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Matrix metalloproteinase-7 as a marker of metastasis and predictor of poor survival in bladder cancer

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Matrix metalloproteinases (MMPs) play an important role in tumor progression and metastasis. Here, we investigated the prognostic relevance of MMP-7 in urinary bladder cancer. MMP-7 gene expression was measured in tissue samples of 101 patients using quantitative real-time PCR. Circulating MMP-7 serum levels of 98 individuals (79 patients and 19 controls) were analyzed by enzyme-linked immunosorbent assay. The results were compared with the clinical follow-up data, performing Kaplan–Meier log-rank test as well as univariate and multivariate Cox analysis. In representative cases, immunohistochemical analysis for MMP-7 was performed. We detected significantly elevated MMP-7 levels both in tissue and serum samples of patients with metastatic disease ($P = 0.001$ and $P = 0.002$). Multivariate analysis revealed that high MMP-7 tissue expression and serum concentration are stage- and grade-independent predictors of both metastasis-free (hazard ratio [HR] = 3.80, 95% confidence interval [CI], 1.29–11.23, $P = 0.016$, and HR = 2.53, 95% CI, 1.01–6.37, $P = 0.048$) and disease-specific survival (HR = 1.89, 95% CI, 1.00–3.55, $P = 0.050$ and HR = 1.95, 95% CI, 1.03–3.71, $P = 0.041$). Based on these findings, we conclude that MMP-7 is a promising marker to detect present and to predict future metastasis. Serum MMP-7 analysis provides information about the risk of metastasis before surgery which could help to optimize therapeutic procedures. Furthermore, high MMP-7 tissue and/or serum levels could identify patients most likely to benefit from early adjuvant chemotherapy. (*Cancer Sci* 2010; 101: 1300–1308)

Urinary bladder cancer (UBC) is the most common malignancy of the urinary tract. Although the majority of patients present with superficial UBC, 20–40% of patients will either present or ultimately develop muscle-invasive disease. Radical cystectomy is the standard treatment for muscle-invasive UBC. However, this 'gold standard' only provides 5-year survival in about 50% of patients.[1] The most reliable prognostic factor in this cystectomy cohort is lymph node status. Patients with organ-confined muscle-invasive UBC enjoy long-term freedom from progression after radical cystectomy, while those with metastatic disease are at an increased risk for progression and mortality. The main cause of relapse is the presence of microscopic metastasis that remains undetected before surgery.[2] The role of conventional imaging modalities is limited due to their poor performance for detecting low-volume lymph node and/or distant metastases.[3] To date there are no markers in the daily routine to identify patients most likely to benefit from radical cystectomy. Therefore there is a clear need for novel prognostic biomarkers to ensure adequate risk stratification in muscle-invasive UBC.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes capable of cleaving extracellular matrix proteins (ECMs). Degradation of ECM is an important step both in normal physiological processes (embryonic development, reproduction, and tissue remodeling) and in pathological processes such as arthritis, tumor progression, and metastasis.[4] Furthermore, MMPs can influence several molecular processes involved in tumor progression through their ability to cleave pro-apoptotic factors, cell surface molecules, cell adhesion molecules, and growth factors.[5–7] These activities alter cell signaling and/or produce active protein fragments. MMPs can mobilize pro-angiogenic factors but are also able to generate angiogenic inhibitors such as endostatin and angiostatin.[8–11] MMP-7, also known as matrilysin, is the smallest MMP; its molecular weight as proenzyme is 28 kDa, which reduces to 19 kDa after an activation step induced by plasmin and trypsin.[12] MMP-7 has been shown to be constitutively expressed in the ductal and glandular epithelium of normal mammary and parotid glands, liver, pancreas, and prostate.[13] In human tumors matrilysin seems to be unique among MMPs because it is produced by the tumor cells themselves and not solely by stromal cells (fibroblasts, macrophages, and endothelial cells) as other MMPs. Matrilysin is overexpressed in a variety of epithelial and mesenchymal tumors such as esophagus, colon, liver, renal, and pancreas, and correlates with unfavorable prognosis.[14–17] Increased circulating levels of MMP-7 were correlated with the presence of metastatic disease and poor patients' survival in colorectal and renal cell cancer.[18,19]

The prognostic value of MMP-7 in UBC is unknown. The aim of the current study was to better understand the role of MMP-7 in the formation, progression, and metastasis of UBC and to assess its prognostic significance. Therefore, we analyzed the tissue expression and serum concentration levels of MMP-7 in samples of UBC patients using quantitative real-time PCR, immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA). Gene expression and serum concentration values were compared with the clinical follow-up data and both univariate and multivariate analysis were performed. Our data demonstrate elevated tissue expression and serum concentration levels of MMP-7 in patients with metastatic UBC. Furthermore, we show here that elevated expression and circulating levels of MMP-7 are stage- and grade-independent risk factors for metastasis and UBC-related death.

Materials and Methods

Clinical samples. A total number of 179 individuals (160 UBC patients and 19 controls) divided in two cohorts were included in this study: (1) frozen tissue samples of 101 UBC patients analyzed by quantitative real-time PCR; and (2) serum samples of 79 UBC patients and 19 healthy individuals (Table 1). In 20 cases, both frozen tissues and sera obtained during surgery were analyzed. The samples were collected in the

