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Proteomic Approach for Identification of IgA Nephropathy-Related Biomarkers in Urine

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Summary

Proteinuria is often used as a surrogate marker in monitoring and predicting outcome in patients with chronic kidney diseases, but it is non-specific. IgAN belongs to the most common primary glomerulonephritis worldwide with serious prognosis. The main aim of this work was to assess differences in urine proteins in patients with IgA nephropathy and to identify abnormal proteins as potential biomarkers of IgA nephropathy or the renal disease. In our pilot project, we selected 20 patients and compared them with 20 healthy volunteers. Protein quantification was performed using iTRAQ (isobaric tag for relative and absolute quantitation) labeling method. The peptides were separated by the isoelectric focusing method (IEF) and nano-LC with C18 column and identified by mass spectrometry using MALDI-TOF/TOF MS. Proteins´ lists obtained from IEF-LC-MS-MS/MS analysis were combined and contained 201 proteins. It was found out that 113 proteins were common in both experiments. 30 urinary proteins were significantly up- or down-regulated in patients with IgA nephropathy. We characterized potential biomarkers such as alpha-1-antitrypsin, apolipoprotein A-I, CD44 antigen or kininogen. Potential biomarkers of IgAN should be validated in further studies.

Key words

Urine proteomics • Kidney disease • IgAN • LC-MS/MS

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Introduction

IgA nephropathy (IgAN) is the most common primary glomerulonephritis worldwide. Diagnosis of IgAN is based on the demonstration of mesangial IgA-dominant staining (by immunofluorescence or by immunohistochemistry).

Among the most important clinical predictors of renal outcome in IgAN belong proteinuria, hypertension, and decreased estimated glomerular filtration rate at the time of the diagnosis as well as histological grading (Reich *et al*. 2007, Lee *et al.* 2012). The Oxford classification of IgAN identified mesangial hypercellularity, endocapillary proliferation, segmental glomerulosclerosis, and tubular atrophy/interstitial fibrosis as independent predictors of outcome (Alamartine *et al.* 2011). Three risk factors at biopsy, 24-h urinary protein excretion ≥1.0 g, hypertension (>140/90 mm Hg), and severe histological lesions, are significantly associated with dialysis or death (Berthoux *et al.* 2011).

Proteins and polypeptides play an important role in our bodies as structural elements, enzymes, inhibitors, neurotransmitters, or hormones. A deeper insight into the functional relevance of different polypeptides under

different physiological and pathophysiological conditions is one of the main challenges in proteome research. The proteome is the entire set of proteins expressed by a genome, cell, tissue or organism at a certain time. Proteome changes, alterations in the entire set of polypeptide concentrations or modifications at a given time point under defined conditions; reflect normal biological and pathological processes. Traditionally, biomarker discovery was hypothesis driven, hence related to extensive biochemical research to characterize pathological processes for identification of potential biomarker candidates. In contrast, the fast-paced technical advancement over the last decades, especially in mass spectrometry and computer sciences, initiated a paradigm shift into the direction of hypothesis-free multi-parametric profiling approaches for biomarker discovery. These techniques provide a patient's protein profile of a specific intra- and inter-cellular compartment under different physiological and pathophysiological conditions. The analysis of the human urinary proteome for diagnostic purposes has reached a technical level with the potential to revolutionize early disease diagnosis, drug discovery, and sensitive monitoring of a response to therapeutic intervention. Due to non-invasive sampling and high pre-analytical stability urine provides several advantages among the clinically important and available body fluids: urine is easily and non-invasively accessible in large quantities. Sample instability is less an issue compared to other body fluids, such as serum or plasma. Standardized protocols for urine sampling to control various pre-analytical influences, such as sampling conditions, storage, freeze-thaw cycles, bacterial interferences, and handling are available. For urinary proteomic profiling, a crude unprocessed urine sample would be ideal. However, the presence of interfering compounds, such as salts or lipids, often limits this approach. The enormous complexity of the urinary proteome prevents its proteomic analysis in a single mass spectrometric step without additional separation to increase overall analytical resolution.

In our study, we focused on patients with IgAN and stable renal function assessed by the level of serum creatinine, eGFR level, and daily proteinuria excretion compared with healthy volunteers. Peptides were separated and identified using nano-LC, mass spectrometry MALDI-TOF/TOF MS and Orbitrap Fusion Tribrid mass spectrometer. Undoubtedly, renal biopsy still plays the crucial role in the diagnosis of IgAN. However, the development of noninvasive diagnostic tests could be useful for detection of subclinical types of IgAN, evaluation of disease activity, monitoring disease progression and assessment of treatment effectiveness. Consequently, these tests will be useful for assessing the activity or prognosis.

