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Matrix metalloproteinase-9 measured in urine from bladder cancer patients is an independent prognostic marker of poor survival

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Abstract

Introduction. Matrix metalloproteinase 9 (MMP-9) is an endopeptidase involved in various cellular processes, such as tumour development and metastatic spread. In biological samples, MMP-9 can occur as pro-MMP-9 and active MMP-9, or these factors complexed with the inhibitor TIMP-1. An assay, which can measure active and total MMP-9 in biological samples, has been used on the urine from bladder cancer patients and demonstrated a significant correlation between MMP-9 and clinical parameters. The prognostic value of these measurements has never been investigated. Using this assay we have investigated the prognostic influence of total and active MMP-9 in urine from bladder cancer patients. *Material and methods.* Fresh voided urines from 188 consecutive patients diagnosed with bladder cancer were collected and frozen at diagnosis. After 15 years follow-up 13 patients were still alive, and 175 patients had died. MMP-9 was measured with an immunocapture activity assay. *Results*. Median $MMP\text{-}9_{total}$ was 173.7 units/10 g creatinine (range 0–34 792), and median $MMP\text{-}9_{active}$ was 14 units/10g creatinine (range, 0–294 757). The two factors were correlated (Spearman's rho 0.74, $p<0.0001$). High $MMP\text{-}9_{total}$ and $MMP\text{-}9_{active}$ were significantly correlated with large tumour size and poor malignancy grade. Increasing tertiles of $MMP\text{-}9_{total}$ and $MMP\text{-}9_{active}$ were associated with poor overall survival ($p<0.0001$ and $p=0.003$, respectively). A Cox multivariate analysis using death as endpoint identified high tertiles of $MMP\text{-}9_{total}$ as independent prognostic markers with a relative risk 2.25 (95% confidence interval, 1.53–3.30). *Conclusion*. MMP-9 measured in urine from bladder cancer patients was a strong independent prognostic marker of poor survival. This is the first time high levels of MMP-9 measured in urine from bladder cancer patients have been linked to poor prognosis. This may reflect MMP-9 playing a role in tumour invasion and metastasis. It may be possible to non-invasively measure tumour response to therapy and identify possible tumour recurrence in an early phase.

More than 100 000 incident cases of bladder cancer were seen in Europe in 2006; the male:female ratio being 4:1. This cancer was the fourth most common cause of cancer death among men. At diagnosis approximately 30% of cases are muscle-invasive, and among patients treated for muscle-invasive bladder cancer approximately one-third of patients have undetected metastasis at the time of primary therapy [1]. One quarter of patients treated with radical cystectomy present with lymph node involvement at the time of surgery. Treatment selection is based on extent of disease, and today staging of bladder cancer is routinely established on detection of tumour cells in the urine and evaluation of tumour during bladder cystoscopy. Bladder cytology is relatively effective in diagnosing carcinoma in situ (CIS) whereas the detection rate is only 20% for a grade I papillary tumour. At cystoscopy virtually all the papillary tumours in the bladder are identified, but CIS can easily be overlooked. Novel effective methods at low costs to identify bladder carcinomas and classify their aggressiveness are needed to better select the appropriate treatment for the individual patient.

Matrix metalloproteinases (MMPs) are a family of 23 members of zinc-dependent endopeptidases involved in tumour development and metastatic spread [2]. One of the MMPs involved in tumour invasion is MMP-9, also called Gelatinase B. This factor has several substrates, collagen type IV being the most important because it is the main component

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of basement membranes. The role of MMP-9 is very complex, but it affects apart from invasion and metastasis, also signal pathways involved both in normal physiology and in disease, growth signalling, angiogenesis, vasculogenesis and lymphangiogenesis [2]. MMP-9 is secreted in an inactive proform, but the mechanism of activation *in vivo* still remains to be elucidated. In biological samples MMP-9 can occur in different forms: latent pro-MMP-9, active MMP-9, active MMP-9 complexed with TIMP-1, and pro-MMP-9 complexed with TIMP-1. TIMP-1 is the inhibitor of MMP-9 [3]. Previously, only gelatin zymography was able to detect both the inactive and the active form of MMP-9, however, an assay has been developed that can measure quantitatively both active and latent MMP-9 in biological samples. This assay has been used to measure the content of MMP-2 and MMP-9 in urine samples from bladder cancer patients [4], and MMP-9 was shown to correlate with other clinical parameters. However, the prognostic significance of these measurements remains to be evaluated. The aim of the present study was to investigate if MMP-9 was detectable in urine samples from patients diagnosed with bladder carcinoma, and furthermore to establish the clinical and prognostic importance of this factor.

