



Utility of cystatin C as a potential bladder tumour biomarker confirmed by surface plasmon resonance technique

Anna Tokarzewicz¹, Tomasz Guszcz², Anna Onopiuk³, Robert Kozłowski² & Ewa Gorodkiewicz¹

¹Department of Electrochemistry, Faculty of Biology & Chemistry, Institute of Chemistry, University of Białystok, ²Department of Urology, J. Sniadecki Provincial Hospital of Białystok, Białystok & ³Department of Technique & Food Development, Division of Food Research & Development, Faculty of Human Nutrition & Consumer Sciences, Warsaw University of Life Sciences, Warsaw, Poland

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Background & objectives: The determination of cystatin C (cysC) may be helpful in diagnosis and monitoring of cancer because the pathogenesis of cancer is linked with an increased activity of cysteine peptidases (cathepsins) and a decrease of cysC concentration. This study was aimed to examine the utility of cysC as a marker of bladder cancer (BCa) to be used in the diagnosis.

Methods: This study was conducted with 90 patients with BCa and 27 healthy people. Patients with other cancers, inflammation process and impaired renal function were excluded from the study. The concentrations of cysC in the plasma and urine were measured by surface plasmon resonance imaging technique.

Results: The concentration of cysC in the serum taken from the patients with BCa [0.35 ± 0.02 $\mu\text{g/ml}$ (range: 0.20-0.78 $\mu\text{g/ml}$)] was significantly ($P < 0.001$) lower than the serum cysC concentration of the healthy people [0.68 ± 0.05 $\mu\text{g/ml}$ (range: 0.52-0.89 $\mu\text{g/ml}$)]. The urinary cysC concentration of the BCa patients [0.19 ± 0.01 $\mu\text{g/ml}$ (range: 0.09-0.34 $\mu\text{g/ml}$)] was not significantly different from the urinary cysC concentration of the healthy people [0.24 ± 0.02 $\mu\text{g/ml}$ (range: 0.16-0.33 $\mu\text{g/ml}$)]. Receiver operating characteristic (ROC) curve showed that BCa patients with cysC concentration < 0.54 $\mu\text{g/ml}$ [sensitivity: 87%; specificity: 92%; area under the curve (AUC) of ROC: 0.927; $P = 0.02$] could be optimally separated from healthy people. The ROC curve further showed that superficial low-grade patients with cysC concentration lower than 0.36 $\mu\text{g/ml}$ (sensitivity: 0.63%; specificity: 0.58%; AUC of ROC: 0.635; $P = 0.08$) could not be optimally separated from high-risk tumour patients.

Interpretation & conclusions: BCa patients have lower serum cysC concentration than the control group. Serum cysC may be considered as a potential marker of BCa but not its aggressiveness.

Key words Biomarkers - bladder cancer - cystatin C - surface plasmon resonance imaging

The human cystatin C (cysC) is a small molecule protein (13.3 kDa), which consists of 120 amino acids. Its concentration in body fluids does not depend

on gender, age or muscle mass. It only depends on the glomerular filtration rate, which makes it a good indicator of urinary tract function. CysC is also known

to inhibit and regulate cathepsins activity. Thus, it plays a protective role in inflammation and pathogenesis of cancers by acting as an inhibitor of cathepsins (cysteine proteases)¹. The reference value in human serum is 0.57-1.79 µg/ml and in urine 0.03-0.29 µg/ml^{1,2}.

Surface plasmon resonance imaging (SPRI), a label-free technique based on the resonance of plasmons, is used to measure the cysC concentration in the samples. The surface of the metal (gold) is coated with cysteamine and papain. Binding of the cysC with the papain causes changes in the resonance condition, which can be observed as changes in the reflectivity. This technique has already been validated and can be used to measure cysC concentration in biological fluids³.

Bladder cancer (BCa) is the most common urinary tract cancer⁴⁻⁶. The treatment of this cancer depends on the stage of the cancer at the time of diagnosis, aggressiveness and degree of differentiation⁷. The search for new BCa markers is important for early diagnosis and prognosis. Because cysC plays an important role in the pathogenesis of the cancer, it may serve as such a marker. This study was aimed to determine the utility of cysC as a potential BCa biomarker for diagnosis and determination of the aggressiveness of the disease.