Methods

Urine samples

The study included twenty patients with IgA nephropathy and the same number of healthy age and sex matched volunteers as control samples. Clinical data of patients with nephropathy and healthy individuals are summarized in Table 1.

Table 2 presents demographic and clinical data of both groups. Adult patients with biopsy-proven IgA nephropathy were included. The inclusion criteria for the control group were as follows: age older than 18 years and absence of any kidney diseases or other chronic diseases requiring treatment. Patients with active infection, history of malignancy, previous organ transplantation, or current pregnancy were excluded from both groups. The glomerular filtration rate was estimated by the Chronic Kidney Disease Epidemiology Collaboration equations (Levey *et al.* 2009).

The study protocol was approved by the local ethics committee and informed consent was obtained from all participants. The study was performed in accordance with the Declaration of Helsinki Principles.

Sample preparation, digestion and iTRAQ labeling

The second morning midstream urine was collected to sterile urine containers. Samples were then vortexed, centrifuged at 3,000 × g at room temperature for 15 min to clear the debris and pipetted into 1 ml aliquots and stored at -80 °C before further use.

Thawed aliquots were desalted and concentrated on membrane filters of the 3 kDa cut-off (Amicon Ultra-2, Millipore, Billerica, United States). Filters were first passivated with 5 % Tween-20 aqueous solution and washed with deionized water prior to use. Urine was centrifuged through the membrane at 4,000 × g for 30 min. Next, the retentate was washed by 2,000 µl of deionized water and centrifugation step was repeated. Bradford assay was used to determine overall protein concentration and aliquoted samples were stored at -80 °C.

Table 1. Clinical data of patients with nephropathy and healthy individuals.

Patient number	Age	Sex	U_proteinuria (g/mmol_crea)	S_creatinine (μmol/l)	U_creatinine (mmol/l)	Hg hemoglobin (g/l)	CRP (mg/l)	Diagnosis
1	36	F	0.007	56	2.8	114	1.4	IgA nephropathy
2	32	F	0.050	65	4.7	146	3.6	IgA nephropathy
3	33	M	0.070	79	9.2	163	1	IgA nephropathy
4	48	M	0.020	98	20.9	155	1	IgA nephropathy
5	48	M	0.070	102	22.1	151	1	IgA nephropathy
6	27	F	0.060	223	2.2	121	4.3	IgA nephropathy
7	67	M	0.007	109	11.9	123	*	IgA nephropathy
8	34	M	0.011	111	17.0	143	4.1	IgA nephropathy
9	60	F	0.043	124	9.6	153	3.9	IgA nephropathy
10	34	M	0.114	132	12.9	159	4	IgA nephropathy
11	50	M	0.054	133	8.51	144	2.3	IgA nephropathy
12	39	M	0.081	161	9.3	157	2.0	IgA nephropathy
13	37	M	0.032	164	11.5	158	6.0	IgA nephropathy
14	51	M	0.133	170	9.7	156	3.8	IgA nephropathy
15	32	M	0.050	176	9.1	129	9.3	IgA nephropathy
16	68	M	0.137	181	11.4	148	2.2	IgA nephropathy
17	46	F	0.220	57	5.7	*	1	IgA nephropathy
18	29	M	0.110	92	15.0	*	1	IgA nephropathy
19	21	M	0.170	104	7.6	*	1	IgA nephropathy
20	43	M	0.280	149	4.2	*	1	IgA nephropathy
21	57	F	0.010	91	10.7	137	2.5	healthy control
22	55	F	0.003	90	14.3	123	1.3	healthy control
23	67	F	0.017	85	8.4	129	2.1	healthy control
24	47	M	0.016	121	17.2	156	1.3	healthy control
25	45	M	0.020	122	3.0	152	1.3	healthy control
26	48	M	0.007	114	14.2	143	3.2	healthy control
27	50	F	0.006	77	8.8	137	1	healthy control
28	54	F	0.003	76	8.6	134	1	healthy control
29	50	M	0.005	99	15.2	148	2.8	healthy control
30	56	M	0.009	93	5.7	148	2.2	healthy control
31	49	M	0.023	70	1.7	148	1	healthy control
32	60	M	0.006	91	12.5	157	2.2	healthy control
33	50	M	0.003	91	10.1	152	2.7	healthy control
34	53	M	0.005	88	16.9	151	1	healthy control
35	44	M	0.006	64	5.3	145	1.8	healthy control
36	65	M	0.011	80	6.2	156	3.2	healthy control
37	47	M	0.008	84	11.9	154	1.1	healthy control
38	48	M	0.002	82	8.5	162	1.1	healthy control
39	42	M	0.004	80	15.9	147	7.8	healthy control
40	50	F	0.010	57	22.9	135	3.2	healthy control

* Data not available. Proteinuria in urine was measured using the pyrogallol red by turbidimetry, determination of the substance concentration of creatinine using photometric Jaffe method without deproteinization on analyzer Modular. Hemoglobin was measured by photometry, determination of the mass concentration of CRP using immunoturbidimetric method. F – female, M – male.

