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Newborn Screening for X-linked Adrenoleukodystrophy: Further evidence high throughput screening is feasible

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Abstract

X-linked adrenoleukodystrophy (ALD) is characterized by adrenal insufficiency and neurologic involvement with onset at variable ages. Plasma very long chain fatty acids are elevated in ALD; even in asymptomatic patients. We demonstrated previously that liquid chromatography tandem mass spectrometry measuring C26:0 lysophosphatidylcholine reliably identifies affected males.

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We prospectively applied this method to 4689 newborn blood spot samples; no false positives were observed. We show that high throughput neonatal screening for ALD is methodologically feasible.

Keywords

Newborn screening; Adrenoleukodystrophy; Adrenomyeloneuropathy; Adrenal insufficiency; Peroxisomal disorders; Tandem mass spectrometry

1. Introduction

Adrenoleukodystrophy (ALD) result from mutations in *ABCD1* which encodes an ATPase binding cassette protein that localizes to the peroxisome membrane and is involved in the beta-oxidation of saturated very long chain fatty acids (VLCFA) [1]. Other peroxisomal disorders are less common and are due to other gene mutations leading to peroxisome biogenesis disorders or VLCFA single enzyme defects. They have separate phenotypes and typically present in infancy. While dysfunction of peroxisomal beta-oxidation results in accumulation of VLCFA in all tissues, clinically ALD affects the nervous system and the adrenal glands with onset of symptoms at variable ages [1].

Primary adrenal insufficiency occurs in 90% of males affected by ALD (which is an X-linked recessively inherited disease). Biochemical signs of adrenal insufficiency maybe detected in boys screened because of family history as early as in the first months of life [2]. Adrenal insufficiency may present as adrenal crisis with potentially catastrophic presentation [3-7] or lead to a number of hospitalizations before a diagnosis is made [8]. Adrenal insufficiency is effectively treated with maintenance and stress dose replacement steroids. The neurologic presentation of ALD is variable. Approximately 35% of at risk males develop an inflammatory demyelination in childhood (typically at 4-8 years of age) and progress to a vegetative state within 1-2 years; others develop myelopathy later in life [1]. In cerebral ALD, MRI changes precede neurological symptoms and hematopoietic cell transplantation is an effective therapy if performed early [9-13]; individuals monitored by MRI show best transplant outcomes [14]; after onset of neurological symptoms patients do not fare well.

Given this potential to intervene at an asymptomatic stage, identification of at-risk individuals early in life is desirable. We have advocated extended family screening, but at risk individuals may be missed and new mutations occur in 5-7% of identified cases [15;16]. As individuals with ALD are asymptomatic at birth but are biochemically and genetically identifiable, addition of ALD biochemical testing to newborn screening panels should be considered. As an essential step towards ALD newborn screening, we aim to establish and validate the necessary laboratory methodology. A method using standard newborn blood spots (NDBS) and high throughput tandem mass spectrometry (MS/MS) already used by most newborn laboratories is most desirable. We introduced liquid chromatography MS/MS (LC-MS/MS) measurement of C26:0 lysophosphatidylcholine (26:0-lyso-PC) isolated from NDBS [17]. This method accurately diagnosed affected individuals from venous blood spotted on newborn cards as well as retrieved NDBS samples of known affected children [18]. We now prospectively applied this method to 4689 NDBS samples collected over a 21 months period.

2. Subjects and Methods

2.1. Subjects

Newborns were enrolled at three Maryland hospitals: Johns Hopkins Hospital, Greater Baltimore Medical Center and Frederick Memorial Hospital. Permission to perform ALD screening on an unused portion of the NDBS was requested through a separate consent. Families were informed that in the event of a positive determination they would be contacted by the Newborn Screening Program for confirmation, further testing and counseling provided by the genetics staff of the Maryland State Newborn Screening Program and the Kennedy Krieger Institute. The study was approved by the Investigational Review Boards of participating hospitals and the Maryland Department of Health and Mental Hygiene.

