Metabolomics-based screening of salivary biomarkers for early diagnosis of Alzheimer’s disease†

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Early diagnosis of Alzheimer’s disease (AD) is an attractive strategy to increase the survival rate of patients. Metabolomics has a great potential for identifying useful biomarkers for early diagnosis, and prognosis. In this work, faster ultra-performance liquid chromatography (FUPLC) mass spectrometry (MS) coupled with multivariate statistical method were employed to find the metabolic changes of the salivary metabolome from AD patients. Saliva samples were obtained from patients with AD (n = 256) and age-matched healthy controls (n = 218). The metabolic differences among AD and control subjects were identified based on principal component analysis (PCA). Sphinganine-1-phosphate, ornithine, phenyllactic acid, inosine, 3-dehydrocarnitine, and hypoxanthine in the AD subjects were significantly different from the control subjects. To demonstrate the utility of salivary biomarkers for the early diagnosis of AD, 3 metabolites (AUC > 0.8) comprising sphinganine-1-phosphate, ornithine, and phenyllactic acid were selected as candidate biomarkers. The major contributor to the predictive model was sphinganine-1-phosphate, which was upregulated in AD, yielded satisfactory accuracy (AUC = 0.998), sensitivity (99.4%) and specificity (98.2%), indicating potential diagnosis in the AD. Our data provided highlights the potential advantages of the application of salivary metabolomics in clinical setting for the diagnosis of AD.

Introduction

Alzheimer’s disease (AD) is the most prevalent cause of dementia among older people and can be considered as a major public health problem.1,2 Although AD probably starts 20–30 years before the first clinical symptoms become noticeable, nowadays it cannot be diagnosed accurately in its early stages. Most previous studies have focused on the examination of Aβ and Tau in cerebrospinal fluid as markers in AD patients.3 Reports on other molecules of Aβ generation, transportation and degradation as markers for early diagnosis of AD are limited.4,5 Owing to lack of early-warning signs, AD patients are often diagnosed late. Unfortunately, AD is hard to discover in the early stages because of a lack of screening method, which would generally result in a low survival rate. Therefore, novel diagnostic technologies are urgently needed to diagnose AD. Saliva as diagnose medium offers an easy, inexpensive, safe, and noninvasive approach. Saliva is a complex secretory fluid that contains trace metals, metabolites, biochemicals, proteins, glycoproteins, lipids, etc., that serve a spectrum of physiological needs. In recent years, attention has been focused to the utility of this fluid in terms of monitoring and diagnosis of diseases.6–9 Saliva samples can be easily collected through noninvasive means and has been considered tool to monitor general disease status, because of its potential to mirror systemic health conditions. Recent research has identified a wide diversity of biomolecules in saliva that can provide information for identifying potential biomarkers.10

Metabolomics is a rapidly evolving technology that has been increasingly used to discover early markers of disease.11 It is the systematic study of small-molecular-weight substances, as modern high-throughput technology has developed rapidly, and provides a readout that is closely linked to the phenotype of interest, opens a door to biomarker discovery.12 LC-MS based metabolomics approach has been successfully used as a tool in the diagnosis of diseases with excellent reproducibility and sensitivity.13 Application of metabolomics could help to identify biomarkers for early AD diagnosis, to discover novel therapeutic targets, and to monitor therapeutic response and disease progression. Recently, salivary metabonomics analysis demonstrates its applicability for the diagnosis and prognosis of AD.14 A faster ultrahigh performance liquid chromatography-mass spectrometry (FUPLC-MS) metabolomics analysis technique has been used as to diagnose oral cancer, diabetes, colorectal cancer, hepatocellular carcinoma, and chronic renal failure.15,16 However, an integrated FUPLC-MS method based saliva metabolomics analysis of AD has not been reported hitherto as

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far as we know. Salivary metabolomics is an emerging and promising field that offers unique perspective on the use of oral fluids in molecular diagnostics. Application of salivary metabolomics is still in the preliminary stages, but its influence continues to grow each year. To explore salivary biomarkers of AD, we applied FUPLC-MS metabolomics in negative ion mode to investigate saliva samples from 256 AD patients and 218 healthy volunteers in a large Asian cohort. Multivariate data analysis was performed to highlight discriminated variables. The purpose of this study is to develop a saliva metabolomics analysis for identifying potential biomarkers to diagnosis of AD.

