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Application of surface plasmon resonance imaging biosensors for determination of fibronectin, laminin-5 and type IV collagen in serum of transitional bladder cancer patients

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ABSTRACT

The urothelial basement membrane (UBM) contains type IV collagen, laminin-5, and fibronectin. Urothelial neoplasm can break through the UBM underlying the urothelium to invade the lamina propria. Conceptually, all bladder cancer staging over Ta (T1–T4) may demonstrate disturbances in the UBM structure, as well as alterations in the serum concentrations of the studied components. The aim of this study was to determine the blood serum concentration of collagen IV, laminin-5 and fibronectin in bladder cancer patients. Quantification of their concentrations and correlation with various clinicopathological parameters may be useful for making more accurate predictions and identifying high-risk patients. The study included 96 patients with bladder cancer confirmed by transurethral resection or cystectomy and 26 patients with diagnosed cystitis chronica or BPH (benign prostate hyperplasia). Collagen IV, laminin-5 and fibronectin were detected using Surface Plasmon Resonance Imaging biosensors. Significant differences in blood serum concentrations of the studied biomarkers were observed between the control group and bladder cancer patients, as well as between nonmuscle-invasive and muscle-invasive groups. ROC analysis gave satisfactory results for differentiation between the control group and bladder cancer group (AUC 0.92–0.99), with lower values only for collagen IV between nonmuscle-invasive and muscle-invasive patients (AUC 0.71), and a statistically insignificant difference for laminin-5. Laminin-5 concentration was more closely correlated to tumour grade, size and recurrence rate; fibronectin to tumour stage, size and morphology; and collagen IV to tumour stage, grade and recurrence rate. The relations between serum concentrations of the presented biomarkers of the urothelial basement membrane may be useful for bladder cancer detection and for determination of the tumour stage, hence simplifying the making of therapeutic decisions.

1. Introduction

Urinary bladder cancer (BCa) is the tenth most common cancer in the world. There are an estimated 549,393 diagnosed cases and 200,000 deaths annually. It is the sixth most common malignancy in males and the seventeenth in females with age; the standardized risks are 9.6 % and 2.4 %, respectively [1].

The disease can present as nonmuscle-invasive bladder cancer (NMIBC), muscle-invasive bladder cancer (MIBC), and the metastatic form of the disease. Tobacco smoking is the most important risk factor for BCa, responsible for approximately half of BCa cases and associated with poor oncological outcomes for both NMIBC and MIBC [2]. Quitting smoking reduces the risk of developing BCa by almost 40 % within 5 years [3]. Bladder cancer remains a highly prevalent and lethal malignancy. The optimal selection of treatment depends on early diagnosis and accurate staging and grading.

Biomarkers are critical in routine clinical practice. They are indicators for the detection of bladder carcinoma and for the prediction of its recurrence and progression. The literature describes a number of bladder cancer markers [4].

The urothelial basement membrane (UBM) contains collagen IV, laminin, and fibronectin. Integrins are found in or near the UBM in the extracellular matrix (ECM) [5]. The objects of particular interest in this study are laminin-5, collagen IV, and fibronectin.

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Table 1a
Clinical characteristic of patients.

Group	Control group	BPH (benign prostate hyperplasia)	Cystitis chronica	Bladder cancer	NMIC (nonmuscle invasive)	MI (muscle invasive)
Number of patients	***26***	***11***	***15***	***92***	***59***	***33***
Average age	67 (36–86)	71 (55–84)	64 (36–86)	70 (36–96)	69 (36–96)	71 (41–91)
Sex	F-13 M-13	F-0 M-11	F-5 M-10	F-25 M-67	F-20 M-39	F-5 M-28

F- female, M- men

Table 1b
Demographic and clinicopatological characteristic of patients.

