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Proteomic approaches in the discovery of potential urinary biomarkers of mucopolysaccharidosis type II

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ABSTRACT

Mucopolysaccharidosis type II (MPS II) is a rare lysosomal storage disorder caused by deficient or absent activity of the iduronate-2-sulfatase (IDS) enzyme, which leads to pathological accumulation of the glycosaminoglycans(GAGs). The absence of early diagnosis can result in irreversible developmental, neurological, and physiological damage. The lack of clear understanding of the etiology of physiological dysfunction in MPS II has been a major obstacle to the development of new treatment. Therefore, a reliable biomarker for early diagnosis and exploration of pathogenic mechanism are of great importance. Proteomics provides powerful tool for protein expression alterations and study of complicated pathological process. This study was performed to identify the differential protein profile in urine of MPS II patients using two-dimensional gel electrophoresis(2D-PAGE) combining with MALDI-TOF/TOF and a total of 15 differentially expressed proteins were identified. Content of alpha1-antitrypsin, Gm2 activator and lipocalin-type prostaglandin D synthase was measured by ELISA method. The value of urinary α1-AT/Cr in MPS II group was 0.79 ± 0.10 mg/mmol, significantly higher than 0.42 ± 0.05 mg/mmol in healthy control group; whereas the value of GM2A/Cr and L-PGDS/Cr in MPS II group was 1.30 ± 0.12 μg/mmol and 9.86 ± 1.16 ng/mmol respectively, which was significantly lower than 2.19 ± 0.19 μg/mmol and 13.98 ± 1.48 ng/mmol in healthy control group. The proteins can be considered as accessory diagnostic biomarkers for MPS II. This approach helped to discover early diagnostic markers and provided a better understanding of the pathogenic mechanism of MPS II.

1. Background

Mucopolysaccharidosis type II (MPS II), also known as Hunter disease, is a rare lysosomal storage disorder caused by deficient or absent activity of the iduronate-2-sulfatase(IDS) enzyme, which leads to pathological accumulation of the glycosaminoglycans(GAGs), e.g., heparan sulfates(HS) and dermatan sulfte(DS), in any tissues and organs [1]. MPS II affects 0.3–0.75 in 100,000 live birth [2]. The widespread accumulation of GAGs results in cellular damage, multiple organ failure and reduced life expectancy. The main clinical symptoms, including coarse facial features, short statue, hepatosplenomegaly and mental retardation, do not appear immediately after birth but grow progressively with age [3]. Even though MPS II patients may exhibit great variations in the age of onset, symptoms and severity, the average life expectancy of most patients is one to two decades without treatment. The absence of early diagnosis, especially in infants, can result in irreversible developmental, neurological, and physiological damage [4]. Thus, identification of simple, reliable early diagnosis biomarkers is of undeniable importance.

However, up until now, there is no simple and rapid test for the diagnosis of MPS II. Urinary GAG analysis, the most common conventional screening test for MPSs, suffers from a few drawbacks. The instability of GAGs could lead to inaccurate result. The test itself provides limited information on the improvement of disease pathology [5]. Moreover, the etiology of physiological dysfunction in MPS II is unclear, which hampers the development of new treatment. For all reasons above, GAGs are not considered as an ideal biomarker, and there is a great need for simple, reliable biomarker for early diagnosis and exploration of pathogenic mechanism.

The rapid development and wide application of proteomics provides powerful tool for protein expression alterations clarifications and for the study of complicated pathological process [6]. For its advantages of high throughput, high sensitivity and wide detection range,2D-PAGE is one of the main applied methods in proteomics for protein separations.

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Furthermore, it is able to identify proteins and protein isoforms that may be neglected by other methods [7]. Combing 2D-PAGE with mass spectrometry is a common approach for screening of biomarkers, but it has not been used in analyze MPS II urine sample analysis.

This study was performed to identify the differential protein profile in urine of MPS II patients using 2D-PAGE combining with MALDI-TOF/TOF and a total of 20 differentially expressed proteins were identified. This approach helped to discover early diagnostic markers and provided a better understanding of the pathogenic mechanism of MPS II.

