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Original Article

Distinguishment of patients with mesangial proliferative glomerulonephritis from healthy subjects by urine volatile metabolites

Mingao Wang[1], Changsong Wang[2], Weiwei Wang[2], Xin Pi[2], Lin Zhu[1], Enyou Li[2], Ruichan Liu[1]

Departments of [1]Nephrology, [2]Anesthesiology, First Affiliated Hospital of Harbin Medical University, Harbin, China

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Abstract: Aims: Urinary volatile organic compound (VOCs) has received growing interest because it was convenient and non-invasive and it can successfully provide unique biomarker profiles for many diseases, especially in cancers. Materials and methods: Urinary samples were collected from 16 MsPGN patients and 15 healthy controls. Solid phase microextraction-chromatography-mass spectrometry (SPME-GC-MS) was used to analyse the urinary metabolites. The principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLSDA) were performed to deal with the data. Results: Six specific VOCs biomarkers were present at decreased levels in the urine of MsPGN patients. These VOCs included pyrrole, 2-pentanone, 4-heptanone, silanediol, dimethyl- and carbamic acid, a monoammonium salt. Conclusions: These significant decreases indicate that they are potential biomarkers for distinguishing the differences between MsPGN and healthy subjects.

Keywords: Mesangial proliferative glomerulonephritis, volatile organic compounds, solid-phase microextraction, gas chromatography-mass spectrometry, diagnostic method

Introduction

Defining mesangial proliferative glomerulonephritis (MsPGN) as an essentially uniform increase in mesangial cells (more than 3 mesangial cells per mesangial area) is more than 80% of the glomeruli [1]. The clinical manifestations of MsPGN are typically hematuria and proteinuria, and approximately 1/3 or more of patients also exhibit hypertension [2]. MsPGN can cause glomerular sclerosis and obliteration, and if it is not treated properly and promptly, it can lead to severely impaired renal function [3]. Therefore, it is necessary to screen and diagnose MsPGN as early as possible. Clinically, MsPGN diagnosis relies on biopsy, in which the disease is characterized by diffuse mesangial proliferation and depositions of immunoglobulin and complement in the mesangial area, with no damage to endothelial cells, tubules and renal interstitium [1-3]. However, MsPGN diagnosis techniques involving renal biopsy still have shortcomings. (1) Based on the characteristics of different clinical manifestations and pathological changes, the majority of primary MsPGN can be diagnosed; however, the pathological attributions of MsPGN, minimal change nephropathy and focal segmental sclerosis remain controversial, as the three conditions are clinically and pathologically similar to each other to a large extent [4]. (2) Renal biopsy is an invasive procedure that involves a risk of infection; the choice of biopsies must not only address the indications properly but also exclude contraindications, e.g., significant bleeding tendency, severe hypertension, etc. [4, 5]. (3) This treatment is time-consuming, expensive, and not readily accepted by patients. Owing to these limitations, it is meaningful for us to identify an adequate, sensitive, reproducible, specific, non-invasive, rapid and inexpensive method that can reliably be used for MsPGN screening and diagnosis.

In recent years, volatile organic compounds (VOCs) and gases that emanate from urine have received growing interest because evaluating them is convenient and non-invasive. Currently, more than 230 volatiles have been iden-

Table 1. Demographic characteristics of the study subjects

	Normal	MsPGN
Subjects (n)	15	16
Age	35 (7.6)	37 (12.0)
Male	7	5
Female	8	11
Smokers (n)	3	4
SCR (μmol/L)		91.7 (44.7)
ALB (g)		33.8 (7.6)
24 h urine protein (g)		2.7 (2.0)

Abbreviations: SCR, serum creatinine; ALB, plasma albumin.

