Matrix metalloproteinase-2 and -9 in the sera and in the urine of human oncocytoma and renal cell carcinoma

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Abstract. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases, capable of degrading all the molecular components of extracellular matrix. MMPs have been shown to play critical roles in tumor cell invasion and metastasis. We verified the activity of MMPs in the sera and in the urine of patients with kidney carcinoma by gelatin zymography. Of these patients, 16 had clear cell renal carcinoma (ccRCC) and 4 patients had oncocytoma. The sera and the urine of 16 healthy subjects were used as controls. In the sera, zymography analysis showed gelatinolytic bands at 72 kDa (gelatinase A) at 92, 130 and 240 kDa (gelatinase B). MMP-9 activity was slightly enhanced in sera from ccRCC compared with oncocytoma patients. Serum MMP-2 activity was similar in ccRCC and in oncocytoma patients. In the urine, 2 oncocytoma patients and 3 (33%) of the ccRCC patients showed gelatinolytic activity, whereas MMPs could not be detected in the concentrated urine of healthy subjects. The most abundant lytic activity was at 92 kDa, whereas MMP-2 was present in lesser quantities. However, there was broad overlap of the data and we did not find any correlation to type, stage or grade. Therefore, despite previous evidence, MMP-2 and -9 activity in serum and urine may not be useful biomarker for kidney carcinomas.

Introduction

Kidney tumors may be benign or malignant. Benign tumors are incidentally findings at autopsy and are rarely of clinical significance with the exception of oncocytoma. On the other hand, malignant tumors arise from renal epithelial cells and are of great clinical importance. They are the third most common malignancy of the urinary tract after prostate and bladder cancer (1). The renal cell carcinomas (RCC) can be pathologically classified into subtypes: the clear cell type, which constitutes 80% of all cases, the papillary type, at around 15% and the remaining 5% of other histological types (chromophobe, collecting duct and unclassified RCC). The subtype chromophobe RCC cases have a better prognosis compared to those of clear cell RCC (ccRCC) due to the high incidence of cell invasion in the ccRCC subtype. Early stage RCC is relatively asymptomatic, and the classical triad of flank pain, hematuria and of renal mass only manifests very late in the course of the disease. The diagnosis is confirmed with imaging studies, and many cases are now accidentally discovered during routine imaging. Moreover, kidney biopsy is an invasive technique that may result in complications and will not be able to provide accurate diagnosis in certain situations (2). Therefore, there is a pressing need for non-invasive methods to diagnose carcinoma of the kidney as well as for follow-up surveillance.

Matrix metalloproteinases (MMPs) form a family of >25 endopeptidases known to degrade extracellular matrix and basal membrane. MMPs depend on a Zn^{2+} ion to degrade components of the extracellular matrix such as fibrillar and non-fibrillar collagen, proteoglycans, glycoproteins and denatured collagen. Furthermore, MMPs are involved in direct and indirect release of growth factors enhancing tumor growth and tumorigenicity. In particular, the ability to degrade type IV collagen, the major component of the basement membrane, is unique to MMP-2 and MMP-9 also known as gelatinase A (72 kDa) and gelatinase B (92 kDa), respectively. These two MMPs are most often linked to the malignant phenotype of tumor cells, and are commonly used as markers of malignant cancer (3-5).

In the present study, we determined MMP-2 and MMP-9 activity levels in sera and urine from patients with oncocytoma and clear renal cell carcinoma using gelatin zymography in order to analyze the pattern of gelatinolityc activities and to verify whether they may have potential as non-invasive biomarker in providing useful clinical information in kidney cancer.

Materials and methods

Patients. Peripheral venous blood samples and first morning urine were collected from patients before surgical or other therapeutic intervention.

Specimens were obtained from patients who underwent surgical procedure. Diagnosis of tumors was made by usual

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clinical laboratory criteria and confirmed postoperatively by histopathological findings. The age of the patients was between 40 and 73 years (mean ± SD, 59.2±9.7) and there were 11 males and 19 females. The tumors were classified for grade and stage according to the pTNM classification (6). All patients provided written informed consent. The study was approved by the local ethics committee. Sixteen healthy volunteers with no concomitant illnesses were used as controls. The age of the healthy volunteers was between 30 and 70 years (mean ± SD, 57±11) and there were 9 males and 7 females. Healthy volunteers gave their permission verbally. The subjects in the controls had no sign of infections, gastrointestinal hepatic or renal disease, tumors or immunologic disease. The values of the basic laboratory parameters of these participants were within the reference limits.