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Table 1. Patient characteristics and gene expression

	n	MMP–7 gene exp. values Median (range)	*P*-values	*n*	MMP–7 serum cc. Median (range)	*P*-values
Age						
≤65 years	46	17.25 (1.11–6155.42)	0.102	37	5.16 (0.22–36.95)	0.302
>65 years	55	14.87 (0.15–892.42)		42	5.59 (2.36–32.25)	
Gender						
Male	78	14.55 (0.15–2350.13)	0.262	60	5.52 (0.22–36.95)	0.900
Female	23	19.24 (0.18–6155.42)		19	5.10 (3.07–32.56)	
Stage						
Ta	32	11.72 (0.15–554.63)	0.793	9	4.03 (2.65–13.63)	0.486
T1	18	8.95 (0,36–891.42)	0.411	22	5.83 (2.29–12.28)	0.397
T2	9	14.23 (1.12–82.23)	0.106	12	4.65 (2.08–28.51)	0.107
T3	30	34.06 (2.02–2350.13)	0.486	24	6.59 (0.22–36.95)	0.615
T4	12	15.82 (0.18–6155.42)		12	9.76 (3.14–32.25)	
Non-invasive	50	10.25 (0.15–891.27)	**0.006**	31	4.80 (2.29–13.63)	0.105
Invasive	51	23.06 (0.18–6155.42)		48	5.40 (0.22–36.95)	
Grade						
G1	17	8.1 (0.47–42.59)	0.337	6	4.28 (2.93–12.28)	0.689
G2	37	15.26 (0.15–891.42)	0.234	25	5.10 (2.29–26.32)	0.269
G3	47	17.73 (0.18–6155.24)		48	5.71 (0.22–36.95)	
Low grade (G 1–2)	54	12.41 (0.15–891.42)	0.078	31	4.80 (2.29–26.32)	0.174
High grade (G 3)	47	17.73 (0.18–6155.42)		48	5.71 (0.22–36.95)	
Lymph node						
N0/Nx	87	14.17 (0.15–2350.13)	**0.001**	65	4.88 (0.22–32.25)	**0.002**
N+	14	301.84 (3.96–6155.42)		14	13.92 (3.94–36.95)	
Primer	48	29.43 (0.36–6155.42)	**0.001**	44	5.74 (2.08–36.95)	0.500
Recurrent	53	9.40 (0.15–2028.44)		35	5.22 (0.22–32.25)	
Smoking						
Yes	49	17.73 (0.18–2350.13)	0.232	40	5.39 (0.22–32.65)	0.519
No	37	11.90 (0.15–6155.27)		34	5.24 (2.08–36.95)	
Unknown	15	–		5	–	
Control	16	69.38 (0.20–987.44)	0.678	19	2.64 (1.07–6.10)	**<0.001**
Tumor	16	39.26 (0.52–993.43)		79	5.35 (0.22–36.95)	

P-values of statistical significance are shown in bold. –, unknown.

Department of Urology of the University Hospital of Essen between 1990 and 1997. The criteria for study enrollment were histopathological diagnosis of transitional cell carcinoma of the bladder, no history of other tumor, no chemotherapy before surgery, availability of sufficient tumor/serum sample, and the potential to follow up. The study was performed in accordance with the ethical standards of the Helsinki Declaration and was approved by the ethical board of the hospital. Tissue sections from each biopsy were stained with H&E and reclassified by a pathologist according to the 2004 World Health Organization classification.[20] Only biopsies containing ≥70% tumor cells were selected for further analysis. In 16 cases corresponding normal-appearing bladder tissues from radical cystectomy were available and used as controls. Blood samples of 79 UBC patients and 19 healthy controls without history of cancer were collected between 1990 and 1997. In 13 cases of radical cystectomy postoperative serum samples were available for analysis. Samples were centrifuged at 400*g* for 15 min, immediately aliquoted, and kept at −80°C until assayed.

RNA isolation and cDNA synthesis. RNA was isolated using the RNeasy mini Kit (Qiagen, Hilden, Germany). DNA was digested with the RNase-free DNase Set (Qiagen), as recommended by the supplier. Total RNA was quantified using an ultraviolet spectrophotometer (Peqlab ND-1000; Peqlab, Erlangen, Germany) and the quality and integrity of samples were assessed on a 1.5% agarose gel. RNA was reverse transcribed (RT) in a final volume of 20 μL containing 200 ng RNA, 1 × RT Buffer, 0.5 mM dNTPs, 1.8 μM oligo dT, 10 units RNase inhibitor, and 40 units Omniscript RTase. The cDNA synthesis was performed at 37°C for 60 min.

TaqMan reverse transcription–polymerase chain reaction assay (RT–PCR). Quantitative real-time PCR was performed using the StepOne Real-time PCR System (Applied Biosystems, Foster City, CA, USA). To provide high reproducibility, we used the TaqMan Gene Expression Assay (Applied Biosystems). The assay IDs were as follows: MMP-7, Hs01042794_g1; TBP, 4333769. The expressions were related to Universal Human Reference RNA (Stratagene, CA, USA), composed of pooled RNA from 10 human cell lines. This allows a reliable lab-to-lab comparison of gene expression data independently of the actually used control sample.

Choosing the right housekeeping gene as an endogenous control is essential in data analysis of real-time PCR. Ohl *et al.* found TATA box binding protein (TBP) to be the most appropriate endogenous control in UBC.[21] Therefore we used TBP to normalize the expression of our target genes.

PCR reactions contained 2.5 μL cDNA, 1 × TaqMan Gene Expression Assay (containing primers and probe), and 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), in a final volume of 25 μL. The thermal cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was tested in duplicate. The threshold cycle (C_t) was determined on the amplification linear area of the target and control genes. The normalized quantity of the target gene was obtained by subtracting C_t for the internal control (TBP) from the C_t for the target gene (ΔC_t sample). The same calculation was performed with Human Reference RNA (ΔC_t reference). Then $\Delta\Delta C_t$ was calculated as the difference of these values ($\Delta\Delta C_t = \Delta C_t$ sample $- \Delta C_t$ reference). Finally, the result was expressed as $2^{-\Delta\Delta C_t}$.[22] The intra-assay

precision for duplicate measurements was 0.7% and the inter-assay precision was 11.1%.

Measurement of serum MMP-7 levels. Serum levels of MMP-7 were quantified by sandwich ELISA using the Quantikine ELISA kit from R&D Systems (Wiesbaden, Germany), according to the manufacturer's instructions. All samples were examined in duplicate, and the mean values were used for statistical analysis. The intra-assay variability for duplicate measurement was 4.5% while the inter-assay variability was 7.7%.