Table 2. Characterization of the studied group including renal function.

Parameters	Study group	Control group
n	20	20
Male/female (%)	15 (75 %)/5 (25 %)	14 (70 %)/6 (30 %)
Mean age in years (range)	43.5 (21-67)	51.9 (42-67)
Mean serum creatinine in μmol/l (range)	122 (56-149)	74.3 (59-90)
GFR in ml/min (range)	57.3 (23-106)	87.8 (57-122)
Mean proteinuria g/l	0.75	0.08

Fig. 1. The work design: 8 pooled samples analyzed using 2 IEF strips in two LC-MS/MS experiments.

IEF/LC-MS/MS experiment	**1**				**2**			
iTRAQ label	114	115	116	117	114	115	116	117
Pooled sample	HC IA	HC IB	IgA IA	IgA IB	HC IIA	HC IIB	IgA IIA	IgA IIB

Twenty IgAN samples and twenty healthy control samples were randomly divided into two biological replicates, disease pooled samples IgA I and IgA II, and control pooled samples HC I and HC II. The aliquots (corresponding to 10 μg of protein) of 10 urine samples were pooled to obtain 100 μg of total protein content in each sample group. In addition, two technical replicates of each HC and IgA were prepared, denoted as A or B to control intragroup technical variability (Fig. 1).

Pooled samples were reduced, alkylated, digested with trypsin and labeled with 114, 115, 116 and 117 iTRAQ chemistry according to the manufacturer's instructions (AB Sciex, USA). Four labeled samples 114-115 HC and 116-117 IgA were then combined and the volume of the final sample was reduced to 40 μl in a vacuum concentrator (Eppendorf, Hamburg, Germany). In total, two independent analyses of the IgA IA, IgA IB, HC IA and HC IB samples were performed including digestion, labeling and IEF/LC-MS/MS analysis (Fig. 1).

IEF-IPG of peptides

For each of the two LC-MS/MS experiments 2 IgAN and 2 HC iTRAQ-labeled samples were combined, evaporated to 40 μl and then 360 μl of rehydratation buffer was added [8 M urea, 1 % IPG buffer pH 3-11 NL (GE Healthcare, Little Chalfont, United Kingdom), 0.002 % bromophenol blue in 50 mM Tris-HCl, pH 8.0]. The solution was applied to 18 cm IPG strip with pH 3-11 NL gradients (GE Healthcare) for isoelectrofocusing (IEF). The IPG strip was rehydrated overnight in an IPG box (Bio-Rad, Hercules, California, USA). The next day, the strips were isoelectrofocused using a Protean IEF Cell system (Bio-Rad) as follows: Stage_1: 250V ↑ 6 h, Stage_2: 2000V ↑ 18 h, Stage_3: 6000V ↑ 26 h; 22 μA/gel.

After focusing, strips were briefly washed in water, cut into 15 pieces and peptides were extracted from individual strip pieces into 150 μl of 80 % acetonitrile with 0.5 % trifluoroacetic acid, for one hour at room temperature. The volume of all fractions was reduced to 5-10 μl by evaporation in a vacuum concentrator. Aliquots with extracted peptides were desalted on 100 μl C18 OMIX tips (Agilent, Santa Clara, California, USA) and stored at -80 °C for LC-MS/MS analysis.

Nano-LC/MALDI-MS/MS

LC-MALDI analyses were performed on the EASY-nLC II system (Bruker Daltonics, Billerica, Massachusetts, USA) coupled to the PROTEINEER fc II fraction collector (Bruker Daltonics). Extracted post-IEF fractions were individually loaded (max. 10 ug) onto a trapping EASY-Column (L 2 cm, ID 100 μm, 5 μm, 120 Å, ReproSil-Pur C18-AQ) using water containing 0.1 % trifluoroacetic acid as the mobile phase and the EASY-Column (L 10 cm, ID 75 μm, 3 μm, 120 Å, ReproSil-Pur C18-AQ) using an acetonitrile gradient (2-45 % acetonitrile over 145 min) in the presence of 0.1 % trifluoroacetic acid with a flow rate of 300 nl/min. The separated peptides were mixed with the α-cyano-4-