Variable	Range	Number of patients
Age	< 65	33
	> 65	59
Gender	Women	25
	Men	67
Tumor stage	Non-muscle invasive	33
	Muscle invasive	59
Tumor grade	Low grade	55
	High grade	37
Tumor size	< 30	53
	> 30	3
Recurrence	Primary	35
	Recurrent	47
Multiplicity	Single	46
	Multiply	36

Table 2
Summary of the kinetic characteristics of the tested molecular interaction in biological system of biosensors.

Ligand–Antibody system	**K_A** [dm3/mol]	**K_D** [mol/dm3]
Fibronectin – antibody	$1.22{\cdot}10^{8}$	$8.20{\cdot}10^{-9}$
Laminin-5 – antibody	$1.04{\cdot}10^{7}$	$9.65{\cdot}10^{-8}$
Collagen IV - antibody	$3.58{\cdot}10^{8}$	$2.80{\cdot}10^{-9}$

The obtained values of the kinetic constants prove the long stability of the molecular complexes, which form the basis of the good operation of biosensors.

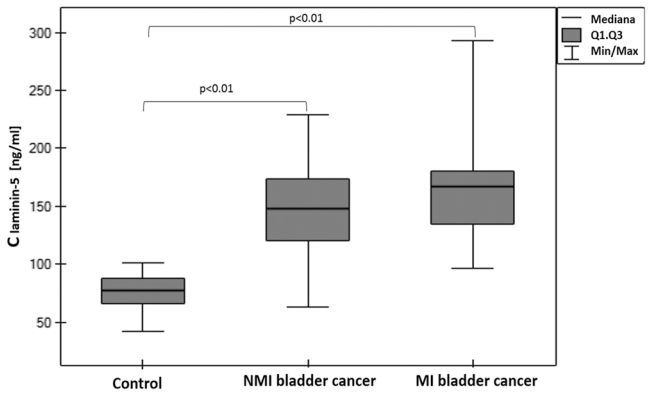

Fig. 1. Concentration of laminin-5 in serum of bladder cancer patients and the control group.

Urothelial neoplasms can break through the UBM underlying the urothelium to invade the lamina propria [6]. Conceptually, all bladder cancer staging over Ta (T1–T4) may demonstrate disturbances in the UBM structure, as well as alterations of the serum concentrations of the studied components.

Tumorigenesis leads to dynamic interactions between tumour cells and mesenchymal stroma. Numerous enzymes, including collagenases, elastases, cathepsins and metalloproteinases, form a cascade system facilitating EMC breakdown.

Previous studies have demonstrated the possible significance of

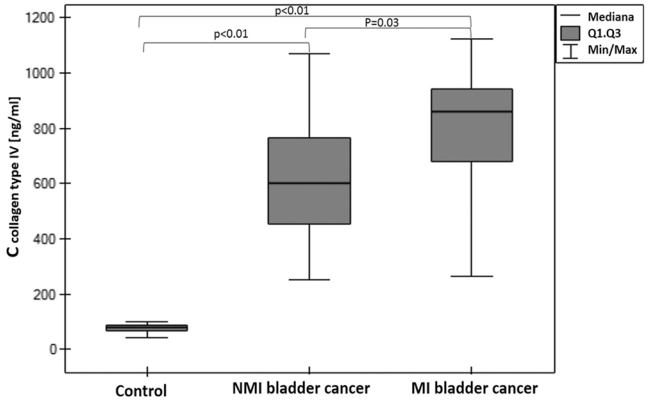

Fig. 2. Concentration of collagen IV in serum of bladder cancer patients and the control group.

