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Label-free mass spectrometric profiling of urinary proteins and metabolites from paediatric idiopathic nephrotic syndrome

Mirela Sedic [a], Lee A. Gethings [b], Johannes P.C. Vissers [b], John P. Shockcor [c], Stephen McDonald [c], Olga Vasieva [d], Maja Lemac [e], James I. Langridge [b], Danica Batinić [e], Sandra Kraljević Pavelić [a,*]

[a] Department of Biotechnology, University of Rijeka, Rijeka, Croatia
[b] Waters Corporation, MS Technologies, Manchester, United Kingdom
[c] Waters Corporation, Milford, MA, USA
[d] Department of Functional and Comparative Genomics, Institute of Integrative Biology, University of Liverpool, United Kingdom
[e] Division of Nephrology, Department of Pediatrics, Zagreb University Hospital Centre, Zagreb, Croatia

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ABSTRACT

Idiopathic nephrotic syndrome (INS) is caused by renal diseases that increase the permeability of the glomerular filtration barrier without evidence of a specific systemic cause. The aim of the present work was to reveal inherent molecular features of INS in children using combined urinary proteomics and metabolomics profiling. In this study, label-free mass spectrometric analysis of urinary proteins and small molecule metabolites was carried out in 12 patients with INS *versus* 12 sex- and age-matched control subjects with normal renal function. Integration and biological interpretation of obtained results were carried out by Ingenuity IPA software. Validation of obtained proteomics data was carried out by Western blot method. Proteomics data have been deposited to the ProteomeXchange Consortium with the data set identifier PXD000765. This study indicates for the first time that paediatric INS is associated with up-regulation of afamin, hydroxyphenylacetate and uridine, and concomitant down-regulation in glutamine and phenylalanine levels, and many of these molecular species were previously shown to be involved in oxidative stress. Further studies in larger patient population are underway to investigate the role of oxidative stress in renal injury in paediatric INS.

1. Introduction

Nephrotic syndrome (NS) can be generally divided into primary (idiopathic nephrotic syndrome, INS) reflecting glomerular diseases intrinsic to the kidney and unrelated to any systemic cause, and secondary NS that can be induced by several different pathological conditions (e.g. autoimmune and vasculitic diseases, infectious diseases, etc.). INS is considered the most common form of NS in children, wherein more than 90% of cases are between 1 and 10 years of age, and around 50% after 10 years of age [1]. Major clinical features that characterise INS develop as a consequence of compromised integrity of the glomerular filtration barrier, in particular altered cell morphology and motility of podocytes, terminally differentiated cells that line the outer portion of the glomerular basement membrane. Although the molecular trigger for the onset of INS still remains largely unknown, several lines of evidence demonstrate that altered T-lymphocyte responses could play a central role in the INS pathogenesis. It has been postulated that T cells produce a circulating permeability factor that interferes with the expression, function or both of the key podocyte proteins to induce proteinuria [2]. However, the nature of this circulating factor is not known, and many cytokines and inflammatory molecules have been implicated including interleukins, Interferon-γ, TGF-β, vascular permeability factor, NF-κB and TNF-α.

Urinary proteomics has garnered much attention lately as a novel tool for biomarker discovery in kidney diseases, because urine can be collected easily, in large amounts and non-invasive manner. Additionally, urinary proteome closely mirrors pathological changes associated with the function of the kidney and the urogenital tract, and is generally more stable than the blood proteome [3]. Similarly, metabolomics has emerged as a valuable analytical platform for identification of urinary and serum biomarkers for renal diseases. The comprehensive investigation of the metabolome has thus proved as an important tool to study phenotype and changes in phenotype triggered by environmental factors, pathological condition, or alterations in genotype [4].

* Corresponding author. Address: Department of Biotechnology, University of Rijeka, Radmile Matejčić 2, 51000 Rijeka, Croatia. Fax: +385 51 584 599.
E-mail address: sandrakp@biotech.uniri.hr (S.K. Pavelić).

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A limited number of previous proteomics studies in paediatric idiopathic nephrotic syndrome were directed towards the identification of either differentially expressed proteins in urine and plasma during NS compared with remission, or of predictive biomarkers for steroid therapy response in the treatment of idiopathic nephrotic syndrome. Currently, there is neither a report on the urinary proteomics nor on the metabolomics profiling to discriminate between paediatric idiopathic nephrotic syndrome patients and healthy subjects with functional kidneys to identify inherent molecular features of INS. In the present study, we applied for the first time a combined -omics approach using label-free mass spectrometry-based profiling of urinary proteins and metabolites to reveal molecular signatures underlying INS and identify putative pathogenic factors of this disease.

2. Material and methods

2.1. Patients

Twelve subjects with INS and twelve sex- and age-matched patients with normal renal function (control subjects) were recruited at Division of Nephrology, Department of Paediatrics, Zagreb University Hospital Centre, Croatia (Supplementary Table 1). Diagnosis of INS was established by standard laboratory tests and histological examinations of renal biopsy specimens. Both, INS and control patients did not receive any therapy prior to the commencement of this study. The University Hospital Centre Zagreb Ethical Committee approval was received for the study, and the informed consent of children's parents was signed. First morning urine samples were collected from all subjects using sterile urine containers, portioned into 1 ml aliquots to avoid freeze/thaw cycles in repeated experiments of the same sample, and stored at −20 °C for further use.

