Discrimination of type 2 diabetes mellitus corresponding to different traditional Chinese medicine syndromes based on plasma fatty acid profiles and chemometric methods

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ARTICLE INFO

ABSTRACT

Aims of the study: Traditional Chinese medicine (TCM) has a long history and particular advantages in the diagnosis and treatment of type 2 diabetes mellitus (T2DM). Syndrome differentiation is the foundation and essence of TCM theories. The aims of the study are to discriminate T2DM corresponding to different syndromes (Qi-deficiency, Qi and Yin-deficiency and Damp heat) and discover syndrome-related biomarkers using metabolomics technology.

Materials and methods: Plasma fatty acid profiles of 85 clinical samples were established by high performance liquid chromatography (HPLC). Moreover, some of the lipid parameters, including total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL), were obtained through clinical testing methods. Orthogonal signal correction-partial least squares (OSC-PLS) and uncorrelated linear discriminant analysis (ULDA) were employed to establish two-class models for three different syndromes.

Results: Compared with the plasma fatty acid profiles of healthy controls, the characteristic fatty acids of three TCM syndromes were discovered ($p < 0.01$). Totally, the plasma fatty acids of T2DM were up-regulated, while significant differences existed in different syndromes. Results of ULDA indicate that the three TCM syndromes could be effectively separated by the plasma fatty acid profiles and the syndrome-related biomarkers were also screened.

Conclusions: The fact that three TCM syndromes can be separated indicates certain metabolic differences in different TCM syndromes of T2DM really exist and such differences can be manifested by fatty acids and lipid parameters. The results benefit modern biological interpretation of the three TCM syndromes and in a sense the diagnosis and treatment of diabetes.

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1. Introduction

Traditional Chinese medicine (TCM) is a healthcare-focused medical system with over 3000 years of continuous practice experience and refinement through treatment observations (Zhang et al., 2010) and therefore has its own advantages and characteristics in early intervention and personalized treatment. In TCM theories, syndrome differentiation (also called as Zheng differentiation or pattern classification) is the foundation and essence (Jiang et al., 2012). The diagnostics mainly depend on overall observation of human symptoms including observation, listening smelling, touching and background investigation (Jiang, 2005; Ma et al., 2010) rather than "micro" level tests. For the same disease, different

therapeutic methods would be sometimes employed to treat different pathological states. These correlations between syndromes and corresponding treatments are from practice experience and refined by long-term treatment observations. However, TCM faces severe challenges and suffers from insufficient modern research owing to lack of scientific and technologic approaches (Lu et al., 2011; Wang et al., 2011). Thus, it is necessary to investigate the changes of compounds (including metabolites, proteins, fatty acids etc.) among several symptoms of the same disease to validate these experiences and extend the understanding of disease.

Type 2 diabetes mellitus (T2DM) is one of major causes of morbidity all over the world. It is a typical metabolic disorder with a chronic turbulence. To date, several candidate mechanisms have emerged in the past decade, such as lipotoxicity and inflammation (Taubes, 2009), though the curative effect is still limited. In TCM, T2DM was regarded as Xiaokezheng with symptomatic polydipsia, according to the TCM theory (Ning et al., 2009). Chinese practitioners further divide Xiaokezheng into different syndromes with

corresponding clinical manifestation, including Qi-deficiency, Qi and Yin-deficiency and Damp heat (Zheng, 2002). Chinese medicines could supply more effective personalized treatment for their pathological features. To date, the advantages of TCM in the treatment of diabetes mellitus are valued by an increasing number of people.

Strategy of metabolomics might be employed to provide a feasible method for the modernization of traditional Chinese medicine, namely, the changes of metabolites were investigated among several syndromes of the same disease (Nicholson and Lindon, 2008). Among metabolites, fatty acids not only provide an important energy source as nutrients, but also act as signaling molecules in various cellular processes (Han et al., 2000; Perassolo et al., 2003). It was reported that the plasma fatty acid concentration in subjects with diabetes and pre-diabetes was higher than that in healthy controls (Bergman and Ader, 2000). Additionally, elevated fatty acid concentration would clearly influence the insulin sensitivity (Funaki, 2009) and metabolized to various molecules which may participate or interfere with insulin-mediate signal transduction (Corcoran et al., 2007). Therefore, the combination of fatty acid profiles and serum parameters, such as total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) might be a powerful tool for elucidating the pathology of disease, screening for potential biomarkers and monitoring clinical pharmacologic therapy.