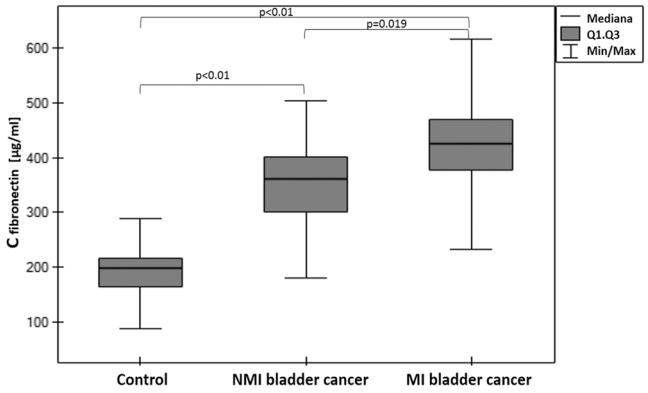

Fig. 3. Concentration of fibronectin in serum of bladder cancer patients and the control group.

laminin-5 [7], fibronectin [8] and collagen IV [9] as bladder cancer biomarkers. Plasma fibronectin is a large dimeric structural glycoprotein that has two biological types termed plasma and cellular fibronectin. Plasma fibronectin is synthesized by hepatocytes and released into circulation, while cellular fibronectin constitutes the extracellular matrix and is found in all kinds of tissues [10]. Laminin-5, which consists of alpha 2, beta 3 and gamma 2 chains, is a major component of transitional and stratified squamous epithelia, lung mucosa and other epithelial glands [11]. Type IV collagen is a major structural protein of basement membranes and is chemically and genetically distinct from stromal collagens (types I and III) and cartilage collagen (type II). Type I, II and III collagenases fail to degrade type IV collagen [12].

The most common methods used for laminin-5, fibronectin or collagen IV detection are enzyme-linked immunosorbent assay (ELISA) [13,14], immunohistochemistry [9,15] or chemiluminescence immunoassay (CLIA) [7]. However, these techniques are time-consuming and labour-intensive and require labels or the addition of another reagent.

Biosensors are devices with great potential to change patients' lives. Currently, surface plasmon resonance (SPR) biosensors are gaining popularity. SPR is an optical nondestructive method that can detect even

