Technical note

Detection of bladder cancer with aberrantly fucosylated ITGA3

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ABSTRACT

We describe a simple, non-invasive assay to identify fucosylated-glycoisoform of integrin alpha-3 (ITGA3) directly from unprocessed urine. ITGA3 was detected directly from the urine of bladder cancer (BlCa) (n = 13) and benign prostatic hyperplasia (BPH) (n = 9) patients with the use of lectins coated on europium-doped-nanoparticles (Eu^{3+}-NPs). Lectin *Ulex europaeus agglutinin-I* (UEA) showed enhanced binding with BlCa-derived ITGA3. The evaluation with individual samples showed that a glycovariant ITGA3-UEA assay could significantly discriminate BlCa from BPH patients ($p = 0.007$). The detection of aberrantly fucosylated-isoform of ITGA3 from urine can be used to distinguish BlCa from age-matched benign controls in a simple sandwich assay.

1. Introduction

Bladder cancer (BlCa) is the second most occurring urological malignancy after prostate cancer. Currently, the diagnosis and surveillance of bladder cancer is dependent on voided urine cytology and direct cystoscopic examination of the bladder [1]. Even though cystoscopy and cytology are considered as the golden standard investigation, these tests have limitations in either sensitivity or specificity [2,3]. Moreover, numerous commercially available FDA-approved biomarkers and kits for the detection of BlCa have been developed. These include assays based on the detection of bladder tumor antigen (BTA) and nuclear matrix protein 22 (NMP22). Also, the ImmunoCyt + test, relying on the detection of mucins and carcinoembryonic antigen (CEA). Unfortunately, none of them have been accepted for the diagnosis or follow-up of BlCa in routine practice due to the limited sensitivities or specificities [4]. Thus, there is an urgent need for the development of a non-invasive, sensitive, and specific diagnostic tool that could be used for the detection and follow-up of bladder cancer.

Integrins are heterodimeric transmembrane proteins consisting of an alpha (α) and a beta (β) chain. In mammals, integrins have twenty-four α and nine β subunits. Several types of integrins or their parts have also been found on the surface of cells and extracellular vesicles [5,6] and as soluble proteins which can be detected from urine [7]. Some integrins (e.g.-α6β4, α5 and β3) are highly glycosylated and linked with a variety of glycan structures [8, 9]. Previously ITGA3 has been shown to contain aberrant glycosylations, particularly in BlCa [10]. As such, targeting the glycosylation patterns of ITGA3 in BlCa could provide novel tools for cancer diagnosis.

Lectins are a large group of glycan binding proteins with diverse structures and origin. Due to their capacity for specific recognition of glycan structures they are widely used research tools in glycobiology. *Ulex europaeus* agglutinin-I (UEA), with a molecular weight of 63 kDa, is a plant lectin having affinity to various fucosylated glycans, particularly for structures containing Fucα1-2Galβ moiety [11]. UEA and other fucose-specific lectins such as *Aleuria aurantia* lectin (AAL; specific for fucα1-6/3GlcNAc) [12] have previously been reported for the detection of the fucosylation status of prostate specific antigen (PSA) in prostate cancer [13, 14]. The strength of lectin-glycan interaction is usually relatively weaker for example in comparison to typical antibody-antigen interactions [15]. However, it has been shown that the problem of low affinity of lectins can be compensated with the avidity effect when coated on nanoparticles (NPs) [16, 17]. We previously demonstrated that the use of macrophage galactose lectin when conjugated on Eu-NPs, enhances the cancer specificity of a conventional CA125 assay used for

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the detection of ovarian cancer (OvCa) [18]. In the present study, we report the detection of ITGA3 glycoisoform with the use of UEA conjugated on nanoparticles. The assay is capable of discriminating BlCa from clinically challenging age-matched benign conditions directly from unprocessed urine.

2. Materials and methods

2.1. Urine samples

This study was conducted with the approval of the Ghent University Hospital ethics committee (B670201420715) and in accordance with the guidelines and regulations of the Helsinki Declaration. Participants had given written informed consent. Urine samples from patients with bladder cancer (n = 13) were collected by catheterization prior to surgical treatment by transurethral resection of the bladder tumor (TURBT) or radical cystectomy. Similarly, age matched urine samples from with benign prostatic hyperplasia (BPH) patients (n = 9) were collected prior to transurethral resection of the prostate (TURP) or simple prostatectomy and used as control. All patients were in a fasting state. Patients characteristics are presented in Table 1. Both bladder and non-bladder cancer samples were histologically classified and urological co-morbidities possibly influencing immunoassay results were listed (Supplementary Table 1). Urine samples were stored at −80 °C until use. Prior to the assay, urine samples were thawed at room temperature and centrifuged at 2000 g for 5 min to remove cells and cell debris.

