Original Article

An integrated metabonomic and proteomic study on Kidney-Yin Deficiency Syndrome patients with diabetes mellitus in China

Ning JIANG¹, Hong-fang LIU², Si-di LI¹, Wen-xia ZHOU^{1, *}, Yong-xiang ZHANG^{1, *}, Qi ZHANG³, Xian-zhong YAN³

¹Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China; ²Dongzhimen Hospital of Beijing University of Chinese Medicine, Beijing 100050, China; ³National Center of Biomedical Analysis, Beijing 100850, China

Aim: To investigate specific changes in metabolites and proteins of Kidney-Yin Deficiency Syndrome (KYDS) patients with diabetes mellitus (DM) in China.

Methods: KYDS ($n=29$) and non-KYDS ($n=23$) patients with DM were recruited for this study. The KYDS was diagnosed by two senior TCM clinicians separately. The metabonomic and proteomic profiles of the patients were assessed using a metabonomic strategy based on NMR with multivariate analysis and a proteomic strategy based on MALDI-TOF-MS, respectively.

Results: Eighteen upregulated peptides and thirty downregulated peptides were observed in the plasma of the KYDS patients. Comparing the proteomic profiles of the KYDS and non-KYDS groups, however, no significantly differentially expressed peptides were found. At the same time, major metabolic alterations were found to distinguish the two groups, including eight significantly changed metabolites (creatinine, citrate, TMAO, phenylalanine, tyrosine, alanine, glycine and taurine). The levels of creatinine, citrate, TMAO, phenylalanine and tyrosine were decreased, whereas the levels of alanine, glycine and taurine were increased in the KYDS patients. These biochemical changes were found to be associated with alterations in amino acid metabolism, energy metabolism and gut microflora.

Conclusion: The identification of distinct expression profiles of metabolites and signaling pathways in KYDS patients with DM suggests that there are indeed molecular signatures underlying the principles of ‘Syndrome Differentiation’ in traditional Chinese medicine.

Keywords: Kidney-Yin Deficiency syndrome; diabetes mellitus; traditional Chinese medicine; metabonomics; proteomics

Acta Pharmacologica Sinica (2015) 36: 689–698; doi: 10.1038/aps.2014.169; published online 4 May 2015

Introduction

Differentiation of syndromes in traditional Chinese medicine (TCM), which is also referred to as ‘Zheng differentiation’, is the profiling of a combination of symptoms or clinical phenotypes^[1]. ‘Syndrome’ is one of the most important concepts in the theoretical and clinical research of TCM. It not only establishes a path for disease diagnosis on a microcosmic level but also subtypes a disease and provides a basis for individualized therapy^[2]. Understanding ‘syndromes’ is essential to the establishment of disease classification, diagnostic criteria, and efficient therapeutic strategies for patients. However, in the era of complementary and alternative medicine, the concept of TCM syndromes has been difficult to comprehend from

a modern biomedical perspective due to a shortage of evidence-based theoretical interpretations and solid evidence of syndrome-based effects^[3]. Thus, it is necessary to comprehensively investigate the intrinsic mechanisms of TCM syndromes to validate and further understand this concept.

Systems biology, which consists of genomics, epigenomics, proteomics, and metabonomics, seeks to describe complex interactions between components of biological systems and predict biological behaviors^[4]. By assessing the profiles of genes, proteins and metabolites, systems biology provides a holistic approach to biological research. This property of systems biology is analogous to the holistic philosophy of TCM; therefore, systems biology may be a useful tool for studying the basis of Chinese medicine, including its concepts of syndrome differentiation and disease diagnosis. Within systems biology, metabolic profiling is defined as the quantitative measurement of the dynamic multiplex response of a living system to pathophysiological stimuli or genetic modifica-

* To whom correspondence should be addressed.

E-mail zhouwx@bmi.ac.cn (Wen-xia ZHOU);

zhangyx@bmi.ac.cn (Yong-xiang ZHANG)

Received 2014-10-06 Accepted 2014-12-08

tions^[5]. Nuclear magnetic resonance (NMR) spectroscopy-based metabolomics is an attractive method for a nonselective and comprehensive analysis of metabolites. Metabolomics analysis may facilitate the modernization of TCM syndrome research and provide an in-depth understanding of the TCM theory^[6]. Proteomics technology is based on the vast analytical power for protein identification and quantification by modern mass spectrometry coupled with hyphenated separation techniques^[7].

The rapid growth of the proteomics field has resulted in an array of new tools for studying TCM, and proteomic analysis may also facilitate the modernization and globalization of TCM^[8]. A fractionation method that selectively separates peptides on the chromatographic surfaces of magnetic beads according to chemical differences has been developed to be used in conjunction with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis. The method can be applied to human body fluids, such as blood, saliva and urine, which offer the advantages of minimal invasiveness, low cost, and ready acquisition and processing^[9]. Being aware of the intrinsic relationship between TCM and systems biology, some researchers have already proposed that metabonomic and proteomic approaches may be applicable to the scientific validation of TCM theories^[10–13].

Diabetes mellitus (DM) is one of the most prevalent chronic diseases in the world. It is characterized by insulin resistance coupled with a failure of pancreatic cells to compensate by adequate insulin secretion. According to the theories of TCM, diabetes is considered to be a condition of disharmony in the body. Kidney-Yin Deficiency Syndrome (KYDS) is one of the elementary syndrome patterns in patients with DM and is closely related to defects in multiple metabolic pathways. Moreover, studies have found that the characteristics of KYDS may be manifested throughout all stages of DM. However, there has been no research on the physiology of KYDS from the perspective of systems biology, and the proteomic and metabolic signatures underlying the pathophysiology of KYDS are still unknown. Therefore, the present study was designed to integrate metabonomic and proteomic analyses to construct KYDS-specific profiles of urine metabolites and plasma proteins, which may reveal specific metabolic and protein changes in KYDS patients that can be used to objectively differentiate clinical syndromes. To our knowledge, this is the first report of using an integrated metabonomic and proteomic method to investigate KYDS from a holistic perspective.

