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Low molecular weight proteins and enzymes in the urine of patients with bladder cancer – a pilot study

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Introduction A steady increase in cases of bladder cancer (BC) has been observed. Detection of unfavorable changes, especially in the early stages of disease, is crucial to medical procedure. There is still a need to search for new, non-invasive biomarkers of BC. The aim of this study was to estimate the levels of selected low molecular weight proteins (LMWP) and enzymes in the urine of patients at different BC stages and grades.

Material and methods Urine samples from 46 patients with BC and 16 healthy controls were examined. We measured levels of LMWP such as: retinol-binding protein (RBP), β2-microglobulin (β2M), enzymes: N-acetyl-β-D-glucosaminidase (NAG), isoform (NAG-B) and also neutrophil gelatinase-associated lipocalin (NGAL).

Results The levels of all examined parameters differed between patients and healthy subjects. Levels of NAG ($p = 0.031$), NAG-B ($p = 0.023$) and NGAL ($p = 0.008$), and total protein ($p = 0.007$) concentrations, were significantly higher in the BC patients than in the control group. Among the examined parameters, positive significant correlations were observed only between urinary NGAL concentration and tumor stages and grades. The highest percentages of changes in NGAL concentration were observed in tumor in situ (TIS) and G3grade patients.

Conclusions Our study showed that urinary NGAL concentrations, as well as NAG and NAG-B activity, could be helpful noninvasive parameters for the diagnosis of BC. The most promising seems to be NGAL determination, but further study is needed on a larger group of participants in order to confirm this observation.

Key Words: bladder cancer ‹› urine ‹› NGAL ‹› NAG ‹› low molecular weight proteins

INTRODUCTION

Worldwide cancer statistics indicate that bladder cancer (BC) constitutes about 3% of all cancer cases. BC represents the most expensive type of cancer per patient lifetime, and generates very high cost-of-illness and health care (about 5 billion EUR per year in the European Union) [1, 2, 3]. Although bladder cancer incidence and mortality rates have been dropping slightly in recent years worldwide, this tendency has not been observed among Poles. The incidence of BC in Poland rises around 5% per annum, due to the detection of BC late, when the disease is already in a high stage of advancement [4, 5].

Bladder cancer demonstrates a close causative relationship with exposure to occupational and environmental factors. Moreover, chronic inflammation of the urinary bladder, e.g. induced by Schistosoma haematobium or genetic predisposition, is not without significance [6, 7]. The main symptoms, such as hematuria or an urgent need to urinate, which may be accompanied by dysfunction such as pain or micturition, may suggest pre-existing bacterial cystitis rather than a cancerous process [1, 6]. No specific symptoms appear at the early stages of bladder

cancer, hence it is necessary to search for new diagnostic methods or biomarkers (e.g. proteins, enzymes, cytokines, geens), which could enable the early detection of BC as well as improving the diagnostic utilization of the existing ones. Research is needed from which markers will emerge which could be useful in the diagnosis of BC, and from the patient's point of view, it is important that these diagnostic methods are non-invasive [8–11].

Cystoscopy remains the gold standard for the diagnosis and monitoring of BC disease, whereas cytology urine sediment is the standard non-invasive test performed on urine [2, 6]. Although many markers are under investigation, no spectacular breakthrough has been achieved so far. The Food and Drug Administration (FDA) currently approve several tests for the diagnosis and/or monitoring of bladder cancers: BTA stat and BTA TRAK, NMP 22 BladderChek, UroVysion and ImmunoCyt/uCyt+ is recommended for monitoring relapse. These tests are not performed routinely in Europe, but are available in some laboratories. However these tests still have low specificity when compared to urinary cytology [12, 13]. To date, the concept of a single marker as a base for clinical decisions has not proven sufficient and has not been recommended [14]. A good solution to this problem seems to be the creation of a panel of the best non-invasive markers, with the highest sensitivity and specificity.

Some low molecular weight proteins (LMWP) and some hydrolytic enzymes, such as β_2-microglobulin (β_2M) and retinol binding protein (RBP), as well as N-acetyl-β-D-glucosaminidase (NAG) and its isoform B (NAG-B) respectively, have a well-known presence in kidney diseases. These biomarkers are especially related to renal tubular damage in different renal disorders [15, 16], however data about their changes in bladder cancer are still insufficient. The neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2, has also recently been of increasing interest to scientists, especially regarding human cancer, and is considered to be one of the most promising next-generation biomarkers in clinical nephrology, as well as other diseases and pathological conditions [17, 18]. Higher levels of NGAL and its complex with matrix metalloproteinases (MMP) were observed in breast cancer, gastric cancer and BC with metastasis to the lymph nodes and the prostate [19, 20, 21].

