Original Article
Sputum metabolomic profiling of bronchial asthma based on quadruple time-of-flight mass spectrometry

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Abstract: To improve diagnosis of asthma, we tend to confirm potential biomarkers by comparing sputum metabolome profiles between asthma patients and healthy controls, using ultra-high-performance liquid chromatography coupled to quadruple time-of-flight mass spectrometry (UHPLC-QTOF/MS). Thirty endogenous metabolites contributing to the separation of asthma patients and healthy controls were tentatively identified in positive mode, such as 1-hexadecanoyl-sn-glycerol, glycerol 1-stearate, sphingosine, Phe-Ser, Tyr-Ala and Phe-Gln, and 12 endogenous metabolites were identified in negative mode, such as cytidine 2’,3’-cyclic phosphate, 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1’-rac-glycerol), 1-octadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoserine, thymidine, gamma-L-glutamyl-L-valine and adenine. Those differential metabolites were mainly participated in glycerophospholipid metabolism, retrograde endocannabinoid signaling and metabolic pathways in positive mode and 2-oxocarboxylic acid metabolism, biosynthesis of amino acids, phenylalanine, tyrosine and tryptophan biosynthesis, valine, leucine and isoleucine degradation and metabolic pathways in negative mode. Importantly, several metabolic pathways including glycerophospholipid metabolism, inositol phosphate metabolism, and glycolysis or gluconeogenesis were found most important. These findings suggest sputum metabolomics can be used for the early diagnosis and risk prediction of asthma.

Keywords: Asthma, UHPLC-QTOF/MS, metabolomics, sputum

Introduction
Asthma is a complicated chronic airway allergic disease involving many cells and cytokines characterized by airway inflammation, hyperresponsiveness and remodeling [1]. Asthma is caused by a variety of inflammatory cell infiltrates, with symptoms of episodic dyspnea and reversible airway obstruction [2, 3]. Recently, more than 300 million people suffer from asthma worldwide, with the prevalence rate in children and adult were 3%-38% and 2%-12%, respectively, and their morbidity and mortality are increased annually [4, 5]. Recurrent attacks of asthma may cause a variety of complications, such as chronic obstructive emphysema, chronic pulmonary heart disease, pulmonary fibrosis, respiratory arrest, respiratory failure, pneumothorax and mediastinal emphysema [6]. Genetic variation and environmental changes have shown to trigger asthma, which genetic susceptibility and environmental exposure has significant influence on the development of asthma, and the interaction of genes and the environment can also lead to asthma and allergies [7].

Metabolomics are not only goal of qualitative and quantitative analysis of all metabolic components in particular biological samples, but also explain all the information about the metabolism of organisms from the point of view of systematic biology [8]. It is a science that analyzes the changes in the concentration and density of small molecular metabolites in biological cells, fluids (e.g. blood and urine), and tissue or tissue extracts [9]. Small molecule metabolites are the end product of the body's metabolic activity, and the concentration changes of metabolites can therefore reaction of biochemical functions due to disease. Although metabolomics technology mainly includes the determination of nuclear magnetic resonance (NMR), mass spectrometry (MS), gas (GC) and
high performance liquid chromatography (HP-LC) and ultra HPLC (UHPLC). The ultra high pressure system can improve the separation and sensitivity of chromatographic peaks significantly, and UHPLC coupled to quadruple time-of-flight mass spectrometry (UHPLC-QTOF-MS) is more efficient and rapid in the analysis of complex samples [10, 11]. Mayr et al. [12] combined proteomic with metabolomics techniques and found increased acyl coenzyme A dehydrogenase and decreased alanine and cytosolic malate dehydrogenase in blood vessel of mouse with coronary heart disease, suggesting that the metabolism of vascular fat cells may replace glucose metabolism, and the decrease in effective energy synthesis and glucose as well as oxidative stress is of great importance in the pathogenesis of coronary heart disease. Beger et al. [13] analyzed 40 patients with acute kidney injury in children after cardiac surgery, in which the vanillic acid was significantly increased in urine, suggesting that vanillic acid can be used as an early and sensitive diagnostic marker for acute kidney injury after cardiac surgery.