Materials and methods

Subjects

Patients of KYDS ($n=23$) and non-KYDS ($n=29$) with DM were recruited from the Dongzhimen Hospital of Beijing University of Chinese Medicine between May 1st, 2008 and December 30th, 2009. All patients provided informed consent prior to the collection of any data. The study was approved by the Ethical Committee of Beijing Institute of Pharmacology and Toxicology, and it complied with the provisions of the Good Clinical Practice Guidelines and the Declaration of Helsinki.

Diagnostic criteria for DM

DM patients who met the diagnostic criteria from the 1999 issue of “Standard for Diagnosing Diabetes and Other Types of Hyperglycemia” by WHO were included in the study.

Diagnostic criteria for DM in Traditional Chinese Medicine

The criteria for diagnosing diabetes in TCM were from the teaching materials of Traditional Chinese Internal Medicine (6th edition).

Participants were eligible if they had independent clinical diagnoses of a deficiency of kidney Yin (Table 1) by two senior TCM clinicians who assessed the patients separately. The exclusion criteria include (1) major neuropsychiatric disorders, such as schizophrenia, epilepsy, alcohol abuse, and anorexia; (2) plans for pregnancy; (3) cardio-cerebral vascular diseases, insufficiently controlled hypertension or hypotension, thromboembolic diseases, gastrointestinal diseases affecting drug absorption, hematopoietic system diseases, or autoimmune system diseases; (4) abnormal liver or renal function; and (5) other criteria that the investigators deemed to be unsuitable for the study. Syndrome differentiation was independently performed by two associate professors on each patient, and a patient would only be included in the study if the two diagnoses were consistent with one another. Fifty-two paired plasma and urine samples from twenty-nine KYDS and twenty-three non-KYDS patients were taken. The distributions of gender and age in KYDS and Non-KYDS groups are shown in Table 2. The differences in age and gender were not statistically significant between the groups ($P>0.05$).

Table 1. Diagnosis criteria of Kidney-Yin deficiency. Patients who have at least 3 of the following items can be diagnosed as Kidney-Yin deficiency.

-
- (1) Dizziness
 - (2) Tinnitus
 - (3) Flaccid waist and knees
 - (4) Hectic fever
 - (5) Dry mouth and throat
 - (6) Night sweat
 - (7) Spermatorrhea
 - (8) Thirst and drink
 - (9) Red tongue and less fur
 - (10) Thready and rapid pulse
-

Sample preparation

Urine collection

Tools for urine collection and transport were provided to the patients. The patients were asked to collect urine into sterile containers, pipette it into transport tubes, and immediately store the tubes in insulated ice packs. The packed samples were not re-opened until they were received by the investigators. Upon arrival at the laboratory, the samples were centrifuged at 10000 rounds/min for 10 min at 4°C to remove any solid debris. The supernatants were transferred to 1.5 mL polypropylene tubes and stored at -80°C until further analysis.

Table 2. Demographics of patients included in the study. Data were presented as the mean±SD.

Group	n	Age (year)	Gender proportion		Glucose level
			Female	Male	
Non-KYDS	29	59.07±8.57	65.5% (19/29)	34.5% (10/29)	9.86±1.74
KYDS	23	58.74±6.88	65.2% (15/23)	34.8% (8/23)	9.80±1.05

Plasma collection

Fasting blood samples were collected from the subjects in the morning by venipuncture and stored in heparin-containing green-top tubes (BD, 366480). The samples were then centrifuged at 3000×g for 10 min at 4°C to isolate plasma. The plasma samples were stored at -80°C until further processing and analysis.

Proteomic study

Isolation of peptides

Plasma were thawed at room temperature for 2 min with only one freeze-thaw cycle and processed immediately by weak cation exchange (WCX) pre-purification. As recommended in the manufacturer's protocol (ClinProt™, Bruker Daltonics, state, Billerica, MA, USA), the samples were purified and isolated in three steps: binding, washing, and elution. First, 10 mL beads, 10 mL WCX binding solution and 5 mL plasma samples were added to a tube, mixed carefully and incubated for 5 min. Second, the tube was placed on a magnetic bead separation device (Bruker Daltonics, Billerica, MA, USA) for 1 min to collect the beads onto the tube wall. The supernatant was then removed and 100 mL washing solution was added and mixed thoroughly with the magnetic beads. After washing three times and removing the supernatant, 5 mL eluting buffer was added and the beads were collected onto the tube wall for 2 min. After transferring the clear supernatant into a fresh tube, 5 mL of stabilizing solution was added and the tube was mixed thoroughly.

MS analysis

To prepare the MALDI target, we spotted 1 µL of a mixture containing 10 µL of 0.3 g/L α-cyano-4-hydroxycinnamic acid in 2:1 ethanol/acetone (v/v) and 1 µL of the eluted peptide fraction onto a MALDI AnchorChip™ (Bruker Daltonics) sample target platform (384 spots). For the proteomic analysis and peptidome profiling, we used a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS; Autoflex, Bruker Daltonik) with the following settings: linear positive mode; ion source 1, 20 kV; ion source 2, 18 kV; lens, 7.5 kV; pulsed ion extraction, 210 ns. We removed excess matrix particles with 10 shots at a laser power of 50% before data acquisition at 45%. For each MALDI spot, 400 spectra were acquired (50 laser shots at eight different spot positions). External calibration was performed using a set of peptide/protein standards (Bruker Daltonics) to calibrate the spectrum in the range of 1000–17000 Da.

