Biochimica et Biophysica Acta 1832 (2013) 1207–1216

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Metabolic signatures of esophageal cancer: NMR-based metabolomics and UHPLC-based focused metabolomics of blood serum

Xiaoli Zhang [a], Luan Xu [a], Jianmin Shen [a,b], Bei Cao [c], Ting Cheng [a], Tong Zhao [a], Xiaoyan Liu [a], Haixia Zhang [a,*]

[a] State Key Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou 730000, China
[b] Institute of Biochemistry and Molecular Biology, School of Life Science, Lanzhou University, Lanzhou 730000, China
[c] Key Lab of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 210009, China

ARTICLE INFO

Article history:
Received 28 August 2012
Received in revised form 28 January 2013
Accepted 10 March 2013
Available online 20 March 2013

Keywords:
Metabolomics
Focused metabolomics
Proton nuclear magnetic resonance (^{1}H NMR)
Ultra high performance liquid chromatography (UHPLC)
Esophageal cancer (EC)

ABSTRACT

Focused metabolic profiling is a powerful tool for the determination of biomarkers. Here, a more global proton nuclear magnetic resonance (^{1}H NMR)-based metabolomic approach coupled with a relative simple ultra high performance liquid chromatography (UHPLC)-based focused metabolomic approach was developed and compared to characterize the systemic metabolic disturbances underlying esophageal cancer (EC) and identify possible early biomarkers for clinical prognosis. Serum metabolic profiling of patients with EC (n = 25) and healthy controls (n = 25) was performed by using both ^{1}H NMR and UHPLC, and metabolite identification was achieved by multivariate statistical analysis. Using orthogonal projection to least squares discriminant analysis (OPLS-DA), we could distinguish EC patients from healthy controls. The predictive power of the model derived from the UHPLC-based focused metabolomics performed better in both sensitivity and specificity than the results from the NMR-based metabolomics, suggesting that the focused metabolomic technique may be of advantage in the future for the determination of biomarkers. Moreover, focused metabolic profiling is highly simple, accurate and specific, and should prove equally valuable in metabolomic research applications. A total of nineteen significantly altered metabolites were identified as the potential disease associated biomarkers. Significant changes in lipid metabolism, amino acid metabolism, glycolysis, ketogenesis, tricarboxylic acid (TCA) cycle and energy metabolism were observed in EC patients compared with the healthy controls. These results demonstrated that metabolic profiling of serum could be useful as a screening tool for early EC diagnosis and prognosis, and might enhance our understanding of the mechanisms involved in the tumor progression.

1. Introduction

Worldwide, in 2008, an estimated 482,300 patients have been diagnosed with esophageal cancer (EC) and resulted in 406,800 deaths, making this disease the eighth leading cause of cancer death in men [1]. Higher incidence rates have been reported in South and East Africa, Eastern Asia, North-Central China, and North Iran [2]. Despite recent improvements in diagnosis, the prognosis and survival of the patients remain universally poor, with overall 5-year survival rates of only 5%–15% [3,4]. It is believed that detecting cancer at an early stage would result in a decrease in mortality. To achieve this, various approaches such as upper gastrointestinal endoscopy, barium esophagram, non-endoscopy-based balloon cytology, and serological tumor markers (i.e., PTHrP and Cyfra 21-1) tests for cancer screening and surveillance have been taken. However, each of them has specific limitations in their own ways. For instance, the accuracy of endoscopic screening and surveillance relies on taking adequate biopsies of the esophageal mucosa, although it remains to be the most commonly used method; and serological tumor marker tests were not useful as a screening or surveillance test for their low specificity and sensitivity [5,6].

Metabolomics, a relatively novel methodology arising from the post-genomics era, has been increasingly recognized as a valuable complementary approach to other well-established 'omic' sciences (genomics and proteomics), to aid in the assessment of disease and toxicity [7–9]. Metabolic profiling is the comprehensive studying of large numbers of all endogenous low molecular weight metabolites and their roles in various disease states in a global view [10,11]. An important goal of metabolomics is the identification of useful biomarkers for disease diagnosis and prognosis. Metabolic profiling based on mainly nuclear magnetic resonance (NMR) [12–14], chromatographic techniques (liquid chromatography (LC), gas chromatography (GC)) or their hyphenated techniques [15–18] can be used to monitor multiple metabolic changes simultaneously in pathological processes and characterize the dynamic metabolic response of key intermediary biochemical pathways. Quantitative metabolic profiling may significantly improve the chances for the discovery of disease-related biomarkers. With the development of metabolomic technology in the past few years, large

* Corresponding author. Tel.: +86 931 8912510; fax: +86 931 8912582.
E-mail address: zhanghx@lzu.edu.cn (H. Zhang).

0925-4439/$ – see front matter.
http://dx.doi.org/10.1016/j.bbadis.2013.03.009

improvements have been made in cancer diagnosis and prognosis based on metabolomic analysis. Metabolic profiling has been used to assess various cancer progressions and many studies have attempted to find novel biomarkers for the early detection of these diseases [19–23].

Although metabolomic analysis is a promising approach in screening for diseases such as cancer, certain limitations including the requirements to measure most endogenous metabolites existing, data-redundancy problems, false discovery problems, and cost constraints remain major challenges to metabolomic research [24,25]. To overcome these problems, many excellent platforms known as focused metabolomics which limit the objects of the analysis to a class of the targeted metabolites that play important roles in general metabolism exist. Focused metabolomics that restrict their target analytes to those measured well by the focused analytical technologies can produce data with high quality that maximize sensitivity and minimize the false discovery problem. The most developed focused metabolomic fields include lipid profiling, amino acid profiling, amine profiling, simple sugars profiling, etc. [26–29]. In the present study, we also used a focused metabolomics, ultra high performance liquid chromatography (UHPLC) analysis of serum free amino acids (SFAA), for the development of a more simple classification model and insights into the altered metabolic pathways in EC. As is well known, profiling of SFAA is also a promising approach because amino acids link all organ systems and play essential roles in metabolism. Furthermore, amino acid profiles are known to be influenced by metabolic variations induced by specific diseases, including cancers [30]. Therefore, a UHPLC method was used to determine the characteristics of the amino acid profiles in EC patients and the possibility of using this information for the early detection. Metabolomics and focused metabolomics exhibit different analytical strengths and weaknesses, and give complementary information. Here, both of the two methods have been used for our metabolomic analysis.

In the present study, using a more global NMR-based metabolic profiling approach and a relative simple UHPLC-based focused metabolic profiling approach, respectively, we investigated serum samples from twenty-five subjects with primary EC, as well as serum from twenty-five healthy individuals with the aids of multivariate statistical analysis. Following the recently reported studies [18,31,32] on plasma or serum metabolic profiling, the present work aims to discover novel biomarkers for EC and determine if distinguished metabolites for the establishment of improved clinical biomarkers which reflect the presence of the disease can be found and, thus, if the simple UHPLC-based amino acid profiling may be useful in EC detection.

2. Materials and methods

2.1. Volunteer recruitment and sample collection

The present work was approved by the Ethical Committee of First Affiliated Hospital of Lanzhou University, and was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans http://www.wma.net/en/30publications/10policies/b3/index.html.

A total of twenty-five patients with primary esophageal tumors (13 females, 12 males, mean age 56 ± 5 years) and twenty-five healthy control subjects (10 females, 15 males, mean age 49 ± 8 years) were enrolled in this study following informed consent. Only subjects who had never received any antitumor therapy prior and without any other complicating diseases were included as EC cases in this study. Volunteers in the control group were included on the basis of a physicians' assessment of their general health status (body mass index, normal values in blood plasma and urine standard clinical tests, as well as absence of major illness or chronic medication). Each subject had fasted overnight, and blood sample was collected in the morning pre-prandial. Venous blood samples were collected into plastic serum tubes (5 mL) and allowed to clot by standing tubes vertically at room temperature for 60 min. Then, tubes were centrifuged at 3000 rpm for 10 min at room temperature. The supernatant (serum) was transferred and immediately frozen and stored at −80 °C until analysis. The collection and storage conditions were identical for cases and controls.