Materials and methods

Patients and urine collection

Surgery was performed at Aarhus University Hospital, Skejby, in Denmark, on 188 consecutive patients (143 male and 45 female with a median age of 69 years (range 44–90)). Clinical characteristics of the patients are given in Table I. All tumours were histologically classified for grade and stage according to the TNM-classification [5]. Fresh voided urines were collected before surgery and centrifuged 1 700–1 800 rpm for five minutes and then frozen at −80°C until use. Before measurements the urines were slowly thawed at 5°C and 100 µl were taken for analysis and frozen once more. Urinary creatinine levels were used to correct for the dilutions of the samples. Creatinine was measured at the Department of Biochemistry, Aarhus University Hospital, Noerrebrogade, Denmark, using the Jaffé method according to the manufacturer's instructions (Roche). Urine samples from 29 patients diagnosed with prostate hyperplasia were used as controls.

Table I. Clinical parameters stratified by MMP-9 total and MMP-9 active corrected for creatinine.

		MMP-9total			MMP-9active		
	N	<66	66-480	>480	<4.3	4.3-39.0	>39.0
Number of patients	188	63	61	64	63	61	64
Tumour size							
Below 3 cm	10	69	37	22	10	32	27
Above 3 cm	46	101	21	35	45	27	28
			P<0.0001			P<0.0001	
T classification							
Ta	20	13	6	1	14	3	3
T1	18	8	8	2	9	6	3
T2-4	150	42	47	61	40	52	58
T2	30	10	11	9	10	12	8
T3a	79	22	27	30	18	30	31
T3b	17	6	2	9	5	5	7
T4a	6	1	2	3	2	1	3
T4b	18	3	5	10	5	4	9
			P=0.01			P=0.02	
Grade							
I	8	1	6	1	6	1	1
II	15	11	3	1	7	7	1
III	120	36	41	43	39	37	44
IV	43	11	15	17	11	15	17
Unknown	2	0	0	2	0	1	1
			P=0.01			P=0.09	
Gender							
Male	143	51	44	48	49	47	47
Female	45	12	17	16	14	14	17
			P=NS			P=NS	

Units for MMP measurements: units per 10 g creatinine.
NS: not significant.
The background for p-values is given in Statistics.

MMP-9-specific immunocapture activity assays

MMP-9 activity was determined with a commercially available kit (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK: product: Biotrak RPN2634, or QuickZyme Biosciences, Leiden, The Netherlands, product: MMP-9 (human) activity assay), and the assay has previously been described in detail [6,7]. In short: ninety-six-well plates (Costar) coated with specific anti-MMP-9 monoclonal antibodies were used to capture MMP-9 from urine samples (diluted 1:2 in 1% BSA-PBS) by overnight incubation at 4°C. The wells were washed and incubated with assay buffer, and subsequently a MMP specific detection enzyme UKcol [6] and chromogenic urokinase substrate were added. Colour development was recorded in a Titertek plate reader at 405 nm (Flow Laboratories, Irvine, Scotland). For measurement of total activity (already active plus latent MMP-9) the immobilised MMP-9 was incubated with assay buffer containing 0.5 mM APMA for two hours, after which detection enzyme and chromogenic urokinase substrate were added and activity was recorded.

Statistics

Urinary MMP-9 antigen levels are expressed as units per 10 g creatinine. An independent samples t-test was used to compare the differences of MMP-9 measurements in the urines from patients diagnosed with cancer and prostate hyperplasia. The range of the MMP-9 measurements was quite high, thus the logarithm of the values was used because they were normally distributed. A Spearman´s rho was used to investigate the correlation between MMP-9 in tertiles and other known ordinal clinical-pathological parameters, whilst a χ^2 test was used to investigate the correlation between MMP-9 in tertiles and gender. Survival functions were made according to the Kaplan-Meier method and the differences among the survival curves were calculated according to the log-rank test with a test for trend. All time estimates were made using the date of primary surgery as initial value. A multivariate Cox's proportional hazards regression analysis was used to investigate the prognostic value of the clinical-pathological parameters regarding overall death. Proportional hazards rates were controlled graphically using log-minus-log survival plots from the multivariate analysis data stratified by the controlled variable, and proportional hazards were seen for the first five years for the investigated variables; the statistical method was backward Likelihood Ratio. Univariate and multivariate analyses were done using the SPSS 18 program package. All p-values were based on two-sided testing and the level of statistical significance was 5%. The date for evaluation of survival status of the patients was April 1, 2010.