2. Materials and methods

2.1. Samples

Urine samples were collected from MPS II patients attending the genetics clinic at PLA General Hospital ($n = 12$) from March to December in 2017. Patients were confirmed by elevated urine GAGs and reduced IDS activity. The urinary GAG was measured using the Dimethylene Blue assay, while the IDS activity was measured in leukocytes using fluorogenic assay. The tests were performed according to the previous literature [2]. Control samples were collected from healthy volunteers with matched gender and age($n = 12$). Mid-stream of the second morning urine were collected by subjects using clean urine container and stored at −80 °C. All test subjects in both groups provided urine samples voluntarily with informed consents.

2.2. 2D-PAGE and image analysis

Six urine samples were randomly selected from each gorup. They were then mixed and centrifuged to remove cellular debris, and the urinary protein was then isolated by cold acetone precipitation after dialysis. Protein concentrations were determined using the Bradford assay. Before separation, the samples were diluted to 450 μl with 9 μl DTT, 2.25 μl IPG buffer(GE Healthcare)and rehydration buffer(7 M urea, 2%CHAPS, 0.002% bromphenol bule). In isoelectric focusing, proteins were separated on 24 cm pH 3–10 IPG strips and focused at ≤ 50 μA per strip at 20 °C, using progressively increasing voltage for a total of 80kVh. After equilibration and alkylation, the strips were laid on SDS-PAGE gel, and the run were performed at constant voltage (4 W). Each sample was processed in triplicate to reduce variability and detect differences with real statistical significance. Images of the stained gels were taken and scanned with laser densitometer, and then analyzed using ImageMaster 7.0. Spot volumes of the desired spots were normalized using the standard spots that were present in all gels, to calculate the relative density of a spot in a sample. Dunnett's test was used to assess whether corresponding spots in different groups were different.

2.3. In-gel tryptic digestion and mass spectrometry

Protein spots of interest were selected based on a fold increase or decrease of ≥1.5 and p-value < 0.05. Selected spots were destained followed by in-gel tryptic digestion. The gel pieces were washed for 30 min with destaining solution containing 100 mM $Na_2S_2O_3$. For the dehydration step, the gel pieces were rinsed in 100% acetonitrile for 15 min and dried with lyophilizer equipment. After incubated in trypsin on ice for 45 min, gel pieces were digested at 37 °C over night. Peptides were then extracted and run on a MALDI TOF/TOF(Bruker ultrafleXtreme™ MALDI TOF/TOF). MS of the digested peptides was done in positive ion mode. Autotryptic and common keratin peaks were excluded from the analysis. The mass spectra were acquired over a mass range of 1000–4000 Da. Most intense peptides from each spot were subjected to MS/MS analysis and the data was searched against NCBI databases using MASCOT. The database search parameters included one missed cleavage, error tolerance of ± 100 ppm for PMF and ± 0.3 Da for MS/MS ion search.

2.4. ELISA analysis

Three of the significantly changed proteins, alpha1-antitrypsin, Gm2 activator and lipocalin-type prostaglandin D synthase, were further analyzed using ELISA kits obtained from Cloud-Clone Corp (SEB687Hu,SEL122Hu,SEB640Hu) and following the instructions of the manufacturer. After preparation, 100 μl standard and samples were added to each well and incubated at 37 °C for 1 h. Then 100 μl Detection Reagent A(first antibody) was added per well and incubated at 37 °C for 1 h. Following 3 times washes for 5 min in wash buffer, 100 μl Detection Reagent B (secondary antibody) was added per well and incubated at 37 °C for 30 min. After 5 times washes for 5 min, Substrate Solution and Stop Solution were added for color development and termination reaction. All standards and samples were measured with microplate reader at a wavelength of 450 nm. Urine creatinine in every sample was measured in the meantime.

2.5. Statistical analysis

All data were presented as the mean ± standard deviation (SD). The significant differences were statistically assessed by one-way analysis of variance (ANOVA) followed by Dunnett's test, and the difference was considered statistically significant when $P < .05$.

3. Result

3.1. 2D-PAGE of urinary samples

Urinary samples from MPS II patients and healthy individuals were collected to identify differentially expressed protein by 2D-PAGE combined with MALDI-TOF/TOF.All 3 silver-stained gels of each group were run to ensure reproducibility (Fig. 1). A total of 25 spots with a fold change ≥ 1.5 and a p-value < 0.05 between MPS II and controls were selected for in-gel digestion(Table 1).

