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The Differential Urinary Metabolite Profiling of Intellectual Disability Disorders Using Untargeted Metabolomics Approach

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Abstract

Intellectual disability (ID) is a disorder characterized by cognitive delays. Inborn errors of metabolism constitute an important subgroup of ID for which a number of treatments have become available. We aimed to identify potential urine biomarkers of inherited metabolic disorders from the children with intellectual disability using an untargeted Ultra Performance Liquid Chromatography coupled to Mass Spectroscopy (UPLC-MS) based metabolomics approach. All of the patients were having ID, with IQ less than 70 and no recognizable symptoms for the condition. Eleven compounds have been identified that are up regulated in the ID cohort, three of which are previously reported namely Dopamine, Glutarylglycine and Suberic acid. The physiological significance of these markers is discussed. In conclusion, the results of the study indicate that urinary metabolite analysis of ID using sophisticated analytical high throughput techniques may be an important tool to achieve an early diagnosis and therapy of several metabolic defects, and reflect the underlying biology of ID.

Keywords: Mass Spectroscopy; Metabolic diseases; Metabolomics; Urinalysis

Introduction

Metabolomics is a powerful technique used for the comprehensive assessment of global low molecular weight metabolites from a biological sample. It has shown a great potential to facilitate biomarker discovery to differentiate between diseased and non-diseased patients. Metabolite profiling of body fluids has been used for improving the diagnosis, prognosis and therapy of diseases [1]. Metabolites being the end product of cellular processes are the direct outcome of protein and enzymatic activity. Hence, their proximity is more towards phenotype or disease than genetic or proteomic information. It can be referred as a complete catalogue of the human metabolome [2].

ID refers to a group of developmental conditions characterized by significant impairment of cognitive functions, which are associated with limitations of learning, adaptive behaviour and skills [3]. It affects 1 to 3 percent of general population [4]. It is a complex, variable and heterogeneous disorder. The aetiology is multiple and diagnosis poses a formidable challenge worldwide. Metabolic conditions comprise an important subgroup of ID [5]. Inborn errors of metabolism appear to be a rare

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cause of developmental delay or intellectual disability. The percentage of patients with identifiable metabolic disorders as cause of the ID ranges from 1 to 5 percent in published reports [6]. In a systematic literature review, it has been reported a total of 81 ‘treatable inborn errors of metabolism’ presenting with intellectual disability as a major feature, including disorders of amino acids, cholesterol and bile acid, creatine, fatty aldehydes; glucose homeostasis and transport; hyperhomocysteinemia; lysosomes, metals, mitochondria, neurotransmission; organic acids, peroxisomes, pyrimidines, urea cycle, and vitamins/co-factors [7].

Global metabolomics is based on non-biased quantitative analysis of metabolites in biological samples obtained from healthy (control) and diseased individuals with the aim to identify difference in the endogenous metabolites [8]. Untargeted metabolomics approach aims at measuring simultaneously as many metabolites as possible in a biological sample providing long range of compounds to identify potential biomarkers. Global metabolite profile of urine samples captures the global changes and overall physiological status in biochemical networks and pathways that can be a promising means to identify biomarkers of diseases. Biomarker metabolites can be therapeutic targets [9]. Urine provides a pattern of polar metabolites excreted from the body due to catabolic processes. It is a useful medium for disease diagnosis since it poses various advantages that include non-invasive sample collection, ease of sample repetition and not volume-limited. Urine analysis has easier sample preparation due to lower sample complexity because of low protein and peptide levels. Being a waste product, urine reflects metabolic disregulation and provides insights to changes in response to disease processes [10].

UPLC-MS is an emerging high throughput powerful tool in the field of metabolomics of biological fluids. It uses smaller particles, speed and peak capacity that results in increase in the resolution, sensitivity and speed of analysis of hundreds of metabolites in a single run [11]. There are no reports for a valid methodology for the detection of urinary markers from ID children for the detection of inborn errors of metabolism that may help in the treatment of the associated metabolic disorders [12-25].

