Enhanced Urinary Gelatinase Activities (Matrix Metalloproteinases 2 and 9) Are Associated with Early-Stage Bladder Carcinoma: A Comparison with Clinically Used Tumor Markers

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ABSTRACT

Matrix metalloproteinases (MMPs) are involved in tumor growth and metastasis, promoting the migration and invasion of cells. In this study, the amount of MMP-2 and MMP-9 activity was measured in urine from superficial bladder carcinoma patients (pTa, pT1) to evaluate their possible diagnostic value. The active and total amount of MMP-2 and MMP-9, respectively, in urine from tumor patients were compared with the levels in urine from age- and gender-matched healthy volunteers. Both MMP-2 and MMP-9 activity levels were significantly enhanced in urine from patients with high invasive cancers (pT2, pT3), whereas in urine from healthy controls no or very low MMP activities were found. More importantly, a substantial number of urine samples from patients with superficial tumors contained elevated MMP-2 and MMP-9 activities, suggesting that enhanced urinary MMP activity levels, indeed, might be indicative for early-stage bladder cancer. Overall, urinary MMP-2 and MMP-9 activity levels were significantly correlated to each other, with some individual exceptions. A comparison between urinary MMP-9 activity and a recently proposed urinary marker for bladder cancer, NMP-22, showed slightly lower numbers of patients with elevated levels for MMP-9. But because MMP-9 and NMP-22 levels were not correlated, enhanced urinary MMP activity might be useful as a marker for superficial bladder carcinoma like, or especially in combination with, other markers.

INTRODUCTION

In industrialized countries transitional cell carcinoma of the bladder is a common type of malignancy. Its occurrence is strongly associated with cigarette smoking and the use of certain chemicals. Bladder cancer is usually divided in two categories, superficial tumors (75–80%) confined to the mucosa, and muscle-invasive tumors. Seventy percent of the superficial bladder carcinomas (pTa, pT1) show recurrence after treatment, from which, unfortunately, 30% progress to muscle-invasive tumors (1). The currently used methods to detect early-stage bladder carcinomas or to monitor patients after endovesical treatment for recurrence of invasive cancer, are neither very specific nor easy to perform. Recent studies have focused on the detection of specific bladder carcinoma-associated proteins in urine of patients. Several classes of proteins have been investigated, including growth factors, specific basement membrane components, and nuclear matrix proteins, with variable results (1–6). Recently, several members of the matrix MMP family have been found in urine of bladder carcinoma patients (7–9) and in bladder washes of these patients the level of MMP-9 was found to be enhanced (10).

MMPs are a class of proteolytic enzymes that are directly involved in carcinogenesis (11). MMPs, in particular interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), stromelysin-3 (MMP-11), and the gelatinases MMP-2 and MMP-9, are found in enhanced amounts in tumor tissues, where they regulate tumor growth and metastasis, promoting the invasion of malignant cells (12, 13). Increased amounts of MMPs in various cancer tissues are found to be associated with bad survival (14, 15), corresponding with the hypothesis that MMPs promote the initiation and tumor growth of metastatic foci (reviewed in Ref. 13). Enhanced levels of MMPs have also been found in blood from cancer patients (16–20). The elevated MMP concentrations in the circulation might be a reflection of the presence of these enzymes in the actual cancer tissue. Urinary MMP levels

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1 The abbreviations used are: MMP, matrix metalloproteinase; APMA, p-aminophenylmercuric acetate; MMP-2, gelatinase-A; F, 72,000 type IV collagenase; MMP-9, gelatinase-B; M, 92,000 type IV collagenase; NED, no evidence of disease; MMP-22, nuclear matrix protein-22; S-2444, pyro-Glu-Gly-Arg-paranitroanilide HCl; Mab, monoclonal antibody; TNO-S22.2, MMP-9-specific MAb; UKcol, modified pro-uromodulin; MW, molecular weight.
in cancer patients could in their turn reflect the enhanced presence in the circulation, but in the case of bladder cancer might also directly originate from the tumor and could, therefore, be particularly indicative for superficial bladder tumors (21).

