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Expression and activity of matrilysins – matrix metalloproteinases 7 and 26 in human urinary bladder cancer

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Introduction Urinary bladder cancer occurs mainly in the elderly and is the fourth most common cancer in humans. The process of metastasis depends on the degradation of the extracellular matrix. The cleavage of its constituent proteins is initiated and prolonged by matrix metalloproteinases (MMPs) present in both pathological and normal tissues, i.e. MMP-7 and MMP-26, which belong to the matrilysin group. The study aimed to evaluate the expression and activity of both MMPs present at different stages of human bladder cancer.

Material and methods We used western blot and ELISA to determine the expression and content of both MMPs in two different types of tissue: healthy and bladder cancer tissue. The fluorimetric method was used to measure the activity (specific and actual) of the enzymes in the tissues.

Results The content of MMP-7 and MMP-26 was very similar in all tested tissues. Their activities differed in both grades of bladder cancer. In all tested tissues, the specific activity of MMP-7 was significantly higher than that of MMP-26.

Conclusions The determined differences in the activities of the two matrilysins demonstrate their opposing involvement in different phases of extracellular matrix remodeling and at different stages of tumour development.

Key Words: urinary bladder cancer ‹› matrilysin ‹› MMP-7 ‹› MMP-26

INTRODUCTION

Metalloproteinases

The extracellular matrix (ECM) consists of collagen (mainly type IV), elastin, proteoglycans, and glycoproteins. It is degraded by matrix metalloproteinases (MMPs), which facilitates cell migration. As a result of the above-mentioned processes, other components, such as peptide growth factors that accelerate tumour growth processes, can be released. Interleukins and growth factors induce the synthesis of MMPs in tumour cells.

Increased activity of metalloproteinases is associated with the risk of progression of the cancer process [1–4]. On the other hand, studies in mice have shown that decreased metalloproteinase activity is associated with reduced tumour growth, as well as a lower risk of metastasis [5, 6].

Urinary bladder cancer

The urinary bladder acts as a reservoir for the urine and drains it from the body to the outside. It consists of 4 layers, which play an important role in determining the depth of tumour invasion and the final stage of the cancer. The epithelium lining the bladder and being in contact with the urine is called transitional or urothelial epithelium. Most bladder cancers originate from transitional

epithelial cells. Beneath the epithelium is the lamina propria – a layer consisting of connective tissue and blood vessels. The third layer is the muscularis propria. For bladder cancer staging purposes, this layer is divided into a superficial half and a deep half. The fourth, outermost layer consists of fat, fibrous tissue, and blood vessels [7–11].
The type of tumour is also described by its grade under the microscope. This feature describes how the cancer cells differ from healthy cells. When tumour tissues are similar to healthy tissue, they are referred to as well-differentiated or low-grade (LG) cells. Tumour cells that show morphological differences from normal cells are called poorly differentiated cells or cells with a high degree (HG) of malignancy. Low-grade cancers have a much lower risk of progression than high-grade cancers [2–4, 10].

Matrilysins

A characteristic feature of matrilysins is the difference in their structure. The lack of a hemopexin domain makes these enzymes much lighter than others. Matrilysins include MMP-7 and MMP-26. These enzymes degrade various components of the ECM, as well as molecules present on the cell surface [12]. According to many authors, MMP-7 is active mainly in the first phase of the carcinogenic process [13–17].
The action of MMP-7 has a wide range. It inhibits apoptosis [18] and inducts osteolysis, which plays a key role in the formation of bone metastases [19]. An increase in MMP-7 activity in the tissues, blood, and urine of bladder cancer patients was observed by Svatek et al. [20].
The main substrates of MMP-26 are type IV collagen, gelatin, and fibronectin [21–23]. So far, no significant involvement of MMP-26 in the development of bladder cancer has been demonstrated, which may be due to the methods used or the type of tested material. The usefulness of the determination of various MMP concentrations in blood as prognostic factors has been analysed [24].

Aim of the study

Studies conducted on the content and activity of MMPs in bladder cancer have shown different results. The differences may be due to the methods and the tissue material used. The predominant results were determined in the blood and urine of cancer patients. These results do not seem to be adequate for the contribution of MMPs in tissue altered by carcinogenesis. Therefore, we aimed to evaluate the expression, content, and activity of MMP-7 and MMP-26 in cancer tissue compared to control tissue.