Results

We had access to overall survival data and at analysis 175 patients had died at a median of 15.8 months (range, 0.1–188.1 months), and 13 patients were still alive after a median follow-up of 181.5 months (range, 138.5–188.1 months). The median MMP-9 total was 173.7 units per 10 g creatinine, (range 0–294 757 units per 10 g creatinine), and the median MMP-9 active was 14 units per 10 g creatinine (range 0–34 792 per 10 g creatinine). In urine from 29 patients diagnosed with prostate hyperplasia the median MMP-9 total was 21 units per 10 g creatinine (range 0–2 893 units per 10 g creatinine), and the median MMP-9 active was 0 units per 10 g creatinine (range 0–98 units per 10 g creatinine). Compared to urine from patients diagnosed with prostate hyperplasia the measurements from the cancer patients were significantly higher regarding MMP-9 total ($p=0.002$) and almost significantly higher for MMP-9 active ($p=0.06$).

Figure 1 illustrates the significant correlation between MMP-9 total and MMP-9 active plotted logarithmically. Table I shows the distribution of MMP-9 total and MMP-9 active divided into tertiles and the known clinical-pathological parameters. High tertiles of both MMP-9 measurements were significantly correlated to large tumour size, while MMP-9 total was also correlated to increasing tumour grade. Figure 2 highlights the Kaplan-Meier survival plots where the MMP-9 measurements have been separated in tertiles and associated to overall survival. For both MMP-9 total and MMP-9 active a significant association was seen to poor overall survival. Finally, a

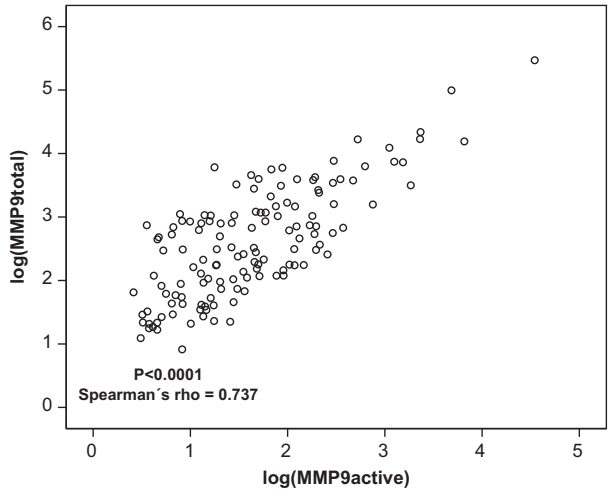

Figure 1. Correlation plot showing MMP-9 total as a function of MMP-9 active.