Experimental

Chemicals

Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Fisher (USA). Distilled water (18.2 MΩ) was purified using a Milli-Q system (Millipore, Billerica, USA). Formic acid (HPLC grade), lactic acid, and carnitine were purchased from J&K Chemical Ltd (Beijing, China). Leucine enkephalin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Ethics statement

The Ethical Committee of the Heilongjiang University of Chinese Medicine, approved the protocol (approval number: HUCM-2014-12749). All of the volunteers and patients signed an Ethical Committee consent form agreeing to serve as saliva donors for the experiments. The study was approved by the Ethical Committee of the Heilongjiang University of Chinese Medicine and complied with the provisions of the Good Clinical Practice Guidelines and the Declaration of Helsinki.

Study participants

All patients and all control individuals were recruited from the First Affiliated Hospital, Heilongjiang University of Chinese Medicine. The diagnosis of AD was based on the National Institute of Neurological and Communicative Disorders and Stroke and the AD and Related Disorders Association criteria. 256 AD patients diagnosed provided the test group; 218 healthy subjects provided the control group. The control subjects did not have any neurologic or cognitive disease. The detailed clinical characteristics of saliva samples used in this study were provided in the ESI Table 1.†

Saliva collection and preparation

All the donors were asked to refrain from smoking, eating, drinking, or oral hygiene procedures for at least 2 hour prior to samples collection, and then rinse their mouth thoroughly with water. Saliva samples were collected between 9:00 and 11:00 a.m. in a private room using standard techniques. The samples were centrifuged at 10 000 rpm for 20 min at 4 °C to remove insoluble materials, cell debris and food remnants. The supernatant (200 μL) was transferred to fresh tubes and frozen at −80 °C until the FUPLC-MS analysis. A mixture of acetonitrile/methanol (75:25 v/v, 400 μL) was added to saliva (200 μL) in a 1.5 mL Eppendorf tube to precipitate proteins. The mixture was allowed to stand for 10 min, and then the samples were centrifuged at 10 000 rpm for 20 min at 4 °C. The supernatant were filtered through 0.22 μm syringe filters before FUPLC-MS analysis.

FUPLC-MS analysis technology

Chromatography. Saliva separation was performed on an ACQUITY UPLC™ BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm, Waters, Milford, USA). The column was maintained at 40 °C. The injected sample volume was 2 μL for each run. The flow rate of the mobile phase was 0.4 mL min⁻¹. Gradient elution was performed with the following solvent system: (A) 0.1% formic acid–water, (B) acetonitrile (ACN). The gradient were started as follows: 0–2.0 min, linear increasing from 1–15% A; 2.0–5.0 min, 15% to 25% A; 5.0–7.0 min, 25–50% A; 7.0–8.5 min, 50–95% A; 8.5–10 min, 95% A, 10–14 min, 1% A.

Mass spectrometry. Mass spectrometry experiments were performed on an Acquity UPLC™ Waters (Milford, USA) equipped with an electrospray ion source. Data were acquired in negative ion mode and collected in centroid mode, the scan range was from m/z 50 to 1500 in the full scan mode. The source temperature was 120 °C, and desolvation gas temperature was 400 °C. Nitrogen was used as cone and desolvation gas. The flow rates of cone and desolvation gas were set at 80 L h⁻¹ and 500 L h⁻¹, respectively. Capillary, cone and extraction cone voltages were set at 3.0 kV, 25 V and 5.0 V.

Data pretreatment and analysis

FUPLC-MS data from saliva samples were analyzed to identify potential discriminant biomarkers. Data acquisition and handling were performed by Masslynx 4.1 (Waters). MarkerLynx application manager (Waters, Manchester, UK) was used for peak finding, filtering, and alignment. The MS matrix was then introduced to EZinfo 2.0 software for principal component analysis (PCA), partial least-squares-discriminant analysis (PLS-DA), and orthogonal partial least-squared discriminant analysis (OPLS-DA). In PCA, R²Y (cum) and Q² (cum) parameters were used for the evaluation of the models, indicating the fitness and prediction ability, respectively. Q² (cum) > 50% shows that the mode is useful; if the Q² (cum) > 90%, the mode is excellent. VIP-plot was calculated the discriminating variables in saliva between AD patients and healthy individuals. MassFragment™ manager (Waters corp., Milford, USA) was used to facilitate the MS/MS fragment ion analysis process by way of chemically intelligent peak-matching algorithms. Metabolites were identified by searches of databases using exact molecular weights. Commercial standard reagents were used to support identification of metabolites.

