The High Resolution MASS Spectrometry in Personalised Medicine: Retinol-Binding Protein 4 as a Candidate Biomarker Predictor of Progression in Bladder Urothelial Carcinoma

A Espectrometria de Massa de Alta Resolução na Medicina Personalizada: Proteína 4 de Ligação ao Retinol como Candidata a Biomarcador Preditor de Progressão no Carcinoma Urotelial da Bexiga

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Abstract:

Introduction: Bladder cancer (BC) is the seventh most commonly diagnosed cancer in males.

A large proportion of T1 cases and some Ta cases are under-staged and the significant risk of residual tumour after initial TURB of TaT1 lesions has been demonstrated.

A second TURB is recommended in T1 tumours because it can increase recurrence-free survival (RFS) providing prognostic information.

Non-invasive methods differentiating bladder cancer stages would be essential for diagnosis of under staged cases.

Previously we demonstrated that a panel of urinary proteins measured by high resolution mass spectrometry could predict bladder cancer stages namely Ta, T1 and T2 cases.

Our aim is to increase the accuracy of the biomarker panel for BC stage differentiation using a more patients, also intending to identify proteins that could be a signature to bladder cancer progression.

Methods: Forty-eight urine samples were collected from volunteers of these groups: 20 patients with bladder cancer stage Ta; 19 patients with BC stage T1 and 9 patients with BC stage T2+ (T2, T3 and T4). Urinary proteome was cleaned and digested using the Filter-Aided Sample Preparation methodology and analysed by liquid chromatography-mass spectrometry. For protein identification and label-free quantification we used MaxQuant, the data was further interrogated with the bioinformatics platform Perseus.

Results: A biomarker panel was developed which consists of 87 proteins which were down-regulated between three different urothelial bladder carcinoma stages evaluated. Retinol-binding protein 4 (RBP4) consistently increases from Ta to T1, to T2+ (p <0.001) and in high grade tumours (p = 0.006).

Conclusion: Our results showed a proteomic biomarker panel capable to differentiate bladder cancer stages. Besides, retinol--binding protein 4 can be a candidate signature of progression.

Keywords: Biomarkers, Tumor; Retinol-Binding Proteins, Plasma; Urinary Bladder Neoplasms

Resumo

Introdução: O carcinoma urotelial da bexiga é a sétima neoplasia mais comum dos homens. Uma proporção significativa de T1 e Ta são subestadiados e o risco de doença residual já foi demonstrado. Assim, a segunda RTU-V recomenda-se nos tumores T1 e está associada ao aumento da taxa livre de recidiva fornecendo informação prognóstica.

Por isso, métodos não invasivos, capazes de diferenciar os estadios assim como diagnosticar os casos subestadiados são cada vez mais necessários.

Num estudo prévio, desenvolvemos um painel de proteínas identificadas por espetrometria de massa de alta resolução capaz de diferencias os estadios Ta, T1 e T2+ (T2 a T4).

O objetivo deste estudo consiste em aumentar a eficácia deste painel em diferenciar os estadios do carcinoma urotelial da bexiga e associadamente identificar candidatos promissores a biomarcadores de progressão.

Métodos: Colheram-se 48 urinas de voluntários dos seguintes grupos: 20 doentes com carcinoma urotelial da bexiga Ta; 19 doentes com T1 e 9 doentes com T2 + (T2, T3 e T4). O proteoma urinário foi preparado usando a metodologia *filter-aided sample* e analisado através da cromotografia liquida e espetrometria de massa de alta resolução. Para a identificação e quantificação das

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proteínas utilizamos o MaxQuant e os resultados analisados pela plataforma bioinformática Perseus.

Resultados: Um painel de biomarcadores proteicos com 87 proteinas sobre e sub expressas nos diferentes estadios foi conseguido. A proteína de ligação ao retinol 4 aumentou consistentemente de Ta para T1 e T2+ ($p < 0,001$) e nos tumores de alto grau comparativamente aos de baixo grau ($p = 0,006$).

Conclusão: O estudo mostrou um painel de biomarcadores proteicos capaz de diferenciar os estadios do carcinoma da bexiga. Além disso, a proteína de ligação ao retinol 4 poderá ser um candidato promissor a biomarcador de progressão.

