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Glycan affinity magnetic nanoplatforms for urinary glycobiomarkers discovery in bladder cancer

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ABSTRACT

Bladder Cancer (BC) presents one of the highest recurrence rates amongst solid tumours and constitutes the second deadliest disease of the genitourinary track. Non-invasive identification of patients facing disease recurrence and/or progression remains one of the most critical and challenging aspects in disease management. To contribute to this goal, we demonstrate the potential of glycan-affinity glycoproteomics nanoplatforms for urinary biomarkers discovery in bladder cancer. Briefly, magnetic nanoprobes (MNP) coated with three broad-spectrum lectins, namely *Concanavalin A* (ConA; MNP@ConA), Wheat Germ Agglutinin (WGA; MNP@WGA), and *Sambucus nigra* (SNA; MNP@SNA), were used to selectively capture glycoproteins from the urine of low-grade and high-grade non-muscle invasive as well as muscle-invasive BC patients. Proteins were identified by nano-LC MALDI-TOF/TOF and data was curated using bioinformatics tools (UniProt, NetOGlyc, NetNGlyc, ClueGO app for Cytoscape and Oncomine) to highlight clinically relevant species. Accordingly, 63 glycoproteins were exclusively identified in cancer samples compared with healthy controls matching in age and gender. Specific glycoprotein sets exclusively found in low-grade non-muscle invasive bladder tumours may aid early diagnosis, while those only found in high-grade non-invasive and muscle-invasive tumours hold potential for accessing progression. Amongst these proteins is bladder cancer stem-cell marker CD44, which has been associated with poor prognosis. Orthogonal validation studies by slot-blotting demonstrated an elevation in urine CD44 levels of high-grade patients, which became more pronounced upon muscle-invasion, in mimicry of the primary tumour. These observations demonstrate the potential of MNP@lectins for identification of clinically relevant glycoproteomics signatures in bladder cancer. Future clinical validation in a larger and well characterized patient subset is required envisaging clinical translation of the results.

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1. Introduction

Bladder cancer (BC) is the second most deadly malignancy of the urinary tract [1]. Patients diagnosed with low-grade non-muscle invasive BC (NMIBC) generally face better prognosis, whereas those with high-grade lesions are frequently burdened by significant recurrences, generally accompanied by progression to muscle invasion (MIBC) and metastasis [2]. As such, these patients are subjected to several post-operative cycles of intravesical therapy proceeded by intensive and invasive follow-up interventions [2]. The development of non-invasive tools supporting the differentiation between low and high-grade lesions, as well as the early identification of patients at risk of progression, remains a critical aspect for disease management [3].

Urine is easily accessible and, in bladder cancer cases, is in direct contact with the tumour, thereby providing a key source of biomarkers for addressing the above mentioned difficulties [4]. Moreover, the human urine proteome is well characterized, supporting proteomics-based biomarker studies [5]. Interestingly, the most relevant targets arising from these studies include several glycoproteins, such as members of the apolipoprotein family, fibrinogen chains, alpha-1-antitrypsin, alpha-2-macroglobulin, and uromodulin [5,6]. Nevertheless, these biomarkers may be reflective of haematuria and have also been associated with kidney disease (e.g. apolipoproteins, uromodulin, or alpha-1-antitrypsin) and other pathologies [6]. As such, current urine biomarkers lack the necessary sensitivity and specificity for clinical use; however, their incorporation in multi-biomarker panels may provide the necessary molecular context for more effective applications. However, the lack of efficient enrichment strategies remains one of the most critical aspects underlying the identification of cancer-specific biomarkers, generally present in minute amounts (nano-femtomolar range) in bodily fluids such as urine [7].

Targeting glycan moieties in cancer-associated glycoproteins holds tremendous potential to overcome these limitations, while providing an important source of clinically relevant biomarkers [8]. Moreover, the identification of disease-associated glycoforms may further improve the biomarker potential of already proposed urine glycobiomarkers. Accordingly, several decades of research, mostly using lectins and antibodies for tissue screening, have disclosed a plethora of alterations in membrane proteins glycosylation associated with bladder cancer [3]. Namely, we have reported that disease progression to invasion and dissemination is accompanied by the overexpression of short-chain sialylated *O*-glycans, associated with poor prognosis [9–11]. Lectin-enrichment targeting these glycans further enabled the identification of clinically relevant glycoproteins in patient samples, including several integrins, cadherins, the stem cell marker CD44 and MUC16 [12,13]. Taken together, our studies demonstrated the importance of pre-enrichment strategies for downstream comprehensive glycoproteomics approaches envisaging biomarker discovery [12–14]. Concomitantly to *O*-glycan alterations, advanced stage bladder cancer cells also experience *N*-glycome remodelling, including changes in mannose core, branching, elongation and degree of fucosylation and sialylation [3]. However, the nature of the glycoproteins exhibiting these alterations is still poorly understood. Nevertheless, we hypothesize that abnormally glycosylated proteins may be secreted or shed from the primary tumour into bodily fluids, constituting an important source of potentially relevant urine glycobiomarkers.

