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Podoplanin serum and urine concentration in transitional bladder cancer

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Abstract.
BACKGROUND: Podoplanin (PDP) is a mucin – a type of transmembrane protein expressed in numerous tissues during ontogeny and in adult animals, including the brain, heart, kidney, osteoblasts and lymphoid organs.
OBJECTIVE: The aim of this study was to determine podoplanin concentration in the blood serum and urine of patients with bladder cancer. Quantifying podoplanin concentration and its correlation with various clinicopathological parameters may be useful for more accurate predictions and identifying high-risk patients.
METHODS: The present study included 82 patients with bladder cancer confirmed by transurethral resection or cystectomy and 27 healthy volunteers. The Surface Plasmon Resonance Imaging biosensor was applied for the detection of podoplanin in the serum and urine samples.
RESULTS: Significant differences in serum and urine podoplanin concentration levels were observed between bladder cancer patients. The statistically significant higher values of PDP were detected in serum of patients with invasive, more aggressive, larger, multifocal tumors.
CONCLUSIONS: The association between podoplanin concentration and clinicopathological features indicates that it might be useful while making therapeutic decisions.

Keywords: Podoplanin, bladder cancer, surface plasmon resonance imaging biosensor, transitional cell carcinoma

1. Introduction

Urinary bladder cancer (UBC) is the ninth most common cancer worldwide. It is the seventh most common malignancy among males and seventeenth in case of females [7]. Transitional bladder cancer is the predominant histologic type of bladder cancer in the United States and Europe and accounts for 90% of cases [21].

Bladder cancer is more common with men than women and it is more prevalent in older persons. Many different risk factors have been identified for bladder cancer. It has a clear correlation with environmental exposures, such as smoking, industrialization and environmental pollutants [25].

Approximately 75% of the cases are confined to the mucosa (stage Ta, CIS) or submucosa (T1). The other 25% of the patients present with the muscle-invasive disease (T2–T4). In 2004 new WHO grading was introduced. It differentiates between papilloma, papillary urothelial neoplasm of low malignant potential (PULMP), low-grade and high-grade urothelial carcinomas. First two grading forms are extremely rarely recognized and in our study population they are not present [5].

Bladder cancer remains a highly prevalent and lethal malignancy. An early diagnosis, accurate staging and grading is important factors to select optimal treatment. Therefore, molecular diagnostic assessment be-

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comes an integral part of the routine clinical management of patients with cancer. Currently, there is a need for new prognostic molecular biomarkers that can help clinicians to identify patients requiring early, aggressive treatment. Biomarkers are potential tools for the detection of bladder cancer but also for the prediction of tumour recurrence and progression and for the development of metastases. The literature describes a number of bladder cancer markers. They are usually measured at DNA, RNA, and/or protein levels [8,17,19]. Especially the proteins involved in molecular pathways and implicated in tumorgenesis and metastasis are studied (i.e. cadherins, cathepsin D, matrix metalloproteinase-7 [23], thrombomodulin [29], podoplanin [24]).