To investigate a possible clinical use of urinary MMP analyses as a bladder tumor marker, we determined MMP-2 and MMP-9 activity levels in urine from patients with low-stage transitional cell bladder carcinoma. Gelatin zymography indicated that both gelatinases MMP-2 and MMP-9 are abundantly present in a majority of urine samples from bladder cancer patients, whereas hardly any MMP was detectable in healthy controls. Zymography is still the most widespread method for measuring MMP activity and, although very useful as a qualitative method, it is not suitable for quantitation of large numbers of samples. The available ELISAs for MMP-2 and MMP-9 have the disadvantage that they do not distinguish between active and latent forms of (pro)MMPs, which might be relevant with respect to carcinogenesis. Therefore, in this study a recently developed method for the detection of specific MMP activities in human body fluids was used (22, 23). MMP-2 and MMP-9 activities in urine from low invasive stage bladder carcinoma patients were compared with the levels in healthy volunteers and in patients with high invasive bladder cancer. Finally, the diagnostic value of the detection of bladder carcinoma by measuring urinary MMP-2 and MMP-9 activity, respectively, was compared with traditional urinary cytology analysis, with the urinary antigen levels of uPA and Cathepsin B. This assay was performed as described previously (22, 25). In brief, 96-well plates (Costar) were coated with anti-MMP-9 MAb S22.2 (TNO-PO, G, Leiden, the Netherlands; Ref. 25) and washed with PBS and 0.05% Tween 20 (v/v). Purified standard MMP-9 and urine samples (1/2 diluted in 1% BSA-PBS) were incubated overnight at 4°C. The wells were washed and incubated with assay buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM CaCl₂, 1 μM ZnCl₂, and 0.01% (v/v) Brij-35], to which 15 μl (50 mg/ml) of UKcol (TNO-PO; Ref. 22) and 10 μl (6 mg stock) of chromogenic substrate S-2444 (Chromogenix, Mödling) were added. Color development was recorded in a Titerpak plate reader at 405 nm (Flow Laboratories, Irvine, Scotland). For measurement of total activity (already active plus latent MMP-9), the immobilized MMP-9 was incubated with assay buffer containing 0.5 mM APMA for 2 h, after which UKcol and S-2444 were added and activity was recorded. The MMP-2 activity assay was purchased from Amersham Pharmacia Biotech, which is based on the same technology as described for MMP-9.

**MMP-9 and MMP-2-specific Immunocapture Activity Assays.** The MMP-9 activity assay was performed as described previously (22, 25). In brief, 96-well plates (Flow Laboratories) were coated with 100 μl of 5 μg/ml MAb S22.2 in PBS overnight at 4°C. After washing, the plates were blocked with 0.1% (w/v) casein for 1 h, washed, and incubated with biological sample. After an overnight incubation at 4°C, the plates were washed and incubated for 2 h with 100 μl of biotin-labeled anti-MMP-9 polyclonal antibody (B21, TNO-PO; Ref. 25). After washing, bound biotin-labeled MAb was assessed by incubation with 100 μl of avidin/ horseradish peroxidase (Pierce Chemical Co., Rockford, IL). The chromogenic substrate used was 3,3’5,5’-tetramethyl benzidine. The reaction was stopped by the addition of 2 M H₂SO₄, and the absorption was measured at 450 nm on a Titerpak Multiskan spectrophotometer (Flow Laboratories). Both the MAb S22.2 and the polyclonal antibody B21 were specific for MMP-9 in both ELISA, Western blotting (polyclonal only; monoclonal did not recognize MMP-9 on Western blots), and activity assay and were found to recognize both latent and active MMP-9 (25) and MMP-9 complexed to TIMP-1 or TIMP-2.

**Gelatin Zymography.** Gelatin zymography on urine samples was carried out as described previously (23). The amount of urine loaded on the gel was between 5 and 15 μl depending on the creatinine content.