3.2. Identification of differentially expressed proteins

From 2D gels, 25 spots were analyzed by MALDI-TOF/TOF and 15 proteins were found to exhibit differential expression in the urine samples of patients and control (Table 1). 7 proteins such as alpha1-antitrypsin were up-regulated in MPS II patient; while 8 proteins such as lipocalin-type prostaglandin D synthase were down-regulated. Several representative gel image of identified proteins spots and their relative quantitative value is shown in Fig. 2.

3.3. Confirmation of proteomics data using ELISA method

Contents of alpha1-antitrypsin, Gm2 activator, lipocalin-type prostaglandin D synthase were measured by ELISA method, in order to validate the densitometric data. The results were normalized using protein-to-creatinine RATIO. The value of urinary α1-AT/Cr in MPS II group was 0.79 ± 0.10 mg/mmol, significantly higher than 0.42 ± 0.05 mg/mmol in healthy control group; whereas the value of GM2A/Cr and L-PGDS/Cr in MPS II group was 1.30 ± 0.12 μg/mmol and 9.86 ± 1.16 ng/mmol respectively, which were significantly lower than 2.19 ± 0.19 μg/mmol and 13.98 ± 1.48 ng/mmol in healthy control group(Fig. 3). This result was in concordance with proteomics examination. Receiver operating characteristic (ROC) curvewas drawn, and the area under the curve (AUC), contrast sensitivity and specificity were analyzed(Table 2 and Fig. 4). The AUC of AAT/Cr, GM2A/Cr and L-PGDS/Cr was 0.833, 0.896 and 0.750, respectively.

4. Discussion

The current laboratory diagnosis of MPS II disease includes analysis of urinary GAG species, enzymatic tests or gene sequencing. Unlike

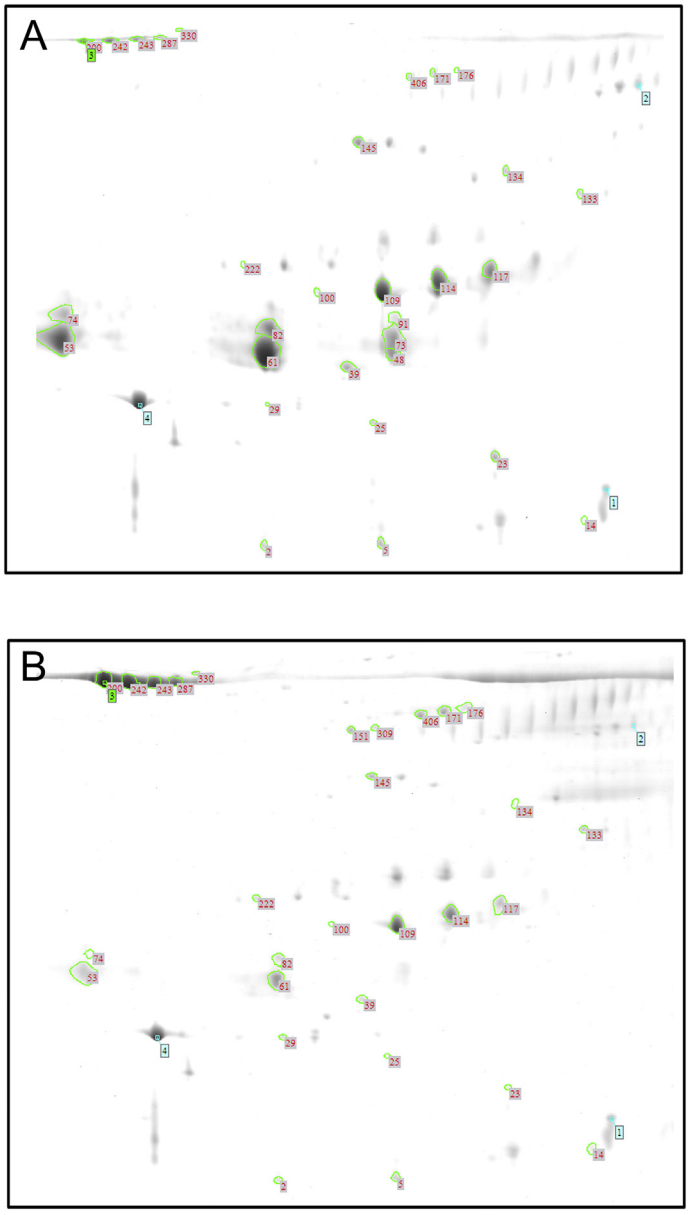

Fig. 1. Representative 2D gels of urine samples.(A) MPSII patients sample,(B) control sample. Spots of differentially expressed potein are outlined.