2.2. ^{1}H NMR spectroscopic analysis of blood serum

Serum samples were thawed at room temperature and homogenized using a vortex mixer. Then 200 μL D_2O was added to 400 μL serum. After centrifugation at 12 000 rpm for 5 min at 4 °C, 550 μL of the supernatants was transferred into 5-mm NMR tubes and stored at 4 °C until analysis. The ^{1}H NMR spectra for all specimens were acquired in a random order at 298 K on a Bruker AVANCE 600 spectrometer (Rheinstetten, Germany) equipped with a 5 mm TCI cryogenic probe. One-dimensional (1D) spectra were recorded using the Carr–Purcell–Meiboom–Gill (CPMG) spin echo pulse sequence with a fixed total spin relaxation delay ($2n\tau$) of 35 ms to attenuate broad resonances from high molecular weight compounds such as proteins and retain those from low-molecular weight compounds and some lipids. Water resonances were suppressed with irradiation at the water frequency during both the relaxation delay of 2 s and the mixing time of 100 ms. For each sample, a total of 64 scans were collected into 32 000 data points with a spectral width of 11999.4 Hz and an acquisition time of 1.36 s.

All ^{1}H NMR free induction decays (FID) were imported into Mnova NMR Suite Version 6.0.2 (Mestrelab Research, S.L, Registro Mercantil de A Coruna, Spain) for processing and binning. The FID was zero filled to 64 K and an exponential weighting function corresponding to 0.3 Hz line broadening was applied prior to Fourier transformation. Both phase correction and baseline correction were manually performed carefully. The spectra were referenced to the methyl doublet signal of lactate ($\delta = 1.33$ ppm). Each spectrum (0–9.0 ppm) was divided into 0.04 ppm bins excluding the residual water region from 5.1 to 4.7 ppm. The remaining bins of each spectrum were normalized to a total spectral area of unity prior to pattern recognition.

2.3. UHPLC analysis of serum free amino acids

At the time of UHPLC analysis of SFAA, a UHPLC method coupled with a diode array detector (DAD) was used for the simultaneous determination of 23 amino acids in serum samples after pre-column derivatization with 2,4-dinitrofluorobenzene (DNFB). 600 μL of acetonitrile was added to 200 μL of the serum sample and vortexed vigorously for 1 min. Then, the mixture was centrifuged at 13000 rpm for 10 min at 4 °C, and 600 μL of the supernatant was evaporated to dryness at 45 °C under a gentle stream of nitrogen. Then, to the residue, 200 μL of 0.2 M sodium borate buffer (pH 9.0) and 100 μL of 72 mM DNFB in acetonitrile were added and the derivatization reaction was carried out on a water bath (60 °C) for 60 min in the dark. After cooling in ice-cold water, 700 μL of phosphate buffer solution (pH 7.0) was added to the reaction solution. The resulting solution of DNFB amino acid derivatives was then briefly vortexed again and transferred through a 0.45 μm nylon filter into autosampler vials for injection.

All the specimens were carried out in a random order on an Agilent 1260 series rapid resolution liquid chromatography system (Agilent Technologies, Waldbronn, Germany) which consisted of a G1311B quaternary pump equipped with on-line vacuum degasser, a G1329B refrigerated model SL autosampler, a G1316A column oven and a G1315C diode array detector. Chromatographic separations were performed on an Agilent ZORBAX Eclipse Plus C_{18} column (4.6 mm × 50 mm, 1.8 μm; Agilent Technologies). Column temperature was maintained at 45 °C. The mobile phase consisting of 10 mM (A) ammonium acetate solution, (B) acetonitrile and (C) methanol was carried out at a flow rate of 1.5 mL·min^{-1}. The gradient program was as follows: 0–4 min, 90%–78% A and 5%–17% B; 4–5 min, 17%–22% B and 5%–0% C; 5–7 min, 7–9 min, and 9–10 min, linear gradient of 78%–67% A and

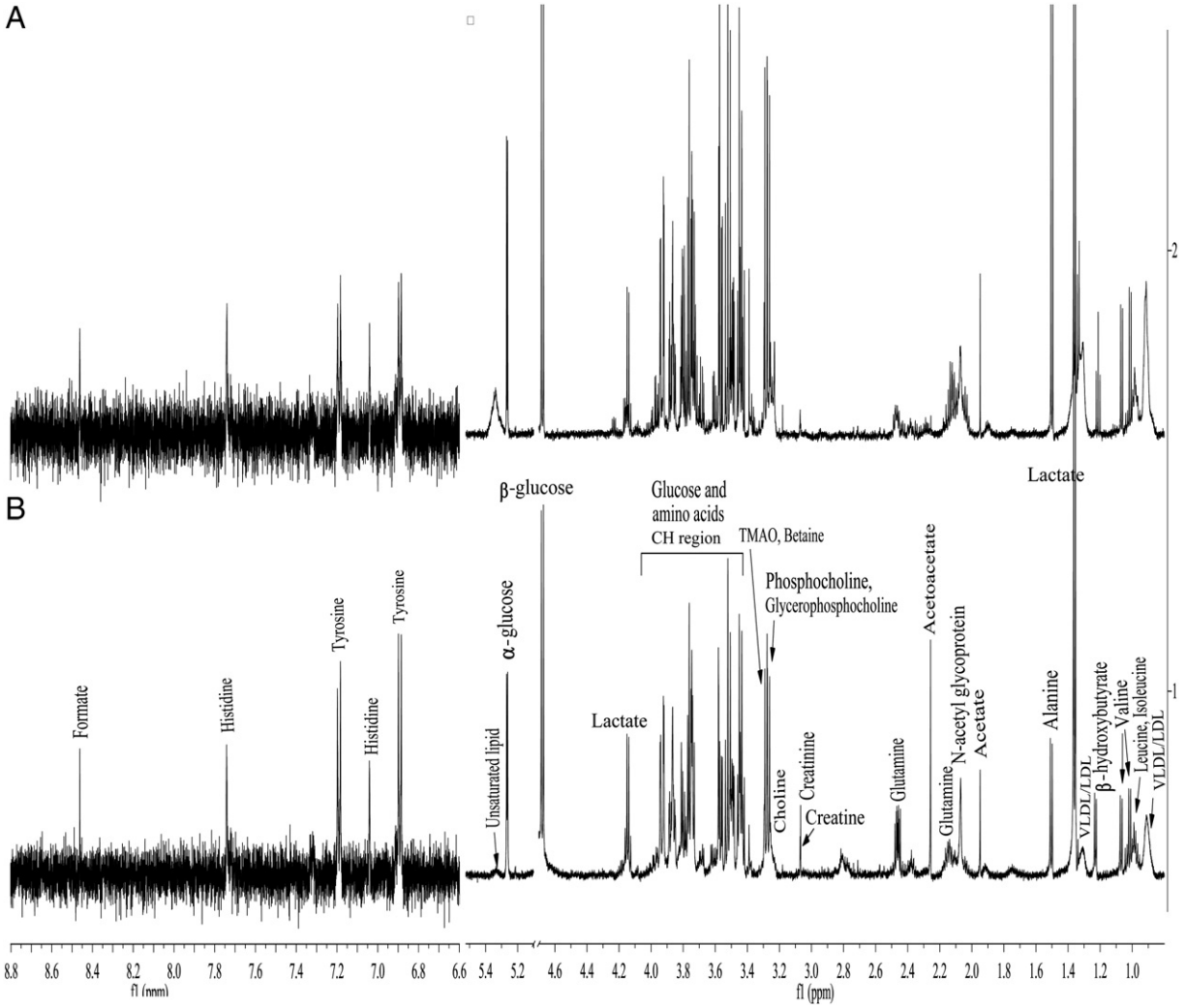

Fig. 1. Representative 600 MHz one-dimensional Carr–Purcell–Meiboom–Gill (1D-CPMG) ^{1}H NMR spectra of serum samples from a healthy control (A) and an EC patient (B) indicating key metabolites (chemical shifts δ 6.6–8.8 ppm were expanded 8 times).

22%–33% B; 67%–50% A and 33%–50% B; and 50%–90% A, 50%–5% B and 0%–5% C; respectively. UV detection was set at 360 nm. The filtration was injected at 4 °C using a temperature controlled autosampling device, and the injection volume was 5 μL. The experimental programs and data processes of quantification were both performed with the software Agilent OpenLAB Control Panel Version A.01.02 (Agilent Technologies). Areas under the peaks were integrated to calculate the concentration of amino acids. Based on six-point (from 1 to 500 μmol·L^{-1}) calibration curves, the concentrations of SFAA were determined, expressed in μmol·L^{-1} serum.

