Matrix Metalloproteinase-9 (MMP-9) Immunoreactive Protein in Urinary Bladder Cancer: A Marker of Favorable Prognosis

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Abstract. *Backround: The purpose of this study was to explore whether changes in matrix metalloproteinase-9 (MMP-9) are involved in the progression of urinary bladder cancer and whether they have any prognostic value. Patients and Methods: Overexpression of MMP-9 was evaluated in the primary tumor of 87 urinary bladder cancer cases with immunohistochemical staining. Results: Of the urinary bladder carcinomas, 38% showed an overexpression (>25%) of MMP-9 immunoreactive protein. Increased positivity for MMP-9 correlated with favorable overall survival of the urinary bladder cancer patients. The 5-year overall survival and relapse-free survival was 68% and 36% in patients showing high MMP-9 expression and 48% and 19% in patients with low (<25%) or negative MMP-9 expression (p=0.006, p=0.08, respectively). In multivariate analysis, MMP-9 overexpression seems to retain its independent prognostic value. Conclusion: This study shows that MMP-9 expression in urinary bladder carcinoma is associated with better prognosis.*

Incidence of urinary bladder cancer has been increasing in recent years in European Union countries. It is now the fourth most frequent cancer among men (1). The 5-year relative survival rate is 66% in both sexes. When diagnosed, 75% of the urinary bladder cancers are superficial (1). After treatment, however, the recurrence rate is 50-80% and in 10-25% of cases it will progress into a muscle-invasive bladder cancer (1, 2). One general problem in urinary bladder cancer treatment is how to recognize patients in different risk groups who would need more tailored treatment or follow-up even at an early stage.

Matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases which degrade the extracellular matrix (ECM). They are known to play an important role in degrading matrix boundaries, for instance type IV collagen which forms the backbone of basement membranes. Besides tumor invasion, metalloproteinases, particularly gelatinases (gelatinase A, MMP-2 and gelatinase B, MMP-9), are also known to participate angiogenesis and tumor growth (3, 4). The fact that both tumor cells and stromal cells produce proMMP-9 is well recognized. ProMMP-9 is activated by many proteolytic enzymes, but the detailed regulating process is still under keen study (5). The overexpression of gelatinases has been found to correlate with the grade and stage in several types of cancer and is also related to the relapse-free or cancer-specific survival (6-9). MMP-2 is more clearly associated with the aggressive clinical course in several types of solid tumor. The role of MMP-9 is, however, not that clear particularly in bladder cancer (10, 11). The results have also been conflicting in other types of cancer and the expression of MMP-9 has been linked to both favorable and poor prognosis (12-14).

In light of these facts, our study aimed to explore whether MMP-9 is involved in urinary bladder cancer and has any prognostic significance.

Patients and Methods

Patients. Eighty-seven urinary bladder carcinoma cases with paraffin embedded tissue samples were obtained from the files of the Department of Pathology, Oulu University Hospital between years 1987 and 1992. The samples were all primary samples and were evaluated by a pathologist to represent a transitional cell carcinoma (TCC of the urinary bladder). The histological grade of the tumors was reviewed and classified according to the WHO Classification for urological tumors (15). The median age of the patients was 67 years (21-86). Sixty-eight of the patients were men and 19 women. The clinical stage was defined according to the 1997 International Union against Cancer tumor staging system (16). The minimum follow-up time was 5 years. The patient characteristics are shown in Table I.

Most of the patients were treated endoscopically. In the case of patients with high risk tumor (*In situ*, T1G3, plural tumor) or superficial recidival tumors, immunotherapy with intravesical Bacillus Calmette-Guérin (BCG) or intravesical chemotherapy with farmorubicin or mitomycin-C was given. Radical cystectomy was performed on eleven high-risk patients with multifocal, grade 3 tumor. Seven of the cystectomy patients received preoperative radiotherapy and intravenous chemotherapy. Three patients, who could not be operated on because of high age and risk for

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Table I. *MMP-9 overexpression (≥25% of cells) in bladder cancer.*

Patient characteristic	Total	MMP-9 overexpression n (%)
All patients (n)	87	33 (38)
Men	68	28 (41)
Women	19	5 (26)
Stage		
I	50	21 (42)
II	19	7 (37)
III	11	3 (27)
IV	7	2 (29)
Grade		
1	24	11 (46)
2	48	19 (40)
3	15	3 (20)

comorbidity, were treated with radical radiotherapy. Five patients received only palliative radiotherapy.