In the previous study, we researched the differences between TCM syndromes patients and healthy controls (Xu et al., 2010; Xu et al., 2012). Discrimination of different syndromes and the discovery of syndrome-related biomarkers were more meaningful for revealing the essence of syndrome. In this study, a metabolomics approach was used to evaluate the levels of plasma fatty acids in patients with the three TCM syndromes of T2DM for investigating whether the fatty acid profiles could distinguish the different syndromes. Compared with the commonly used method of partial least squares (PLS), uncorrelated linear discriminant analysis (ULDA) is much more efficient for revealing discriminatory information in metabolomics data (Ye et al., 2006; Yuan et al., 2008). Thus, this multivariate analysis method was employed to discriminate three TCM syndromes and discover syndrome-related biomarkers..

2. Experimental

2.1. Solvents and standards

All the fatty acid standards (more than 99.0% purity) used in this experiment were purchased from Sigma (St. Louis, MO, USA), including myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), α-linolenic acid (α-C18:3), γ-linolenic acid (γ-C18:3), eicosadienoic acid (C20:2), arachidonic acid (C20:4), eicosapentaenoic acid (C20:5), docosahexaenoic acid (C22:6). Heptadecanoic acid was used as internal standard. Phase

transfer catalyst 18-Crown-6 and derivation reagent α-bromoacetophenone (phenacyl bromide) were also purchased from Sigma. Acetonitrile of chromatographic grade was obtained from Burdick & Jackson (USA). Other solvents were of analytical grade and purchased from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). The stock solution of fatty acids was prepared by dissolving individual standards in acetone and store in refrigerator. The working solution was further diluted prior to HPLC analysis.

2.2. Plasma sample collection

The study was approved by the ethics committee of institute and adhered to the tenets of the Declaration of Helsinki. Additionally, informed consent to the study protocol was obtained from patient.

Plasma samples were collected from 25 healthy adults and 60 type 2 diabetic patients with different TCM syndromes, i.e., 20 cases of Qi-deficiency, 20 cases of Qi and Yin-deficiency and 20 cases of Damp heat patients. In three experimental groups, all the patients selected were diagnosed by both syndrome differentiation of TCM and western medicine. They were provided by Affiliated Hospital of Tianjin University of Traditional Chinese Medicine under the guideline of the University Human Subjects Committee. The fasting plasma glucose (FPG) values of the patients were above 7.0 mmol/L and some of the lipid parameters, such as total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were obtained through clinical testing methods. It was observed by an authentic Chinese doctor in accordance with "Diabetes TCM Diagnostic Criteria" (Zheng, 2002). The clinical features of the three TCM syndromes are shown in Table 1.

2.3. Fatty acid extraction and derivatization

The collected plasma sample were immediately anticoagulated with EDTA-Na and then centrifuged at 3000 rpm for 10 min. The isolated plasma was stored at $-70\,^{\circ}\text{C}$ until analysis. In the experiment, the samples were firstly separated from protein by trichloroacetic acid and then extracted with n-hexane three times. The derivatization procedure was referenced in the report by Mehta et al. (1998). The details were not described here.

2.4. Fatty acid analysis

After derivatization, the samples were analyzed on Agilent 1200 HPLC system equipped with a multi-wavelength UV detector (Germany). The HPLC separation was performed on Symmetry C18 column (250 mm × 4.6 mm, 5 μm, Waters, Ireland). Acetonitrile and pure water were used as the mobile phase. The elution gradient program started with 74% acetonitrile and then increased linearly to 84% in 40 min, then to 95% in the next 10 min and kept this proportion for 6 min. The flow rate was set to 1.0 mL/min, while the column temperature was maintain at $55\,^{\circ}\text{C}$ in the analytic process.