Serum. Native serum was prepared using plastic tubes without coagulation accelerators, to prevent the release of gelatinase during platelet activation. Tubes were centrifuged at 1600 x g for 10 min, 30 min after blood collection. For each sample, determination of protein concentration was performed using the Bradford method (7). Sera were aliquoted and stored at -20˚C until used. Each aliquot was used only once in order to prevent enzyme activation due to freeze-thawing processes.

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 frozen (-20˚C) until assay. The samples were thawed and an aliquot of each sample (15 ml) were 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 GmbH, Goettingen, Germany). An aliquot (12 μl) of concentrate urine was used to determine MMP-2 and MMP-9 by gelatin zymography.

Materials. Gelatinase A and gelatinase B were purchesed from Hoffmann-La Roche Ltd (Basel, Switzerland). Calcium chloride ($CaCl_2$) glycerol, gelatin, ethylenediaminetetraacetic (EDTA), Triton X-100, phenylmethylsulphonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO, USA). Ultra filtration spin columns were from Sartorius Stedim Biotech GmbH. 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 (8,9). Briefly, serum samples or concentrated urine samples were mixed with 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

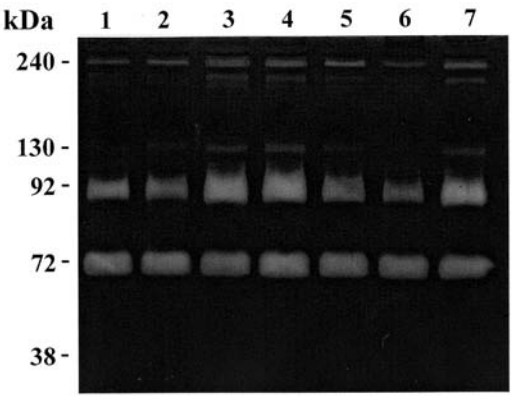

Figure 1. Gelatin zymography of serum specimens from patients with oncocytoma and ccRCC. Molecular weights standards are shown on the left. In all serum sample 25 μg of protein was loaded onto the gel. Lane 1, oncocytoma T2N0M0 G1 in presence of 2 mM Pefabloc (patient P3); lane 2, oncocytoma T1N0M0 G1 (patient P2); lane 3, ccRCC T3N0M0 G3 (patient P20); lane 4, ccRCC T2N0M0 G2 (patient P13); lane 5, ccRCC T1N0M0 G2) (patient P7); lane 6, oncocytoma T2N0M0 G1 (patient P3); lane 7, oncocytoma T1N0M0 G1 (patient P4).

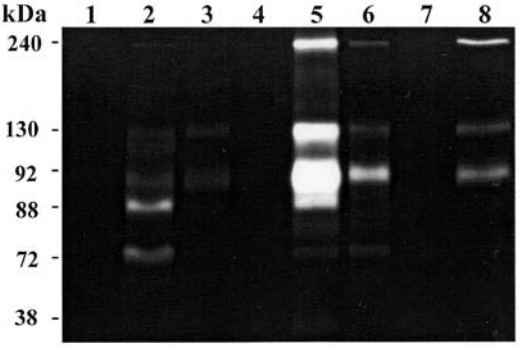

Figure 2. Gelatin zymography of urine specimens from patients with oncocytoma and ccRCC. Molecular weights standards are shown on the left. Lane 1, oncocytoma T1N0M0 G1 (patient P4); lane 2, ccRCC T2N0M0 G3 (patient P15); lane 3, ccRCC T1N0M0 G1 (patient P5); lane 4, ccRCC T1N0M0 G2 (patient P9) in the presence of 1,10 phenanthroline 10 mM; lane 5, ccRCC T1N0M0 G2 (patient P9); lane 6, oncocytoma T1N0M0 G1 (patient P1): lane 6, oncocytoma (patient P1); lane 7 ccRCC T3N0M0 G3 (patient P20); lane 8 ccRCC T1N0M0 G2 (patient P7).

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 for several hours. The 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 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 serum or urine sample from a patient was incorporated in every gel.

