Matrix metalloproteinase-2 and -9 in the urine of prostate cancer patients

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Abstract. The matrix metalloproteinase family of enzymes is comprised of critically important extracellular proteases whose activity has been implicated in a number of key normal and pathological processes. The latter include growth, progression and metastasis as well as dysregulated angiogenesis that is associated with these events. The MMPs are secreted by all types of cells, and they also carve through the extracellular matrix, allowing cancer cells to take root and metastasize. Endogenous inhibitors typically hold MMPs in check but in cancer, the balance shifts against the inhibitors and in favor of MMPs, which ultimately spill over from blood into urine. By gelatin zymography we verified MMP activity in concentrated urine of patients with prostate disease. Of these patients, 30 had cancer, consisting of 13 with Gleason score 6, 12 with Gleason 7, 2 with Gleason 8, 3 with Gleason 9 and 8 had benign prostate hyperplasia. Zymography showed 4 dominant gelatinolityc bands of 240, 130, 92 and 72 kDa in prostate disease. The most abundant lytic activity is at 92 kDa (MMP-9), whereas MMP-2 is present in lesser quantities. Moreover, MMP-9 activity is enhanced in the urine from patients with benign prostate hyperplasia compared with cancer patients. No correlation between gelatinolytic activity and Gleason score or pathological findings was found.

Introduction

Carcinogenesis of tumors is a process of multiple steps in which there are genetic affections that leads to deregulation of cell metabolism and disruption of intra- and intercellular homeostatic regulation. Once the homeostatic balance is lost and malignant transformation has occurred, microenvironmental factors such as degradation of matrix components and host-tumor interactions are essential for survival and growth of malignant cells. Proteolytic enzymes play a fundamental role in cancer invasion and progression, providing tumor cells with access to vascular and lymphatic system that supports tumor growth and constitutes an escape route for further dissemination. Among all proteolytic enzymes potentially associated with tumor invasion, members of matrix metalloproteinase (MMPs) family are of vital importance due to their ability to cleave virtually any component of the extracellularmatrix and basement membranes, thereby allowing cancer cells to penetrate and infiltrate the subjacent stromal matrix (1). MMPs are an important family of zinc-dependent endopeptidases and their basic action, degradation of proteins, regulates cell behaviour playing key roles in tissue remodelling (2-4). They also are involved in several processes associated with cancer development. These include cancer cell growth, differentiation, apoptosis, migration and invasion, and the regulation of tumor angiogenesis and immune surveillance (3,5). Endogenous inhibitors typically hold MMPs in chek, but in cancer, the balance shifts against the inhibitors and in favor of MMPs (6). Among the matrix metalloproteinase members, an accumulating body of evidence supports a positive correlation between gelatinase (MMP-2 and -9) activity and tumor cell invasion. These two MMPs are most linked to human tumor aggressiveness and overall survival (7-11). Thus they are commonly used as serum markers of the malignant phenotype. Furthermore, it has been reported that MMPs can be detected in urine from patients with a variety of cancer and are independent predictor of disease status (12). In prostate cancer, urine represents an interesting fluid in which to seek biomarkers; it is readily available and obtainable non-invasively and it can be used to detect either exfoliated cancer cells or secreted prostatic products that could indicate the presence of prostate cancer.

In the present preliminary study, we determined MMP-2 and MMP-9 (also known as gelatinase A and gelatinase B) activity levels in urine from patients with prostate carcinoma,

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and benign prostatic hyperplasia (BPH) using gelatine zymography in order to analyze the urinary pattern of gelatinolytic activities and to verify whether urine forms of MMPs might have potential as non-invasive biomarkers in providing useful clinical information in the prostate cancer.

Materials and methods

Patients and urine collections. Patients were chosen for the study and their first morning urine were collected before surgical or other therapeutic intervention. Specimens were obtained from patients underwent biopsy or radical prostatectomy at the Department of Urology of the Faculty of Medicine of the University 'Federico II' of Naples. Pathological diagnosis was performed by usual clinical laboratory criteria and confirmed postoperatively by histopathological findings. The age of patients was between 51 and 75 years (mean 64 ± SD 5.7). The tumors were classified for Gleason score and pathological UICC 2002 pTNM stage by genitourinary pathologist (13,14). All men provided informed consent to participate in the study and allowed their biological samples to be analyzed. Approval for the study was provided by institutional board of ethics.

