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Glycovariant-based diagnosis of bladder cancer using urinary mucin 1 and carcinoembryonic antigen

Parvez Syed [a], Md. Khirul Islam [b,c,*], Henna Kekki [b,c], Joonas Terävä [b,c], Urpo Lamminmäki [b,c], Peter J. Boström [d], Janne Leivo [b,c], Kamlesh Gidwani [b,c]

[a] Olo Health Oy, Satakunnantie 8C, Turku 20100, Finland
[b] Department of Life Technologies and FICAN West Cancer Research Center, Division of Biotechnology, University of Turku, Finland
[c] InFLAMES Research Flagship Center, University of Turku, Finland
[d] Department of Urology, Turku University Hospital, Turku, Finland

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ABSTRACT

Glycan alterations, or glycovariants (GV), are well-recognized features of malignant transformation and are commonly observed in several cancers, including bladder cancer (BlCa). However, their integration into practical diagnostic assays remains limited. Here, we investigated whether glycan modifications on the urinary glycoproteins mucin 1 (MUC1) and carcinoembryonic antigen (CEA) could serve as diagnostic indicators for BlCa diagnosis. MUC1 and CEA were captured directly from urine samples of patients with benign prostate disease (n = 20) and BlCa (n = 19) by immobilizing monoclonal antibodies against these glycoproteins on microtitration wells. Cancer-associated glycan structures were detected using wheat germ agglutinin (WGA) lectin and glycan-specific antibody C241 (a clone of anti-carbohydrate antigen 19–9), both conjugated to Eu^{+3}-doped nanoparticles. This glycovariant-based detection approach enables selective recognition of disease-associated glycoforms. By combining assay results, several models were constructed to evaluate marker panels with improved sensitivity and selectivity. A combined diagnostic model incorporating MUC1-WGA and CEA-C241 immunoassays, designed to detect N-acetylglucosamine (GlcNAc) on MUC1 and sialyl-Lewis^a (sLe^a) structures on CEA, achieved approximately 95% sensitivity and 95% selectivity for identifying bladder cancer. The MUC1-WGA assay alone distinguished cases with 79% sensitivity and 95% selectivity, while other individual GV assays demonstrated comparatively lower sensitivities. In contrast, conventional protein-level measurements showed lower diagnostic performance. Concurrent detection of glycan alterations on MUC1 and CEA improved discrimination between bladder cancer and benign conditions. This GV-assay framework provides a modular platform that can be extended to incorporate additional urinary biomarkers. However, validation in larger patient cohorts is required to confirm clinical applicability.

1. Introduction

Bladder cancer (BlCa) was reported as the ninth most frequently diagnosed cancer in 2022, with about 613,791 new cases and 220,349 associated deaths worldwide [1]. The disease shows that nearly three-quarters of cases occur in men [1]. Early detection is crucial for improving patient survival; therefore, there is an urgent need for sensitive, non-invasive diagnostic tools capable of detecting BlCa at early stage. Moreover, BlCa is associated with a high recurrence rate, with 50–70% relapse within five years, requiring lifelong monitoring. As a result, BlCa has become one of the costly malignancies to manage on a per-patient basis compared with other cancers [2]. BlCa also imposes an estimated annual financial burden of €4.9 billion in the EU, with healthcare costs alone representing 5% of total EU health expenditures, highlighting the need for more sensitive diagnostic tools [3].

The most common symptom to prompt investigations is hematuria, and the frequently used method for BlCa diagnosis is cystoscopy. However, cystoscopy is an invasive procedure that can cause patient anxiety and discomfort and may also produce false-negative results. Urine cytology, another widely used method, has suboptimal sensitivity ranging from 12—50% depending on tumor stage. Inter-observer variability is another issue associated with urine cytology. However, the

* Correspondence to: Biotechnology Division, University of Turku, Kiinamyllynkatu 10, Turku FI-20014, Finland.
E-mail address: mdkhis@utu.fi (Md.K. Islam).

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sensitivity of urine cytology can be further enhanced to 78—100% by using the immunoCyt/uCyt + test (Scimedx, Dover, New Jersey) as an adjunct to urine cytology [4]. The immunoCyt/uCyt + test involves quantification of tumor-associated antigens using fluorescently labeled antibodies targeting mucin−1 (MUC1; clone 19A211), other carcinoma-associated glycoproteins (e.g., detected by clone LDQ10), and carcinoembryonic antigen (CEA: clone M344)-related epitopes. Although the immunoCyt/uCyt + test improves the performance of urine cytology, it still detects total protein-level antigens, which may limit the sensitivity. Other available tests based on proteins like nuclear matrix protein 22 (NMP22) and bladder tumor antigen (BTA) show high false positive rates for benign conditions of the urinary tract [5,6]. Abogunrin et al. [7] showed that, with multivariate algorithms and a panel of markers detection of BlCa in patients with hematuria can be achieved with sensitivity and selectivity of 91% and 79%, respectively. However, separation of benign prostate hyperplasia (BPH), a significant confounding pathology, remained a challenge. The limitations arising due to the invasiveness of cystoscopy and the inconsistent sensitivities of urine-based tests make the need for highly sensitive and specific minimally invasive tests even more important.

