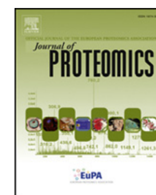
Journal of Proteomics 130 (2016) 26–32

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot



Urine proteome analysis in Dent's disease shows high selective changes potentially involved in chronic renal damage

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ARTICLE INFO

Article history:
Received 10 July 2015
Received in revised form 12 August 2015
Accepted 27 August 2015
Available online 11 September 2015

Keywords:
CLCN5 gene
Dent's disease
Fibrosis
Chronic renal failure
Metalloproteases
TIMPs

ABSTRACT

Definition of the urinary protein composition would represent a potential tool for diagnosis in many clinical conditions. The use of new proteomic technologies allows detection of genetic and post-trasductional variants that increase sensitivity of the approach but complicates comparison within a heterogeneous patient population. Overall, this limits research of urinary biomarkers. Studying monogenic diseases are useful models to address this issue since genetic variability is reduced among first- and second-degree relatives of the same family.
We applied this concept to Dent's disease, a monogenic condition characterised by low-molecular-weight proteinuria that is inherited following an X-linked trait. Results are presented here on a combined proteomic approach (LC-mass spectrometry, Western blot and zymograms for proteases and inhibitors) to characterise urine proteins in a large family (18 members, 6 hemizygous patients, 6 carrier females, and 6 normals) with Dent's diseases due to the 1070G > T mutation of the *CLCN5*.
Gene ontology analysis on more than 1000 proteins showed that several clusters of proteins characterised urine of affected patients compared to carrier females and normal subjects: proteins involved in extracellular matrix remodelling were the major group. Specific analysis on metalloproteases and their inhibitors underscored unexpected mechanisms potentially involved in renal fibrosis.
Biological significance: Studying with new-generation techniques for proteomic analysis of the members of a large family with Dent's disease sharing the same molecular defect allowed highly repetitive results that justify conclusions. Identification in urine of proteins actively involved in interstitial matrix remodelling poses the question of active anti-fibrotic drugs in Dent's patients.

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1. Introduction

Proteinuria is the direct biomarker of a kidney defect and may represent the unique symptom in several renal diseases. Practically, all renal diseases are characterised by proteinuria, and behind any generic consideration, it is believed that a good characterisation of urine proteins represents a necessary starting point in diagnosis [1,2]. Technology evolution in the field of protein analysis has implemented potentiality and strengthened the logics of the urinary approach. In fact, urine protein heterogeneity can be analysed in great details with new proteomic approaches based on mass spectrometry, urine fractionation, exosomes, etc. [3,4]. Variability in single protein structure due to genetic reasons and/or post-trasductional modification introduces a problem in studies that utilise proteinuria as biomarker. Studying monogenic diseases and large families sharing the same molecular defect as a model of investigation seems a possible approach to define main urinary features since inter-personal variability is reduced at the minimum.

Low-molecular-weight proteinuria is a specific field of application: it includes a spectrum of defects affecting the proximal renal tubule [5,6] and underscores the failure of tubular cells to re-absorb filtered proteins [7–9]. Dent's disease (OMIM 300009) is a typical example low-molecular-weight proteinuria that is inherited following an X-linked trait and manifests with features of proximal tubulopathy or Fanconi syndrome [10–13]. Nephrolithiasis and nephrocalcinosis with rickets are common symptoms and progressive renal failure may occur [14]

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http://dx.doi.org/10.1016/j.jprot.2015.08.024
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in which case severe tubulo-interstitial fibrosis and glomerulosclerosis are the pathology hallmarks of a degenerative conditions characterised by collagen accumulation [15,16].

Dent's disease is caused by mutation of *CLCN5* located on Xp11.22 and coding for the Cl^-/H^+ exchanger channel ClC-5 [17–19]. Alternative to *CLCN5*, a few patients present specific mutations in *OCRL1* at the 5′ region of the gene (exons 4–15) that are associated with a phenotype similar but less renal involving (Dent's 2) [20,21].

Mechanisms for renal symptoms of Dents' disease had not received unequivocal explanations. Human and animal studies have shown that impairment of ClC-5 is associated with loss of megalin and cubulin from the proximal tubule brush-border [13,22–24]; corresponding effects are loss of megalin/cubulin ligands (albumin, PTH hormone, vitamin D-binding protein) into urine [25], which explains low-molecular-weight protenuria and impairment of Ca^{2+} metabolism (nephrocalcinosis/rickets). Inactivation of lysososomes may further contribute to defective endocitosis and urinary loss of both lysosomal enzymes and LMWP. How alteration of ClC-5 and loss of megalin/cubulin are linked to glomerulosclerosis and tubulo-interstitial fibrosis is not clear. Since megalin and cubulin form the endocytic complex of the proximal tubules that fulfils absorption of proteins and delivery to lysosomes for degradation, it is conceivable that more ligands than expected are not reabsorbed and lost in urine that represents a potential key step in pathogenesis of degenerative renal lesions in Dent's.

We had the opportunity to characterise molecular and clinical features of members of the same three-generation large pedigree with Dent's overall including 18 subjects (6 affected, 6 carriers, and 6 non–affected); inter-familial variability as a source of error was reduced in this way. Results on urine protein composition were, in fact, highly repetitive and showed specific features of the disease.