**MATERIALS AND METHODS**

 Patients and Urine Collection. Surgery was performed at the Department of Urological Pathology, Institute of Nephro-Urology (Turin, Italy), on 82 patients with bladder carcinoma (69 males and 13 females, ages 46–92 yr). All tumors were histologically classified for grade and stage according to the pTNM classification (24): 43 pTa, 28 pT1, 6 pT2, and 4 pT3. Urine samples were collected before operation and urine samples from gender- and age-matched healthy volunteers (14 females and 14 males, ages 34–80 yr) were used as controls. Moreover, 20 urine samples were collected from previously treated patients with bladder cancer (29 males and 1 female, ages 53–82 yr), who were with NED at the time of sampling (i.e., 3–6 months after surgery) a negative cystoscopy and cytology. For cytology, fresh urine samples of the second morning micturition were diluted (50%) with ethanol, centrifuged, and the sediment was stained according to the Papanicolaou test. Cytological analysis for inflammatory, dysplastic, and malignant cells was done by two independent pathologists.

All urine samples were snap-frozen and kept at −80°C. Before measurements, the samples were quickly thawed at 37°C and centrifuged at 5000 × g for 5 min. Urinary creatinine levels were used to correct for the dilution of the samples. Creatinine was measured at the Department of Clinical Biochemistry, Ospedale San Raffaele (Milan Italy), using a modification of the Jaffé method, according to the manufacturer’s instructions (Boehringer Mannheim), with a BM/Hitachi 747 analyzer. All patients and controls had no known renal problems and the creatinine values of urine samples from the patients used in this study did not vary from the control samples (P > 0.05, ANOVA). Urinary NMP-22 levels were evaluated with a commercially available kit (Matritech).

**Gelatin Zymography.** Gelatin zymography on urine samples was carried out as described previously (23). The amount of urine loaded on the gel was between 5 and 15 μl depending on the creatinine content.

**MMP-9 and MMP-2-specific Immunocapture Activity Assays.** The MMP-9 activity assay was performed as described previously (22, 25). In brief, 96-well plates (Costar) were coated with anti-MMP-9 MAb S22.2 (TNO-PO, Leiden, the Netherlands; Ref. 25) and washed with PBS and 0.05% Tween 20 (v/v). Purified standard MMP-9 and urine samples (1/2 diluted in 1% BSA-PBS) were incubated overnight at 4°C. The wells were washed and incubated with assay buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM CaCl₂, 1 μM ZnCl₂, and 0.01% (v/v) Brij-35], to which 15 μl (50 mg/ml) of UKcol (TNO-PO; Ref. 22) and 10 μl (6 mg stock) of chromogenic substrate S-2444 (Chromogenix, Mödling) were added. Color development was recorded in a Titerpak plate reader at 405 nm (Flow Laboratories, Irvine, Scotland). For measurement of total activity (already active plus latent MMP-9), the immobilized MMP-9 was incubated with assay buffer containing 0.5 mM APMA for 2 h, after which UKcol and S-2444 were added and activity was recorded. The MMP-2 activity assay was purchased from Amersham Pharmacia Biotech, which is based on the same technology as described for MMP-9.

**ELISAs for MMP-9, uPA, and Cathepsin B.** The MMP-9 ELISA was carried out as described previously (25). In brief, microtiter plates (Flow Laboratories) were coated with 100 μl of 5 μg/ml MAb S22.2 in PBS overnight at 4°C. After washing, the plates were blocked with 0.1% (w/v) casein for 1 h, washed, and incubated with biological sample. After an overnight incubation at 4°C, the plates were washed and incubated for 2 h with 100 μl of biotin-labeled anti-MMP-9 polyclonal antibody (B21, TNO-PO; Ref. 25). After washing, bound biotin-labeled MAb was assessed by incubation with 100 μl of avidin/ horseradish peroxidase (Pierce Chemical Co., Rockford, IL). The chromogenic substrate used was 3,3’,5,5’-tetramethyl benzidine. The reaction was stopped by the addition of 2 M H₂SO₄, and the absorption was measured at 450 nm on a Titerpak Multiskan spectrophotometer (Flow Laboratories). Both the MAb S22.2 and the polyclonal antibody B21 were specific for MMP-9 in both ELISA, Western blotting (polyclonal only; monoclonal did not recognize MMP-9 on Western blots), and activity assay and were found to recognize both latent and active MMP-9 (25) and MMP-9 complexed to TIMP-1 or TIMP-2.