2.4. Multivariate statistical analysis

After mean-centering and scaling the ^{1}H NMR and UHPLC data sets to default unit variance, multivariate statistical analysis [33–35] was conducted using SIMCA-P version 12.0 software package (Umetrics, Umeå, Sweden). First, the unsupervised principal component analysis (PCA) was performed to observe intrinsic clusters and find obvious outliers. Then, the supervised orthogonal projection to least squares discriminant analysis (OPLS-DA) was employed to visually discriminate between EC patients and healthy controls. The OPLS-DA model removes variability not relevant to class separation. Thus, normally only one predictive component is used for the discrimination between two classes [36,37]. The quality and reliability of the models were assessed by the parameters R^2 and Q^2. R^2 represents the explained variation in the data and indicates goodness of fit, and Q^2 is the cross validation parameters and indicates predictability of the model. The cumulative values of total Y explained variance (R^2) and the Y predictable variation (Q^2) approaches 1 indicated proper modeling. The default 7-round cross-validation procedure in SIMCA-P software package with one-seventh of the samples being excluded from the mathematical model in each round was applied to the OPLS-DA models in order to guard against over fitting, and the reliabilities of the models were further rigorously validated by the permutation tests ($n = 100$) [38]. Additionally, CV-ANOVA (analysis of variance testing of cross-validated predictive residuals) tests were performed to determine significant differences between groups in the OPLS-DA models. The Pearson product–moment correlation coefficient and level of significance were determined as described [39].

On the basis of a variable importance in the projection (VIP) threshold of 1 from the 7-fold cross-validated OPLS-DA model, metabolites responsible for discriminating between the metabolic profiles of EC patients and healthy controls could be obtained. Variables with high VIP values (>1.0) were highlighted to be important for discrimination in the model whereas those with values closer to 0 indicate minimal discriminatory power [40,41]. In parallel, independent samples *T*-test from SPSS, version 16.0, was used to determine if different biomarker candidates obtained from the OPLS-DA models were statistically significant between the two groups at the univariate level. A p-value < 0.01 (confidence level 99%) was considered statistically significant.

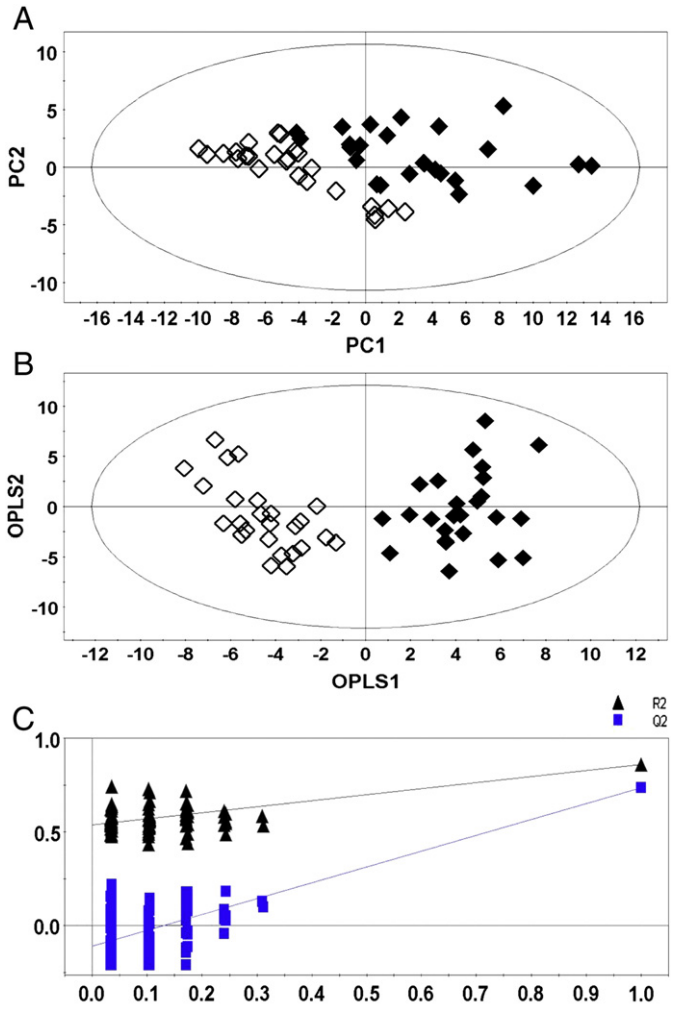

Fig. 2. Multivariate statistical analysis from NMR-based metabolic profiling. (A) PCA score plot with all variable unit variance scaled. (B) OPLS-DA score plot of EC group versus healthy control. (C) Cross-validation plot with a permutation test repeated 100 times (⬥, healthy controls; ◇, EC patients).

3. Results

3.1. Discrimination between esophageal cancer patients and healthy controls by NMR-based metabolic profiling

Representative ^{1}H NMR spectra from serum of healthy control and EC patients were shown in Fig. 1, with metabolites indicated based on their chemical shifts. Assignment of metabolites was achieved using in-house databases and published literatures [42–44], and confirmed by 2D ^{1}H–^{1}H correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) spectra. The 1D-CPMG ^{1}H NMR spectra of serum showed signals mainly from lipids, glucose, amino acids, glycoproteins, trimethyl-amine N-oxide (TMAO), betaine, ketone bodies and choline metabolites. Major metabolites were labeled in the spectra. A number of perturbations in endogenous metabolites were observed in the ^{1}H NMR spectra of serum from EC patients. Most visual inspection of the spectra suggested that prominent changes in the EC patient group compared with the healthy control group were the increases in lactate, ketone bodies (β-hydroxybutyrate and acetoacetate), and the decreases in lipids, glucose, and amino acids.

The unsupervised PCA was initially utilized on the normalized ^{1}H NMR spectral data, and the PCA score plots (Fig. 2A) showed a clear trend of group clustering between the EC patients and healthy controls. To maximize the group separation and identify discriminating metabolites, the supervised OPLS-DA classification model using one PLS component and one orthogonal component was established, and even clearer class discrimination was obtained. The OPLS-DA method not only correctly separated the sera of healthy subjects from the EC patients, but also was able to classify the samples into EC and control groups in its score plot (Fig. 2B). Goodness of fit values and predictive ability values (R^2 X, R^2Y, and Q^2) were 0.461, 0.858, and 0.736, respectively. These values indicated that the model possessed a satisfactory fit with good predictive power. A random permutation test with 100 permutations was performed with OPLS-DA to further evaluate the robustness of this method and statistically validate the biomarkers. As shown in Fig. 2C, the validation plots strongly assured that our original OPLS-DA models were not random and over fitting because both the permuted R^2 and Q^2 values to the left were significantly lower than the original points to the right and Q^2 regression lines have a negative intercepts (intercepts: R^2 0.0, 0.528; Q^2 0.0, −0.067). Furthermore, the CV-ANOVA test was performed to examine the statistical significance of the differences between the two groups in the OPLS-DA model, this resulted in a score of $p = 3.76 \times 10^{-15}$, indicating that the differences between the groups within the model were highly significant.

Top twenty five variables with high VIP values (VIP > 1.0) that picked out from analysis of the OPLS-DA model were responsible for the discrimination in the score plot. The *T*-test ($p < 0.01$) was then applied to test the significance of these differential metabolites, and to show that if these metabolites were also significant after controlling for the false discovery rate. Finally, twelve metabolites were identified as potential biomarkers for esophageal cancer (Table 1). As compared with healthy controls, a number of metabolites showed increased concentration in serum of EC patients, such as lactate, acetoacetate, β-hydroxybutyrate, glutamine, glutamate, and histidine, while several others such as tyrosine, lipids, acetate and glucose, were observed in decreased levels in the EC patients.

3.2. Discrimination between esophageal cancer patients and healthy controls by UHPLC-based focused metabolic profiling

Method validation and evaluation for measurement of 23 amino acids were according to the published literature we have reported [45]. Linearity, precision and recovery results of 23 amino acids in serum of healthy controls were given in Supplementary Table S1. Typical UHPLC chromatograms of a standard mixture of 23 amino acids, serum samples obtained from a healthy control and an EC patient were shown in Fig. 3. The concentration of amino acids in serum of EC patients and healthy controls was given in Supplementary Table S2.