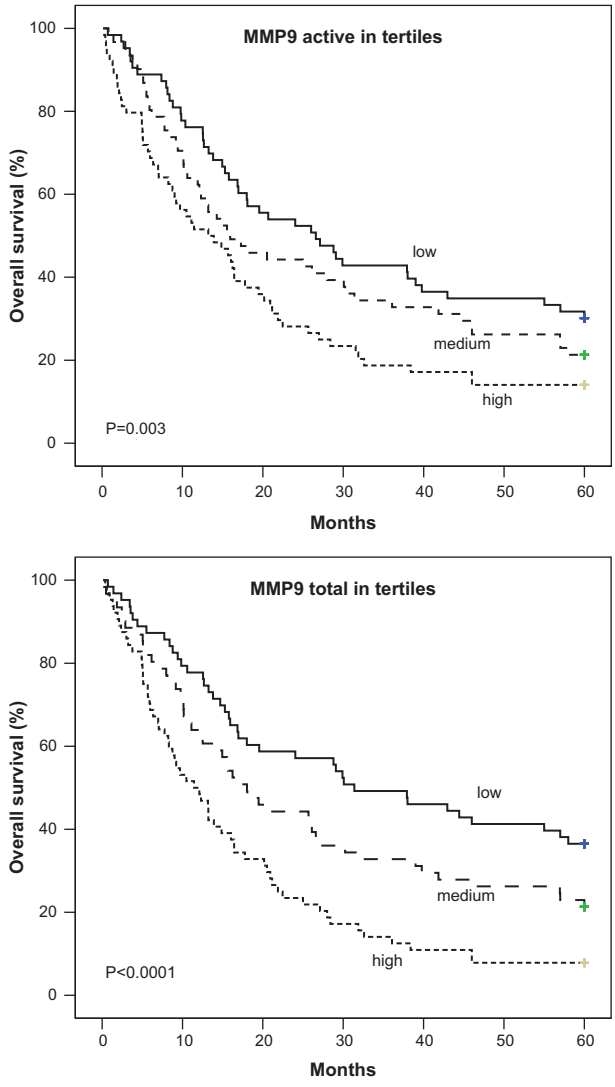

Figure 2. Kaplan-Meier survival plot showing the association between MMP-9 total and MMP-9 active in tertiles and overall survival in months.

Cox multivariate analysis using overall death as endpoint was made and is shown in Table II. In this cohort, T2-4 and MMP-9 total evaluated as a continuous parameter were identified as independent markers of death, whilst MMP-9 active, grade, age, and gender did not add independent information to the analysis. If MMP-9 measurements were included in the analysis separated in tertiles the HR for MMP-9 total was 2.25 (95% CI 1.53–3.30).

Table II. Cox Multivariate analysis using overall death as the endpoint. The final model is demonstrated.

Parameter	Subgroup	Hazard ratio	(95% CI)	P-value
T- classification	Ta	1		
	T1	1.24	(0.54-2.83)	0.61
	T2-4	2.18	(1.16-4.10)	0.02
MMP-9 total	continuous	1.002	(1.001-1.003)	<0.0001
MMP-9 active	continuous			NS
Age	continuous			NS
Grade				NS
Gender				NS

CI: confidence interval. NS: not significant.

Discussion

This study was undertaken to investigate the presence and clinical value of urinary MMP-9 in a group of patients diagnosed with bladder carcinoma. Using an assay which can measure both active and total content of MMP-9 in biological samples we identified urinary MMP-9 as a prognostic parameter in bladder carcinoma.

There are some limitations in this study. Since the patients included were operated in the mid-1990s where the surgery was not as thorough as today, the lymph node status in the patients was not systematically evaluated. Also, we do not have access to data on the type of therapy the individual patient received. However, the guidelines at that time recommended patients diagnosed with a Ta or T1 tumour had resection of the tumour, or in select T1 cases a cystectomy. Patients in good condition with operable muscle-invasive tumours were offered cystectomy, whilst patients with co-morbidities were offered radiotherapy with curative intent. Patients with non-operable bladder cancer and in good shape were treated with chemotherapy (MVAC: Methotrexate + vincristine + adriamycin + cisplatin or cisplatin+gemcitabine) and if the patient had a very good response a cystectomy was then performed in some patients. The treatment offered to bladder cancer patients today, however, is more complex both to increase the anti-tumour effect [8] and to spare normal tissues [9,10]. Matrix metalloproteinases have even been linked to the effect of preoperative radiotherapy of rectal cancer, but this has not been investigated for bladder cancer [11].

MMP-9 is produced in various non-malignant stromal cells such as neutrophils, macrophages, lymphocytes, mast cells, fibroblasts, and dendritic cells [12]. We have previously demonstrated that intense inflammation can be seen in invasive bladder carcinomas, and it is likely that the MMP-9 measured in this study is derived from these inflammatory cells infiltrating the tumour [13]. The proteolytic activity of MMP-9 is regulated at many levels: the gene level, enzyme level, and at the level of the inhibitor TIMP-1 [3]. Since TIMP-1 is produced by the same cells that produce MMP-9, and therefore likely was present in the urine it is important that the assay used in this study was able to measure both active and pro-MMP-9 complexed with TIMP-1.

Identifying and measuring a prognostic factor in urine opens new possibilities for the future strategy

for managing bladder carcinoma. Besides acting as an additional prognostic parameter, optimising the process of staging the patient at diagnosis when deciding choice of therapy, measurements of MMP-9 in urine may be used to evaluate response to treatment during, for example, chemotherapy. Also a urine investigation at control visits post-operatively may be able to identify patients with relapse but still without evidence of disease. It may even be possible to spare the patient repeated cystoscopies in the follow-up program.

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