Urine sample preparation. Prior to analysis urine samples were tested using 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. Samples were frozen immediately after collection and stored at -20˚C until assay. The samples were thawed and aliquot of each sample (15 ml) was centrifuged at 1000 x g for 10 min at 4˚C. An aliquot of the supernatant of each sample (2 ml) was concentrated by ultrafiltration using Vivaspin 2 spin column membrane molecular weight cut-off (MWCO): Mr 30000 according to manufacturer's instructions (Sartorius Stedim Biotech). Aliquot (12 μl) of concentrate urine was used to determine MMP-2 and MMP-9 (gelatinase A and B).

Materials. Gelatinase A and gelatinase B were purchased from Hoffmann-La Roche, Ltd. (Basel, Switzerland). Triton X-100, Calcium chloride ($CaCl_2$) glycerol, gelatin, ethylenediaminetetraacetic (EDTA), phenylmethylsulphonyl fluride (PMSF) were from Sigma Chemical Co. (St. Louis, MO, USA). Ultrafiltration spin columns were from Sartorius Stedim Biotech GmbH (Goettingen, Germany). Total prostate specific antigen (t-PSA) and free prostate specific antigen (f-PSA) were determined using the Immulite analyzer with a commercial kit (Diagnostic Products Co., Los Angeles, CA, USA). 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 gelatine as previously described. Briefly, 12 μl of concentrated urine samples were mixed with 5X sample buffer (10 mM Tris-HCl pH 6.8, 12.5% SDS, 5% sucrose, 0.1% bromophenol blue) and applied directely without prior heating or reduction to the gel. After removal of 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 G 250 and destained in the same solution without dye. The gelatinolytic activity of each gelatinase was evident as a clear band against the blue background of stained gelatin. The molecular size of bands displaying enzymatic activity were identified by comparison with prestained standard protein, as well as with purified gelatinase A or gelatinase B. To normalize the possible difference between zymograms an internal urine sample from a patient was incorporated in every gel.

Control gels for MMPs. Control gels contained either the MMP selective inhibitors, 20 mM EDTA or 10 mM 1,10-phenanthroline, in the MMP incubation buffer to confirm that lysis band were the results of MMPs. Furthermore, the character of proteolytic bands was analyzed by incubating the identical zymograms in 0.1 mg/ml of PMSF, a serine protease inhibitor; or 2 mM Pefabloc, an irreversible serine protease inhibitor.

Analysis of the gels. Following zymography, the degree of gelatin digestion was quantified as previously described. Briefly, we used an image analysis software (ImageQuant TL, Amersham Bioscience, Chicago, IL, USA) according to the manufacturer's specifications. The image of the gel was inverted to reveal dark bands on a white background. The molecular weight, volume and background of each band were determined. The relative amounts of the different forms of the urine gelatinases were expressed as the integrated density $x10^{-3}$ (volume) of all the pixels above the background of each band.

Results

During a 1-year period a total of 38 patients with prostate disease were evaluated. Of these 8 had benign prostate hyperplasia (BPH) and 30 had carcinoma. The tumor samples were: 13 cases with Gleason 6, 12 patients with Gleason 7 (cases from 14 to 25), 2 patients with Gleason 8 (cases 26 and 27), and 3 patients Gleason 9 (cases 28-30). The patients were untreated before surgery. The serum percentage of f-PSA/ t-PSA ratio ranged from 4.7 to 28.6 (mean 12.7±SD 6.2) in cancer patients, and from 15 to 54.4 (25.3±13.4) in BPH subjects.