The aim of this study was to examine a panel of low molecular weight proteins and enzymes such as β_2M and RBP, NAG and its isoform NAG-B and NGAL in the urine of patients with bladder cancer, and to attempt to establish their relationship to the stage and grade of BC.

MATERIAL AND METHODS

The biological material for this study was obtained from 46 bladder cancer patients (37 men, 9 women), aged from 36 to 87 years old (mean age 69). All participants were examined in the Department of Urology and Urological Oncology of Wroclaw Medical University. Bladder cancer patients had clinical diagnoses of bladder cancer confirmed by histopathology examinations, and no additional diseases of the urinary tract had been detected. The tumors were classified by grade and stage according to TNM Classifications of Malignant Tumours [22]. The histopathological characteristics of the examined patients were as follows: 21 of them were diagnosed as Ta staging, 13 as T1, 6 as T2, and 6 as TIS. According to BC grading, 16 of them were identified as G1, 21 as G2, and 9 as G3. The control group comprised 16 adults (11 men and 5 women) aged from 54 to 81 years old (mean age of 67). Controls were selected from participants with no history of cancer or other chronic inflammation, which was excluded by clinical examination of the cytology of urine sediment and a urine strip test. The BC patients and subjects from the control group were of similar socioeconomic status and there were no significant differences between these groups. All participants were informed of the aim of the study and gave written consent to participate. The approval of the Local Bioethics Committee of Wrocław Medical University was obtained (Nr-360/2016).

A morning sample of urine was collected from all participants, in polyethylene containers without preservatives. Morphological elements were removed by centrifugation for 10 min at 1440 rpm. The remaining urine was stored at -80°C until the determination of low molecular weight proteins and enzyme levels took place. Urinary concentration of β_2M and RBP, as well as NGAL, were measured immunoenzymatically with appropriate, specific methods (Demeditec Diagnostics GmbH, Geramny; Immundiagnostik, Germany; BioPorto Diagnostics, Denmark, respectively). The activity of NAG was measured spectrophotometrically using p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate. Its thermostable isoform – NAG-B, was measured using the same substrate but after previous sample incubation at 500C for 120 minutes [23]. All results were calculated per mg of creatinine in urine samples. Creatinine concentration was measured by the Jaffe method using reactions with picric acid in acid conditions, using the routine laboratory method [24]. Total protein concentration was also measured spctrophotometrically using Total Protein Kit Micro Pyrogallol Red Method (Sigma, USA).

Statistical analysis

Statistical analysis of the obtained results was carried out using Statistica PL version 12.0. Quantitative variables are provided as the mean ±standard deviation and median with minimum (min) and maximum (max) values. Data analysis was performed using Student's t-test or ANOVA test after checking normal distribution of results by Kolmogorov-Smirnov and Lilliefors tests. The associations between continuous variables were analyzed by the Spearman for nonparametric data and Pearson for parametric data tests. In all of the performed analyses, a p-value less than 0.05 was considered as statistically significant.

RESULTS

Concentrations of total protein and low molecular weight proteins (β_2M, RBP), enzyme NAG and its isoform (NAG-B) activity, and concentration of NGAL, expressed as mean and standard deviations, as well as medians with min and max values are presented in Table 1, along with statistical analysis. In the patients with bladder cancer the levels of all examined parameters differed in comparison to the control group, but significantly higher mean activity of NAG and NAG-B, as well as concentration of NGAL and total proteins were observed only in patients. The greatest difference was revealed in NGAL concentrations, which were almost twice as high in bladder cancer patients than in the control group. The activities of NAG and its isoform were higher in BC patients, from 49.2 to 64.3%, respectively. Moreover, statistically significant positive correlations ($r = 0.9486$, $p < 0.001$) between NAG and NAG-B activities were observed. The value of RBP in patients was higher compared to the control group, but not significantly. Only mean value of β_2M was slightly lower in the patients compared to the control group, but this did not exceed the reference values recommended by the manufacturer of the test (Immunodiagnostic Company), and significant differences were not observed.