Immunohistochemistry. Immunohistochemical staining was performed on 5-μm thick sections obtained from formalin-fixed and paraffin-embedded tissue blocks of bladder cancer of different tumor stages ($n = 15$) and both positive and negative lymph nodes. In paraffin sections of some blocks ($n = 5$) tissue areas with normal transitional epithelium and those with superficial and muscle invasive tumor stages were identified side by side in the same section by histopathological evaluation. Immunostaining was carried out after heat-based antigen retrieval (20 min, 95°C water bath, citrate buffer [pH 6]) using mouse monoclonal antibody against MMP-7 (MAB-9071, final dilution 1:600; R&D Systems). Automated immunohistochemistry was performed using the Dako Autostainer Plus System (DakoCytomation, Carpinteria, CA, USA) with the antimouse IgG EnVision Plus detection kit (DakoCytomation,) for secondary and tertiary immunoreactions. Reaction products were developed with diamino-benzidine (DAB), according to general protocols. Negative control sections with the omission of the primary antibody were included in each run.

Statistical analysis. The lack of normal distribution of gene expression data (controlled by Shapio–Wilk test) indicated the use of non-parametric two-sided Wilcoxon rank sum test (Mann–Whitney test) for paired group comparisons. Univariate recurrence-free, metastasis-free, and disease-specific survival analysis was done using both Kaplan–Meier log-rank test and univariate Cox analysis. For multiple analysis, the Cox proportional hazards regression model was used. Variables with effect on survival in univariate analysis ($P \leq 0.05$) were included in the Cox proportional hazards regression models. According to the test of Schoenfeld's residuals, proportional hazards assumption was fulfilled for each variable. The relationship of the tissue expression and serum levels was examined by using Spearman's correlation coefficients. Nonparametric receiver–operating curves (ROC) in which the value for sensitivity is plotted against false-positive rate (1-specificity) were generated. In all tests a P-value of at least 0.05 was considered to be statistically significant. All statistical analyses were done with the SPSS software package (version 17.0; SPSS, Chicago, IL, USA).

Results

Clinical background. The main characteristics of patients are given in Table 1. The median follow-up period was 41 months with a maximum of 196 months. Seventy-five of 87 patients with muscle-invasive UBC were treated by radical cystectomy. In five cases, partial cystectomy, and in seven cases, transurethral, resection of the bladder was performed with palliative intent. Forty-eight of 73 patients with Ta/T1 cancers were treated by transurethral resection, while in 25 patients with high-risk non-muscle-invasive cancer radical cystectomy was performed. Serum samples of 19 age-matched healthy individuals with no history of cancer were used as controls.

Comparison of MMP-7 between tumor and control samples. *(a) Tissue expression.* MMP-7 mRNA expression was measured in tumor and corresponding normal-appearing bladder tissues from 16 radical cystectomies. We did not find any significant difference between tumor and corresponding normal tissue. No significant relationships were observed between MMP-7 mRNA abundance and gender, age, or smoking consumption (Table 1).

(b) Serum concentration. Seventy-nine preoperatively collected serum samples of UBC patients and 19 healthy controls were analyzed using MMP-7 ELISA. The MMP-7 concentration was significantly elevated in serum samples of tumor patients ($P < 0.001$) (Table 1). There were no significant differences in MMP-7 concentration between males and females or between patients with primary or recurrent tumors. Serum MMP-7 concentrations did not correlate with age and smoking consumption (Table 1).

MMP-7 tissue expression/blood concentration and clinicopathological parameters. *(a) Tissue expression.* We could not detect any correlation between tumor grade and MMP-7 mRNA expression ($P = 0.078$). On the other hand, in muscle-invasive (T2–T4) tumors, MMP-7 gene expression was significantly higher than in superficial (Ta/T1) cases ($P = 0.006$). Most important, we found that MMP-7 expression is 21-fold significantly higher (median) in tumor samples of patients with metastatic UBC than in those of patients without known metastasis ($P = 0.001$) (Table 1). Furthermore, we found significant lower MMP-7 gene expression ($P = 0.041$) in lymph node-negative patients without evidence of metastasis in their follow-up compared to patients with a history of metastasis within a 2-year follow-up period after surgery (Fig. 1). ROC analysis was performed to estimate whether MMP-7 gene expression can be used to differentiate between patients with metastases and those with local disease (Fig. 2A). MMP-7 achieved an AUC (area under the curve) of 0.78 (95% CI, 0.62–0.94) and this was significant compared to the reference AUC of 0.50 ($P = 0.001$). The point with the highest diagnostic accuracy determined by ROC analysis was at the MMP-7 expression of 63 (relative expression) with a sensitivity and specificity of 71% and 84%.

(b) Serum concentration. We found no significant correlation between MMP-7 serum concentration and tumor stage or grade ($P = 0.105$, $P = 0.174$); however, MMP-7 concentration was slightly higher in high-stage, high-grade tumors. According to the observations in UBC tissues, we detected a 2.9-fold and

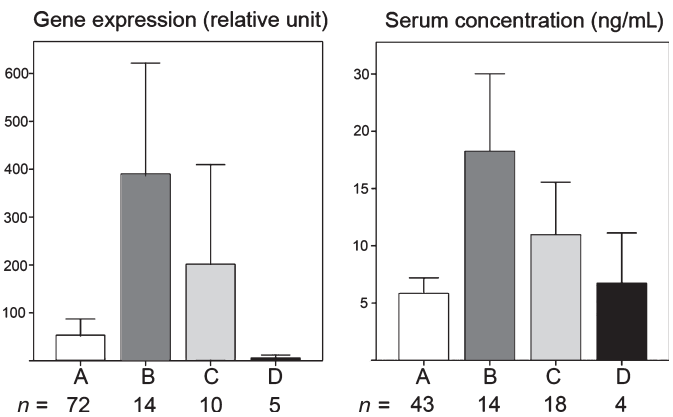

Fig. 1. Matrix metalloproteinase 7 (MMP-7) gene expression, serum concentration, and metastasis. (A) Patients with non-metastatic urinary bladder cancer (UBC) and no metastasis in follow-up; (B) patients with lymph node-positive tumors (at surgery); (C) patients with lymph node-negative tumor but metastasis in a 24-month follow-up period; (D) patients with lymph node-negative cancer but metastasis more than 24 months after surgery. MMP-7 gene expression and serum concentration is the highest in patients with present metastasis (B) and in patients with metastasis in a 24-month follow-up period (C). In contrast, MMP-7 gene expression and serum concentration is lower in patients without present or later metastasis (A) and in those with a history of metastasis more than 24 months after surgery (D).