MATERIAL AND METHODS

Tissue material

The material consisted of tissues of the most common type of bladder cancer – urothelial carcinoma – at two stages of morphological malignancy. It was collected during surgical procedures in the Department of Urology at the Medical University of Bialystok. The study included 20 patients who underwent radical cystectomy or transurethral resection of a bladder tumour and were histopathologically diagnosed with urothelial carcinoma. The age of the patients ranged from 47 to 91 years, with an average of 70.3 years. The tissue samples were taken from macroscopically visible tumours after bladder resection in a classic open procedure. The study included two groups of patients: 10 patients with LG tumours and 10 patients with HG tumours. Of these, 16 were male and 4 were female. In terms of local extent, patients with low-grade tumours had lesions limited to the bladder mucosa of pTa or pT1. In contrast, patients diagnosed with high-grade cancer were characterised by a diagnosis of infiltration of the bladder muscle membrane – pT2. Control tissues (C) were obtained after radical open cystectomy from the side opposite the tumour, while an attempt to obtain healthy tissue during transurethral resection of the bladder tumour was unsuccessful.

Proteolytic activity

Carboxymethyl transferrin was prepared by the Satomi method [25] and used as a substrate to evaluate proteolytic activity. Its degradation revealed MMP-7 activity and was evaluated using the method of Yu and Woessner [26]. A representative zymogram is shown.

Matrilysin content

MMP-7 content in the materials was measured by Quantikine ELISA Human Total MMP-7 Kit (provided by R&D Systems, USA), and MMP-26 content was measured by Enzyme-linked Immunosorbent Assay Kit for Matrix Metalloproteinase 26 (Cloud-Clone Corporation, USA) according to the manufacturer's instructions.

Western blot analysis

Samples (2 μg or 10 μg of protein) of tissue extracts from the normal bladder and both types of cancer were electrophoresed on 10% SDS-polyacrylamide

gels using the Laemmli method [27] and blotted onto nitrocellulose membranes (Sigma; USA) according to the standard procedure. A monoclonal antibody directed against human MMP-7 (catalogue number MAB9071; R&D Systems, USA) or a human monoclonal antibody directed against MMP-26 (catalogue number sc-100558; Santa Cruz Biotechnology Inc., USA) were used for detection of respective MMPs. The molecular weight of MMPs was estimated using pre-stained molecular weight markers (BioRad, USA). Representative blots are shown.

Matrilysin activity

The actual and specific activity of MMP-7 and MMP-26 was measured in a black 96-well flat-bottom microplate (Greiner Bio-One, Austria), which was pre-coated with appropriate specific MMPs antibodies (the same antibodies used in the western blot analysis) [28]. One hundred microlitres of the appropriate sample were added to each well to immobilise the matrilysin. The microplate was incubated overnight at 4ºC. All remaining proteins were washed with TBS-T buffer (50 mM Tris/HCl pH 7.4, 0.9% NaCl, 0.05% Tween 20). MMP activity was measured in 100 ml of 50 mM Tris/HCl buffer pH 7.5 containing 10 mM $CaCl_2$, 150 mM NaCl, and 0.025% Brij 35 with MCA-Pro-Leu-Ala-Cys (p-OMeBz)-Trp-Ala-Arg(Dpa)-H2 (Merck, Germany) as fluorogenic substrate (final concentration 4 mM). Then, the microplate was incubated at 37°C for 60 minutes with gentle shaking. The reaction was stopped by adding 25 ml of 100 mM EDTA.Na_2. The degradation of the fluorogenic substrate was measured using a multi-mode microplate reader (Tecan Infinite® 200 PRO, Tecan, USA) with excitation and emission wavelengths set at 325 nm and 393 nm, respectively. The amount of degraded substrate was calculated from a calibration curve obtained under the same conditions with 7-amino-4-methylcoumarin (Sigma-Aldrich, USA) as a standard.

Determination of protein concentrations

Protein concentrations were determined using the Bradford method [29].

Statistical analysis

The statistical analysis was based on mean values obtained from 10 samples with the determination of the standard deviations (SD). The content of matrilysins was determined in milligrams per kg of protein. Their activity was expressed in microkatals/kg protein. Statistical analysis was performed using Student's t-test, taking $p < 0.05$ for statistical significance.

Bioethical standards

The study was conducted in accordance with the Declaration of Helsinki and approved by the Bioethical Committee of the Medical University of Bialystok (No. approval: R-I-002/220/2015, 28 May 2015).

