Identification of a Disease-Specific Metabolite Panel As a Systematic Biomarker for Primary Nephrotic Syndrome

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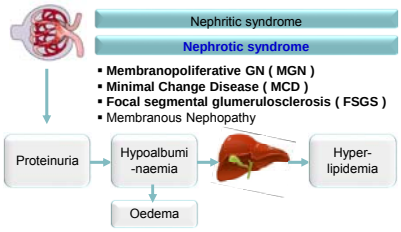
Abstract

Nephrotic syndrome is nonspecific kidney condition characterized by proteinuria, hypoalbuminemia, dyslipidemia, and edema, which are represented by minimal change disease (MCD), FSGS (focal segmental glomerulosclerosis), and MGN (membranous glomerulonephritis). To date, it is mandatory to confirm the diagnosis using biopsy because clinical parameters cannot reliably differentiate the disease status. In this study, we explored the distinctive metabolic changes using urine samples that can lead to biomarker discovery for practical clinical application. A total of 102 samples which are composed of 48 discovery set and 72 validation set, were analyzed by gas chromatography mass spectrometry-based metabolite profiling. The statistical result demonstrated 22 urine metabolites were significantly changed, and particularly branch-chained amino acids showed dynamic alteration compared to control. Consequently, we developed the disease-specific metabolite panel in the discovery set, and the strength of the biomarker panel was validated in the validation set using multiplex bioinformatics platform.

Introduction

Glomeruloephriti



Objectives

1. Compare nephrotic syndrome groups with the control based on urine metabolites
2. Evaluate the differences in pathological physiology among nephrotic syndromes (MGN, MCD, and FSGS)
3. Discover nephrotic syndrome-associated biomarkers in urine metabolites

Materials

Study Participants

Discovery set : 12 control and 36 patients
(Validation set : 54 patients)

Biological source : Urine

Table1. Clinical characteristics of the patients (All values are mean±SD)

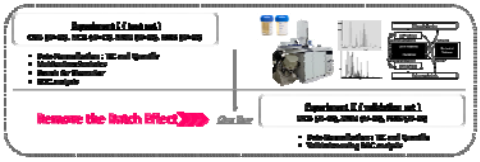
Patient Characteristics	Nephrotic syndrome groups (NS)		
	MGN	MCD	FSGS
Sex (men / women)	7 / 5	8 / 4	7 / 5
Age (yr)	51.8 ± 10.7	49.3 ± 21.9	47.9 ± 15.5
Serum concentrations			
Protein (g/dl)	5.6 ± 1.1	5.2 ± 1.1	6.2 ± 0.7
Albumin (g/dl)	3.1 ± 0.9	2.6 ± 0.9	3.6 ± 0.6
BUN (mg/dl)	15.9 ± 4.6	20.7 ± 6.6	22.4 ± 10.8
Creatinine (mg/dl)	1.0 ± 0.4	1.1 ± 0.4	1.4 ± 0.4
Hb (g/dl)	13.4 ± 2.8	14.8 ± 2.0	12.9 ± 2.4
Uric acid (mg/dl)	7.2 ± 2.3	6.9 ± 2.4	7.1 ± 1.7
Na (mEq/l)	140.8 ± 3.0	138.3 ± 2.5	140.5 ± 4.2
Cholesterol (mg/dl)	260.2 ± 100.4	323.9 ± 111.5	215.3 ± 43.4
TG (mg/dl)	257.9 ± 96.6	235.9 ± 119.6	335.0 ± 130.1
HDL (mg/dl)	45.3 ± 6.9	54.4 ± 10.3	42.5 ± 12.9
LDL (mg/dl)	184.2 ± 46.7	222.0 ± 88.0	145.6 ± 34.6
Urine concentrations			
Urine protein (mg/24hr)	6.6 ± 4.3	9.3 ± 7.2	5.8 ± 3.7
Spot urine protein / Cr ratio (g/g)	5.4 ± 3.7	7.0 ± 3.5	5.7 ± 4.4
UPCR	7.7 ± 3.4	7.3 ± 3.7	8.0 ± 4.9