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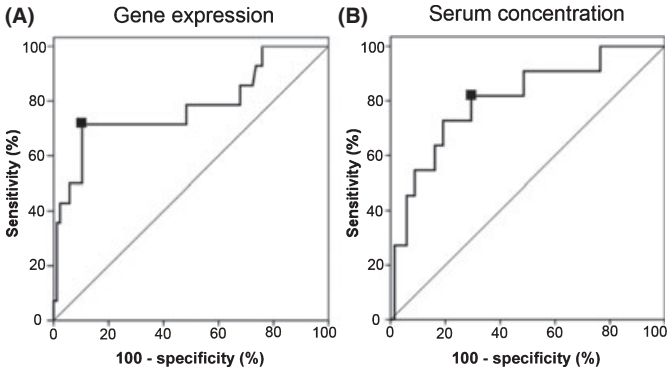

Fig. 2. Receiver–operating curve (ROC) analysis of matrix metalloproteinase 7 in the detection of metastasis in bladder. ROC analysis included patients without metastases (N0M0) as a negative group, and metastatic urinary bladder cancer patients (N1M0 and M1) as a positive group. Black quadrates show the relative gene expression (A) and serum concentration (B) levels provide the highest specificity and sensitivity in the detection of bladder cancer metastasis.

significantly elevated MMP-7 serum concentration in samples of patients with lymph node-positive tumors ($P = 0.002$) (Table 1). ROC analysis identified the serum concentration of 7.15 ng/mL as the cut-off value with the highest sensitivity (82%) and specificity (71%). AUC was 0.71 (95% CI, 0.58–0.83) and this significantly rejected the null hypothesis ($P = 0.003$) (Fig. 2B).

Immunohistochemical analysis. Immunostaining for MMP-7 showed a diffuse cytoplasmatic positivity in epithelial cells of normal-appearing urothelium, also smooth muscle cells of the bladder wall and blood vessels showed MMP-7 expression. The MMP-7 expression profile in superficial (Ta) bladder cancer was similar to that of normal-appearing urothelium. In contrast muscle-invasive cancers showed a largely inhomogeneous MMP-7 expression pattern characterized by strongly positive tumor cell clusters and single-positive tumor cells side by side with tumor cells appearing negative for MMP-7. In tumor-free lymph nodes, no considerable MMP-7 staining was seen, while in metastatic lymph nodes, both tumor cells and lymphocytes were MMP-7-positive (Fig. 3).

Correlation between MMP-7 gene expression and serum concentration. In 20 cases, both frozen tissue and serum samples from the same patient were available for analysis. Spearman's analysis revealed a significant correlation between serum and tissue levels of MMP-7 ($P = 0.019$). Nine of these 20 tissue samples showed high expression for MMP-7 while in 11 cases MMP-7 abundance was low. Eight out of nine cases (89%; 8/9) with high MMP-7 mRNA levels (relative expression >63) exhibited elevated MMP-7 serum concentration (>7.15 ng/mL). In contrast, only 36% (4/11) of cases with low MMP-7 tissue expression showed high serum levels. Three of these four patients (with low tissue expression, but high serum level) had metastasis at the time of surgery.

Univariate analysis. Results of univariate analysis and prognostic endpoints (overall, disease-specific, and metastasis-free survival) are listed in Table 2. Figures 4 and 5 show the Kaplan–Meier survival curves. Patients were subdivided into low or high groups for MMP-7 mRNA expression and serum concentration. The cut-off values of 63 relative gene expression, and of 7.15 ng/mL were defined by ROC analysis as described above.

Association of MMP-7 with patients' prognosis. Patients' age, gender, and smoking habit (yes *vs* no) did not influence overall-, disease-specific, or metastasis-free survival (Table 2). In contrast tumor stage, grade (low *vs* high) and lymph node status (positive *vs* negative) have a strong impact on overall ($P < 0.001$ each), and also disease-specific ($P < 0.001$ each) survival. Similarly, tumor stage and grade are predictors of metastasis ($P = 0.001$ each). Furthermore, high MMP-7 gene expression is a significant predictor of disease-specific and metastasis-free survival ($P = 0.010$, $P = 0.007$). In accordance, high MMP-7 serum concentration correlated with reduced overall, disease-specific-, and metastasis-free survival ($P = 0.009$, $P = 0.008$, $P = 0.008$ respectively) (Table 2, Fig. 3). The risk stratification of patients treated by radical cystectomy is of particular interest. Therefore, we also analyzed the prognostic significance of MMP-7 levels focusing solely on this group. High MMP-7 serum and gene expression levels were significantly associated with poor overall, disease-specific, and metastasis-free survival in patients treated by radical surgery ($P = 0.016$, $P = 0.007$, $P = 0.011$ for serum concentration and $P = 0.003$, $P = 0.002$, $P = 0.016$ for gene expression) (Table 2, Fig. 5).

Multivariate analysis. Multivariate analysis revealed that high MMP-7 gene expression and serum concentration are stage- and grade-independent prognostic factors of metastasis (HR = 3.804; 95% CI, 1.288–11.229; $P = 0.016$, HR = 2.534; 95% CI, 1.008–6.373; $P = 0.048$ respectively). Furthermore, high MMP-7 gene expression and serum levels are significant predictors of disease-specific survival (HR = 1.885; 95% CI, 1.000–3.551; $P = 0.050$, HR = 1.953; 95% CI, 1.027–3.712, $P = 0.041$ respectively) (Table 3).