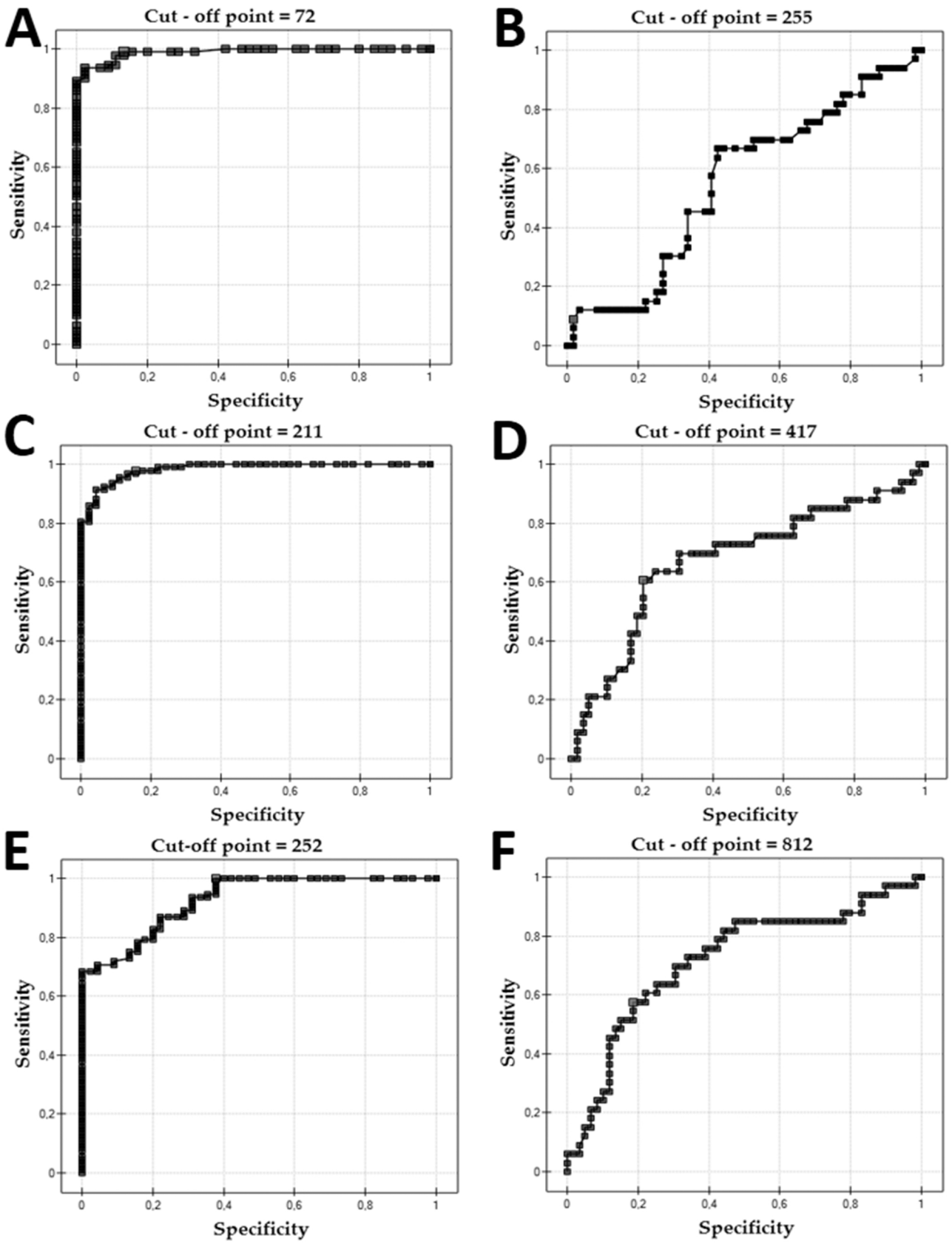

Fig. 4. ROC curves for potential markers in serum as diagnostic tests: (A) laminin-5, control vs. bladder cancer; (B) laminin-5, MI vs. NMI bladder cancer; (C) fibronectin, control vs. bladder cancer; (D) fibronectin, MI vs. NMI bladder cancer; (E) collagen IV, control vs. bladder cancer; (F) collagen IV, MI vs. NMI bladder cancer.

Table 3
Diagnostic efficiency of laminin-5, fibronectin and collagen IV.

Fig	*AUC*	*p*	*Sensitivity*	*Specificity*	*PPV*	*NPV*	*Cut-off*
4A	0.99	< 0.01	98	92	97	96	72
4B	0.55	0.41	9	98	75	65	255
4 C	0.98	< 0.01	97	84	92	95	211
4D	0.67	0.004	60	79	62	78	417
4E	0.92	< 0.01	100	62	84	100	252
4 F	0.71	0.0005	57	81	63	77	812

low concentrations of disease markers. One version of the SPR technique is the imaging version (surface plasmon resonance imaging (SPRI)). This technique can be applied in a stationary, nonfluidic system with multi-array measuring points. SPRI biosensors based on selective antibody–antigen or inhibitor–enzyme interactions have shown promise as potential tools for clinical application.

SPRI has been successfully used for the determination of fibronectin [16], collagen IV [17,18], and laminin-5 [19,20]. However, to date, these components of the extracellular matrix have not been determined simultaneously.

The aim of this study was to assess serum concentrations of laminin-5, collagen IV, and fibronectin in a control group and in bladder cancer patients, the latter being divided into nonmuscle-invasive and muscle-invasive subgroups.

2. Materials and methods

2.1. Biological materials

Samples were obtained from patients with bladder cancer (transitional cell cancer (TCC)) who were under observation at the J. Sniadecki Provincial Hospital of Bialystok (Bialystok, Poland). The subjects were divided into two groups: malignant and control. Serum samples were obtained from patients diagnosed with bladder cancer (initial cystoscopy or computer tomography). The cancer diagnosis was confirmed by histological examination of tumour specimens after transurethral resection or cystectomy. The control group comprised patients with diagnosed benign prostate hyperplasia (BPH diagnosed on USG) or chronic cystitis (persistent bladder inflammation diagnosed based on histopathological examination). Patients' clinical characteristics are presented in Table 1a.1b.