Control gels for MMPs. Control gels contained either of the MMP selective inhibitors, 20 mM EDTA or 10 mM 1,10 phenanthroline, in the MMP incubation buffer to confirm that the lysis band was the result 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

Table I. Serum MMP content in healthy subjects.

Case	Age (years)	Gender	Volume x10^{-3}			
			MMP (240 kDa)	MMP (130 kDa)	MMP (92 kDa)	MMP (72 kDa)
H1	58	F	340	0	554	456
H2	54	M	186	0	507	455
H3	63	M	119	33	663	440
H4	67	F	175	0	749	520
H5	55	M	88	0	367	142
H6	61	M	0	0	785	414
H7	34	M	30	0	710	294
H8	70	M	51	69	775	127
H9	44	M	281	115	577	161
H10	56	F	235	73	538	250
H11	43	F	267	87	642	128
H12	68	M	36	106	515	408
H13	65	F	85	18	659	296
H14	64	F	334	117	505	893
H15	69	F	141	67	746	501
H16	39	M	263	86	532	630

Table II. Serum MMP content in oncocytoma.

Case	Age (years)	Gender	Stage	Grade	Volume x10^{-3}			
					MMP (240 kDa)	MMP (130 kDa)	MMP (92 kDa)	MMP (72 kDa)
P1	42	F	T1N0M0	G1	208	147	1259	1125
P2	66	M	T1N0M0	G1	19	6	133	190
P3	59	F	T2N0M0	G1	12	0	98	175
P4	59	F	T1N0M0	G1	28	14	229	183

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 both serum and 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 20 patients with kidney disease were evaluated. Of these patients, 4 had oncocytoma and 16 had clear cell renal carcinoma (ccRCC). All patients provided venous blood samples; among these the 4 patients with oncocytoma and 9 patients with ccRCC collected their first morning urine.

To investigate the gelatinolytic activity present in the serum and in concentrate urine, substrate gel zymography was performed. This method allows the detection of the metalloproteinases that exhibit gelatinolytic activity (gelatinase A and B). Representative zymography results are shown in Figs. 1 and 2. Polyacrylamide gels were evaluated for the presence of clear zone representing degradation of gelatine by proteolysis. The nature of lytic bands was confirmed by inhibition assay with a selective inhibitor of serine proteases (Fig. 1, lane 1) and with selective inhibitors of MMPs (Fig. 2, lane 4). In the sera of all patients, the gels revealed the existence of four clear zones representing degradation of gelatin by proteolysis migrating at approximately 240, 130, 92 kDa (MMP-9) and 72 kDa (MMP-2), respectively. 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

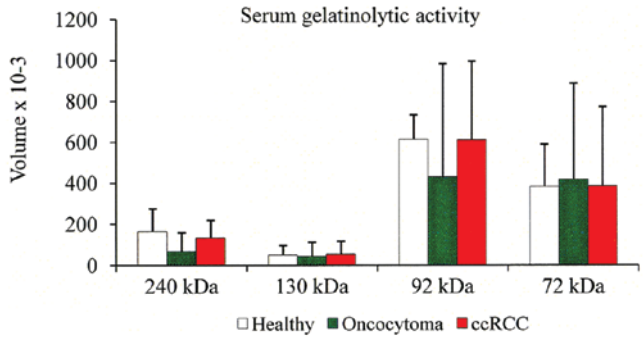

Figure 3. Mean expression + SD of serum matrix metalloproteinases.

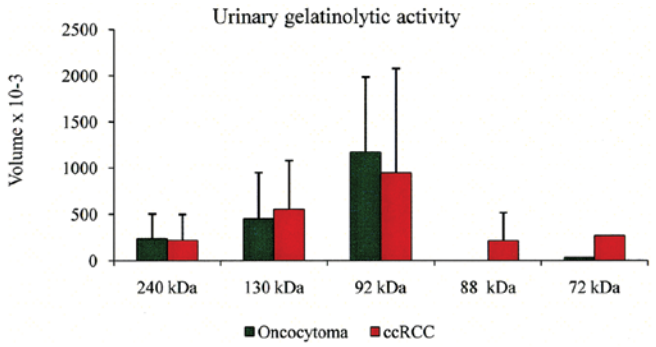

Figure 4. Mean expression + SD of urinary matrix metalloproteinases.