Immunohistochemistry. For immunohistochemistry, 4 μm paraffin sections were cut onto slides coated with poly-l-lysine (Sigma Chemicals, St Louis, MO, USA) and incubated overnight at 37˚C. The slides were deparaffinized in a histological clearing agent (Histo-Clear, National Diagostics, Atlanta, GA, USA) and hydrated. Endogenous peroxidase activity was blocked by incubating the slides in 0.3% hydrogen peroxidase in absolute methanol and any non-specific binding was blocked with 10% goat serum.

Mouse monoclonal antibody (CA-4001 and GE 231; Diabor Ltd., Oulu, Finland) against MMP-9 (10 μg /ml) was used as the primary antibody. The slides were incubated with primary antibody at room temperature overnight in a humidified atmosphere after which immunohistochemical staining was continued using the Histostain-plus bulk kit (Zymed Laboratory Inc., San Francisco, CA, USA). The slides were washed thoroughly with PBS after each step in the procedure. The antibody reaction was visualized using a fresh substrate solution containing AEC (aminoethyl carbazole substrate; Zymed Laboratory Inc., San Francisco, CA, USA). The sections were counterstained with hematoxylin and mounted in Immu-Mount (Shanon Inc., Pittsburgh, PA, USA). For negative controls the primary antibody was replaced either with the mouse non-immuno IgG or PBS.

Evaluation of MMP-9 immunostaining scores. Three independent observers scored the immunostaining for MMP-9 in two replicate experiments. Two slides per patients were stained. When staining was <5% it was scored as negative, over 5% but under 25% was scored as low staining (+), or over 25% but under 50% intermediate staining (++) and 50% or over as high staining (+++). The interobserver variability was good. The clinical data were not analyzed until the immunostaining scores were available. When more than 25% of the tumors cells showed positive staining, this was considered as overexpression.

Statistical analysis. The SPSS software system for Windows was utilized for statistical analyses (version 12.0; SPSS, Chicago, IL, USA). The association of the immunoreactivity of MMP-9 with the tumor grade, stage and age of the patients was tested using the chi-square test. The relapse-free survival, cancer-specific survival and the overall survival were determined with Kaplan-Meier analysis and the statistical differences in survival among subgroups were compared by log-rank test (17). *P*-values <0.05 were considered statistically significant. Cancer-specific survival was defined from the diagnosis to the date of death from urinary bladder carcinoma. In these analyses the cases were censored at the last follow-up or when dying from another disease. Relapse-free survival was determined as the time elapsed from the diagnosis to the relapse, and the cases were censored at the time of the last follow-up. Cox regression analysis was used in multivariate analysis.

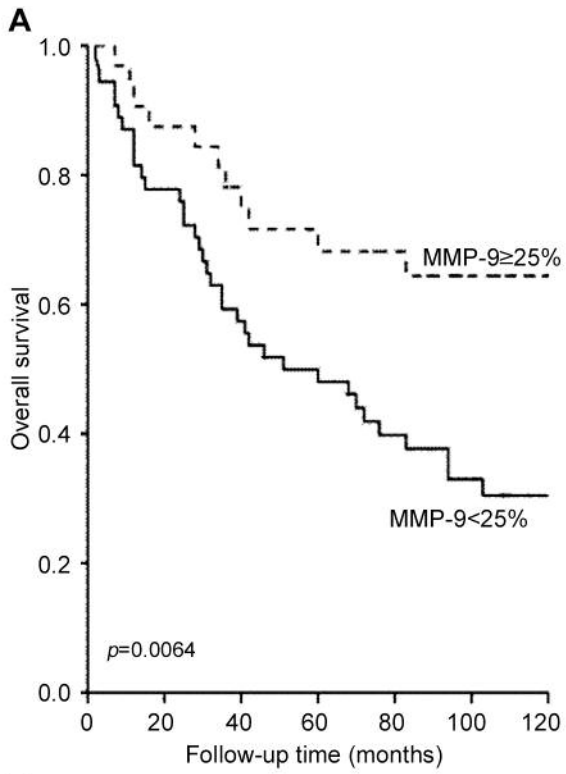
Table II. *First site of relapse and occurrence of overexpression of MMP-9 immunoreactive protein in bladder cancer.*