Folkman in 1971 advanced the hypothesis that tumor growth is angiogenesis-dependent. Recent studies of tumor pathophysiology have focused attention on role of lymphangiogenesis in the progression of muscle-invasive transitional cell carcinoma (TCC) of the bladder. Some glycoproteins (VEGF-C, VEGF-D) belonging to the family of vascular endothelial growth factor (VEGF) are considered the most important regulators of lymphangiogenesis [13]. Studies of highly specific marker for lymphatic endothelial cells enable the identification and the quantitative assessment of lymphatic vessels in tumors. Podoplanin is one of the specific markers of lymphangiogenesis [4]. Human podoplanin (PDP) also known as T1alpha-2, PA2.26 antigen and gp36 is a mucin-type transmembrane protein made up of 162 amino acids, 9 of which create an intracellular domain. It is expressed in multiple tissues during ontogeny and in organs of adult animals, including the brain, heart, kidney, lungs, osteoblasts, and lymphoid organs [16,26]. The protein demonstrates expression in numerous pathological conditions such as: lymphangiomas, Kaposi sarcomas, in epithelioid mesotheliomas hemangioblastomas and seminomas and in many tumors in humans [16,18,20]. High podoplanin expression was observed in the endothelial cells of the lymphatic vessels in contrast to the endothelium of the blood vessels [3,20]. PDP is studied most extensively in cancers. PDP is upregulated on tumor cells themselves in several cancer types, including squamous cell carcinoma of the lung, head, and neck, malignant mesothelioma, brain tumors [26] and bladder carcinoma [24]. PDP is often expressed as the leading invasive edge of tumor. It plays a crucial role in process of regulation of epithelial-mesenchymal transition and therefore also in process invasion and metastasis [1]. It is the endogenous ligand for the platelet C-type lectin like receptor. Recent reports indicate that podoplanin association with C-type lectin-like receptor 2 (CLEC2) induces platelet aggregation and contributes to cancer progression [15,22]. Moreover, podoplanin expression in tumor-initiating cells also suggests a significant role in cancer progression [2]. Currently, the mechanism of podoplanin in tumor invasion is being studied. The results of recent clinicopathological studies of human cancer seem to be controversial. Some scientists postulate that a higher expression level of podoplanin in cancer cells significantly correlates with poor prognosis [30]. Other results of studies demonstrate that lower expression of podoplanin in cancer cells indicates a poorer prognosis [12].

The podoplanin expression in bladder cancer was investigated [24]. The results of these studies suggest that PDP would be useful as a biomarker for prediction of metastatic transitional cell carcinomas (TCC) and squamous cell carcinomas (SCC) of the bladder. An alternative approach seems to be determination of podoplanin concentrations in serum and urine and associating PDP levels with the respective stage of the disease.

The podoplanin concentration in the serum and urine can be investigated using the surface plasmon resonance imaging (SPRI) biosensor [9]. The combination of SPRI technique with the sensitive biosensors proved to be very valuable for the quantitative determination of podoplanin in biological samples. SPRI is label-free method that quickly provides real-time information on the concentration of biologically active species. The SPRI technique is based on the optical phenomenon that occurs when polarised light is reflected by a glass surface coated with a thin layer of metal [11]. The aim of this study was to determine podoplanin concentration in the blood serum and urine of patients with bladder cancer and to correlate the results with clinicopathological parameters. Moreover, the possible efficiency of the podoplanin in the diagnosis of invasive form of bladder cancer and its non-muscle invasive form with higher risk of progression was evaluated.

Quantifying podoplanin for prognostication leads to more accurate predictions and may be useful for identifying high-risk patients and the optimal time of treatment, respectively.

2. Materials and methods

2.1. Preparation of biological samples

A prospective study to determine podoplanin in serum and urine of patients with TCC was performed. The subjects were divided into a malignant and a control group. Urine and serum samples of patients with initial (after cystoscopy) or confirmed (prior transurethral resection of bladder tumor – TURBT) diagnosis of bladder cancer were obtained prior to surgery at admission to the J. Sniadecki Provincial Hospital of Bialystok (Bialystok, Poland). Individuals with additional malignant or inflammatory disease (also negative urine cultures) were excluded.

Blood samples taken from healthy donors were supplied by the Blood Donor Centre of Bialystok, Poland. Urine samples were obtained from healthy volunteers. Blood samples were obtained from the median cubical vein. The urine and serum samples were frozen immediately and maintained at -70°C.

Prepared serum samples were diluted ten-fold with phosphate-buffered saline (PBS) (BIOMED, Lublin, Poland). Urine samples were centrifuged at 1,850 $\times$ g for 15 min and the supernatant was separated. Finally, the samples were filtered through a paper filter of medium density.

The creatinine (CREA) concentration in serum and urine was determined using Jaffe's Method.