2.2. Urinary metabolomics analysis

For metabolomics analysis, urine samples were purified using Oasis HLB solid phase extraction (SPE) cartridges (Waters Corporation). SPE was performed according to manufacturer's instructions with slight alterations using 1 ml of paediatric urine as a starting amount. Water/methanol (90/10) washes were performed followed by neat methanol analyte elution. The resultant eluent solutions were evaporated to dryness using a vacuum centrifuge concentrator, reconstituted in 200 μL aqueous 0.1% formic acid solution and vortexed prior to LC–MS analysis. The chromatographic separation of the metabolite samples was conducted with an ACQUITY system equipped with a 1.7-μm bridged ethylene hybrid (BEH), 10 cm × 2.1 mm C18 column (Waters Corporation) and the column was maintained at 45 °C. The metabolites were resolved with a 10 min gradient from 10% to 50% acetonitrile (0.1% formic acid) at 500 μL/min. 5 μL of each sample was injected in triplicate on column and analysed in a random order. A quality control (QC) sample was created from a pool of all samples and injected every tenth injection. MS data were acquired in positive ion mode using a hybrid ion mobility-oaToF Synapt G2 mass spectrometer (Waters Corporation) operated in v-mode of analysis with a resolving power of 20,000 FWHM. Data were real-time lock mass corrected using the singly charged precursor ion of Leu-Enkephalin, which was acquired with a sampling frequency of 30 s. The capillary and cone voltages were 4 kV and 40 V, respectively. Accurate mass data were collected in a data-independent acquisition (DIA) mode [5,6] by alternating the energy applied to the collision cell between a low and elevated state. In low energy MS mode, data were collected at constant collision energy of 4 eV (per unit charge). In the elevated energy mode, the collision energy was ramped from 15 eV to 40 eV (per unit charge) during each integration. The spectral acquisition time in each mode was 0.18 s with a 0.02 s interscan delay. One cycle of low and elevated energy data was acquired every 0.4 s. The quadrupole mass analyser was operated in non-resolving mode and the LC-DIA-MS acquisition range from 20 to 1000 *m/z*. The metabolomics LC–MS data were aligned and normalized using CoMet (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom). Total ion current normalization was conducted using a two group experimental design with each group the data from thirty-six (patient)/thirty-three (control) (three technical replicates per twelve (patient)/eleven (control) subject samples) LC-DIA-MS runs. The complete data set was imported into EZinfo (Umetrics, Umeå, Sweden) for multivariate statistics, using orthogonal partial least squares-discrimination analysis (OPLS-DA) to examine the multidimensional data and identify group differences. Pareto scaling was used in which each variable was centered and multiplied by $1/\sqrt{S_k}$, where S_k is the standard deviation of the variable. Identification of major metabolic perturbations within the pattern recognition models was achieved by analysis of the corresponding contrasting loadings OPLS-DA results plots. Identification was based on accurate mass and fragmentation spectra using a combination of compound databases (Human Metabolite Database version 3.0 and ChEBI 2012).

2.3. Urinary proteomics analysis

For proteomics analysis, urine samples for protein digest analysis were prepared as previously described with minor modifications using 1 ml of sample as a starting amount [7]. The samples were treated with 1% RapiGest at 80 °C for 45 min prior to reduction and alkylation. The proteins were reduced in the presence of 5 mM dithiothreitol at 60 °C for 30 min and alkylated in the dark with 10 mM iodoacetamide at room temperature for 30 min. The aliquots were incubated with anti-HSA resin and centrifuged using Vivaspin 5,000 MWCO filters (Millipore, Billerica, MA). A series of washes using water were implemented to ensure adequate recovery followed by a final wash with 0.1% RapiGest in 50 mM ammonium bicarbonate (pH 8.5). The supernatants were collected and proteolytic digestion initiated by adding modified trypsin at a ratio of 1:10 (w:w) followed by incubation overnight at 37 °C. The RapiGest surfactant was hydrolyzed by the addition of 2 μl of trifluoroacetic acid to the sample. The samples were incubated at 37 °C for 30 min, centrifuged for 30 min at 13,000 rpm and the supernatants collected. Label-free LC–MS was used for qualitative and quantitative protein digest analyses. The experiments were conducted using a 90 min gradient from 5% to 40% acetonitrile (0.1% formic acid) at 300 nL/min using a nanoACQUITY system and a BEH 1.7 μm C18 reversed phase 75 μm × 20 cm nanoscale LC column (Waters Corporation). Prior to separation, peptides were focused and desalted on a 5 μm Symmetry C18, 180 μm × 2 cm trapping cartridge (Waters Corporation). 1 μL of each sample was injected in triplicate on column and analysed in random order. The column was interfaced to the source of the mass spectrometer, which was maintained at 70 °C, using an electrospray ionization emitter tip (New Objective, Woburn, MA). MS data were acquired in positive ion mode using a hybrid ion mobility-oaToF Synapt G2 mass spectrometer, operated in v-mode of analysis with a resolving power of 20,000 FWHM. Data were post-acquisition lock mass corrected using the doubly charged precursor ion of [Glu^1]-Fibrinopeptide B, which was acquired with a sampling frequency of 60 s. The capillary and cone voltages were 3.5 kV and 30 V, respectively. Peptide data were acquired in ion mobility assisted data independent analysis (IM DIA) mode [8]. In low energy MS mode, data were collected at constant collision energy of 4 eV (per unit charge). In the elevated energy mode, the collision energy was ramped from 15 eV to 40 eV (per unit charge) during each integration. The spec-