The pathogenesis of asthma is very complex, varies or overlaps in different types of asthma and has so far not been fully understood. Metabonomics can detect differences in small molecule metabolites in asthma patients and normal controls and find out the metabolic markers associated with pathogenesis of asthma, thus explaining the pathogenesis of asthma and providing a suitable treatment method [14, 15]. NMR-based metabonomics applied to exhaled breath condensate can clearly identify biochemical metabolism in asthma patients with different severity [16]. LC-MS (liquid chromatography mass spectrometry) based metabolomics was also applied to the detection of urine samples from asthmatic patients, and representative markers were identified as the basis for the diagnosis of asthma [17]. Ried et al. [18] collected the serum samples from asthmatic patients and found that changes in lecithin and phosphatidylcholine concentrations may be used to identify and diagnose asthma.

As a direct secretion in the airways, sputum can reflect airway inflammation and is therefore used to identify and diagnose asthma and chronic obstructive pulmonary disease [19]. In the present study, UHPLC-QTOF/MS based metabolomics was performed to analyze the differential metabolites in sputum between patients with asthma in childhood and healthy controls. Biological correlates of metabolic pathways and potential biomarkers have been studied in depth to understand the metabolic disturbance of asthma in childhood. Insight obtained from these studies will be useful for developing novel diagnostic biomarkers and aiding in the prevention and control of asthma.

Materials and methods

Patient recruitment

35 clinical samples including 15 healthy controls and 20 asthma patients were collected from patients hospitalized in Child’s Hospital of Nanjing Medical University, stored in -80°C and prepared for UHPLC-QTOF-MS analysis. This prospective study was approved by the Ethics Committee of Affiliated Nanjing Children’s Hospital, Nanjing Medical University. Informed consent was obtained from all participants.

Sample collection and preparation

Sputum samples were collected from the 35 clinical samples and added into 900 μL extraction liquid and 10 μL L-2-chlorophenylalanines (1 mg/mL stock in dH₂O) as internal standard to the sample in 1.5 mL EP tubes. After vortex mixing for 30 s, samples were ultrasound treated for 10 min (incubated in ice water), incubated 1 h at -20°C and centrifuged for 15 min at 12000 rpm at 4°C. The supernatant (0.7 mL) fresh were transferred into a 2 mL LC/MS glass vial, dried in a vacuum concentrator without heating and added 100 μL extraction liquid. After vortex for 30 s and sonicate for 10 min (4°C water bath), samples were centrifuged for 15 min at 12000 rpm at 4°C, and then transfer the supernatant (60 μL) into a fresh 2 mL LC/MS glass vial, take 10 μL from each sample and pooling as QC samples and take 60 μL supernatant for the UHPLC-QTOF-MS analysis.

UHPLC-QTOF-MS analysis

LC-MS/MS analyses were performed using an UHPLC system (1290, Agilent Technologies) with a UPLC BEH Amide column (1.7 μm, 2.1×100 mm, Waters) coupled to TripleTOF 6600 (Q-TOF, AB Sciex). The mobile phase consisted of 25 mM NH₄OAc and 25 mM NH₄OH in
Figure 1. The sputum PCA (A) and OPLS-DA score plot (B) between healthy controls and asthma patients in childhood in both positive and negative mode. P: positive mode; N: negative mode. C: healthy control; A: asthma patients.
Figure 2. Different metabolites between asthma patients in childhood and healthy controls. (A) Volcano plot between asthma patients and healthy controls in positive and negative mode. Top 3 increased (B) and decreased metabolites (C) in healthy controls compared with asthma patients in positive mode. Top 3 increased (D) and decreased metabolites (E) in healthy controls compared with asthma patients in negative mode. P: positive mode; N: negative mode.
Metabolites and asthma

Table 1. Different metabolites in asthma patients compared with healthy control using UHPLC-QTOF-MS in positive ion mode