hydroxycinnamic acid MALDI matrix directly in the Proteineer fc II and spotted onto a MALDI target plate AnchorChip 384 TF (Bruker Daltonics) with an interval of four spots per minute and 384 fractions were collected. Spectra were acquired on an autoflex II MALDI TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a nitrogen laser (337 nm, frequency 50 Hz). All spots were first measured in MS mode from m/z 800 to 4,000 and then up to 15 strongest precursors were selected for MS/MS analysis. The MALDI-TOF/TOF instrument was operated in the positive ion mode and controlled by the Compass for Flex software, version 1.3 (FlexControl 3.0, FlexAnalysis 3.0, Bruker Daltonics). The spectrometric analysis was performed in an automatic data-dependent mode. The non-redundant precursor peptides were selected for MS/MS using the WARP-LC 1.2 software (Bruker Daltonics) with a signal-to-noise threshold of 15. The MS spectra were externally calibrated using the Peptide Calibration Standard mixture (Bruker Daltonics).

Mass spectrometry data processing

The acquired MS/MS spectra were recalibrated with PeptideShaker version 1.3.2. Mass spectrometry data from both parallel analyses were merged and processed as a single dataset. Protein identification and quantitation were performed using Protein Pilot 5.0 with the Paragon algorithm (AB Sciex, Framingham, Massachusetts, USA) (Shilov *et al.* 2007). Protein identification was conducted against a concatenated target/decoy version of the Homo sapiens complement of the UniProtKB (version of 01-Nov-2015, 20193 (target) sequences) with the following settings: trypsin digestion, methyl methanethiosulfonate modification of cysteines, iTRAQ 4-plex labeled peptides, default iTRAQ isotope correction settings, quantification, bias correction, biological modifications and thorough ID parameters selected. The detected protein threshold (unused protein score and confidence of results) was set to 2.0 and 99.0 % and false discovery rate analysis was enabled. Proteins sharing a set of peptides were grouped automatically with the default Pro Group™ Algorithm. Ratios of iTRAQ were calculated with default Protein Pilot setting, protein fold change (iTRAQ ratio for an individual protein) was calculated automatically by the Protein Pilot software as a weighted average of Log iTRAQ ratios determined for individual peptides belonging to the particular protein after background subtraction. To estimate the false discovery rate (FDR) a decoy database search was performed. The fold-change of differentially expressed proteins (p-value <0.05) was calculated as the average value from the protein iTRAQ ratios reported by Protein Pilot. To be considered as differentially expressed, individual proteins with at least 2 peptides had to fulfill the following statistical criteria p-value <0.05 and EF (error factor) <2.

Western blotting

Pooled urinary protein samples (10 μg) were separated on 4-12 % Bis-Tris minigel in MOPS SDS running buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA). Electrophoresis was performed at a constant voltage 200 V. Proteins were then transferred to PVDF membrane (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) in wet Mini Blot module from ThermoFisher Scientific at a constant voltage 20 V. Membrane was blocked for one hour in 5 % milk in tris-buffered saline containing 0.1 % Tween 20, and incubated overnight with the primary antibody in 5 % milk. Rabbit primary antibodies against human uromodulin (1:6000) from BioVendor, Brno, Czech Republic and human α-1-antitrypsin (1:1000) from Sigma were used. Anti-rabbit horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) was added to the membrane for one hour and a signal was detected using a chemiluminescent luminol-based substrate (Cell Signaling Technology, Danvers, Massachusetts, USA).

Results

In our pilot project, we analyzed protein composition of urine in 20 patients with IgAN and matched healthy control group using combined IEF-LC-MS-MS/MS relative quantitation of iTRAQ labeled tryptic peptides. We chose the methodology of partial pooling strategy to overcome difficulty with a huge number of LC-MS-MS/MS runs.

Two independent labeling and separation experiments resulted in a total 30 LC-runs, collecting nearly 33,000 MS/MS spectra. Mass spectrometry data from both parallel analyses were merged and processed as a single dataset by Protein Pilot software. At high confidence, we identified 201 individual proteins with a false discovery rate (FDR) below 1 %. For the differential expression analysis, we considered only those proteins that were identified with at least two peptides, each peptide with at least 95 % confidence. The statistical

analysis of the quantitative results of the two proteomic experiments revealed 30 proteins that were significantly differentially expressed (with p-value <0.05) in the urine of patients with IgAN as compared with healthy controls, 9 of which were downregulated and 21 were upregulated.

These proteins were quantified with $p<0.05$ (Table 3) as calculated by Protein Pilot software based on two-tailed t-tests where the degree of freedom is equal to the number of distinct peptide minus one.