Because diagnosed bladder cancer differs in terms of overall tumor mass and depth of invasion, according to the TNM classification, the values of the panel of examined biochemical parameters were estimated in subgroups of patients with different tumor stages (TIS, Ta, T1, T2). The results of urinary levels of low molecular weight proteins and enzymes in subgroups of patients at different tumor stages with statistical comparison to the control group as well as differences between subgroups, are given in Table 2. The highest mean values of NGAL, NAG, and NAG-B were detected in the urine of patients in stage T2 BC, and they were significantly higher compared to the control group. Additionally, NAG activity in stage TIS was significantly higher compared to healthy people. The highest value of RBP presented in patients with tumor stage T2 was significantly different in comparison to those at stages Ta and TIS, and was about 2.8-times and 3.7-times higher, respectively. Concentration of β_2M was the highest in the subgroup of patients with non-invasive tumors (T1), but no differences between subgroups of patients were observed. Interestingly, mean NGAL concentration was the most significantly higher (around threefold) in TIS in contrast to healthy subjects. NGAL values in patients with stage T2 were also significantly higher, about 2.6 times, in relation to the control group. Moreover, in patients with TIS, concentrations of NGAL were considerably higher than concentrations observed in patients with both Ta and T1 tumor stages. Additionally, among the examined parameters, a significant positive correlation between urinary concentration and bladder cancer stages was demonstrated only for NGAL ($R = 0.3606$, $p < 0.05$).

Based on these significant differences of NGAL in subgroups of patients with different stages of bladder cancer we also examined whether bladder cancer grade can be connected with the level of this enzyme.

Table 1. *Urinary levels of examined parameters in bladder cancer patients and control group. Results were expressed as mean and standard deviation as well as median with minimum and maximum values*

	Control group (n = 16)	Patients group (n = 46)	[p] value
Protein [mg/ml]	0.152 ±0.091 0.133 (0.057–0.385)	0.465 ±0.498 0.229 (0.010–1.901)	p = 0.0072
β_2M [µg/mg creat.]	0.246 ±0.278 0.126 (0.043–0.911)	0.136 ±0.072 0.120 (0.040–0.420)	p >0.05
RBP [µg/mg creat.]	0.128 ± 0.089 0.104 (0.022–0.314)	0.192 ±0.180 0.151 (0.005–1.151)	p >0.05
NAG [U/mg creat.]	1.770 ±0.694 1.635 (0.811–3.403)	2.468 ±1.694 2.271 (0.656–10.671)	p = 0.0315
NAG-B [U/mg creat.]	1.125 ±0.605 1.119 (0.212–2.540)	1.778 ±1.454 1.455 (0.244–8.809)	p = 0.0225
NGAL [ng/mg creat.]	25.587 ± 14.133 26.501 (7.433–50.730)	47.735 ±45.110 32.235 (1.691–184.261)	p = 0.0080

β_2M – β_2-microglobulin; RBP – retinol-binding protein; NAG, NAG-B – N-acetyl-β-D- glucosaminidase and isoensyme B of N-acetyl-β-D-glucosaminidase, respectively; NGAL – neutrophil gelatinase-associated lipocalin: p – statistically significant difference

Table 2. *Mean urinary levels of examined biochemical parameters in subgroups of patients depending on the staging of bladder cancer and in the control group*

Parameters		B_2M [µg/mg cr.]	RBP [µg/mg cr.]	NAG [mU/mg cr.]	NAG-B [mU/mg cr.]	NGAL [ng/mg cr.]
Stage of bladder cancer	Ta	0.101 ±0.036	0.092 ±0.089	2.420 ±1.114	1.689 ±1.077	30.468 ±28.270
	T1	0.116 ±0.037	0.174 ±0.083	2.578 ±0.917	1.936 ±0.902	35.584 ±27.919
	T2	0.107 ±0.041	0.261 ±0.019	3.402 ±2.102	2.259 ±1.268	53.960 ±13.456
	TIS	0.071 ±0.010	0.074 ±0.050	3.083 ±2.100	2.257 ±2.055	68.592 ±3.604
Control group		0.161 ±0.002	0.120 ±0.065	1.770 ±0.694	1.125 ±0.605	23.906 ±14.381
Statistical analysis			p^b <0.001 p^d = 0.0024 p^e = 0.0017	p^a = 0.0464 p^b = 0.0244	p^a = 0.0155 p^b = 0.0153	p^a <0.001 p^b <0.001 p^e = 0.0581 p^f = 0.0652

Ta, T1, T2, TIS – appropriate cancer staging; β_2M – β_2-microglobulin; RBP – retinol- binding protein; NAG, NAG-B – N-acetyl-β-D-glucosaminidase and isoensyme B of N-acetyl-β-D-glucosaminidase, respectively; NGAL – neutrophil gelatinase-associated lipocalin; p^a – cancer stage TIS vs. control group; p^b – cancer stage T2 vs. control group; p^c – cancer stage T1 vs. control group; p^d – cancer stage T2 vs. Ta; p^e – cancer stage T2 vs. TIS; p^f – cancer stage T1 vs. TIS