Statistical analysis

The areas under curve (AUC) of receiver operating characteristic (ROC) curves were performed to determine the diagnostic effectiveness of important metabolites using GraphPad Prism Version 5.00 (GraphPad Software, San Diego, California, USA). The t test and analysis of covariance were performed using SPSS.
software (version 19.0; SPSS, Inc., Chicago, IL). The P values less than 0.05 were considered significant.

**Results**

**Clinical characteristics**

Clinical characteristics of participants in the training set are provided in ESI Table 1.† The mean ages, sex, body mass index, 2 hour postprandial glucose, total cholesterol, triglycerides, systolic blood pressure, and diastolic blood pressure were not significantly different between patients with AD (n = 256) and age-matched healthy controls (n = 218). The mean Mini-Mental State Examination score was 14.4 ± 3.2 for AD patients, indicating a fairly advanced stage of this disease. Additionally, the average level of A-amyloid protein was significantly higher than that of the healthy controls.

**Typical base peak intensity chromatogram**

In this study, the saliva samples were analyzed by FUPLC-MS. The separation conditions of saliva samples were optimized. Typical UPLC-TOF/MS base peak intensity (BPI) chromatograms of saliva samples from the controls and the AD in negative ion mode was shown in Fig. 1. From the BPI chromatograms, more marked variations can be seen in the patient group than in the control group. The utilization of pattern recognition could enlarge metabolite identification.

**Pattern recognition analysis**

Using MarkerLynx software for peak detection, 3319 peaks were obtained and these peaks can be used as a comprehensive saliva metabonomics profiling. The variables were exported into EZinfo 2.0 software for multivariate data analysis to detect metabolite. In Fig. 2, the classification resulted in excellent modeling and predictive abilities (R2(X) = 64.7%, R2(Y) = 98.3%, Q2(Y) = 96.9%). From these results, we can find that the PCA model was valid for negative ion mode. All saliva samples were divided into two groups: healthy controls as AD group. As can be seen from the Fig. 2, there are obvious separation trend observed for patients with AD and age-matched healthy controls. The satisfactory clustering trends are observed in the scores plot, indicating that the possibility of using saliva metabonomics for discriminating AD.

**Early AD biomarker discovery**

In order to identify discriminating variables used in the early stage detection of AD, an VIP-plot model was used. VIP was used as an important parameter to select the interesting variables biomarkers for AD relative to the control group. Variables with VIP value greater than 1 were considered as great value. A t test was performed in variables with significant differences between AD patients and control individuals (P < 0.01) were retained. VIP-plot of AD patients vs. control individuals was shown in Fig. 3, which is a scatter plot that combines the covariance and

![Fig. 1](image-url)  
**Fig. 1** Typical base peak chromatograms of control subjects (up) and AD patients (down) by FUPLC-MS.
correlation for the model variables with respect to model component scores. Therefore, a total of six discriminate variables as interesting biomarker candidates were found in AD relative to the control group. Six variables were highlighted in VIP-plot (VIP > 11 and \( P < 0.01 \)). Elemental composition was calculated using the Masslynx 4.1 analysis software. And then, it was finally confirmed by comparison with standard sample. Eventually, 6 metabolites were tentatively identified as potential biomarkers for early diagnosis of AD and were listed in Table 1. These results suggested that combining FUPLC-MS and pattern recognition techniques can be used for a comprehensive saliva metabonomics analysis and screening biomarkers for the early diagnosis of AD.