Table 1
The clinical features of three traditional Chinese medicine (TCM) syndromes.

	Qi deficiency	Qi and Yin deficiency	Damp heat
Representative	Lethargy	Soreness of waist and knees	Thirsty
	Constipation	Sleep hyperhidrosis	Diuresis
Holistic			Hyperorexia
Symptoms		Palpitations and insomnia	Weight loss
Tongue appearance	Fat tongue	Reddish tongue	Red tongue with yellow fur
Pulse pattern	Thin and weak pulse	Thin and rapid pulse	Stringy pulse

Table 2
Quantitative analysis from fatty acids of TCM syndromes and healthy controls.

Fatty acid	Control (n=25)	Qi-deficiency (n=20)	Qi and Yin-deficiency (n=20)	Damp heat (n=20)
Myristic acid (C14:0)	4.01 ± 0.98	5.69 ± 1.48*	6.44 ± 1.80**	5.43 ± 1.30*
Palmiticacid (C16:0)	113.31 ± 20.21	177.40 ± 40.98***	190.98 ± 58.72**	164.03 ± 44.01***
Palmitoleic acid (C16:1)	2.33 ± 0.63	4.35 ± 1.69*	4.61 ± 1.05***	3.45 ± 1.44
Stearic acid (C18:0)	68.36 ± 11.60	97.80 ± 21.27**	97.83 ± 26.46**	90.32 ± 18.61***
Oleic acid (C18:1)	93.49 ± 24.77	165.28 ± 57.38***	190.23 ± 58.08***	142.49 ± 47.24***
Linoleic acid (C18:2)	91.26 ± 24.86	3.29 ± 36.77**	180.68 ± 55.78***	136.90 ± 44.46*
α-linolenic acid (α-C18:3)	5.25 ± 1.50	6.47 ± 1.77*	7.43 ± 2.04**	6.32 ± 1.84
γ-linolenic acid (γ-C18:3)	1.38 ± 0.31	1.85 ± 0.51*	1.97 ± 0.76*	1.95 ± 0.62*
Eicosadienoic acid (C20:2)	1.67 ± 0.17	2.23 ± 0.58***	2.72 ± 0.81***	2.05 ± 0.58*
Arachidonic acid (C20:4)	5.92 ± 1.57	9.36 ± 2.37**	10.14 ± 2.43**	8.40 ± 2.18**
Eicosapentaenoic acid (C20:5)	1.19 ± 0.04	1.57 ± 0.24***	1.61 ± 0.29***	1.46 ± 0.26***
Docosahexaenoic acid (C22:6)	2.69 ± 0.64	4.83 ± 1.32***	5.59 ± 1.55***	4.59 ± 1.30***
TC	4.42 ± 0.78	5.49 ± 0.90***	7.77 ± 1.85***	8.31 ± 2.64***
TG	1.10 ± 0.52	1.53 ± 0.74*	5.58 ± 1.10***	5.47 ± 1.43***
LDL	1.62 ± 0.39	2.51 ± 0.76***	1.58 ± 0.92	1.80 ± 0.61
HDL	1.51 ± 0.31	1.32 ± 0.18*	2.93 ± 0.83***	2.19 ± 0.73***

Significance of group differences is given by.
TC: total cholesterol; TG: triglycerides; LDL: low-density lipoprotein; HDL: high-density lipoprotein.

* for $P < 0.01$.
** for $P < 0.001$.
*** for $P < 0.0001$.

2.5. Multivariate analysis

2.5.1. Orthogonal signal correction-partial least squares (OSC-PLS)

As the most commonly used multivariate statistical analysis method, partial least squares (PLS) was firstly carried out to identify the difference between patients with three TCM syndromes and healthy controls, respectively (Ebbels et al., 2003; Gavaghan et al., 2002). Orthogonal signal correction (OSC) was implemented before PLS-LDA (linear discriminant analysis) to remove unwanted systematic variation in spectral data in order to enhance the predictive power and low the complexity of the resulting PLS models, and obtain a great simplification in terms of model interpretation (Wold et al., 2001).Variable selection was employed to obtain the best feature combination to discriminate the two groups (Wold et al., 1998). The LVs (latent vectors) generated by PLS explaining at least 85% of the total variance were used as the input to LDA. The recognition ability of OSC-PLS-LDA models was evaluated by five-fold cross validation. All samples are divided into five groups. Each time, one group is selected as test set and the other four groups are train set to train model, which then predicts the classes of samples in test set. This process is repeated five times, so all samples have been used as test data once and the prediction rates are calculated.