<table>
<thead>
<tr>
<th>MS2 name</th>
<th>MS2 score</th>
<th>Type</th>
<th>P-value</th>
<th>Log2 Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Hexadecanoyl-sn-glycerol_1</td>
<td>0.817</td>
<td>MS2 forward</td>
<td>0.001</td>
<td>2.3</td>
</tr>
<tr>
<td>Glycerol 1-stearate_1</td>
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<td>MS2 reverse</td>
<td>0.000</td>
<td>2.0</td>
</tr>
<tr>
<td>Sphingosine_2</td>
<td>0.677</td>
<td>MS2 forward</td>
<td>0.003</td>
<td>1.7</td>
</tr>
<tr>
<td>PC (16:0/16:0)</td>
<td>16.000</td>
<td>MS2 reverse</td>
<td>0.005</td>
<td>1.6</td>
</tr>
<tr>
<td>1-Hexadecanoyl-sn-glycerol_2</td>
<td>0.961</td>
<td>MS2 reverse</td>
<td>0.048</td>
<td>1.5</td>
</tr>
<tr>
<td>Glu-Pro_1</td>
<td>0.729</td>
<td>MS2 reverse</td>
<td>0.010</td>
<td>-1.5</td>
</tr>
<tr>
<td>Pro-Arg_2</td>
<td>0.624</td>
<td>MS2 forward</td>
<td>0.011</td>
<td>-1.5</td>
</tr>
<tr>
<td>L-Arginine_4</td>
<td>0.987</td>
<td>MS2 reverse</td>
<td>0.010</td>
<td>-1.5</td>
</tr>
<tr>
<td>Phe-His_1</td>
<td>0.937</td>
<td>MS2 forward</td>
<td>0.004</td>
<td>-1.6</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine_2</td>
<td>0.643</td>
<td>MS2 forward</td>
<td>0.043</td>
<td>-1.7</td>
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<tr>
<td>Gamma-L-Glutamyl-L-valine_1</td>
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<td>MS2 forward</td>
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<td>-1.7</td>
</tr>
<tr>
<td>Glu-Pro_2</td>
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<td>MS2 forward</td>
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<tr>
<td>Lys-Pro_3</td>
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<td>-1.8</td>
</tr>
<tr>
<td>Phe-Ile</td>
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<td>0.035</td>
<td>-1.8</td>
</tr>
<tr>
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<td>MS2 forward</td>
<td>0.004</td>
<td>-1.8</td>
</tr>
<tr>
<td>Pro-Val_2</td>
<td>0.608</td>
<td>MS2 reverse</td>
<td>0.009</td>
<td>-1.9</td>
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<tr>
<td>Urocanic acid_1</td>
<td>0.933</td>
<td>MS2 forward</td>
<td>0.002</td>
<td>-2.0</td>
</tr>
<tr>
<td>Tyr-Pro_2</td>
<td>0.935</td>
<td>MS2 reverse</td>
<td>0.021</td>
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<tr>
<td>His-Pro</td>
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<td>MS2 forward</td>
<td>0.007</td>
<td>-2.1</td>
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<tr>
<td>Tyr-Pro_1</td>
<td>0.954</td>
<td>MS2 reverse</td>
<td>0.022</td>
<td>-2.1</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine_1</td>
<td>0.908</td>
<td>MS2 reverse</td>
<td>0.047</td>
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</tr>
<tr>
<td>Lys-Phe_1</td>
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<td>MS2 forward</td>
<td>0.020</td>
<td>-2.1</td>
</tr>
<tr>
<td>Thr-Phe_1</td>
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<td>MS2 forward</td>
<td>0.007</td>
<td>-2.2</td>
</tr>
<tr>
<td>L-Citrulline_1</td>
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<td>MS2 reverse</td>
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<td>-2.3</td>
</tr>
<tr>
<td>Arg-Phe_1</td>
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<td>MS2 forward</td>
<td>0.003</td>
<td>-2.5</td>
</tr>
<tr>
<td>Adenine_1</td>
<td>0.834</td>
<td>MS2 reverse</td>
<td>0.007</td>
<td>-2.5</td>
</tr>
<tr>
<td>Phe-Tyr_1</td>
<td>0.802</td>
<td>MS2 forward</td>
<td>0.004</td>
<td>-2.6</td>
</tr>
<tr>
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<td>0.888</td>
<td>MS2 forward</td>
<td>0.001</td>
<td>-2.8</td>
</tr>
<tr>
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<td>0.935</td>
<td>MS2 forward</td>
<td>0.007</td>
<td>-4.4</td>
</tr>
<tr>
<td>Phe-Ser_1</td>
<td>0.856</td>
<td>MS2 forward</td>
<td>0.005</td>
<td>-5.1</td>
</tr>
</tbody>
</table>