To investigate gelatinolytic activity present in concentrate urine substrate gel zymography was performed. This method allows the detection of the metalloproteinases that exhibit significant gelatinolytic activity (gelatinase A and B). Representative zymography results are shown in the figures. Polyacrylamide gels were evaluated for the presence of clear zone representing degradation of gelatin by proteolysis. The nature of lytic bands was confirmed by inhibition assays with selective inhibitors of MMPs (Fig. 1 lane 2, and Fig. 2 lane 4) and with selective inhibitor of serine proteases (data not shown). Four dominant proteinases were reproducibly detected migrating at ~240, 130 and 92 kDa (MMP-9), and 72 kDa (MMP-2). Furthermore, in some urine specimens we revealed

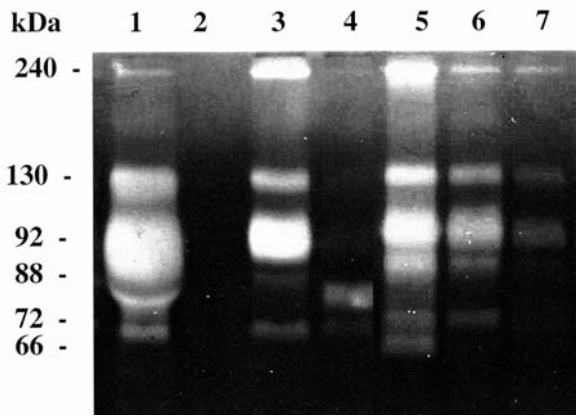

Figure 1. Gelatin zymography of urine specimens from patients with BPH and prostate carcinoma. Molecular weights standards are shown on the left. Lane 1, BPH (patient 4); lane 2, BPH (patient 4) in presence of EDTA 20 mM; lane 3, pT3bN0M0 6 (3+3) (patient 13); lane 4, BPH (patient 1); lane 5, pT2cN0M0 7 (3+4) (patient 17); lane 6, pT2aN0M0 7 (3+4) (patient 15) lane 7, pT3aN0M0 9 (4+5) (patient 28).

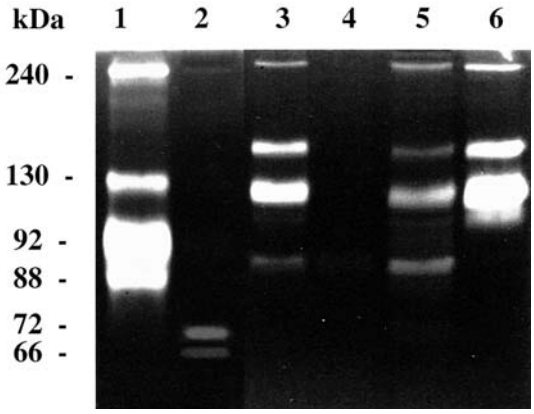

Figure 2. Gelatin zymography of urine specimens from patients with prostate carcinoma. Molecular weights standards are shown on the left. Lane 1, pT2cN0M0 7 (3+4) (patient 16); lane 2, gelatinase A (MMP-2) 120 mU; lane 3, pT3bN0M0 7 (4+3) (patient 25); lane 4, pT3bN0M0 7 (4+3) (patient 25) in presence of 1.10 phenathroline 10 mM; lane 5, pT2aN0M0 7 (3+4) (patient 14); lane 6, pT3aN0M0 6 (3+3) (patient 12).

a lytic band with apparent molecular weight of 88 and 66 kDa. 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 (proMMP-2, 72 kDa, and activated MMP-2, 66 kDa) (Fig. 2, lane 2) and gelatinase B (proMMP-9, 92 kDa; and activated MMP-9, 88 kDa) (data not shown). The clear zones with molecular weight >92 kDa might represent complexes of MMPs that are not dissociated in zymography. MMP-9 can be associated with a 25-kDa protein (lipocalin) giving a band at 125 kDa (15,16) and can form a complex with its endogenous inhibitor TIMP-1 giving a band at ~140 kDa (17). Furthermore, MMP-9 can form dimers or multidimers giving lytic bands at ~215 and 240 kDa (18). Also, several MMPs together can form complexes of high molecular weight (HMW) gelatinase species that can only be identified with specific antibodies in Western blot analysis.