Approval for the study (R-I-002/409/2014) was obtained from the Bioethics Committee of the Medical University of Bialystok (Bialystok, Poland), and written informed consent was obtained from all patients.

2.2. Preparation of samples

Blood samples were obtained from the median cubical vein. The plasma was prepared according to standard protocols [21]. Plasma samples were frozen immediately and maintained at − 70 °C until analysis. Immediately before analysis, the samples were thawed and diluted with PBS buffer to keep the analyte concentration within the linear range of the calibration curve.

2.3. Determination of concentration with SPRI biosensors

The concentrations of fibronectin, collagen IV and laminin-5 were determined using SPRI biosensors. All of the necessary steps in the preparation and optimization of the biosensor have been described in publications by Sankiewicz et al. [16,17,19].

2.3.1. Preparation of biosensors

First, the base of the biosensor was prepared. For that, a chrome layer was sprayed on the glass chip and then covered with a gold layer. Next, the gold layer was covered with photopolymer and hydrophobic paint to separate the active reaction sites. The prepared chip, after being washed with ethanol and water and dried under a stream of argon, was immersed in 20 mM cysteamine ethanolic solution for 24 h. After this time, the chip was again rinsed with ethanol and water and dried under a stream of argon.

The next step was the immobilization of a specific antibody as the biosensor receptor. Depending on the type of analysed biomolecule, an appropriate antibody was used. The antibodies used were monoclonal rabbit-specific anti-fibronectin antibody (Sigma, Germany), monoclonal mouse anti-human collagen type IV (Tebubio, France), and rabbit polyclonal antibody specific to human laminin-5 (Abcam, USA). All biosensors were prepared in the same way. First, the antibody solutions were activated by adding a mixture of NHS (250 mM, N-

Table 4
Diagnostic characteristic of serum concentration values in relation to clinicipathological parameters.

Parameter	Laminin-5 [ng/ml]		Fibronectin [μg/ml]		Collagen IV [ng/ml]	
	Median (range)	p-value	Median (range)	p-value	Median (range)	p-value
Age (Year)						
< 65	139 (63–293)	ns	375 (179–533)	ns	713 (252–1086)	ns
> 65	160 (72–338)		371 (192–627)		688 (266–1123)	
Gender						
Female	160 (63–338)	ns	388 (217–627)	ns	713 (252–1086)	ns
Male	156 (72–269)		371 (179–614)		688 (266–1123)	
Tumor stage						
NMIT	149 (63–338)	ns	363 (179–627)	0.004	602 (268–1070)	0.0005
MIT	164 (72–293)		422 (192–617)		860 (252–1123)	
Tumor grade						
Low grade	124 (75–214)	0.008	350 (179–627)	ns	605 (252–1063)	0.007
High grade	162 (63–338)		378 9211–617)		788 (286–1123)	
Tumor size (mm)						
< 30	139 (63–247)	0.02	359 (192–614)	0.002	648 (252–1086)	ns
> 30	170 (75–338)		422 (179–627)		748 (266–1123)	
Recurrence						
Primary	169 (72–293)	0.03	411 (179–617)	ns	817 (266–1123)	0.005
Recurrent	139 963–338)		373 (225–627)		620 (252–1070)	
Morphology						
Papillary	149 (63–229)	ns	353 (277–502)	0.0001	646 (252–1086)	ns
Fossile	157 (75–338)		423 (179–627)		740 (298–1123)	
Multiplicity						
Single	157 (72–269)	ns	378 (179–617)	ns	670 (268–1063)	ns
Multiply	157 (63–338)		381 (226–627)		732 (252–1123)	

hydroxysuccinimide, Aldrich, Germany) and EDC (250 mM, N-ethyl-N0-(3-dimethylaminopropyl) carbodiimide, Aldrich, Germany) in a carbonate buffer solution (pH 8.5). Three microlitres of activated antibody were placed on the cysteamine-modified surface sites and incubated at 37 °C for 1 h. After this time, the chip was rinsed with water and dried. The prepared biosensor was used to measure the concentration of fibronectin, laminin-5 or collagen IV.