Analysis of spectral profiles

The spectra from all samples were imported into ClinProTools (ClinProt software version 2.0, Bruker Daltonics, Billerica, MA, USA) to subtract the baseline, normalize the spectra (using total ion current) and determine the peak *m/z* values and intensities in the mass range of 600 to 10000 Da. The spectra were processed with the following workflow: (1) spectra normalization to their total ion current; (2) spectra recalibration using the prominent peaks; (3) baseline subtraction, peak smoothing (Savitsky-Golay algorithm) and peak detection; (4) calculation of peak areas for each spectrum. The signal-to-noise (S/N) ratio was set higher than 5. To align the spectra, a mass shift of no more than 0.1% was determined during peak detection. The peak area was used for quantitative standardization, and a comparison of relative peak intensity levels between classes was also performed within the software suite. Statistical analyses of the processed spectra were performed using *t*-tests and Wilcoxon tests. *P* values less than 0.05 were considered to be statistically significant.

Metabonomic study

Sample preparation and ¹H-NMR measurement

Urine samples were prepared by mixing 350 µL of urine with 350 µL of deuterated water (D₂O), which was used as a field-frequency lock solvent, and 30 µL of 0.1% 3-trimethylsilyl-[2,2,3,3-²H₄]-propionate acid (TSP) in D₂O, which was added as a chemical shift reference (δ 0.0), to a total volume of 750 µL. Samples were centrifuged at 12000 rounds/min for 10 min, and the supernatants were transferred into 5-mm NMR tubes. NMR measurements were performed on a Varian INOVA 600 NMR spectrometer operating at 599.73 MHz, using a 5-mm triple resonance probe. Urine samples were analyzed using a 1D NOESY (RD-90°-t1-90°-tm-90°-acquire). On-resonance saturation was used to suppress the water signal during a recycling delay (RD) of 2.0 s and mixing period (tm=0.15 s). A total of 64 transients were collected into 32k time domain data points, with a spectral width of 7000 Hz. Prior to Fourier transform, the free induction decay (FID) was multiplied by an exponential weighting function corresponding to 0.5 Hz line broadening and zero-filled by a factor of 2. Spectra were manually phased and baseline corrected and referenced to TSP at δ=0.0.

Data processing and multivariate analysis

All NMR data acquired as described above were then subjected to multivariate statistical analysis. Before the analysis, the data were first reduced to integral regions of equal width

of 0.04 ppm. The spectral region between δ values of 9.48 and 0.52 was segmented into 224 integrated regions. The regions where δ ranged from 4.6 to 6.2 were excluded to avoid residual water signal and the broad resonance from urea, both of which represent highly variable regions in the spectra. The integrated data were normalized to the total integrals of each spectrum and then exported to text files for multivariate analysis. Multivariate analysis was carried out using SIMCA-P plus software (version 10.0, Umetrics, Umea, Sweden). Principal component analysis (PCA), the most commonly used method for multivariate analysis, was used to establish group separation. Prior to PCA, all NMR data variables were mean-centered and Pareto-scaled. The results of PCA were visualized by scores plot of PCs, which display the separation of groups, and the values of the PC loadings in the loadings plot reflect the spectral regions responsible for group separation in the scores plot. When group separation was not satisfied on the basis of PCA, the data were further processed using orthogonal signal correction (OSC) to remove the variations that were not correlated to the group membership and irrelevant systematic information from the data set^[14].

Metabolite set enrichment analysis and metabolic pathway analysis

In this study, the metabolite set enrichment and metabolic pathway analyses were performed by MetaboAnalyst 2.0 (<http://www.metaboanalyst.ca/MetaboAnalyst/>) with R version 2.15.0. Metabolite set enrichment analysis is a way to identify biologically meaningful patterns that are significantly enriched in quantitative metabolomics data. Metabolic pathway analysis module combines results from powerful pathway enrichment analysis with pathway topology analysis to help researchers identify the most relevant pathways that are involved in the conditions under study.

Results

Creation of plasma protein profiles

MALDI-TOF MS in combination with WCX pre-purification were used to produce the plasma protein profiles from 23 KYDS patients and 29 non-KYDS patients. Clinical characteristics of the patients are presented in Table 2. There were no significant differences in age between the KYDS patients and non-KYDS patients ($P>0.05$). All of the samples were collected using a single standard clinical protocol. Each individual plasma sample was incubated with WCX beads, and the eluted proteins were spotted for MALDI-TOF MS analysis. The entire set of mass spectra of the extracted peptides from fifty-two subjects in the two groups, ranging from 1000 to 10000 Da, was generated using the same instrument settings (Figure 1).

Comparison of plasma protein profiles between KYDS patients and non-KYDS patients

Mass spectra from 1000 to 10000 Da were obtained from linear-mode MALDI-TOF analysis of pre-fractionated plasma of KYDS patients and non-KYDS patients. The protein profiles

Figure 1. Average spectrum profile and pseudo-gel view of training set. (A) Average spectrum profile of KYDS patients (red). X-axis: molecular mass (m/z); Y-axis: relative intensity. The five different signals of the diagnostic model are labeled with the values of m/z . (B) Average spectrum profile of non-KYDS patients (green). (C) Pseudo-gel view of individual spectra of training set. X-axis: molecular mass (m/z); Y-axis: specific samples; relative intensity: grade of blackening. The horizontal line in the view indicated the separation between KYDS (upper) and non-KYDS patients (lower).

of the patients were fairly homogeneous within groups and were reproducible (data not shown). On average, 70 peptide mass peaks per sample were discovered in the plasma samples using ClinProTools software. Differences between the plasma protein profiles from the KYDS and non-KYDS patients were visually inspected, and the peaks specific to the KYDS state were identified. A k-nearest neighbor genetic algorithm was used to identify statistically significant differences in protein peaks among the two groups. Overall, eighteen peptides were upregulated and thirty peptides were downregulated in the

plasma protein profile of KYDS patients. However, no significantly different peptide mass peak was observed between the profiles of KYDS and non-KYDS groups. The top ten peptide mass peaks that were different between the groups are summarized in Table 3.