tified in human urine, such as hydrocarbons, aldehydes, ketones, furans, pyrroles, sulfur-containing compounds and heterocyclic compounds [6]. Clinical trials have confirmed that urinary VOCs can successfully provide unique biomarker profiles for many diseases, especially in cancers [7, 8]. For example, Hanai Y et al. [7] found that urinary VOCs can be used as biomarkers to discriminate between lung cancer patients and normal control patients; Willis CM et al. [8] proposed that volatile biomarkers for bladder cancer exist in the urine headspace and have the potential to be exploited for diagnosis. However, to our knowledge, there has not yet been any research work published addressing whether urinary VOCs can provide unique biomarker profiles for MsPGN. Taking into account the influence of kidney disease itself on the composition and content of urinary VOCs, in our study, we used gas chromatography/mass spectrometry (GC/MS) and multivariate data analysis to compare the VOCs in urine samples from MsPGN patients and healthy subjects to identify potential volatile biomarkers of MsPGN in urinary VOCs.

Materials and methods

Human subjects

The present experiments were conducted in accordance with the Declaration of Helsinki. The protocol in this study was approved by the Ethics Committee at the First Affiliated Hospital of Harbin Medical University (No. 201314). Before study enrollment, written informed consent was signed by patients. This study was conducted between Dec. 2013 and Sep. 2014 at the Department of Anesthesiology in the First Affiliated Hospital of Harbin Medical University.

Men between 19 and 42, and women between 23 and 62 years of age identified as ASA I and II individuals were included in this study, and scheduled for renal biopsy. This study involved 16 patients with histologically confirmed cases of MsPGN and 15 healthy volunteers who were negative for kidney diseases, urinary infection, and their renal function were normal. The demographic characteristics are summarized in **Table 1**.

As detailed in **Table 1**, the normal control group involved 16 patients. The 16 MsPGN patients who were selected included 5 males and 11 females. The mean age of the MsPGN patients was 37 y, with a standard deviation (SD) of 12.0 y, and 4 of these patients were smokers.

Urine collection

Fasting patients' portions of midstream urine samples were collected severally in the morning before analysis. After collected, all samples were analyzed within 1 h.

Solid-phase microextraction (SPME)

Amanual SPME holder with carboxen/polydimethylsiloxane (CAR/PDMS) fibers of 75 um thickness was purchased from Supelco (Bellefonte, USA). The SPME fiber was inserted into the vial and exposed to the gaseous sample for 20 min at 40°C. Subsequently, the desorptions of volatiles occurred in the hot GC injector at 200°C for 2 min.

Gas chromatography-mass spectrometry (GC/MS) analysis

Analysis was performed on a GC/MS (Shimadzu GC-MS QP 2010, Shimadzu, Japan) and be equipped with a DB-5MS (length 30 m *ID 0.250* film thickness 0.25 um; Agilent Technologies, USA) plot column. Injections were performed in the splitless mode. The temperature of injector was 200°C. The flow rate of the helium (99.999%) carrier gas was kept constant at 2 ml min^{-1}. The column temperature was held at 40°C for 1 min to concentrate the hydrocarbons at the head of the column and then increased by 5°C min^{-1} to 200°C for 1 min, and then ramped 15°C min^{-1} to 230°C. The MS analyses were performed in full-scan mode,