2.3.2. SPRI measurements

SPRI measurements were performed using the apparatus built by our laboratory described in a previous paper [16]. Biosensors were used for the measurements described in a previous chapter. The measurement was carried out in several stages. The prepared biosensor with a receptor layer was placed on a prism and exposed to laser light, and the first SPRI signal measurement was performed.

To eliminate nonspecific adsorption, Bovine Serum Albumin (BSA) solution with a concentration of 1 ng/ml was applied to the biosensor's active sites with the receptor, and it was washed again several times with redistilled water. Next, plasma sample solutions were placed directly on the prepared biosensor. The time of interaction with the antibody was a maximum of 10 min. Finally, the biosensor was washed with water and HBS-ES buffer solution, pH = 7.4 (0.01 M 4-(2-hydroxyethyl)piperazine-1-ethane sulfonic acid, 0.15 M sodium chloride, 0.005 % Tween 20, 3 mM EDTA; BIOMED, Lublin, Poland) to remove unbound molecules from the surface, and the second measurement of the SPRI signal was performed. The SPRI signal was measured at a fixed SPR angle on the basis of recorded images by a CCD camera. NIH Image J version 1.32 software was used to evaluate the SPRI images in 2D form and to convert the numerical signal to a quantitative signal. The SPRI signal was obtained by subtraction of the signal after and before interaction with the analyte. Nine serum samples were analysed simultaneously, and 12 measurements were made for each of them. This allows more accurate measurement of the concentration of biomolecules in the serum. Fibronectin, collagen IV and laminin-5 concentrations in the samples were read from calibration curves prepared with the use of standard solutions (human fibronectin and collagen type IV, Sigma, Germany; human protein laminin-5, Abcam, USA). Calibration curves were prepared in the following ranges: 5–400 ng/ml for fibronectin, 0.01–0.25 ng/ml for laminin-5, and 10–300 ng/ml for collagen IV. Analytical parameters of the methods for the determination of fibronectin, laminin 5 and collagen IV were determined and described in previous works. Biosensors were characterized by good selectivity, recovery and precision. The LOQ values for all three biomarkers were 8 ng/ml for kollagen IV, 14 pg/ml for laminin-5 and 5 ng/ml for fibronectin. The LOQ values for all three biomarkers were 8 ng/ml for kollagen IV, 14 pg/ml for laminin-5 and 5 ng/ml for fibronectin. The validation of the methods was performed by comparing fibronectin, laminin-5 and kollagen IV concentrations obtained by the SPRI biosensor with results obtained by commercial ELISA kits. Comparison between the results of both techniques showed good agreement (r = 0.979–0.996).

2.4. Statistical analysis

The results are presented as medians and standard deviations. The Kolmogorov–Smirnov test was performed to test the assumption of a normal distribution. The statistical analyses were performed using the unpaired t-test and ANOVA for independent variables (for normally distributed variables) or the Mann—Whitney U test and ANOVA Kruskal–Wallis test (for variables that failed the normality test). The p-value was automatically calculated, and $p < 0.05$ was considered to indicate a statistically significant difference. The receiver operating characteristic curves with optimal cut-off points were calculated. For the cut-off points, sensitivity, specificity, and positive and negative predictive values were calculated.

2.5. Kinetic studies of the molecular interaction in biological systems of biosensors with the use of QCM

Quartz crystal microbalance (QCM) was used to better characterize the interactions that take place in the biosensors. Based on the frequency changes, the dissociation (K_D) and association constants (K_A) of the forming molecular complexes on the surface of the biosensor were determined.