2.5.2. Uncorrelated linear discriminant analysis (ULDA)

As a conventional method based on Fisher criterion function, ULDA was firstly proposed in the field of face recognition (Jin et al., 2001) and improved by Ye et al. (2006). The improved algorithm of ULDA is briefly described as follows and more information can be found in Ye et al. (2006) and Yuan et al. (2008).

The aim of ULDA is to find a linear transformation matrix G to transform the data matrix X to matrix Z in low-dimension space with the constraint of so-called "S-orthogonality".

$$Z = XG$$

The new variables, uncorrelated discriminant vectors (UDVs), maximizes the class separation and are mutually orthogonal. According to the theory of ULDA, the optimal number of UDVs is k-1, where k represents the number of classes of samples. More importantly, as linear combination coefficients of data matrix for UDVs, the transformation matrix G could render the influence of variables on separating different classes of samples and therefore be employed to evaluate the importance of variables by the transformation matrix plot. All programs used were coded in MATLAB7.6 for windows.

3. Results and discussion

Using the analytical conditions abovementioned, fatty acid profiles from 60 T2DM patients and 25 healthy controls were obtained by the pre-column derivatization HPLC method. Additionally, the precision, recovery and linear ranges of the conditions were also investigated. Relative standard deviation (RSD) ranges from 0.97% to 2.52% for inter-day assay, recoveries are in the range of 93.20–102.81% and the correlation coefficient (R^2) of the calibration curves is higher than 0.991. All these results demonstrate the optimized method was reliable for quantitative analysis of plasma fatty acids. Using these conditions, the fatty acids in plasma from 60 T2DM patients and 25 healthy controls were quantitative analyzed for the following metabonomics study.

3.1. Multivariate statistical analysis

3.1.1. Fatty acid composition of clinical plasma samples

Plasma collected from patients with TCM syndromes and healthy controls contain almost the same fatty acids. The main differences among them are quantitative, i.e. the fatty acid levels in the three TCM syndromes are significantly higher than those of healthy controls. Fundamental statistics of independent-samples T test (2-tailed) was performed to compare the targeted fatty acid levels and lipid parameters (TC, TG, HDL and LDL) of the three TCM syndromes with those of healthy controls, respectively (see Table 2). These results indicate that the average concentrations of some fatty acids were significant differences among the three syndromes, as well as betweenT2DM and healthy samples.

These results reflect the change trends of fatty acids from three TCM syndromes to healthy controls. However, whether these fatty acids could completely separate these three syndromes of diabetes from healthy controls and which fatty acids are most important ones for classification model are still unknown. Thus, it

is necessary to further investigate the fatty acid profiles with the help of chemometric methods.

3.1.2. Three TCM syndromes of T2DM and healthy controls

Long-term complications from high blood sugar can increase the risk of heart attacks, strokes, amputation and diabetic retinopathy. Intuitively, T2DM could be divided into several cases without or with different complications. Unfortunately, till now, there is no classification of T2DM according to clinical parameters. Interestingly, different syndromes of T2DM could be determined by TCM theories though they are just from long-term practice experience. More importantly, the special treatment is usually given under the direction of different syndromes. Thus, to further understand the T2DM, it is necessary to study the different syndromes using modern biological method. In this study, the fatty acid profiles of three syndromes and healthy controls are investigated using the strategy of metabolomics.