Material & Methods

The study included 90 patients with histological confirmation of BCa. Consecutive patients who qualified for transurethral resection of bladder tumour or cystectomy, and were without other recognized tumours or renal insufficiency and who gave consent were included in the study.

Urine and serum samples were taken from patients with BCa during the hospitalization in Urology department of J. Sniadecki Provincial Hospital in Bialystok (Bialystok, Poland) for transurethral resection of the BCa or after cystectomy. The blood samples were collected from the median cubital vein. The blood and urine samples were also taken from 27 healthy people from the Blood Donor Centre of Bialystok, Poland. The age range of the study population was 19-45 years.

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 tumour and the presence of three of the four clinical parameters (size >30 mm, nonpapillary, multifocal, recurrent).

After collection, the serum and urine samples were kept at -70°C. Patients with the other cancer diseases, inflammation process and impaired renal function were excluded from the study. The urine samples were centrifuged at 1850×g for 15 min, and the supernatants were separated. Subsequently, the samples were once filtered through some medium density paper filters before analysis.

The study was approved by the Bioethics Committee of Medical University of Bialystok (R-I 002/409/2014, dated October 30, 2014). The study was conducted between October 2014 and May 2015. Written informed consent was obtained from each patient.

Procedure of cystatin C (cysC) determination: The cysC concentration in the serum and urine samples was measured by the SPRI biosensors (by the SPRI apparatus). The SPRI technique uses a very specific interaction between enzymes and their inhibitors, what enables determination of the tested proteins. All of the necessary steps in the preparation and optimization of the biosensor have been described elsewhere³. In this study, papain (Sigma, Steinheim, Germany) was used as an inhibitor and was immobilized on the biosensor surface. The serum samples taken from both the patients with BCa and the healthy people were two-fold diluted with phosphate-buffered saline (pH=7.4, BIOMED-LUBLIN, Poland). The volume of each sample used to the SPRI measurements was 2 µl. After 10 min of the interaction between cysC from the samples and the papain, the biosensor was washed with water and Hepes-buffered saline-ES buffer solution at 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] to remove other unbound molecules. To control the level of non-specific binding, some of the biosensor sites were covered with buffer. The SPRI signal was measured twice, before the interaction of the cysC with the papain and after this interaction, and was proportional to the amount of the cysC bound to the biosensor surface. The difference between the signals before and after the cysC -papain interaction was used to determine the cysC concentration in the sample. The concentration of serum and urinary cysC was calculated using a previously plotted calibration curve.

Statistical analysis: To compare the obtained data, Student's *t* test was used. The receiver operating

characteristic (ROC) curves were drawn using MedRoc software version 2.0 (StenStat, USA).

Results

Changes in cystatin C (cysC) concentration: Serum cysC concentration in the samples taken from patients with BCa [$0.35 \pm 0.02 \mu\text{g/ml}$ (range: 0.20-0.78 $\mu\text{g/ml}$)] was significantly ($P < 0.001$) lower than serum cysC concentration in the samples taken from healthy people [$0.68 \pm 0.05 \mu\text{g/ml}$, (range: 0.52-0.89 $\mu\text{g/ml}$)]. The urinary cysC level in the BCa patients was $0.19 \pm 0.01 \mu\text{g/ml}$ (range: 0.09-0.34 $\mu\text{g/ml}$), which was not significantly different from the urinary

cysC level in the healthy people [$0.24 \pm 0.02 \mu\text{g/ml}$ (range: 0.16-0.33 $\mu\text{g/ml}$)]. There was a difference between the urine cysC concentrations in the samples from BCa patients and healthy people, but these results are omitted in the further consideration.