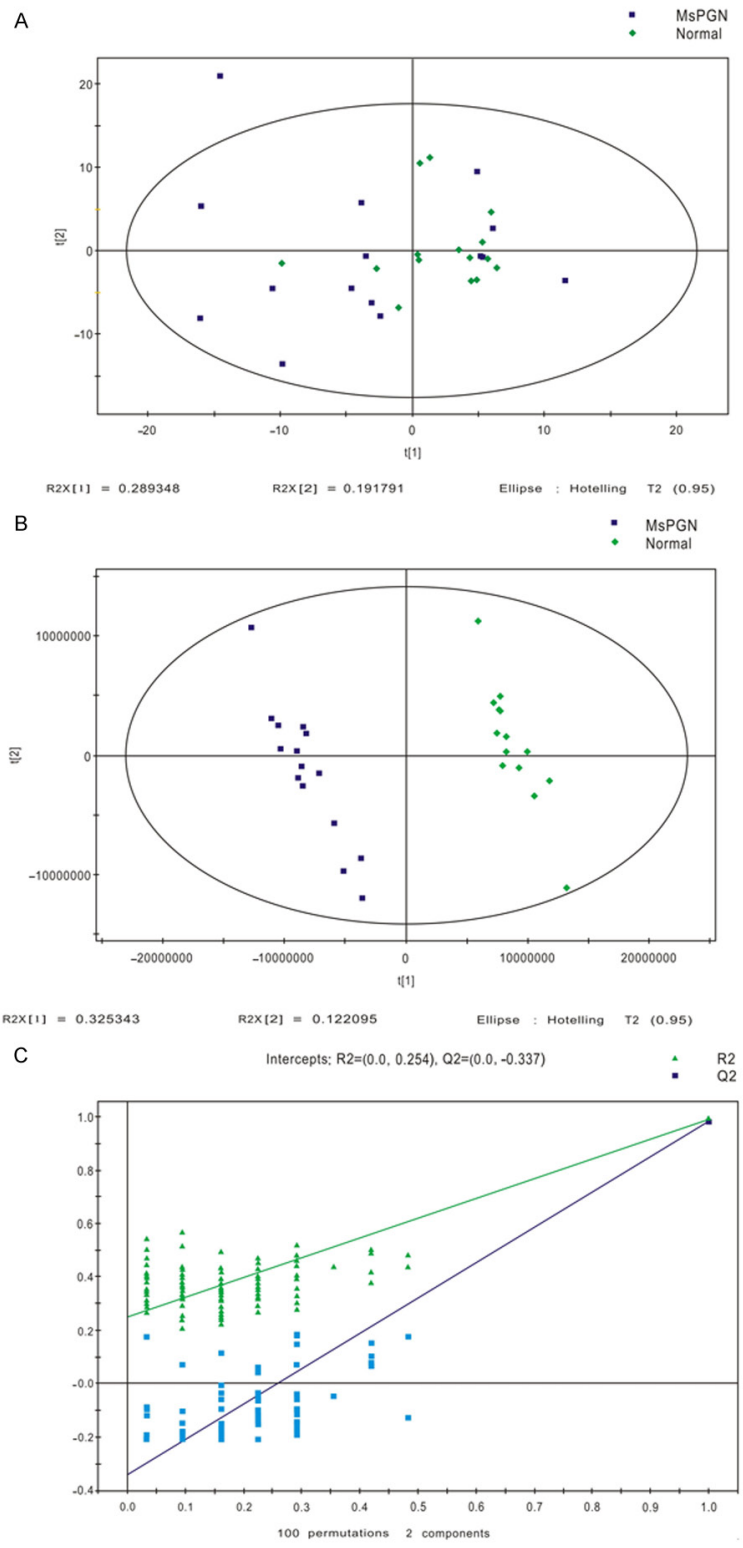

Figure 1. A. PCA score plot: (6 components, R2X = 0.802, Q2 = 0.511); B. OPLSDA score plot: (2 components, R2X = 0.447, R2Y = 0.990, Q2 = 0.981); C. PLSDA validation plot intercepts: R2 = (0.0, 0.254); Q2 = (0.0, - 0.337).

using a scan range from 35-350 amu. The ion source was maintained at 230°C, and an ionization energy of 70 eV was used for each measurement.

Extraction and pretreatment of the GC/MS raw data

Raw GC/MS data were converted into CDF format (NetCDF) files using Shimadzu GCMS Postrun Analysis software and subsequently processed using the XCMS toolbox (http://metlin.scripps.edu/download/). The XCMS parameters consisted of the default settings with the following exceptions: xcmsSet (fwhm = 8, snthresh = 6, max = 200); retcor (method = "linear", family = "gaussian", plottype = "mdevden"); and a bandwidth of eight for first grouping command and four for the second grouping command. The data set of the aligned mass ions was exported from XCMS and could be further processed using Microsoft Excel to normalize the data prior to multivariate analyses.