The uPA ELISA recognized single-chain, two-chain uPA as well as uPA/PAI-1 complex with comparable efficiency and has been described in detail by Koolwijk et al. (26). Cathepsin B was analyzed using an ELISA (KRKA, d.d.) developed at the Jozef Stefan Institute (Ljubljana, Slovenia). The ELISA detects the precursor molecule, the mature form, as well as enzyme-inhibitor complexes and was optimized for the use of human biological fluids (27, 28). The urinary antigen levels of uPA and cathepsin B were corrected for dilution by the creatinine content.
RESULTS

Gelatin zymography identified that MMPs were abun-
dantly present in urine from patients with bladder cancer,
whereas hardly any MMP could be detected in urine from
healthy controls. A representative gelatin zymogram of
urine from five healthy controls (C1-C5) and five bladder
cancer patients (P1-P5) is shown in Fig. 1. Many gelat
inase activity bands were observed (e.g., at MW 150,000; MW
92,000; MW 84,000; MW 72,000; and MW 64,000), the latter four bands
interacting with latent MMP-9, activated MMP-9, latent
MMP-2, and active MMP-2, respectively. In principle, quanti-
fication of specific activities is possible by scanning or densi-
tometry of these lysis bands (15). However, this method is
laborious, time consuming, and, therefore in a clinical setting,
unsuitable. The use of specific ELISAs would have the disad-
antage that possible information about the active/inactive state
of the proteinases would be lost. We, therefore, measured MMP
activities using specific activity assays for MMP-2 and MMP-9,
which are able to distinguish active and activatable enzyme next
to each other, just like zymography, and which have been shown
to be significantly correlated with the results obtained by scan-
nig zymograms (29). For comparison, urinary MMP-9 levels
were also measured in a subgroup of samples by a specific
ELISA able to detect (pro)MMP-9 and MMP-9/inhibitor com-
plexes. Although total MMP-9 activity in the urine samples
correlated significantly with MMP-9 antigen levels (r = 0.616;
P < 0.0001; n = 35), some individual urine clearly showed
relatively low amounts of MMP-9 activity compared with total
antigen amount. This difference is probably due to the presence
of MMP/inhibitor complexes (data not shown).

Fig. 1 Gelatin zymography of urine samples from bladder car-
cinoma patients and healthy controls. Five randomly chosen urine
samples (5 – 15 µl) from patients (Lanes P1-P5) and healthy male
volunteers (Lanes C1-C5) were applied to gelatin zymography.
The positions of the bands for purified latent MMP-9 and latent
MMP-2 are indicated by arrows. Patients P1, P2-P3, and P4-P5
had carcinomas with stages pTa, P1, and pT2/pT3, respectively.

The positions of the bands for purified latent MMP-9 and latent
MMP-2 (e.g., MW 150,000; MW 92,000; MW 84,000; MW 72,000;
and MW 64,000), the latter four bands corresponding with latent
MMP-9, activated MMP-9, latent MMP-2, and active MMP-2,
respectively. In principle, quantification of specific activities is possible by scanning or densitometry of these lysis bands (15). However, this method is laborious, time consuming, and, therefore, in a clinical setting, unsuitable. The use of specific ELISAs would have the disadvantage that possible information about the active/inactive state of the proteinases would be lost. We, therefore, measured MMP activities using specific activity assays for MMP-2 and MMP-9, which are able to distinguish active and activatable enzyme next to each other, just like zymography, and which have been shown to be significantly correlated with the results obtained by scanning zymograms (29). For comparison, urinary MMP-9 levels were also measured in a subgroup of samples by a specific ELISA able to detect (pro)MMP-9 and MMP-9/inhibitor complexes. Although total MMP-9 activity in the urine samples correlated significantly with MMP-9 antigen levels (r = 0.616; P < 0.0001; n = 35), some individual urine clearly showed relatively low amounts of MMP-9 activity compared with total antigen amount. This difference is probably due to the presence of MMP/inhibitor complexes (data not shown).