Metabolic profiles of KYDS patients and non-KYDS patients

^1H -NMR spectra of biological fluids can provide a large amount of biochemical information, which is contained within

the metabolic profile. Although NMR spectra of urine consist of hundreds of peaks, many of them can be assigned based on their chemical shifts and signal multiplicities or with the help of two-dimensional correlation spectra. The chemical shift contains information on molecular structure and can be used to discriminate ^1H -NMR spectra of various molecules. The representative NMR spectra of urine samples from KYDS patients and non-KYDS patients are shown in Figure 2. The spectral region where δ ranged from 0.0 to 10.0 was segmented

Table 3. The top ten different peaks between KYDS and non-KYDS.

Mass	PAD ^a	PTTA ^b	PWKW ^c	Ave1 ^d	Ave2 ^e	SD1 ^f	SD2 ^g
2863.05	0.597	0.572	0.704	353.52	282.4	153.82	121.63
4964.21	0.0152	0.572	0.581	102.07	143.01	38.93	68.99
2082.45	0.0928	0.572	0.581	129.16	91.66	71.59	50.4
2210.49	0.0205	0.572	0.581	113.31	83.99	63.87	49.29
5864.9	<0.000001	0.572	0.581	34.06	48.75	9.22	32.49
4819.39	0.00733	0.572	0.581	22.85	30.94	11.47	17.67
2229.42	0.52	0.572	0.704	63.66	55.79	19	11.76
4940.63	0.00131	0.572	0.581	27.49	35.22	9.25	13.64
4376.29	0.801	0.572	0.692	36.17	42.07	10.87	11.07
7466.73	0.0000635	0.572	0.581	9.37	12.23	3.16	5.01

^aP value was calculated with Anderson-Darling test; values higher than 0.05 indicated normal distribution. ^bP value was calculated with t-test; values lower than 0.05 indicated statistical relevance. ^cP value was calculated with Wilcoxon/Kruskal Wallis test; values lower than 0.05 suggest statistical relevance. ^dAverage area of peak of KYDS patients. ^eAverage area of peak of non-KYDS. ^fSD of the peak area of KYDS patients. ^gSD of the peak area of non-KYDS.

Figure 2. 600 MHz ^1H -NMR spectra of the urine samples from (A) female KYDS patients, (B) female non-KYDS patients, (C) male KYDS patients and (D) male non-KYDS patients.

into regions of 0.04 ppm in width, resulting in 250 integrated regions in each NMR spectrum. Visual inspection of the ^1H -NMR spectra revealed differences between urine samples from KYDS patients and non-KYDS patients, indicative of differences in endogenous metabolites. Most of the metabolites were identified and labeled in the spectra, mainly according to previously published data^[15].

Differential identification of metabolic profiles

Although differences can be detected by visual inspection of the ^1H -NMR spectra, this method is not applicable to the overall differentiation of metabolomics profiles between groups because data interpretation can be complicated by inter-individual variation. Therefore, the metabolic profiles from KYDS patients and non-KYDS patients were analyzed using pattern recognition techniques to detect any subtle metabolic differences. PCA is a commonly used pattern recognition method for identifying the overall clustering patterns and trends within data sets. The original data are reduced to a few latent variables or principal components (PCs) describing maximum variation within the data. Before PCA, the data set was preprocessed with Pareto scaling. Further analysis by simple PCA did not show any clear trend of group clustering between KYDS patients and non-KYDS patients. To maximize group separation and to identify discriminating metabolites, OSC was applied to the data set to remove factors unrelated to group characteristics. This approach resulted in a marked improvement in group separation between KYDS patients and non-KYDS patients, as shown in the scores plot (Figure 3A), which indicated that the metabolic profile of the KYDS group was distinct from that of the non-KYDS group. The scores plot revealed that the KYDS group could be easily distinguished from the non-KYDS group both along PC1 and PC2. Each data point corresponds to one NMR spectrum of one urine sample, and the distance between points in the scores plot is an indication of the similarity between samples. Mean scores

from the urinary samples were plotted for the first two PCs to monitor differences in endogenous metabolites between the KYDS patients and the non-KYDS patients. The corresponding loadings plots are shown in Figure 3B. Each data point represents one bucket (with the chemical shift indicated explicitly). The loadings plot identified the spectral regions that were responsible for the inter-spectra differences that we observed in the scores plot. Thus, it was possible to identify the metabolites that were responsible for differentiating the two groups in PCA. Further analysis of the PCA factor loadings and the ^1H -NMR spectrum itself using OSC-PCA can pinpoint the metabolites associated with group separation. Based on the loadings plot (Figure 3B), the major biochemical differences between the KYDS and the non-KYDS urine samples are summarized in Table 4. We found 8 metabolites to be different between the two groups: creatinine, alanine, citrate, glycine, taurine, TMAO, phenylalanine and tyrosine. The concentrations of 5 metabolites (creatinine, citrate, TMAO, phenylalanine and tyrosine) were decreased while the concentrations of 3 metabolites (alanine, glycine and taurine) were increased in the KYDS group.