traditional dye-binding assays, several methods have been developed to separate specific GAGs, including gas chromatography and high-performance liquid chromatography. Although laboratories are moving to higher throughput LC-MS based systems for quantitative GAG analysis, these are still complicated assays to develop and interpret [8]. In order to make GAGs suitable targets for mass spectrometry, they must first be enzymatically or chemical cleavage after long incubation periods using expensive reagents. Moreover, the enzymes may not completely degrade the total amount of GAGs molecules [9].As definitive diagnosis, enzymatic test is the most reliable method to confirm MPSII. Using fluorometric and LC-MS/MS based methods to detect IDS enzyme activity had been described in previous publications. However, the former requires long incubation times and excess purified α-iduronidase; the substrate of the latter are not commercially available. Moreover they both have disadvantage of low conversion of the substrates(0.1 to 1 μl/L/h) for healthy control samples [10]. To date, about 600 different IDS gene mutations have been detected [2]. Gene polymorphism creates barrier to identify two clearly pathogenic mutation in molecular diagnosis [11]. Therefore, we investigated potential urine protein markers that could provide additional information to diagnose this disorder. Furthermore, the identification of markers for biological systems affected in MPS II would provide evidence for pathological and physiological mechanism of the disease, for which current methods furnish no such useful information.

Only a few studies have been performed to identify biomarkers that can be used to detect pathological mechanisms associated with MPS. Heparin cofactor II–thrombin complex is proved to be a biomarker using serum proteomic studies, which appears to correlate with disease severity [12,13]. Several researches also identified differentially expressed proteins in the murine MPS models [6,14,15]. However, there is still no recognized protein marker for MPS as chitotriosidase for Gaucher disease(another lysosomal storage disorder). The only report of urinary proteomics of MPS II is that Heywood and his team analyzed urine samples of MPS I, II and VI patients using label-free quantitative proteomics and targeted multiplexed peptide MRM LC-MS/MS assay [16]. Urine samples have many advantages over blood samples in proteomics analysis. Collecting urine samples is a simple and non-invasive procedure without causing any discomfort, so that samples can

Table 1
Identification of differentially expressed proteins using MALDI/TOF/TOF. in urine samples of MPSII patients.

Spot[a]	Protein name	[b]Protein MV (kd)	Matched peptide number	[c]Masccot score	[d]Fold change	*P-value*
2,5	Secreted and transmembrane 1 precursor variant(SECTM1)	27,277	9	332	−1.62	0.007
14	Chain A, solution structures of The Fn3(Fibronectin type 3) Domain Of Human Ephrin Type- B Receptor 1(EPHB1)	11,842	4	45	2.87	0.0008
23	Chain A, Human Gm2 Activator Structure(GM2A)	17,994	7	164	−6.41	0.00009
39	Immunoglobulin kappa 1 Light Chain(IGKC1)	23,523	18	457	−3.54	0.001
48 , 53 , 61 , 73 , 74 , 82 , 91	Crystal Structure Of The Human Lipocalin-type Prostaglandin D Synthase Crystallised(L-PGDS)	19,939	15	475	8.95	0.00009
100 , 117	Protein AMBP preproprotein	39,886	17	334	−1.56	0.0007
109	Complex-forming Glycoprotein HC(A1M)	20,592	3	103	−2.14	0.002
114	Chain A, Crystal Structure Of Protein Hc From *Homo sapiens* At 2 Angstrom	22,757	18	504	−2.28	
133	Chain A, Structure Of Recombinant Alphaec Domain From Human Fibrinogen-420(FIB)	22,924	9	146	1.74	0.01
134	Chain B, Human Zinc-Alpha-2-Glycoprotein(ZAG)	31,854	20	338	−4.08	0.009
145	Similar to Gelsolin (amyloidosis, Finnish type)(GSN)	31,052	20	344	−2.01	0.025
171	Alpha1-Antitrypsin(AAT)	44,280	33	607	5.78	0.0002
200	Albumin-like(ALB)	53,416	5	313	14.95	0.01
242 , 243 , 287	Chain A, Structure Of Human Serum Albumin With S-Naproxen And The Ga Module	654	43	654	12.97	0.001
330	Lysosomal alpha-glucosidase preproprotein(GAA)	106,112	5	131	6.36	0.008