Fig. 2 shows the urinary levels of, respectively, active (Fig.
2a) and total MMP-9 activity (Fig. 2b) and active (Fig. 2c) and
total MMP-2 activity (Fig. 2d) in individual bladder carcinoma
patients in comparison with healthy controls. All data are normal-
ized for urinary creatinine content to correct for dilution effects and expressed as activity units/10 µg creatinine. Active
MMP-9 is detectable in the majority of carcinoma patients
(range, 0 – 202), whereas in healthy controls active MMP-9 was
present in only 9 of 28 urine samples (range, 0 – 1.4; Fig. 2a).
Active MMP-2 is found in 11 of 80 patients and in none of the
healthy volunteer urine (Fig. 2c). The APMA-treated urine
samples, corresponding with the total MMP-9 or MMP-2 activity
(i.e., active plus latent enzyme), show higher levels, as expected (Fig. 2, b and d). The patients in Fig. 2 are classified
according to the stage of the tumors (TNM), and the horizontal bars represent the median values of each subgroup. Significant
differences between the different stages of the disease compared
with the control urine samples are indicated (Mann-Whitney U
test, not significant). The MMP-9 activities in urine’s from all
stages of tumor patients were significantly higher than in those
from the healthy controls. Regarding MMP-2, only active
MMP-2 in pTa patients was not significantly elevated (Fig. 2c).
As a comparison, the urinary levels of 2 other proteinases
known to be involved in carcinogenesis are presented in Fig. 2,
e and f. The levels of cathepsin B and urokinase were not
enhanced (Mann-Whitney U test) in the total group of bladder
cancer patients, nor in any of the subgroups except for the
urokinase levels in pT2/pT3 tumor patients (Fig. 2f).

Active and total activity of MMP-9 were highly correlated
(r = 0.889; P < 0.0001; see Table 1), but active and total
activity of MMP-2 were only weakly correlated (r = 0.314; P = 0.09).
Surprisingly, MMP-2 and MMP-9 activity levels were
also significantly correlated as shown in Fig. 3 for total activities
(r = 0.609, P < 0.0001; n = 109), but some individuals showed
specific elevation for either MMP-2 or MMP-9.

As arbitrary cutoff values for active and total MMP-activity
we used the mean value of the healthy control group plus twice the

Statistics. The MMP data are expressed as the ratio of
urinary activity:creatinine in units per milliliters divided by
mg/dl (units/10 µg). Urinary uPA and cathepsin B and MMP-9
antigen levels are expressed as ng/µg creatinine. Group results
are given as median values. The Mann-Whitney U test was used
to compare differences between groups. One-way ANOVA was
used to establish the variance between creatinine values of
control and patient urine samples. Correlation coefficients are
according to Pearson. Ps lower than 0.05 were considered
significant. Cutoff values for MMP activities and other param-
eters were calculated as the mean of the healthy control group
plus twice the SD, unless indicated elsewhere.
SD (i.e., 0.79 and 1.90, respectively, for MMP-9 active and total activities and 1.14 for total MMP-2 activity). For active MMP-2, no activity was detected in all control samples. The cutoff values were used to divide the urine samples into groups with normal or elevated MMP-2 or MMP-9 activity content (see Fig. 2). For both, active and total MMP-9 activity, 2 out of 28 healthy controls had enhanced levels (specificity, 93%). In a substantial number of patients with early-phase low-invasive tumors (pTa/pT1), enhanced MMP-9 activity values (30–42%) were observed. Eighty percent of high-invasive tumors (pT2/pT3) showed high levels (Table 2). The ability to detect bladder cancer patients based on the amount of enhanced urinary