Table 4. The different metabolites between KYDS and non-KYDS.

Chemical shifts (ppm)	Compounds	KYDS versus non-KYDS
1.46, 1.50	Alanine	↑
3.42, 3.26	Taurine	↑
3.54	Glycine	↑
2.54, 2.66	Citrate	↓
3.06, 4.06	Creatinine	↓
3.26	Trimethylamine-N-oxide (TMAO)	↓
7.34, 7.42	Phenylalanine	↓
6.82, 7.22	Tyrosine	↓

Figure 3. OSC-PCA scores plot from ^1H -NMR spectra of urine samples obtained from (●) KYDS patients and (■) non-KYDS patients (A) and corresponding loadings plot (B).

Metabolite set enrichment analysis and pathway analysis

The pathway analysis was performed by MetaboAnalyst 2.0 (<http://www.metaboanalyst.ca/MetaboAnalyst/>) with R version 2.15.0 to identify the most relevant pathways that are involved in the study conditions. The enrichment analysis indicated that 12 sets of metabolites that were different between the KYDS and non-KYDS groups were putatively associated with KYDS. Three cellular processes including phenylalanine and tyrosine metabolism, protein biosynthesis, and catecholamine biosynthesis were significantly enriched with *P* values of 0.006, 0.013, and 0.048, respectively (Figure 4, Table 5). The pathway analysis showed that 19 pathways involving the differentially expressed metabolites were highly associated with KYDS. Among these pathways, taurine and hypotaurine metabolism, glycine, serine and threonine metabolism, and phenylalanine metabolism had the highest impact factors of 0.33, 0.19, and 0.12, respectively (Figure 5, Table 6).

Figure 4. Metabolite enrichment analysis on the 12 putative sets of metabolites that differed between the KYDS group and the non-KYDS group. Detailed results from this enrichment analysis are shown in Table 5.

Figure 5. Summary of pathway analysis on the 19 quantified variances from the KYDS and non-KYDS groups. Detailed results of the analysis are shown in Table 6. *P* values were calculated from enrichment analysis. The pathway impact values were calculated from pathway topology analysis. 1, taurine and hypotaurine metabolism; 2, glycine, serine and threonine metabolism; 3, phenylalanine metabolism.

Discussion

Although TCM has long been practiced effectively, TCM syndrome differentiation is still a controversial subject because it relies on clinical observation and the TCM practitioner's experience, which can be subjective and unrepeatable. This nature of TCM syndromes makes this concept extremely challenging to study with modern research methods, and there has been a severe lack of scientific and technological approaches to TCM syndrome research. The emergence of systems biology is bringing forth a new set of tools for advancing personalized TCM globally. Given that metabonomics and proteomics analyze different types of molecules, they complement each other when applied to the studying of TCM. The combination of

Table 5. Result from over representation analysis.

	Total	Expected	Hits	Raw <i>P</i>	Holm <i>P</i>	FDR
Phenylalanine and tyrosine metabolism	13	0.13	2	6.09×10^{-3}	0.487	0.487
Protein biosynthesis	19	0.18	2	1.30×10^{-2}	1.00	0.519
Catecholamine biosynthesis	5	0.05	1	4.77×10^{-2}	1.00	1.00
Taurine and hypotaurine metabolism	7	0.07	1	6.62×10^{-2}	1.00	1.00
Bile acid biosynthesis	49	0.47	2	7.69×10^{-2}	1.00	1.00
Glutathione metabolism	10	0.10	1	9.33×10^{-2}	1.00	1.00
Ammonia recycling	18	0.17	1	0.162	1.00	1.00
Porphyrin metabolism	22	0.21	1	0.195	1.00	1.00
Citric acid cycle	23	0.22	1	0.203	1.00	1.00
Methionine metabolism	24	0.23	1	0.211	1.00	1.00
Glycine, serine and threonine metabolism	26	0.25	1	0.227	1.00	1.00
Tyrosine metabolism	38	0.37	1	0.315	1.00	1.00

Table 6. Result from pathway analysis.

	Total	Expected	Hits	Raw <i>P</i>	-log (<i>P</i>)	Holm adjust	FDR	Impact
Nitrogen metabolism	39	0.13	4	3.94×10^{-6}	12.4	3.15×10^{-4}	3.15×10^{-4}	0.00
Cyanoamino acid metabolism	16	0.05	2	1.13×10^{-3}	6.78	8.96×10^{-2}	3.88×10^{-2}	0.00
Aminoacyl-tRNA biosynthesis	75	0.25	3	1.45×10^{-3}	6.53	0.113	3.88×10^{-2}	0.00
Thiamine metabolism	24	0.08	2	2.57×10^{-3}	5.96	0.198	5.15×10^{-2}	0.00
Phenylalanine, tyrosine and tryptophan biosynthesis	27	0.09	2	3.26×10^{-3}	5.73	0.247	5.21×10^{-2}	0.01
Methane metabolism	34	0.11	2	5.14×10^{-3}	5.27	0.386	6.86×10^{-2}	0.00
Phenylalanine metabolism	45	0.15	2	8.91×10^{-3}	4.72	0.659	9.70×10^{-2}	0.12
Primary bile acid biosynthesis	47	0.16	2	9.70×10^{-3}	4.64	0.708	9.70×10^{-2}	0.02
Citrate cycle (TCA cycle)	20	0.07	1	6.47×10^{-2}	2.74	1.00	0.517	0.06
Taurine and hypotaurine metabolism	20	0.07	1	6.47×10^{-2}	2.74	1.00	0.517	0.33
Ubiquinone and other terpenoid-quinone biosynthesis	36	0.12	1	0.114	2.17	1.00	0.798	0.00
Glutathione metabolism	38	0.13	1	0.120	2.12	1.00	0.798	0.00
Lysine degradation	47	0.16	1	0.146	1.92	1.00	0.826	0.00
Glycine, serine and threonine metabolism	48	0.16	1	0.149	1.90	1.00	0.826	0.19
Glyoxylate and dicarboxylate metabolism	50	0.17	1	0.155	1.87	1.00	0.826	0.00
Tyrosine metabolism	76	0.25	1	0.227	1.48	1.00	1.00	0.05
Arginine and proline metabolism	77	0.26	1	0.229	1.47	1.00	1.00	0.01
Purine metabolism	92	0.31	1	0.268	1.32	1.00	1.00	0.00
Porphyrin and chlorophyll metabolism	104	0.35	1	0.298	1.21	1.00	1.00	0.00