Since BlCa is a genitourinary tract cancer, soluble and membrane-bound glycoproteins released into urine serve as an attractive target for developing a non-invasive diagnostic tool. Glycovariants of both MUC1 and CEA are reported to be present at elevated levels in the majority of breast and colorectal cancers, respectively [8]. MUC1 is heavily O-glycosylated transmembrane mucin that is overexpressed and aberrantly glycosylated in BlCa, with cancer-associated glycoforms including truncated O-glycans such as Tn, sialyl-Tn, and GlcNAc-terminating structures well documented in urological malignances [18,19]. CEA is a glycoprotein aberrantly elevated in BlCa patients and its overexpression has been associated with malignant transformation [21]. Overexpression of *N*-acetylglucosamine (GlcNAc), sialylation, and sialylated Lewis antigens such as sialyl Lewis^{a} (sLe^{a}) and sialyl Lewis^{x} (sLe^{x}), is frequently observed in cancers [9,10]. Although many FDA-approved cancer biomarkers are glycoproteins with extensive glycosylation, techniques to exploit disease-associated glycosylation changes are lacking [8]. This study explores the simultaneous detection of disease-associated glycans on MUC1 and CEA, rather than relying on total protein measurements alone. To our knowledge, although MUC1 and CEA are established cancer biomarkers, their disease-associated glycovariants in urine have not been systematically studied for bladder cancer diagnosis. In this study, we report a simple immunoassay that targets cancer-associated glycan alterations on these clinically relevant glycoproteins, MUC1 and CEA, to improve diagnostic selectivity beyond conventional protein-level measurement.

2. Materials and methods

2.1. Urine specimens

The study was authorized by the local Ethics Committee of the Hospital District of Southwest Finland (ethical committee statement for BlCa samples: TO3/010/13) and performed in accordance with the Declaration of Helsinki. After written informed consent, urine samples, collected via catheter or cystoscope at the time of surgery at Turku University Hospital, from 19 bladder cancer patients were obtained from the Turku Prostate Cancer Consortium (TPCC). From the BlCa patients 14 underwent transurethral resection of bladder tumor (TUR-BT) (in 2013–2015) and five patients had radical cystectomy (in 2014–2017) at Turku University Hospital. The tumors were graded according to the WHO/International Society of Urinary Pathologist (ISUP) 2004 classifications and staged according to the 2010 TNM staging system. During a median follow up of 2.7 years (3 days−3.8 years) three patients were deceased due to bladder cancer and two patients undergoing TUR-BT had a recurrence.

For control samples, urine samples from 20 consecutive men with clinical suspicion of prostate cancer (PSA range of 2.5–20 ng/mL) in a prospective controlled trial investigating the role of pre-biopsy MRI in prostate cancer diagnosis (IMPROD, ClinicalTrials.gov identifier NCT01864135) were included. Written consent was obtained from the participants. According to systematic 6 + 6 biopsies and two ultra-sound -targeted biopsies in cases of a suspected lesion based on MRI, 19 patients were diagnosed as having benign prostate tissue and one patient had a low volume Gleason 6, clinically non-significant prostate cancer. The urine samples were collected directly into sampling containers between 2013 and 2015 at Turku University Hospital. During the median follow-up of 0.65 years (24 days to 1.9 years) the status for all of the 20 patients remained unchanged.

All urine samples were stored at -70 °C. The clinicopathological characteristics, including information regarding the patient and tumor characteristics are presented in Table 1.

2.2. Standard materials

Standards for MUC1 and CEA used for the quantification of analytes in urine samples were purified from a breast cancer cell line and from a colorectal cancer patient with liver metastases, respectively. Commercial CA15–3 (MUC1) and CEA standards were obtained from Fujirebio Diagnostics AB (Gothenburg, Sweden). For analytical assessment, MUC1 and CEA were evaluated over concentrations of 1–200 U/mL and 1–200 ng/mL, respectively. The limit of detection (LOD) was defined as the concentration corresponding to the mean signal of the blank calibrator plus three times the standard deviation. The assay standard curves were evaluated across the tested concentration range, showing a linear response (Supplementary Figure S1).

Table 1
Clinicopathological characteristics of bladder cancer patients (n = 19) and men with benign prostate disease (n = 20).

Characteristics			Number (%)
Cases (Bladder cancer)			19 (49)
Gender		Male	18 (95)
Age		Mean/ median (range)	69/70 (57, 80)
Smoking (current, previous)		Yes	10 (53)
		Unknown	6 (32)
Follow up (months)		Mean/ median (range)	27.6/ 34.3 (0.1, 51.6)
Status			
	Alive, no evidence of disease		13 (68)
	Alive with cancer relapse		1 (5)
	Death due to bladder cancer		3 (16)
Grade	Low		6 (32)
	High		13 (68)
pT category	pTa, pT1		12 (63)
	pT2		3 (16)
	pT3, pT4		4 (21)
Controls (men with benign prostate disease)			20 (51)
Age		Mean/ median (range)	65/ 65 (49, 78)
Histology of prostate biopsy specimen			
	Benign		19 (95)
	Gleason 6 (3 +3)		1 (5)
total PSA (μg/L)		Mean/ median (range)	5.5/ 6.8 (1.7, 17)
Follow up (months)		Mean/ median (range)	9.8/ 8.0 (0.8, 22.8)

2.3. Labeling of antibodies and WGA lectin

The Eu^{3+}-NPs were Fluoro-Max™ fluorescent carboxylate-modified particles, 0.1 μm in diameter, and were purchased from Seradyn Inc. (Indianapolis, IN, USA). Sulfo-NHS (N-Hydroxysulfosuccinimide sodium salt) was from Sigma-Aldrich (St. Louis, MO, USA) and EDC (EDC-HCl Novabiochem®, 1-Ethyl-3-(3′-dimethylaminopropyl) carbodiimide ·HCl) was from Millipore (Darmstadt, Germany). Nanosep 300 K Omega filters were purchased from Pall Life Sciences (Port Washington, NY, USA) and the tip sonicator UP100H and tube sonicator Vial Tweeter were from Hielscher (Teltow, Germany).