RESULTS

Proteolytic activity

A representative MMP-7 zymogram is shown in Figure 1, where lane 1 was obtained for control tissue, lane 2 for LG tissue, and lane 3 for HG tissue. A molecular weight standards lane is shown on the left side of the zymogram. The discoloured bars indicate proteolytic activity and are marked with the letters A, B, and C. Band A represents a molecular weight of about 17 kDa. Band B indicates a mass of 28 kDa. MMP-7 in its active form weighs 19 kDa, while the inactive form weighs 28 kDa.

Matrix metalloproteinases 7 and 26 content

The content of both tested matrilysins was determined in milligrams per kg of protein. The determined content of MMP-7 in control bladder tissue was 1.537 mg/kg of protein. The content of this

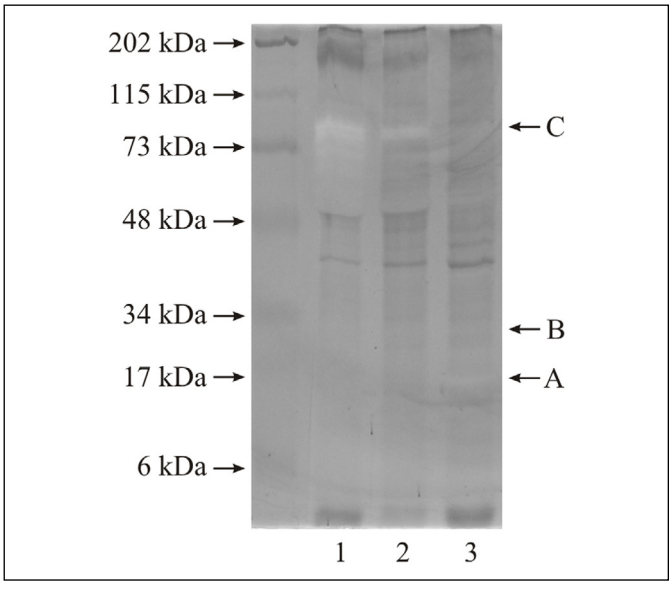

Figure 1. *A representative MMP-7 zymogram; lane 1 – control tissue, lane 2 – LG tissue, lane 3 – HG tissue; a molecular weight standards lane is shown on the left side of the zymogram.*

enzyme in both LG and HG bladder cancer tissues was significantly higher. Almost 85% more of this enzyme was determined in LG cancer tissue (2.827 mg/kg protein) and about 400% more in HG cancer tissue (7.699 mg/kg protein) compared to control tissue (Figure 2A).

Control bladder tissue contained 5.760 mg/kg of MMP-26 protein – almost 3 times more than MMP-7. LG bladder cancer tissue contained almost twice as much MMP-26 (5.530 mg/kg of protein) as MMP-7. Similar amounts of both matrilysins were determined in high-malignancy bladder cancer tissue; the amount of MMP-26 was 8.730 mg/kg of protein (Figure 2B).

Western blot of matrilysins

Western blot analysis was used to evaluate the expression of the tested MMPs. It was performed under both non-reducing and reducing conditions. The amount of the applied samples was 20 and 10 μg of protein for MMP-7 and MMP-26, respectively.

