Discovery of serum metabolites for diagnosis of mild cognitive impairment to Alzheimer's disease progression using an optimized metabolomics method

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Discovery of serum metabolites for diagnosis of mild cognitive impairment to Alzheimer’s disease progression using an optimized metabolomics method

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Abstract

Mild cognitive impairment (MCI) is considered to represent early AD. Currently, there is a great need of sensitive tools to monitor the progression of MCI to AD. In this work, a nontargeted metabolomics approach was developed to examine metabolic differences in serum samples from the MCI subjects and the age-matched AD subjects. Based on principal component analysis, metabolic differences among AD and MCI subjects were identified. Nine metabolites in the serum of the AD subjects were significantly different from the MCI subjects. Two metabolites were selected as the candidate biomarkers and validation in separate and independent patient cohorts. The major contributors to the predictive model were upregulated sphinganine-1-phosphate and 7-ketocholesterol, yielded satisfactory sensitivity, and specificity, indicating potential value for the predicting conversion of MCI to probable AD. The present study may provide a diagnosis tool to monitoring the progression of MCI to AD.

Keywords:

Alzheimer’s disease; mild cognitive impairment; metabolomics; serum; metabolites; diagnosis
Introduction
Alzheimer's disease (AD) is the most prevalent form of dementia with an estimated worldwide prevalence of over 30 million people, and its incidence is expected to increase dramatically with an increasing elderly population [1,2]. It is a growing challenge to the health care systems and economies of developed countries [3]. Mild cognitive impairment (MCI) is believed to be an intermediate state between normal cognition and AD. MCI confers an increased risk of developing AD [4,5]. The prediction of progression from MCI to AD is also of major interest. Recent research has concentrated on obtaining biomarkers to identify metabolic features that differentiate between those MCI subjects who will develop AD [6-9]. Biomarkers are needed for early and accurate diagnosis, for prediction of conversion from pre-clinical MCI and to monitoring AD progression. An ideal biomarker would be one that was simple and inexpensive to test, with high specificity and sensitivity.

Recently, metabolomics has become a powerful profiling method with the aim to discover novel biomarkers and to understand the disease state [10,11]. It is the systematic study of small molecules with unbiased identification[12,13]. Application of metabolomics could help to identify biomarkers for early AD diagnosis, and to monitor disease progression. Recently, serum metabonomics analysis demonstrates its potential ability for the diagnosis and prognosis of diseases [14]. Serum samples can be easily collected and has been considered potential tool to monitor general disease status, because it can mirror systemic health conditions.

The nontargeted metabolomics approach maybe potentially provide a variety of new biomarkers to monitor AD. A faster ultrahigh performance liquid chromatography-mass spectrometry (FUPLC-MS) technique has been used as to diagnose oral cancer, diabetes, colorectal cancer, hepatocellular carcinoma, and chronic renal failure [15,16]. In this work, a serum metabolomic approach based on FUPLC-MS has been used to obtain representative fingerprints of low molecular weight metabolites from serum samples in order to distinguish between patients with AD and MCI.

EXPERIMENTAL PROCEDURES

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) was purchased from J&K Chemical Ltd (Beijing, China). Leucine enkephalin was purchased from Sigma-Aldrich (St. Louis, MO, USA). All standard chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Ethics statement
The study was carried out in accordance with the provisions of the Good Clinical Practice Guidelines and the Declaration of Helsinki. The protocol was reviewed and approved by the Ethics Committee at Heilongjiang University of Chinese Medicine. The informed consent was obtained from all subjects. All patients and healthy individuals were approached using approved ethical guidelines in this study.
Subjects

AD and MCI patients were recruited from the First Affiliated Hospital, Heilongjiang University of Chinese Medicine. Cases were classified as AD (AD group) or MCI (MCI-AD group) based on standard diagnostic criteria [17,18]. Demographic characteristics and clinical diagnosis of studied subjects are detailed in Supporting Table 1. All the patients were asked to refrain from smoking, eating, drinking, or oral hygiene procedures for at least 2 hours prior to samples collection, and then rinse their mouth thoroughly with water. Participants were not received any drugs before one week.