Anti-carbohydrate antigen 19–9 (CA19–9) antibody C241, anti-MUC1 antibodies Ma552 and Ma695, and anti-CEA antibodies 12–140–1 and 12–140–10 were acquired from Fujirebio Diagnostics (Gothenburg, Sweden). Unconjugated wheat germ agglutinin (WGA) was purchased from Vector laboratories (Burlingame, CA, USA). Biotin-isothiocyanate had been synthesized at the University of Turku (Turku, Finland).

2.3.1. Biotinylation and Eu chelate labeling of antibodies

Antibodies Ma552 and 12–140–10 were biotinylated utilizing the methods described by Eriksson et al. [11] with the difference that a 40-fold molar excess of biotin-isothiocyanate (BITC) was used in the reaction. The antibodies-BITC mixture was then incubated for 4 h at room temperature. The unreacted BITC was removed by gel filtration with NAP−5 column using TSA buffer (pH 7.5, 150 mmol/L NaCl, 50 mmol/L Tris-HCl, and 0.5 g/L NaN_3). Here NAP−5 column separates biotinylated antibodies from unreacted BITC by size-exclusion chromatography, retaining small BITC molecules (MW: 390 Da) within the gel matrix while larger antibody molecules (MW: 150 kDa) elute in the void volume.

Antibodies Ma695 and 12–140–1 were labeled with methods described by Väisänen *et al.* [12], for antibody fragments, with minor differences. The Eu^{3+}-chelate molar excess used was 125-fold for 12–140–1 and 40-fold for Ma695. The reaction mixtures were first incubated for two hours at room temperature, followed by overnight incubation at + 4 °C. The purification of unreacted Eu^{3+} chelate was done in the same manner as the purification of unreacted biotin-isothiocyanate for biotinylated antibodies.

2.3.2. Coating of Eu^{3+}-doped nanoparticles

The coating was performed by utilizing well-established carbodiimide crosslinking chemistry which enables linking the carboxylate groups of the Eu^{3+}-NPs to the primary amines of proteins [28,29]. The steps were performed at room temperature unless mentioned otherwise. All washes were performed with 200—300 μL of buffer by centrifuging the Eu^{3+}-NPs in the filters at 5400 × g until no distinct liquid droplet could be observed by eye, but the membrane is still moist. All Eu^{3+}-NP recovery steps were performed by sonicating the filter membranes from a 2—5 mm distance for 30 pulses at 60—80% amplitude and recovering the solution from the membrane. Approximately 3.3×10^{11} Eu^{3+}-NPs were used per reaction.

The Eu^{3+}-NPs were washed twice with 200 μL of conjugation buffer (50 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.1) and recovered in 50 μL of conjugation buffer. The particles were activated by vortexing for 15 min with EDC and NHS in the final concentration of 1.3 mM and 8 mM, respectively. The Eu^{3+}- NPs were conjugated with 150 μg of protein in the reaction buffer (50 mM MES, pH 6.1, 100 mM NaCl) by vortexing for 30 min. The pH of the reaction was adjusted to 8.0 with 0.5 M carbonate buffer (pH 9.8) and the mixture was vortexed for 30 min. Bovine serum albumin (BSA), for blocking reactive carboxylate groups, was added to a final concentration of 10 mg/mL and the mixture was vortexed for 30 min and stored at + 4 °C overnight in rotary mixing.

The mixtures were centrifuged at 5400 × g until dry and washed three times with 300 μL of storage buffer (25 mM Tris, pH 7.8, 150 mM NaCl, 0.1% NaN3). The particles were recovered in 150 μL of storage buffer, mixed thoroughly, and BSA was added to a final concentration of 2 mg/mL. The Eu^{3+}-NPs were stored at + 4 °C for two days. The solution was mixed and then sonicated for seven pulses at 100% amplitude. The supernatant was collected after centrifuging at 500 × g for five minutes. This conjugation to Eu^{3+}- NPs was performed for C241 and WGA in separate reactions. Successful conjugation of lectins and antibodies to Eu^{3+}-NPs was confirmed through specific concentration-dependent binding activity and high signal-to-background ratios observed during assay optimization, consistent with our previous publications of this well-established platform [16,23,35].