Fig. 2 Urinary levels of active and total MMP-9 (a and b), active and total MMP-2 (c and d), cathepsin B antigen (e), and urokinase antigen (f) in individual bladder carcinoma patients in comparison with healthy controls. All data are normalized for urinary creatinine content and expressed as units/10 μg creatinine for MMPs and ng/μg creatinine for cathepsin B and uPA. The patients are subgrouped by the stage of the tumor. The horizontal bars represent the median values of each subgroup. The dotted lines represent the arbitrary cutoff values (i.e., the mean value of the healthy controls plus twice the SD for each parameter).
MMP-2 activity seemed less effective (Table 2). Similar results are found for the relation between MMP activity and the differentiation grade of the tumors. In general, urine from grade 3 tumor patients contained most active or total MMP activity, either MMP-9 or MMP-2. The patients (17–43%) with low-grade tumors already showed enhanced MMP-9 activity levels (Table 3). Total MMP-2 activity showed a trend to increase with the tumor grade.

The MMP-9 activities in urine from NED patients (previously treated bladder cancer patients, but, at present, with NED) are also shown in Fig. 1. Although 50% of these patients had normal range values, overall MMP-9 activity was significantly enhanced in this group compared with control urine samples \(P = 0.0001\) for active as well as total MMP-9). For MMP-2 this analysis was not done.

MMP-9 activity is not significantly correlated with NMP-22, a promising recently introduced bladder tumor marker (Table 1). The relation between active MMP-9 and NMP-22 in urine from only early-stage (pTa and pT1) bladder carcinoma patients is shown in Fig. 4. Also in this subgroup of patients, both parameters were not significantly correlated. In the figure, the arbitrary cutoff value of 0.79 for MMP-9 is indicated, dividing the patients in two groups with normal and enhanced levels. The established cutoff value for NMP-22, 10 units/ml (15), is also marked. Enhanced urinary MMP-9 activity is present in 20 of 48 patients with low-invasive (early-stage) transitional cell bladder carcinoma (sensitivity, 42%), which is less as compared with NMP-22 (64%). However, because both parameters are not correlated, the combination of MMP-9 and NMP-22 provides additional information. This is shown in Table 4 in which a comparison is made between both parameters and cytology, the analysis of the presence of malignant cells in urine from the same patients. The actual number of cancers that would have been detected by the different assays is similar for

### Table 1
Correlation coefficients and corresponding Ps for the levels of several proteinases and NMP-22 measured in urine from 82 bladder carcinoma patients and urine from 28 healthy volunteers

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>Active (n = 109)</th>
<th>Total (n = 109)</th>
<th>uPA (n = 108)</th>
<th>Cathepsin B (n = 83)</th>
<th>NMP-22 (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2 active</td>
<td>0.314</td>
<td>0.0009</td>
<td>0.120</td>
<td>0.131</td>
<td>0.053</td>
</tr>
<tr>
<td>MMP-2 total</td>
<td>0.323</td>
<td>0.379</td>
<td>0.365</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MMP-9 active</td>
<td>0.889</td>
<td>&lt;0.0001</td>
<td>0.104</td>
<td>0.071</td>
<td>0.199</td>
</tr>
<tr>
<td>MMP-9 total</td>
<td>0.315</td>
<td>0.0009</td>
<td>0.226</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>uPA</td>
<td>0.625</td>
<td>0.4</td>
<td>0.169</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>0.009</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

### Table 2
Percentage of urine samples with enhanced MMP activities in patients with bladder carcinomas subgrouped according to the stage of the tumor.

 amended as follows:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Controls (n = 28)</th>
<th>pTa (n = 43)</th>
<th>pT1 (n = 28)</th>
<th>pT2 and pT3 (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td>7%</td>
<td>42%</td>
<td>39%</td>
<td>80%</td>
</tr>
<tr>
<td>Total</td>
<td>7%</td>
<td>30%</td>
<td>32%</td>
<td>80%</td>
</tr>
<tr>
<td>MMP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td>0%</td>
<td>7%</td>
<td>14%</td>
<td>50%</td>
</tr>
<tr>
<td>Total</td>
<td>7%</td>
<td>36%</td>
<td>25%</td>
<td>50%</td>
</tr>
</tbody>
</table>
NMP-22 and cytometry with 36 of 54 patients being detected (sensitivity, 69%). The selection of patients that are either enhanced in MMP-9 activity or in NMP-22 resulted in the detection of nine additional pTa/pT1 stage tumors (17%) with a final sensitivity of 85%.