Objectives of the Study

The aim of the present work was to develop an untargeted UPLC-MS based metabolomics approach with multivariate analysis, to screen and identify urine metabolic changes in intellectually disabled children. The focus of the study was on the global metabolite profiling method optimization and validation to identify relevant and putative biomarkers.

Materials and Methods

Chemicals and reagents

HPLC grade Acetonitrile and Formic acid were obtained from Sigma-Aldrich Co., St. Louis, MO, USA. Water was produced by a TKA Smart2Pure Ultra-pure water system (TKA, Germany). All other chemicals used were of HPLC grade.

Subjects and consent

The details of the study were explained to the parents or guardians and school representatives of the participating children, and written informed consent was obtained. Patient samples were collected from Kamayani School for Mentally Challenged, Pune, Maharashtra. Ten participants with ID (n=10; male: 5, female: 5) and control subjects (n=10, male: 5, female: 5) were

recruited in the study. Inclusion criterion for the patients was age (9-13 years) with IQ score below 70 and deficit in adaptive functioning that define them as ID. Clinical data was obtained from all the probands including patient's name, age, gender, IQ score, epilepsy and any other known neuropsychiatric disorders. Under the guidance of a clinician, children without any recognizable syndromes were included in the study as the non-syndromic cohort. Children were not taking any type of medication which can alter the metabolites secreted in the urine. Control subjects included in the study were from a school of the same geographical area not undergoing any special diet or medication. To avoid influence of ageing and gender on metabolic profiles, subjects in the healthy control cohort were matched to corresponding subjects in the ID cohort.

Preparation of urine samples

Random urine samples were collected in sterile urine containers. Sodium azide (0.05% w/v) was added to prevent bacterial contamination. The samples were centrifuged at 10000 rpm for 10 min at 4°C to remove any particulates. The supernatant was transferred to a 2 mL polypropylene tube followed by 1:4 dilution with water and 3 µl of this processed sample was transferred to appropriate LC vials for further analysis.

UPLC-MS conditions

A 1290 Infinity UHPLC System, 1260 infinity Nano HPLC with Chipcube, 6550 iFunnel Q-TOF (Agilent technologies, USA) was used. Details of the settings for optimized instrument operation and parameters of the protocol are given in TABLE 1.

Data processing and analysis

Data was collected using Agilent Mass Hunter Workstation Data Acquisition software and processed by Agilent MassHunter Qualitative Analysis software (Agilent Technologies, CA, USA). All the raw data .D files were converted to compound exchange format file (.CEF) and were imported to the Mass Profiler Professional (MPP) software (Agilent Technologies, CA, USA) for analysis. Data mining was done using the Molecular Feature Extractor (MFE) algorithm in the MassHunter workstation software. Experimental grouping was done by segregation of patients into two groups according to the parameter healthy (control) versus patients (diseased) respectively. The first filter (abundance filtering) was set at minimum absolute abundance at 5000 counts to reject low-intensity data. All the entities that presented with less than this abundance were considered as noise. The second filter of frequency selected entities that present in at least 50% of all samples. Principal component analysis (PCA) was the algorithm used to detect major trends in the data and a sample quality control check by examining the values of the entities. Un-paired t-test with Benjamini-Hochberg multiple testing correction was used for statistical analysis to filter the entities based on the significant differences in the abundance of compounds between the two groups. Potential metabolite markers were extracted using a statistically significant threshold of fold change values larger than 2.0 and p-values less than 0.05. METLIN Personal Metabolite Database was used for the identification of endogenous and exogenous metabolites. The structure and functional information was then obtained by searching freely accessible database of HMDB (The Human Metabolomic Database), KEGG (Kyoto Encyclopaedia of Genes and Genomics) and HMP (Human Microbiome Project) database.