2.4. Time resolved fluorometry immunoassay configuration

The schematic representation of the time resolved fluorometry (TRF) assays is shown in Fig. 1. In brief, biotinylated antibodies were diluted in Uniogen assay buffer (Uniogen Oy, Turku, Finland) to a final concentration of 100 ng/well and were immobilized on streptavidin-coated microtiter wells (Uniogen, Turku, Finland) by incubating for 60 min at room temperature. The final volumes of 50 μL and 25 μL of biotinylated antibody were used when Eu^{3+}-chelate labeled Ma695 or 12–140–1 and C241 or WGA conjugated Eu^{3+}-NPs were used as tracers, respectively. Washing of the wells was performed to remove the excess unbound biotinylated antibody. Then, 50 μL of diluted standard or urine sample was added to the wells in triplicates. Dilution of the standard and the urine samples was done in Uniogen assay buffer. 1:40 dilution of standard/sample was performed for the immunoassays where biotinylated Ma552 antibody was used. On the other hand, when biotinylated 12–140–10 antibody was used, standards/samples were diluted 1:5 and 1:20 for assays where Eu^{3+}-chelate labeled 12–140–1 and C241 antibody conjugated Eu^{3+}-NPs were used as tracers, respectively. The diluted standards and samples were incubated in the wells for 60 min on a plate shaker which was followed by washing of the wells. The bound analytes were detected using TRF. In the conventional immunoassays, the sialylated carbohydrate epitope expressed on the MUC1 and protein epitope on CEA were targeted by Eu^{3+}-chelate labeled Ma695 and 12–140–1 antibodies, respectively. The antibodies were diluted in 50 μL of Uniogen assay buffer and incubated for 60 min at room temperature with shaking. After washing the wells, 200 μL of europium fluorescence intensifier (Uniogen, Turku, Finland) was added to each well and incubated on a shaker for 10 min at room temperature. On the other hand, the assays where the glycan epitopes on MUC1 and CEA were targeted using WGA and C241 antibody conjugated Eu^{3+}-NPs. The NP-bioconjugates were diluted in 25 μL of Uniogen assay buffer and incubated for 60 min at room temperature with shaking. The TRF for europium was measured (λ_{ex}: 340 nm; λ_{em}: 615 nm) using Victor™ 1420 Multilabel counter from Perkin-Elmer Life Sciences (Waltham, MA, USA).

2.5. Statistical analysis

Statistical significance between groups was assessed using a one-tailed Mann-Whitney *U* test to evaluate the ability of the assays to differentiate samples [30,31]. Significance level of 0.95 (p-value <0.05) was considered to be statistically significant. R version 3.3.3 was used for generating and testing logistic regression models, based on the assay results, for classifying controls and cases [13]. The whole dataset was used to build the models. A global logistic regression model was generated for ranking all possible models. The models were ranked by second order bias corrected Akaike Information Criterion (AICc) [14] using the MuMIn package after removing the models with negative correlations [15]. Cutoffs were used to assess the assays/models while keeping 95% selectivity as a constant. The diagnostic performance of the assays/models was evaluated by receiver operating characteristic (ROC) curve analysis using R [32,33]. AUC values are reported with 95% confidence intervals (CI) calculated using the pROC package. Intra- and

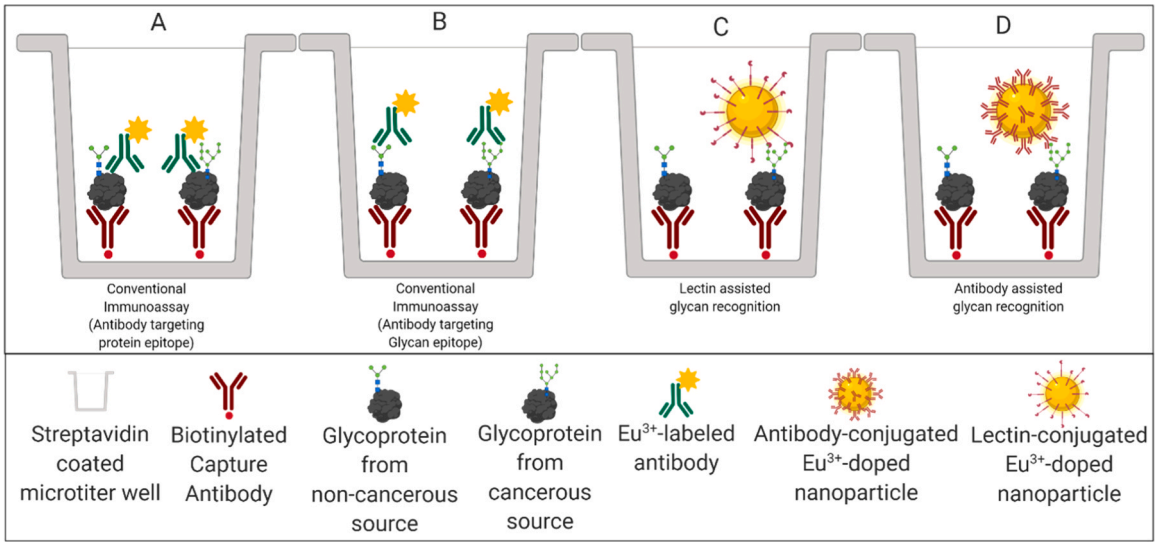

Fig. 1. Schematics of the assays. Biotinylated antibodies against a specific protein epitope are immobilized on the surface of streptavidin coated well for capturing the target glycoprotein from urine sample. The captured analyte is then detected either by targeting protein or glycan epitopes. **A** and **B** represent the detection of captured analyte by targeting protein and glycan epitopes with Eu^{3+}-chelate labeled antibodies, respectively. **C** and **D** represent the detection of altered glycans, present on the analyte originating from cancerous source, with lectins or antibodies conjugated to Eu^{3+}-doped polystyrene nanoparticles, respectively. The time-resolved fluorescence is recorded for data analysis.

inter-assay variation were assessed by calculating the coefficient of variation (CV%) from triplicate measurements within a single run and across independent runs of different days, respectively. All CV% values below 15% are considered acceptable.