Building on these insights, our group has developed lectin functionalized magnetic nanoprobes for glycoproteomics research, which have demonstrated enhanced sensitivity in comparison to conventional enrichment strategies [15,16]. Herein, we aim to explore the potential of these nanoplatforms to highlight the existence of glycoprotein signatures associated with bladder cancer stage and grade. We envisage that this preliminary approach may provide the necessary analytical rationale for more in depth translational studies.

2. Material and methods

2.1. Patient samples

Between 2010 and 2011, thirty-one first void urine samples without visible signs of haematuria were prospectively collected before surgery of bladder cancer male patients with mean age of 70 (range, 45–89) years, attending the Portuguese Institute of Oncology of Porto (IPO-Porto; Portugal). Only patients that had not been previously submitted to neoadjuvant therapy were included. Corresponding formalin-fixed paraffin embedded (FFPE) tumours were also obtained for this study. Based on the World Health Organization urothelial carcinoma grading and staging criteria, three different groups were considered in this study, namely low-grade (n = 15) and high grade (n = 9) non-muscle-invasive bladder tumours (NMIBC) and muscle-invasive (n = 7) tumours (MIBC). An additional 15 urines were obtained from healthy control male volunteers, mean age of 68 (range 41–82) years. All procedures were performed under the approval of the institution ethics committee and upon patients' informed consent. All clinicopathological information was obtained from patients' clinical records.

2.2. Isolation and quantification of urine proteins

Five to forty millilitres of collected urine were centrifuged at 5000*g* for 40 min at 4 °C to remove cells and debris, desalted on Amicon Ultra 10 kDa centrifugal filters (Merck KGaA, Darmstadt, Germany) and proteins were resuspended on 50 mM Ammonium bicarbonate (pH 7.8, Sigma-Aldrich, St. Louis, MO, EUA). Protein quantification was accessed using the DC Protein assay (Bio-Rad, Hercules, CA, USA) and protein content was normalized in relation to creatinine, as previously described [17,18]. Creatinine content was determined using a colorimetric Creatinine Assay Kit (Abcam, Cambridge, UK). Protein/Creatinine ratio was used to disclose sample proteinuria as result of disease.

2.3. Total sialic acids

Total sialic acids (TSA) were determined by fluorimetry as previously described [15,16]. All solutions were precooled in an ice bath. Twenty microliters of sodium periodate solution (10 mM, Sigma-Aldrich) were added to 30 µl of glycoconjugate sample (10–200 µg) placed in a 2 mL polypropylene test tube. The solution was chilled in the ice bath for 45 min. The reaction was stopped by adding 100 µl of 50 mM sodium thiosulfate (Sigma-Aldrich), 500 µl of 4.0 M ammonium acetate (pH 7.5, Sigma-Aldrich) and 400 µl of an ethanolic solution of 100 mM acetoacetanilide (Sigma-Aldrich), followed by incubation for 10 min at room temperature. The fluorescence intensities of the solution were measured at 471 nm with an excitation wavelength of 388 nm. Detection limits of the determination were obtained according to the relative fluorescence of sample solution to a sample blank prepared under identical conditions. TSA content was normalized in relation to the creatinine content in the samples.

2.4. Synthesis of lectin functionalized magnetic nanoparticles (MNP@ConA; MNP@WGA; MNP@SNA)

Lectin functionalized ferromagnetic nanoparticles were synthetized as previously described by Ferreira et al. [15] and Cova et al. [16]. Briefly, the iron oxide magnetic core of the MNPs was synthesized by coprecipitation of $FeCl_2$ and $FeCl_3$ (Sigma-Aldrich) under alkaline conditions and coated with amorphous silica to prevent particle clustering and ensure their chemical stability. Encapsulation of the magnetic core was achieved by hydrolysis and condensation of tetraethyl orthosilicate (TEOS, Sigma-Aldrich) under alkaline conditions with trimethylamine (Sigma-Aldrich) as catalyst. Subsequently, the silica-coated nanoparticle surface was functionalized with amine groups with 3-aminopropyltrimethoxysilane (APS, Sigma-Aldrich) to reduce