with molecular weight >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 (lipocalin) giving a band at ~125 kDa (10,11) and can form a complex with its endogenous inhibitors TIMP-1 giving a band at ~140 kDa (12). Furthermore, MMP-9 can form dimer or multidimer giving lytic bands at approximately 215 and 240 kDa (13). 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. 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 serum standard, were expressed as the integrated density of all the pixels of each band (volume $x10^{-3}$). A summary of expression patterns of each proteinase is shown in the tables. Considering the volume average of each individual band, we observed that the 92 kDa band is slightly higher in the sera from ccRCC patients compared with that of oncocytoma patients. The second point is that the serum 72 kDa band is similar both in ccRCC and oncocytoma patients as well as in normal individuals (Fig. 3).

As it concerns urine specimens, gelatin zymography identified that MMPs were present in the concentrate urine of 2 oncocytoma patients and in 3 out of 9 (33%) of ccRCC patients (Tables IV and V), whereas MMP could not be detected in the concentrate urine of healthy subjects with no evidence of disease (data not shown). In the oncocytoma group, one sample (patient P1) showed faint small lytic bands at 240, 130 and 72 kDa and a more strong lytic band at 92 kDa (Fig. 2, lane 6 and Table IV). Another sample (patient P3, T2N0M0, G1) showed a more intense lytic activity at 240, 130 and 92 kDa with a value of 425, 805 and 1749, respectively and no lytic band at 72 kDa (Table IV). Regarding ccRCC patients, 2 specimens

Table III. Serum MMP content in clear cell renal carcinoma.

Case	Age (years)	Gender	Stage	Grade	Volume $x10^{-3}$ MMP (240 kDa)	MMP (130 kDa)	MMP (92 kDa)	MMP (72 kDa)
P5	69	M	T1N0M0	G1	183	24	1153	1017
P6	54	M	T1N0M0	G1	94	29	463	100
P7	53	M	T1N0M0	G2	23	7	160	188
P8	60	F	T1N0M0	G2	135	0	632	791
P9	51	F	T1N0M0	G2	295	215	1151	1070
P10	63	F	T1N0M0	G2	237	124	1185	913
P11	63	M	T2N0M0	G2	125	40	343	113
P12	60	F	T2N0M0	G2	128	73	634	105
P13	40	M	T2N0M0	G3	29	15	241	207
P14	73	M	T2N0M0	G3	87	0	558	107
P15	70	M	T2N0M0	G3	279	168	1341	886
P16	61	M	T2N0M0	G3	117	47	623	114
P17	73	F	T2N0M0	G3	221	47	432	97
P19	43	M	T2N0M0	G3	99	45	411	117
P19	67	M	T3N0M1	G3	34	9	214	186
P20	57	F	T3bN0M1	G3	35	13	253	179

Table IV. Urine MMP content in oncocytoma.

Case	Age (years)	Gender	Stage	Grade	Volume x10^{-3} MMP (240 kDa)	MMP (130 kDa)	MMP (92 kDa)	MMP (72 kDa)
P1	42	F	T1N0M0	G1	48	98	602	34
P2	66	M	T1N0M0	G1	0	0	0	0
P3	59	F	T2N0M0	G1	425	805	1749	0
P4	59	F	T1N0M0	G1	0	0	0	0

Table V. Urine MMP content in clear cell renal carcinoma.

Case	Age (years)	Gender	Stage	Grade	Volume x10^{-3} MMP (240 kDa)	MMP (130 kDa)	MMP (92 kDa)	MMP (88 kDa)	MMP (72 kDa)
P5	69	M	T1N0M0	G1	0	0	0	0	0
P6	54	M	T1N0M0	G1	0	0	0	0	0
P7	53	M	T1N0M0	G2	91	180	418	0	0
P9	51	F	T1N0M0	G2	540	927	2246	26	0
P13	40	M	T2N0M0	G3	0	0	0	0	0
P14	73	M	T2N0M0	G3	0	0	0	0	0
P15	70	M	T2N0M0	G3	23	0	181	430	270
P19	67	M	T3N0M1	G3	0	0	0	0	0
P20	57	F	T3bN0M1	G3	0	0	0	0	0