Cancer diagnosis was determined by histological examination of tumor specimens obtained from transurethral resection or cystectomy. Finally the malignant group consisted of 82 patients with confirmed TCC. The control group included 27 healthy volunteers. The other main variables collected were creatinine, podoplanin to creatinine ratio, clinical parameters including stage, grade, size, tendency to reoccurrence, pattern of growth and multifocal nature.

Table 1 presents the clinical characteristics of patients.

Patients with non-muscle invasive tumor were divided into two subgroups: high and low risk of invasiveness. Two factors were taken into account the aggressiveness of the cancer (grading) and the presence of particular clinical parameters. The high risk patients were characterized by high – grade tumor and presence of 3 out of 4 clinical parameters (size $>$ 30, nonpapillary, multifocal, recurrent).

Approval for this study was obtained from the Bioethics Committee of the Medical University of Bialystok (Bialystok, Poland) and written informed consent was obtained from all the patients and donors.

2.2. Procedure of podoplanin determination

The podoplanin (human recombinant podoplanin Fc chimera, R&D System. Inc.) concentration was determined using the SPRI (Surface Plasmon Resonance Imaging) biosensor. The SPRI technique allows sensitive determination of proteins using highly specific protein-antibody interactions. The sheep polyclonal antibody (IgG) specific for human podoplanin was purchased from R&D System. Inc. The exact description of the methodology of measurements and biosensor design is set out in the previous paper [9]. The samples of plasma and urine were placed directly on the prepared biosensor for 10 min to allow interaction with the antibodies. The volume of the sample applied on each measuring field was 2 μl.

The biosensor was washed with water and HBS-ES buffer solution pH = 7.4 (0.01 M 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, 0.15 M sodium chloride, 0.005% Tween 20, 3 mM EDTA), BIOMED, Lublin, Poland) to remove unbound molecules from the surface. The SPRI signal was measured twice on the basis of registered images, following the immobilization of antibodies and then following interaction with podoplanin from the samples. The signal, which is proportional to coupled biomolecules, was obtained by calculating the difference between the signal prior to and following the interaction with biomolecules. The concentration was determined using the calibration curves of the SPRI signal depending on the concentration of podoplanin.

2.3. Statistical analysis

The results are presented as the median $\pm$ standard deviation. Statistical analyses were performed using Student's T-Test and $P < 0.05$ and $P < 0.01$ were considered to indicate a statistically significant difference. The receiver operating characteristic curves with optimal cutoff points were calculated by MedRoc (Version 2.0), Software for ROC analysis of biomedical data. For cutoff points sensitivity, specificity, positive and negative predictive values were calculated.

3. Results

3.1. Changes in podoplanin concentration

A significant difference in serum concentration of podoplanin was observed between bladder cancer pa-

Table 1
Demographic and clinicopathological characteristic of patients

Variable	Range	Number of patients
Age (year)	< 65	34
	> 65	48
Gender	Women	23
	Men	59
Tumor stage	Non-muscle invasive (Ta + T1)	51
	Muscle invasive (T2 + T3 + T4)	31
Tumor grade	Low grade	35
	High grade	47
Tumor size (mm)	< 30	49
	> 30	33
Recurrence	Primary	35
	Recurrent	47
Multiplicity	Single	46
	Multiply	36

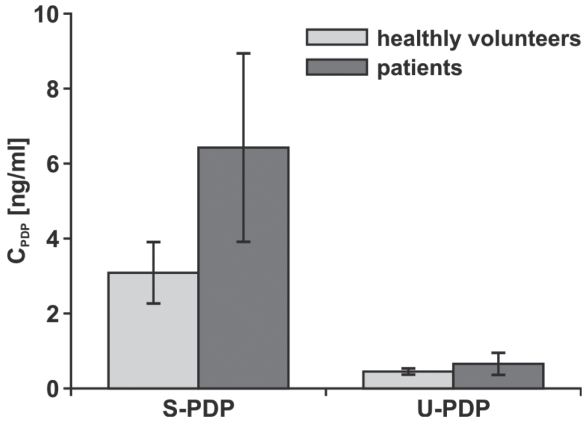

Fig. 1. Concentration of podoplanin in blood serum and urine of bladder cancer patients and healthy subjects. Confidence bars were calculated at $p = 0.001$. PDP-podoplanin; S-serum; U-urine.