Table 3. Proteins differentially expressed in urines of IgAN patients versus healthy controls with p-value <0.05 (IgAN vs. control) obtained by iTRAQ-IEF-LC-MALDI-TOF/TOF MS approach. P-value <0.05 was calculated by Protein Pilot 5.0 software based on two-tailed t-tests where the degree of freedom is equal to the number of distinct peptides minus one.

Protein ID	Protein Name	Fold change iTRAQ-IEF-LC-MALDI-TOF/TOF	Regulation
ALBU_HUMAN	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2	4.19*	upregulated
A1AT_HUMAN	Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3	3.56*	upregulated
A1BG_HUMAN	Alpha-1B-glycoprotein OS=Homo sapiens GN=A1BG PE=1 SV=4	3.50*	upregulated
IGHA1_HUMAN	Ig alpha-1 chain C region OS=Homo sapiens GN=IGHA1 PE=1 SV=2	2.76*	upregulated
TRFE_HUMAN	Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=3	3.38*	upregulated
APOA1_HUMAN	Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1	2.13*	upregulated
VTDB_HUMAN	Vitamin D-binding protein OS=Homo sapiens GN=GC PE=1 SV=1	2.72*	upregulated
HBB_HUMAN	Hemoglobin subunit beta OS=Homo sapiens GN=HBB PE=1 SV=2	2.51*	upregulated
A1AG1_HUMAN	Alpha-1-acid glycoprotein 1 OS=Homo sapiens GN=ORM1 PE=1 SV=1	2.24*	upregulated
AFAM_HUMAN	Afamin OS=Homo sapiens GN=AFM PE=1 SV=1	2.06*	upregulated
IGHG2_HUMAN	Ig gamma-2 chain C region OS=Homo sapiens GN=IGHG2 PE=1 SV=2	2.41*	upregulated
HBA_HUMAN	Hemoglobin subunit alpha OS=Homo sapiens GN=HBA1 PE=1 SV=2	2.26*	upregulated
A1AG2_HUMAN	Alpha-1-acid glycoprotein 2 OS=Homo sapiens GN=ORM2 PE=1 SV=1	2.19*	upregulated
A2GL_HUMAN	Leucine-rich alpha-2-glycoprotein OS=Homo sapiens GN=LRG1 PE=1 SV=2	2.19*	upregulated
IGLL5_HUMAN	Immunoglobulin lambda-like polypeptide 5 OS=Homo sapiens GN=IGLL5 PE=4 SV=2	1.94*	upregulated
IGHG1_HUMAN	Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1	1.87*	upregulated

CO3_HUMAN	Complement C3 OS=Homo sapiens GN=C3 PE=1 SV=2	1.79*	upregulated
CERU_HUMAN	Ceruloplasmin OS=Homo sapiens GN=CP PE=1 SV=1	1.76*	upregulated
HPT_HUMAN	Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1	1.75*	upregulated
IGHA2_HUMAN	Ig alpha-2 chain C region OS=Homo sapiens GN=IGHA2 PE=1 SV=3	1.69*	upregulated
C9JV77_HUMAN	Alpha-2-HS-glycoprotein OS=Homo sapiens GN=AHSG PE=1 SV=1	1.66*	upregulated
PEPA3_HUMAN	Pepsin A-3 OS=Homo sapiens GN=PGA3 PE=1 SV=1	0.22*	downregulated
AMPN_HUMAN	Aminopeptidase N OS=Homo sapiens GN=ANPEP PE=1 SV=4	0.45*	downregulated
UROM_HUMAN	Uromodulin OS=Homo sapiens GN=UMOD PE=1 SV=1	0.74*	downregulated
ITIH4_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4 OS=Homo sapiens GN=ITIH4 PE=1 SV=4	0.27*	downregulated
MASP2_HUMAN	Mannan-binding lectin serine protease 2 OS=Homo sapiens GN=MASP2 PE=1 SV=4	0.51*	downregulated
KNG1_HUMAN	Kininogen-1 OS=Homo sapiens GN=KNG1 PE=1 SV=2	0.65*	downregulated
FINC_HUMAN	Fibronectin OS=Homo sapiens GN=FN1 PE=1 SV=4	0.64*	downregulated
CD44_HUMAN	CD44 antigen OS=Homo sapiens GN=CD44 PE=1 SV=3	0.55*	downregulated
EGF_HUMAN	Pro-epidermal growth factor OS=Homo sapiens GN=EGF PE=1 SV=2	0.46*	downregulated

The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System was used to classify proteins (and their genes) in order to facilitate high-throughput analysis. Proteins have been classified according to: *Family and subfamily*: families are groups of evolutionarily related proteins; subfamilies are related proteins that also have the same function; *Molecular function*: the function of the protein by itself or with directly interacting proteins at a biochemical level, e.g. a protein kinase; *Biological process*: the function of the protein in the context of a larger network of proteins that interact to accomplish a process at the level of the cell or organism, e.g. mitosis; *Pathway*: similar to biological process, but a pathway also explicitly specifies the relationships between the interacting molecules. Most of our found proteins are involved in metabolic processes, cellular processes and in the pathway of blood coagulation. According to the protein class most belongs to the enzyme modulators, hydrolases, or transfer/carrier proteins (Fig. 2).