Comparison of pre- and post-operative MMP-7 serum concentrations. In 13 cases, both pre- and post-operative serum samples were available. The median time to the second blood sample collection was 3.4 (in a range from 1.0 to 9.2 months). Four of 13 preoperatively collected sera showed high MMP-7 concentrations (12–40 ng/mL). In three of these four cases MMP-7 dropped by 14–23 ng/mL after radical surgery, while in one case, high MMP-7 remained unchanged. In this patient, prostate cancer was diagnosed at the time of surgery. In eight of nine patients with low preoperative MMP-7 concentration, the serum concentrations remained low, while in one patient, with known lymph node metastasis, MMP-7 level elevated (by 10 ng/mL) after cystectomy.

Discussion

The present study shows that MMP-7 expression is significantly elevated in tissue samples of metastatic UBC compared to those with local disease. High tissue expression levels are accompanied by elevated serum MMP-7 concentrations. Furthermore, high tissue and serum MMP-7 levels are stage- and grade-independent risk factors of metastasis and cancer-related death.

Matrix metalloproteinases (MMPs) are the most important proteolytic enzymes catalyzing the degradation of ECM, connective tissue, and basement membrane, which is essential for tumor invasion and metastasis. Matrilysin (MMP-7), the smallest member of the MMP family, is involved in the regulation of cellular processes, such as apoptosis, cell growth, and angiogenesis, beside of its primary ECM-degradation effect.[5–11] Furthermore, Kioi and coworkers showed that matrilysin specifically binds to colon cancer cells and induces tumor cell aggregation due to processing of membrane proteins. These aggregated cells showed a dramatically enhanced metastatic potential.[23] In accordance, increased circulating levels of MMP-7 were correlated with the presence of metastatic disease and poor patients' survival in colorectal and renal cell cancer.[18,19] Little is known about the role of MMP-7 in UBC. The only publication dealing with this issue reported a tendency of MMP-7 to be more strongly expressed in high- than in low-grade tumors by immunohistochemistry on paraffin-embedded tissue sections of 20 UBC patients.[24] Our results on gene expression level confirmed this observation. Immunohistochemical analysis identified MMP-7 expression in both