2.2. Conjugation nanoparticles with lectins

An overview of the lectins tested in this study, and their major glycan-binding specificities is provided in supplementary Table 2. Lectins were coated on Eu^{3+}-NPs, as previously described with minor

Table 1
Patient and tumor characteristics. Abbreviations: TURBT: transurethral resection of bladder tumor; TURP: transurethral resection of the prostate.

Patients characteristics	Bladder Cancer (n = 13)	Benign Prostatic Hyperplasia (n = 9)	P-value
Age (years) Median (range)	70 (54–80)	73 (58–85)	0.37
Sex, n (%)			
Male	10 (76.9%)	9 (100%)	0.24
Female	3 (23.1%)	0 (0%)	
Tumor stage, n (%)			
Ta	2 (15.4%)		
Tis	2 (15.4%)		
T1	1 (7.7%)		
T2	2 (15.4%)		
T3	4 (16.7%)		
T4	2 (30.8%)		
Tumor grade, n (%)			
Low grade	0 (0%)		
High grade	13 (100%)		
Nodal stage, n (%)			
N0	9 (69.2%)		
N1	2 (15.4%)		
N2	2 (15.4%)		
Neo-adjuvant chemotherapy, n (%)			
Yes	3 (23.1%)		
No	10 (76.9%)		
Local treatment, n (%)			
TURBT	2 (15.4%)		
Radical cystectomy	11 (84.6%)		
TURP		4 (44.4%)	
Simple prostatectomy		5 (55.6%)	
Urological comorbidity, n (%)			
Yes	2 (15.4%)	3 (33.3%)	
No	11 (84.6%)	6 (66.7%)	0.61

modifications [17]. In brief, Eu^{3+}-NPs (Seradyn Indianapolis IN, USA) are monodispersed polystyrene beads that produce long-life time fluorescence. Approximately 1×10^{12} Eu^{3+}-NPs were used per reaction. The lectins, through their amino groups, were covalently coupled with activated carboxyl groups present on the Eu^{3+}-NPs. Concentration of lectins was 0.1 mg/mL in the coupling reaction. The surface of the nanoparticles was activated with 8 mmol/L NHS and 1.3 mmol/L EDC (Sigma-Aldrich, St. Louis, Mo, USA). Then, lectins were coupled with activated particles by vortexing for 30 min in MES buffer (50 mM, pH 6.1, 100 mM NaCl). Bovine serum albumin (BSA), with a final concentration 10 mg/mL, was added in the reaction-mixture, followed by vortexing for 30 min, to block remaining active sites on the particles. Next, the reaction-mixture was incubated at +4 °C overnight in a rotary mixer. The following day, the mixture was concentrated using a Nanosep centrifugal filter device (300 K) at 6000 g and washed again in storage buffer (25 mM Tris, pH 7.8, 150 mM NaCl, 0.1% NaN_3). The bio-conjugated NPs were recovered in storage buffer and stored at +4 °C by adding BSA (with a final concentration of 2 mg/mL) for two days in order to remove the aggregates. After sonication with Vail Tweeter (7 pulses, 0.5 cycle, amplitude 100%) and centrifugation (500 g, 5 min), the aggregate-free supernatant was collected and stored at +4 °C until use.

2.3. Capture-antibody labeling with biotin

Following antibody was used for immunocapturing: monoclonal anti-ITGA3 (clone IA3; catalogue number MAB1345, R&D systems, Abingdon, UK). Antibody was biotinylated as described previously, with minor modifications [17,18]. Briefly, the pH of the antibody solution was adjusted to 9.8 with 0.5 M of carbonate buffer. Biotin isothiocyanate (BITC) was dissolved in absolute ethanol to a final concentration of 10 mM. A 40-fold excess of biotin to antibody was added to the final reaction volume (antibody concentration: ~2 mg/mL). After 4 h incubation at RT, unreacted BITC was removed by gel filtration with NAP-5 columns using TSA buffer (pH 7.5, 50 mmol/L Tris-HCL, 150 mmol/L NaCl, and 0.5 g/L NaN_3). Then, biotinylated antibodies were stored at +4 °C in 1 g/L BSA.