PCA and OPLS-DA were also performed on the SFAA profiles. The score plots of PCA and OPLS-DA models and the validation of permutation tests were shown in Fig. 4. The PCA score plot from all the samples using 3 components clearly showed trend of group clustering between the EC patients and healthy control group (R^2 X = 0.525, Q^2 = 0.324). The OPLS-DA score plots revealed that the EC patients were statistically distinguishable from healthy controls (R^2 X = 0.591, R^2 Y = 0.973, Q^2 = 0.959), suggesting that the OPLS-DA model was robust. Model validation using permutation test with 100 iterations assured that this OPLS-DA model was not random and over fitting, and was reliable in explaining and predicting the variations in X and Y matrices (Fig. 4C, intercepts: R^2 0.0, 0.258; Q^2 0.0, −0.37). Similarly, a score of $p = 7.22 \times 10^{-27}$ was obtained by the CV-ANOVA test, suggesting that the differences between the EC group and the healthy control group in the OPLS-DA model were highly significant.

Among the statistically significant variables identified using VIP values (VIP > 1.0) in the OPLS-DA model and the *T*-test ($p < 0.01$) (Table 2), a total of seven metabolites from UHPLC analysis of SFAA were identified as potential biomarkers for esophageal cancer. Differential metabolites identified from the focused metabolomic analysis suggested a significant reduction of methionine and tryptophan in

Table 1
Marker metabolites found in OPLS-DA models of NMR-based metabolic profiling.

Chemical shift (ppm)	VIP[a]	p-Value[b]	Metabolites	Variations versus healthy controls[c]	Related metabolic pathways
0.84–0.88	2.81	0.000	LDL/VLDL	↓	Lipid metabolism
0.88–0.92	2.67	0.000			
1.28–1.32	1.98	0.000			
5.30–5.34	2.62	0.000	Unsaturated lipids	↓	
5.34–5.38	2.36	0.000			
1.20–1.24	1.80	0.000	β-Hydroxybutyrate	↑	Ketogenesis, lipid metabolism
2.25–2.29	1.31	0.005	Acetoacetate	↑	
1.93–1.97	1.58	0.000	Acetate	↓	
3.05–3.09	1.70	0.000	Creatine	↑	Energy metabolism
3.05–3.09	1.70	0.000	Creatinine	↑	
5.26–5.30	6.78	0.000	α-Glucose	↓	Glycolysis
4.66–4.70	4.06	0.009	β-Glucose	↓	
1.32–1.37	3.04	0.002	Lactate	↑	
4.12–4.16	3.35	0.000			
2.29–2.33	1.31	0.001	Glutamate (Glu)	↑	Glutamine/glutamate metabolism
2.01–2.05	1.52	0.000			
2.41–2.45	2.06	0.000	Glutamine (Gln)	↑	
2.45–2.49	1.19	0.001			
2.12–2.16	1.80	0.000			
6.87–6.91	2.16	0.000	Tyrosine (Tyr)	↓	Phenylalanine and tyrosine metabolism
7.15–7.19	2.11	0.000			
7.19–7.23	1.23	0.002			
7.03–7.07	2.47	0.001	Histine (His)	↑	Histine metabolism
7.71–7.75	1.17	0.000			

[a] Variable importance in the projection (VIP) was obtained from OPLS-DA with a threshold of 1.0.
[b] The p-value was calculated from independent samples *T*-test.
[c] The arrows ↑ and ↓ indicate increase and decrease of levels in the EC group compared with healthy control group, respectively.

the serum of EC patients but elevation of aspartate, cysteine, leucine, phenylalanine and lysine.

4. Discussion

4.1. Systemic metabolic changes in esophageal cancer patients

Identification of specific metabolites displaying altered levels and their associative metabolic pathways can improve the understanding of the biology and pathology in the process from normal to esophageal disease and eventually cancer. Fig. 5 showed a more detailed pathway map associated with marker metabolites identified using both NMR-based metabolomics and UHPLC-based focused metabolomics. Altered pathways include changes in glycolysis (glucose and lactate), amino acid metabolism (down-regulation in methionine and tryptophan, and up-regulation in aspartate, cysteine, leucine, phenylalanine and lysine), ketone bodies (β-hydroxybutyrate and acetoacetate) synthesis and degradation, tricarboxylic acid (TCA) cycle, energy and lipid metabolism. Among these selected biomarker metabolites, we found that some of them were worthy of further investigation. We would like to discuss their roles during the process of tumor metabolism associated with EC.

4.1.1. Glucose metabolism and related metabolites

In this study, one of the most obvious observations is the marked reduction of blood glucose level together with increased level of lactate in EC patients in comparison with the healthy controls, which suggested that the disturbance of glycolysis and the Cori cycle. The Cori cycle, also known as lactic acid cycle, refers to the metabolic pathway in which lactate produced by anaerobic glycolysis in the skeletal muscle moves to the liver and is converted to glucose through gluconeogenesis, which then returns to the skeletal muscle and is converted back to lactate [46]. Since lactate is the endpoint product of glycolysis, its accumulation implied an increased anaerobic glycolysis. Additionally, as the well-known Warburg effect [47] describes, cancer cells result in an increase of glycolysis, which eventually lead to an abnormal reduction of glucose level and accumulation of lactate in the serum of EC patients.

4.1.2. Lipids metabolism and related metabolites

Several metabolites in lipid metabolism, such as apolipoproteins (low density lipoprotein (LDL) and very low density lipoprotein (VLDL)) and unsaturated lipid, were observed to be significantly decreased in the serum of EC patients, suggesting an altered lipid metabolism associated with EC morbidity [48]. Furthermore, the levels of the two main ketone bodies, acetoacetate and β-hydroxybutyrate, were found to be significantly increased in the serum of cancer patients relative to the healthy controls. The ketone bodies, including β-hydroxybutyrate, acetoacetate and acetone, are formed via β-oxidation of fatty acids in mitochondria and elevations of them suggested a promoted β-oxidation in EC patients. The levels of ketone bodies could be raised when acetyl-CoA derived from β-oxidation exceeds the capacity of the TCA cycle [49], which explained higher levels of β-hydroxybutyrate and acetoacetate in the serum of EC patients. Thus, both enhanced lipid β-oxidation and malabsorption could explain lower levels of lipids in serum, and the observed increase in β-hydroxybutyrate and acetoacetate was an indication of ketogenesis from EC patients. Since the liver is the main tissue for ketone body production, liver dysfunction might also contribute to lipid metabolism disorders. Additionally, a reduction of acetate was observed in the serum of EC patients relative to healthy controls. Acetate is the end product of lipid metabolism, and its decrease also supports the preliminary hypothesis of lipid metabolism disorders.

4.1.3. Energy metabolism and related metabolites

Serum creatine and creatinine levels were significantly elevated in EC patients compared with healthy controls. Creatine and its phosphorylated form are well recognized as key intermediates in energy metabolism, and the increase in creatine is associated with energy demand. Creatine is primarily synthesized mainly in the liver and kidney and mostly stored in muscle as phosphocreatine. Phosphocreatine has a direct function in cellular energy transport. With increased energy demand, the muscle rapidly re-synthesizes ATP from ADP through the phosphocreatine–creatine kinase system and creatine. Creatinine production stems from creatine and creatine phosphate metabolism [50]. Many researchers have reported elevated creatine and creatinine levels in cancer patients [20–22,32].

