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Experimental study

Decreased levels of urinary free amino acids in children with autism spectrum disorder

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ABSTRACT

Autism spectrum disorder (ASD) is a range of neurodevelopmental problems without certain causes. Conventional diagnostic or screening tools for ASD rely on the observation of children's behavioral presentations. Novel methods are focused on the alterations of some important biochemical matters in ASD patients, which are applicable in the screening for ASD. This study investigated and compared amino acids in the first morning urine from age and sex matched ASD and non-ASD children using high performance liquid chromatography. Significantly lower urinary free methionine, phenylalanine, valine, tryptophan, and leucine plus isoleucine were observed in ASD children. The effects of using urinary free amino acids (UFAAs) singly or conjointly to classify participants into ASD or control group were analyzed and compared. ROC curves on these UFAAs singly in classification performed the sensitivity of 0.593–0.889 and the specificity of 0.704–0.963. Binary-logistic regression analysis of these UFAAs obtained a final regression model comprised of urinary free valine and tryptophan. The ROC curve established by the linear combination of the two amino acids achieved a sensitivity of 0.926 and a specificity of 0.889, which showed superiority to single UFAA and comparability to existing diagnostic or screening tools. It was suggested that the multivariate model based on UFAAs was possibly applicable in screening for children at higher risk of ASD.

1. Introduction

Autism spectrum disorder (ASD) describes a range of conditions classified as neurodevelopmental disorders in the fifth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-5). ASD is characterized by two symptom dimensions of impairments in social communication and interaction as well as restricted, repetitive patterns of behavior, interests or activities. UP to now, the cause of ASD is unclear. Early behavioral and educational intervention are efficacious treatment methods, which can improve ASD children's development rather than curing the disease. The median estimated worldwide prevalence of ASD is 62/10 000 [1]. Supporting an individual with ASD in the whole life span costs $2.4 million in the US and $2.2 million in the UK [2]. Therefore, it has been particularly important to identify ASD patients accurately.

The newest diagnostic criteria for ASD is published in DSM-5. The specificity and sensitivity of DSM-5 in diagnosing ASD is 0.97 and 0.81 [3]. Some other diagnostic tools were also developed, such as Diagnostic Interview for Social and Communication Disorders (DISCO), Autism Diagnostic Interview (ADI), Autism Diagnostic Observation Schedule (ADOS) and Childhood Autism Rating Scale (CARS), which provided operational diagnostic schedules. Autism Spectrum Screening Questionnaire (ASSQ) and Autism Behavior Checklist (ABC) are screening instruments for ASD. All the above tools are used by psychiatrists and psychologists based on observations of children's behavioral performances.

Recently, there are growing studies trying to combine the identification of ASD with various active molecules involved in different functions in human body. This orientation screens

Abbreviations: ASD, Autism spectrum disorder; R. Time, Retention time; DSM-5, Diagnostic and Statistical Manual of Mental Disorders, fifth edition; DISCO, Diagnostic Interview for Social and Communication Disorders; ADI, Autism Diagnostic Interview; ADOS, Autism Diagnostic Observation Schedule; ASSQ, Autism Spectrum Screening Questionnaire; CARS, Childhood Autism Rating Scale; ABC, Autism Behavior Checklist; AA, Amino acid; TYR, Tyrosine; MET, Methionine; PHE, Phenylalanine; VAL, Valine; TRP, Tryptophan; LEU, Leucine; ILE, Isoleucine; BCAA, branched-chain amino acid; CRE, Creatinine; UFAA, Urinary free amino acid; HPLC, High Performance Liquid Chromatography.

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biomarkers for the anomalies in different systems, which are possibly applicable in the diagnosis or screening of ASD. Reported experiments have included the biomarkers for brain function [4], metallothionein system [5], oxidative stress [6] and immune system [7].

Generally, most reported diagnostic markers for ASD are single indicators. Jointly using multiple markers to diagnose is also recommended [8], which has been applied in the diagnosis of hepatitis [9] and cardiovascular diseases [10].

Amino acids (AA) participate in various metabolisms, some are altered in ASD patients. Tryptophan (TRP) played a role in the metabolism of serotonin, melatonin, quinolinic and kynurenic acid; ASD children's are reported of showing decreased tryptophan metabolism [11]. Tyrosine (TYR) and phenylalanine (PHE) are involved in the biosynthesis of dopamine in human body [12]; Dopaminergic system anomaly is a hypothetical neurophysiologic mechanism of autism [13]. Additionally, tyrosine acts as the precursor for urinary p-cresol through the activity of gut bacteria, which is also altered in ASD children [14]. Methionine (MET) is the biomarker of oxidative stress and methylation. In ASD children, increased oxidative stress and impaired methylation capacity are observed, as well as decreased plasma methionine [15]; Valine (VAL), leucine (LEU) and isoleucine (ILE) are known as branched-chain amino acids (BCAA) which played important roles in the function of skeletal muscles [16]. Deficient BCAAs and impaired motor skills are reported in ASD children [17,18]. Therefore, the purpose of this study was to test the feasibility of using multiple urinary amino acids for the screening for children at higher risk of ASD.