### Diagnostic values of differential metabolites

In all biomarkers, 4 potential biomarkers were up-regulated in saliva of AD patients and 2 potential biomarkers were down-regulated. In order to characterize these potential biomarkers in early stage of AD, receiver operating characteristic (ROC) analysis was performed. The six identified potential biomarkers were divided into two groups, 4 up-regulated in AD patients and 2 down-regulated. The detailed parameters of potential salivary biomarkers for AD prediction were provided in Table 2. Table 2 shows the detailed sensitivity, specificity levels and 95% confidence interval of the 6 identified potential salivary biomarkers for AD early prediction. The metabolites, sphinganine-1-phosphate, ornithine, phenyllactic acid, inosine, 3-dehydrocarnitine, hypoxanthine provided the areas under curve (AUC) values of 0.998, 0.927, 0.831, 0.740, 0.669, and 0.674, respectively in AD patients vs. control individuals. As a single biomarker in saliva, sphinganine-1-phosphate had a sensitivity of 99.4% and a specificity of 98.2% for early predicting AD. To demonstrate the utility of salivary biomarkers for the early diagnosis of AD, 3 metabolites (AUC > 0.8) comprising sphinganine-1-phosphate, ornithine, and phenyllactic acid were selected to form a biomarker group. Therefore, these

### Table 1 Information of AD biomarkers detected by FUPLC-Q-TOF/MS in negative ion mode

<table>
<thead>
<tr>
<th>No</th>
<th>( R_i ) (min)</th>
<th>( m/z )</th>
<th>Compound ID</th>
<th>Formula</th>
<th>Mass error (ppm)</th>
<th>Description</th>
<th>Anova (p)</th>
<th>Max fold change</th>
<th>Trend</th>
<th>VIP</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>3.64</td>
<td>380.2556</td>
<td>HMD801383</td>
<td>C(<em>{18})H(</em>{40})NO(_5)P</td>
<td>−4.06</td>
<td>Sphinganine-1-phosphate</td>
<td>0.0001</td>
<td>12.11</td>
<td>↑</td>
<td>14.18</td>
</tr>
<tr>
<td>2</td>
<td>0.63</td>
<td>131.0825</td>
<td>HMD800214</td>
<td>C(<em>{6})H(</em>{12})NO(_2)</td>
<td>−0.52</td>
<td>Ornithine</td>
<td>0.0030</td>
<td>3.94</td>
<td>↑</td>
<td>13.94</td>
</tr>
<tr>
<td>3</td>
<td>1.54</td>
<td>165.0558</td>
<td>HMD800779</td>
<td>C(<em>{6})H(</em>{12})O(_3)</td>
<td>0.69</td>
<td>Phenyllactic acid</td>
<td>0.0045</td>
<td>3.44</td>
<td>↑</td>
<td>13.53</td>
</tr>
<tr>
<td>4</td>
<td>2.04</td>
<td>267.0731</td>
<td>HMD800195</td>
<td>C(<em>{10})H(</em>{16})N(_2)O(_4)</td>
<td>−1.49</td>
<td>Inosine</td>
<td>0.0000</td>
<td>18.58</td>
<td>↓</td>
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<tr>
<td>5</td>
<td>0.98</td>
<td>158.0824</td>
<td>HMD812154</td>
<td>C(<em>{6})H(</em>{12})NO(_3)</td>
<td>1.14</td>
<td>3-Dehydrocarnitine</td>
<td>0.0030</td>
<td>5.76</td>
<td>↓</td>
<td>11.94</td>
</tr>
<tr>
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<td>135.0299</td>
<td>HMD800157</td>
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<td>Hypoxanthine</td>
<td>0.0064</td>
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<td>↓</td>
<td>11.80</td>
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</table>
salivary biomarkers in combination might have important clinical value for the diagnosis of AD in its early stage.

Discussion

AD is the most common neurodegenerative dementia, with the accumulation of extracellular amyloid-β and formation of neurofibrillary tau tangles as leading explanations of pathology. Despite decades of research, no early-onset biomarkers are currently available for AD, a cureless neurodegenerative disease affecting millions worldwide. It is hoped that identifying the effect of AD on the metabolism composition of saliva will improve both diagnosis and treatment. Saliva testing is inexpensive, and easy to use and rapidly advancing in recent years. The collection of saliva could reduce the discomfort for patients, particularly if repeated sampling is necessary. For the discovery of new biomarkers, the application of omics is emerging, especially metabolomics. Previous studies have demonstrated altered metabolites in plasma samples of AD patients. However, the sample size from many of them is relatively small and the metabolites are relatively limited.