Sex and Age of **Healthy Control group (CON)** : 7 / 5 and 46.8 ± 11.7

Methods

Sample extraction and derivatization

1. Urine 30㎕ + 750㎕ of methanol : isopropanol : water, 3:3:2, v/v/v
2. Vortexed (1 min) and Sonicated (10 min)
3. Centrifuged (5 minutes at 13,200 rpm) at 4℃
4. Supernatants 700㎕ : concentrated with speed vacuum concentrator
5. Derivatization
   a. Methoxyamination (shaken at 30°C for 90 min)
   b. Trimethylsilylation with internal retention index (RI) markers (shaken at 37°C for 60 min)



Mass-spectrometry analysis and Data processing *Agilent 7890B GC (Agilent Technologies, Wilmington, DE) with Pegasus HT TOF MS (LECO, St. Joseph, MI)*

1. Injection : 0.5㎕, splitless mode
2. Oven temperatures : 50 °C for 1 min, followed by ramping to 330 °C at 20 °C/min, and a final holding for 5 min.
3. LECO Chroma TOF software (ver. 3.34; St. Joseph, MI) : GC/TOF MS data were pre-processed to detect peaks and deconvolute the mass spectra
4. BinBase : The processed data were processed, in-house programmed database built for metabolite identification.

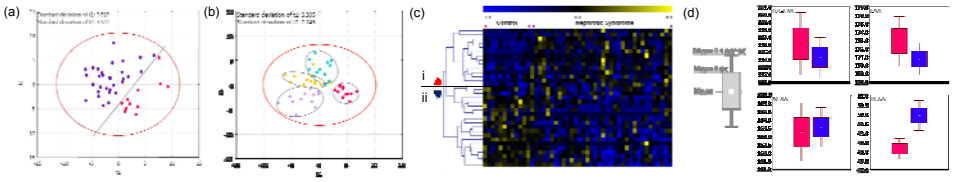
Statistical analysis

MS data analysis were accomplished using the various software. We normalized the raw data set by genomics Workbench (geWorkbench 2.5.1 version). Data was replaced logarithm and transformed by using quantile normalization method. Statistica (ver. 7.1; StatSoft, Tulsa, OK) was used for multiple comparisons using by univariate and multivariate analysis. Also, MS data distributions were shows box and whisker plots using Statistica. Hierarchical clustering analysis (HCA) and significance analysis of microarray (SAM) were performed using MultiExperiment Viewer (MeV, ver.4.8.1) to visualize and organize metabolite profiles. The receiver operating characteristic (ROC) curve was displays using MedClac software version 14.8.1.0 (Broekstraat, Mariakerke, Gelgium).

Results

A. (a) Unsupervised multivariate statistics using principal component analysis (PCA) between control and nephrotic syndrome groups. (b) Supervised multivariate statistics using partial least square analysis (PLS) among the 4 groups of GC-TOF MS data. (c) Hierarchical clustering analysis (HCA) i. Up-regulation: sugar(hexose), sugar alcohol, and nucleotide ii. Down-regulation : sugar(pentose), amino acid, organic acid, nucleobases. (d) Nephrotic syndrome-specific integrative compositional alterations of amino acids (Student's t-test, *P*<0.05)

● Control ● Nephrotic syndrome (● FSGS ● MGN ● MCD)

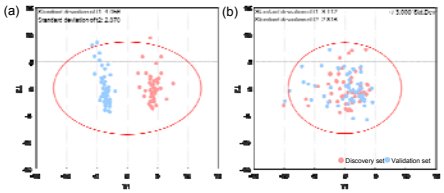


B. Metabolite combinations with highest predictive potentials according to ROC AUC values

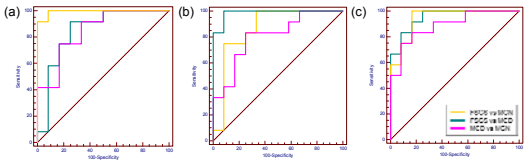
Table2. Abbreviation : receiver operating characteristic; AUC, area under the ROC curve; PLS, partial least squares; VIP, variable influence on projection; SAM, significance analysis of microarray; ANOVA, one-way analysis of variance.