these two 'omics' technologies is a powerful tool for elucidating pharmacological responses, biological pathways and novel biomarkers^[3]. Considering the complexity of TCM syndromes, one recommendation is that modern research on TCM syndrome differentiation should start with those syndromes that have the most obvious diagnostic evidence such as discernable symptoms and signs^[16]. The non-targeted analyses of metabolomics and proteomics may provide a global view of the organism and be used to monitor dynamic metabolic alterations that occur in different pathological processes.

Recently developed proteomic technology features high-throughput parallel analysis of all expressed proteins, thus it can potentially provide more details on molecular mechanisms at a global level^[13]. Proteomic analysis permits investigation of the qualitative, quantitative, and functional characteristics of protein profiles, and MALDI-TOF-MS is one of the most effective and sensitive approaches for the identification of potential disease biomarkers^[17, 18]. The bead-based fractionation method, which selectively separates certain peptides according to different chemical chromatographic surfaces on the outer layers of magnetic beads, has been developed for direct use in MALDI-TOF-MS analysis^[19, 20]. This mass spectrometry-based proteomics technique that selectively targets proteins to remove any undesired phenotypes is a powerful method for protein target discovery in TCM^[21]. This method does not rely on the identification of a single peptide difference; instead, it uses the profiles of a series of peptide peaks. Previous studies have already established the diagnosis models of spleen-Qi asthenia syndrome, liver-kidney Yin deficiency syndrome, and blood-stasis syndrome in HBC patients using the method above^[22]. Therefore, proteomic analysis can further our knowledge on the molecular bases of TCM syndromes.

In this study, plasma protein profiles of KYDS patients and non-KYDS patients were established. However, differential peaks in the plasma profiles between KYDS and non-KYDS patients were not discovered. The most likely reason may be that the plasma profiling method was not sensitive enough to detect KYDS-specific protein changes. Current proteomic technologies can only detect hundreds of proteins, which are far less than the whole proteome. In some cases^[23], there was only a <30% overlap in the proteins that were identified from proteomic analyses using different protein separation methods. In this study, another likely reason may be that certain biomarkers of KYDS were out of the detection range of mass-based proteomics. Although it is a high-throughput method for the analysis of blood proteomics, mass-based proteomics mainly detects low molecular weight proteins in blood (1–10 kDa). Nevertheless, our work highlights the possibilities and limitations of MALDI-TOF serum-profiling approaches. Therefore, using diverse proteomics technologies in the study of TCM syndromes should be encouraged. In future studies, we will continue to incorporate proteomics in addition to other protein biomarker discovery strategies.

Metabonomics is a powerful new technology that assesses holistic metabolic profiles via a non-targeted approach and facilitates disease biomarker discovery. Metabonomics may serve as a potential bridge between Chinese and Western medicine, and it may help advance the explaining of the theoretical meaning of evidence-based Chinese medicine^[24]. Although metabonomics started later than other 'omics' technologies, it has been successfully applied to TCM syndrome research by multiple domestic and international groups^[13, 25, 26]. In this study, we developed a ¹H-NMR method with PCA for the metabolic profiling of patient urine samples to identify meta-

bolic alterations that are specifically associated with KYDS.

The major metabolic alterations responsible for separating the KYDS from non-KYDS samples were linked to eight differentially expressed metabolites. Biochemical changes are known to be related to dysregulations of amino acid metabolism, energy metabolism and gut microflora. Thus, this finding is helpful to further our understanding of KYDS. Taurine, a sulfur-containing β -amino acid, is a major free intracellular amino acid present in many tissues of human and animals^[27]. Blood taurine concentrations may change under external stimuli or pathological situations. For example, plasma taurine concentrations may decrease in response to surgical injury, trauma, sepsis, and other critical illnesses^[28]. The increases in urine taurine levels are likely related to adaptive cellular responses in patients at various stages of KYDS.

Phenylalanine is an essential amino acid, and phenylacetylglutamine is its metabolite generated by the gut microflora. In our study, we observed a decrease in phenylalanine level in the urine of KYDS patients. In a previous study, elevated concentration of phenylalanine was found in the plasma and urine of a rat model of Kidney-Yang Deficiency Syndrome^[29], and urinary phenylalanine was found to be a biomarker of Kidney-Yang Deficiency Syndrome^[30]. Therefore, those results and the findings from the current study corroborate each other. Tyrosine is a precursor of catecholamines including dopamine, norepinephrine and epinephrine, which has been shown to be increased in the plasma and urine of rats with hydrocortisone-induced Kidney-Yang Deficiency Syndrome^[31]. Phenylalanine is needed for the synthesis of proteins, melanin and tyrosine. A reduction in urinary phenylalanine level in KYDS patients may lead to reduced tyrosine synthesis. In turn, a decrease in tyrosine level may lead to decreased production of dopamine and norepinephrine.