Table 1. Different metabolites in asthma patients compared with healthy control using UHPLC-QTOF-MS in positive ion mode

water (pH=9.75) (A) and acetonitrile (B) was carried with elution gradient as follows: 0 min, 85% B; 2 min, 75% B; 9 min, 0% B; 14 min, 0% B; 15 min, 85% B; 20 min, 85% B, which was delivered at 0.3 mL min⁻¹. The injection volume was 2 μL. The TripleTOF mass spectrometer was used during an LC/MS experiment as previously described [20].

Statistical analysis

The resulted three-dimensional data involving the peak number, sample name, and normalized peak area were fed to SIMCA14.1 software package (V14.1, MKS Data Analytics Solutions, Umea, Sweden) for PCA and OPLS-DA. The variable importance in the projection (VIP) value exceeding 1.0 was first filtered out as the changed metabolites. The remaining variables were then assessed by Student’s t-test (P-value < 0.05). Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) and MetaboAnalyst3.0 (http://www.metaboanalyst.ca) databases were utilized to analyze for the pathways of metabolites.

Results

Multivariate data analysis

First of all, matrices showed 1514 and 651 features for UHPLC-QTOF-MS in positive and negative mode, respectively. For multivariate analysis, PCA that showed the distribution of origin data was performed to prediction of control and asthma groups for UHPLC-QTOF-MS in both positive and negative mode. As shown in Figure 1A, control and asthma groups had no differences in metabolic profiles and no strong outliers were observed.

In order to gain higher levels of population separation and obtain better understandings of variables that are responsible for classification, supervised orthogonal projections to latent structures-discriminate analysis (OPLS-DA) was presented. As shown in Figure 1B, clear separation of groups in UHPLC-QTOF-MS in both positive and negative mode was found obviously. Afterwards, the parameters for the classification from the software were R²Y=0.54 and Q²=0.86 in positive modes and R²Y=0.52 and Q²=1.07 in negative modes. 7-fold cross validation is used to estimate the robustness and predictive power of our model; such permutation experiments are carried out for further verification of the model, which is stable and fitted and predicted well. These results suggest that the method can identify potential biomarkers for distinguishing between asthmatics and healthy people.
Identification and quantization of potential metabolites

Volcano plot analysis using Student’s t-test (P-value < 0.05) with VIP score >1 when compared between asthma patients and healthy control (Figure 2A). Combined with retention time, accurate molecular mass and mass spectrometric analysis of the data provided by the method, the preliminary screening of endogenous metabolites can be made. Our data revealed 30 potential biomarkers in positive mode, among which 5 metabolites were increased and 25 metabolites were decreased in asthma patients compared with healthy controls (Table 1). Meanwhile, there were 12 potential biomarkers in negative mode, among which 4 metabolites were increased and 8 metabolites were decreased in asthma patients compared with healthy controls (Table 2). Top 3 increased and decreased metabolites in healthy controls compared with asthma patients in positive mode, including 1-Hexadecanoyl-sn-glycerol, Glycero-3-phosphocholine, Phos-
shown in Figure 2B and 2C, respectively. Top 3 increased and decreased metabolites in healthy controls compared with asthma patients in negative mode, including Cytidine 2’,3’-cyclic phosphate, 1-Hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1’-rac-glycerol), 1-Octadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoserine, Thymidine, Gamma-L-Glutamyl-L-valine and Adenine, were shown in Figure 2D and 2E, respectively.