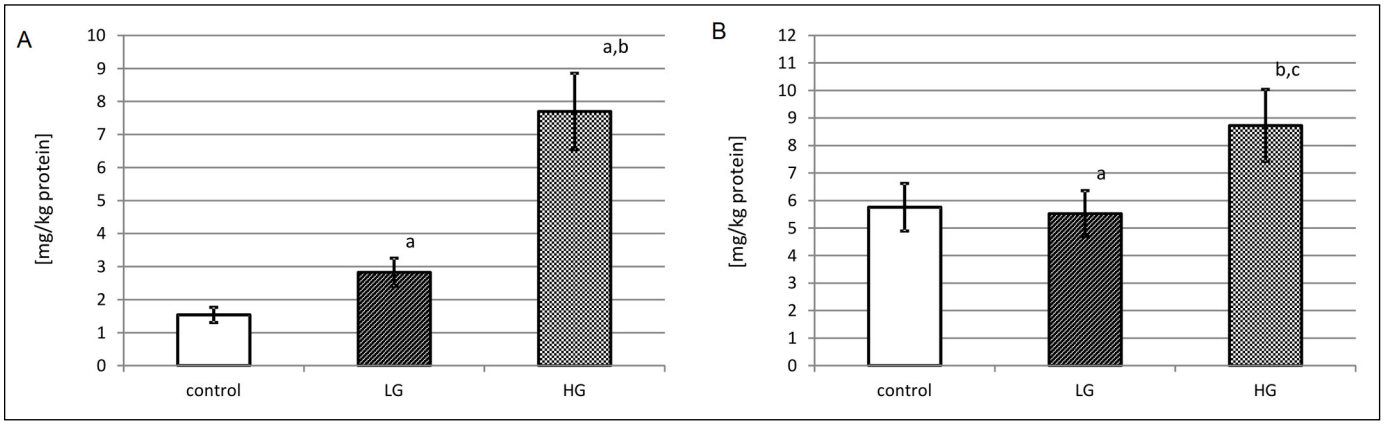

Figure 2. A) *Content of MMP-7 in control tissue, low-grade (LG) and high-grade (HG) bladder cancer tissue: a – p <0.001 control vs urinary bladder cancer, b – p <0.001 HG vs LG bladder cancer tissue.* **B)** *Content of MMP-26 in control tissue, LG and HG bladder cancer tissue: a – p <0.05 LG vs control tissue, b – p <0.001 HG vs control, c – p <0.001 HG vs LG bladder cancer tissue.*

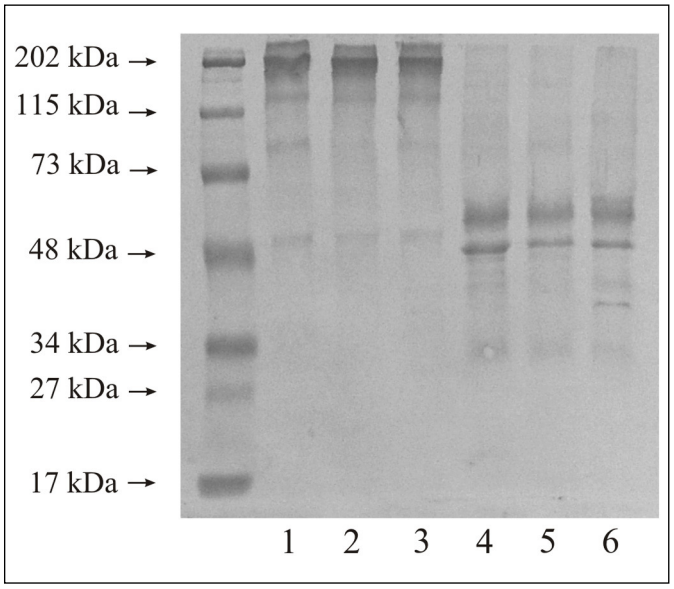

Figure 3. *Western blot of MMP-7 in control tissue, and LG and HG bladder cancer tissues; non-reducing conditions: lane 1 – control tissue, lane 2 – LG tissue, lane 3 – HG tissue; reducing conditions: lane 4 – control tissue, lane 5 – LG tissue, lane 6 – HG tissue; mass of the applied samples was 20 µg of protein.*

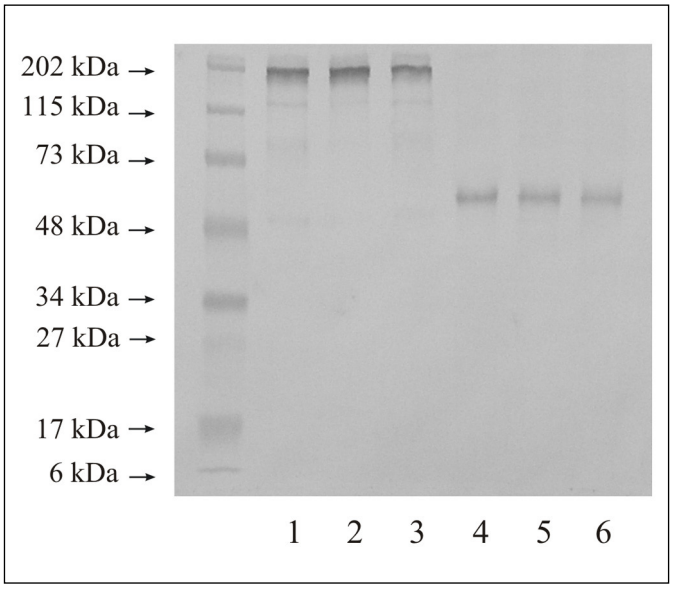

Figure 4. *Western blot of MMP-26 in control tissue, and LG and HG bladder cancer tissues; non-reducing conditions: lane 1 – control tissue, lane 2 – LG tissue, lane 3 – HG tissue; reducing conditions: lane 4 – control tissue, lane 5 – LG tissue, lane 6 – HG tissue; the mass of the applied samples was 10 µg of protein.*