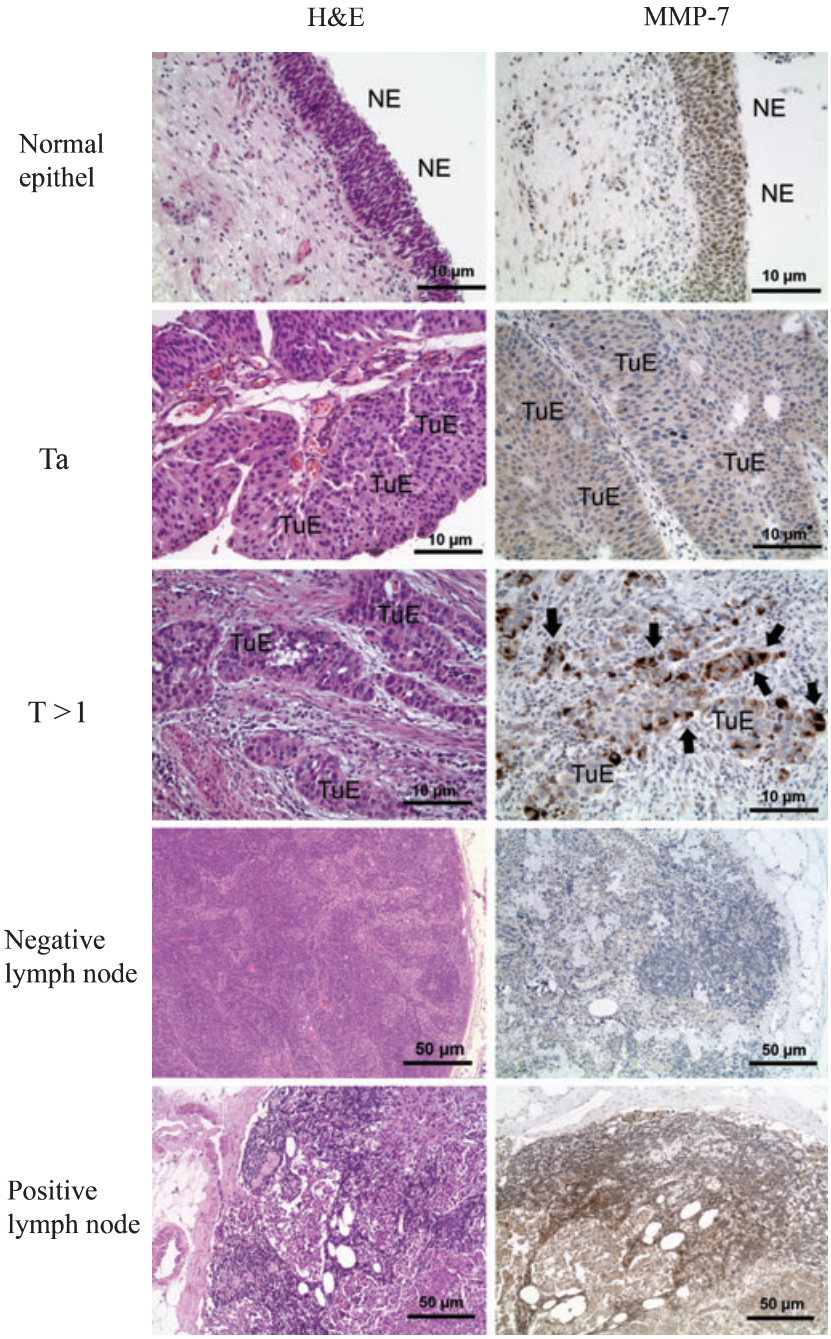

Fig. 3. Hematoxylin–eosin (H&E) staining and matrix metalloproteinase 7 (MMP-7) immunohistochemical analysis of normal-appearing urothelium, bladder cancer tissue, and both positive and tumor-free lymph nodes. Protein expression pattern of MMP-7 in bladder tissue: In normal urothelium (NE) a diffuse staining for MMP-7 is visible. In pTa, the superficial stage of bladder cancer, the tumor epithelium (TuE) shows a diffuse MMP-7 staining. In a part of the invasive urinary bladder cancer (UBC), tumor cell clusters or single tumor cells strongly positive for MMP-7 (arrows) were found within the tumor tissue side by side with tumor cells appearing negative for MMP-7. While lymph nodes without metastatic tumor cells were completely negative for MMP-7, lymph nodes containing metastatic tumor cells exhibited a strong staining for MMP-7. Remarkably, both the metastatic tumor cells as well as the lymphocytes were positive for MMP-7.

superficial and muscle-invasive UBC; however, in a markedly different pattern, while superficial (Ta) tumor cells and normal-appearing epithelial cells showed a weak-to-moderate diffuse cytoplasmatic immunostaining, muscle-invasive tumors were characterized by strongly positive tumor clusters and single-positive tumor cells. These tumor cells strongly positive for MMP-7 might promote tumor invasiveness and metastasis.

The significantly (P = 0.001), 21-fold elevated MMP-7 gene expression in UBC samples of patients with lymph node-positive disease suggests that determination of MMP-7 expression may help to select patients with metastatic carcinoma. Using the cut-off point of 63 we found a sensitivity of 71% and a specificity of 84%. Since all gene expression values (tumors, controls) were related to the Human Reference RNA, this cut-off value provides comparable results to other laboratories when using the same primers (MMP-7 and TBP). Furthermore, multivariate analysis revealed that high MMP-7 expression is a strong predictor of metastasis and disease-specific death (P = 0.016 and P = 0.050, respectively) independently from tumor stage and grade.

Analysis of serum samples offers several advantages when compared to tumor tissue, for example higher sample homogeneity, and its collection is only minimally invasive. Furthermore, analysis of serum samples can provide information prior to surgery with a possible impact on surgical decisions. Therefore, we extended our studies to the circulating MMP-7 serum levels of UBC patients. Based on the results at the gene expression level, we focused mainly on patients with advanced disease, treated by radical cystectomy. In accordance with the findings at the tissue expression level, serum MMP-7 concentration was significantly higher in patients with lymph node-positive tumors (P = 0.002). This is in line with our immunohistochemical findings showing

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Table 2. Cox univariate analysis

Variables	*n*	Overall survival			Disease-specific survival			Metastasis-free survival		
		HR	95% CI	*P*-values	HR	95% CI	*P*-values	HR	95% CI	*P*-values
Age										
≤65 years	77	Ref.			Ref.			Ref.		
>65 years	83	1.103	0.740–1.644	0.631	1.043	0.656–1.656	0.859	1.027	0.501–2.107	0.942
Sex										
Female	37	Ref.			Ref.			Ref.		
Male	123	0.958	0.600–1.530	0.857	0.969	0.481–1.374	0.448	0.663	0.303–1.448	0.316
Stage										
Non-inv. (Ta-T1)	73	Ref.			Ref.			Ref.		
Invasive (T2-T4)	87	2.180	1.441–3.298	<0.001	3.769	2.206–6.439	<0.001	3.761	1.718–8.237	0.001
Grade										
Low grade	76	Ref.			Ref.			Ref.		
Hig–grade	84	2.408	1.603–3.619	<0.001	3.346	2.050–5.462	<0.001	3.940	1.751–8.863	<0.001
Lymph node status										
N–/Nx	137	Ref.			Ref.			–		
N+	23	4.167	2.466–7.040	<0.001	4.638	2.667–8.068	<0.001	–	–	–
Prior recurrence										
Primer	82	Ref.			Ref.			Ref.		
Recurrent	78	1.301	0.870–1.943	0.314	1.270	0.797–2.022	0.314	0.981	0.478–2.012	0.958
Smoking										
No	65	Ref.			Ref.			Ref.		
Yes	75	0.943	0.626–1.423	0.781	0.768	0.479–1.231	0.273	0.605	0.291–1.259	0.178
Unknown	20									
MMP-7 gene exp.										
Low	77	Ref.			Ref.			Ref.		
High	24	1.712	0.993–2.952	0.053	2.270	1.219–4.227	0.010	4.211	1.482–11.965	0.007
in Ta-T1 cases										
Low	44	Ref.			Ref.			–		
High	6	0.855	0.246–2.969	0.805	2.435	0.592–10.021	0.218	–	–	–
in T2-T4 cases										
Low	33	Ref.			Ref.			Ref.		
High	18	1.440	0.766–2.709	0.257	1.374	0.685–2.753	0.371	4.914	1.445–16.717	0.011
Cystectomy	55									
Low	36	Ref.			Ref.			Ref.		
High	19	2.604	1.393–4.871	0.003	3.024	1.480–6.177	0.002	4.475	1.317–15.208	0.016
MMP-7 serum cc.										
Low	50	Ref.			Ref.			Ref.		
High	29	2.087	1.201–3.627	0.009	2.351	1.251–4.418	0.008	3.381	1.370–8.347	0.008
in Ta–T1 cases										
Low	23	Ref.			Ref.			Ref.		
High	8	1.579	0.591–4.224	0.363	1.935	0.544–6.885	0.308	1.957	0.355–10.793	0.441
in T2–T4 cases										
Low	27	Ref.			Ref.			Ref.		
High	21	2.061	1.033–4.111	0.040	2.076	0.993–4.339	0.052	3.819	1.246–11.703	0.019
Cystectomy	58									
Low	35	Ref.			Ref.			Ref.		
high	23	2.084	1.149–3.779	0.016	2.472	1.280–4.775	0.007	3.341	1.321–8.449	0.011

CI, confidence interval; HR, hazard ratio; ref., referent.

a strong MMP-7 expression in metastatic but not in tumor-free lymph nodes. Using the cut-off value of 7.15 ng/mL, determined by ROC analysis, we found a high sensitivity of 82% and a specificity of 71%. Furthermore, high serum concentration of MMP-7 was found to significantly correlate with shorter UBC-specific and metastasis-free survival ($P = 0.041$ and $P = 0.048$) independently from tumor stage and grade.