Metabolic pathway analysis

KEGG pathways representing the differentially expressed metabolites in asthma patients compared with healthy control using UHPLC-QTOF-MS in positive and negative mode were shown in Tables 3 and 4, respectively. KEGG analysis identified only the pathways in which all differential metabolites were involved, but further metabolic pathway analysis was needed to determine whether these pathways were closely related to experimental conditions. Using the comprehensive analysis of the pathway of differential metabolites (including enrichment and topological analysis), the pathway can be further screened to find the most critical pathway associated with the different metabolites. We used the KEGG metabolic pathway as a background repository. Our data showed that several metabolic pathways, including glycerophospholipid metabolism (positive mode), inositol phosphate metabolism (negative mode) and glycolysis or gluconeogenesis (negative mode) with the impact-value 0.23, 0.14 and 0.10, respectively, were found most important (Figure 3A and 3B), with the impact-value ≥ 0.10 as potential target pathway [21].

Discussion

In the present study, we found that 30 potential biomarkers in positive mode and 12 potential biomarkers in negative mode were different between healthy controls and asthma patients, based on UHPLC-QTOF-MS analysis. Metabolic pathways, retrograde endocannabinoid signaling and glycerophospholipid metabolism in positive mode and metabolic pathways, biosynthesis of amino acids, 2-oxocarboxylic acid metabolism, phenylalanine, tyrosine and tryptophan biosynthesis and valine, leucine and isoleucine degradation in negative mode represented the differentially expressed compounds in asthma patients compared with healthy control using UHPLC-QTOF-MS. Moreover, several metabolic pathways, including glycerophospholipid metabolism, inositol phosphate metabolism, and glycolysis or gluconeogenesis, were found most important.

Asthma and related respiratory disease are the most common chronic disease in industrialized countries, with doubled prevalence rate in recent decades, affecting 26 million children in the United States [22]. The development of new treatments for asthma, however, has not kept up with the rise in prevalence, which mainly due to the incomplete understanding of the pathological and physiological mechanism of asthma. Asthma is a heterogeneous syndrome, including many different subtypes and multiple phenotypes. Explaining its complex, underlying biological mechanisms requires new approaches. New biomarkers and therapeutic targets are needed to reflect new approaches to heterogeneity of asthma. Although the metabolomics of asthma has so far promoted studies of biological metabolites and metabolic pathways that are associated with asthma development and performance, the validation of these findings is lacking, which mainly due to the heterogeneity of asthma [23]. To better understand which metabolites are important in asthma, we performed metabolomic analysis of sputum from asthma patients and healthy controls.