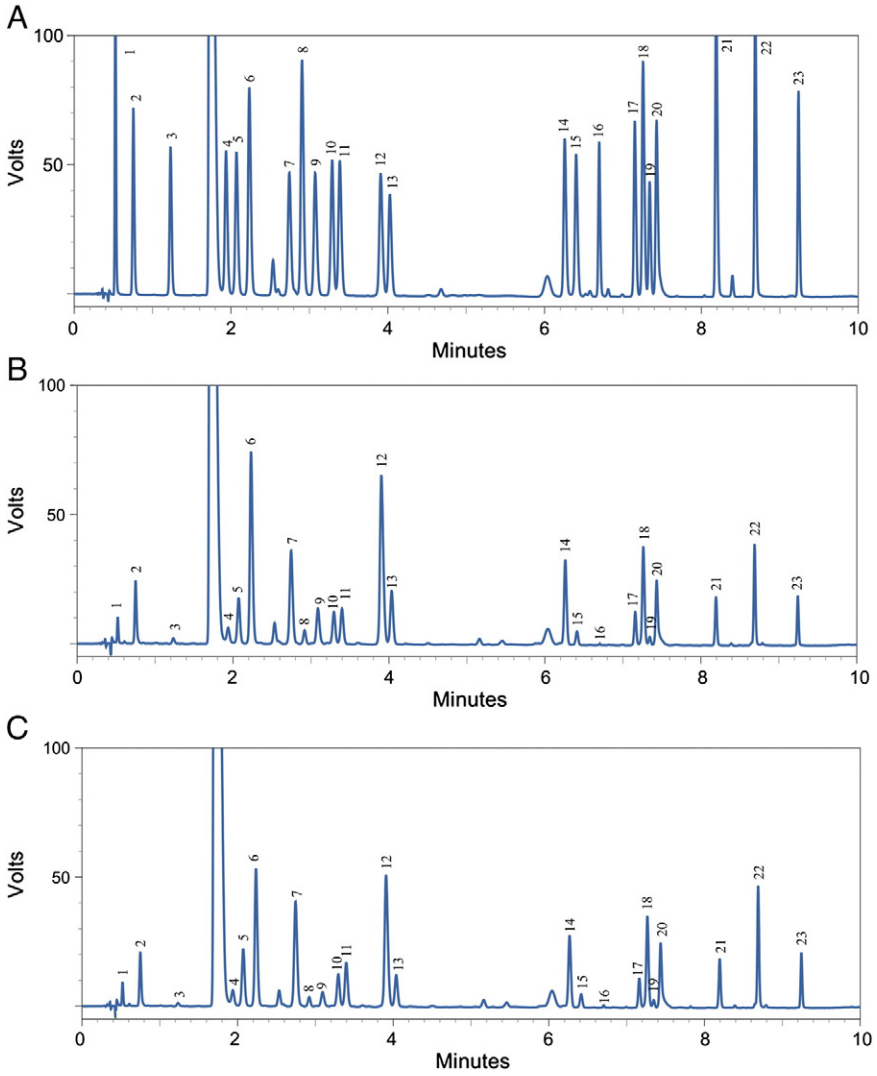

Fig. 3. Typical chromatograms of a standard mixture of 23 amino acids (A), serum sample obtained from a healthy control (B) and an EC patient (C). Peak identification: 1, Aspartic acid; 2, Glutamic acid; 3, Hydroxyproline; 4, Asparagine; 5, Glutamine; 6, Serine; 7, Glycine; 8, Arginine; 9, Threonine; 10, Histidine; 11, Taurine; 12, Alanine; 13, Proline; 14, Valine; 15, Cysteine; 16, Methionine; 17, Ornithine; 18, Isoleucine; 19, Tryptophan; 20, Leucine; 21, Phenylalanine; 22, Lysine; and 23, Tyrosine.

4.1.4. Amino acid metabolism and related metabolites

The levels of glutamine and glutamate were found to be significantly increased in the serum of EC patients relative to healthy controls. Glutamate and glutamine are a group of glucogenic amino acids and have many biological functions. They are considered to be very important for maintenance and promotion of cell function [51]. The demands of the body for glutamine and glutamate are enormous during severe illness, and their blood concentration may significantly decrease or increase, suggesting that they may become a conditionally essential amino acid in EC patients.

An increased level of aspartate was detected in the serum of EC patients as compared with the healthy controls. Aspartate is a nonessential amino acid which can be synthesized by glutamate through aspartate aminotransferase using vitamin B6 or asparagine through the action of asparaginase. Aspartate is a precursor of ornithine and plays important roles in the urea cycle and DNA metabolism. In this study, the increased level of aspartate corroborated that the urea cycle is disrupted in EC patients and the degradation of amino acids is decreased due to the down-regulation of the argininosuccinate synthetase in the urea cycle.

A decreased level of methionine has been observed in EC group. Methionine is an essential amino acid and a precursor of cysteine, a metabolite shown higher levels in the serum of EC patients. Because regulation of the methionine metabolic pathway is based on the availability of methionine and cysteine, the reduced levels of methionine and increased levels of cysteine observed in EC patients could be explained by altered homocysteine–methionine conversion as well [52]. In addition, the results from the previous study suggested that methionine enhanced the in vivo catabolism of histidine by stimulating one-carbon metabolism. In our study, the decreased level of methionine might lead to a down-regulation of histidine catabolism which eventually resulted in the increased level of histidine in the serum of EC patients [53].

One interesting finding from our experiment was that the level of leucine was significantly higher in patients with EC than in healthy controls. Leucine, one of the branched chain amino acids (BCAA), along with isoleucine and valine are indispensable amino acids and essential nutrients in the synthesis of body proteins and represent the major nitrogen source for glutamine and alanine syntheses in the muscle. Our hypothesis is that the tumors have activated proteolysis of the skeletal muscle and enhanced BCAA oxidation [54].

Another interesting finding from our study was that the serum tryptophan levels were significantly lower in patients with EC than in healthy controls. Tryptophan is an essential amino acid required not only for the synthesis of proteins but also for important biological functions such as mood, stress response, sleep and appetite regulation,

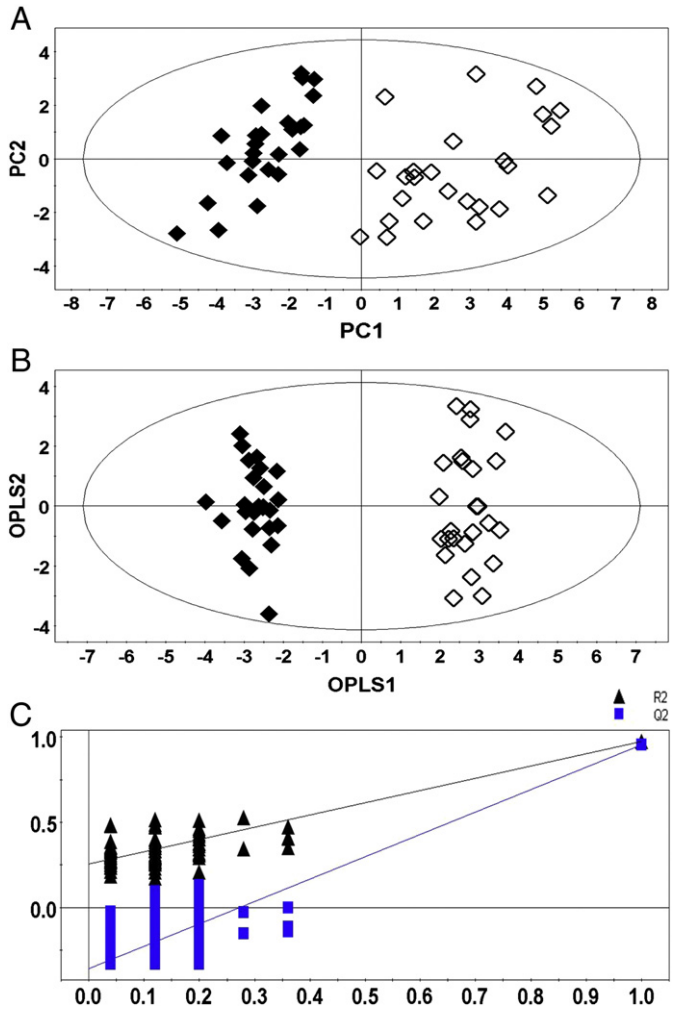

Fig. 4. Multivariate statistical analysis from UHPLC-based targeted metabolic profiling. (A) PCA score plot with all variable unit variance scaled. (B) OPLS-DA score plot of EC group versus healthy control. (C) Cross-validation plot with a permutation test repeated 100 times (◆, healthy controls; ◇, EC patients).

glucose homeostasis and immune function, and most of them being associated with metabolic pathways involved in tryptophan catabolism. Some previous studies have investigated the role of tryptophan and its metabolites involving in cancer progression [55]. The obvious reduction of tryptophan revealed a disordered tryptophan metabolism in EC patients.

Serum phenylalanine was significantly increased in EC patients, while tyrosine was decreased in comparison with healthy controls. Phenylalanine is an essential aromatic amino acid which must be supplied in the dietary proteins. Once in the body, most of the phenylalanine is usually converted to tyrosine which in turn is degraded to acetoacetate and fumarate [56]. The obvious increase in phenylalanine and decrease in tyrosine might reveal disorders of phenylalanine and tyrosine metabolism and reduced phenylalanine hydroxylation in patients with EC.

