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Urine proteome analysis to evaluate protein biomarkers in children with autism

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ABSTRACT

Background: Autism is a complex developmental disability for which no specific diagnostic markers have been identified so far. The present study aimed to evaluate whether there is any abnormal protein(s) excreted in the urine of autistic children by proteome analysis which may act as diagnostic marker.
Methods: Urine proteome analysis was carried out in first void urine samples of autistic and normal children ($n = 30$) in the age group of 4–12 years by 2D-PAGE followed by MALDI–TOF–MS analysis.
Results: Comparison of 2D-PAGE gels revealed that many urinary proteins are expressed differentially in autistic children. Total numbers of spots observed were 250 and 159 in autism and normal samples respectively, out of which 95 matches were observed. In addition, 3 spots of abnormally expressed peptides were selected, excised and analyzed. Peptide sequence with significant match score was for kininogen-1 (KNG-1)–50 (spot-1), IgG1 heavy chain variable region-35(spot-2) and mannan-binding lectin serine protease-2 isoform-2 precursor-45(spot-3). All the autistic children showed significant increase ($p < 0.001$) in urinary kininogen level measured quantitatively by ELISA, when compared to normal children.
Conclusion: Increased urinary kininogen-1 level in all the autistic children and the possibility of this protein as a diagnostic marker need further investigation.

1. Introduction

Autism is a complex developmental disability where symptoms are discernible in the affected infant's life in the first 3 years of age and manifest throughout the life span. Based on the Diagnostic and Statistical Manual of Mental Disorders (DSM)-5 criteria approximately four times as many males as females are diagnosed with autism [1]. Autism is not a specific disease but rather a neurological disorder that has an effect on normal brain function with strong genetic basis, affecting development of the person's communication, social interaction skills, with language changes and restricted/repetitive behaviors and activities [2]. The etiology and pathogenesis of ASD remain elusive, although some brain regions and neural systems have been suggested to be associated with the disorder [3,4]. Autism is diagnosed based on behavior and not on cause or mechanism. Based on observation of children, the severity of autism is assessed by the Childhood Autism Rating Scale (CARS) [5].

Autism has shown to have strong association with various complications including immunological and gastrointestinal disturbances [6,7]. Many children with autism show symptoms such as inflammation, diarrhea, gastroesophageal reflux, abdominal pain, and increased gastrointestinal permeability that leads to entry of certain peptides into the circulation that cross the blood brain barrier and exert opioid-like effects in the brain [8–10]. Though many neuropeptides which are exogenous and endogenous in origin have been identified in the urine of autistic children, reproducible results are not presented and studies on the pathophysiology of autism and the associated biological markers in Indian autistic children are very much limited.

Developing novel and specific biomarkers is pivotal in improving the clinical management of children with autism. So far there is no specific protein markers identified for early intervention and for understanding the mechanism underlying the pathophysiology of autism. Proteomic based approaches have great potential in explaining the biological defects in heterogeneous neurodevelopmental disorders such as autism by examining the abnormally expressed proteins in tissues and body fluids [11]. One of the applications of proteome analysis is qualitative and semiquantitative measure of proteins in a wide variety of biological samples such as saliva, blood, urine and cerebrospinal fluid (CSF) which provide promising opportunities to identify specific diagnostic and prognostic biomarkers [12]. Application of proteomics for identification of protein biomarker in neurodevelopmental disorders, such as autism

Abbreviations: IPG, immobilized pH gradient strips; TFA, trifluoro acetic acid; KNG-1, kininogen-1; HMWK, high molecular weight kininogen; LMWK, low molecular weight kininogen; MET RTK, MET receptor tyrosine kinase; ASD, autism spectrum disorder; PLAUR, urokinase plasminogen activator receptor; MBL, mannan-binding lectin; MASP, mannan-binding lectin-associated serine protease.

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spectrum disorder is comparatively less well-established and only few studies have shown proteomic based research on autism [13–16]. This technique provides an improved method to generate the urinary protein database [17]. The present study compares the urinary proteome of autistic children with that of age- and sex-matched normal children to examine the abnormally excreted protein(s) which could act as urinary markers for autism. Urine is a non-invasive biological sample used for biomarker identification.

2. Materials and methods

2.1. Reagents and chemicals

Immobilized pH gradient strips (IPG) of pH 3–10, 11 cm, sodium dodecyl sulfate (SDS), ammonium persulfate (APS), acrylamide, methylene bis-acrylamide, N, N, N′, N′ tetramethylethylenediamine (TEMED), tris, glycine, urea, thiourea, CHAPS, iodoacetamide, dithiothreitol (DTT), IPG buffer (pH 3–10), 2D clean-up kit, 2D quant kit, protein molecular weight marker, phast gel blue R-350 tablets (Coomassie stain) and other reagents were bought from GE Healthcare Bioscience Limited. Human kininogen ELISA kit is from Assaypro LLC. Other reagents and chemicals used were of analytical grade.