The results of MMP-7 expression performed by western blot in control tissue and bladder cancer tissues are shown in Figure 3. Control bladder tissues showed at least 4 bands with molecular weights of 202 kDa, 120 kDa, 80 kDa, and 50 kDa (lane 1). The LG (lane 2) and HG (lane 3) bladder cancer tissues also showed similar results to those obtained for control tissue. The use of reducing conditions resulted in a reduced number of visible bands and the obtaining of bands with reduced molecular weights. All 3 tissue types revealed 2 bands with molecular weights of 55 and 48 kDa (lanes 4–6), as shown in Figure 3.

The results of MMP-26 expression performed by western blot for control tissue and bladder cancer tissues are shown in Figure 4. In control tissue, only 2 bands with the following molecular weights were observed: a narrow band of 115 kDa and a broad band of 202 kDa (lane 1). LG tissues (lane 2) and HG tissues (lane 3) showed similar results to those obtained for control tissue. Reduction of disulfide bonds resulted in a reduction of visible bands, of which only one was separated. The presence of a 55 kDa band in the control tissue was observed (lane 4). LG and HG tissues showed similar results (lanes 5 and 6).

Matrilysin activities

Actual activity of matrix metalloproteinases 7 and 26

The actual activities of the matrilysins were measured by fluorimetric assay with oligopeptides as substrates. Each enzyme was isolated on a microplate pre-coated with an antibody specific for a particular matrilysin; the same as used for western immunoblot.

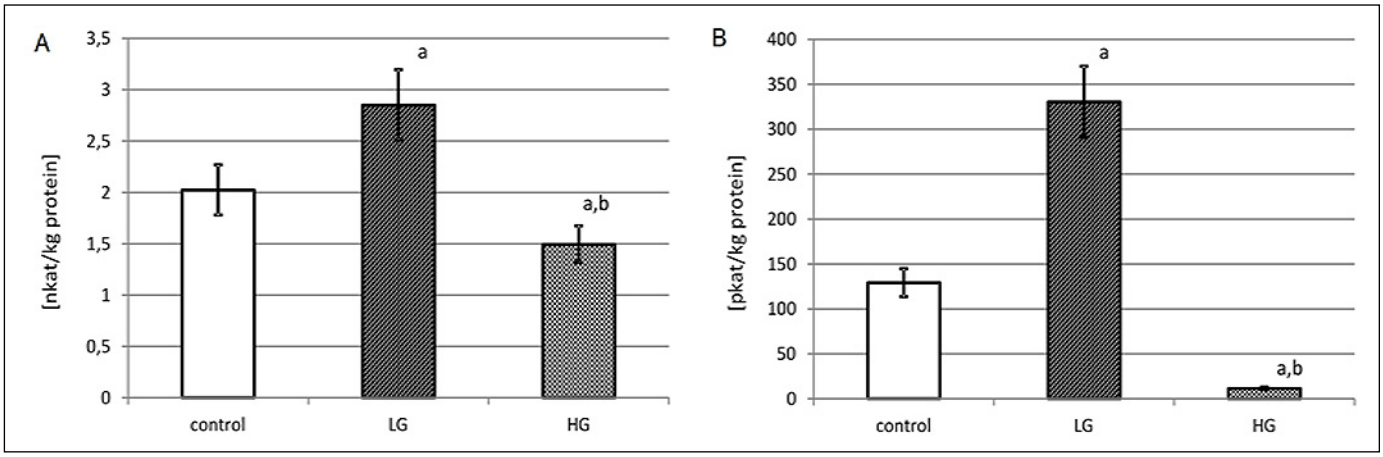

Figure 5. A) *MMP-7 actual activity in control tissue, and LG and HG bladder cancer tissues; a – p <0.001 cancer vs control; b – p <0.001 HG vs LG urinary bladder cancer.* **B)** *MMP-26 actual activity in control tissue, LG and HG bladder cancer tissues; a – p <0.001 cancer vs control; b – p <0.001 HG vs LG urinary bladder cancer.*