**DISCUSSION**

In this study, we present data showing the presence of MMP-9 and MMP-2 in urine, metalloproteinases known to be involved in the invasive processes during carcinogenesis. We found that in a substantial group of bladder carcinoma patients (±40%, depending of the stage) active and total MMP-9 activity levels and, to a lesser extent, MMP-2 activity levels were enhanced as compared with age- and gender-matched healthy controls. These data are comparable with what has been found in bladder tumor tissue homogenates using zymography (i.e., enhanced MMP-2 and MMP-9 activity, respectively, in approximately 50% and 40% of all bladder cancer homogenates; Ref. 30) and confirm that at least part of the urinary MMPs might actually derive from the actual tumor (21). Immunohistochemical studies using bladder tumor tissue showed a significant correlation between the amount of MMP-9 and the invasiveness of the carcinoma (30). A similar relation was found for urinary MMP-9 activity levels. The relatively high urinary MMP-9 content of some pTa and pT1 stage bladder carcinomas in the present study might be explained by the fact that these tumors were spread through the whole bladder and, hence, were rather voluminous. Another aspect to take into consideration is that although a tumor can be superficial and still localized at the bladder wall, it might nevertheless be aggressive and, hence, releasing relative high amounts of MMP directly into the urine. This fact could be an advantage for the use of MMP-9 as a

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**Table 3** Mean and median urinary MMP activity levels in bladder carcinoma patients, subgrouped according to the tumor grade

All subgroups are compared with normal healthy controls (Mann-Whitney U tests). Enhanced: percentage of patients with urinary MMP-9 and MMP-2 activity levels is higher than the arbitrary cut-off values based on normal healthy controls (see Table 2).

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 28)</th>
<th>Grade 0* (n = 6)</th>
<th>Grade 1 (n = 14)</th>
<th>Grade 2 (n = 40)</th>
<th>Grade 3 (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9 active</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.14</td>
<td>1.03</td>
<td>5.70</td>
<td>1.30</td>
<td>13.02</td>
</tr>
<tr>
<td>Median</td>
<td>0.00</td>
<td>0.06</td>
<td>0.54</td>
<td>0.42</td>
<td>1.36</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>0.0001</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Enhanced</td>
<td>7%</td>
<td>33%</td>
<td>43%</td>
<td>42%</td>
<td>57%</td>
</tr>
<tr>
<td>MMP-9 total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.57</td>
<td>2.13</td>
<td>14.50</td>
<td>5.31</td>
<td>18.90</td>
</tr>
<tr>
<td>Median</td>
<td>0.30</td>
<td>0.69</td>
<td>1.43</td>
<td>0.97</td>
<td>2.98</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>0.003</td>
<td>0.007</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>Enhanced</td>
<td>7%</td>
<td>17%</td>
<td>43%</td>
<td>27%</td>
<td>57%</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.00</td>
<td>0.14</td>
<td>0.00</td>
<td>0.06</td>
<td>1.10</td>
</tr>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>0.03</td>
<td>NS</td>
<td>NS</td>
<td>0.0004</td>
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<td>17%</td>
<td>0%</td>
<td>10%</td>
<td>38%</td>
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<tr>
<td>MMP-2 total</td>
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</tr>
<tr>
<td>Mean</td>
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<td>0.51</td>
<td>0.78</td>
<td>1.75</td>
<td>2.01</td>
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<td>Median</td>
<td>0.14</td>
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<td>0.59</td>
<td>0.39</td>
<td>0.69</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>0.003</td>
<td>0.0002</td>
<td>0.0001</td>
<td></td>
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<tr>
<td>Enhanced</td>
<td>4%</td>
<td>17%</td>
<td>14%</td>
<td>31%</td>
<td>29%</td>
</tr>
</tbody>
</table>

* Dysplasia.