[a] Spot number as given in 2-DE gels.
[b] MW = Molecular Weight.
[c] Masccot score higher than 67 were considered significant.
[d] Positive fold changes represent an up-regulation while negative values represent a down-regulation of protein expression in urine of patients.

be repeatedly collected for a period of time to monitor disease progression and estimate treatment effect. As urine remains in bladder for hours, almost all endogenous proteolytic activity is completed before voiding, making it a relatively stable specimen [17]. Moreover, small proteins and polypeptides in urine can be directly analyzed, free from removing high abundance proteins and hydrolysis. Furthermore, urinary GAG test is a routinely screening test of MPS, so using the same type of sample will be convenient.

The biomarker discovery method used in this study is combined 2D-PAGE with mass spectrometry, which is a common proteomic approach for high throughput screening of potential biomarkers, but it has not been used in MPS II urine. Only proteins found in spots with a p value < 0.05 and a fold change ≥ 1.5 were considered significant, in order to ignore the artificial errors and select the real difference. This study was performed on just a small cohort of MPS II samples because the MPS disorders are so rare that large number of samples are impossible to collect [16]. Even so, we were able to find 25 differential protein spots and identify 15 corresponding differentially expressed proteins. Mascot computes a score based on the probability that the peptides from a sample match those in the selected protein database. The more peptides Mascot identifies from a particular protein, the higher the Mascot score for that protein. Thus, all of the identified proteins had the relatively highest Masccot score. Masccot score higher than 67 were considered significant. The average values of Masccot score was 329.8 while the number of the matched piptide was 15.07. It proved that the results are reliable. In order to further verify the results, the significant change of alpha1-antitrypsin, Gm2 activator and lipocalin-type prostaglandin D synthase were confirmed by ELISA assay. The high sensibility and the AUC illustrated by the ROC curve > 0.5 indicates good validity of each prediction model in detecting MPS II. The specificity of three biomarkers may not ideal due to the small sample size. However, this finding still confirmed that markers of the underlying pathological features of this disease can be detected in patient's urine.

As one of an acute phase reaction protein, alpha1-antitrypsin has many biological activities. The elevation of alpha1-antitrypsin in urine samples of MPS II patients may be caused by inflammation reaction. Inflammatory processes triggered by activation of immune effectors has been found in several LSDs including MPS [18–20]. Although the precise mediators of the inflammatory response are still unknown [21], it is known that GAGs have proinflammatory properties related to the stabilization and release of cytokines and their oligomerization to achieve their biological action, as well as to cellular signaling processes [22]. One mechanism proposed for the trigger of inflammatory response in MPS diseases is through activation of the TLR4 receptor pathology. HS resemble the LPS, and other incomplete GAG degradation products, which can bind to TLR4 receptor that cause the secretion of pro-inflammatory cytokines and trigger an innate immune response In turn, its accumulation in the extracellular fluid could activate this receptor [23]. Animal models of various MPSs exhibiting accumulation of HS showed an increase in the expression of several genes involved in signaling through TLR4, among which, are the genes encoding for TLR4 itself, the LPS binding protein and MyD88 [24].

Ganglioside GM2 activator binds gangliosides and catalyzes the degradation of the ganglioside GM2. It extracts single GM2 molecules from membranes and presents them in soluble form to beta-hexosaminidase A for cleavage of *N*-acetyl-D-galactosamine and conversion to GM3 [25]. Accumulation of GM2 and GM3 gangliosides is observed primarily in neurons of MPS, which occurs very early in the course of the disease and may constitute a causal factor determining the dysfunctional progress of centralnervous system [26]. However, its mechanism has not been clearly elucidated. Degradation of GM is from GM2 to GM3 and GM2A are mainly effective in this process, thus the decrease of GM2A may explain the accumulation of GM2 in neurons of MPS II.