In this study,12 fatty acids and 4 lipid parameters (TC, TG, HDL and LDL) were detected to investigate the T2DM patients with different syndromes. OSC-PLS was employed to establish the discriminant models for T2DM patients with three TCM syndromes and healthy controls respectively and subsequently investigate the relationships between metabolites and syndromes. OSC-PLS-LDA is a sophisticated and commonly used supervised clustering method for establishing the optimal discriminant surface to separate best classification (Nordhausen, 2009; Bylesjö et al., 2006). The correct

rate of three TCM syndromes and healthy controls was 98.2%, 99.3% and 96.8%, respectively. Thus, the statistic analysis results indicate plasma fatty acid metabolic profiles could reflect part of the perturbations of three syndromes in TCM and healthy controls.

3.1.3. Comparison of three TCM syndromes

In TCM theories, different syndromes represent the different stages of one disease, or a variety of clinical symptoms. Whether the differences among syndromes have an internal relationship with the metabolic network and whether these differences can be manifested by fatty acid metabolic profiles is the research question. With these thoughts, we try to analyze the diversity of three syndromes to find the biomarkers which can be used to distinguish these syndromes. The difference among metabolic profiles of different syndromes in diabetics was smaller than the difference between T2DM patients and healthy controls, thus it is very difficult to have good classification results using common multivariate statistical methods, such as OSC-PLS. From Fig.1 A–C, many samples in different groups were also scattered with each other, especially Qi and Yin-deficiency vs Damp heat. Therefore, new multivariate statistical methods might be necessary to overcome this difficulty. Uncorrelated linear discriminant analysis (ULDA) is a powerful tool for exploration of metabolomics and proteomics data (Yi et al., 2008; Zhang et al., 2011).

According to the ULDA theory, the optimal number of uncorrelated discriminant vectors (UDVs) is k-1, thus only the first UDV is plotted. As shown in Fig. 2, ULDA could obtain a better
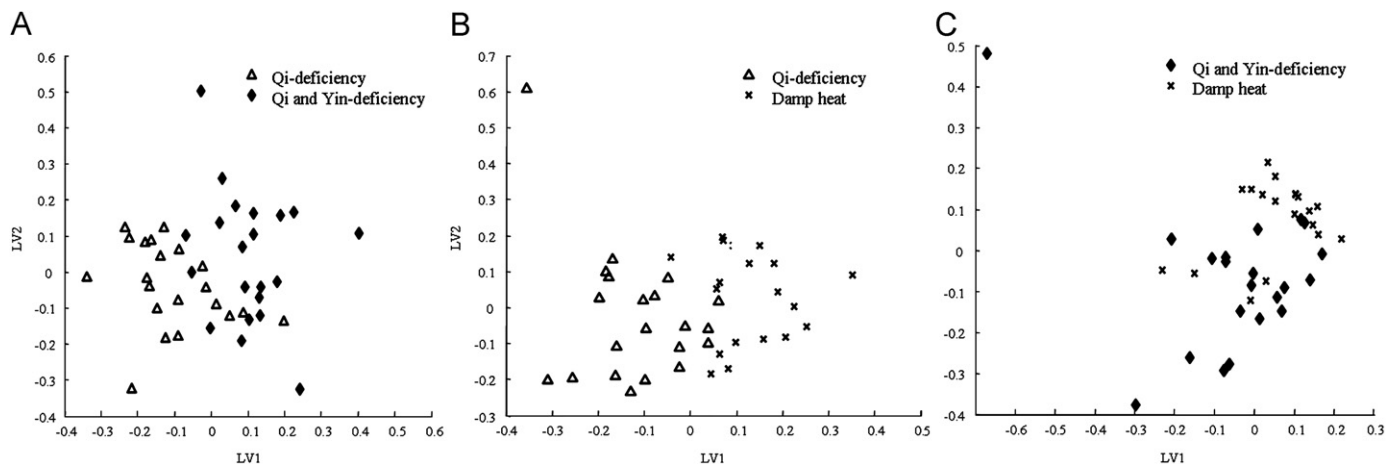