TABLE 1. **UPLC-MS conditions for global urine metabolite profiling.**

UPLC-MS conditions		
LC Column	Agilent C18 100/150 mm × 2.1 mm 1.7 µm	
Ionisation	Positive ion Electrospray ionisation (ESI)	
Injection volume	3 µl	
Flow rate	0.300 mL/min	
Mobile phases:		
A: 0.1% formic acid in water		
B: 90% acetonitrile, 10% water and 0.1% formic acid.		
LC Gradient program:		
Time (min)	**A (%)**	**B (%)**
2	95	5
20	5	95
26	95	5
30	95	5
MS Conditions:		
Desolvation gas	Nitrogen	
Sheath gas flow	11 L/min	
Gas temperature	250°C	
Gas flow rate	13 L/min	
Nebulizer pressure	35 psi	
Capillary voltage	3500 V	
Nozzle voltage	1000 V	

Results

Twenty subjects, ten patients and ten controls successfully completed the study. Multiple classes of metabolites were successfully detected and identified including peptides, lipids and small molecules. To investigate differences in the metabolic expression signals between healthy volunteers and patients, PCA was first conducted on the datasets under positive ESI ion mode. Score plot obtained from the PCA was used for the unsupervised pattern analysis of all the urine samples (FIG. 1). In the score plot clustering was not clear and PCA did not show any apparent separation between the patients and healthy controls. However, metabolic profiles of some ID group patients significantly changed indicating that there was significant biochemical perturbation. Potential metabolite markers were extracted using a statistically significant threshold of fold values larger than 2.0 and p-values less than 0.05 (unpaired t-test) constructed from the PCA.

Metabolic analysis revealed a total number of 101 metabolites that were differently expressed between the healthy controls and ID patients. Further MPP analysis revealed that among the identified metabolites, eleven metabolites were significantly

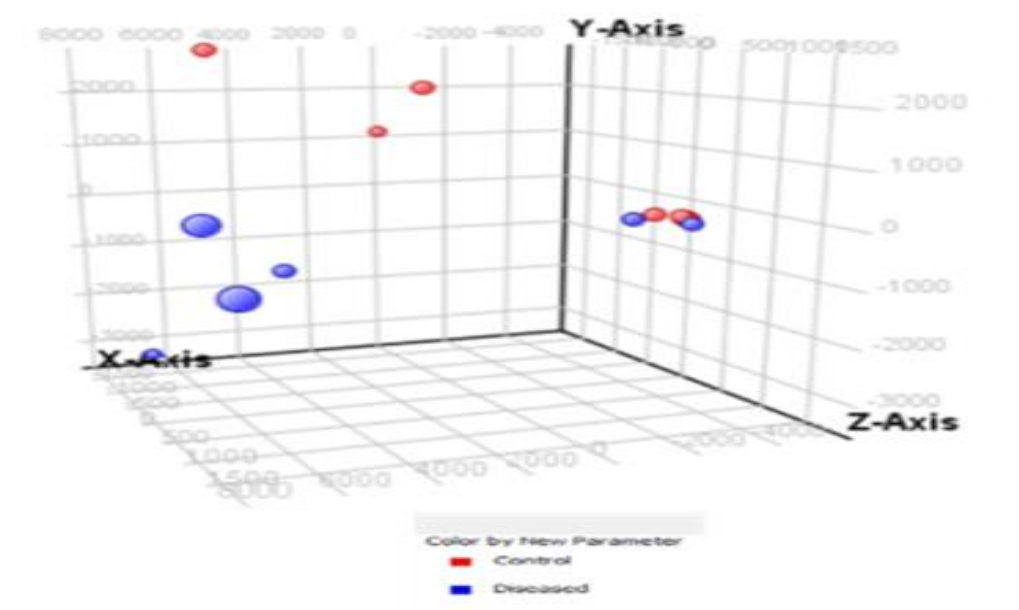

FIG. 1. **3D PCA score plot of urinary metabolite profile segregating healthy and ID cohort in a population.**

different in their expression levels. Summary of the metabolites and their regulation is shown in TABLE 2. UPLC-MS platform could provide the compound name, accurate mass, retention time, CAS number, annotations and regulation information on the identification of these potential biomarkers.

