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The significance of calprotectin, CD147, APOA4 and DJ-1 in non-invasive detection of urinary bladder carcinoma

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Aim of the study is to define the diagnostic accuracy of selected urinary protein biomarkers in the non-invasive detection of primary and recurrent urothelial carcinoma of the urinary bladder. The urinary levels of calprotectin, CD147, APOA4 and protein deglycase DJ-1 were examined in 255 individuals, including 60 controls with non-malignant urological disease, 61 patients with a history of urinary bladder cancer with negative cytology and negative cystoscopy and 134 patients with urinary bladder cancer. Urinary concentrations of biomarkers were determined by Enzyme-Linked Immunosorbent Assay (ELISA). During the follow-up of patients with non-muscle invasive bladder cancer (NMIBC), a group of 44 patients with cancer recurrence was compared to the group of 61 patients with a history of NMIBC but with no evidence of disease. Urinary concentrations of the evaluated markers did not reveal any significant difference between these groups. During the primary diagnosis, a group of 90 patients with primary bladder cancer and 60 subjects with benign disease were compared. Urinary levels of CD147 were not significantly higher in patients with tumors. The greatest diagnostic accuracy was observed in APOA4 (sensitivity 55.6, specificity 83.3, AUC 0.75), and lesser in calprotectin (sensitivity 39.4, specificity 87.7, AUC 0.66) and in DJ-1 (sensitivity 61.1, specificity 66.7, AUC 0.64), respectively. Apolipoprotein A4 may be used potentially as a supplemental urinary marker in the diagnosis of primary bladder cancer.

Key words: bladder cancer, diagnosis, CD147, calprotectin, APOA4, DJ-1

At the time of presentation, about 75% of patients are diagnosed with non-muscle invasive bladder cancer (NMIBC). These tumors are characterized by a high incidence of recurrence, even after a long interval without the disease. This fact is reflected in the intensity and length of the surveillance, which is, in most patients, long-term [1]. In NMIBC, the follow-up is based on regular cystoscopy, which plays a crucial role in bladder cancer diagnosis. Even though we know that the cystoscopic approach is unpleasant for the patients and costly for the healthcare system, it has not been replaced by any less invasive method thus far, despite the long-term, intensive research in this area.

The use of urinary biomarkers in the bladder cancer diagnosis is not recommended by current guidelines [1]. None of the urinary markers provide sufficient diagnostic accuracy to replace cystoscopy [2]. In standard clinical practice, urine cytology has been used to complement cystoscopy, i.e. to detect diseases that may be overlooked by routine cystoscopy (carcinoma *in situ*, upper tract tumors, prostatic urethra involvement). Cytology yields high specificity and sufficient sensitivity in high-grade carcinomas. However, in low-grade carcinomas, it provides only 4–31% sensitivity [3]. Urine cytology has been used as criteria of usefulness in new urinary biomarkers. An applicable biomarker should yield at least the same sensitivity and specificity as urine cytology.

Specific soluble proteins tested as potential indicators of the presence of urinary bladder carcinoma can be examined easily by relatively cheap immunoassays. This method allows fast, qualitative, point-of-care testing, but also multiplex testing for a panel of protein biomarkers. In this study, we assessed the diagnostic accuracy of 4 potential biomarkers with a reported combined sensitivity and specificity (sensitivity + specificity/2) ≥80%. The 80% limit is relatively high and corresponds to the combined sensitivity and specificity of cystoscopy, the gold standard to which biomarkers are usually referenced [4]. From the potentially promising protein molecules that could be tested by the available assays using the ELISA method, we chose the following biomarkers:

apolipoprotein A4 (APOA4), calprotectin, cluster of differentiation 147 (CD147) and protein deglycase DJ-1.

Calprotectin is a heterodimer comprised of S100A8 and S100A9 proteins with antibacterial properties. In the study of 46 patients and 40 controls, it provided 80% sensitivity at 92% specificity. The median urinary calprotectin level was 10-fold higher in bladder cancer patients than healthy controls [5]. Urinary protein CD147 was reported as a potential marker in the study of 30 patients and 30 controls. In this study, CD147 (also known as Basigin or EMMPRIN), showed 97% sensitivity at 100% specificity [6]. Two other proteins, DJ-1 and apolipoprotein A-4, were designated as potential urinary markers with 83.3% sensitivity at 100% specificity in DJ-1 and 79.2% sensitivity at 100% specificity in apolipoprotein A4, respectively. These results were reported by Kumar *et al.* on a population of 173 patients and 212 controls [7].

In our study, we decided to verify the high diagnostic accuracy of selected protein biomarkers in a separate analysis for the detection of primary and recurrent tumors that were originally NMIBC.