Fig. 1. OSC-PLS score plots of three TCM syndromes. Qi-deficiency (♦), Qi and Yin-deficiency (Δ), Damp heat (×).
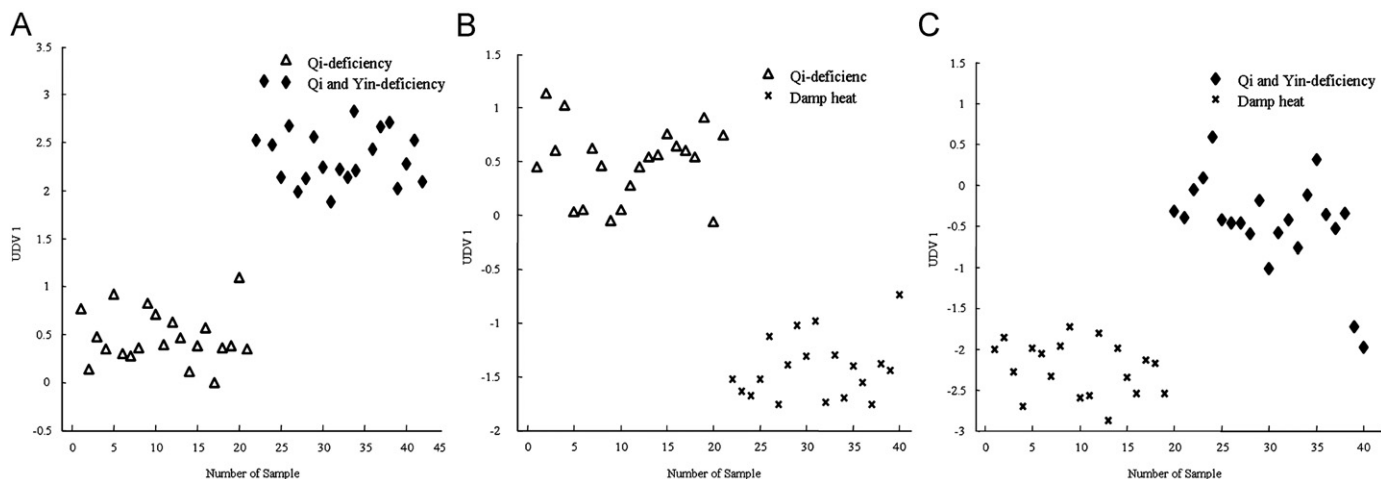

Fig. 2. The results of ULDA for classification three TCM syndromes. Qi-deficiency (♦), Qi and Yin-deficiency (Δ), Damp heat (×).
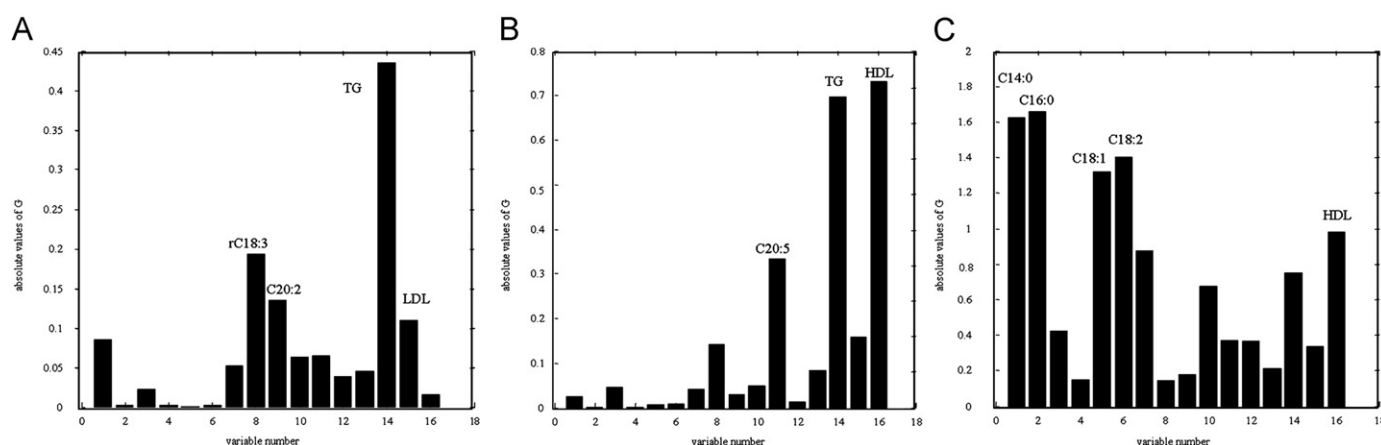
Fig. 3. Syndrome-related biomarkers for three TCM syndromes based on ULDA. (A) Qi-deficiency vs Qi and Yin-deficiency (B) Qi-deficiency vs Damp heat (C) Qi and Yin-deficiency vs Damp heat Variable order from 1 to 16: C20:5, α–C18:3, γ-C18:3, C22:6, C14:0, C20:4, C18:2, C16:1, C16:0, C18:1, C20:2, C18:0, total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) and high-density lipoprotein (HDL).