Interestingly, the average values of NGAL concentration raised in particular subgroups depended on the cancer grade (G1, G2, G3) and amounted to: 24.610 ng/mg cr., 49.735 ng/mg cr. and 70.928 ng/mg cr., respectively. The concentrations of NGAL in the grade G3 subgroup were significantly higher than those observed in patients with G2 and G1 ($p = 0.0351$ and $p = 0.0295$, respectively). Additionally, a significant positive correlation was observed between urinary concentration of NGAL and subgroups of patients with different bladder cancer grades ($R = 0.3787$, $p < 0.05$). In Figure 1 the percentage of changes of NGAL levels is presented, depending on the stage and grade of bladder cancer, and with respect to the control group. In TIS stage as well as G3 grade of bladder cancer patients, similar percentages of differences between NGAL concentrations in relation to the control group were observed (286.92 % and 296.70%, respectively).

Estimating the influence of smoking, age and gender on the levels of examined parameters in the urine of bladder cancer patients, a lack of statistically significant differences between men and women, smoking and non-smoking patients and older (>67 years old) and younger (<67 years old) patients was observed (data not shown).

DISCUSSION

Cancer pathogenesis and development is very complex process and is connected with many different agents – oxidative, immune, inflammatory, nutritional, environmental, and others [25–28]. The search for different, noninvasive parameters, which could reflect the carcinogenic process occurring within the bladder is still of great relevance. Up to now the best universally applicable method for the detection of bladder cancer has been cystoscopy with biopsy, but this is an invasive examination for patients [2]. In our study we used noninvasive biological material, such as morning urine samples, to estimate levels of selected LMWP, such as β_2M and RBC, and enzymes, such as NAG and its isoenzyme NAG-B, as well as NGAL, in BC patients with different stages and grades, to estimate whether they are connected with BC development. Analysis of 38 publically available microarray datasets and the Human Protein Atlas tool showed that NGAL transcripts were significantly higher in the majority of solid tumors compared to relatively normal tissues for every dataset analyzed. Furthermore, concordance of NGAL at both mRNA and protein levels was observed for 6 cancer types, including bladder, colorectal, liver, lung, ovarian, and pancreatic. All metastatic tumors showed a decrease in NGAL expression when compared to matched primary lesions (analysis of mRNA and NGAL expression, an immunohistochemistry). According to these results, the authors stated that NGAL is a candidate marker for tumor growth in a fraction of solid tumors. Further investigations are required to elucidate the function of NGAL in tumor development and metastatic processes [29, 30]. We observed varying degrees of change in levels of all the examined parameters between patients with bladder cancer and the control group, as well as among patients with different bladder cancer stages and grades, but the most significant differences were observed for NGAL concentration and additionally for the activity of NAG and its isoenzyme – NAG-B. Although the levels of low molecule proteins (RBP and β_2M) in the subgroup of patients we examined were slightly different, significantly higher levels of RBP were found in stage T2 in comparison to stage Ta and TIS. In patients with inva-

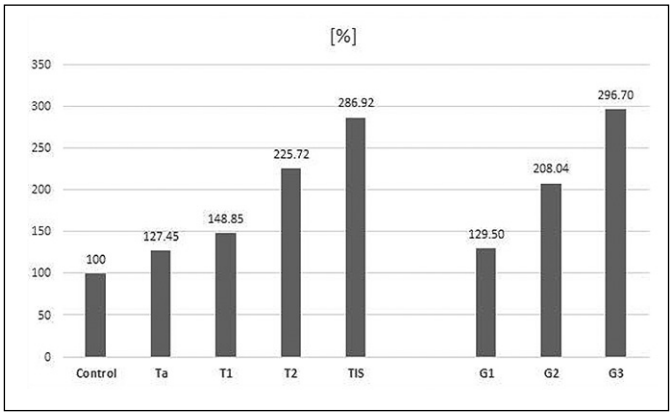

Figure 1. *The percentage of changes of NGAL (gelatinase-associated lipocalin) concentration in relation to staging and grading of cancer, in comparison to the control group. Ta, T1, T2, TIS and G1, G2, G3 – appropriate bladder cancer staging and grading, respectively.*