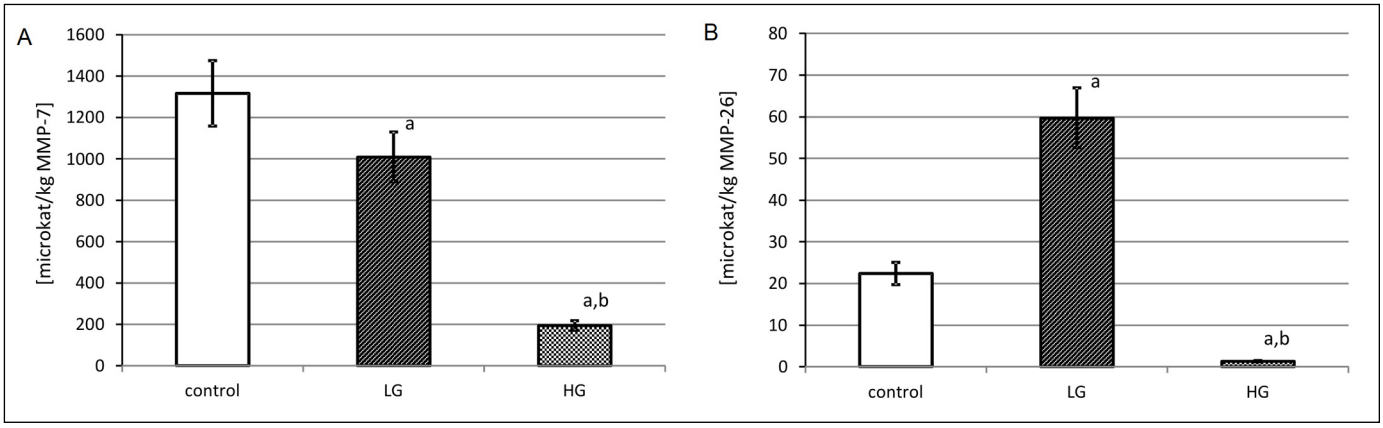

Figure 6. A) *MMP-7 specific activity in control tissue, and LG and HG bladder cancer tissues; a – p <0.001 cancer vs control; b – p < 0.001 HG vs LG urinary bladder cancer.* **B)** *MMP-26 specific activity in control tissue, and LG and HG bladder cancer tissues; a – p <0.001 cancer vs control; b – p <0.001 HG vs LG urinary bladder cancer.*

Actual male activity was expressed in katals per kg of total protein content in the tissue extract.
As shown in Figure 5A, the MMP-7 activity was 2 nanokatals per kg of total protein content in normal human bladder. LG cancer tissues were characterised by a slight increase in actual MMP-7 activity. As the grades of bladder cancer increased, the measured activity decreased. The differences between both grades of cancer and control tissue were significant.
The results shown in Figure 5B indicate that the actual activity of MMP-26 was about 129 picokatals per kg of total protein in the control tissues. In LG bladder cancer tissue, the actual activity of MMP-26 was more than twice as high. An increase in tumour grade resulted in a drastic 30-fold decrease in actual enzyme activity. The differences in the actual activity of MMP-26 between both cancer grades and control tissue were significant.

Specific activity of matrix metalloproteinases 7 and 26

The specific activity of each matrilysin was calculated from the results of the actual enzyme's activity and its content measured by ELISA, separately for each patient. The average value of specific activity was calculated, and it was expressed in katals per kg of the content of a given enzyme in the tissue extract.
As shown in Figure 6A, the highest specific activity of MMP-7 was found in control tissue. The specific activity of this enzyme decreased as the grade of bladder cancer increased. The differences between both cancer grades and control tissue were significant.
Figure 6B shows the results of the specific activity of MMP-26 in microkatals per kg of the enzyme. The determined enzyme activity value for the control tissue was intermediate. LG tissue was characterised by a significant increase in specific MMP-26 activity, while HG tissue was characterised by a 40-fold decrease in this activity.