tients (range 1.28–11.71 ng/ml) and healthy subjects (range 0.99–7.83 ng/ml). In urine podoplanin concentration for bladder cancer patients and healthy donors was in the range of 0.12–1.20 ng/ml and 0.20–0.93 ng/ml, respectively. For urine samples the differences between these two groups are not evident. The results are presented in Fig. 1.

Table 2 shows the serum concentration of the podoplanin (PDP) in correlation to clinicopthological characteristics. The high values of PDP were observed in invasive, multifocal, more aggressive (high grade) ($p < 0.001$) and larger tumors ($p < 0.0095$) For more aggressive (high-grade) and invasive cases (T2-T4) the podoplanin higher concentration was confirmed by corresponding PDP/S-CREA ratio.

Table 3 shows podoplanin urine concentration and PDP/ CREA ratio. In contrary to the results obtained in serum, we observed almost no correlation of clinicopathological parameters to raw podoplanin values. Noticeably, PDP/CREA ratio was higher for invasive, high-grade (both $p < 0.0001$) and multiple ($p < 0.011$).

3.2. Analysis of the risk group

The relationship between podoplanin concentration in patients high and low risk of invasiveness were analyzed. In Table 4 the results of podoplanin and PDP/CREA ratio in serum and urine of patients with only non-muscle invasive TCC, divided into high – and low-risk group were presented. As it was mentioned, these two groups differed with probability of progression. Several clinical parameters predispose TCC tumors to higher progression ratio (e.g. multifocality, larger size, non-papillary pattern of growth, greater reoccurrence rate and, most importantly, high-grade). There were no differences in urine between the groups. On the contrary, in serum the PDP concentration and PDP/CREA ratio was high in the risk group.

3.3. ROC analysis

Analysis of receiver operating characteristic curves revealed an optimal cut-off point of podoplanin in serum for diagnosis of TCC (cut-off point between healthy and TCC patients) see ROC Figs 2A, 2B in Table 5. Differentiation between non-muscle invasive (Ta-T1) and invasive stage (T2-T4) is shown in Figs 2C, 2D, as well as for non-muscle invasive high-risk group patients is shown in Figs 2E, 2F. The results are presented below.

Table 2
Diagnostic characteristic of serum podoplanin S-PDP, S-PDP/S-CREA concentration ratio compared with various clinocopathological parameters

Parameter	Concentration S-PDP [ng/ml]			Concentration S-PDP/S-CREA [ng/mg]		
	Range	Mean ± SD	p-value	Range	Mean ± SD	p-value
Primary/recurrent						
Primary (35)	2.18–11.41	6.45 ± 0.96	NS	149-1369	691 ±107	NS
Recurrent (47)	2.84–11.71	6.75 ± 0.59		149-1502	671 ± 83	
Multiplicity						
Single (46)	1.28–11.71	6.13 ± 0.66	< 0.01	149–1502	671 ± 80	NS
Multiply (36)	2.18–11.41	6.85 ± 0.90		149–1369	668 ± 98	
Stage						
Non-muscle invasive (51) (Ta + T1)	2.19–10.32	6.26 ± 0.63	< 0.01	149–1343	633 ± 81	< 0.01
Muscle invasive (31) (T2 + T3 = T4)	4.17–11.71	7.02 ± 0.91		267–1502	713 ±117	
Grade						
Low-grade (35)	1.28–8.94	5.23 ± 0.70	< 0.01	149–994	545 ± 78	< 0.01
High-grade (47)	4.17-11.71	7.35 ± 0.67		268–502	752 ± 89	
Size (mm)						
< 30 (49)	1.29–11.71	6.14 ± 0.64	< 0.01	149–1502	657 ± 88	< 0.05
> 30 (33)	2.20–11.40	6.57 ± 0.99		250–1369	713 ± 106	

S-PDP – serum podoplanin, S-CREA – serum creatinine, NS – no statistically significant values. Creatinine concentration was determined by Jaffe's method.