Patients and methods

Patients. To detect urinary biomarker levels, urine samples from 255 consecutive individuals were taken in the period from 9/2011 to 7/2014. In 134 patients, urothelial bladder carcinoma was confirmed; for 90 patients it was the initial diagnosis and in 44 patients it was a recurrence of the original NMIBC. The control population comprised of 60 individuals with non-malignant urological diseases other than urolithiasis (prostatic hyperplasia, hydrocele, etc.), and 61 patients with a history of NMIBC but with no evidence of disease (with negative cystoscopy and urine cytology). Patients with macroscopic hematuria, urinary infection, and those that were presently being treated by intravesical chemotherapy or the bacillus Calmette-Guerin (BCG) vaccine were not included in the study. Other exclusion criteria included urinary catheter, urolithiasis and histological types other than urothelial carcinoma. The characteristics of patients included in the study are provided in Table 1.

Sample and data collecting. After obtaining the local ethics committee's approval, urine samples were collected, always from the second urination in the morning. Part of the urine sample was used to examine urine sediment, urine culture and voided urinary cytology. The urine sample for the analysis of biomarkers were then evenly divided and kept frozen at –80 °C.

Urinary biomarker analysis. The following assays were used to detect individual protein markers by ELISA: Human Apolipoprotein A-IV ELISA Kit (Abcam plc, Cambridge, Great Britain), S100A8/A9 (Calprotectin) Human ELISA (Biovendor-Laboratorní medicína, a.s., Brno, Czech Republic), Human DJ-1/PARK7 ELISA Kit (Circulex, MBL International Corporation, Woburn, Massachusetts, USA) and Human EMMPRIN/CD147 Quantikine ELISA Kit (Bio-Techne R&D Systems Inc., USA, Minneapolis, Minn., USA). For urinary cytology, slides were stained according to Papanicolaou and Marshall and microscopically reviewed by a single cytopathologist in accordance with the recommendations of the Papanicolaou Society of Cytopathology [8]. Specimens with suspected carcinoma were labeled as clinically positive.

Data analysis. Basic statistical data, i.e., mean, standard deviation, median, and inter-quartile range, minimum and maximum, were calculated for the obtained parameters. Spearman's rank correlation coefficient was used to determine the correlation between the studied parameters. Correlations between individual biomarkers and bladder cancer were determined by the Wilcoxon test. Non-parametric ROC (receiver operating characteristic) curves were created. The relative potential of the biomarkers to identify bladder carcinoma was specified by the calculation of the area under the

Table 1. Demographic and clinicopathological characteristics of patients.

	Patients with primary bladder cancer (n=90)	Patients with recurrent bladder cancer (n=44)	Control subjects with non-malignant disease (n=60)	Patients with a history of NMIBC but with no evidence of disease (n=61)
Age, years; median (min-max)	67 (30–90)	70 (47–88)	65 (24–84)	71 (25–92)
Men:women	61:29	35:9	49:11	41:20
Tumor stage				
Ta	40	36	–	–
T1	31	5	–	–
T2–4	19	3	–	–
Tumor grade				
G1	21	31	–	–
G2	35	7	–	–
G3	34	6	–	–
Average tumor size (cm)	3.5	1.5	–	–
Average number of tumors	2.2	3.4	–	–

NMIBC – Non-muscle invasive bladder cancer

ROC curve (AUC). Statistical significance was set at $p<0.05$, and all reported p values were 2-sided. Statistical analysis was carried out using SAS 9.4 software (SAS Institute Inc., Cary, N.C., USA).

Results

When comparing the urinary levels of all four tested biomarkers, no significant difference was observed between the group of 44 patients with recurrent NMIBC and the group of 61 patients in surveillance with a history of NMIBC but with no evidence of disease (Table 2).

We observed significantly higher urinary values of calprotectin, APOA4 and DJ-1 in the 90 patients with primary tumors, when compared to the group of 60 patients with benign urologic diseases, whereas the urinary values of CD147 did not significantly differ between the groups (Table 2). We determined the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and AUC in biomarkers that were significantly elevated in bladder cancer patients (Table 3). Table 3 also provides the values of sensitivity, specificity, NPV and PPV in urinary cytology as a standard urinary diagnostic test, in order to provide comparison. The likelihood ratio (LR) of positive and negative test results presented in Table 3 can be used for diagnostic test interpretation in LR nomogram.