Statistical analysis

Normalization to total peak area for each sample was performed before statistical analysis. The normalized data were exported to SIMCA-P 11.5 for principal component analysis (PCA) to detect grouping trends and outliers. Then Orthogonall partial least-squares discriminant analysis (OPLSDA) was employed and variable importance in the projection (VIP) values calculated. To prevent overfitting, the default seven-round cross-validation was applied in the SIMCA-P software.Tofurthervalidatethesupervised model, permutation tests with 100 iterations were performed later. Besides, to determine the significance of each metabolite, the nonparametric Kruskal-Wallis rank sum test was performed. Potential metabolic biomarkers were selected

Table 2. Related metabolites that exist at abnormal levels in the urine from MsPGN patients

Potential biomarker	RT	*P*-value	FC	VIP
Pyrrole	184.45	0.000055	3.968001	2.774970
Silanediol, dimethyl-	179.37	0.000432	4.256574	3.728240
2-Pentanone	138.75	0.003443	3.195844	4.618810
Carbamic acid, mono ammonium salt	68.79	0.014255	1.654572	4.620970
4-Heptanone	328.82	0.010189	2.954116	3.038430
2H-1,4-Benzodiazepin-2-one, 7-chloro-1,3-dihydro-5-phenyl-1-(trimethylsilyl)-	1067.99	0.015899	8.447179	1.546900

based on VIP values and nonparametric *P* values, using thresholds of 1.2 and 0.05, respectively.

Results

In this study, the VOCs in the exhaled breath of 16 patients with MsPGN and 15 healthy subjects were analyzed using GC-MS. Between the patients with MsPGN and the healthy subjects, we obtained two-dimensional principal component analysis (PCA) score plots using 250 parameters, with good separation tendency (**Figure 1A**). A partial least squares Discriminant Analysis (PLS-DA) score plot was then used to separate these two groups with three components (R2X = 0.447, R2Y = 0.990, and Q2 = 0.981) (**Figure 1B**). A validation plot obtained from 100 permutation tests revealed that all of the R2 and Q2 values calculated from the permutated data were lower than the original values (**Figure 1C**). All of the variable importances in projection (VIP) values of the examined factors in either the PLS-DA model or the orthogonal signal correction (OSC)-PLS-DA model were calculated. The distinct metabolic biomarkers were selected based on the standard of a VIP value greater than 1.2 using the NIST 11 mass spectral database with a similarity threshold of 75% (**Table 2**).

Discussion

Currently, several methods have been reported for screening and diagnosing for MsPGN through urine, including transcriptomic, genomic, proteomic, and metabolomic analyses. Because of urine's complexity it is suitable for analysis; with its many components, including intermediate metabolites [9], metabolomics may be a better choice for MsPGN diagnosis. Metabolites are the end-products in various metabolic pathways [10]; thus, any changes in path = ology or physiology can be reflected in the metabolic profiling [11]. In addition, compared with genes, transcripts, and proteins in their respective omics fields, there is a relatively small number of metabolites to examine; therefore, metabolomics data are more easily handled and analyzed [12]. Urine-based metabolomics has been applied in research studies of early detection and renal toxicity, diagnosis, and biomarker identification [13, 14]. In our study, we sought to identify potential volatile biomarkers of MsPGN in urinary VOCs using metabolomics strategies. Several potential VOC biomarkers were detected.