Lipocalin-type prostaglandin (PG) D synthase (L-PGDS) is a dually functional protein, acting both as a PGD2-synthesizing enzyme and as an extracellular transporter of various lipophilic small molecules [27]. In brain, L-PGDS is found in arachnoid cell, meningocyte and oligodendrocyte, and it has the effect of anti-apoptosis and of neuron early damaged repair [28]. Moreover, it has been demonstrated that L-PGDS plays a role in transporting small molecule and neurotoxic agent. Surface plasmon resonance analyses revealed that L-PGDS binds to GM1 and GM2 gangliosides [29]. Thus, reduction of L-PGDS may contribute to the aggravation of neurological damage in MPS II.

Taken together, we report for the first time a urine proteomic study

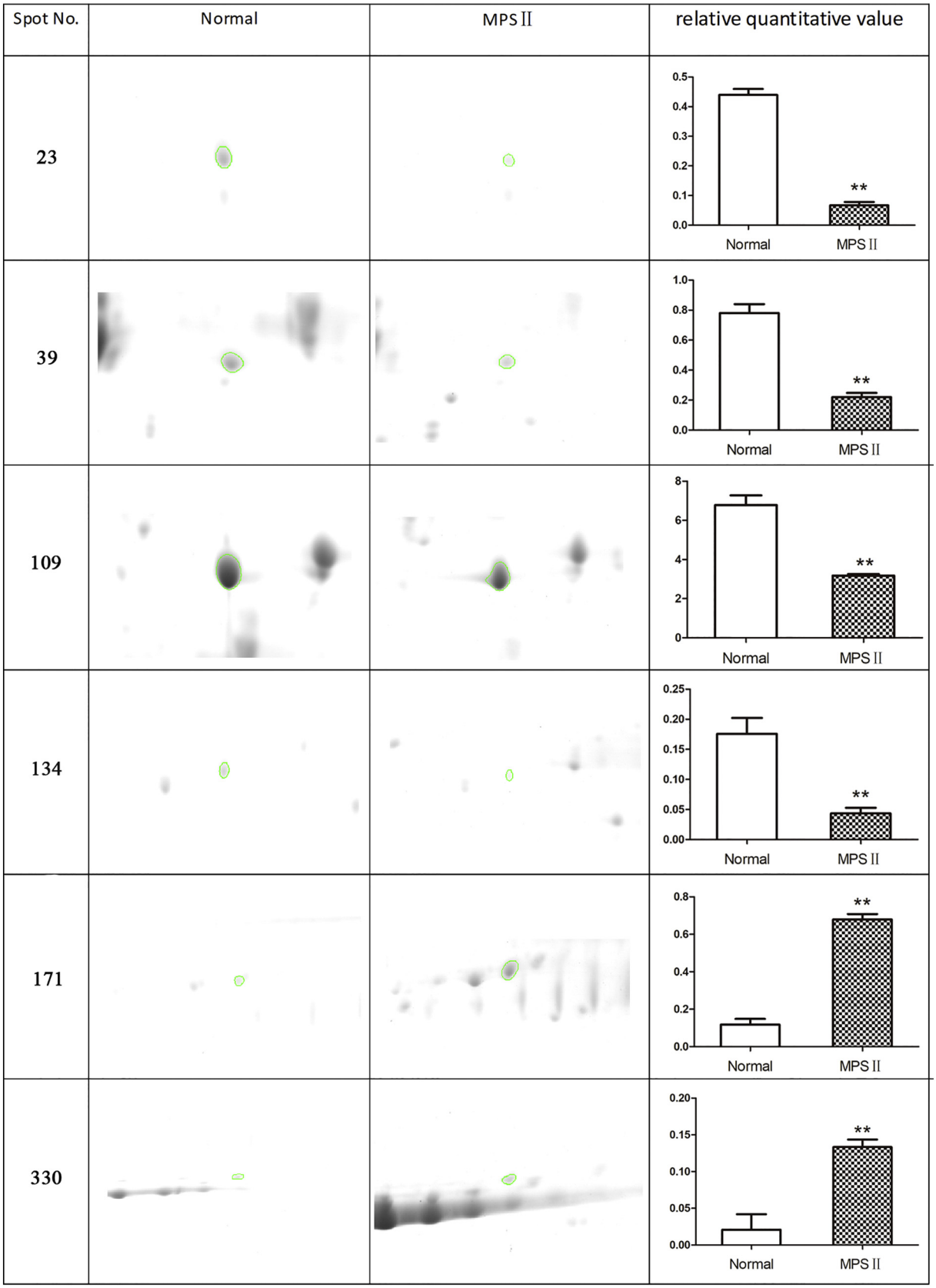

Fig. 2. Image of representative protein spots differentially expressed between MPSIIand normal urinary samples. Relative quantitative value is shown using column charts.
**:$P < 0.01$.