The QCM investigation was carried out using a quartz crystal microbalance coupled with a PGSTAT 302 N potentiostat/galvanostat (Methrom Autolab B.V.). The crystal placed in the measuring cell (3 ml) had a resonance frequency of 6 MHz and an area of 0.361 cm2. The gold layer was 100 nm thick. QCM analysis was supported by dedicated NOVA 2.1 software.

The results obtained for the kinetic characteristics of the studied ligand–antibody system, obtained by means of QCM, are summarized in the table below (Table 2).

3. Results

3.1. Changes in serum concentration

A significant difference in the serum concentration of the analysed parameters was observed for the control group and bladder cancer patients. The second cohort was divided into two subgroups: nonmuscle-invasive and muscle-invasive. In the case of fibronectin and collagen IV, statistically significant differences were observed between all cohorts (Figs. 1, 2, 3), but for laminin-5, there was no difference in serum concentration between NMI and MI patients.

3.2. ROC analysis

Analysis of the receiver operating characteristic curves revealed optimal cut-off points of the serum concentrations of the studied substances for the diagnosis of bladder cancer (cut-off points between healthy and TCC patients and between nonmuscle-invasive and muscle-invasive groups). For the ROC for laminin-5, see Fig. 4A, 4B; for fibronectin, see Fig. 4C, 4D; for collagen, IV see Fig. 4E, 4F.

The efficiency of the studied substances was quite satisfactory for differentiation between the control group and bladder cancer group (AUC 0.92–0.99) and for collagen IV only between nonmuscle-invasive and muscle-invasive patients (AUC 0.71) (Table 3).

3.3. Changes in serum concentration

Table 4 shows the serum concentrations of the studied substances in juxtaposition with clinicopathological characteristics. High values of laminin-5 were obtained for high-grade, larger and primary tumours. The fibronectin concentration was significantly elevated in muscle-invasive, larger and fissile tumours. Collagen IV was higher in muscle-invasive, high-grade, recurrent lesions.

4. Discussion

Research into new malignancy markers is crucial for the development of clinical oncology. Although urine cytology and cystoscopy are used to diagnose bladder cancer, there are no reliable markers for its screening and for the detection of muscle invasion, which can be valuable for the making of therapeutic decisions.

In this study, we concentrated on the urothelial basement membrane (UBM) and extracellular matrix components, namely, laminin-5, type IV collagen and fibronectin, in bladder cancer patients. To the best of our knowledge, the plasma concentrations of the aforementioned substances have not previously been evaluated together in a single study. A further innovation is the application of SPRI.

To date, this technique has been used to determine the level of the

abovementioned biomolecules in only a few studies. Similar concentration ranges were obtained. The concentration of fibronectin ranged from 101.4 to 208.0 μg/ml for healthy donors and 473.0–865.5 μg/ml for patients after injury [16]; 300–438 ng/ml of collagen IV in patients after burns [20]; and 74.743–104.379 ng/ml of laminin-5 after burns [20] and 49–89 ng/ml in boys with cryptorchidism [18].

Some previous studies have reported changes in the concentrations of these substances in bladder cancer. The fibronectin urine concentration has been a subject of interest in numerous previous studies [14,22,23]. Plasma fibronectin concentration (FPc) was assessed in bladder cancer by Hegele et al. [24] and in gastrointestinal and neck cancer by Warawdekar et al. [25]. Tissue levels of laminin P1 and fibronectin were presented by Kirkali [26] and Di Maida [27].

Hegele's results showed higher plasma concentrations of fibronectin than those found in the present study: for the control group, a mean of 404 μg/ml (range 181–746), compared with our result (median) of 198 μg/ml (range 87–288); for nonmuscle-invasive cancer, 463 μg/ml vs. 362 μg/ml; and for muscle-invasive cancer, 944 μg/ml vs. 426 μg/ml. Elevated fibronectin concentration was dependent on progression of the tumour and on clinicopathological parameters (large, fissile and muscle-invasive).