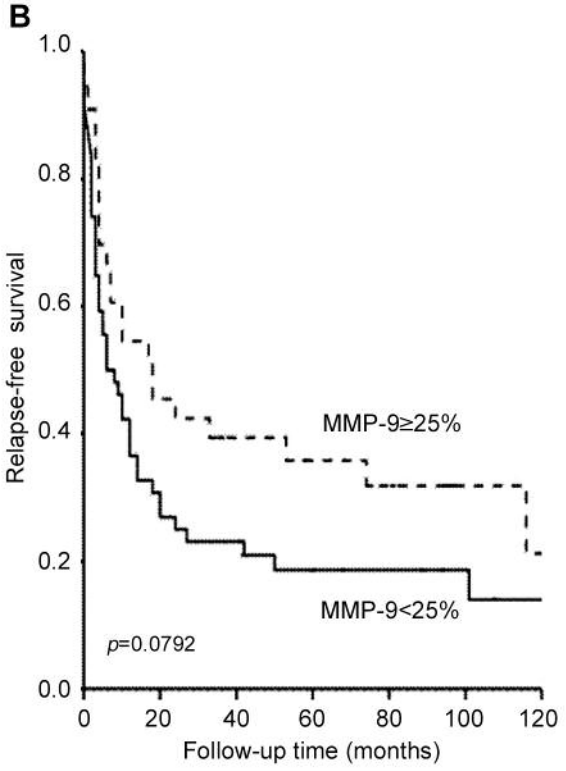
Type of relapse	n	MMP-9 overexpression n (%)
No relapse	20	10 (50%)
Relapse	67	23 (34%)

Results

A positive immunoreaction was most often seen in cancer cells and appeared as diffuse cytoplasmic positivity. Thirty-eight percent (33 of 87) of the urinary bladder cancer samples showed an overexpression of MMP-9 protein (>25% of the cells appearing as positive) (Table I). We could not find any association between the overexpression of MMP-9 and the tumor stage or grade (p=0.78 and p=0.25, respectively, χ^2 test). There was also no statistically significant association between the overexpression of MMP-9 and sex (p=0.24) or age (p=0.06, χ^2 test) (Table I). The urinary bladder cancer relapsed in 67 of the 87 patients. Most of the relapses were local (47 of 67). Ten patients had lymph node relapses and ten had a hematogenous relapse. Twenty-three out of the 67 relapsed cases showed an overexpression for MMP-9 (Table II).

MMP-9 overexpression was significantly correlated with overall survival of the patients with urinary bladder cancer. Patients with MMP-9 posititivity >25% had a five year overall survival rate of 68% when it was only 48% in those with no or low (<25%) expression of MMP-9 (p=0.006, Figure 1A). The prognostic variables stage and tumor grade were analyzed with log rank univariate analysis. They both appeared to be significant indicators (p=0.0006, and p=0.002, respectively). Multivariate Cox regression analysis was then used to evaluate whether the correlation between MMP-9 expression and overall survival depended on these two important prognostic factors. The MMP-9 expression was found to be the most significant factor for overall survival. Patients with low (<25%) or no MMP-9 expression had a 2.18-fold relative risk for death compared to the patients with the MMP-9 overexpression (≥25%). (p=0.02, 95% confidence interval 1.15 - 4.16, Table III).