2.4. Immunoassay

The schematic representation of the time-resolved fluorescence (TRF) immunoassay is displayed in Fig. 1. Briefly, 150 ng of biotinylated capture antibody (anti-ITGA3) was diluted in assay buffer (Kaivogen, Turku, Finland) in a final volume of 30 μL/well and immobilized in a 96-wells-streptavidin-coated plate (KaiSA96, Kaivogen, Turku, Finland) for 1 h at RT, followed by washing of the wells 4 times with wash buffer (Kaivogen, Turku, Finland). Then, 50 μL of urine sample and 100 μL of assay buffer were added to each well. After 1 h incubation at RT on a plate shaker at 600 rpm, the wells were washed 4 times with wash buffer. Altered glycosylation on the captured glycoproteins was detected using lectin conjugated Eu^{3+}-NPs (lectin-NPs). In a final volume of 30 μL, 1×10^7 of lectin-NPs were added to each well and incubated for 1.5 h at RT on a plate shaker with slow shaking. Next, the wells were washed 4 times with wash buffer in a DELFIA plate washer. Signal measurement was performed (λ_{ex}: 340 nm; λ_{em}: 615 nm) with a Victor™ 1420 multilabel counter (PerkinElmer) using the program europium from surface. All measurements were conducted in triplicate and signal/background was picked up for analysis.

2.5. Data analysis

Statistical analysis was performed using GraphPad Prism software, lnc (version 6). Comparison of medians was performed using a Mann–Whitney rank sum test. Contingency tables were analyzed using a Fisher's exact test. A two-tailed P value < 0.05 indicated statistical significance.
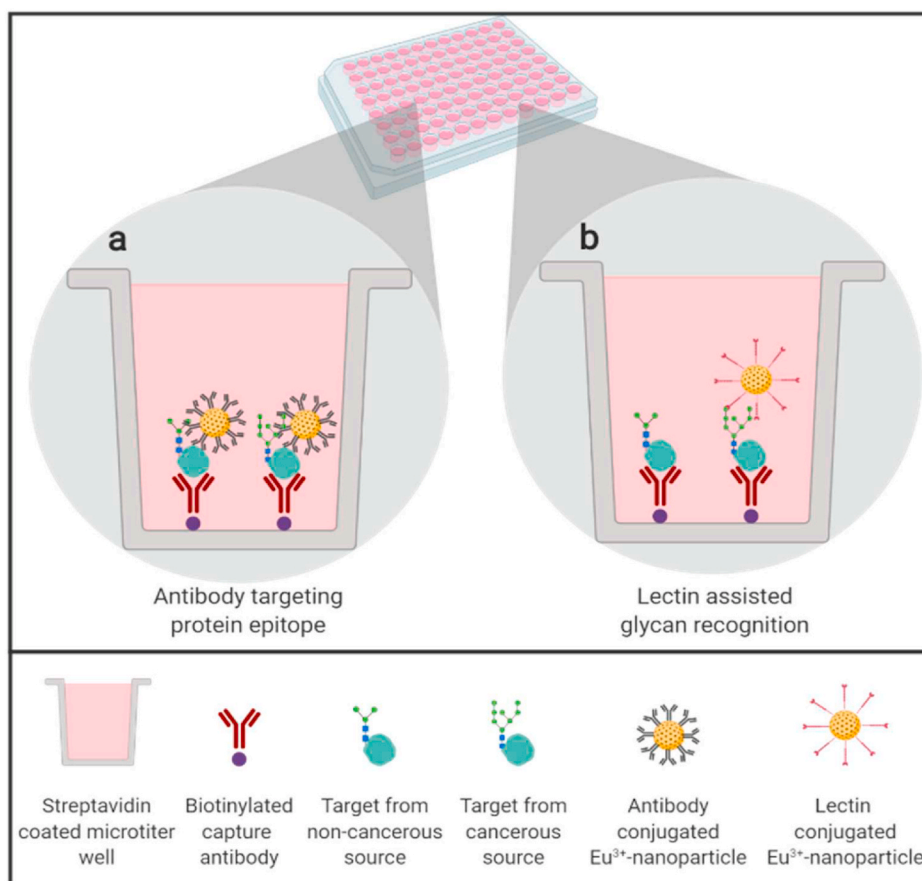
Fig. 1. Schematic representation of assay: Biotinylated antibody (anti-ITGA3) was immobilized on the surface of streptavidin coated microtiter wells to capture ITGA3 from urine samples. The captured ITGA3 was detected with Eu^{3+}-doped polystyrene nanoparticles conjugated either with (a) anti-ITGA3 antibody or (b) lectins. This figure was created using BioRender.com (2020).