Two-fold significantly elevated serum levels of MMP-7 were detected in cancer patients compared to controls ($P < 0.001$). No such correlation was observed in tissue samples between gene expression ($P = 0.678$) and protein level. A possible explanation for the lack of difference in the gene expression between tumor and tumor-adjacent normal-appearing tissue is the 'field cancerization' or 'field effect', which has been described in bladder cancer.[25] In preneoplastic, histologically normal-appearing fields, early genetic alterations or gene expression changes may occur. Therefore, normal-appearing epithelium from a cancerous bladder does not necessarily represent normal epithelial cells. Furthermore, tumor cells can influence the mRNA and protein expression of normal cells in a paracrine manner.

The significant correlation between circulating and tissue MMP-7 levels in cases with high tissue matrilysin expression observed, suggests that serum MMP-7 originates mainly from the UBC cells. On the other hand, in four of 11 cases with low MMP-7 tissue expression, we detected elevated circulating matrilysin levels. Three of these four patients had metastasis at the time of surgery, indicating that the primary tumor is not the

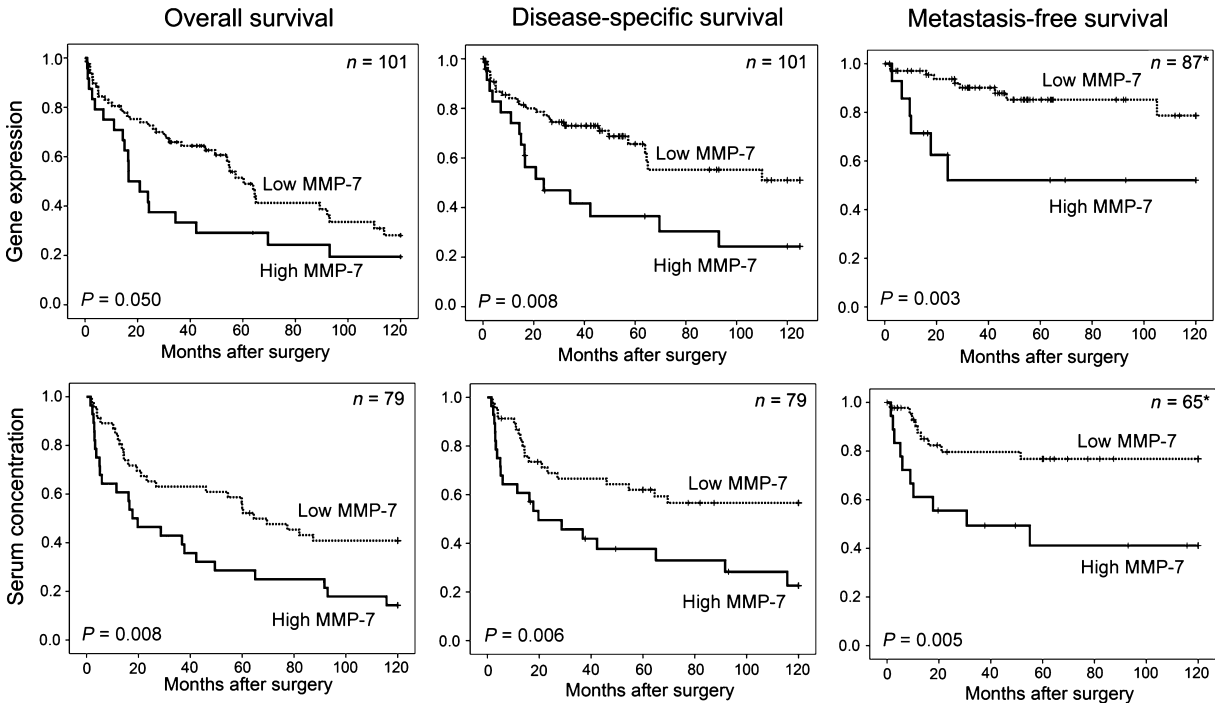

Fig. 4. Kaplan–Meier survival curves. Kaplan–Meier curves of overall, cancer-specific, and metastasis-free survival stratified by matrix metalloproteinase 7 (MMP-7) gene expression. Overall, disease-specific-, and metastasis-free survival is significantly shorter in patients with high MMP-7 gene expression and serum concentration. *Patients with metastasis at surgery were not included in this analysis.