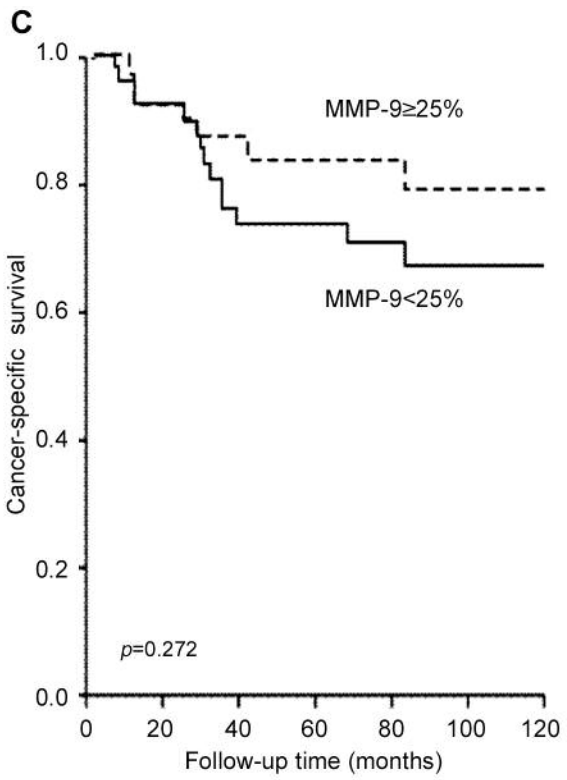


Figure 1. *Survival analysis (Kaplan-Meier) for patients with bladder cancer according to the expression of MMP-9 immunoreactive protein. (A) Overall survival, (B) Relapse-free survival and (C) Cancer-specific survival of patients with bladder carcinoma showing MMP-9 overexpression and negative or low MMP-9 expression.*

Table III. *Cox regression analysis including the MMP-9 status and the stage and the grade of disease.*

Covariate	β[a]	SE risk	Relative	95% CI	*p*-value
MMP-9					
MMP-9 ≥25%	0				
MMP-9 <25%	0.781	0.329	2.184	1.15-4.16	0.02
Stage					
I	0				
II	0.625	0.358	1.867	0.93-3.77	0.08
III	0.281	0.515	1.324	0.48-3.63	0.59
IV	1.122	0.524	3.072	1.099-8.59	0.03
Grade					
1	0				
2	0.535	0.408	1.708	0.77-3.80	0.19
3	0.933	0.561	2.542	0.85-7.63	0.10

[a]Coefficient; CI: confidence interval; SE: standard error.

The 5-year relapse-free survival was 36% in patients with no or low (<25%) MMP-9 expression and 19% in patients with MMP-9 overexpression (≥25%) in the tumor (p=0.08, Figure 1B). Due to the small sample size, the correlation between relapse-free survival and the overexpression of the MMP-9 protein in immunohistochemical staining did not

quite reach statistical significance. In log rank univariate analysis the stage correlated significantly with relapse free survival but not with the grade (p=0.003, p=0.26, respectively). In multivariate Cox regression analysis stage was the most predictive factor, although no statistical significance was reached.

Both tumor grade and stage correlated here with cancer-specific survival in univariate analysis (p<0.0001, p=0.0006, respectively). Patients with MMP-9 overexpression (≥25%) in the tumor tended to have a better cancer-specific survival than those with no or low (<25%) MMP-9 expression; 83% versus 73% (Figure 1C). This difference did not quite reach statistical significance, however. In multivariate analysis, stage was the most significant factor for cancer-specific survival.

Discussion

In the present study, we evaluated the role of MMP-9 expression as a prognostic factor in urinary bladder cancer. The association of MMP-2 with an aggressive behavior is well defined in this cancer type. Recently some studies have shown gelatinase-A (MMP-2) to be linked to the clinical progression of bladder carcinoma (18). MMP-2 expression seems to correlate with poor outcome (19). The present study is, to our knowledge, the first to report that MMP-9 overexpression could, unlike MMP-2 expression, be a favorable prognostic factor in urinary bladder carcinoma. The overall survival of urinary bladder cancer patients with no or low (25%) grade MMP-9 expression in primary tumors was poor. In multivariate Cox regression analysis, MMP-9 overexpression was the most significant factor for overall survival.