Table 3
Diagnostic characteristic of urine podoplanin U-PDP, U-PDP/U-CREA concentration ratio compared with various parameters of urotelial cancer

Parameter	Concentration U-PDP [ng/ml]			Concentration U-PDP/ U-CREA [ng/mg]		
	Range	Mean ± SD	p-value	Range	Mean ± SD	p-value
Primary/recurrent						
Primary (35)	0.11–1.15	0.63 ± 0.10	NS	0.06–2.33	0.73 ± 0.19	NS
Recurrent (47)	0.17–1.20	0.65 ± 0.08		0.09–3.03	0.77 ± 0.16	
Multiplicity						
Single (46)	0.17–1.20	0.65 ± 0.08	NS	0.06-2.15	0.68 ± 0.15	< 0.01
Multiply (36)	0.11–0.15	0.64 ± 0.11		0.12–3.03	0.83 ± 0.20	
Stage						
Non-muscle invasive (51) (Ta + T1)	0.11-1.20	0.64 ± 0.08	NS	0.06–2.33	0.71 ± 0.15	< 0.05
Muscle invasive (31) (T2 + T3 = T4)	0.23–1.14	0.64 ± 0.09		0.25–3.03	0.81 ± 0.21	
Grade						
Low-grade (35)	0.11–1.15	0.64 ± 0.10	NS	0.06–2.32	0.73 ± 0.20	< 0.01
High-grade (47)	0.17–1.20	0.64 ± 0.08		0.08–3.03	0.99 ± 0.16	
Size (mm)						
< 30 (49)	0.12–1.18	0.63 ± 0.08	NS	0.09-2.15	0.72 ± 0.14	NS
> 30 (33)	0.11–1.20	0.66 ± 0.11		0.06–3.03	0.75 ± 0.21	

U-PDP – urine podoplanin, U-CREA – urine creatinine, NS-not significant. Creatinine concentration was determined by Jaffe's method.

Table 4
Diagnostic characteristic of serum and urine podoplanin and podoplanin/ CREA ratio for patients with non-muscle invasive TCC with high and low risk for progression to muscle invasive stage

Risk group	U-PDP [ng/ml]			U-PDP/U-CREA [ng/mg]		
	Range	Mean ± SD	p-value	Range	Mean ± SD	p-value
Low risk group (41)	0.12–1.17	0.63 ± 0.20	NS	0.06–2.33	0.71 ± 0.18	NS
High risk group (10)	0.17–1.20	0.65 ± 0.09		0.09–1.54	0.69 ± 0.28	
		S-PDP [ng/ml]			S-PDP/S-CREA [ng/mg]	
Low risk group (41)	1.28–10.28	5.50 ± 0.71	< 0.01	149–1231	575 ± 87	< 0.01
High risk group (10)	5.05–11.24	7.83 ± 1.10		561–1343	868 ± 135	

U-PDP – urine podoplanin, U-CREA – urine creatinine, NS – not significant. Creatinine concentration was determined by Jaffe's method.

Diagnostic efficiency for S-PDP is quite satisfactory ranging from AUC = 0.82 to AUC = 0.89 and for U-PDP diagnostic efficiency is poor ranging from AUC = 0.51 to AUC = 0.66 (details in Table 5).