separation effect and Qi-deficiency vs Qi and Yin-deficiency (A), Qi-deficiency vs Damp heat (B) and Qi and Yin-deficiency vs Damp heat (C) were separated in the first UDV. In contrast to PLS-DA, the objective function of ULDA could maximize the ratio of variance between groups to the variance within groups. Since the composite variables in low-dimensional space obtained by ULDA are mutually uncorrelated, they have large discriminatory ability but small redundancy. The fact that three TCM syndromes can be effectively separated indicates certain metabolic differences in different TCM syndromes of T2DM really exist and such differences can be manifested by fatty acids and lipid parameters. Studies on different syndromes of T2DM might provide some improvement and supplement to the optimum diagnosis and treatment of diabetes, even revealing the pathogenesis.

3.2. Potential biomarkers of different TCM syndromes

The biomarkers reflect the metabolic abnormality of disease to some extent. The different stages of one disease might have different pathological features and therefore have corresponding biomarkers. Screening potential biomarkers related to these syndromes has important practical significance. It can not only provide effective information to elucidate different metabolic pathways for these three syndromes of diabetes, but also assist TCM clinical diagnosis and individualized treatment based on accurate classification of different syndromes.

Furthermore, the absolute value of transformation matrix G was used to evaluate the influence of variables on separating different syndromes. The advantage of ULDA in biomarker screening is that it could collect the correct information efficiently for discriminant analysis. For biomarker discovery, ULDA mainly emphasizes the variables with best discriminatory ability, while OSC-PLS involves all the variables with explanatory information. As shown in Fig. 3, it is found that various potential biomarkers of different syndromes were discovered, indicating that different pathological stages of disease might be closely linked to metabolism status. For the two groups Qi-deficiency vs Qi and Yin-deficiency (A), γ-C18:3, C20:2 TG and LDL were the potential biomarkers, C20:5, TG and HDL were the candidate biomarkers of Qi-deficiency and Damp heat (B), while for Qi and Yin-deficiency and Damp heat (C), C14:0, C16:0, C18:1, C18:2 and HDL provided the main classification information. These biomarkers represent the major features of the syndromes and their content changes are responding to T2DM development and can be used to distinguish different states. Dividing disorders into different stages according to different syndromes followed by subsequent

symptom-specific treatment is an innovative and efficient strategy to treat complex diseases. Meanwhile, discovery of biomarkers associated with syndromes is particularly important for personalized treatment. The syndrome-related biomarkers can assist clinical diagnosis in TCM, in addition, it maybe promote the modernization of TCM and provide some reference to reveal the pathogenesis of diabetes.

4. Conclusions

Syndrome differentiation is the foundation and essence of TCM and also a difficult issue in modernization of TCM. In this paper, fatty acid profiles and clinical lipid parameters were employed to reflect the metabolic differences between three TCM syndromes and healthy controls, respectively. Different biomarkers discovered by ULDA for the different syndromes might provide with a clue to investigate TCM syndromes. Further studies on the characterization of fatty acids will contribute to the understanding of the three TCM syndromes of T2DM and the development of new diagnosis strategies for them. This article is the paradigm of metabolomics applied in TCM research to offer new ideas and practical approaches in modernization of TCM.

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