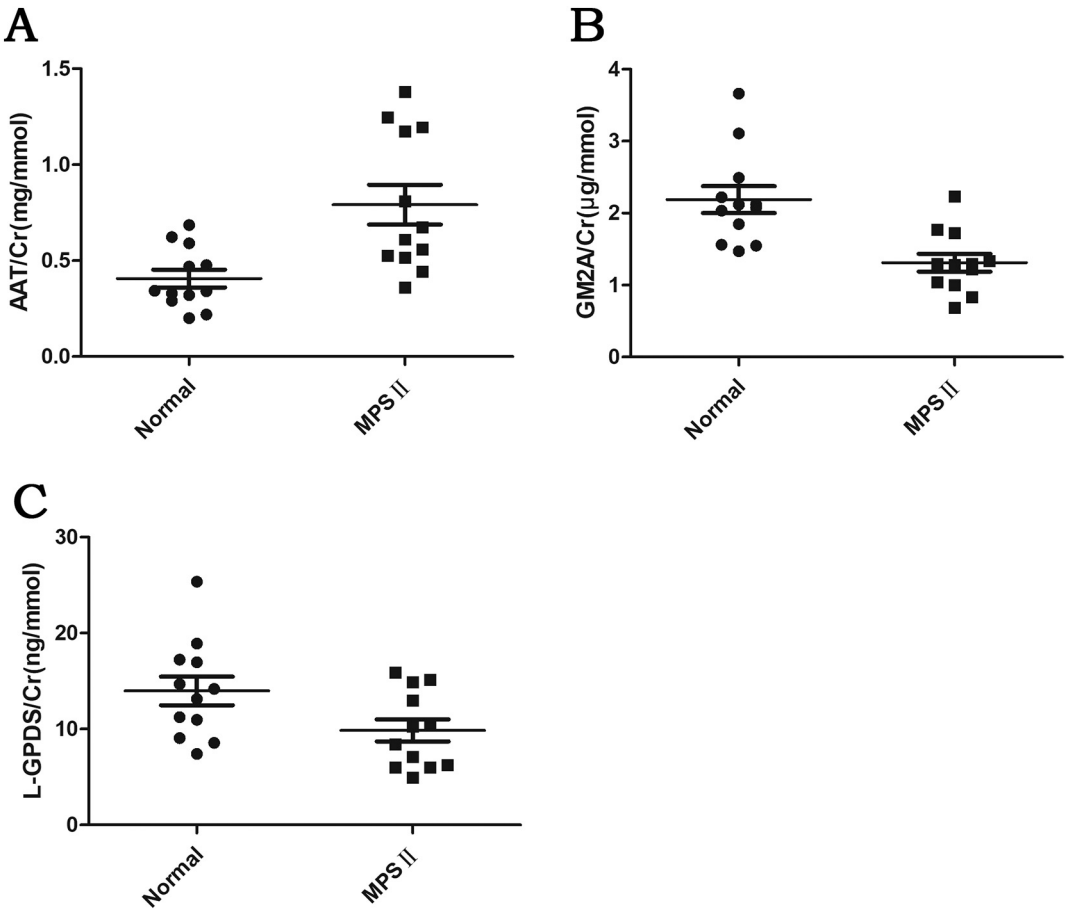

Fig. 3. Content of three proteins in MPSIIand normal urinary samples detected by ELISA method.

of MPSII in China. This study shows that expression of proteins change significantly in urine of MPSII patients. A collective consideration of these set of proteins taken together may provide a system to diagnose MPSII in a non-invasive method. It is well known that confirmation of initial biomarker findings needs larger cohorts, since biomarker discovery is low throughput and confirmation of potential markers should be performed using high throughput targeted techniques [16]. However, the collection of adequate samples, due to the low morbility and high misdiagnosis rate of this disease, are not possible to achieve in a few short years. Further studies are warranted to validate the biomarkers in a larger cohort of patients. Additionally, it would be interesting to investigate the possible cause of differential expression of proteins.