4. Discussion

The podoplanin protein proved to play a crucial role in the development and physiological function of many

Table 5
Diagnostic efficiency of podoplanin

	Sample	AUC (95%Cl)	Cut-off	Specificity %	Sensitivity %	PPV %	NPV %
ROC Fig. 2A	S-PDP	0.82 (0.71–0.92)	4.74	69.47	72.46	75.64	87.74
ROC Fig. 2B	U-PDP	0.66 (0.55–0.77)	0.42	72.41	43.66	79.61	34.26
ROC Fig. 2C	S-PDP	0.83 (0.73–0.92)	6.65	62.90	81.58	70.83	75.61
ROC Fig. 2D	U-PDP	0.51 (0.37–0.64)	0.47	73.57	78.72	72.55	80.65
ROC Fig. 2E	S-PDP	0.89 (0.69–0.94)	8.20	51.64	84.33	46.93	86.67
ROC Fig. 2F	U-PDP	0.54 (0.35–0.74)	0.42	72.00	74.56	70.73	80.00

ROC (Receiver Operating Characteristic); S-serum; U-urine; PDP-podoplanin; AUC (area under the curve); Cl-confidence limits; PPV-positive predictive value; NPV-negative predictive value.

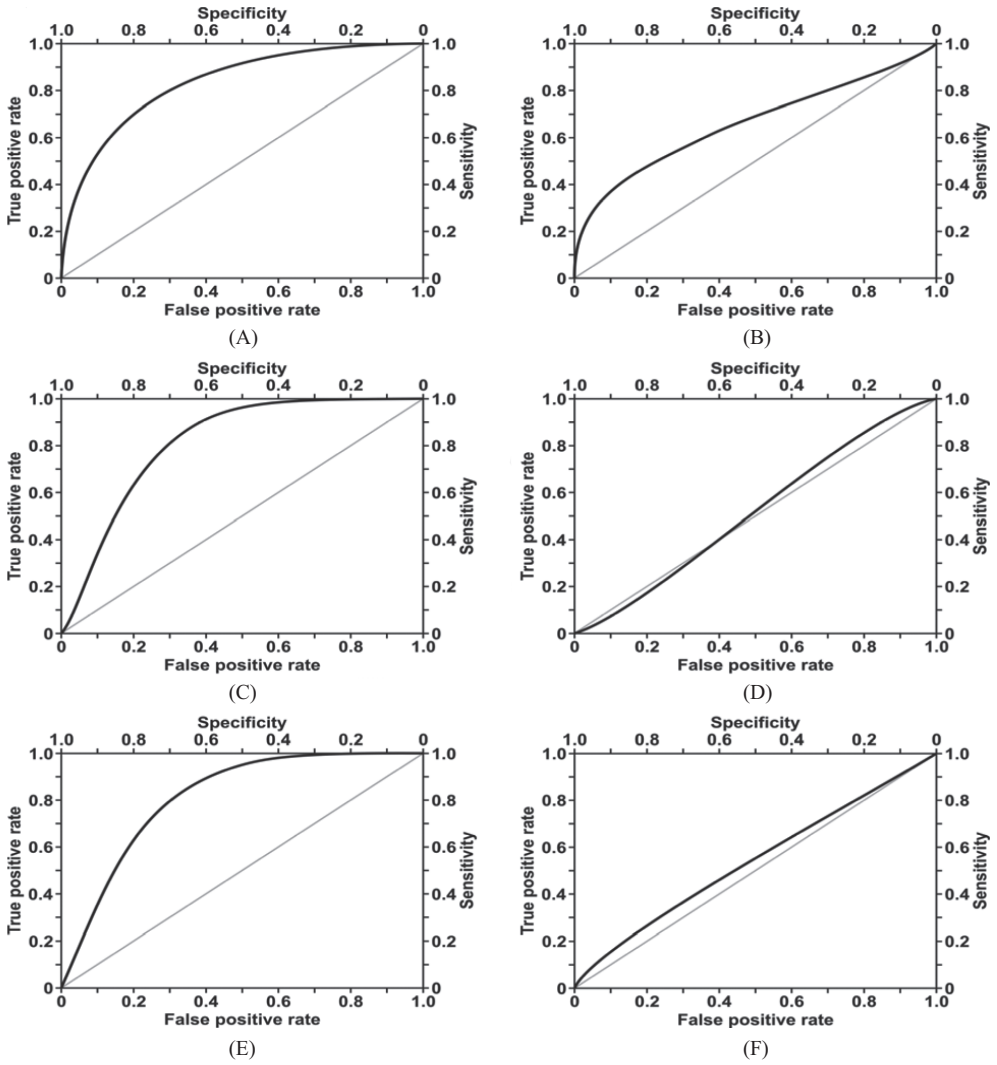

Fig. 2. Diagnostic efficiency of (A) S-PDP (B) U-PDP in detection of bladder TCC, (C) S-PDP (D) U-PDP in detection of bladder TCC invasive forms, (E) S-PDP (F) U-PDP in detection of patients with high-risk of the progression.

organs [6,28]. On the other hand it seems to participate in tumor invasion and metastasis [27]. Podoplanin expression in vessels is often used as a diagnostic marker [3].

This study tries to determine the relation between podoplanin and TCC of bladder cancer. In the literature there is only one paper concerning the role of podoplanin in TCC of bladder cancer [24]. Takagi et al.

showed the correlation between podoplanin expression and tendency to pulmonary metastasis.

In this studies, the high values of podoplanin (PDP) were observed in invasive, multifocal, more aggressive (high grade) ($p < 0.001$) and larger tumors ($p < 0.0095$). For more aggressive (high-grade) and invasive cases (T2-T4) the podoplanin greater concentration was confirmed by corresponding PDP/S-CREA ratio.

Podoplanin concentration was adjusted to creatinine to take into account alternation in renal function and their possible impact on final results. Patients with impaired glomerular filtration may have diminished elimination of podoplanin. Hence, was proposed to introduce the U-PDP ratio as additional parameter.