The data available concerning the prognostic role of MMP-9 in urinary bladder cancer is conflicting. Durkan *et al*. showed a correlation of MMP-9 immunoreactivity with poor disease-specific survival. The median follow-up time of their 106 patients was only 25 months. Most of their cases represented a superficial tumor and staining with 3+ or 4+ were considered as MMP-9 positive (10). Grignon *et al*. studied both tissue inhibitor of MMPs (TIMP) and MMP-9 and found a statistically significant correlation between the stromal TIMP-2 staining and disease-specific survival in bladder cancer in a study of 43 patients with a median follow-up time of 18 months. They did not, however, find any statistically significant correlation between MMP-9 and survival (11).

One study on breast cancer suggests MMP-9 expression to be an indicator for a better prognosis (12). In that study by Scorilas *et al*. patients had an early node-negative breast carcinoma and MMP-9 overexpression was shown to predict a more favorable survival. In another study by Rahko *et al*., MMP-9 positive early breast cancer did not show any increase in aggressive clinical behavior (14).

MMP-9 similarly to other MMPs is produced as an inactive, latent form that requires activation for being enzymatically active (14). The MMP-9 antibody used in the present study measures the proMMP-9 protein, both free and in complex with TIMP-1. The samples were primary samples collected prior to treatment and most of the patients had stage I disease. The tumors were also of grade 1 or 2. Most of these cases represented thus early urinary bladder cancer. It was suggested by Scorilas *et al*. that the balance between latent and active MMP-9 could shift towards active when the grade of breast carcinoma would progress (12). Our results are in line with this suggestion. Since the association in the present study was stronger with the overall survival rather than the cancer-specific survival also other explanations may be likely, such as a possible role of MMP-9 in immunological host reactions in cancer patients or in controlling the tumor angiogenesis. It is possible that these factors might dominate in early tumor progression.

One of the most effective activators for MMP-9 is stromelysin-1 (MMP-3) (20). In addition other MMPs such as MMP-2, MMP-7 and MMP-13 and some serine proteinases are able to activate proMMP-9. MMPs are known to be needed in degradation of extracellular matrix and basement membranes, but they also play a role in the formation of new capillaries, which is essential for tumor progression (21). TIMPs regulate MMP activity, but have also been found to modulate tumor angiogenesis. It seems that MMPs have a complex role in this process and certain MMPs also take part in inhibition of neovascularization (22). Heljasvaara *et al*. indicated recently that certain cancer-related MMPs, such as MMP-9, may participate in the inhibition of endothelial cell proliferation and angiogenesis. This is due to the fact that MMPs, including MMP-9, generate antiangiogenic endostatin-containing peptides from collagen XVIII (23).

In the present study, we did not find a statistical correlation between MMP-9 staining and tumor grade or stage. This is in line with Durkan *et al*. who also did not found any correlation between MMP-9 staining and histological grade or stage (10). Some data using zymography has shown correlation between MMP-9 activity and tumor grade (24, 25). Zymography cannot be considered, however, as a quantitative method for assaying the amounts of gelatinases. The role of MMPs and their inhibitors as clinical progression markers differs in different carcinomas and is still far from clear. MMP-2 seems to be more established as a marker of aggressive clinical course and poor prognosis in different types of cancer.

The role of MMP-9 is, on the other hand, more complicated and somewhat controversial. The present study shows that MMP-9 overexpression in urinary bladder carcinoma could be a marker of favorable prognosis especially for overall survival. Potentially,

MMP-9 might offer new information along with markers associated with aggressive behavior. Further studies are still needed to confirm the prognostic value of MMP-9 in urinary bladder cancer.

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