TABLE 2. **Metabolites that were differentially expressed in the healthy and patient cohort.**

Sr. no.	Compound	Regulation	Annotations	Mass	Retention time
1.	1-Methyl-5-imidazoleacetic acid	Up regulation	C6 H8 N2 O2, CAS ID=4200-48-0, KEGG ID=, METLIN ID=3776	140.058	3.97887
2.	6-Hydroxydopamine	Up regulation	C8 H11 N O3, CAS ID=1199-18-4, KEGG ID=, HMP ID=HMDB01537, METLIN ID=6307	169.074	5.24107
3.	Dopamine	Up regulation	C8 H11 N O2, CAS ID=51-61-6, KEGG ID=C03758, HMP ID=HMDB00073, METLIN ID=64	153.078	8.08946
4.	Erythrono-1,4-lactone	Up regulation	C4 H6 O4, CAS ID=17675-99-9, KEGG ID=, HMP ID=HMDB00349, METLIN ID=5338	118.027	5.53193
5.	Glutarylglycine	Up regulation	C7 H11 N O5, CAS ID=17686-38-3, KEGG ID=, HMP ID=HMDB00590, METLIN ID=5569	189.064	5.09047
6.	Malonylcarnitine	Up regulation	C10 H17 N O6, CAS ID=853728-01-5, KEGG ID=, HMP ID=HMDB02095	247.106	4.7218

7.	O-Acetylserine	Up regulation	C13 H21 N2 O7 P S, CAS ID=5147-00-2, KEGG ID=, HMP ID=HMDB03011, METLIN ID=6809	380.081	5.11285
8.	Orinapterin	Up regulation	C9 H11 N5 O3, CAS ID=13039-82-2, KEGG ID=, HMP ID=HMDB00817, METLIN ID=5781	237.087	5.89467
9.	Phosphocreatine	Up regulation	C4 H10 N3 O5 P, CAS ID=67-07-2, KEGG ID=, METLIN ID=326	211.036	4.84671
10.	Suberic acid	Up regulation	C8 H14 O4, CAS ID=505-48-6, KEGG ID=C08278, HMP ID=HMDB00893, METLIN ID=4243	174.089	9.69879
11.	Succinylacetone	Up regulation	C7 H10 O4, CAS ID=51568-18-4, KEGG ID=, HMP ID=HMDB00635, METLIN ID=5608	158.058	3.00343

Among these eleven differentially expressed metabolites, three of them are reported earlier to be associated with inborn errors of metabolism along with intellectual disability disorders. The list of these metabolites along with their functions is given in TABLE 3.

TABLE 3. **Metabolites reported to be elevated in the urine associated with different inborn errors of metabolism along with intellectual disability.**

Sr. no.	**Compound name**	**Description**	**Reference**
1.	Dopamine	Aromatic L-amino acid decarboxylase (AADC) deficiency: rare inborn error of neurotransmitter biosynthesis associated with hyperdopaminuria characterized by global developmental delay.	[22], [12], [23]
2.	Glutarylglycine	Glutaric acidemia Type 1: autosomal recessive inborn error of lysine, hydroxylysine, and tryptophan metabolism. It is reported with urinary elevated levels of glutarylglycine and delayed treatment leading to mental retardation.	[24], [16], [17]

3.	Suberic acid	Medium-chain acyl-CoA dehydrogenase deficiency (MCADD): autosomal recessive hereditary metabolic disorder of mitochondrial fatty acid β-oxidation classified as a treatable disorder with major preventable outcome of intellectual disability.	[19], [25]

Discussion

Eleven metabolites were found to be up regulated in the ID patients. These compounds are identified in normal human urine samples. Five of these are not associated with any specific disease. 1-Methyl-5-imidazoleacetic acid is a known metabolite of Histamine. Erythrono-1,4-lactone has been identified as a lactone of tetronic acid in the urine from normal adults and neonates. O-Acetylserine is an acylated amino acid derivative that arises from either microbial metabolism in the gut or through consumption of foods containing O-Acetylserine. Orinapterin is a threo diastereomer of biopterin found in human urine. 6-Hydroxydopamine is a naturally occurring amine in human urine.