Raw U-podoplanin results show no significant differences between cancer samples and healthy subjects. However, U-podoplanin/U-creatinine ratio showed the positive correlation for more aggressive, invasive and multiply forms. It is noteworthy that this ratio helps to extract some more information, where podoplanin concentration between two groups is slightly different and not statistically significant.

The similar observation in terms of serum and urine concentration of another potential marker (Cathepsine B) was made by Kataska et al. [14] and Gorodkiewicz et al. [10].

In addition, the non-muscle invasive TCC group with high and low risk of progression are separated. S-podoplanin and S-podoplanin/creatinine ratio concentration were higher in the high risk group.

The study shows that concentration of podoplanin can be both utilized for early detection of bladder cancer and for determination of its invasive form and non-invasive form with the high risk of progression. The ROC curves were constructed in order to calculate cut off values. Diagnostic efficiency of S-podoplanin was fair, while U-podoplanin was poor. Clinically applicable cut-off values were determined at the maximum specificity and sensitivity. The S-podoplanin concentration 4.74 ng/ml is the threshold point for presence of TCC of bladder. The S-podoplanin value might be potentially useful for diagnosis patients with first time hematuria or even replace cystoscopy after TURBT, but further prospective studies should be conducted on a larger scale. Cut-off value 6.65 ng/ml suggests the presence of invasive form and imposes far more radical treatment. Interestingly, non-muscle invasive tumors with high risk of progression, were characterized by higher concentration of S-podoplanin (8.2 ng/ml) than nuscle-invasive form (6.65 ng/ml). It can be explained by its higher aggressiveness or presence of the occult invasion process. In this case, treatment decision should be advocated by clinicopathological findings and we propose to apply the lower cut off value (6.65 ng/ml).

The association between podoplanin concentration and clinicopathological features indicates that it might be useful in making therapeutic decisions. Further studies on larger groups should be performed to confirm these results.

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