Blood serum analysis: The following parameters were significantly related to a higher cysC concentration: Age <65 yr, male, non-muscle invasive, low-grade and fossil tumours (Table). This indicated that cysC could be used to distinguish healthy individuals from BCa patients by measuring the cysC concentration and comparing it with the listed parameters. On the other hand, the presence of inflammation, multiplicity and

Table. Diagnostic characteristic of serum cystatin C and cystatin C/mass of a protein ratio compared with various demographic and clinical parameters of bladder cancer

Parameter	CysC ($\mu\text{g/ml}$)		P	CysC/mass of protein ($\mu\text{g/mg}$)		P
	Average	Range		Average	Range	
Age (yr)						
<65 (n=42)	0.37	0.23-0.78	<0.03	0.0052	0.0032-0.0109	<0.01
≥ 65 (n=48)	0.34	0.19-0.61		0.0047	0.0026-0.0085	
Gender						
Male (n=59)	0.37	0.23-0.78	<0.01	0.0052	0.0033-0.0109	<0.001
Female (n=31)	0.32	0.23-0.70		0.0045	0.0023-0.0098	
Stage						
Non-muscle invasive (Ta + T1) (n=54)	0.36	0.25-0.65	<0.05	0.0050	0.0035-0.0091	NS
Muscle invasive (T2 + T3 + T4) (n=36)	0.33	0.23-0.42		0.0047	0.0033-0.0059	
Primary/recurrent						
Primary (n=44)	0.35	0.23-0.59	NS	0.0049	0.0033-0.0083	NS
Recurrent (n=46)	0.35	0.24-0.50		0.0049	0.0034-0.0070	
Multiplicity						
Single (n=54)	0.35	0.24-0.54	NS	0.0049	0.0034-0.0076	NS
Multiple (n=36)	0.35	0.23-0.50		0.0048	0.0032-0.0070	
Size (mm)						
<30 (n=53)	0.37	0.25-0.59	NS	0.0051	0.0035-0.0083	NS
≥ 30 (n=37)	0.34	0.23-0.47		0.0048	0.0033-0.0066	
Morphology						
Fossil (n=42)	0.38	0.25-0.61	<0.001	0.0053	0.0035-0.0085	<0.001
Papilloma (n=48)	0.33	0.23-0.50		0.0045	0.0032-0.0070	
Grade						
Low grade (n=38)	0.38	0.25-0.61	<0.01	0.0052	0.0035-0.0085	NS
High grade (n=52)	0.34	0.23-0.50		0.0048	0.0032-0.0070	
Inflammation						
Not present (n=48)	0.35	0.19-0.65	NS	0.0049	0.0026-0.0091	NS
Present (n=42)	0.37	0.16-0.78		0.0051	0.0023-0.0109	

CysC, cystatin C; NS, not significant; T1-4, tumour node metastasis (TNM) stages 1-4

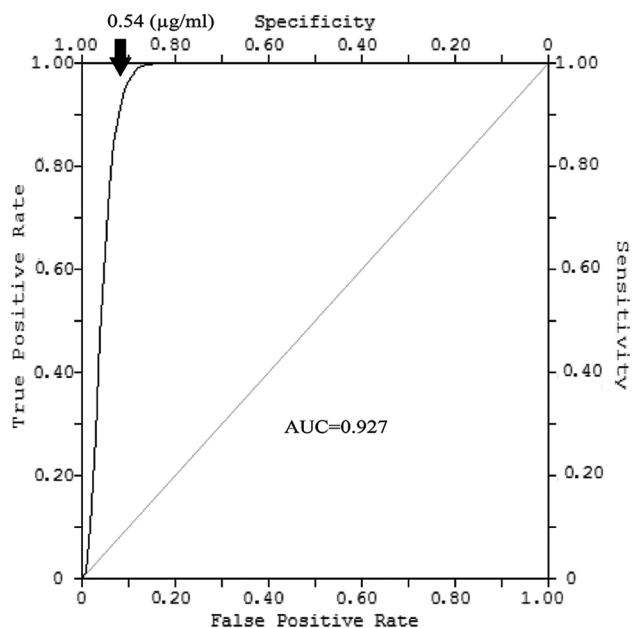


Fig. 1. Diagnostic efficiency of serum cystatin C concentration in detection of bladder cancer determined by receiver operating characteristic curve.

recurrence rate were not significant. For this reason, it was not possible to diagnose BCa using cysC concentration alone.