In patients with primary bladder cancer, the highest diagnostic accuracy was found in marker APOA4; the ROC curve is shown in Figure 1. The urinary value of markers in the group of patients with primary bladder carcinoma corresponded to the tumor stage; the significant correlation was observed in calprotectin ($p=0.0007$), APOA4 ($p<0.0001$) and in DJ-1 ($p=0.0045$). Although we did find a significant correlation between the tumor grade and the urinary values of calprotectin ($p<0.0001$), APOA4 ($p=0.0002$) and DJ-1 ($p=0.0082$), and between the tumor size and urinary value of calprotectin ($p=0.0081$), APOA4 ($p=0.0001$) and DJ-1 ($p=0.0037$), we did not observe any significant correlation between the urinary values of the tested biomarkers and the number of tumors.

Discussion

The aim of this study was to confirm the high diagnostic accuracy of several selected protein biomarkers in patients with urothelial bladder carcinoma. The selection criteria for biomarkers were combined sensitivity and specificity ≥ 80% and the available immunoassays. Based on these criteria, the biomarkers calprotectin, CD147, DJ-1 and APOA4 were selected, in which the diagnostic accuracy was tested separately in a group of patients with primary diagnosis and with recurrent bladder cancer. In patients with primary

Table 2. Mean and median values of CD147, calprotectin, APOA4 and DJ-1 in urine tested by the ELISA method. Comparison of 90 patients with primary tumor and 60 non-malignant control subjects, and of 44 patients with cancer recurrence and 61 patients with a history of NMIBC but with no evidence of disease.

Recurrent tumors	Case		Control		p-value
Biomarker	mean ± SD	median [min., max.]	mean ± SD	median [min., max.]	
CD147 (ng/ml)	15.35±7.80	14.63 [2.33, 39.50]	16.62±9.75	16.18 [2.50, 40.00]	0.6226
Calprotectin (ng/ml)	645.58±516.91	543.52 [32.78, 1600.00]	692.09±512.73	535.85 [32.78, 1600.00]	0.5836
APOA4 (ng/ml)	41.90±6.16	16.02 [3.13, 200.00]	41.72±63.47	12.26 [3.13, 200.00]	0.5722
DJ-1 (pg/ml)	174.25±289.49	47.42 [25.00, 1600.00]	216.61±377.02	31.20 [25.00, 1600.00]	0.9647
Primary tumors	**Case**		**Control**		**p-value**
Biomarker	**mean ± SD**	**median [min., max.]**	**mean ± SD**	**median [min., max.]**	
CD147 (ng/ml)	15.00±9.42	13.30 [2.59, 40.00]	15.33±8.92	15.76 [1.59, 40.00]	0.5340
Calprotectin (ng/ml)	678.20±616.21	320.67 [25.00, 1600.00]	364.67±470.68	143.27 [25.00, 1600.00]	0.0055
APOA4 (ng/ml)	84.69±8.13	38.16 [3.13, 200.00]	29.01±48.78	8.98 [3.13, 200.00]	<0.0001
DJ-1 (pg/ml)	498.93±628.68	115.31 [25.00, 1600.00]	222.04±415.19	39.77 [25.00, 1600.00]	0.0046

SD – standard deviation

Table 3. Sensitivity, specificity, negative predictive value, positive predictive value, likelihood ratio of positive and negative test results and area under the curve of urinary diagnostic tests calprotectin, APOA4, DJ-1 and urine cytology in the group of patients with an initial diagnosis of bladder cancer.

Urinary diagnostic test	Sensitivity	Specificity	NPV	PPV	LR^+	LR^-	AUC	95% CI
Calprotectin	39.4	87.7	42.9	86.1	3.20	0,69	0.66	0.56–0.76
APOA4	55.6	83.3	55.6	83.3	3.33	0,53	0.75	0.67–0.83
DJ-1	61.1	66.7	53.3	73.3	1.83	0,58	0.64	0.55–0.72
Cytology	67.2	98.0	54.4	98.9	33,6	0,34	–	–

NPV – negative predictive value, PPV – positive predictive value, LR^+ – likelihood ratio of positive test, LR^- – likelihood ratio of negative test, AUC – area under the curve, CI – confidence interval

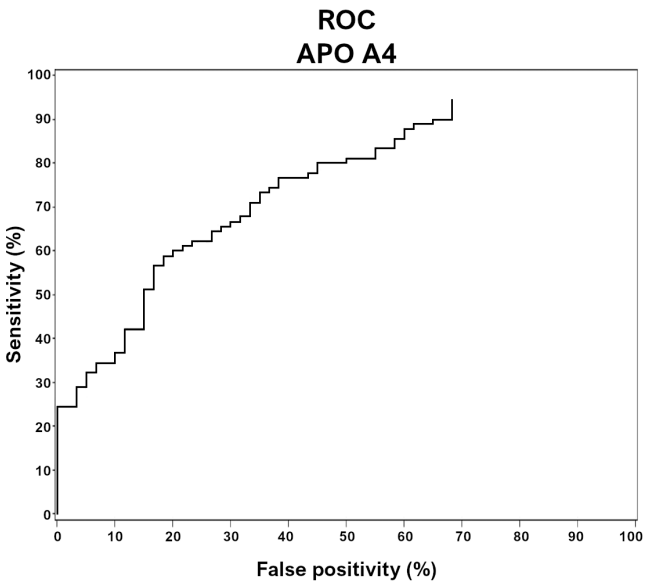

Figure 1. Urinary APOA4 level. Comparison of patients with primary bladder cancer and non-malignant control subjects. AUC=0.75. APOA4 – apolipoprotein A4; AUC- area under the curve.