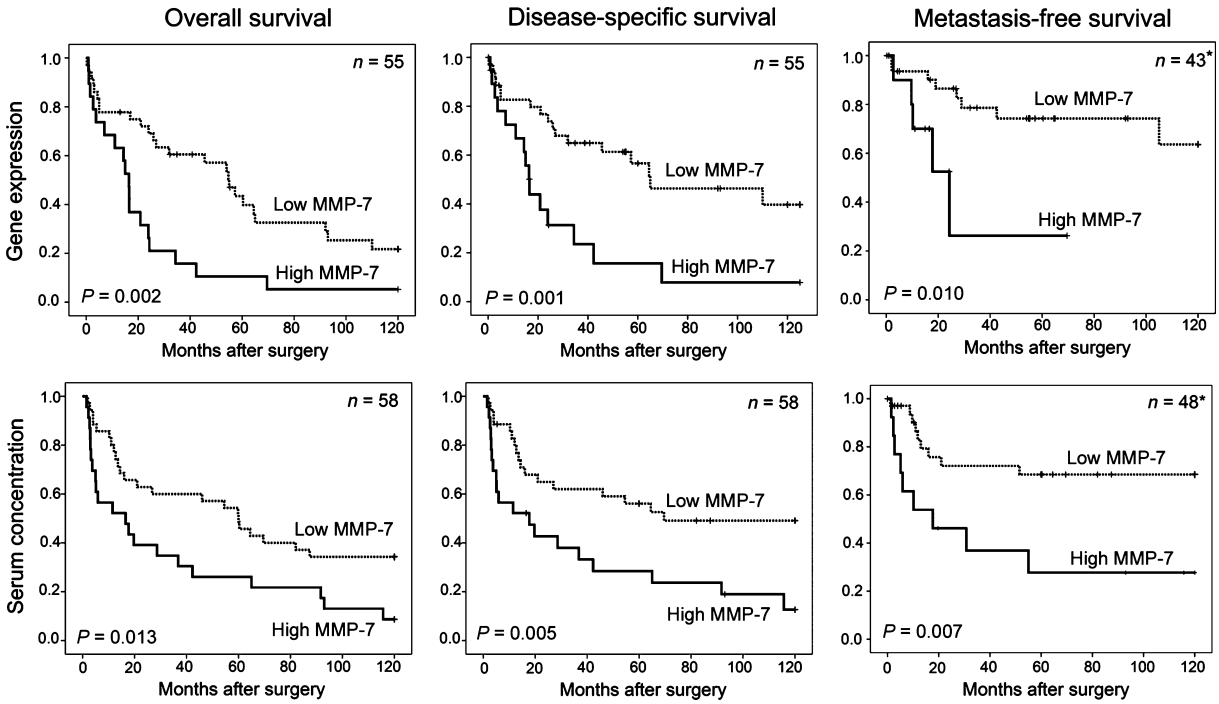

Fig. 5. Kaplan–Meier survival curves. Kaplan–Meier curves of overall, cancer-specific, and metastasis-free survival stratified by matrix metalloproteinase 7 (MMP-7) gene in patients treated by radical cystectomy. Overall, disease-specific-, and metastasis-free survival is significantly shorter in patients with high MMP-7 gene expression and serum concentration. *Patients with metastasis at surgery were not included in this analysis.

only possible source of serum MMP-7. In our opinion, residual tumor and/or undetected (micro)metastases could also be responsible for high circulating MMP-7 levels. This idea is in line with the findings of *Zeng et al.* who have shown that MMP-7 is significantly overexpressed in liver metastases of colorectal cancer compared to adjacent normal-appearing liver tissue.[26]

In muscle-invasive UBC treated by radical cystectomy, lymph node status is a more reliable predictor of cancer-specific

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Table 3. Cox multivariate models

Variables	Disease-specific survival		
	HR	95% CI	*P*-values
Model 1			
Stage (T2-T4)	2.221	0.979–5.040	0.056
Grade (G2-G3)	1.679	0.711–3.962	0.237
MMP-7 serum (>7.15 ng/mL)	1.953	1.027–3.712	0.041
Model 2			
Stage (T2-T4)	2.595	1.035–6.508	0.042
Grade (G2-G3)	2.557	1.089–6.008	0.031
MMP-7 expression (>63 rel.exp.)	1.885	1.00–3.551	0.050
Variables	Metastasis-free survival		
	HR	95% CI	*P*-values
Model 3			
Stage (T2-T4)	1.323	0.430–4.075	0.626
Grade (G3)	3.699	0.859–15.926	0.079
MMP-7 serum (>7.15 ng/mL)	2.534	1.008–6.373	0.048
Model 4			
Stage (T2-T4)	7.427	1.521–36.258	0.013
Grade (G3)	1.035	0.272–3.933	0.960
MMP-7 expression (>63 rel.exp.)	3.804	1.288–11.229	0.016

CI, confidence interval; HR, hazard ratio.

survival than is pathological stage. Patients without metastasis do have a relative favorable prognosis with 5-year survival rate of 80%.[27] In contrast, only 30% of patients with lymph node-positive UBC survive the 5-year period. A subset of patients with lymph node-positive tumors may be cured by extended lymph node dissection alone, while most of them would benefit from early adjuvant chemotherapy.[28,29] The common techniques used for the monitoring of metastasis are computer tomography (CT) and nuclear magnetic resonance imaging. The sensitivity of these imaging methods is limited particularly in the detection of low-volume metastases.[30] In this context, the significantly elevated MMP-7 level in sera of patients with metastatic UBC and its stage- and grade-independent prognostic relevance indicates that this marker may be a valuable tool for the identification of patients with present and/or with high risk of metastasis. These patients could be subjected to a more extensive lymph node dissection. Our results suggest that high MMP-7 expression is able to predict later metastasis as much as 2 years before diagnosis (Figs 1,3,4). These high-risk patients may benefit from strict surveillance or from early adjuvant chemotherapy. However, this assumption must be verified in a prospective study.

Comparing the pre- and postoperative serum levels of matrilysin in 13 cases of radical surgery, we observed a strong reduction in circulating MMP-7 level after cystectomy in three of four cases with elevated preoperative matrilysin concentrations; however, the postoperative concentrations were still high in two cases with lymph node-positive tumors. This change in MMP-7 serum levels confirms the former notion that circulating MMP-7 originates mainly in the tumor cells. The dynamic change of MMP-7 serum concentration we observed makes this marker a promising tool for therapy monitoring. Further analysis with a larger number of patients is required to confirm this idea.

Conclusions

Analyzing the prognostic value of MMP-7 in UBC, we demonstrate here for the first time that elevated tissue expression and serum concentration levels of MMP-7 are associated with the presence of metastatic disease. Furthermore, elevated MMP-7 expression and circulating levels are stage- and grade-independent risk factors for metastasis and UBC-related death. This information is of particular interest in the risk stratification of patients with advanced UBC and may be a useful tool to optimize therapy. A further prospective study (including both training and validation subsets) is needed to validate MMP-7 analysis in UBC.

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Disclosure Statement

The authors have no conflict of interest.

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