In this study, urinary alanine level in the KYDS patients was significantly greater than in the non-KYDS patients. Alanine is an important intermediate regulator in glucose metabolism. Normal blood alanine is transported to liver via the glucose-alanine cycle to generate pyruvate, which is also an important source for gluconeogenesis. Proper regulation of glucose catabolism and anabolism is achieved by the glucose-alanine cycle and Cori (glucose-lactate) cycle in peripheral tissues and liver. The significant increase in alanine level suggested that the glucose-alanine cycle was impaired in KYDS patients. Furthermore, elevated urinary alanine concentration may potentially serve as a biomarker of renal cortical toxicity, especially during the later stages of HgCl_2 -induced nephrotoxicity^[32].

Markedly lower levels of citrate and creatinine were observed in the KYDS patients. Citrate and creatinine are associated with energy metabolism. Citrate is a major intermediate in the tricarboxylic acid (TCA) cycle. Therefore, depletion of urinary citrate is attributed to the impairment of the TCA cycle^[33] and a deficit in mitochondrial respiration. Creatinine was also significantly decreased in the urine of KYDS patients. Creatinine is a nonenzymatic breakdown product of creatine and phosphocreatine, and the creatine-phosphocreatine system is crucial for cellular energy transpor-

tation. The decreased levels of creatinine and citrate indicated that KYDS is associated with energy metabolism dysfunction. Mitochondrion is a cellular organ with metabolic activities, which supply energy for other cellular activities via oxidative phosphorylation in the process of energy conversion. Therefore, decreased levels of urinary citrate and creatinine in the KYDS patients also suggested that a mitochondrial defect had impaired the TCA cycle. Furthermore, taurine and hypotaurine metabolism, glycine, serine and threonine metabolism, and phenylalanine metabolism were the top functions listed by MetaboAnalyst that were altered in the KYDS patients. Further investigation of these metabolic pathways may facilitate the development of noninvasive biomarker detection methods and become more efficient therapeutic strategies for KYDS.

Conclusion

In summary, we report for the first time a comprehensive analysis of proteomic and metabolic profiles that are associated with KYDS. The results indicated that there is indeed a molecular basis for syndrome differentiation. The expression profiles of proteins and metabolites showed differences between KYDS and non-KYDS patients with DM. These differences are associated with multiple molecules, cellular functions and pathways. These findings suggested that 'omics' technologies are suitable for the evaluation of TCM syndromes and syndrome biomarker screening. The combination of metabonomics and proteomics allows for the integration of multidimensional data sets to improve our understanding of the principles of TCM. We believe that future advancement in proteomics and metabonomics technologies could help further clarify the mechanisms underlying TCM principles, thus facilitating the modernization of TCM globally.

Acknowledgements

This study was supported by grants from Chinese Scientific and Technological Major Special Project (Grant No 2014ZX09J14103-03A), the Key Program of Natural Science Foundation of China (Grant No 90709012) and the National Natural Science Foundation of China (Grant No 30701073).

Author contribution

Ning JIANG designed the experiments and wrote the manuscript; Ning JIANG, Hong-fang LIU, Si-di LI, Qi ZHANG, and Xian-zhong YAN performed the experiments; Hong-fang LIU contributed to sample collection; Wen-xia ZHOU and Yong-xiang ZHANG designed the experiments.

References

- 1 Ma T, Tan CG, Zhang H, Wang MQ, Ding WJ, Li S. Bridging the gap between traditional Chinese medicine and systems biology: the connection of Cold Syndrome and NEI network. *Mol Biosystems* 2010; 6: 613-9.
- 2 Dai JY, Sun SJ, Cao HJ, Zheng NN, Wang WY, Gou XJ, et al. Applications of new technologies and new methods in ZHENG differentiation. *Evid Based Complement Alternat Med* 2012; 2012: 298014.