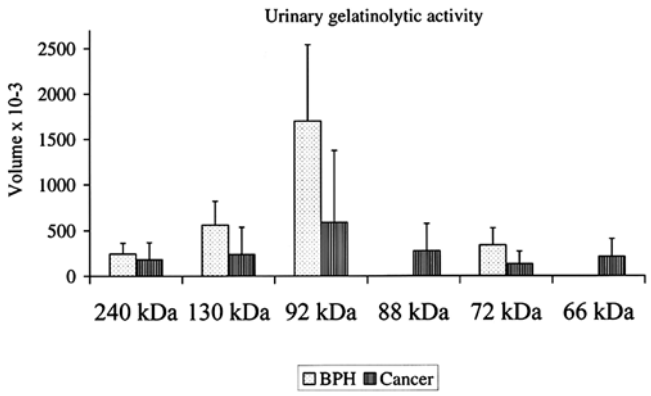

Figure 3. Mean expression ± SD of urinary matrix metalloproteinases.

However, because zymography is much more sensitive than Western blot analysis, it has been difficult to find antibodies that were sensitive enough to detect small amounts of MMPs. Following gelatin zymography, the proteolytic bands were subjected to densitometric analysis and the data, normalized to an internal urine standard, were expressed as the integrated density of all the pixels of each band (volume $x10^{-3}$).

Considering the volume average of each individual band we observed that the 130 and 92 kDa bands as well as the 72 kDa band are higher (about 2-fold) in the urine from BPH patients compared with those of cancer patients. The second point is that the urinary MMP-9 is the most abundant of all the bands both in BPH and cancer with a density value of 1700±842 and 591±783, respectively. In particular MMP-9 is 2.9-fold higher in benign prostate hyperplasia patients compared with the cancer patients. Moreover, MMP-2 is present in lesser quantities than MMP-9 with a density value of 339±188 in BPH and of 132±139 in cancer patients. Finally, the active form of gelatinase B (88 kDa) and of gelatinase A (66 kDa) are revealed only in cancer (Fig. 3). A summary of expression patterns of proteinases in both benign and malignant concentrate urine specimens is shown in the tables. It is evident that in urine there is gelatinolytic activities at different molecular weight. A second point is that the density of lytic activities varies when comparing tumors of the same grade and/or stage as well as comparing cancer and benign hyperplasia. As shown in Table I, in the benign hyperplasia 1 sample showed only a lytic band at 72 kDa with a value of $609x10^{-3}$ (Fig. 1, lane 4), 6 samples showed lytic bands at ~240, 130 and 92 kDa as well as at 72 kDa (MMP-2), (Fig. 1, lane 1), and 1 specimen showed lytic bands at 240, 130 and 93 kDa but no lytic activity at 72 kDa. Regarding Gleason score 6 (3+3) patients, zymographic analysis revealed the existence of lytic band at 72 kDa in 11/13 (84%) specimens with a value ranging from 14 to 520 (mean ± SD, 147±161) and a faint lytic band with lower molecular weight (66 kDa) in one specimen with a value of 77. All patients of this group showed lytic activity at 92 kDa (proMMP-2, gelatinase B) with a density value ranging from 31 to 2914 (mean 687 ±DS 1006 volume $x10^{-3}$). Moreover, 5 (38%) of these specimens showed a lytic band at 88 kDa (active MMP-9) presumably due to an autoactivation during renaturation period; 11 (84%)

Table I. Urinary MMP content and serum percentage of f-PSA/t-PSA ratio in benign prostatic hyperplasia.

Case no.	Age (years)	f-PSA/t-PSA (%)	Volume x10^{-3}			
			MMP 240 kDa	MMP 130 kDa	MMP 92 kDa	MMP 72 kDa
1	62	33.3	0	0	0	609
2	62	23.6	83	55	653	39
3	57	16.8	408	512	1220	496
4	64	26.9	202	767	2595	207
5	61	15.7	198	480	1050	290
6	66	16.4	210	650	1300	10
7	68	54.4	404	820	2790	320
8	68	15.0	200	651	2295	0

Table II. Urinary MMP content and serum percentage of f-PSA/t-PSA ratio in human prostate cancer Gleason 6.