3. Results

3.1. Glycoprofiling with Lectin-NPs

To explore if particular glycosylations of ITGA3 differ between the two patient groups, we used a time-resolved-fluorometry sandwich bioaffinity-assay with 9 different lectins (UEA, AAL, SBA, SNA, GSL-1, DC-SIGN, WGA, BPL, and DSL) coated on Eu^{3+}-doped nanoparticles. With the use of pooled urine samples (benign $n = 9$, BlCa $n = 13$), we observed distinct binding intensities for different lectins on immuno-captured urinary ITGA3 (Fig. 2). We also detected the total ITGA3 with the use of an assay combining an anti-ITGA3 antibody as both capture and tracer (ITGA3-ITGA3). The total ITGA3 showed a 2-fold higher signal-to-background (S/B) ratio in pooled BlCa compared to benign samples, whereas the ITGA3 assay with lectin UEA as a tracer (ITGA3-UEA) showed over 6-fold higher S/B ratio (Fig. 2). ITGA3 assays constructed with five other lectin-NPs (AAL, SNA, GSL-1, DC-SIGN, and BPL) showed 2-, 2-, 2-, 1.5-, and 3-fold higher S/B ratios, respectively. The remaining three lectin-NPs (SBA, WGA, and DSL) showed no discrimination between pooled BlCa compared to benign samples.

3.2. Detection of bladder cancer with ITGA3-UEA assay

Next, the performance of the ITGA3-UEA assay was evaluated with the use of individual urine samples from BlCa patients and patients with the benign condition BPH. The performance of the ITGA3-ITGA3 assay was also studied with the corresponding samples. The difference between both groups was considered statistically significant when the two-tailed P value < 0.05. Both the total ITGA3-ITGA3 and the ITGA3-UEA
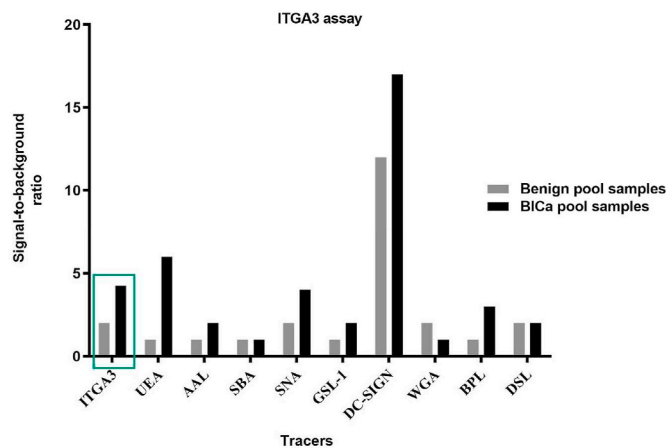
Fig. 2. Screening of different lectin-NPs for the detection of immunocaptured ITGA3 antigen from pooled urine samples of BPH and BlCa patients. The levels of total ITGA3 (blue box) as detected with Eu-NPs coated with anti-ITGA3 antibody tracer are also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