Laminin-5 has been shown to increase the motility and invasiveness of bladder cancer and to be correlated with the aggressiveness of the tumour [28]. Increased laminin-5 expression and synthesis have been reported in bladder cancer [29]. Tumour motility is increased due to the influence on cell receptors. Laminin-5 stimulates integrin signalling, which activates the guanosine triphosphate (GTP) binding protein Rho and leads to cytoskeletal reorganization [30]. The utility of laminin-5 in urine for bladder cancer diagnosis (particularly < pT1) was proven in a previous study (ROC curve analysis: AUC 0.86) [7]. Interestingly, our results similarly demonstrated that laminin was not related to the muscle-invasive stage but was correlated with size, higher aggressiveness, and primary tumours. It should be noted that a majority of newly diagnosed lesions are larger and more invasive.

Several previous reports have demonstrated that collagen IV plays an important role in tumour angiogenesis and progression [30]. Collagen IV produced by tumour cells activates the intracellular AKT signalling pathway, which leads to an E/N-cadherin switch. Its overexpression is associated with a high risk of tumour progression and poor prognosis [31]. The urine concentration of collagen IV was reported to be elevated in patients with bladder tumours, with higher urine concentrations associated with greater progression [32]. We have not found any reports in PUBMED on collagen IV concentration in cases of bladder tumours. In our study, we demonstrated that collagen IV was significantly higher in bladder tumour patients.

Prior to invasion and metastasis, tumour cells must penetrate various host connective tissue barriers. Previous studies have reported that malignant epithelial cells develop elastinolytic activity [33]. Elastase degrades not only elastin but also complex molecules such as type IV collagen, fibronectin and proteoglycans [34]. The higher plasma concentration of the studied substances may result from alterations in ECM caused by lysis (in the course of tumour proliferation and invasion), increased accumulation of matrix components by host cells in response to the local presence of the tumour, and synthesis of matrix components by tumour cells [35].

We may speculate, based on our results, that the higher plasma concentrations of fibronectin and collagen IV result from a more intensive process of tumorigenesis, as reflected in the tumour characteristics (higher stage, aggressiveness, size). Laminin-5 levels in serum seem to increase at an earlier stage of tumour growth, where the lesion is massive and aggressive, with a tendency to recur, but not muscle-invasive.

The proposed SPRI biosensor method may be an alternative to the standard methods for the diagnosis of bladder cancer. This is a relatively quick, label-free, inexpensive technique. Biosensor SPRI can be applied in a stationary, nonfluidic system with multiarray measuring points. This enables the determination of many biomarkers without signal enhancement or the preliminary preconcentration of analyte, which is an advantage of this method over the other techniques used.

5. Conclusion

This study has demonstrated that serum levels of laminin-5, fibronectin and collagen IV are significantly elevated in TCC of the human bladder. Surprisingly, high sensitivity (98–100 %) and satisfactory specificity (62–92 %) were obtained for bladder cancer detection.

Further studies, including urine levels, should be performed to analyse the value of these substances as tumour markers in a larger group of patients.

The combination of the SPRI technique with biosensors proved to be a rapid, high-throughput and valuable tool for the detection of fibronectin, laminin-5 and collagen-IV in samples from patients with bladder cancer.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

not applicable.

Author contributions

Conceptualisation, T.G., A.S., and E.G.; methodology, A.S. and E.G.; data collection, A.S. and T.G.; statistical analysis T.G and A.S.; data interpretation, T.G, A.S., and E.G.; writing—original draft preparation, A.S. and T.G.; writing—review and editing, E.G; visualisation, T.G. and A.S.; supervision, E.G. All authors have read and agreed to the published.

Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Bioethics Committee at the Medical University of Bialystok, nr R-I-002/409/2014.

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

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