagnostic Products Co., Los Angeles, CA, USA).

Results

During a 1-year period, a total of 25 urine samples from patients with bladder cancer were evaluated. The patients were divided into three groups according to tumor grade: grade 1, well-differentiated; grade 2, moderately differentiated; and grade 3, poorly differentiated. Further, on the basis of cytology results carried out as a routine laboratory procedure, the cases were classified as low, medium or high for the presence of malignant cells.

To investigate the gelatinolytic activity present in urine, substrate gel zymography was performed. Representative zymography results are shown in the figures. Polyacrylamide gels were evaluated for the presence of a clear zone representing degradation of gelatin by proteolysis. Gelatin zymography identified that MMPs were present in the urine of many patients with bladder carcinoma, whereas only traces of MMP could be detected in the urine of healthy subjects with no evidence of disease (NED). Five dominant proteinases were detected migrating at approximately 240, 220, 130 and 92 kDa (MMP-9) and 72 kDa (MMP-2). Comparison of these gelatinolytic bands with prestained standard protein and purified gelatinase A (MMP-2) and gelatinase B (MMP-9) clearly identified the MMP-constituting bands as gelatinase A (MMP-2; 72 kDa) and gelatinase B (MMP-9; 92 kDa). The clear zones with a molecular weight higher than 92 kDa might represent complexes of MMPs that are not dissociated in zymography. In fact, MMP-9 can be associated with a 25-kDa protein with a band at 125 kDa (14,15) that can form a dimer or multidimer with lytic bands at approximately 215 and 240 kDa (16). Several MMPs together can also form complexes that can only be identified with specific antibodies in Western blot analysis. However, because zymography is much more sensitive than Western blot analysis, it has been difficult to find antibodies that are sensitive enough to detect small amounts of MMPs. Figs. 1 and 2 show a panel of zymograms from bladder cancer urine specimens at various stages and grades. The nature of lytic bands observed in zymograms was confirmed by inhibition assays. Selective inhibitors of MMPs excluded the presence of gelatinolytic activities due to serine or cysteine proteinases. In the presence of EDTA 20 mM or 1,10 phenathroline 10 mM, two selective inhibitors of MMPs, the gelatinolytic bands disappeared (Fig. 1, lanes 2, 6 and 7). In contrast, bands of gelatinolytic activity were unaffected by the addition of PMSF enzyme buffer, an inhibitor of serine proteases (data not shown). This inhibition profile clearly attributes gelatinolytic activity in urine to the presence of gelatinases.

Following gelatin zymography, the proteolytic bands were subjected to densitometric analysis. The data were normalized to an internal urine standard and expressed as the integrated density of all pixels of each band (volume x10^{-3}). A summary of expression patterns of each proteinase by tumor grade is shown in the tables. In the well-differentiated group (grade 1), zymographic analysis revealed the existence of a 72-kDa lytic band in two urine specimens (20%) (Fig. 1, lane 4; Fig. 2, lane 4) with a value of 732 and 142, respectively, whereas the 92-kDa lytic band was detected in 5 (50%) samples and lytic activity ranged between 41 and 109. Three samples showed a lytic band of approximately 130 kDa and one showed a band at the top of the gel of 240 kDa (Table I). In the moderately (grade 2) differentiated group, two samples (25%) showed a small lytic band of 72 kDa (Fig. 1, lanes 5 and 8), four (50%) showed a lytic band of 92 kDa, two showed a band of approximately 130 kDa and only one showed a band at the top of the gel (240 kDa; volume 51x10^{-3}) (Table II). In the poorly differentiated group (grade 3), the lytic activity

Table II. Urinary MMP content related to UBC, TPS, and NMP22 in grade 2 human bladder cancer.

					Volume x10^{-3}							
Patient	Sex	Age (years)	Stage	Malignity	MMP 240 kDa	MMP 220 kDa	MMP 130 kDa	MMP-9 92 kDa	MMP-2 72 kDa	UBC ng/mg cr	TPS mU/mg cr	NMP22 U/mg cr
AG	M	81	T1	Medium	0	0	0	0	0	15.4	821	4.7
DM	M	46	Ta	Medium	51	0	87	245	39	264.0	7682	54.4
DP	M	86	T1	Medium	0	0	0	30	15	4.4	282	16.8
MM	M	63	Ta	Medium	0	0	0	0	0	17.0	648	6.1
GA	M	80	T1	Medium	0	0	0	0	0	10.0	299	7.7
DT	M	50	Ta	Medium	0	0	0	0	0	13.0	345	12.1
PP	M	71	T1	Medium	0	0	0	21	0	8.0	204	5.8
DD	M	61	Ta	Medium	0	0	32	103	0	184.0	1280	35.8

Table III. Urinary MMP content related to UBC, TPS, and NMP22 in grade 3 human bladder cancer.