Case no.	Age (years)	f-PSA/ t-PSA (%)	pTNM	Gleason score	Volume x10^{-3}					
					MMP 240 kDa	MMP 130 kDa	MMP 92 kDa	MMP 88 kDa	MMP 72 kDa	MMP 66 kDa
1	66	19.1	pT1bN0M0	6 (3+3)	0	0	64	0	133	0
2	51	13.3	pT2aN0M0	6 (3+3)	218	20	75	0	22	0
3	53	9.9	pT2aN0M0	6 (3+3)	106	286	663	0	73	0
4	65	28.6	pT2aN0M0	6 (3+3)	668	629	1835	0	520	0
5	68	13.9	pT2aN0M0	6 (3+3)	25	23	69	0	14	0
6	67	20.6	pT2cN0M0	6 (3+3)	0	0	31	21	26	0
7	61	6.6	pT2cN0M0	6 (3+3)	102	41	46	15	45	0
8	63	10.4	pT2cN0M0	6 (3+3)	18	14	134	0	73	0
9	66	18.3	pT2cN0M0	6 (3+3)	0	75	253	0	301	0
10	62	11.1	pT2cN0M0	6 (3+3)	0	16	52	0	0	0
11	74	7.9	pT3aN0M0	6 (3+3)	202	101	409	345	104	77
12	67	7.0	pT3aN0M0	6 (3+3)	281	1177	2914	698	0	0
13	65	12.8	pT3bN0M0	6 (3+3)	674	586	2392	73	310	0

samples showed high molecular weight lytic band at ~130 kDa, and 9 (69 %) at ~240 kDa. In particular, case 12 (pT3aN0M0) (Fig. 2, lane 6) showed very strong lytic bands at 240, 130 and 92 kDa and a faint diffuse band below the 92 kDa band, but no lytic band at 72 kDa. Another specimen (case 13, pT3bN0M0) (Fig. 1, lane 3) showed lytic bands at 240, 130, 92 kDa with a faint lytic band at 88 kDa and a band at 72 kDa (Table II). In cancer with Gleason 7 score, 8 patients (66%) showed lytic band at 72 kDa ranging in density between 22 and 409 volume x10^{-3} with a mean value of 161±139; and 2 samples showed lytic bands at 66 kDa with a value of 433 and 318, respectively. In this group the 92 kDa lytic band ranged from 26 to 1658 (mean 675±625). Of these, 10 samples (83%) showed lytic band at 130 kDa and all specimens had band at 240 kDa, whereas only 3 (25%) specimens showed the lytic activity at 88 kDa (Fig. 1, lanes 5 and 6; Fig. 2, lanes 1, 3 and 5) (Table III). The two Gleason 8 (4+4) specimens showed lytic band at 240, 130, 92 and 72 kDa; and one had also lytic activity at 66 kDa. Regarding Gleason 9 cancer, all samples analysed showed urinary gelatinolytic activities (Table IV). In conclusion, the lytic band at 92 kDa was detected in all cancer specimens analysed, whereas the 72 kDa was present in 80% of samples. Moreover, only 4 cancer patients showed the active form of gelatinase A and 9 cancer specimens the active form of gelatinase B.

Discussion

It is widely recognized that the serum prostate-specific antigen (PSA) level as a biomarker of prostate cancer is imperfect, in that it can have many false positive elevations attributable to BPH, subclinical prostatic inflammation and

Table III. Urinary MMP content and serum percentage of f-PSA/t-PSA ratio in human prostate cancer Gleason 7.

Case no.	Age (years)	f-PSA/ t-PSA (%)	pTNM	Gleason score	Volume x10^{-3} MMP 240 kDa	MMP 130 kDa	MMP 92 kDa	MMP 88 kDa	MMP 72 kDa	MMP 66 kDa
14	68	4.7	pT2aN0M0	7 (3+4)	218	278	1063	0	409	0
15	63	16.7	pT2aN0M0	7 (3+4)	233	437	1372	0	226	0
16	68	9.2	pT2cN0M0	7 (3+4)	261	493	1506	678	0	0
17	66	22.3	pT2cN0M0	7 (3+4)	552	509	1085	578	221	433
18	75	14.8	pT2cN0M0	7 (4+3)	86	0	26	0	0	0
19	70	6.9	pT2cN0M0	7 (4+3)	25	61	89	0	98	318
20	74	9.5	pT2cN0M0	7 (4+3)	48	24	96	0	32	0
21	60	13.3	pT2cN0M0	7 (4+3)	167	41	114	0	22	0
22	63	4.8	pT2cN0M0	7 (4+3)	79	0	165	32	28	0
23	74	6.3	pT2cN0M0	7 (4+3)	35	64	266	0	0	0
24	71	13.5	pT2cN0M0	7 (4+3)	94	228	660	0	0	0
25	53	5.9	pT3bN0M0	7 (4+3)	129	798	1658	0	250	0

Table IV. Urinary MMP content and serum percentage of f-PSA/t-PSA ratio in human prostate cancer Gleason 8 and 9.