Palavras-chave: Biomarcadores Tumorais; Neoplasias da Bexiga Urinária; Proteínas Plasmáticas de Ligação ao Retinol

Introduction

Bladder urothelial carcinoma is one of the most frequent malignant tumours in the western world.[1]

After the TURB (transurethral resection of the bladder), the clinical staging of the tumour, based on the histology analysis, is fundamental in order to define the recurrence and progression risk, as well as, the therapeutic orientation.[2-6]

Unfortunately, a significant proportion of high grade non muscle invasive bladder carcinoma are understaged. In a meta-analysis, in a subgroup of 1565 T1 tumours with detrusor muscle present, persistent tumour was found in 58% and understaging occurred in 11% of cases.[7]

Non-invasive methods capable of diagnosing understaged tumours and prognostic biomarkers are becoming more important.

The high resolution mass spectrometry has come to revolutionise the method to identify new proteins as potential biomarkers.

Proteome analysis has taken a key role on the understanding of the carcinogenesis involved in bladder carcinoma and other diseases. Knowing that proteins have a close connection with the phenotype, translating cellular functions/processes, the usage of these as potential diagnostic biomarkers and therapeutic targets, has been developed over the years.[8]

On a previous pilot study, we had identified a panel of 83 candidate biomarkers for bladder cancer (BC) diagnostics and staging (Ta n=6, T1 n=6, T2+ n=6) with two control groups (LUTS patients n=6 and non-urinary condition patients n=6). Despite this breakthrough, the number of samples used in the pilot study was low (Fig. 1).[4]

The first biomarkers panel allows us to identify urothelial bladder carcinoma patients, whereas with the second panel we can identify the different stages Ta, T1 and T2+ (T2/T3/T4) patients.

The aim for this study consists in validating the panel for staging differentiation, increasing the number of patients and identifying the potential predictive biomarkers for progression.