2. Materials and Methods

2.1. Participants

Participants were recruited from two kindergartens in Suzhou, China. ASD children with ASD were from a special kindergarten, while non-ASD children were from a regular kindergarten. Participants were recruited through several physical check projects. The recruitment was done in Mar 2015. For ASD group, children were previously diagnosed by psychiatrists and psychiatrists before they were enrolled into the kindergarten according to their clinical manifestations and DSM-5. In addition, ASD children took the autism behavior checklist (ABC) [19] and all scored over 70. For the control group, the exclusion criteria included history of psychiatric disease, metabolic disease, or recent acute illness. All the subjects were confirmed of taking no medications in a recent month.

This work was carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Children's caregivers were informed of the purposes and processes and signed a written consent form. Approval of the ethics committee of Zhongda Hospital, Southeast University was taken.

2.2. Samples

First morning urine has the representative value to assess the excretion of urinary amino acids [20]. Urines were collected using aseptic polypropylene tubes by children's caregivers when the children were waked up in the morning on a regular school day at about 8:00 am. Samples were labelled and sent to researchers at school before 9:00 am. Then samples were stored at −20 °C in a freezer.

Prior to assay, samples were thawed and centrifuged at 12,000 rpm for 5 min to separate aqueous component. The supernates were filtered using 0.45 μm organic filter membranes. Then 50 μL of the filtered supernate was taken out for derivatization at 30 °C for 20 min. The derivatization solution was 0.25 mL ethanol solution containing 1 mol/L sodium sulfite (Na_2SO_3) and 0.4 mol/L o-Phthalaldehyde mixed with 5 mL 0.1 mol/L aqueous solution of sodium tetraborate ($Na_2B_4O_7$). Finally, the treated samples were injected into HPLC for determination.

2.3. Materials and chromatographic conditions

Instruments were LC-20AD HPLC, L-ECD 6A electrochemical detector (Shimadzu Co., Ltd. Japan), Shimadzu chromatography workstation (Shimadzu Co., Ltd. Japan), Shimadzu VP-ODS chromatographic column (4.6 × 150 mm, 5 μm) (Shimadzu Co., Ltd. Japan).

HPLC grade methanol, analytical grade standards tyrosine, methionine, phenylalanine, valine, leucine, isoleucine and tryptophan were from Sigma Aldrich. Analytical grade standard creatinine was from National Institutes for Food and Drug Control, China. Analytical pure (AR) sodium tetraborate, sodium sulfite, o-Phthalaldehyde, sodium hydroxide, hydrochloric acid and picric acid were from sinopharm chemical reagent, China. Triple-distilled deionized water was used throughout.

The mobile phase was 68:32 methanol/deionized water (V/V) containing 0.1 mmol/L EDTA and 32.5 mmol/L KH_2PO_4. It was filtrated with 0.45 μm organic membranes. Detection potential, flow rate of mobile phase, injection volume and column temperature were 0.7 V, 1.0 mL/min, 20 μL and 30 °C respectively. The levels of urinary amino acids were adjusted by computing the ratio of amino acids to creatinine (CRE) and defined as urinary free amino acids (UFAAs) [21]. The analytical method for urinary creatinine was a national standard method (WS/T97-1996).

2.3.1. Linearity, RSD, limit of detection and recovery

The R^2 of the working curves for the urinary amino acid were 0.9939–0.9995. The inter-day RSD for each amino acid was 0.8–9.4%, the inner-day RSD for each amino acid was 0.6–8.9%. The limit of detection and quantification were 0.01–0.04 and 0.05–0.2 μg/mL. The recoveries were 86.6–107.5%.

2.4. Statistics

IBM SPSS Statistics 22.0 was used for statistical analysis. UFAAs were compared between ASD children and normal children. The effects of using these UFAAs to distinguish ASD and non-ASD children separately or conjointly were tested and compared. $P < 0.05$ was considered statistically significant.

3. Results

There are 27 ASD children (M/F = 20/7) and 27 (M/F = 20/7) non-ASD children in this study. The average ages of ASD group and non-ASD group are 5.22 ± 1.09 (M ± SD) and 5.56 ± 0.51, respectively. The two groups are matched on age ($Z = -1.683$, $p = 0.091$) and sex.

Typical HPLC chromatograms of urinary amino acids were shown in Fig. 1. Seven amino acids were consecutively detected in a single run. The levels of UFAAs were compared between the two groups using Mann-Whitney *U* test and shown in Table 1.

The levels of urinary free methionine, phenylalanine, valine, tryptophan and leucine plus isoleucine were significantly lower in ASD group compared with non-ASD group. ROC curves of these UFAAs (except for TYR) were analyzed to test the effect of using each UFAA singly to classify the subjects into ASD or non-ASD group. The results were shown in Fig. 2 and Table 2. The optimum sensitivity and specificity were observed on TRP.

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