Phosphocreatine is a phosphorylated creatine molecule that serves as a rapidly mobilizable reserve of high-energy phosphates in skeletal muscle and the brain. It is reported with Eosinophilic esophagitis when elevated in the urine. Succinylacetone, a tyrosine metabolite is a specific marker for Tyrosinemia type I which is an inherited metabolism disorder. However its primary effects are liver and kidney dysfunction and is not associated with intellectual disability disorders. Malonylcarnitine is a metabolite that is found to be accumulated in the blood in malonyl-CoA decarboxylase (EC 4.1.1.9) deficiency (OMIM 248360) which typically appears with developmental delay. It is been reported in the human urine, however the effect of its elevated levels are not reported (HMDB). Total three compounds namely, dopamine, glutarylglycine and suberic acid are already reported to be linked to developmental delay.

Dopamine is a member of the catecholamine family in the brain, and is a precursor to epinephrine and norepinephrine. Dopamine is synthesized in the body first by hydration of the amino acid tyrosine DOPA by tyrosine hydroxyalse followed by its decarboxylation by aromatic-L-amino-acid decarboxylase. (PubChem CID: 681) In Aromatic L-amino acid decarboxylase (AADC) deficiency (OMIM 608643), in spite of deficiency of dopamine the patients are found with increased levels of dopamine in their urine and characterised by mental retardation. The reason behind this is explained by Wassenberg et al. [12] being the combination of residual renal AADC-activity and pathologic increase of L-dopa substrate availability in AADC-deficient patients. Coexistence of cognitive deficits and neuropathology indicates that defective neurotransmission in the central nervous system may lead to intellectual disability disorders [13].

Glutarylglycine is an acyl glycine (PubChem CID: 23592950). Acyl glycines are normal intermediates of amino acid and fatty acid metabolism. However, in elevated concentrations they are biochemical markers in several inborn errors of metabolism. Glutarylglycine is involved in lysine metabolism (HMDB). Glutaric acidemia type 1 (OMIM 231670) is an inherited disorder in which body is unable to break down the amino acids lysine, hydroxylysine and tryptophan caused by deficiency of mitochondrial glutaryl-CoA dehydrogenase. Excessive levels of their intermediate breakdown products

(Glutaric acid, hydroxyglutaric acid, glutaconic acid and glutarylglycine) accumulate and cause damage to the brain, particularly basal ganglia causing irreversible neurological damage. These intermediates are found elevated in the urine presenting as potential biomarkers for the identification of the condition. Delayed treatment worsens the condition of the patient resulting in significant mental retardation.

Suberic acid, also octanedioic acid, is a dicarboxylic acid (PubChem CID: 10457). It is a metabolic breakdown product derived from oleic acid. Suberic acid is detected in the urine of patients with fatty acid mitochondrial disorders [18]. Substantial excretion of suberic acid has been reported in the urinary analysis of patients with medium-chain acyl-CoA dehydrogenase deficiency [19]. Medium-chain Acyl-CoA dehydrogenase (EC 1.3.8.7) is one of the enzymes involved in mitochondrial fatty acid oxidation, which fuels hepatic ketogenesis. Deficiency of the enzyme causes incomplete beta-oxidation resulting in increased levels of suberic acid in the urine. Medium-Chain Acyl-CoA Dehydrogenase Deficiency (MCADD; OMIM 607008) is the most common inherited disorder of mitochondrial fatty acid b-oxidation in humans. Various developmental disabilities are found associated with MCADD including intellectual disability, cerebral palsy and autism spectrum disorders.

Literature suggests variable diagnostic yield of metabolic testing ranging from 0.8 to 13 percent. However, inborn errors of metabolism represent largest group of genetic defects associated with ID amenable to causal therapy [20]. Without early detection, at least 50 percent of the children will experience metabolic crisis and up to 10 percent of the survivors will develop a serious developmental disability. There are reports for method validation for metabolic profiling analysis of urine samples using UPLC–TOFMS [21-25]. Although there are no reports of global urine metabolite profiling using UPLCMS technique to identify inborn errors of metabolism associated with intellectual disability.

Conclusion

Our findings indicate that urinary metabolites may allow the segregation of ID patients from normal individuals and may reflect the underlying biology of ID, which is still largely unknown. Further validation of the methodology and parameters on a larger population may provide novel instincts into the etiology of ID and help in the identification of urinary metabolites as biomarkers by a non-invasive technique.

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