sive BC, Tyler et al. [31] showed a significantly lower serum level of RBP in comparison to healthy people. Some authors indicate the role of disturbances of vitamin D and RBP in the etiology not only of bladder but also other cancers [32]. NAG is especially well known as a marker of diabetic nephropathy and other renal diseases [33, 34]. NAG is present in free form in the lysosomes of many cells. The source of its increased activity in the urine of patients may be not only the damaged lysosomes of epithelial cells, but also phagocytic cells comprising NAG in their azurophilic granules, which could be involved in the ongoing local inflammatoryprocess [35, 36]. In explaining the pathomechanism of increased urinary excretion of NAG, its isoenzyme – NAG-B, which is attached to the lysosomal membrane, is very helpful. Its increased level in urine is a result of the rupture of the membrane integrity of these organelles, and may suggest deeper destructive changes in the cells during the development of BC. In the present study, excretion of this isoenzyme remains highly correlated with the activity of NAG, indicating extensive cell damage in patients with urinary BC. Elevated levels of urinary NAG were previously confirmed by Youssef et al. [35] in rat bladder carcinogenesis. Różanski et al. [36], evaluating the usefulness of NAG in the diagnosis of bladder cancer in postmenopausal women, showed an increased expression at the mRNA level of NAG in the urine of the examined women. The connection and influence of the enzyme on cancer development is not fully understood. It is believed that increased production of NAG can lead to disruption of the mitotic cell cycle [36, 37]. Our research found significantly higher levels of NGAL in the urine of patients with BC in comparison to healthy subjects, as well as the most significant differences in the subgroups of patients with different stages and grades of bladder cancer. Additionally we revealed that urinary NGAL concentrations increased with tumor stages and grades. This suggests that NGAL increase may result from the induced expression of this lipocaine by damaged epithelium cells. Induction of NGAL synthesis may also be caused by factors stimulating cancer development, e.g. polyomaviruses, hepatocyte growth factors (HCF) or nuclear transcription factor NF-kappa B (NF-κB) [38, 39, 40]. Moreover we found that the urinary concentrations of NGAL in patients with stage TIS of bladder cancer were significantly higher than its concentrations in the control group or in patients at stages Ta and T1. In this light, NGAL appears to be a promising biomarker, which in the future may facilitate the diagnosis of this tumor stage. Interestingly the highest NGAL concentration was observed not only in patients at TIS stage, but also at G3 grade.

Interestingly NGALR, a cell surface receptor for NGAL, was also identified and the co-expression of both NGAL and NGALR has been implicated in different cancers [17, 30]. Moreover, most studies concern changes of NGAL concentration in the serum of bladder cancer patients, while fewer studies refer to urine, so the changes revealed are interesting and promising. Increasing attention is paid to NGAL as a cancer biomarker, but differential expression patterns are indicated. Monier et al. [41] examined levels of metalloproteinases and tissue inhibitors of metalloproteinase (TIMP-1 and TIMP-2) in different urothelial carcinomas. The authors observed, by western blot densitometry, an imbalance in MMP-9/TIMP-1 and MMP-2/TIMP-2 pairs, and changes in NGAL concentration in an unselected cohort of transitional cell carcinoma patients. An imbalance in MMP-9/TIMP-1 and MMP-2/TIMP-2 correlated with histological progression and clinical events, and was more pronounced at latter stages of progression compared to normal urinary enzyme profiles, but such a clear relationship was not observed for NGAL. The authors concluded that the obtained results were not straightforward, as a decrease in active MMP-9 and lack of NGAL are also associated with disease progression, and confirmation is required from a larger and more homogenous cohort, although it can be confirmed already that the degradation process of the epithelial basement membrane provides clues for understanding underlying cancer mechanisms. It is indicated that NGAL complex, with metalloproteinase-9, may be responsible for a procarcinogenic effect, and on the other hand may protect from the proteolytic degradation of enzymes, thus enhancing its activity. MMP-9 is responsible

for extracellular matrix destruction, which increases the risk of invasiveness of cancer and the risk of metastasis [19, 42, 43]. This is confirmed by Mohammed et al. [44], who showed that the concentration of these complexes in the urine of BC patients was elevated in patients with higher grades of cancer, and in those who have metastases to the lymph nodes and the prostate. The results we obtained also indicate the potential use of NGAL as an early diagnostic and prognostic marker of BC, and, importantly, when estimated in urine.

CONCLUSIONS

In our study, we paid attention to a new aspect, which has not previously been considered, the existence of interrelations between changes in urinary NGAL concentrations and NAG and NAG-B activity, and the stages and grades of BC. Urinary NGAL level seems promising due to its highest concentration in patients with TIS stage and G3 grade. Our findings indicate that it might have diagnostic value and may be used as prognostic marker after deeper research. It is, however, important to estimate the sensitivity and specificity of NGAL by performing further studies of a larger number of patients with different stages and grades of BC.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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