Serum collection and preparation

Serum was extracted according to a procedure described by Bruce et al [19]. Serum samples were collected between 9:00 and 11:00 a.m. in a private room using standard techniques. The samples were centrifuged at 10000 rpm for 20 min at 4 °C to remove insoluble materials, cell debris and food remnants. The supernatant (200 µL) were 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 serum (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 10000 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

Serum 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. Gradient elution was performed with the following solvent system: (A) 0.1% formic acid-water, (B) acetonitrile (ACN). The gradient was 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. Each sample was mixed to generate a pooled quality control (QC) sample. Before analyzing the sample sequence, five QC samples were run. During analysis of the sample sequence, one QC sample was run after every 10 injections in order to ensure the stability and repeatability of the UHPLC system.

Mass Spectrometry

Mass spectrometry experiments were performed on quadrupole-time-of-flight mass spectrometer (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 and 500 L/h, respectively. Capillary, cone and extraction cone voltages were set at 3.0 kV, 25 V and 5.0 V. All the data were acquired using a reference lock mass (Leucine enkephalin) to ensure accuracy and reproducibility during the MS analysis.
Data Processing and Multivariate Statistical Analysis

FUPLC-MS data from serum samples were analyzed to identify potential discriminant biomarkers. Automatic peak detection, migration time alignment, and peak integration was performed using Masslynx 4.1 (Waters, Manchester, UK). The MS matrix was then introduced to EZinfo 2.0 software for principal component analysis (PCA) and orthogonal partial least-squared discriminant analysis (OPLS-DA). R2Y (cum) and Q2 (cum) parameters were used for the evaluation of the models, indicating the fitness and prediction ability, respectively. VIP-plot was calculated the discriminating variables in serum between AD and MCI-AD groups. 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 (MassBank, http://www.massbank.jp/; HMDB, http://www.hmdb.ca/) using exact molecular weights. Standards were then used to confirm metabolite identification by means of subsequent LC/MS analysis.

Statistical analysis

The areas under curve (AUC) of receiver operating characteristic curves (ROC) were performed to determine the diagnostic effectiveness of the metabolites using GraphPad Prism Version 5.00 (San Diego, California, USA). The t test was performed using SPSS software (version 20, Chicago, IL) for Windows were used for data processing and the p values less than 0.05 were considered significant.

Results

Clinical Characteristics

Clinical characteristics of participants in the training set are provided in Supplemental Table 1. The mean ages, sex, body mass index, diastolic blood pressure and systolic blood pressure were not significantly different between patients with MCI and age-matched AD. The mean Mini-Mental State Examination score was 15.7±2.8 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 MCI. Then, a second set of MCI (n = 340) patients and control subjects (n = 328) to be blindly selected and tested using our approach.

Typical base peak intensity chromatogram

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

Pattern recognition approach

Using MarkerLynx software for peak detection, 5348 peaks were obtained and these peaks can be used as a
comprehensive serum metabonomics profiling. The variables were exported into EZinfo software for multivariate data analysis to detect metabolite. In Figure 2, the classification resulted in excellent modeling and predictive abilities (R2(X) = 87.3%, R2(Y) = 97.2%, Q2(Y) = 98.8%). From these results, we found that the PCA model was valid in negative ion mode. As can be seen from the Figure 2, interestingly, we could observe a difference between AD and MCI, indicating satisfactory clustering trends in the scores plot.

**Marker metabolite discovery**

In order to identify discriminating variables used in the early stage detection of AD, a VIP-plot model was used. Variables with VIP value greater than 11 were considered as great value. A T test was performed in variables with significant differences between AD patients and MCI-AD patients (P < 0.01) were retained. VIP-plot of AD vs MCI patients was shown in Figure 3, which is a scatter plot that combines the covariance and correlation for the model variables to model component scores. A total of 9 discriminate variables as interesting biomarker candidates were found in AD relative to the MCI group. Nine variables were highlighted in VIP-plot (VIP > 11 and P < 0.01). Elemental composition was calculated using the Masslynx 4.1 analysis software. Finally, 9 marker metabolites were tentatively identified as potential biomarkers and were listed in Table 1.