showed a lytic band at 88 kDa (active MMP-9) presumably due to an autoactivation during renaturation period. In particular, case P9 (T1N0M0, G2) (Fig. 2, lane 5) showed very strong lytic band at 92 kDa (value 2246x10^{-3}). Another specimen (case P15, T2N0M0, G3) (Fig. 2, lane 2) showed a very faint lytic band at 240 kDa, a lytic activity at 92 kDa (value 181x10^{-3}) and a more strong lytic band at 88 kDa (value 430x10^{-3}). Furthermore, this patient showed lytic activity at 72 kDa (value 270x10^{-3}), whereas all the other specimens showed no activity at 72 kDa (Table V). The volume average of each individual band of the positive urine specimens is shown in Fig. 4. It is evident that the most abundant lytic activity was at 92 kDa band. Finally, specimens P19 (T3N0M1, G3) and P20 (T3bN0M1) had metastasis in the bony skeleton. We found that the serum lytic activity of these patients was not increased compared with the serum levels in healthy subjects as well as with those of cancer patients. Moreover, in the concentrate urine of these patients we did not find any gelatinolytic activity (Tables III and V).

Discussion

Due to its asymptomatic clinical course RCC is being detected incidentally in two-thirds of patients (14). Therefore, the diagnosis of RCC is a critical issue in the management of the patients. One of the strategies to improve this situation is the identification of biomarkers in serum or urine samples whose levels are sensitive to detect tumor forms and to monitor for disease progression. Several tumor markers have been tested in the past, but there are no definitive biomarkers available for such purpose (15). Among protein markers, MMP-2 and MMP-9 have been investigated with variable results. Tissue MMP-2 and MMP-9 were found to be overexpressed in tumors and more frequently in non-ccRCC (16,17). In particular, by immunochemistry Kallakury *et al* (17) found overexpression of MMP-2 and MMP-9 in 43% of tumors and this increased expression correlated with poor prognostic variables. Moreover, by immunostaining Abdel-Wahed *et al* found a positive correlation between MMP-2 expression and tumor size, histologic type and high levels of cellular proliferation (18). However, although these studies on tissue markers are highly promising, there are some limitations. In fact, immunochemistry is semi-quantitative and highly dependent on a range variables such as choice of antibody, antibody concentration, fixation techniques, variability in the interpretation and stratification criteria, and inconsistency in specimens handling and technical procedures. Using the RT-PCR technique, Kugler *et al* analyzed MMP-2 and-9 and their inhibitors in 17 RCC patients and demonstrated a strong correlation between increased gene expression and tumor stage and aggressiveness (19). By *in situ* zymography, Kamiya *et al* found that the lytic activity is higher at the peripheries of tumors in inflammatory sites (20). As it concerns peripheral blood, Lein *et al* measured MMP-2 and -9 and their inhibitors using ELISA technique in 36 RCC patients and found that plasma MMP-2 levels were higher in healthy controls, whereas MMP-9 concentrations

were significantly higher in RCC patients than in healthy controls with a sensitivity of only 36% in detecting RCC and found no correlation with tumor type, grade or stage (21). The results shown here indicate that MMP-2 (72 kDa lytic band) and MMP-9 (92 kDa lytic band) are present in the sera of all the patients analyzed. The mean values of MMP-2 are similar in ccRCC and oncocytoma patients, whereas the mean values of MMP-9 are slightly higher in ccRCC patients compared with those of oncocytoma patients. However, there was a broad overlap of the data and we found no correlation to type of carcinoma, pathological TNM stage or histological grading.

The idea to follow localized tumors or to monitor drug-based therapy results by simply analyzing tumor-specific markers in the easily available excretory product of the kidney is desirable. However, to the best of our knowledge there is only scant literature on urine markers for RCC. In the urine samples, we found that only few specimens have lytic activity. Our data are in keeping with the data of Cannon and Getzenberg (22) but in contrast with those of Sherief *et al* (23).

So far, despite the tissue evidence, serum and urine MMP activities seem to be not an adequate test to identify renal cancer. Nevertheless, due to the small number of patients included in the studies, the conclusion may not be transferable to the general population and therefore merits further evaluation. Future investigation involving gelatine zymography in larger cohorts of patients could clear up if MMP-2 and MMP-9 are useful biomarkers for RCC.

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