					Volume x10^{-3}							
Patient	Sex	Age (years)	Stage	Malignity	MMP 240 kDa	MMP 220 kDa	MMP 130 kDa	MMP-9 92 kDa	MMP-2 72 kDa	UBC ng/mg cr	TPS mU/mg cr	NMP22 U/mg cr
DS	M	58	T3	High	0	0	0	0	0	16	948	6.8
MA	M	73	T4	High	491	129	0	6103	0	53	540	3.0
DG	M	60	T1	High	0	135	148	6985	0	164	3224	6.0
SS	M	55	T3	High	0	0	60	1289	0	81	554	45.0
PC	M	74	T4	High	0	35	216	1404	0	76	6111	85.0
TT	M	57	T3	High	0	0	0	850	0	62	899	94.0
RL	M	67	T4	High	282	0	625	1826	934	407	11375	91.0

was more evident than that observed in the other two groups (grades 1 and 2) (Fig. 1, lanes 1, 3 and 9; Fig. 2, lanes 1, 6 and 7) (Table III). In particular, two specimens showed a very strong lytic band ladder. The band ranged in size from approximately 130 to 80 kDa in one sample (Fig. 1, lane 1) and approximately 130 to 72 kDa in the other (Fig. 1, lane 3). In these two patients, cytological analysis revealed the existence of a high number of malignant cells but different tumor stages (T4 for the first and T1 for the second). Another specimen, pT4G3 (Fig. 2, lane 7), showed a lytic band at the top of the gel and a series of bands with molecular weights ranging from approximately 130 kDa to the 72,000 molecular weight standard. In this sample, we noted faint diffuse bands below the two main bands.

Three tumor markers, urinary bladder cancer (UBC), tissue polypeptide-specific antigen (TPS) and nuclear matrix protein 22 (NMP22), were measured in the urine specimens of healthy volunteers and patients (Tables I-III). For the urinary tumor markers, we established cut-off values by the mean ± 2SD of the values obtained in the urine of 112 healthy subjects; the following cut-off values were considered: 12 ng for UBC, 250 mU for TPS and 6 U for NMP22 per mg of creatinine. Samples with tumor marker values higher than the cut-off value were considered positive. In the specimens from cancer patients, the mean values of all three tumoral markers were higher than the cut-off values. In particular, in the well-differentiated group (grade 1), UBC values ranged from 5.4 to 259.0 ng/mg creatinine with a mean value ± SE of 59.1±25.0; TPS values from 82 to 3292 mU/mg creatinine (mean, 683±321), and NMP22 from 1.8 to 9.1 U/mg creatinine (mean, 4.70±0.73) (Table I). Among this group, the highest value of UBC and TPS was obtained in patient FE who showed a strong lytic band of 72 kDa and a faint visible band below the 92 kDa band (Fig. 1, lane 4). In the grade 2 group, the values of UBC (range, 4.4-264.0; mean, 64.5±32.0 ng/mg creatinine) were similar to those observed in grade 1 tumors, whereas the values of TPS (range, 204-7682; mean, 1445±934 mU/mg creatinine) and NMP22 (range, 4.7±54.4; mean, 18.0±6.2 U/mg creatinine) were 2- and 4-fold higher, respectively, than those found in grade 1 tumors. In this group, patient DM had more intense lytic activity as well as the highest values of tumor markers (Table II). In the grade 3 bladder cancer group, the values of UBC (range, 16-407; mean, 123±56 ng/mg creatinine), TPS (range, 540-11375; mean 3379±1548 mU/mg creatinine) and NMP22 (range, 3-94; mean, 47.2±13.0 U/mg creatinine) were higher than those found in the other two groups (Table III). Moreover, we observed that the percentage of positivity of all three tumor markers in the poorly differentiated tumors was higher than those found in the well- and moderately differentiated cancers.

Discussion

For bladder cancer, both the recurrence rate of treated tumors and the tumor progression to a higher stage or grade are high. Tumor stage is a strong prognostic factor and provides information when selecting therapeutic modalities. However, there seems to be some discrepancy between tumor stage and malignant potential in this disease. Therefore, the establishment of adequate biological parameters is mandatory to elucidate additional useful information about stage.