**Diagnostic values of marker metabolites**

In all biomarkers, 6 potential biomarkers were up-regulated in serum of AD patients and 3 potential biomarkers were down-regulated. To assess the feasibility of diagnosis, we performed a ROC analysis model selection in the ‘Methods’ section. Then, a second set of MCI (n = 340) patients and AD subjects (n = 328) to be blindly selected and tested. The detailed parameters of marker biomarkers for AD prediction were provided in Table 1 which shows the detailed sensitivity, specificity levels and 95% confidence interval for prediction. The metabolites, sphinganine-1-phosphate and 7-ketocholesterol provided the AUC values of 0.998 and 0.972, in AD vs MCI-AD patients. ROC analysis between MCI and AD, using these metabolites shows a score of 0.90, indicating a good discrimination power and confirms the potential usefulness of measuring marker metabolites levels for diagnosis. The sphinganine-1-phosphate had a sensitivity of 98.4% and a specificity of 99.1% for early predicting AD. Two metabolites (AUC > 0.9) comprising sphinganine-1-phosphate and 7-ketocholesterol were selected to form a biomarker group, 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 [20,21]. Diagnosis of AD in its early stage is still challenging and demands the development of new analytical strategies. LC-MS metabolomics method makes it possible to identify differences in the serum metabolome from patients with MCL related to AD progression. Indeed, AD is not a homogeneous disorder, and uncovering the etiologies of MCL could be of great importance for a proper
diagnosis, evaluation of disease progression, and adjusting future therapeutical strategies. Serum testing is inexpensive, and easy to use and rapidly advancing in recent years. The collection of serum 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 [22]. However, the sample size from many of them is relatively small and the metabolites are relatively limited.

In this study, FUPLC-MS combined with pattern recognition approach could be an advanced tool to help us find metabolites with classifying of sample groups between AD patients and MCI patients. In our experiment, nine metabolites were obtained including sphinganine-1-phosphate, 7-ketocholesterol, 3-methoxytyrosine, deoxyribose 5-phosphate, L-phenylalanine, ornithine, D-phenyllactic acid, deoxyribose 5-phosphate, L-phenylalanine, ornithine, D-phenyllactic acid, lysoPC(15:0), and L-glutamic acid. Furthermore, by using our platform, a panel of two candidate markers was found to differentiate the AD, may serve as a diagnostic tool for AD biomarker detection. In line with current thinking, sphinganine-1-phosphate and 7-ketocholesterol showed promise as potential biomarkers. This study offers hope that the metabolome will yield useful, accessible biomarkers and that these in turn will support curative research. The next steps have been formulated directly investigated a mechanistic link between the metabolic changes observed in this study with the disease process of MCI to AD.

In our work, an integrated separation approach by combining FUPLC with MS has been developed for performing global metabolomics analysis in serum and identified potential biomarkers for the early diagnosis of AD.A total of 9 potential biomarkers have a close relationship with early stage of AD. Six potential biomarkers were up-regulated in serum of AD patients and 3 potential biomarkers were down-regulated. Two serum biomarkers yielded satisfactory accuracy, sensitivity, and specificity in distinguishing AD patients from the MCL. The major contributors to the predictive model were sphinganine-1-phosphate and 7-ketocholesterol, which was upregulated in AD, yielded satisfactory accuracy (AUC = 0.998), sensitivity (98.4%), and specificity (99.1%), indicating potential early diagnosis. Our research provided highlights the potential advantages of the application of serum metabolomics in real clinical diagnostics. Given the key metabolite from the metabolic signature predictive of MCL progression to AD is abundant in serum, Further investigations should validate them in other cohort studies, as well as in experimental models. Looking for predictive biomarkers such as ours may not only facilitate early diagnosis, also help identify new therapeutic strategies.

Conclusions

Here, we applied a global metabolomics platform to analyze serum samples from MCI and AD patients. Metabolomic data were subjected to pattern recognition approach analysis, aim to discriminate MCL and AD patients, and then some metabolites were identified as possible biomarkers of AD. PCA model yielded the separation for AD and MCI
group. Nine metabolites in the serum, of the AD subjects were significantly different from the MCI subjects. ROC analysis revealed sphinganine-1-phosphate and 7-ketocholesterol to be potent discriminators of the between AD and MCI-AD groups. Predictive power of them was confirmed and, reaching >95 % diagnostic accuracy, indicating potential diagnosis in the AD. These findings also suggest the potential of LC/MS-based metabolomics as a tool to identify serum biomarkers for AD, which could be confirmed by future translational research with human patients.

Acknowledgments
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Competing financial interests
The authors declare no competing financial interests.

References


**Figures Legends**

**Fig.1** Typical base peak chromatograms of MCL subjects (up) and AD patients (down) by FUPLC-MS.

**Fig.2** Score plot of the PCA model of the FUPLC-MS data from MCL patients (black) and AD group (red).

**Fig.3** VIP-plot for selection of interesting variables for patients with AD and age-matched MCL patients.
Table 1. Information of serum metabolites for the predicting conversion of MCI to probable AD, detected by FUPLC-Q-TOF/MS.

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<th>Description</th>
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<th>Max Fold Change</th>
<th>Trend</th>
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