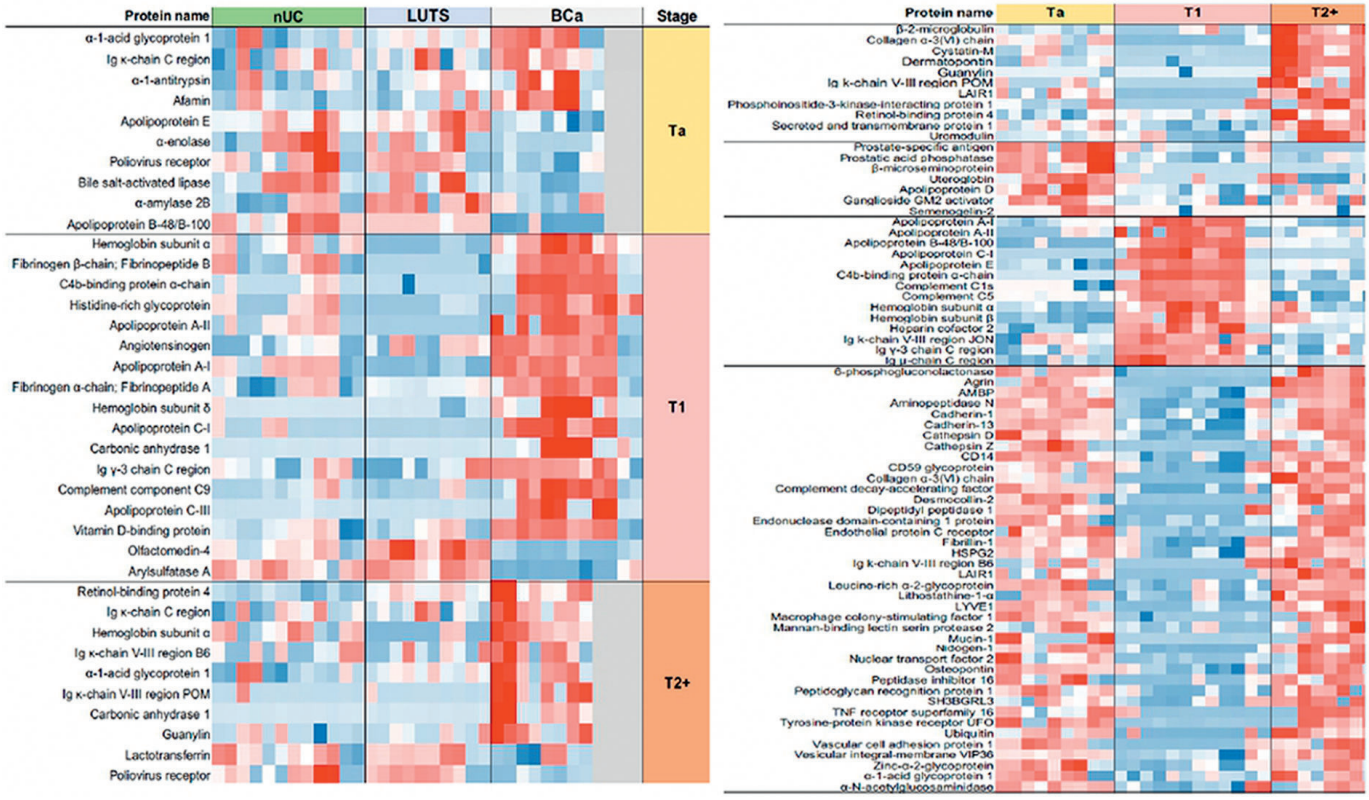

Figure 1 – Panel of biomarkers for diagnostic of bladder urothelial carcinoma and for staging differentiation

We also intended to find a protein that could identify very high risk of progression that would have the potential of being considered a signature of progression in specific patients.

Methods

Firstly, 48 urine samples were collected from voluntaries of the following groups:

Twenty patients with bladder carcinoma Ta, 19 patients with T1 tumours and 9 patients with T2/T3/T4 (T2+) tumours.

From the analysed urine, a treatment was conducted removing possible residuals by performing a low-speed centrifugation.

The total protein in the urine samples was quantified using a Bradford assay and stored at -60ºC.

After carrying out a bottom-up proteomic analysis, using the filter aided sample preparation (FASP) method which has been developed for rapid urine processing. The proteins were washed, reduced, alkylated and finally digested by the trypsin protease having a peptides sample as the final result.

The total amount of peptides was quantified by a colorimetric method and then analysed individually by Nano-liquid chromatography (Ultimate 3000 nano LC system, Bruker Daltonics) coupled with a quadrupole mass-spectrometer – Time-of-Flight (Impact HD, Bruker Daltonics) of high resolution.

After the peptides analysis it was possible, through the data processing, to identify the proteins and their relative quantification levels using MaxQuant software, statistically processed by Perseus software.

Results

A more significant biomarker panel can be seen in Fig. 2 which consists of 87 proteins that were up or down-regulated between the three different BC stages evaluated.

By acting together, the proteins are able to define a panel capable of differentiating the bladder urothelial carcinoma stages.

Despite the number of proteins with increased or decreased expression in the different stages, only a few of them increased consistently over the stages progression (Table 1, Fig. 2).

Retinol-binding protein 4 (RBP4) was one of the proteins whose average LFQ intensity consistently increases according to stage progression and it was the protein that has shown more significance in stage differentiation (ANOVA test, $p < 0.001$).

RBP4 also showed an association with the grade of tumour. (Mann-Whitney U Test, $p = 0.006$).

Other variables like “number of tumours”, “size of tumour”, “age” and “prior recurrence rate” didn´t show statistic association with RBP4 LFQ – intensity (Table 2).

Table 1 identifies the proteins that increase in LFQ intensity over the stages, as well as their function and the pathways involved.[9]

Discussion

Proteins reflect a molecular phenotype, have high potential as biomarkers, and also are key targets for intervention. Given the ease of collection and proximity to certain tumours, the urinary proteome is a rich source of biomarkers and several proteins have been already implemented.[8]

There are some studies that have identified proteomic biomarkers,[10] through proteomic analysis, for example Bryan *et al* that showed that EGFR (epidermal growth factor receptor), detected in the urine, was an independent indicator of poor prognosis in regards to specific survival of bladder carcinoma and in another study (Egloff *et al*) the activated leukocyte cell adhesion molecule (ALCAM) has revealed itself as an independent predictor of overall survival.[11,12] Unfortunately, these studies lack the necessary validation in order to be used in clinical practice.