It is now recognized that during the development of invasive tumors, tumor cells disobey the social order of organ boundaries and cross into tissues where they do not belong. The ability of cancer cells to invade other tissues and spread to distant organs is an often fatal characteristic of malignant tumors. Proteolytic enzymes play a fundamental role in cancer progression, providing tumor cells with access to the vascular and lymphatic system that supports tumor growth and constitutes an escape route for further dissemination (17,18). The complexity of proteolytic systems operating in human tissues is impressive, as demonstrated by the finding that more than 500 genes encoding proteases or protease-like proteins are present in human genome (19). However, among all proteolytic enzymes potentially associated with tumor invasion, members of the MMP family are of vital importance due to their ability to cleave virtually any component of the ECM and basement membranes, thereby allowing cancer cells to penetrate and infiltrate the subjacent stromal matrix. In particular, the ability to degrade type IV collagen, the major component of the basement membrane, is unique to MMP-2 and MMP-9. These two MMPs are most often linked to the malignant phenotype of tumor cells. Moreover, proteolytic processing of bioactive molecules by MMPs contributes to the formation of a complex microenvironment that promotes malignant transformation in the early steps of tumor evolution, including stimulation of cell proliferation and modulation of angiogenesis (8,9). Thus, MMPs are involved in several steps of cancer development and are potential markers of malignant cancer.

As the correlation of urinary MMPs in patients with bladder cancer is logical since the tumor itself is in direct contact with the urine specimens, we measured the gelatinolytic levels of urinary forms of MMP-2 and MMP-9 by zymography, and correlated the data with other urinary biochemical markers currently used in bladder cancer. Zymography has some advantages over immunological assay, such as lower cost, a more rapid time of execution and the possibility of simultaneously detecting multiple forms of the same enzyme.

Our results demonstrated that the majority of specimens show total gelatinolytic activity and MMP-9, but only a few urine samples contain MMP-2. Moreover, MMP-9 content is enhanced in patients with a high number of malignant cells and poorly differentiated cancer.

Numerous individual molecular markers have been identified in tissue, serum and urine specimens that correlate to some extent with tumor stage and/or grade and possibly with prognosis in bladder cancer. Although some markers have the potential for future clinical use, there is currently no urinary marker or test that can replace the need for cystoscopy (6,20,21). UBC, TPS, and NMP-22 are a few of the most widely used tumor markers worldwide for bladder cancer (22-25). We previously reported that cytosol NMP-22 was higher in poorly differentiated bladder cancer than in well- and moderately differentiated bladder tumors (20). In this study, we showed that the urinary levels of TPS and NMP-22 were higher in G3 bladder cancer than in G1 and G2 bladder neoplasias. In particular, the urinary NMP-22 content was strongly increased in poorly differentiated samples. Furthermore, the levels of urinary biomarkers correlated with the increase of MMP-9 lytic activity in high-grade and advanced-stage bladder cancer, indicating that MMP-9 may be an important enzyme in the progression of neoplasias. To our knowledge, two other groups using enzyme-linked immunosorbent assay determined urinary MMP lytic activity in the urine of bladder cancer patients. One group showed that urinary MMP-9 was significantly higher in histologically diagnosed bladder cancer compared to benign urological disorders or normal controls (26). The second group focused on patients with stage Ta and T1 tumors and concluded that MMP-9 was a better marker than MMP-2 for predicting bladder carcinoma (27). Although, enzyme-linked immunosorbent assays offer some analytical advantages, we believe that zymography is a more economical alternative to identify matrix metalloproteinases. The detection of high levels of this enzyme in urine, regarding tumor grade and stage, indicates that MMP-9 expression and activity should be investigated for their possible prognostic significance in the invasive progression of bladder cancer. Therefore, our plan is to begin prospective studies using MMPs to estimate tumor recurrence and progression.

Our results suggest that the inexpensive measurement of MMP-9 in urine may serve as a suitable supplementary tool to identify patients with bladder cancer, and the addition of this enzyme to currently available urinary tumor markers, such as NMP-22 and/or TPS, might provide clinicians with additional objective information on bladder human neoplasias. Furthermore, these observations suggest that MMP-9 should be considered a drug development target for the treatment of bladder cancer.

In conclusion, we showed that MMP lytic activities are easily detected in urine specimens by zymography without requiring concentration of urine samples. We believe that the dosage of matrix metalloproteinases in the urine of bladder cancer patients could be a useful tool for the oncologist in managing these patients. More studies are necessary to determine whether urinary MMP activity is useful in diagnosing and prognosticating the evolution of other genito-urinary tract neoplasias and verifying the effectiveness of treatments.

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