The metabolomic is a biochemical phenotype of a cell or tissue, and metabolite, the component of the metabolomic, is the final product of gene expression and is produced under the action of metabolic enzymes. Many life activities in cells actually occur at the metabolite level, such as cell signaling, energy delivery and intercellular communication, are regulated by metabolites [24]. By analyzing the metabolic groups of different physiological states, we can fully understand the biochemical state of the organism or cell. The metabolomic analysis of phenotype from biological or physiological state of recently acquired information, so metabolomics analysis provided more information to reveal the relationship between gene and phenotype and to achieve the purpose of monitoring and inferring gene function [25]. By using NMR based metabolomics to study saliva in patients with intractable asthma, it was found that metabolomics successfully predicted 86% of the patients; however, the rate of success was 81% by using traditional exhaled NO con-
Figure 3. Summary of pathway analysis of sputum specimens of asthma patients. A. Pathways based on positive mode. (a) Glycine, serine and threonine metabolism; (b) Valine, leucine and isoleucine degradation; (c) Arginine and proline metabolism; (d) Tyrosine metabolism; (e) Sphingolipid metabolism; (f) Purine metabolism; (g) alpha-Linolenic acid metabolism; (h) Phenylalanine metabolism; (i) Arachidonic acid metabolism. B. Pathways based on negative mode. (a) Glycolysis or Gluconeogenesis; (b) Valine, leucine and isoleucine biosynthesis; (c) Pantothenate and CoA biosynthesis; (d) Histidine metabolism; (e) Pyrimidine metabolism; (f) Phenylalanine metabolism; (g) Ubiquinone and other terpenoid-quinone biosynthesis; (h) Fatty acid metabolism; (i) Tryptophan metabolism; (j) Arginine and proline metabolism; (k) Purine metabolism; (l) Citrate cycle (TCA cycle); (m) Fatty acid elongation in mitochondria; (n) Pyruvate metabolism; (o) Galactose metabolism; (p) Ascorbate and aldarate metabolism; (q) Fatty acid biosynthesis; (r) Tyrosine metabolism. P: positive mode; N: negative mode.
tent and forced expiratory volume in one second as the index [26]. Metabolomics study of hydrocortisone induced kidney deficiency in rats showed that the model group showed obvious metabolite difference compared with the normal group [27]. Kim et al. [28] collected urine samples from 50 kidney cancer patients and 13 normal controls and found that a profile analysis of low molecular weight metabolites in urine, including cluster analysis, PCA and linear discriminant analysis, can be used to screen for patients with kidney cancer, and it therefore provides a basis for the feasibility of metabolomics in the diagnosis of kidney cancer.

Mattarucchi et al. [17] applied the LC-MS technology to analyze the urine of 41 cases of children with asthma, which showed significant differences in urine metabolomics between asthmatic and normal children, with the discrimination rate of 98%, and the changes of metabolites are closely related to airway inflammation in asthma. Moreover, to assess the association between urinary romotyrosine level and children with asthma, the HPLC with online electro spray ionization tandem MS (HPLC-ESI-MS) was utilized and showed that increased romotyrosine levels contributed to the severe asthma, suggesting that urinary romotyrosine can be used to assess the risk of asthma in children and to predict the risk of an acute attack of asthma [29]. The HPLC-QTOF-MSF method was developed previously for urine metabolite profiling study and showed nineteen differential metabolites between the Shao-yao-Gancao decoction treatment group and the asthma group [30].

In the present study, metabolic pathways, retrograde endocannabinoid signaling and glycerophospholipid metabolism in positive mode and metabolic pathways, biosynthesis of amino acids, 2-oxocarboxylic acid metabolism, phenylalanine, tyrosine and tryptophan biosynthesis and valine, leucine and isoleucine degradation in negative mode were represented the differentially expressed compounds in asthma patients compared with healthy control. Moreover, several metabolic pathways including glycerophospholipid metabolism, inositol phosphate metabolism, and glycolysis or gluconeogenesis were found most important. Glycerophospholipid metabolism was the most significantly perturbed pathways in experimental allergic asthma [31]. Inositol phosphates are important intracellular second messengers in eukaryotic cells and are of great importance in diverse cellular functions, such as Ca^{2+}-signaling pathways, cell growth, cell differentiation, apoptosis, endocytosis, cell migration, mRNA exportation and maintenance of genomic stability [32, 33]. These findings demonstrated obvious correlation between these metabolic pathways and the development of asthma. However, some of the different metabolites in asthma patients should be further validated in our further investigation by using Real-time PCR and western blot analysis.

Our study illustrates metabolic characteristics of sputum specimens in asthma patients and emphasizes the importance of metabolomics by using UHPLC-QTOF/MS. Several metabolites, signaling pathways and metabolic pathways were found difference between healthy controls and asthma patients. The observations presented here reveal that metabolomic analysis of sputum on basis of UHPLC-QTOF/MS may have clinical implications for the early diagnosis and risk prediction of asthma.

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Metabolites and asthma

Disclosure of conflict of interest

None.

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References


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