assay showed statistically significant discrimination between sample groups (Fig. 3). However, the discrimination was clearly more distinct in the case of ITGA3-UEA assay as indicated e.g., by the difference between median values of BlCa and BPH samples being 4.8 and 2.8-fold for the ITGA3-UEA and ITGA3-ITGA3 assay, respectively.
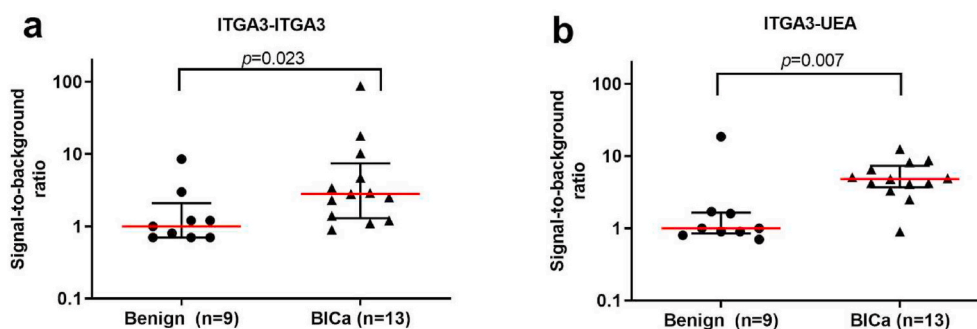
Fig. 3. Discrimination of bladder cancer patients (n = 13) from benign controls (n = 9) using nanoparticles coated with (a) ITGA3 and (b) UEA as a tracer. Immobilized anti-ITGA3 antibody was used for the immunocapture of the analyte. The *p*-values were calculated with Mann-Whitney *U* test from the signal-to-background (S/B) values.

4. Discussion

In this study, we describe a simple, lectin-based detection of aberrant glycosylation on ITGA3 as a potential tool for the discrimination of BlCa from benign urological conditions like BPH.

ITGA3, which has previously been reported to be overexpressed in cancer [17], was used as a starting point for the assay. With the use of various lectin-NPs, we investigated what kind of glycovariants of ITGA3 could be used to increase the cancer specificity of the assay. Among the lectins, we found UEA in combination with ITGA3 (ITGA3-UEA) to have significantly higher S/B ratio of pooled BlCa samples compared to pooled BPH samples. The ITGA3-UEA assay was further evaluated using individual urine samples from BlCa and BPH patients. To confirm that the enhanced cancer specificity was in fact a result of changes in the glycosylations, we also performed an assay where the total ITGA3 was detected using anti-ITGA3 antibody both as a capture and a tracer (Fig. 3a). This same approach to detect a membrane associated protein with the same monoclonal capture antibody has previously been used to detect extracellular vesicles and exosomes from various biofluids [19]. Although the full characterization whether ITGA3 is on EVs was out of the scope of this study, integrins have previously been reported to be found on EVs by several studies [20–22].

UEA has previously been characterized to have specificity towards fucosylated glycans, particularly Fucα1-2Galβ. The fucosylation changes found on ITGA3 are to some extent in line with characterized epitopes found using monoclonal antibodies [23]. Although the previously characterized epitope also consist of fucosylated glycans, the main glycan moiety was found to be Lewis x antigen (Galβ1-4(Fucα1-3) GlcNAc). Although the specific structures and particularly the mechanism leading to the fucose changes in bladder cancer remains elusive, targeting fucosylations in diagnosis holds great promise both in diagnosis and treatment of BlCa [24].

By combining ITGA3 capture and the detection of fucosylated glycans with UEA, we were able to significantly (*p* = 0.007) discriminate BlCa from BPH (Fig. 3b). This ITGA3-UEA-NP assay shows promise in the non-invasive detection of bladder cancer directly from unprocessed urine samples and calls for further studies with a larger patient cohort. The assay concept can also be easily modified by explore the biomarker potential of other bladder cancer associated glycoproteins.

5. Conclusion

We developed an assay combining immunocapture of urinary integrin and the detection of fucosylated glycans on the integrin with the use of lectins. This assay can be used to non-invasively discriminate BlCa from benign conditions with no preanalytical processing of the samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2021.114283.

Author contribution

Md. Khirul Islam: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Software Formal analysis, Writing – original draft Writing – review & editing: Parvez Syed: Conceptualization; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Roles/Writing – original draft Writing – review & editing. Bert Dhondt: Data curation; Formal analysis; Clinical sample Resources; Software; Validation; Visualization; Writing – review & editing. Kamlesh Gidwani: Conceptualization; Formal analysis; Funding acquisition; Methodology; Project administration; Resources; Writing – review & editing. Kim Pettersson: Conceptualization; Funding acquisition; Resources; Supervision; Visualization; Writing – review & editing. Urpo Lamminmäki: Conceptualization; Funding acquisition; Investigation; Project administration; Resources; Supervision; Validation; Visualization; Roles/Writing – original draft Writing – review & editing: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing – original draft Writing – review & editing.

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