Accurate diagnosis of AD may facilitate effective prevention and treatment of AD disease. Saliva has been extensively examined in attempts to assess the AD disease status. FUPLC-MS combined with pattern recognition approach could be an advanced tool to help us find metabolites with classifying of sample groups. With our experiment, the metabolites obtained included sphinganine-1-phosphate, ornithine, phenyllactic acid, inosine, 3-dehydrocarnitine, and hypoxanthine. By using our platform, furthermore, a panel of 3 candidate markers was found to differentiate the AD in saliva, may serve as a diagnostic tool for AD biomarker detection.

Description for the selected biomarkers of AD was grouped as up-regulated and down-regulated metabolites. For up-regulated metabolites, sphinganine-1-phosphate is an intermediate in the metabolism of glycosphingolipids and sphingolipids. It is a critical substrate for sphingosine kinase. Sphingolipid metabolism is thus seen to be abnormal in AD patients compared to controls. The salivary sphinganine-1-phosphate content is higher with healthy subjects probably due to the increased expression of the process of sphingosine kinase. Ornithine is an amino acid produced in the urea cycle by the splitting off of urea from arginine. It is a central part of the urea cycle, which allows for the disposal of excess nitrogen. We found that the levels of ornithine were markedly higher in patients with AD than in healthy controls. Over expression of ornithine has also been reported in other researches. Ornithine is also a precursor of citrulline and arginine. In order for ornithine produced in the cytosol to be converted to citrulline, it must first cross the inner mitochondrial membrane into the mitochondrial matrix where it is carbamylated by ornithine transcarbamylase. Compared with control, a higher level of phenyllactic acid was observed in the early stage of AD patients. Phenyllactic acid, a product of phenylalanine catabolism, is likely produced from phenylpyruvate via the action of lactate dehydrogenase. Metabolites of this transamination reaction include phenylacetate, phenylpyruvate and phenethylamine. Phenylalanine accumulation disrupts brain development, leading to mental retardation. Elevated level of phenyllactic acid appears in the saliva of AD patients probably due to the disorders of phenylalanine metabolism. For down-regulated metabolites, inosine is a purine nucleoside that has hypoxanthine linked by the N9 nitrogen to the C1 carbon of ribose. It is an intermediate in the degradation of purines and purine nucleosides to uric acid and in pathways of purine salvage. Hypoxanthine is a spontaneous deamination product of adenine. The obvious decrease of inosine and hypoxanthine reveals an abnormal purine metabolism in AD patients. 3-Dehydrocarnitine is a member of the carnitine family that is an intermediate in carnitine degradation. Carnitine is a quaternary ammonium compound biosynthesized from the amino acids lysine and methionine. In living cells, it is required for the transport of fatty acids from the cytosol into the mitochondria during the breakdown of lipids for the generation of metabolic energy.

In our work, an integrated separation approach by combining FUPLC with MS has been developed for performing global metabolomics analysis in human saliva and identified potential biomarkers for the early diagnosis of AD. A total of 6 potential biomarkers have a close relationship with early stage of AD. Three potential biomarkers were up-regulated in saliva of AD patients and potential biomarkers were down-regulated. Three salivary biomarkers (sphinganine-1-phosphate, ornithine, and phenyllactic acid) yielded satisfactory accuracy, sensitivity, and specificity in distinguishing AD patients from the controls. Our research provided highlights the potential advantages of the application of salivary metabolomics in real clinical diagnostics.

Conclusions

Saliva is a readily available biofluid that may contain metabolites of interest for diagnosis of diseases. Here, we applied a comprehensive platform using LC/MS to analyze saliva samples from AD patients and normal controls. Metabolomic fingerprints were subjected to multivariate analysis in order to discriminate between groups of AD patients and healthy controls, and then some key-compounds were identified as possible markers of AD. PCA model yielded class separation for AD and controls. The sphinganine-1-phosphate, ornithine, phenyllactic acid, inosine, 3-dehydrocarnitine, hypoxanthine in the saliva, of the AD subjects were significantly different from the control subjects. ROC analysis revealed sphinganine-1-phosphate, ornithine, and phenyllactic acid to be potent discriminators of the between AD and control groups. Predictive power of sphingosine was confirmed and, reaching 99.8% diagnostic accuracy, indicating potential diagnosis in the AD. These findings also suggest the potential of LC/MS-based metabolomics as a method to identify saliva biomarkers for AD, which could be confirmed by future translational research with human patients.

Conflict of interest

The authors declare no competing financial interests.
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