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

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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

Original Article

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-

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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 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) Spermatonhea |
| (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 metabonomics 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 10 000 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 10 000 Da were obtained from linear-mode MALDI-TOF analysis of pre-fractionated plasma of KYDS patients and non-KYDS patients. The protein profiles 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**

$^1$H-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 $^1$H-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 $\delta$ ranged from 0.0 to 10.0 was segmented.

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

| Mass    | PAD   | PTWA | PWKW | Ave1  | Ave2  | SD1  | SD2  |
|---------|-------|------|------|-------|-------|------|------|
| 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| 63.87 | 49.29 |
| 2082.45 | 0.0928| 0.572| 0.581| 129.16| 91.66 | 63.87 | 50.4  |
| 2210.49 | 0.0205| 0.572| 0.581| 113.31| 83.99 | 63.87 | 49.29 |
| 5864.9  | <0.00001| 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 | 0.5  | 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 | 0.000635| 0.572| 0.581| 9.37  | 12.23 | 3.16 | 5.01  |

$^a$P value was calculated with Anderson-Darling test; values higher than 0.05 indicated normal distribution. $^b$P value was calculated with t-test; values lower than 0.05 indicated statistical relevance. $^c$P value was calculated with Wilcoxon/Kruskal Wallis test; values lower than 0.05 suggest statistical relevance. $^d$Average area of peak of KYDS patients. $^e$Average area of peak of non-KYDS. $^f$SD of the peak area of KYDS patients. $^g$SD of the peak area of non-KYDS.

Figure 2. 600 MHz $^1$H-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 $^1$H-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\cite{147}.

**Differential identification of metabolic profiles**

Although differences can be detected by visual inspection of the $^1$H-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 $^1$H-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.

| 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](image-url). OSC-PCA scores plot from $^1$H-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).

Table 5. Result from over representation analysis.

| Metabolite set                                           | Total | Expected | Hits | Raw P       | Holm P   | 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 |

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
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 metabonomics 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-

Table 6. Result from pathway analysis.

| Pathway                                | Total | Expected | Hits | Raw P        | -log (P) | 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   |
| Cyanobiochemical metabolism            | 16    | 0.05     | 2    | 1.13×10^{-3} | 6.78     | 8.96×10^{-2} | 3.88×10^{-2} | 0.00   |
| Aminoacy-H-RNA biosynthesis            | 75    | 0.25     | 3    | 1.45×10^{-4} | 6.53     | 0.113       | 3.88×10^{-2} | 0.00   |
| Thiamine metabolism                    | 24    | 0.08     | 2    | 2.57×10^{-4} | 5.96     | 0.198       | 5.15×10^{-2} | 0.00   |
| Phenylalanine, tyrosine and tryptophan biosynthesis | 27    | 0.09     | 2    | 3.26×10^{-4} | 5.73     | 0.247       | 5.21×10^{-2} | 0.01   |
| Methanol metabolism                    | 34    | 0.11     | 2    | 5.14×10^{-4} | 5.27     | 0.386       | 6.86×10^{-2} | 0.00   |
| Phenylalanine metabolism               | 45    | 0.15     | 2    | 8.91×10^{-4} | 4.72     | 0.659       | 9.70×10^{-2} | 0.12   |
| Primary bile acid biosynthesis         | 47    | 0.16     | 2    | 9.70×10^{-4} | 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   |
| Ubiquitinone 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   |
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 phenylacetylglucine 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[30], and urin ary 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 catabolism and anabolism is achieved by the glucose-alanine cycle. Normal blood alanine is transported to liver via the glucose-alanine cycle and Cori (glucose-lactate) cycle in peripheral tissues and anabolism is achieved by the glucose-alanine cycle. Alanine is an important intermediate regulator in glucose metabolism. In the current study, we observed a decrease in alanine level in the urine of KYDS patients. In a previous study, elevated urinary alanine level was found in the plasma and liver of rats exposed to phenylalanine[28]. The increases in alanine levels are likely related to adaptive cellular responses in patients at various stages of KYDS.

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.

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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.

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