We found out, that our 30 studied proteins are especially involved in the complement and coagulation systems and regulation of actin cytoskeleton. List of 30 proteins with the most significant changes in quantity compared to healthy control selected as potential biomarkers of IgAN are marked in Table 3, where 9 of them were downregulated and 21 upregulated. The quantity of alpha-1-antitrypsin and serotransferrin was increasing more than three times, on the other hand, the quantity of pepsin decrease more than seven times. The data were evaluated at the significance level $p<0.05$ as a statistically significant for the difference between the quantities of proteins in patients with IgAN compared to healthy controls.

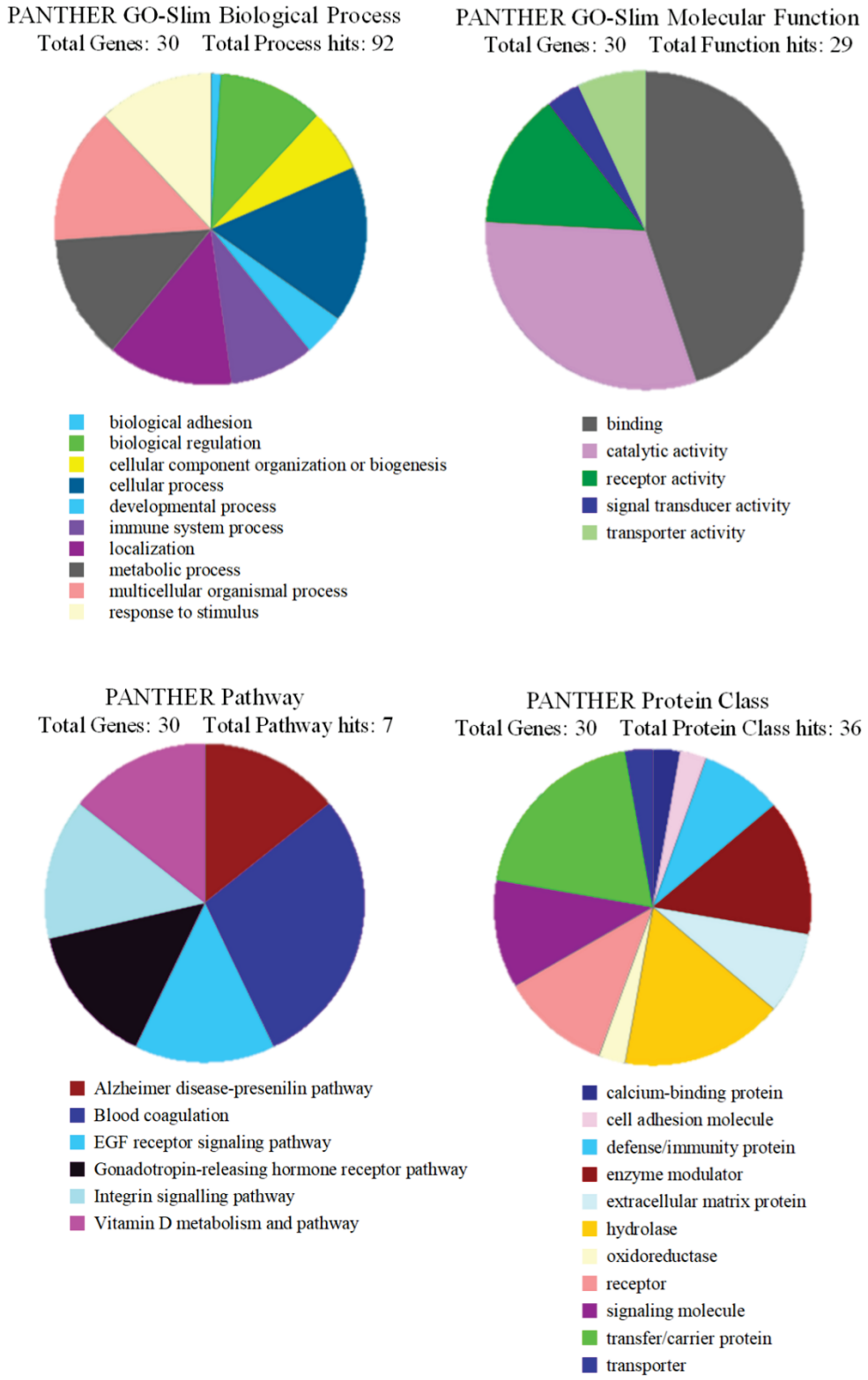

Fig. 2. The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System.