**Table 4** Number of bladder carcinoma patients detected by enhanced urinary MMP-9 activity levels compared with bladder tumor marker NMP-22 and urine cytology for the presence of malignant cells*

<table>
<thead>
<tr>
<th>Stage</th>
<th>n</th>
<th>MMP-9</th>
<th>NMP-22</th>
<th>MMP-9 or NMP-22</th>
<th>Cytology</th>
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</thead>
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<tr>
<td>pTa</td>
<td>28</td>
<td>12</td>
<td>18</td>
<td>24</td>
<td>15</td>
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<td>pT1</td>
<td>20</td>
<td>8</td>
<td>13</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>pT2</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>pT3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
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<tr>
<td>Total</td>
<td>54</td>
<td>24 (44%)</td>
<td>37 (69%)</td>
<td>46 (85%)</td>
<td>37 (69%)</td>
</tr>
</tbody>
</table>

* For cut-off values see Fig. 3.
diagnostic tool especially for aggressive early-phase bladder carcinoma.

A high correlation was found in this study between active and total MMP-9 activity. This could indicate that the amount of active MMP-9 present in urine is a reflection of the total amount of MMP-9. As a consequence the predicting value of both parameters was very similar. Nevertheless some patients were noticed with exceptionally high amounts of latent MMP-9 considering the active MMP-9 present, or on the contrary, cases with relatively high amounts of active MMP-9. The significance of this phenomenon could not be established in this study due to the limited number of patients in each group.

The activity levels of MMP-2 in urine were, in general, lower than for MMP-9. Active MMP-2 was detectable in only 11 of 80 tumor urine samples, which makes it, in principle, unfit for diagnostic purposes. The total MMP-2 activity results were more comparable with MMP-9 activity. MMP-2 is constitutionally expressed by many cell types and is most likely regulated at the level of proenzyme activation, whereas MMP-9 is transcriptionally regulated by inflammatory cytokines such as tumor necrosis factor α and interleukins (31). The latter are frequently found in tumors, which confirms that particularly MMP-9 levels could be enhanced in urine of patients with bladder carcinoma. The rather disappointing results of the other proteinases in the same urine samples, uPA and cathepsin B, confirm the usefulness of MMPs as urinary tumor markers. Both enzymes are expected to be implicated in invasion. High serum cathepsin B levels have been shown to be enhanced in cancer patients and even prognostic for bad survival (28), but apparently the urinary cathepsin B level does not reflect the state of the tumor. With respect to, especially, MMP-9, it should be noted that it is possible that inflammatory cells within the tumors are also contributing to the enhanced urinary MMP levels. Unfortunately, our qualitative data about tumor inflammatory cells did not allow us to show a correlation.

A direct comparison between urinary MMP-9 activity, tumor marker NMP-22, and urine cytometry revealed that as a bladder tumor marker the measurement of MMP-9 activity is less sensitive. However, because a number of patients were detected exclusively by MMP-9, indicating the independent predicting value of this proteinase as compared with other markers, the use of MMP-9 activity in addition to other markers could improve the number of patients detected substantially. This phenomenon could be due to the fact that metalloproteinases are actually involved in carcinogenic processes and might, therefore, reflect active cell migration/invasion. In this respect, it should also be noted that about 50% of the NED patients showed increased MMP-9 activity values. This could mean that in NED patients with high MMP-9 activity values (Fig. 2) the disease could, in fact, be present, but that it is just not yet detectable using the conventional methods (i.e., histology after cystoscopy and urine cytology). Also, NMP-22 levels were enhanced in about 50% of these NED patients (data not shown), but not all patients with enhanced MMP-9 were high in NMP-22 and vice versa. This information would be crucial information for a urologist during monitoring of early-stage bladder cancer patients after endovesical therapy. Therefore, measurement of urinary MMP-9 activity might have clinical applications, but this hypothesis has still to be confirmed in follow-up studies.

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Enhanced Urinary Gelatinase Activities (Matrix Metalloproteinases 2 and 9) Are Associated with Early-Stage Bladder Carcinoma: A Comparison with Clinically Used Tumor Markers

Cornelis F. M. Sier, Giovanni Casetta, Jan H. Verheijen, et al.


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