2. Materials and methods

2.1. Materials

Acrylamide, Piperazine diacrylamide (PDA), N,N′-methylenebis-acrylamide (Bis), Ammonium Persulfate (APS), N,N,N′,N′-tetra-methyl-ethylenediamine (TEMED), Coomassie G-250, Sodium Dodecyl Sulphate (SDS), broad range molecular weight calibration kit, Protean II xi cell system, PD-Quest software analysis programme vs.8.3, VersaDoc 4000 and Molecular Imager GS-800 calibration densitometer were purchased from BioRad (Hercules, CA, USA). Complete protease inhibitor cocktail tablets were from Roche Diagnostics, (Basel, CH). Sequencing grade bovine trypsin was from Promega (Madison, WI, USA). All other chemicals of analytical grade were from J. T. Baker (Deventer, Holland). Monoclonal mouse anti-human MMP 2, anti-human MMP 3, anti-human MMP 9, anti-human TIMP 1, anti-human TIMP 2 antibodies were obtained from Immunological Sciences (Rome, Italy) and polyclonal rabbit anti-human transferrin, anti-human beta2-microglobulin, anti-human retinol-binding protein, anti-human alpha1-microglobulin antibodies were purchased from Dako (Copenhagen F., Dk); anti-mouse and rabbit peroxidise secondary antibodies were obtained from Immunological Sciences (Rome, Italy); nitrocellulose Protran membrane (Whatman, Boston, Ma); Super-Signal West Pico chemiluminescent Substrate (Pierce, Rockford, IL).

2.2. Sample collection and urine preparation

Second morning urines (approximately 50 ml) were collected and immediately added with tablets of protease inhibitor, chilled on ice, and centrifuged at 4 °C for 10 min at $1000 \times g$ to eliminate cell debris in accord to Standard Protocols. An aliquot, after Bradford protein assay, was dialysed three times against 25 mM sodium phosphate pH 7.4 in 3500 MWCO Spectra/Por® cellulose membranes at 4 °C. After lyophilisation, this material was stored at −80 °C until use.

The study was carried out in the frame of a large study on renal inherited diseases for which we had permission of the ethical committee of the Giannina Gaslini Institute.

2.3. Mass spectrometry

A linear Trap Quadropole (LTQ) Orbitrap Velos Pro Mass Spectrometry was used for analysis of all urinary samples. Samples were processed by in-Stage Tip method using enclosed Stage Tip, containing 2 poly (styrenedivinylbenzene) reverse phase sulfonate discs (SDB-RPS). Briefly, pellets were solubilised in 25 μL of 6 M guanidinium chloride (GdmCl), 10 mM tris(2-carboxyethyl)phosphine (TCEP), 40 mM 2-chloroacetamide (CAA), 100 mM Tris pH 8.5, reduced, alkylated then loaded into StageTip and diluted with 10% (*v*/*v*) acetonitrile (ACN), 25 mM Tris pH 8.5 containing 1 μg of lys-C. Following an overnight digestion at 37 °C, 0.5 μg of trypsin was added to the samples and a second digestion was performed. The samples were acidified with 100 μl of 1% (*v*/*v*) trifluoroacetic acid (TFA) and washed three times with 0.2% (*v*/*v*) TFA. Elutions were performed with 60 μl of 5% (*v*/*v*) ammonium hydroxide (NH_4OH), 80% (*v*/*v*) ACN [26].

The mass spectrometer operated in the positive ionisation mode. Single MS survey scans were performed in the Orbitrap, recording a mass window between 350 and 1650 m/z using a maximal ion injection time of 250 ms. The resolution was set to 60,000 and the automatic gain control was set to 1,000,000 ions. The lock mass option was enabled allowing the internal recalibration of spectra recorded in the Orbitrap by polydimethylcyclosiloxane background ions (protonated $(Si(CH_3)_2O))_6$; m/z 445.120025). The experiments were done in data-dependent acquisition mode with alternating MS and MS/MS experiments. The minimum MS signal for triggering MS/MS was set to 500 ions, with the most prominent ion signal selected for MS/MS using an isolation window of 2 Da. The m/z values of signals already selected for MS/MS were put on an exclusion list for 60 s using an exclusion window size of ± 10 ppm. In all cases, one micro-scan was recorded. CID was done with a target value of 5000 ions in the linear ion trap, a maximal ion injection time of 150 ms, normalised collision energy of 35%, a Q-value of 0.25, and an activation time of 10 ms. A maximum of 20 MS/MS experiments were triggered per MS scan.

2.3.1. Mass spectrometry data analysis

Raw MS files were processed with the Thermo Scientific Proteome Discoverer software version 1.3. Peak list files were searched by the MASCOT and SEQUEST search engine against Uniprot human database (Release 2012_07) containing both forward and reversed protein sequences. The resulting peptide hits were filtered for a maximum 1% FDR using percolator, the peptide mass deviation was set to 10 ppm and a minimum of six amino acids per identified peptide were required. The database search parameters were mass tolerance precursor 20 ppm mass tolerance fragment CID 0.8 Da with dynamic modification of deamidation (N, Q), oxidation (M) and static modification of alkylation with IAM (C). For all searches, the option trypsin with two missed cleavages was selected. Proteins were grouped by applying the maximum parsimony rule. The computational methods used to determinate the absolute protein quantification was the average of the three most abundant peptides.

2.4. Western blot analysis of proteases and inhibitors

For WB, proteins were trans-blotted to nitrocellulose membranes Protean BA (Schleicher & Schuell, Dassel, Germany) with a Novablot semidry system (GE Healthcare, Milan, Italy) using a continuous buffer system with 2-amino 2-idroxymethyl 1,3-propanediol, 38 mM tris, 39 mM glycine, 0.035% SDS, and 20% methanol. The transfer was achieved at 1.55 mA/cm^2 for 1.5 h.

Hybridisation was preceded by an overnight incubation at room temperature with a blocking solution of 3% BSA in TBS, 0.15% Tween. As a role, incubation with primary antibodies (i.e. monoclonal mouse

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