3. Results and discussion

3.1. Quantification of conventional MUC1 and CEA

Quantification of urinary MUC1 and CEA from patients with BlCa (n = 19) and benign prostate disease (n = 20) was performed using the standards, as shown in the supplementary file (Figure S1). The conventional MUC1 (conv. MUC1) assay, in which sialylated carbohydrate epitope expressed on MUC1 was targeted, segregated the samples with low sensitivity of 21% at 95% selectivity (Fig. 2a). On the other hand, elevated levels of CEA were found in BlCa urine samples compared to benign prostate disease samples. The conventional CEA (conv. CEA) assay discriminated BlCa from benign prostate disease patients with a sensitivity of 57.9% at 95% selectivity (Fig. 2b). The evaluation of performance of these conv. MUC1 and CEA assays using receiver operating characteristic (ROC) curve analysis revealed an area under the curve (AUC) of 0.647 and 0.816, respectively (Fig. 3a). The misclassification rate was calculated based on the cutoffs which were 67 U/mL and 6 ng/mL for conv. MUC1 and conv. CEA assays, respectively. The diagnostic accuracy of conv. MUC1 and conv. CEA assays were 59% and 77%, respectively (Table 2).

Although serum CEA has been reported to be elevated in BlCa [7], contradicting findings also exist which demonstrate the inability of CEA for primary diagnosis of BlCa [21]. The urinary CEA levels alone, from our study, allowed poor detection of BlCa with 57.9% sensitivity and 95% selectivity. Detection of sLe^a on CEA yielded similar results. However, this finding tells us that glycan-specific detection may require integration with additional biomarkers to achieve clinically meaningful performance.

3.2. Detection of MUC1 and CEA glycovariants

MUC1 is known to be differently glycosylated in cancers. For the detection of such cancer-specific glycans such as GlcNAc on MUC1 and sialylated $Lewis^a$ (sLe^a) epitope on CA19–9, WGA and C241 antibody were used, respectively. The MUC1-WGA and MUC1-C241 assays discriminated BlCa from benign prostate disease patients with relatively higher sensitivities of 78.9% and 52.6%, respectively, at 95% selectivity (Fig. 2c, d & Table 2). The MUC1-WGA and MUC1-C241 assays correctly identified 87% and 74% of samples (Table 2). The cutoffs for the MUC1-WGA and MUC1-C241 assays were 65 U/mL and 66 U/mL, respectively. Similarly, the presence of the CA19–9 glycan on CEA was probed with the anti-CA19–9 antibody, resulting in discrimination of BlCa samples from benign samples with a sensitivity of 57.8% at 95% selectivity (Fig. 2e). The CEA-C241 assay correctly identified 77% of samples at an 18 ng/mL cutoff (Table 2). The diagnostic performance of the MUC1-WGA, MUC1-C241 and CEA-C241 assays was evaluated using ROC analysis, which yielded AUC values of 0.897, 0.853 and 0.832, respectively (Fig. 3a).

A study conducted by Nielsen et al. [17], showed that only the BlCa patients with overexpression of HER3 along with overexpression of MUC1 showed favorable survival compared to the patients with overexpression of MUC1 alone. Also, contradictory studies reporting the pattern, intensity and depth of MUC1 immunostaining correlating with the bladder cancer grade exist [18]. One possible explanation for such contradicting observations could be presence of various forms of MUC1 glycoforms which include underglycosylated, sialylated and fully glycosylated forms [19]. These glycoforms differ in biological function and may differentially interact with lectins and antibodies, emphasizing the need to target glycan-specific epitopes rather than total protein levels. Presence of N-glycan structures such as GlcNAc is well documented [20] and through our study we have shown that BlCa can be detected with 79% sensitivity and 95% selectivity by targeting GlcNAc on MUC1 using WGA. In our recent published study, we observed that WGA showed enhanced binding with *N*-Acetylglucosamine on MUC1 from breast cancer patients as opposed to healthy volunteers [23].

C241-based detection targets sLe^a on both MUC1 and CEA, as reflected in the MUC1-C241 and CEA-C241 assays. However, it is reported that approximately 10% of individuals with a Lewis-negative phenotype are unable to synthesize sialyl Lewis A (CA19–9), which may affect the performance of C241-based detection [25]. Another limitation of this study is the lack of creatinine normalization, which may introduce variability due to differences in urine dilution. Creatinine normalization for glycan-based biomarkers is less standardized and often depends on the study design and clinical context [26]. However, in our previously