Rafael Stroggilos *et al,* recently, classified non-invasive urothelial carcinoma based on its tissue proteome, through the analysis of 117 tissue specimens.[8]

Table 1 – Proteins that increase in LFQ intensity over the stages

Protein	Function	Pathways
Complement factor D (CFD)	Catalytic activity	Alternative complement activation; Platelet degranulation; Neutrophil degranulation;
Retinol-binding protein 4 (RBP4)	Transport activity	Retinol transport and metabolic process; Positive regulation of immunoglobulin secretion;
Beta-2-microglobulin (B2M)	Binding activity	Protein homodimerization activity Regulation of immune response
Proteoglycan 4 (PRG-4)	Binding activity	Polysaccharide binding; Scavenger receptor activity; Regulation of immune response;

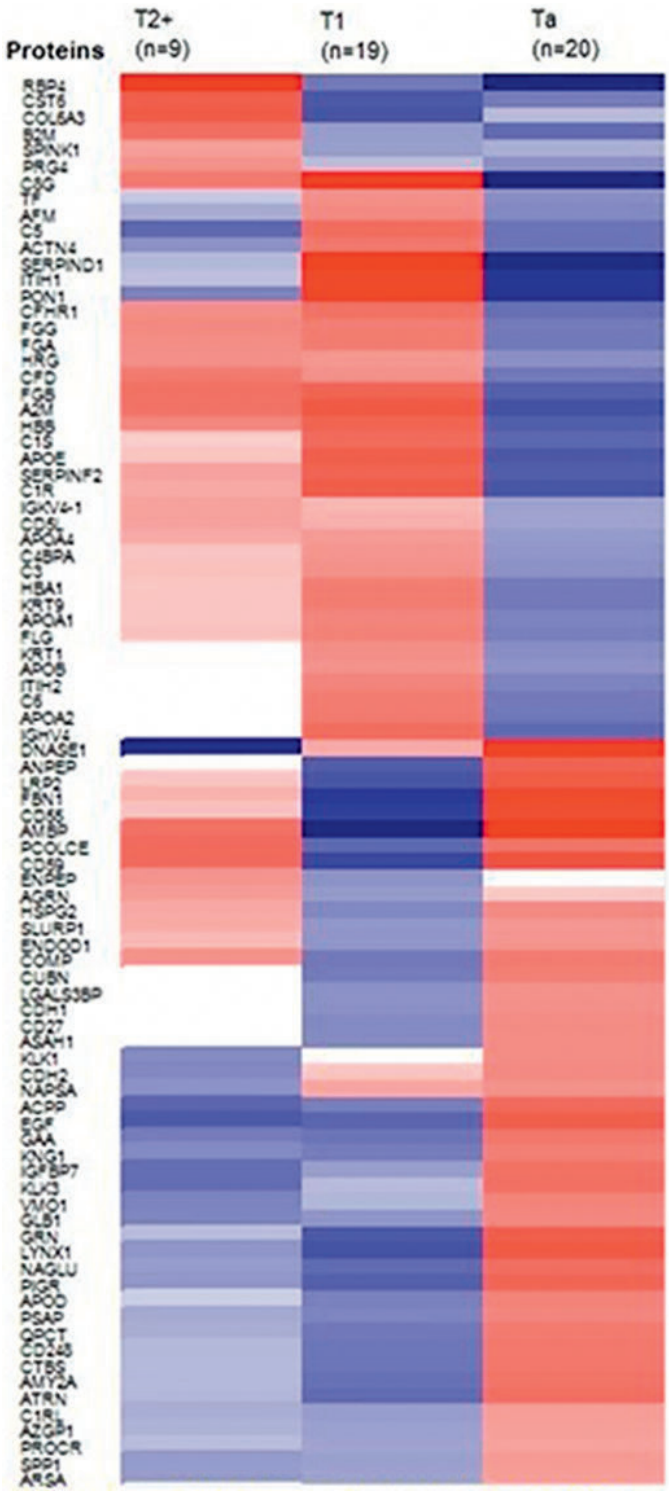

Figure 2 – Cluster analysis presentig the variation of protein levels between the different bladder urothelial carcinoma stages. ANOVA test and significant proteins were normalised by Z-scores and analysed by a Post hoc test FDR 0.05

In this study, it was found that the highest risk samples had an overexpression of proteins of immune/inflammatory phenotype,

Table 2 – Univariate analysis between RBP4 LFQ – intensity and demographic characteristics of patients.