- 3 Jiang M, Zhang C, Zheng G, Guo HT, Li L, Yang J, et al. Traditional Chinese medicine Zheng in the Era of evidence-based medicine: a literature analysis. *Evid Based Complement Alternat Med* 2012; 2012: 409568.
- 4 Oberg AL, Kennedy RB, Li P, Ovsyannikova IG, Poland GA. Systems biology approaches to new vaccine development. *Curr Opin Immunol* 2011; 23: 436–43.
- 5 Nicholson JK, Connelly J, Lindon JC, Holmes E. Metabonomics: a platform for studying drug toxicity and gene function. *Nat Rev Drug Discov* 2002; 1: 153–61.
- 6 Zhang A, Sun H, Wang Z, Sun W, Wang P, Wang X. Metabolomics: towards understanding traditional Chinese medicine. *Planta Med* 2010; 76: 2026–35.
- 7 Bakal C, Lindring R, Lense F, Heffern E, Martin-Blanco E, Pawson T, et al. Phosphorylation networks regulating JNK activity in diverse genetic backgrounds. *Science* 2008; 322: 453–6.
- 8 Desiderio C, Rossetti DV, Iavarone F, Messana I, Castagnola M. Capillary electrophoresis – mass spectrometry: recent trends in clinical proteomics. *J Pharm Biomed Anal* 2010; 53: 1161–9.
- 9 Dai W, Wei C, Kong H, Jia Z, Han J, Zhang F, et al. Effect of the traditional Chinese medicine tongxinluo on endothelial dysfunction rats studied by using urinary metabonomics based on liquid chromatography-mass spectrometry. *J Pharm Biomed Anal* 2011; 56: 86–92.
- 10 Yu BB, Zhou CX, Zhang JY, Ling Y, Hu QF, Wang Y, et al. Latest study on the relationship between pathological process of inflammatory injury and the syndrome of spleen deficiency and fluid retention in Alzheimer's disease. *Evid Based Complement Alternat Med* 2014; 2014: 743541.
- 11 Sun S, Dai J, Wang W, Cao H, Fang J, Hu YY, et al. Metabonomic evaluation of ZHENG differentiation and treatment by Fuzhenghuayu Tablet in hepatitis-B-caused cirrhosis. *Evid Based Complement Alternat Med* 2012; 2012: 453503.
- 12 Wang Q, Yao S. Molecular basis for cold-intolerant yang-deficient constitution of traditional Chinese medicine. *Am J Chin Med* 2008; 36: 827–34.
- 13 Wang X, Zhang A, Han Y, Wang P, Sun H, Song G, et al. Urine metabolomics analysis for biomarker discovery and detection of jaundice syndrome in patients with liver disease. *Mol Cell Proteomics* 2012; 11: 370–80.
- 14 Wold S, Antti H, Lindgren F, Ohman J. Orthogonal signal correction of near-infrared spectra. *Chemometr Intell Lab Syst* 1998; 44: 175–85.
- 15 Lindon JC, Nicholson JK, Everett JR. NMR spectroscopy of biofluid. *Annual Reports on NMR Spectroscopy* 1999; 38: 1–88.
- 16 Jiang M, Lu C, Zhang C, Yang J, Tan Y, Lu A, et al. Syndrome differentiation in modern research of traditional Chinese medicine. *J Ethnopharmacol* 2012; 140: 634–42.
- 17 Ransohoff DF. Opinion - Bias as a threat to the validity of cancer molecular-marker research. *Nat Rev Cancer* 2005; 5: 142–9.
- 18 Hu J, Coombes KR, Morris JS, Baggerly KA. The importance of experimental design in proteomic mass spectrometry experiments: some cautionary tales. *Brief Funct Genomic Proteomic* 2005; 3: 322–31.
- 19 McLerran D, Grizzle WE, Feng Z, Bigbee WL, Banez LL, Cazares LH, et al. Analytical validation of serum proteomic profiling for diagnosis of prostate cancer: sources of sample bias. *Clin Chem* 2008; 54: 44–52.
- 20 McLerran D, Grizzle WE, Feng Z, Thompson IM, Bigbee WL, Cazares LH, et al. SELDI-TOF MS whole serum proteomic profiling with IMAC surface does not reliably detect prostate cancer. *Clin Chem* 2008; 54: 53–60.
- 21 Yue QX, Cao ZW, Guan SH, Liu XH, Tao L, Wu WY, et al. Proteomics characterization of the cytotoxicity mechanism of ganoderic acid D and computer-automated estimation of the possible drug target network. *Mol Cell Proteomics* 2008; 7: 949–61.
- 22 Zhou YW, Xu PC, Cheng Y. Basic pathogenesis of asthenia of healthy energy and blood stasis in liver cirrhosis studied by serum proteomics. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 2011; 31: 595–602.
- 23 Van Eyk JE. Overview: the maturing of proteomics in cardiovascular research. *Circ Res* 2011; 108: 490–8.
- 24 Wang X, Sun H, Zhang A, Sun W, Wang P, Wang Z. Potential role of metabolomics approaches in the area of traditional Chinese medicine: as pillars of the bridge between Chinese and Western medicine. *J Pharm Biomed Anal* 2011; 55: 859–68.
- 25 Jian WX, Yuan ZK, Huang XP. Detection and analysis on plasma metabolomics in patient with coronary heart disease of Xin-blood stasis syndrome pattern. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 2010; 30: 579–84.
- 26 Jiang HQ, Li YL, Xie J. Urine metabonomic study on hypertension patients of ascendant hyperactivity of gan yang syndrome by high performance liquid chromatography coupled with time of flight mass spectrometry. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 2012; 32: 333–7.
- 27 Kerai MD, Waterfield CJ, Kenyon SH, Asker DS, Timbrell JA. Reversal of ethanol-induced hepatic steatosis and lipid peroxidation by taurine: a study in rats. *Alcohol Alcohol* 1999; 34: 529–41.
- 28 Chiarla C, Giovannini I, Siegel JH, Boldrini G, Castagneto M. The relationship between plasma taurine and other amino acid levels in human sepsis. *J Nutr* 2000; 130: 2222–7.
- 29 Huang DX, Yang J, Lu XM, Deng Y, Xiong ZL, Li FM. An integrated plasma and urinary metabonomic study using UHPLC-MS: intervention effects of Epimedium koreanum on 'Kidney-Yang Deficiency syndrome' rats. *J Pharm Biomed Anal* 2013; 76: 200–6.
- 30 Lu XM, Xiong ZL, Li JJ, Zheng SN, Huo TG, Li FM. Metabonomic study on 'Kidney-Yang Deficiency syndrome' and intervention effects of Rhizoma Drynariae extracts in rats using ultra performance liquid chromatography coupled with mass spectrometry. *Talanta* 2011; 83: 700–8.
- 31 Chen MJ, Zhao LP, Jia W. Metabonomic study on the biochemical profiles of a hydrocortisone-induced animal model. *J Proteome Res* 2005; 4: 2391–6.
- 32 Holmes E, Nicholson JK, Nicholls AW, Lindon JC, Connor SC, Polley S, et al. The identification of novel biomarkers of renal toxicity using automatic data reduction techniques and PCA of proton NMR spectra of urine. *Chemometr Intell Lab Syst* 1998; 44: 245–55.
- 33 Akira K, Masu S, Imachi M, Mitome H, Hashimoto M, Hashimoto T. ¹H NMR-based metabonomic analysis of urine from young spontaneously hypertensive rats. *J Pharm Biomed Anal* 2008; 46: 550–6.