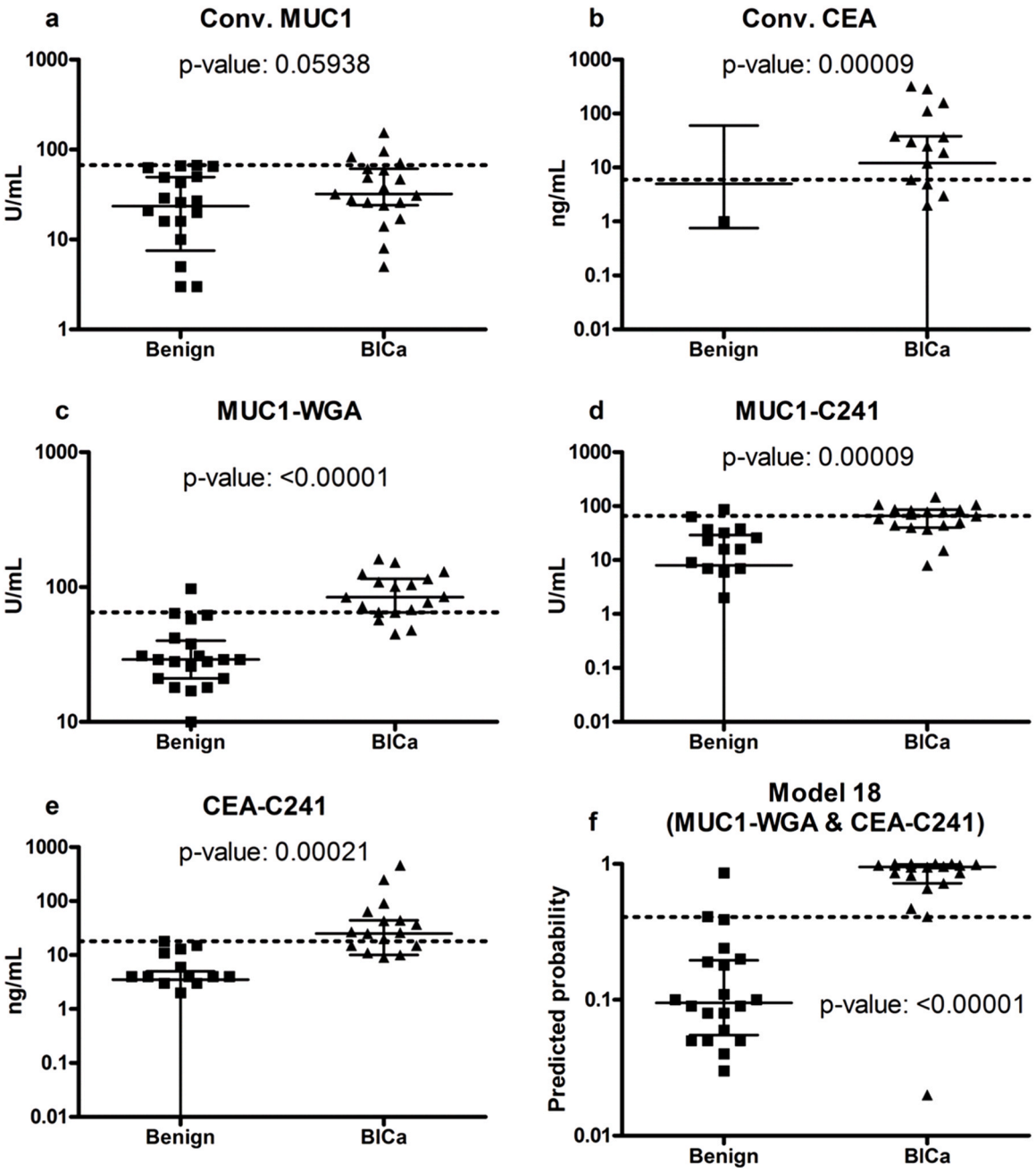

Fig. 2. Performance of immunoassays. **a** and **b** represent conventional immunoassays where the concentrations of MUC1 and CEA in the urine samples from benign prostate disease and BlCa patients were measured, respectively. Antibodies conjugated with Eu^{3+}-chelates were used in these assays as tracers. **c** represents WGA lectin-immunoassay indicating the presence of *N*-Acetylglucosamine on MUC1. **d** and **e** represent the immunoassays performed for the identification of CA19−9 antigen on MUC1 and CEA using C241 antibodies conjugated to Eu^{3+}-nanopartcle, respectively. **f** represents the combined performance of **c** and **e** assays (Model 18) for discriminating BlCa from benign prostate disease. The dotted lines represent the cutoffs. All individual data points are displayed. In panel b, overlapping benign values appear as a single point due to similar measurements. Horizontal solid lines indicate the median values, while vertical error bars represent the interquartile range. The dashed horizontal lines indicate the cutoff values at 95% selectivity. Herein, y-axis represent values on log10 scale. Statistical significance was calculated using the Mann-Whitney *U* test.

published studies using similar glycovariant assay platform with urine samples, no significant dilution effect was observed [24,34–37], suggesting that GV-based TRF detection may be less susceptible to urine dilution variability than conventional protein immunoassays.

3.3. Performance of the models

Thirty-two models were identified through logistic regression modeling which comprised of various permutations and combinations of the assays (Suppl. table S1). After eliminating the models which presented negative correlation values, sixteen models were identified and were ranked according to their AICc values (Table 3). Performance of model 18, which consists of MUC1-WGA and CEA-C241 assays, outranked the rest of the models. Using model 18, BlCa samples were correctly identified with 94.7% sensitivity and 95% selectivity at a predicted probability cutoff of 40.7% (Fig. 2f). The AUC value was found to 0.934 when the diagnostic accuracy of model 18 was evaluated using ROC curve analysis (Fig. 3).