We further verified our results by Western-blotting analysis of two proteins with potential biomarker relevance uromodulin and α-1-antitrypsin (Fig. 3). The results confirm the downregulation of uromodulin and upregulation of α-1-antitrypsin identified by proteomics.

We established proteomic methodology to identify potential urine biomarkers of kidney diseases. Study of changes in urinary proteins could help to establish the diagnosis, assess the disease activity and/or predict the prognosis of different renal diseases.

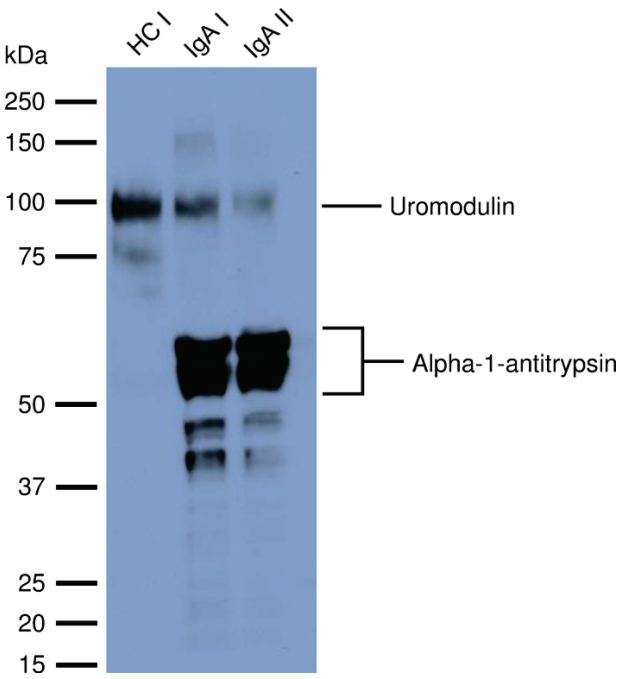

Fig. 3. Western blotting confirmation of the expression changes of human uromodulin and human α-1-antitrypsin in the pooled urinary protein samples. Ten micrograms of total protein content were loaded per lane. Detection of uromodulin is reduced and a signal of α-1-antitrypsin is enhanced in the IgA nephropathy patient samples (IgA I, IgA II) in comparison with healthy controls (HC I). Lines denote the uromodulin- and α-1-antitrypsin-specific bands and protein molecular weight markers.

Discussion

The aim of our study was to evaluate urine proteomics of patients with IgAN. Recently identified biomarkers are related to the pathogenesis of IgAN (Gd-IgA1, IgG and IgA anti-Gd IgA1 Ab, sCD89, urinary sTfR) (Suzuki *et al.* 2009, Novak *et al.* 2007, Boyd and Barratt 2010, Vuong *et al.* 2010, Delanghe *et al.* 2013) or to the degree of renal damage in IgAN (e.g. IL-6/EGF, MCP-1/EGF ratio) (Ranieri *et al.* 1996, Torres *et al.* 2008).

Glycoproteins play the crucial role in cell-to-cell interaction and urinary losses of glycolipoproteins (such as alfa-1-microglobulin and beta-2-microglobulin) are known to be a sign of tubular damage (Woo *et al.* 1997, Woo *et al.* 1981). The role of apolipoproteins as risk factors for progression of IgAN was demonstrated (Lundberg *et al.* 2012). Also, apolipoprotein A-I was found as a urinary biomarker for progression of IgAN (Julian *et al.* 2007). The upregulated urinary levels of apolipoprotein A-I in advanced forms of patients with IgAN were found (Florquin *et al.* 2002), while in the recent study (Kalantari *et al.* 2013) decreased urinary excretion of apolipoprotein A was demonstrated in patients with IgAN. Contrary to this, our study demonstrated apolipoprotein A-I upregulation in IgAN patients with stable renal function.