One more metabolite of interest, lysine, increased in serum of EC patients as compared with healthy controls. Lysine, another essential amino acid, is metabolized in mammals via an initial transamination with α-ketoglutarate. The ultimate end-product of lysine catabolism is acetoacetyl-CoA. That is, the elevation of lysine concentration in serum of EC patients may also be a perturbation of TCA cycle and energy metabolism.

4.2. Comparison of metabolomics and focused metabolomics

In the current study, we have shown that the metabolic profiling of serum using a combination of ^{1}H NMR-based metabolomics and UHPLC-based focused metabolomics along with multivariate statistical methods allowed a detailed picture of metabolic changes in EC patients compared with healthy controls. In order to evaluate the performance of the two different profiling methods, the goodness of fit values (R^2Y) and predictive ability (Q^2) values obtained from the OPLS-DA models and numbers of biomarkers were compared. Both R^2Y and Q^2 values in OPLS-DA score plots of the UHPLC-based focused metabolic profiling were larger than those of NMR-based metabolic profiling, indicating that the focused metabolic profiling technique can provide a better classification and predictive ability for EC. For the biomarker discovery, twelve marker metabolites were identified from NMR-based metabolomics, and seven were from UHPLC-based focused metabolomics. Glutamine, glutamate, tyrosine, and histidine were detected as marker metabolites in NMR-based metabolomics but not in UHPLC-based focused metabolomics owing to a VIP < 1 or a p value > 0.01, although they were indeed detected altered levels by both the NMR-and UHPLC-based metabolomics. This result is likely due to the relatively poor sensitivity of the NMR detected markers in the challenging task of distinguishing EC from healthy controls.

Because of the chemical and physical diversity of the compounds present in the complex biofluid, subtle differences in pH, ionic strength, temperature, etc., will hamper the NMR analysis. Furthermore, each metabolite is differentially sensitive to these effects, which finally leads to a different sensitivity in NMR analysis. While UHPLC analysis can provide good chromatographic resolution, high sensitivity and high efficiency. That is, the global metabolic NMR profiling is advantageous in the identification of more metabolites while the focused UHPLC profiling provides good selectivity, quantification and identification of metabolites. Thus, reliance on focused platforms that produce quantitative data on known metabolites is a logical and efficient route forward. Focused metabolic profiling is highly simple, accurate and specific, and should prove equally valuable in metabolomic research applications. Also, the accuracy and sensitivity of 'omics' platforms often must be sacrificed to gain the required breadth, while the need for such sacrifices is not present in

Table 2
Marker metabolites found in OPLS-DA models of UHPLC-based metabolic profiling.

Retention time (t_R, min)	Metabolites	VIP[a]	p-Value[b]	Variations versus healthy controls [c]	Related metabolic pathways
0.52	Aspartate (Asp)	4.18	0.000	↑	Asparagine/aspartate metabolism
6.41	Cysteine (Cys)	1.33	0.000	↑	Glutathione metabolism
6.71	Methionine (Met)	3.32	0.000	↓	
7.44	Leucine (Leu)	2.49	0.000	↑	Branched chain amino acid (BCAA) degradation
7.35	Tryptophan (Trp)	3.35	0.000	↓	Tryptophan metabolism
8.20	Phenylalanine (Phe)	1.77	0.004	↑	Phenylalanine metabolism
8.69	Lysine (Lys)	1.97	0.000	↑	Lysine metabolism

[a] Variable importance in the projection (VIP) was obtained from OPLS-DA with a threshold of 1.0.
[b] The p-value was calculated from independent samples *T*-test.
[c] The arrows ↑ and ↓ indicate increase and decrease of levels in the EC group compared with healthy control group, respectively.

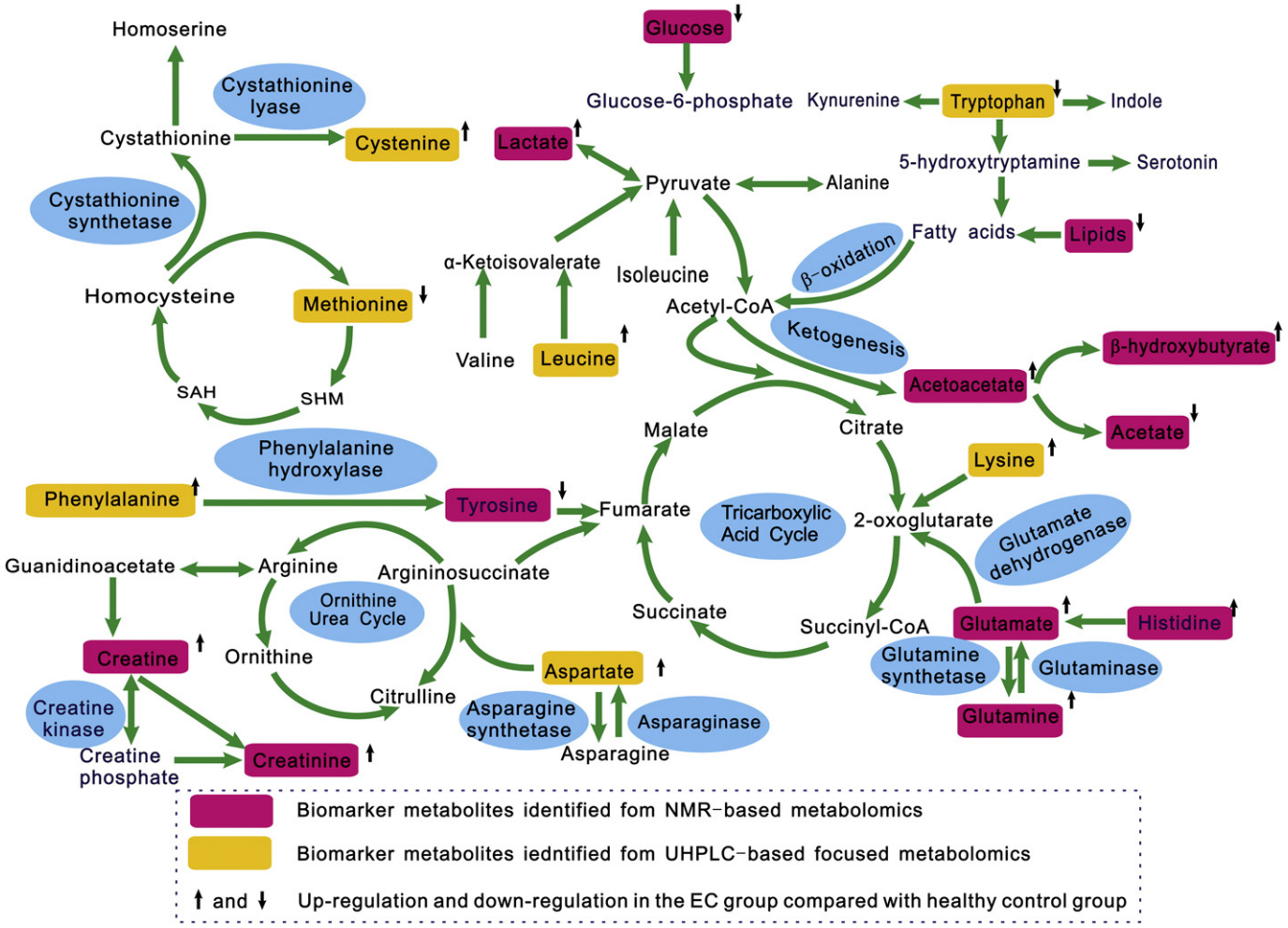

Fig. 5. Map illustrating the most predominant disturbed metabolic pathways and the biochemical linkages among the biomarker metabolites identified in EC group.

focused metabolomics. Herein, focused metabolic profiling is routine to get high throughput, accurate and highly parallel data. Progress in these areas that enables different sets of focused metabolomic data to be integrated and compared will go far toward enhancing the value of metabolomics.

4.3. Comparison with previous studies

In concurrence with our results, previous metabolomic analysis of serum samples from EC patients also found significant increase in glutamine, phenylalanine, and leucine levels along with decreases in methionine, tyrosine and tryptophan levels in EC patients [32]. Furthermore, there are also many similarities between our results and those of previous studies. We noticed that alterations in the glucose, lactate, creatinine, and ketone body levels in our present study were consistent with the results of the previous studies [18,32].