Compared to healthy subjects, patients with MsPGN showed decreased levels of urinary VOCs, including pyrrole, 2-pentanone, 4-heptanone, silanediol, dimethyl- and carbamic acid, a monoammonium salt. 2-pentanone and 4-heptanone are two types of ketones. Ketones may be produced through potential pathways: (1) the oxidation of secondary alcohols catalyzed by alcohol dehydrogenases (ADHs) and (2) the β-oxidation of branched-chain fatty acids [15]. The ketone contents of human urine are relatively high [16]. Therefore, damage to renal function exerts a substantial impact on ketone metabolism. The metabolic pathways of 4-heptanone have been characterized. In hemodialysis patients, di (2-ethylhexyl) phthalate metabolizes to 2-ethylhexanol, and 2-ethylhexanol is then oxidized to 2-ethylhexanoic acid and finally to 4-heptanone [16, 17]. Liebich HM [18] et al. found that the serum level of 4-heptanone in normal subjects was lower by a factor of approximately 100 compared to urine. This result indicates a high renal excretion of 4-heptanone and low re-absorption. In cases of renal insufficiency, the excretion of total 4-heptanone is reduced. This finding is consistent with the hypothesis proposed by Mochalski et al. [19]. The decrease in 4-heptanone levels detected in the urine of the MsPGN patients suggests that it is difficult for MsPGN patients to excrete 4-heptanone though the kidney; therefore, 4-heptanone remains in other tissues, which leads to increased levels of 4-heptanone in the blood and breath. However, the mechanism of

this phenomenon is unclear. The source of 2-pentanone, a methylketone, remains unknown in humans but is assumed to be from fatty acid β-oxidation [20, 21]. Fatty acids are a series of carbolic acid compounds, and three long-chain fatty acids and glycerol form triacylglycerols, the main component of fat. Patients with chronic kidney disease often exhibit abnormal lipid metabolism in the form of hypertriglyceridemia [22]. We hypothesize that the decrease in 2-pentanone levels detected in the urine of MsPGN patients was due to abnormal triglyceride metabolism, which led to a reduction in fatty acid β-oxidation and in turn led to decreased 2-pentanone production; when coupled with the decreased 2-pentanone excretion caused by the damage to renal function, this decrease ultimately led to the reduced 2-pentanone content in the urine.

Our results showed decreased urinary levels of pyrrole in patients with MsPGN compared to healthy subjects. How pyrroles are produced in the human body and then appear in the urine is still unclear. The endogenous pyrroles can come from the metabolism of amino sugars and N-acetylneuraminic acid in the central nervous system [15]. They may also be porphyrins, by-products of bile pigment synthesis, or the oxidation products of hemopyrrole and bilirubin [23]. Urine pyrroles can also have exogenous origins. Burdock GA et al. found that most beverages (i.e., coffee, tea, beer), and heat-treated meat (i.e., roasted and fried chicken, beef, pork) contain pyrroles [24]. Pyrroles are also components of antihypertensive and anti-inflammatory drugs. In our experiments, to avoid the effects of exogenous diets and drugs on the experimental results, the urine used in the experiment was fasted morning urine from patients took no drugs and fasted for 8 hours. However, there has been no research on the cause of the decreased pyrrole contents in the urine of patients with MsPGN; more experiments are needed to explore this phenomenon.

Carbamic acid, a monoammonium salt, is an ammonium salt whose source in humans remains unknown. However, based on its chemical formula, it can be speculated that it is the *in vivo* metabolic product of urea or ammonia. Patients with chronic kidney disease often show elevated blood urea nitrogen and blood ammonia levels [25]. Fadel et al. [26] noted that the abnormal protein and amino acid levels and reduced urea and NH3 excretion in urine may contribute to the high blood urea and NH3 contents. For MsPGN patients, the deposition of proliferative immune substances in mesangial cells and inflammation cause ischemia and hypoxia in the glomerular tissue, leading to glomerular sclerosis and, thus, impaired renal function [11]. Additionally, blocked urea and ammonia excretions result in reduced ammonium salt contents in urine, which may be the causes of the decreased levels of carbamic acid, the monoammonium salt detected in the urine of MsPGN patients.

The metabolomics of many of the urinary VOCs we detected are still not clear and require further investigation. Whatever the mechanism of interaction is, their significant decrease indicates that they are potential biomarkers for distinguishing the differences between MsPGN and healthy subjects.

In conclusion, compared with healthy subjects, MsPGN has unique urinary VOCs, suggesting that these profiles may be useful as a diagnostic assay for MsPGN.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ruichan Liu, Department of Nephrology, First Affiliated Hospital of Harbin Medical University, 23 Youzheng Street, Harbin, China. E-mail: trappist@sina.com

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