Looking at the pathogenesis of IgAN, specific immune complexes made of GdIgA1-IgG and GdIgA1-IgA1, before reaching the kidneys can bind to the CD89 receptor on the monocytes/macrophages cells in the circulation (Launay *et al.* 2000). This tri-molecular complex can bind directly to the transferrin receptor (CD71) in the mesangium (Moura *et al.* 2004), initiating locally the expression of a new molecule the transglutaminase 2 (Berthelot *et al.* 2012) with a local amplification loop (increased expression of CD71 receptors with more IgA1 deposition). Recent data indicated soluble transferrin receptor (sTfR) as an important marker for progression of IgAN (Delanghe *et al.* 2013, Maixnerova *et al.* 2016). Median levels of urinary sTfR were demonstrated to be higher in patients with active IgAN or Henoch-Schoenlein purpura nephritis and urinary concentration of sTfR were reported to correlate with proteinuria (Delanghe *et al.* 2013). In our study, the upregulated urinary levels of serotransferrin were detected in accordance with the results of another study (Moon *et al.* 2011).

Recent studies pointed to complement factors as possible biomarkers of IgAN due to their role in the pathogenesis of the disease (Maillard *et al.* 2015, Schmitt *et al.* 2014). The glomerular IgA1-containing immune complexes cause local activation of the complement system (Maillard *et al.* 2015, Schmitt *et al.* 2014), proliferation of mesangial cells, production of extracellular matrix and cytokines (e.g. tumor necrosis factor-α, transforming growth factor-β) (Novak *et al.* 2011), which could alter podocyte gene expression and glomerular permeability (Lai *et al.* 2009). This mesangio-podocyte injury might explain proteinuria and tubulointerstitial changes in IgAN. The level of urinary mannan-binding lectin was significantly associated with renal function and proteinuria in a Chinese study of 162 patients with IgAN (Liu *et al.* 2012). Significantly lower level of mannan-binding lectin at the time of renal biopsy in patients with IgAN was associated with better clinical outcome and histological renal findings (Liu *et al.* 2012) which was in accordance with the results in our group of IgAN patients with stable renal parameters.

Another study indicated urinary IL-6/EGF ratio (interleukin-6/epidermal growth factor ratio) as a useful prognostic marker of the progression of IgAN (Ranieri *et*

al. 1996). Patients with the ratio of IL-6/EGF >1 showed advanced renal insufficiency. Urinary IL-6/EGF ratio was related to the severity of the disease and also predicted renal outcome. The ratio of proinflammatory chemokines of EGF/MCP-1 (monocyte chemotactic protein) in the urine was related to the severity of histologic lesions and predicted renal prognosis in 132 patients with IgAN (Torres *et al.* 2008). Our study of IgAN patients with preserved renal function at the time of renal biopsy showed downregulated urinary levels of pro-epidermal growth factor compared to healthy controls.

Uromodulin, produced by the thick ascending limb of the loop of Henle (Obara *et al.* 2012), might serve as a unique renal regulatory glycoprotein specifically bounded to and regulated a number of potent cytokines, including IL-1 and tumor necrosis factor.

Higher urinary levels of IgA and IgA-IgG complex in patients with IgAN were confirmed (Matousovic *et al.* 2006). It was shown that the IgA-uromodulin complex could be a good clinical diagnostic marker of IgAN (Obara *et al.* 2012). The value of the IgA-uromodulin complex tended to be higher not in inactive IgAN without microhematuria but in the earlier phase of the disease with an inflammatory activity (Obara *et al.* 2012). Other studies (Wu *et al.* 2010, Graterol *et al.* 2013) identified a fragment of uromodulin in urine samples from patients with IgAN compared to healthy controls and patients with other glomerulonephritides (Wu *et al.* 2010). In our group of IgAN patients with preserved renal parameters, urinary levels of uromodulin were downregulated compared to healthy controls.

It is hypothesized that IgAN patients with higher levels of galactose-deficient IgA1 in the circulation, and/or IgG and IgA antibodies, and/or with higher levels of urinary biomarkers, and/or more of risk genetic variants will have worse prognosis, and, consequently, will need close follow up and more aggressive treatment to control clinical risk factors (proteinuria and hypertension). The patients with substantial disease activity (based on the clinical and histological findings) could through an early initiation of immunosuppressive treatment achieve deceleration of the progression of renal function to end stage renal disease. These clinical improvements will ultimately reduce costs otherwise needed to cover renal replacement therapy (hemodialysis, peritoneal dialysis, kidney transplantation).

To date, a whole range of potential promising biomarkers of the most common primary glomerulonephritis was ascertained. Undoubtedly, recently identified biomarkers must be validated in larger cohorts of patients and could be introduced into broader clinical practice only if shown to provide a better estimate of renal outcome of IgAN.

Conflict of Interest

There is no conflict of interest.

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