On the other hand, as known to all, cancer is expected to become the leading cause of death worldwide within a few years. Therefore, it is very important that the methods used for the prevention and early detection should be implemented to reduce mortality. An important goal of our study is focused on identifying and distinguishing metabolites for the establishment of improved clinical biomarkers for EC, and it would also be of interest to find discover novel biomarkers for EC diagnosis and prognosis. Many previous reports have identified many biomarkers for EC, and the two metabolomic methods described in the present study involved a relatively simple serum assay and identified a few more novel marker metabolites for EC, that is cysteine derived from the UHPLC-based focused metabolomic profiles, and lipids (LDL, VLDL, and the unsaturated lipid) derived from the NMR-based metabolic profiles.

5. Conclusion

The present study investigated metabolic variations in patients with EC using NMR-based metabolomics and UHPLC-based focused metabolomics. Clear metabolic differences were observed between EC patients and healthy controls. These variations involved significant perturbations in lipids, glucose, energy and amino acid metabolism, and the TCA cycle. This work has shown that both metabolomics and focused metabolomics were reliable and predictive, and could be used to discriminate between healthy and diseased subjects. The predictive power of the UHPLC-based focused metabolomic methods performed better in both sensitivity and specificity when compared with the results from the NMR-based metabolomic methods, suggesting that the focused metabolomics technique may be of advantage in the future for the determination of biomarkers. In summary, our study indicated that focused metabolic profiling is also a powerful tool in providing valuable biochemical insights into metabolic alterations in EC.

It is important to note that the current study was exploratory and laboratory due to the limited number of samples, and the mechanism of metabolic changes in human blood serum of EC should be further studied and validated on larger patient cohorts. The differential metabolites addressed in this work and changes in their metabolic pathways could also guide further studies on biomarker discovery for EC diagnosis and prognosis and also on the pathophysiology of EC.

Acknowledgements

This work has been supported by the Fundamental Research Funds of Key Laboratory of Chemistry of Northwestern Plant Resources, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou (CNPR-2012kfkt-10), Fundamental Research Funds for the Central Universities (lzujbky-2012-72) and the National Natural Science Foundation of China (NSFC) fund (No. 21105039).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.03.009.