Case no.	Age (years)	f-PSA/ t-PSA (%)	pTNM	Gleason score	Volume x10^{-3} MMP 240 kDa	MMP 130 kDa	MMP 92 kDa	MMP 88 kDa	MMP 72 kDa	MMP 66 kDa
26	65	15.5	pT2bN0M0	8 (4+4)	58	36	98	0	29	22
27	61	10.9	pT3bN1M0	8 (4+4)	80	55	109	0	121	0
28	64	12.2	pT3aN0M0	9 (4+5)	115	164	397	54	83	0
29	62	7.2	pT2cN0M0	9 (5+4)	0	10	30	0	19	0
30	56	26.6	pT3bN0M0	9 (5+4)	86	31	48	0	7	0

after urethral manipulation. Conversely, it can have false negative results resulting in undiagnosed disease. Numerous reports have showed that free-to-total PSA (f-PSA/t-PSA) ratio is relatively lower in men with prostate cancer compared to men with benign disease (19). However, discrepancies have been reported for the f-PSA/t-PSA cut-off ratio (20). Thus, more accurate tests with better specificity are needed to improve detection and monitoring of prostate cancer and to discriminate between BPH and prostate cancer. In this regard, urine testing is considered a promising method for prostate cancer and a wide variety of urine biomarkers are proposed and have been investigated with variable results (21 and refs. therein). Among protein markers the MMP-2 and -9 have gained wide acceptance. The MMPs are secreted by all types of cells, and they carve through the extracellular matrix, allowing cancer cells to take root and metastasize. Endogenous inhibitors typically hold MMPs in check but in cancer, the balance shifts against the inhibitors and in favor of MMPs which ultimately spill over from blood into urine (12,16,17). Because the correlation of urinary MMPs in patients with prostate cancer is logical as the tumor exfoliate cancer cells and/or secreted products itself in the urine specimens, we measured gelatinolytic levels of the urine forms of the MMP-2 and MMP-9 by zymography assay. These zymographyc tests have 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. We have previously reported that urinary MMP-9 is increased in the high grade and advanced stage bladder cancers whereas only traces were detected in urine of healthy subjects (22), indicating that MMP-9 may be one of the important enzymes involved in the progression of neoplasias. Other researchers determined urinary MMPs lytic activity in urine from cancer patients (17) and they reported that MMP-9 was detected with higher frequency in the urine of patients with prostate cancer compared with those with bladder cancer.

The results shown here indicate that MMP-9 (92 kDa lytic band) is the most abundant form of MMPs and its content is enhanced in patient with BPH compared with the cancer

specimens. Conversely, in the sera of the same patients the MMP-9 value is higher in cancer patients than in benign diseases (our unpublished data). Our results suggest that the inexpensive measurement of MMP-9 in concentrated urine may serve as a suitable supplementary tool to distinguish between patients with prostate cancer and patient with BPH, and the addition of this enzyme to the currently available serum PSA and/or f-PSA/t-PSA ratio might provide clinicians additional objective information on prostate human neoplasias. Furthermore, these observations suggest that MMP-9 should be considered as a drug development target for the treatment of prostate cancer. Nevertheless, there are some limitations of our study: i) the small number of patients included in this observational study, ii) we performed our experiments on concentrated urine and we did not confirm the presence of MMPs in tumor tissue to prove that MMPs did not spill into urine via kidney damage. Therefore, in our future research, the next step will be the analysis of tissue content of MMP-2 and -9 by *in situ* zymography. In conclusion, we showed that MMP lytic activities are easly detected in concentrated urine specimens by zymographic tecnique. We believe that the dosage of gelatinase A and B in the urine of prostate disease patients could be a useful non-invasive tool for the oncologist in managing these patients.

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