2.2. Subject selection

The study was approved by the Institutional Ethics Committee, Madras Medical College and Hospital, Chennai, Tamil Nadu, India. The study involved autistic children currently attending special schools like Maruti Seva, Bala Vihar, AIKYA, National Institute for Empowerment of Persons with Multiple Disabilities (NIEPMD) and 3 other centers at Chennai, Tamil Nadu, India. Proper consent to take part in the study was obtained from either parent of each child. The schools for special children were approached with the help of the Social Welfare Board, Government of Tamil Nadu and got prior permission to collect the samples.

The institutions used Checklist of Autism in Toddlers to assess autism in children. The study involved children 4–12 years of age of both sex (n = 30) with confirmed autism with the help of a pediatrician. For comparison, samples were also collected from age- and sex-matched normal healthy children (n = 30). Table 1 shows the clinical history of the children involved in the study.

2.3. Collection of urine

About 20 to 30 ml of first void urine sample was collected from each child in sterile disposable containers followed by adding protease inhibitor cocktail in the ratio of 10:1. Samples were filtered through Whatman filter paper for the removal of insoluble materials and cellular debris and centrifuged at 2000 ×*g*, 4 °C for 10 min. The supernatant was filtered through a syringe filter (0.45 μmol/l). The samples were stored at −40 °C until analysis.

Table 1
Clinical history of autistic and normal children involved in the study.

Characteristic	Group I Autistic children	Group II Normal children
Number of children	30	30
CARS value (15–60)	46–60	<10
Male/female ratio	19/11	18/12
Age in years	4–12	4–12
Children with gluten sensitivity	5/30	–
Children with special talents (dancing, humming, drawing, jumble picture)	19/30	22/30
No. of children on antibiotic treatment	9/30	–
No. of children with gastrointestinal problems	8/30	–
No. of parents who had given their cooperation	30	30
No. of parents who appreciated the study	30	30

2.4. Sample processing

2.4.1. Protein precipitation by acetone

An aliquot of urine (15 ml) was added to double the volume of ice-cold acetone (30 ml) and kept overnight at −20 °C. The samples were centrifuged at 12,000 ×*g*, 4 °C for 10 min. The supernatant was removed and the pellet was air-dried. Then, the pellet was re-suspended in 1 ml of sample buffer containing 7 mol/l urea, 2 mol/l thiourea, 4% CHAPS and 50 mmol/l DTT.

2.4.2. Protein precipitation by 2D clean-up kit

The re-suspended samples were processed for protein precipitation as per 2D clean-up kit protocol (GE Healthcare). For each volume of sample, 3 volumes of precipitant were added, mixed well by vortex and incubated at 4–5 °C for 15 min. For each original volume of sample, 3 volumes of co-precipitant were added, mixed and centrifuged at 8000 ×*g* for 10 min. Small pellet was visible and the supernatant was removed carefully without disturbing the pellet and again centrifuged for 1 min for complete removal of supernatant. Then, co-precipitant was added 3–4 times the size of the pellet and centrifuged as before for 5 min. The pellet was dispersed by adding enough amount of distilled water. For 1 ml of pre-chilled wash buffer, 5 μl of wash additive was added, vortexed and kept at −20 °C for 30 min; vortex was done for 20–30 s every 10 min. It was centrifuged again at 8000 ×*g* for 10 min and the supernatant was discarded. Protein pellets obtained were air-dried and then resuspended in 1 ml of sample buffer (7 mol/l urea, 2 mol/l thiourea, 4% CHAPS, 50 mmol/l DTT, 2% ampholytes (IPG buffer pH 3–10)), vortexed and centrifuged for 10 min at 8000 ×*g* to remove insoluble material and any foam. Processed samples were used for single dimension PAGE and 2D-PAGE analyses.

2.5. Protein quantification

The protein concentration was assessed by 2D quant Kit (GE Healthcare) by using BSA as standard and the samples were stored at −40 °C until analysis.

2.6. Single dimension SDS-PAGE

The proteins present in urine samples were precipitated by acetone precipitation, 2D clean-up process and separated by electrophoresis on 10% polyacrylamide gel containing sodium dodecyl sulfate (SDS) according to the method of Laemmli [18] in dual model of vertical slab gel electrophoresis unit — Biotech, Yercaud, India.

2.7. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

2.7.1. Isoelectric focusing (IEF) — first dimension

Approximately, 250 μg of processed protein was dissolved in sample buffer with a drop of bromophenol blue and loaded onto IPG 11 cm, pH 3–10, immobilized pH gradient (IPG) gel strips (GE Healthcare). The IPG strips were passively rehydrated for 16 h, and then actively rehydrated for 3 h at 20 °C; using Ettan IPG Phor 3 isoelectric focusing (IEF) cell (GE Healthcare). The IEF condition was set at 50 V step up for 3 h, 150 V step up for 4 h, 500 V step up for 1 h, 1000 V gradient up for 5 h, 3000 V gradient up for 5 h, 3000 V step up for 2 h, and finally 500 V step up for 1 h. After IEF, the strips were equilibrated in the equilibration buffer I [6 mol/l urea, 0.375 mol/l Tris–HCl pH 8.8, 2% SDS, 20% glycerol, 1% dithiothreitol (DTT)] for 10 min followed by the equilibration buffer II [6 mol/l urea, 0.375 mol/l Tris–HCl pH 8.8, 2% SDS, 20% glycerol, 2.5% iodoacetamide (IA)] for 10 min [19].