DISCUSSION

The content of the matrilysins in bladder cancer varied significantly and depended on the grade of the tumour. The content of MMP-26 was found to be significantly higher in control tissue – almost 4 times higher than MMP-7. This may indicate that in this group of matrix metalloproteinases, the enzyme plays a more important role in the restoration of the ECM of the bladder. MMP-26 dominates over MMP-7 in tumour tissues as well. The content of MMP-26 is almost twice as high in HG as in LG tumour tissues. HG bladder cancer samples contain almost 3 times as much MMP-7 as the LG. Significantly higher levels of both enzymes in both tumour grades, excluding MMP-26 in LG cancer tissue compared to control tissue, is a result worth analysing. The above results showed that the extracellular synthesis and secretion of the studied metalloproteinases were not inhibited.
The expression of the two matrilysins, as determined by western blot, was different in all tissues. For both enzymes, no bands were observed with a molecular weight of about 20 kDa, which may represent the free active form of the enzymes. Only bands of higher molecular weight under non-reducing conditions were similar for both matrilysins. This demonstrates the presence of metalloproteinases in complexes with other ECM proteins, including TIMPs, and even dimer formation. While MMP-7 expression revealed several visible bands (6 for both conditions), western immunoblot of MMP-26 showed only 2 visible bands under non-reducing conditions and only one after disulfide bond reduction. In the ECM, different proteins interact with each other, often without affecting enzyme activity.
We compared the tested matrilysins' activities based on the measured actual activity. It differs for both matrilysins in control bladder tissue and is significantly higher for MMP-7 than for MMP-26, nanokatals vs picokatals per kilogram of total protein content. Given the constant process of ECM remodeling involving metalloproteinases, it appears that MMP-7 plays a major role in maintaining ECM homeostasis in the non-cancer urinary bladder. The actual activity of MMP-7 in both grades of bladder cancer is higher compared to MMP-26. This may be further evidence supporting the thesis that MMP-7 plays one of the main roles in the process of ECM remodeling.
MMP-7 activity was found in both LG and HG tumour tissues. No relationship was found between the enzyme's expression/activity and tumour progression. An increase in MMP-7 activity in the tissues, blood, and urine of patients with bladder cancer was reported by Svatek et al. [20]. They analysed whether the determination of levels of various MMPs in blood is an appropriate prognostic factor. It seems that only the determination of MMP-7 can be used as such a factor. However, its use requires further research [23]. MMP-26 appears to have little relevance to the cancer process occurring in the bladder [24].
Calculation of specific activity showed what proportion of the enzyme was present in the active form without bound inhibitor. The specific activity

of both MMPs was lower when the histopathological grade of the tumour increased, and they showed opposite results. It was higher for MMP-7 than for MMP-26.

Degradation of modified transferrin was used to complete the determination of MMP-7 activity in all tested tissues. The zymogram with carboxymethyl transferrin confirmed the presence and activity of this enzyme and gave similar results to those obtained for specific activity.

The determined content of both tested matrilysins might indicate that they play a similar role in the development of bladder cancer. On the other hand, the specific and actual activity was significantly higher for MMP-7 than for MMP-26. Despite the similar content of the two matrilysins, their activity showed a significantly greater contribution of MMP-7 in the development of bladder cancer. Interestingly, the catalytic activity of the tested enzymes increases in bladder cancer of LG and decreases in HG tumour. Such results indicate that HG bladder cancer cells may silence MMP activity. The identified differences between the results for both enzymes may suggest their specific involvement in a specific period of tumour growth and differentiation [3–6]. The specific activity of MMP-7 was more than 100 times higher compared to that of MMP-26, suggesting that significantly more MMP-7 molecules were present in active form than MMP-26 molecules. The identified differences in the activity of the two matrilysins showed their opposing involvement in different phases of ECM remodelling and at different stages of bladder tumour development. Control tissues were taken from the same urinary bladder, so a carcinogenic effect on the metabolism of the entire urinary bladder as an organ cannot be excluded. By using tissue material instead of urine or blood serum from the same patient, the role of matrilysins in tissue metabolism is better reflected.

CONCLUSIONS

During the study, similar amounts of matrilysins were determined in healthy and cancerous urinary bladder. Their content was slightly higher for MMP-26. However, compared to MMP-26, the specific activity was significantly higher for MMP-7 in every studied material. Moreover, for both enzymes, specific activity decreased with increasing tumour stage. This suggests differences in the regulation of expression and activation of the studied MMPs.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FUNDING

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ETHICS APPROVAL STATEMENT

The study was conducted in accordance with the Declaration of Helsinki and approved by the Bioethical Committee of the Medical University of Bialystok (No. approval: R-I-002/220/2015, 28 May 2015).

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