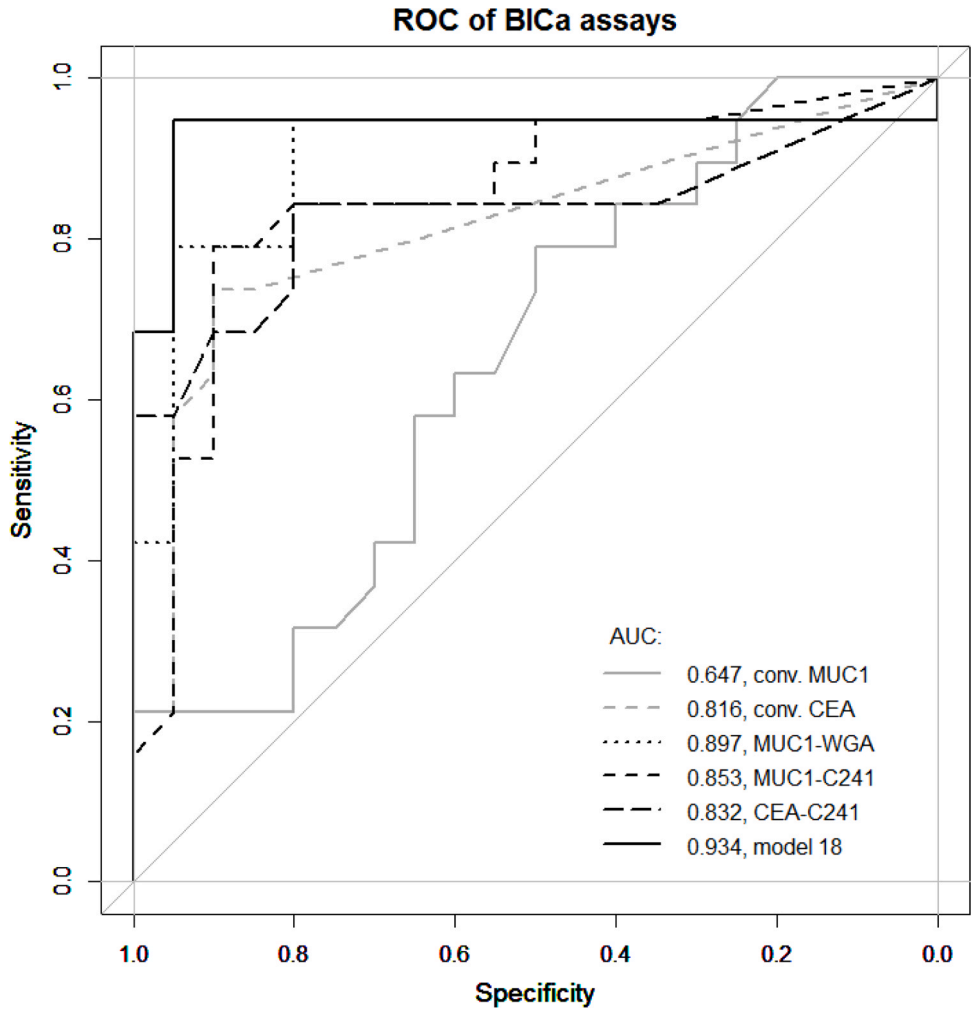

Fig. 3. ROC curve analysis of the individual assays and the model. The performances of the assays were evaluated using ROC curve analysis. "model 18" which consisted of MUC1-WGA and CEA-C241 assays displayed an AUC value of 0.934. "model 18" clearly outperformed the individual assays by correctly identifying the BlCa samples with 94.7% sensitivity and 95% selectivity.

Table 2
Diagnostic accuracy of the assays at 95% selectivity.

Assay	Cutoff	False Negative	Sensitivity	Accuracy
Conv. MUC1	67 U/mL	79% (15/19)	21.1%	59% (23/39)
Conv. CEA	6 ng/mL	42% (8/19)	57.9%	77% (30/39)
MUC1-WGA	65 U/mL	21% (4/19)	78.9%	87% (34/39)
MUC1-C241	66 U/mL	47% (9/19)	52.6%	74% (29/39)
CEA-C241	18 ng/mL	42% (8/19)	57.9%	77% (30/39)

The *dredge* function in the *MuMIn* package for R was used to evaluate all the best fitting logistic regression models that can be established by combining all the measurement results of the five markers. Because all the markers, except conventional MUC1, were significantly elevated in the case group (Table 3), the models with any negative coefficients for marker concentrations were excluded (supplementary Table S1). The models were then ranked by AICc in descending order. AICc is a relative measure of information loss in statistical models that tries to minimize the problems of over- and under-fitting as well as the limitation of small sample sizes by taking the number of variables and the number of samples into account in the AICc equation [14]. Certainly, the small sample size here means that the model is subject to change due to a more thorough evaluation with proper training and test sets from a larger cohort. The AICc of the models negatively correlates with the AUC values of the models and the model ranked best by AICc had the highest AUC which are good indications that the most promising model was selected and so the two most promising markers, MUC1-WGA and CEA-C241, were singled out for further discussion.

Aberrant glycosylation of glycoproteins is commonly observed in many cancers and plays a functional role in tumor progression. Altered glycan structures such as increased branching, sialylation, and truncated O-glycans can influence cellular adhesion and receptor signaling, thereby contributing to cancer development and progression. However, the techniques which specifically target such changes in glycosylation need to be further improved for clinical application. In our previous study, we have shown that by targeting the glycan epitope with macrophage galactose-type lectin (MGL)-coated Eu^{+3}-nanoparticles, an increased sensitivity of cancer antigen 125 (CA125/MUC16) can be achieved for discriminating epithelial ovarian cancer from endometriosis. Such discrimination was not possible with a conventional immunoassay where only the levels of serum CA125 (total protein levels) were measured [16]. In the present study, compared to conventional protein-level immunoassays, the glycovariant-based Eu^{3+}-nanoparticle-sapproach offers improved diagnostic selectivity by targeting cancer-associated glycoforms on MUC1 and CEA while suppressing signals from non-malignant protein forms. This Eu^{3+}-nanoparticle-s-assisted TRF format provides exceptional signal amplification from approximately 30,000 Eu^{3+} chelates per particle, enabling sensitive detection of low-abundance glyco-structures directly from unprocessed urine. Additionally, the high-density coating of lectins or antibodies on

Table 3
Logistic regression modeling. Models with negative coefficients were excluded and AUC for the models was calculated for discriminating controls and cases. The models are ranked by AICc.