bladder tumors, we confirmed elevated values of calprotectin, DJ-1 and APOA4; however, the increase was still significantly lower than in original studies [5–7]. In our population, the AUC in these biomarkers reached values of 0.64–0.75. In patients with recurrent tumors, none of the urinary levels of the tested biomarkers significantly differed from values observed in patients without the recurrence. Our results therefore imply that urinary calprotectin, CD147, DJ-1, or APOA4 are not applicable in the detection of recurrent urinary cancers.

In the study of urinary diagnostic markers, the selection of patients and structure of the study population can have a significant impact on the result. Therefore, the selection of patients should be carried out in such a manner that the study population would maximally correspond to the real population of the clinical practice for which we want to use the diagnostic marker [4]. A common drawback of studies focusing on suitable markers for the initial diagnosis is that they often preferentially include patients with advanced high-grade tumors, which can increase the sensitivity of the test. Furthermore, a comparison with healthy volunteers results in the increased specificity of the test; therefore, patients with primary bladder cancer should be compared to the patients that have same symptoms (usually hematuria), but where the tumor was not confirmed. Urinary markers used for surveillance should be tested on a population of followed-up patients with recurrent tumors, and then compared to patients that are followed-up, but with no evidence of disease [9]. Many studies focusing on the diagnostic benefits of urinary protein markers do not distinguish between patients that have primary or recurrent tumors [10]. With the aim to avoid selection bias and to determine whether the tested biomarkers may be used in clinical practice, we tested their diagnostic accuracy separately in two different clinical situations: during both the primary detection and during the detection of bladder cancer recurrence.

In clinical practice, reliable diagnostic markers should provide the greatest benefit during the follow-up of patients, where it could replace at least a part of the necessary cystoscopies. During the follow-up, we attempt to detect small recurrent tumors. Nevertheless, urinary protein biomarkers often correspond to grade and stage, i.e. they yield higher sensitivity in high-grade and muscle invasive bladder cancer and conversely, a lower sensitivity in low-grade cancer. Thus, low-stage and low-grade diseases are not easily detected by the level of urinary protein biomarkers. By definition, the cancer cells in low-grade diseases are still relatively normal, both genomically and phenotypically, and although some alterations in gene expression have been noted, the processes involved in the releasing of proteins into the urine may still be essentially normal [4]. In our population of patients with recurrent tumors, 36 out of 44 patients had Ta and G1 carcinoma. This may be the reason why we did not find any of the four tested protein biomarkers suitable for the detection of small recurrent tumors and thus, we cannot recommend it as a suitable alternative to cystoscopy.

In original studies, the urinary levels of calprotectin, CD147, DJ-1 and APOA4 provided such a high sensitivity and specificity that, if confirmed, these markers could become a part of everyday clinical practice. However, with just one exception, no studies attempting to verify this exceptional accuracy have been published to date. The one exception is the study of calprotectin, where Yasar *et al.* compared 82 cases that did not receive any prior treatment with 52 healthy controls, and reported 81% sensitivity at 84% specificity and an AUC of 0.9. Diagnostic accuracy of calprotectin was compared to the one of Bladder Tumor Antigen (BTA) tests, which reached 89% sensitivity, 88% specificity and an AUC of 0.95 on the same group of patients [11]. When compared with earlier publications, these results for BTA are unusually good, which further questions the high diagnostic accuracy of calprotectin [4].

The benefits of our study include the fact that it was structured to find a suitable urinary biomarker for the primary diagnosis and for the diagnosis of recurrent tumors. Its limits include a small number of patients in individual groups and the fact that patients with primary bladder cancer were compared with patients with different urological diseases and not directly to patients with a history of hematuria where the urothelial carcinoma was ruled out.

In conclusion, we did not confirm an exceptionally high diagnostic accuracy of CD147, calprotectin, APO A4 and DJ-1. Our results imply that none of the tested biomarkers were suitable for the detection of recurrent bladder carcinoma. However, combined with other urinary markers,

APO-A4 could be used as a supplementary urinary marker in the detection of primary bladder carcinomas.

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