5. Conclusion

We analyzed the differential protein profile in urines of MPSII patients using 2D-PAGE combining with MALDI-TOF/TOF and a total of 15 differentially expressed proteins were identified. Compare to the healthy control group, urinary α1-AT/Cr was found up-regulated in MPS II, while urinary GM2A/Cr and L-PGDS/Cr were found down-regulated, which can be considered as accessory diagnostic biomarkers for MPS II. This approach helped to discover early diagnostic markers and provided a better understanding of the pathogenic mechanism of MPSII.

Ethics

This study was approved by the PLA General Hospital ethical review committee with the following reference number: S2018-108-01.

Data accessiblity

All data have been submitted as figure in the main text.

Authors' contribution

X.Y. performed 2D-PAGE, mass spectrometry, ELISA assays and was the major contributor in writing the manuscript. C.C., S.L., Y.M. and W.J. took part in the sample collection and bioinformatics analysis. Y.M., J.D. and C.W. conceived and supervised the study.

Table 2
AUCs of the ROC for three proteins and the best cut-off values in discriminating MPSIIfrom normal.

	Cut-off value	AUC	Sensitivity(%)	Specificity(%)	Youden' index(%)
α1-AT/Cr(mg/mmol)	0.497	0.833	0.833	0.750	0.583
GM2A/Cr(μg/mmol)	1.404	0.896	1.00	0.750	0.750
L-PGDS/Cr(ng/mmol)	8.484	0.750	0.917	0.500	0.417

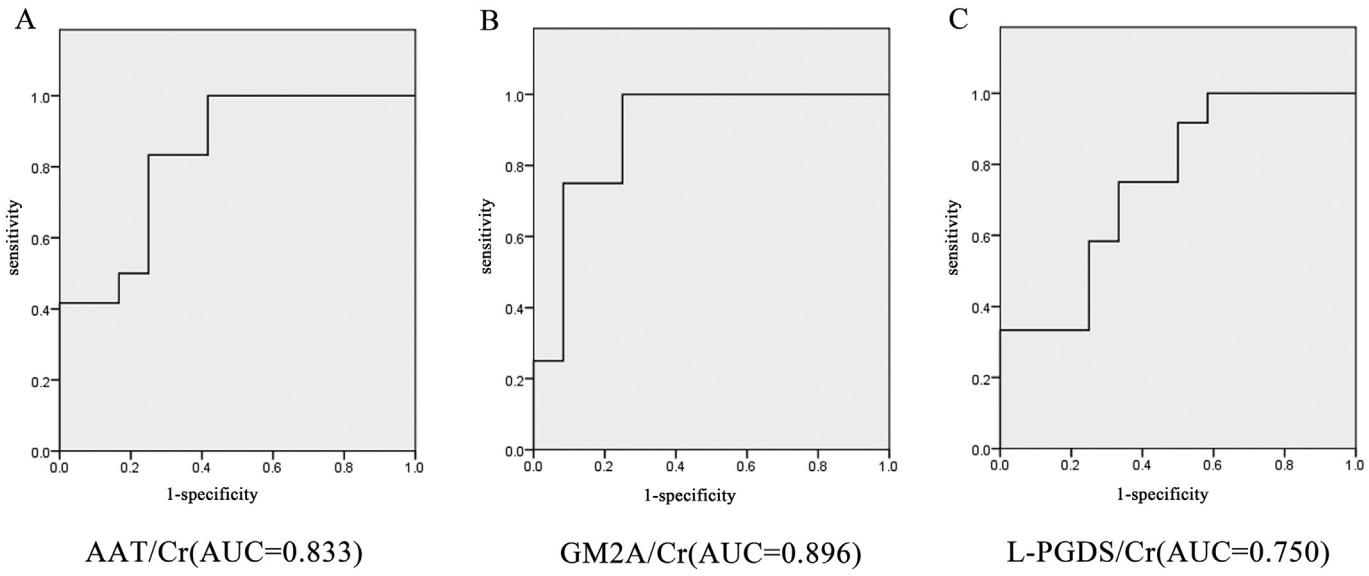

Fig. 4. Area under the receiver-operation characteristic curve of three proteins.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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