Patients (n = 48)	RBP4 LFQ-intensity ($\bar{X} \pm \sigma$)	*P*-value
Age		
≤ 75 years (n =27)	(22.729 ± 0.163)	0.065
> 75 years (n = 21)	(23.412 ± 0.352)	
Tumour stage		
pTa (n = 20)	(22.353 ± 0.181)	< 0.001
pT1 (n = 19)	(23.1385 ± 0.210)	
pT2+ (n = 9)	(24.292 ± 0.589)	
Tumour grade		
Low grade (n = 20)	(22.413 ± 0.186)	0.006
High grade (n = 28)	(23.467 ± 0.258)	
Number of tumours		
1 (n = 23)	(22.533 ± 0.929)	0.089
≥ 2 (n = 15)	(23.070 ± 0.922)	
Tumour size		
< 3cm (n = 24)	(22.638 ± 0.211)	0.373
≥ 3cm (n = 14)	(22.928 ± 0.209)	
Prior recurrence		
Primary (n = 29)	(23.050 ± 0.268)	0.833
Recurrent (n = 19)	(22.994 ± 0.229)	

the intermediate risk samples had a mesenchymal/ infiltrative cell line profile and the lowes t risk had a luminal differentiation profile.

In fact, some of the proteins that increased in label free quantification (LFQ) intensity (Table 1), such as complement factor D (CFD), retinol-binding protein 4 (RBP4), proteoglycan 4 (PRG-4) and beta-2-microglobulin (B2M) act directly or indirectly on the immune response. As a matter of fact, cancer is an inflammatory pathology, and uses immune system characteristics to its advantage, namely, to promote tumour growth and to aid in tumour immune evasion.[13]

On the other hand, proteoglycan 4 (PRG-4) had binding activity. Also, the existence of dysregulations on the extracellular matrix organisation and on the aminoglycan process will affect the cell capacity to engulf and expel molecules. This characteristic is key for tumour cells.[14]

The retinol binding protein 4 (RBP4) increased consistently according to the progression of the different stages. This protein is involved in the transport of vitamin A in the blood and in the positive regulation of immunoglobulin secretion.

This protein mediates retinol transport in blood plasma, delivers retinol from the liver stores to the peripheral tissues and transfers the bound all-trans retinol to STRA6, that then facilitates retinol transport across the cell membrane.

This result is in accordance with other studies, because the overexpression of this protein had already been identified in the urine of patients with BC. It is a negative protein with acute inflammatory phase and its expression is associated with a poorer prognosis.[15,16]

Expression of STRA6 as well as RBP4 is upregulated in various human cancers, including colorectal and breast cancers. The discovery that STRA6 signalling triggered by holo-RBP4 activates a JAK2/STAT3/5 cascade provides a clue to the functional significance of the increased expression of these proteins in tumours. These STATs, considered to be oncogenes, are associated with inflammation, oncogenic transformation, survival, proliferation, invasion, and angiogenesis.[17]

The retinol overexpression characteristics in high risk cases and the fact of being consistently and progressively elevated gives the potential to become a signature of aggressive disease in some patients.

Despite the number of proteins with increased or decreased expression in the different stages, only a few of them increased consistently over the stages progression. This might have to do with the fact that genetic tumour processes change in the different stages and there are several molecular lines that are associated with disease progression which places great importance on the need to find specific progression biomarkers.

Certainly, more studies are necessary in order to investigate the association of these proteins in urine with the overall survival, recurrence-free survival and progression-free survival in a larger number of patients with bladder urothelial carcinoma.

Conclusion

Our results showed a proteomic biomarker panel with the potential to differentiate bladder cancer stages. Besides, retinol-binding protein 4 may be a signature that identifies progression.

Responsabilidades Éticas

Conflitos de Interesse: Os autores declaram a inexistência de conflitos de interesse na realização do presente trabalho.

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MM – Responsável por conceber e planear o estudo, redigir o artigo, sua submissão e revisão

JLCM, HMBCS, LABC – Responsáveis pela análise laboratorial e interpretação de resultados, edição de imagens e revisão de literatura, contribuindo com referências bibliográficas relevantes para apoiar a discussão

LCP – Orientação, supervisão do estudo e revisão do artigo

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