Receiver operating characteristic (ROC) analysis: To determine the diagnostic utility of cysC in BCa, the ROC curve was plotted (Fig. 1). The area under the curve (AUC) was 0.927 ($P=0.02$). The optimum cut-off was 0.54 $\mu\text{g/ml}$, which indicated that people with serum cysC concentration lower than that level might have BCa (sensitivity, 87%; specificity, 92%). From the ROC curve (Fig. 2), it was not possible to separate the patients with an aggressive form of BCa from those with non-aggressive BCa (sensitivity, 0.63%; specificity, 0.58%; AUC of ROC: 0.635; $P=0.08$).

Discussion

The search for new malignancy markers is important in clinical oncology. CysC is a cysteine protease inhibitor, which plays an important role in the pathogenesis of cancer. This study was designed to explore the utility of the serum cysC level as a biomarker of BCa.

The level of serum cysC in the samples from BCa patients was found to be lower than in healthy individuals. This indicates that cysC may be a potential tumour marker for urinary BCa. CysC expression was

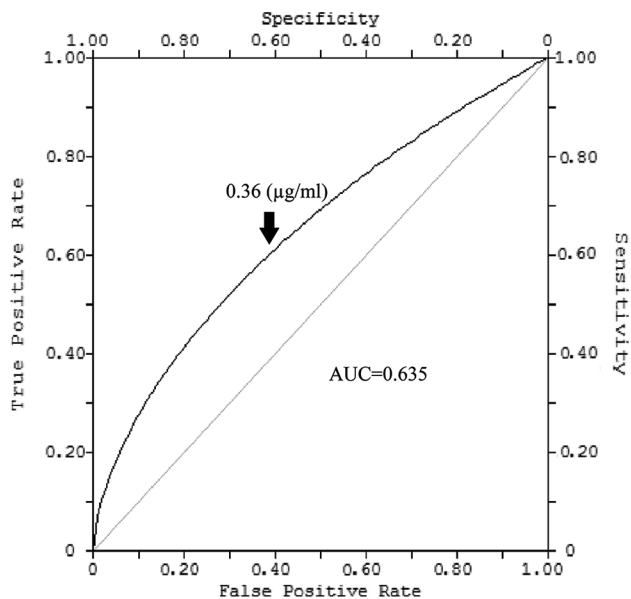


Fig. 2. Diagnostic efficiency of serum cystatin C concentration in prediction of superficial transitional cell carcinomas aggressiveness determined by receiver operating characteristic curve.

found to be declined in glioblastoma⁸, breast cancer⁹, colorectal cancer¹⁰ and adenocarcinoma of prostate, where this was correlated with poorer diagnosis¹¹. The results obtained in this study were in agreement with the studies which indicated that probably a low level of cysC contributed to the pathogenesis of urinary BCa. However, the role of cysC in pathogenesis of cancer has remained controversial as the patients with non-Hodgkin B cell lymphoma¹², multiple myeloma¹³ and liver carcinoma¹⁴ tend to have higher cysC concentration than healthy individuals. Moreover, higher cysC concentration in colorectal¹⁵, lung¹⁶ and melanoma carcinomas¹⁷ was related with the poor outcome.

The ROC curve analysis showed that a cut-off value of 0.54 $\mu\text{g/ml}$ could be used to differentiate BCa patients from a healthy person. The patients with cysC concentration higher than 0.54 ($\mu\text{g/ml}$) can be regarded as the healthy.

In conclusion, our results showed lower level of cysC in patients with BCa compared to healthy individuals. The results obtained by surface plasmon resonance technique need to be validated by a conventionally acceptable method. The findings need to be confirmed in a larger sample.

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Conflicts of Interest: None.

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For correspondence: Ms Anna Tokarzewicz, Department of Electrochemistry, Faculty of Biology & Chemistry, Institute of Chemistry, University of Białystok, Ciołkowskiego 1K, 15-245, Poland
e-mail: anna.tokarzewicz@gmail.com