Model #	Rank	CEA-C241	Conv. CEA	MUC1-C241	Conv. MUC1	MUC1-WGA	AICc	AUC	Sens. (%) at 95% spec.
18	1	0.105				0.045	30.6	0.934	94.7
6	2	0.090		0.035			36.2	0.911	78.9
19	3		0.032			0.053	36.2	0.883	73.7
23	4		0.031	0.023		0.034	36.6	0.913	78.9
17	5					0.056	37	0.897	78.9
7	6		0.040	0.049			37.1	0.893	73.7
21	7			0.021		0.041	37.2	0.9	68.4
8	8	0.067	0.022	0.038			38.2	0.886	73.7
2	9	0.146					38.4	0.832	57.9
10	10	0.134			0.026		38.5	0.883	68.4
4	11	0.143	0.004				40.7	0.841	68.4
12	12	0.131	0.005		0.026		40.9	0.89	68.4
5	13			0.047			41.4	0.853	52.6
11	14		0.047		0.023		48.6	0.796	57.9
3	15		0.050				48.8	0.816	57.9
9	16				0.020		55.3	0.647	21.1

the EuNPs surface creates a multivalent binding effect, that provides a functional avidity power of glycan-binding interactions beyond what individual lectin-or antibody-glycan affinities alone would perform [16, 38]. Compared to alternative glycan detection tools such as lectin microarrays or mass spectrometry-based glycoprotein-omics, the present approach requires no specialized instrumentation or sample pretreatment, making it readily application in routine clinical laboratories. Furthermore, the dual-target glycan profiling strategy, simultaneously targeting GlcNAc on MUC1 and sLe^a on CEA, provides complementary diagnostic information that single-marker conventional assays cannot achieve.

A single marker may lack adequate sensitivity and selectivity which can be overcome by using a panel of markers. ImmunoCyt/uCyt + test is one such test, developed by Fradet and Lockhart, which combined immunofluorescence assays with 3 fluorescent monoclonal antibodies, 19A211, LDQ10 and M344 [22]. The fluorescein labeled 19A211 and LDQ10 antibodies were directed against mucins, primarily MUC1 and related carcinoma-associated mucins, while the M344 targeted CEA glycoproteins. When complemented with urine cytology, ImmunoCyt/uCyt + test has detected BlCa with higher sensitivities compared to cytology or ImmunoCyt/uCyt + test alone [4]. However, a direct comparison with immunoCyt/uCyt + test was not performed in this study. Notably, immunoCyt/uCyt + test primarily detects tumor-associated protein epitopes [27], whereas the present approach targets cancer-associated glycan alterations on MUC1 and CEA. These differences suggest that glycovariant-based assays may provide complementary and more cancer-specific diagnostic information beyond conventional protein-level measurements.

Similarly, through our study, we have illustrated the need for targeting more than one marker; thereby achieving highly sensitive detection of BlCa. In future studies, this biomarker panel will be combined with aberrantly fucosylated integrin α3 (ITGA3), previously reported by our group as a urinary biomarker for bladder cancer, with the aim of further enhancing the sensitivity of urine-based bladder cancer diagnostics [24].

4. Conclusion

In conclusion, glycovariant-based analysis of urinary MUC1 and CEA present the capability for highly sensitive and specific detection of BlCa. The combined MUC1-WGA and CEA-C241 glycovariant assay panel exhibited approximately 95% sensitivity and selectivity, outperforming conventional total protein-based measurements. To our knowledge, this is the first study which attempted to exploit the synergistic effect brought in by simultaneous identification of GlcNAc structures on urinary MUC1 and sLe^a on urinary CEA for discrimination of BlCa from benign urological conditions. Also, it is to be noted that most of the commercially available diagnostic kits offer suboptimal sensitivities and specificities for discriminating BlCa from other benign conditions such as benign prostate disease, which we have achieved on a small cohort, a gap addressed by the present study.

The main advantages of this glycovariant-based approach include the use of non-invasive, unprocessed urine without sample pretreatment, exceptional analytical sensitivity enabled by nanoparticle-assisted TRF detection, and selective detection of cancer-associated glycoforms over benign conditions. This assay operates in a simple immunoassay format that is well-suited for low-abundance analytes in dilute biological matrices such as urine, and is readily adaptable for multiplex biomarker panels.

Key limitations include glycan heterogeneity, potential exclusion of Lewis-negative individuals from C241-based sLe^a detection, absence of creatinine normalization, limited sample diversity, and small cohort size. Direct comparison with approved urine-based bladder cancer test was not performed, and longitudinal evaluation was not included.

Therefore, these findings should be considered as proof-of concept, and require validation in larger, independent, multicenter cohorts. In our future follow up study, we will include healthy controls, clinically relevant benign disease groups, urine normalization strategies, full analytical validation, and integration of additional glycovariant biomarkers.

CRediT authorship contribution statement

Kamlesh Gidwani: Supervision. **Janne Leivo:** Supervision, Resources. **Peter J. Boström:** Resources. **Md. Khirul Islam:** Data curation. **Parvez Syed:** Conceptualization. **Joonas Terävä:** Formal analysis. **Henna Kekki:** Data curation. **Urpo Lamminmäki:** Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2026.117589.

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