References

[1] American Cancer Society, Global Cancer Facts & Figures, 2nd Edition American Cancer Society, Atlanta, 2011.
[2] A. Jemal, T. Murray, A. Samuels, A. Ghafoor, E. Ward, M.J. Thun, Cancer statistics, 2003, CA Cancer J. Clin. 53 (2003) 5–26.
[3] J.A. Ajani, Carcinoma of the esophagus: is biology screaming in my deaf ears, J. Clin. Oncol. 23 (2005) 4256–4258.
[4] A. Ruol, C. Castoro, G. Portale, F. Cavallin, V.C. Sileni, M. Cagol, R. Alfieri, L. Corti, C. Boso, G. Zaninotto, A. Peracchia, E. Ancona, Trends in management and prognosis for esophageal cancer surgery, Arch. Surg. 144 (2009) 247–254.
[5] M. Groome, J. Lindsay, P.E. Ross, J.P. Cotton, T.R. Hupp, J.F. Dillon, Use of oesophageal stress response proteins as potential biomarkers in the screening for Barrett's oesophagus, Eur. J. Gastroenterol. Hepatol. 20 (2008) 961–965.
[6] M. Wakatsuki, Y. Suzuki, S. Nakamoto, T. Ohno, H. Ishikawa, H. Kiyohara, M. Kiyozuka, K. Shirai, Y. Nakayama, T. Nakano, Clinical usefulness of CYFRA 21-1 for esophageal squamous cell carcinoma in radiation therapy, J. Gastroenterol. Hepatol. 22 (2007) 715–719.
[7] J.K. Nicholson, J. Connelly, J.C. Lindon, E. Holmes, Metabonomics: a platform for studying drug toxicity and gene function, Nat. Rev. Drug Discov. 1 (2002) 153–161.
[8] J.K. Nicholson, J.C. Lindon, E. Holmes, 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data, Xenobiotica 29 (1999) 1181–1189.
[9] T.M.D. Ebbels, H.C. Keun, O.P. Beckonert, M.E. Bollard, J.C. Lindon, E. Holmes, J.K. Nicholson, Prediction and classification of drug toxicity using probabilistic modeling of temporal metabolic data: the consortium on metabonomic toxicology screening approach, J. Proteome Res. 6 (2007) 4407–4422.
[10] E.M. Lenz, I.D. Wilson, Analytical strategies inmetabonomics, J. Proteome Res. 6 (2007) 443–458.
[11] A.H. Zhang, H. Sun, P. Wang, Y. Han, X.J. Wang, Recent and potential developments of biofluid analyses in metabolomics, J. Proteomics 75 (2012) 1079–1088.
[12] A.M. Weljie, R. Dowlatabadi, B.J. Miller, H.J. Vogel, F.R. Jirik, An inflammatory arthritis-associated metabolite biomarker pattern revealed by ^{1}H NMR spectroscopy, J. Proteome Res. 6 (2007) 3456–3464.
[13] M.A. Constantinou, E. Papakonstantinou, M. Spraul, S. Sevastiadou, C. Costalos, M.A. Koupparis, K. Shulpis, A. Tsantili-Kakoulidou, E. Mikros, ^{1}H NMR-based metabonomics for the diagnosis of inborn errors of metabolism in urine, Anal. Chim. Acta 542 (2005) 169–177.
[14] K.A. Verwaest, T.N. Vu, K. Laukens, L.E. Clemens, H.P. Nguyen, B. van Gasse, J.C. Martins, A. van der Linden, R. Dommisse, ^{1}H NMR based metabolomics of CSF and blood serum: a metabolic profile for a transgenic rat model of Huntington disease, Biochim. Biophys. Acta Mol. Basis Dis. 1812 (2011) 1371–1379.
[15] K.M. Banday, K.K. Pasikanti, E.C.Y. Chan, R. Singla, K.V.S. Rao, V.S. Chauhan, R.K. Nanda, Use of urine volatile organic compounds to discriminate tuberculosis patients from healthy subjects, Anal. Chem. 83 (2011) 5526–5534.
[16] M. Ciborowski, F.J. Rupérez, M. Martínez-Alcázar, S. Angulo, P. Radziwon, R. Olszanski, J. Kloczko, C. Barbas, Metabolomic approach with LC–MS reveals significant effect of pressure on diver's plasma, J. Proteome Res. 9 (2010) 4131–4137.
[17] F.M. Geier, E.J. Want, A.M. Leroi, J.G. Bundy, Cross-platform comparison of *Caenorhabditis elegans* tissue extraction strategies for comprehensive metabolome coverage, Anal. Chem. 83 (2011) 3730–3736.
[18] H. Wu, R.Y. Xue, C.L. Lu, C.H. Deng, T.T. Liu, H.Z. Zeng, Q. Wang, Metabolomic study for diagnostic model of oesophageal cancer using gas chromatography/mass spectrometry, J. Chromatogr. B 877 (2009) 3111–3117.
[19] A. Ikeda, S. Nishiumi, M. Shinohara, T. Yoshie, N. Hatano, T. Okuno, T. Bamba, E. Fukusaki, T. Takenawa, T. Azuma, M. Yoshida, Serum metabolomics as a novel diagnostic approach for gastrointestinal cancer, Biomed. Chromatogr. 26 (2012) 548–558.
[20] S. Nishiumi, M. Shinohara, I. Atsuki, T. Yoshie, N. Hatano, S. Kakuyama, S. Mizuno, T. Sanuki, H. Kutsumi, E. Fukusaki, Serum metabolomics as a novel approach for pancreatic cancer, Metabolomics 6 (2010) 518–528.
[21] K.K. Pasikanti, K. Esuvaranathan, P.C. Ho, R. Mahendran, R. Kamaraj, Q.H. Wu, E. Chiong, E.C.Y. Chan, Noninvasive urinary metabonomic diagnosis of human bladder cancer, J. Proteome Res. 9 (2010) 2988–2995.
[22] C.M. Rocha, J. Carrola, A.S. Barros, A.M. Gil, B.J. Goodfellow, I.M. Carreira, J. Bernardo, A. Gomes, V. Sousa, L. Carvalho, I.F. Duarte, Metabolic signatures of lung cancer in biofluids: NMR-based metabonomics of blood plasma, J. Proteome Res. 10 (2011) 4314–4324.
[23] D.C. Wedge, J.W. Allwood, W. Dunn, A.A. Vaughan, K. Simpson, M. Brown, L. Priest, F.H. Blackhall, A.D. Whetton, C. Dive, R. Goodacre, Is serum or plasma more appropriate for intersubject comparisons in metabmolomic studies? An assessment in patients with small-cell lung cancer, Anal. Chem. 83 (2011) 6689–6697.
[24] B.J. Blaise, L. Shintu, B. Elena, L. Emsley, M.E. Dumas, Statistical recoupling prior to significance testing in nuclear magnetic resonance based metabonomics, Anal. Chem. 81 (2009) 6242–6251.
[25] W.J. Griffiths, M. Ogundare, C.M. Williams, Y. Wang, On the future of "omics": lipidomics, J. Inherit. Metab. Dis. 34 (2011) 583–592.
[26] J. Dong, X. Cai, L. Zhao, X. Xue, L. Zou, X. Zhang, X. Liang, Lysophosphatidylcholines profiling of plasma discrimination of isomers and discovery of lung cancers biomarkers, Metabolomics 6 (2010) 478–488.
[27] H. Koo, M.A. Wallig, B.H. Chung, T.Y. Nara, B.H.S. Cho, M.T. Nakamura, Dietary fructose induces a wide range of genes with distinct shift in carbohydrate and lipid metabolism in fed and fasted rat liver, Biochim. Biophys. Acta Mol. Basis Dis. 1782 (2008) 341–348.
[28] X. Yuan, S. Yan, J. Zhao, D. Shi, B. Yuan, W. Dai, M. Miao, Lipid metabolism and peroxisome proliferator-activated receptor signaling pathways participate in late-phase liver regeneration, J. Proteome Res. 10 (2011) 1179–1190.
[29] W. Yuan, J.X. Zhang, S.W. Li, J.L. Edwards, Amine metabolomics of hyperglycemic endothelial cells using capillary LC–MS with isobaric tagging, J. Proteome Res. 10 (2011) 5242–5250.
[30] T. Kimura, Y. Noguchi, N. Shikata, M. Takahashi, Plasma amino acid analysis for diagnosis and amino acid-based metabolic networks, Curr. Opin. Clin. Nutr. Metab. Care 12 (2009) 49–53.
[31] D. Yakoub, H.C. Keun, R. Goldin, G.B. Hanna, Metabolic profiling detects field effects in nondysplastic tissue from esophageal cancer, Cancer Res. 70 (2010) 9129–9136.
[32] J. Zhang, J. Bowers, L.Y. Liu, S.W. Wei, G.A.N. Gowda, Z. Hammoud, D. Raftery, Esophageal cancer metabolite biomarkers detected by LC–MS and NMR methods, PLoS One 7 (2012) e30181.
[33] G. Blekherman, R. Laubenbacher, D.F. Cortes, P. Mendes, F.M. Torti, S. Akman, S.V. Torti, V. Shulaev, Bioinformatics tools for cancer metabolomics, Metabolomics 7 (2011) 329–343.
[34] R. Rousseau, B. Govaerts, M. Verleysen, B. Boulanger, Comparison of some chemometric tools for metabonomics biomarker identification, Chemom. Intell. Lab. Syst. 91 (2008) 54–66.
[35] J. Trygg, E. Holmes, T. Lundstedt, Chemometrics in metabonomics, J. Proteome Res. 6 (2007) 469–479.
[36] J. Trygg, S. Wold, Orthogonal projections to latent structures (O-PLS), J. Chemom. 16 (2002) 119–128.
[37] M. Bylesjo, D. Eriksson, A. Sjodin, S. Jansson, T. Moritz, J. Trygg, Orthogonal projections to latent structures as a strategy for microarray data normalization, BMC Bioinform. 8 (2007) 207.
[38] F. Lindgren, B. Hansen, W. Karcher, M. Sjöström, L. Eriksson, Model validation by permutation tests: applications to variable selection, J. Chemom. 10 (1996) 521–532.
[39] L. Eriksson, J. Trygg, S. Wold, CV-ANOVA for significance testing of PLS and OPLS (R) models, J. Chemom. 22 (2008) 594–600.
[40] I.G. Chong, C.H. Jun, Performance of some variable selection methods when multicollinearity is present, Chemom. Intell. Lab. Syst. 78 (2005) 103–112.
[41] G. Quintas, N. Portillo, J.C. García-Cañaveras, J.V. Castell, A. Ferrer, A. Lahoz, Chemometric approaches to improve PLS-DA model outcome for predicting human non-alcoholic fatty liver disease using UPLC–MS as a metabolic profiling tool, Metabolomics 8 (2012) 86–98.
[42] P. Zheng, H.C. Gao, Q. Li, W.H. Shao, M.L. Zhang, K. Cheng, D.Y. Yang, S.H. Fan, L. Chen, L. Fang, P. Xie, Plasma metabonomics as a novel diagnostic approach for major depressive disorder, J. Proteome Res. 11 (2012) 1741–1748.
[43] X.Y. Zhang, Y.L. Wang, F.H. Hao, X.H. Zhou, X.Y. Han, H.R. Tang, L.N. Ji, Human serum metabonomic analysis reveals progression axes for glucose intolerance and insulin resistance statuses, J. Proteome Res. 8 (2009) 5188–5195.
[44] B. Jiménez, C. Montoliu, D.A. MacIntyre, M.A. Serra, A. Wassel, M. Jover, M. Romero-Gomez, J.M. Rodrigo, A. Pineda-Lucena, V. Felipo, Serum metabolic signature of minimal hepatic encephalopathy by ^{1}H-nuclear magnetic resonance, J. Proteome Res. 9 (2010) 5180–5187.
[45] X. Zhang, T. Zhao, T. Cheng, X. Liu, H. Zhang, J. Chromatogr. B 906 (2012) 91–95.
[46] S. Passarella, L. Bari, D. Valenti, R. Pizzuto, G. Paventi, A. Atlante, Mitochondria and L-lactate metabolism, FEBS Lett. 582 (2008) 3569–3576.
[47] R.A. Gatenby, R.J. Gillies, Why do cancers have high aerobic glycolysis, Nat. Rev. Cancer 4 (2004) 891–899.
[48] T.F. Bathen, T. Engan, J. Krane, D. Axelson, Analysis and classification of proton NMR spectra of lipoprotein fractions from healthy volunteers and patients with cancer or CHD, Anticancer. Res. 20 (2000) 2393–2408.
[49] J.D. McGarry, D.W. Foster, Regulation of hepatic fatty acid oxidation and ketone body production, Annu. Rev. Biochem. 49 (1980) 395–420.
[50] M. Wyss, R. Kaddurah-Daouk, Creatine and creatinine metabolism, Physiol. Rev. 80 (2000) 1107–1213.
[51] P. Newsholme, M.M.R. Lima, J. Procopio, T.C. Pithon-Curi, S.Q. Doi, R.B. Bazotte, R. Curi, Glutamine and glutamate as vital metabolites, Braz. J. Med. Biol. Res. 36 (2003) 153–163.
[52] M. Inoue-Choi, H.H. Nelson, K. Robien, E. Arning, T. Bottiglieri, W. Koh, J.M. Yuan, One-carbon metabolism nutrient status and plasma S-adenosylmethionine concentrations in middle-aged and older Chinese in Singapore, Int. J. Mol. Epidemiol. Genet. 3 (2012) 160–173.

[53] D. Fell, R.D. Steele, Effect of methionine on in vivo histidine metabolism in rats, J. Nutr. 113 (1983) 860–866.
[54] M. Holecek, L. Sprongl, F. Skopec, C. Andrýs, M. Pecka, Leucine metabolism in TNF-alpha- and endotoxin-treated rats: contribution of hepatic tissue, Am. J. Physiol. 273 (1997) E1052–E1058.
[55] F. Moroni, Tryptophan metabolism and brain function: focus on kynurenine and other indole metabolites, Eur. J. Pharmacol. 375 (1999) 87–100.
[56] R. Barazzoni, M. Zanetti, M. Vettore, P. Tessari, Relationships between phenylalanine hydroxylation and plasma aromatic amino acid concentrations in humans, Metabolism 47 (1998) 669–674.