Compounds	PLS VIP score	SAM	ANOVA	AUC		
				FSGS vs MCD	FSGS vs MGN	MGN vs MCD
3,6-D-Galactose	○		○	-	0.917	-
Ethanolamine	○	○		-	0.847	-
Citrulline	○	○	○	-	0.972	0.868
Myo-inositol		○	○	-	0.861	-
Uracil	○	○	○	0.896	0.882	-
Xanthine	○	○	○	-	0.917	-

C. Batch effect removal using Surrogated Variable Analysis (SVA). PCA for (a) prior to batch effect remonal (b) after batch effect remobal.



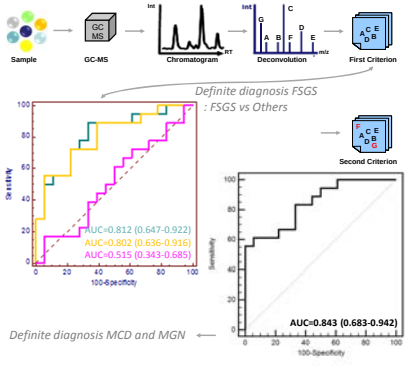
D. Receiver operating characteristic (ROC) curve of urine multiple-metabolite panels in three different NS of discovery set



Groups		AUC[a] ± SE[b] (95% CI[c])	Criterion	Sensitivity (95% CI[c])	Specificity (95% CI[c])
(a)		0.874 ± 0.0874 (0.643 to 0.960)	≤ -0.4337	91.7 (61.5 – 99.8)	75.0 (42.8 – 94.5)
		0.993 ± 0.0098 (0.845 to 1.000)	≤ -0.4337	91.7 (61.5 – 99.8)	100.0 (735. – 100)
		0.847 ± 0.0799 (0.643 to 0.960)	> 0.1092	91.7 (61.5 – 99.8)	66.7 (34.9 – 90.1)
(b)		0.868 ± 0.0833 (0.668 to 0.970)	≤ -0.3555	83.3 (51.6 – 97.9)	75.0 (42.8 – 94.5)
		0.986 ± 0.0168 (0.833 to 1.000)	≤ -0.1968	100.0 (735. – 100)	91.7 (61.5 – 99.8)
		0.806 ± 0.0916 (0.594 to 0.937)	≤ 0.8384	83.3 (51.6 – 97.9)	75.0 (42.8 – 94.5)
(c)		0.951 ± 0.0383 (0.779 to 0.998)	≤ -0.3976	83.3 (51.6 – 97.9)	91.7 (61.5 – 99.8)
		0.944 ± 0.0443 (0.768 to 0.997)	≤ -0.0765	100.0 (73.5 - 100.0)	83.3 (51.6 – 97.9)
		0.889 ± 0.0670 (0.694 to 0.979)	≤ -0.1491	83.3 (51.6 – 97.9)	83.3 (51.6 – 97.9)

[a] : Area under the ROC curve, [b] : standard error, [c] : confidence interval

E. Validation of metabolite multiple factors using ROC analysis and workflow for diagnosis between NSs



Conclusions

1. Hexose and sugar alcohol were up-regulated, and pentose sugar, amino acid, and organic acid were down-regulated in NS groups. Particularly, each of NS groups, MGN, MCD, and FSGS, shows different patterns of hydroxl acid, free fatty acid, and monosaccharide compared to the control group
2. Control and NS groups were separated by component t1, and NS groups were mainly divided by component t2 from un- and supervised multivariate statistics
3. We analyzed NS type-specific metabolites using multiple comparisons and discovered candidates of biomarkers. Also, the candidates were validated using ROC analysis with high sensitivity and specificity of diagnostic accuracy

References

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