2.2. Measurement of 26:0 lysophosphatidylcholine

If completion of routine screening allowed, at least one 1/8 inch punch of the NDBS of consented patients was used for analysis. High-purity grade HPLC solvents were used (J.T.Baker Co or Burdick & Jackson, Inc.). $^2\text{H}_4$ -26:0 lysophosphatidylcholine ($^2\text{H}_4$ -26:0-lyso-PC - internal standard) and 26:0-lyso-PC and 20:0-lyso-PC (reference standard) from Avanti Polar Lipids, Inc.. Lyso-PCs were extracted from the 1/8 inch punched NDBS with 150 μl of methanol solution containing 15 pmoles of the internal standard on a 96 well plate (using batched samples). Blood spots from 2 controls, 2 male patients with X-ALD, 2 patients with peroxisome biogenesis defects and 2 filter paper blanks were assayed with each sample set. Single analysis for screening samples; control samples were done in duplicates with each run and confirmed that run-to-run variability was low. Standard curves of blood spots with known amounts of 26:0-lyso-PC were done with new sets of reagents. Mixing and incubation for 25 min at room temperature were followed by centrifugation at 800G. The 150 μl supernatant was transferred to a 96-well plate; 5 μl were injected for combined LC-MS/MS analysis on an AB Sciex API4000 triple quadrupole mass spectrometer with a Waters UPLC using a Waters X-Terra C8 column (1.0 $\times$ 50 mm, 3.5m) for chromatographic resolution of the analytes and internal standard extracted from the sample matrix. An isocratic 1:1 mixture of mobile phase A comprised of $\text{H}_2\text{O}:\text{CH}_3\text{CN}:\text{HCOOH}$ (54.5:45:0.5) and mobile phase B comprised of $\text{CHCl}_3:\text{CH}_3\text{CN}:\text{HCOOH}$ (10:89.5:0.5), both containing 2mM NH_4HCOOH , was used for elution at flow rate 0.18ml/min; LC MS/MS analysis time was 2 minutes per sample. The MRM transitions monitored were m/z 552 to 104, m/z 636 to m/z 104 and m/z 640 to m/z 104 for 20:0-lyso-PC, 26:0-lyso-PC and $^2\text{H}_4$ -26:0- lyso-PC respectively. Quantitation of 20:0-lyso-PC and 26:0- lyso-PC was performed by analyzing peak areas of the target analyte compared with the area of the internal standard. Data from the calibration curves for 20:0-lyso-PC and 26:0-lyso-PC show a linear response ($R^2= 0.99$) in dynamic range of 0.1-20 pmoles per 1/8 inch NDBS.

3. Results

Between November 26, 2008 and August 27, 2010, a total of 5000 newborns were enrolled: Greater Baltimore Medical Center: 1503 males (M), 1416 females (F); Frederick Memorial Hospital 586 M and 497 F, Johns Hopkins Hospital 519 M, 479 F. We were able to retrieve and analyze 4689 newborn samples, 293 were labeled as insufficient to provide a punch for this study. 18 others were not retrieved, reason unspecified. We determined the 26:0-lyso-PC and the ratios of 26:0-lyso-PC to 20:0-lyso-PC for the 4689 infants (Table 1). All study measurements were within previously established normal ranges for NDBS (18). There was no overlap between the ranges of values measured for the screening samples (normal levels) and XALD samples.

4. Discussion

This report provides further evidence that our previously described LC-MS/MS method [17,18] is amenable to high throughput screening for ALD. Expanding sample size to nearly 5000, we did not encounter any positives.

The specificity remains at 1.0. A high false positive rate of a screening test can pose a major burden on the newborn screening laboratory and clinicians/families. As the incidence of ALD is estimated at 1:17,000, we did not necessarily expect to have true positive during this proof of principle study. A sample size of 5000 was chosen as sufficient to explore feasibility of high throughput analysis in a larger sample size while realistic in relation to planned study duration, yearly birth numbers at the participating hospitals and funding support.

There were no special requirements to obtain, process, or store NDBS samples for ALD screening as the 26:0-lyso-PC is chemically quite stable. With an analysis time of only 2 minutes we estimate that a laboratory with automated equipment and one suitable LC MS/MS would be able to analyze as many as 600 samples per day; of course, this number would vary depending on availability of laboratory staff as well as the number, access times and specification(s) of the instrument(s) used.

A main issue in moving towards universal newborn screening for ALD will be the establishments of protocols for follow-up and counseling of positives screens. For any positive, screening should be duplicated and verified with a second tier test such as expanded lyso-PC fatty acid analysis [18]. Mutation analysis of the ABCD1 gene should be done [15,16]. If either of the additional analyses are inconsistent with the diagnosis of XALD, review of the case by a qualified specialist is needed. If the positive result is confirmed, families should be contacted for follow-up which should provide diagnostic and medical evaluation, referral to all needed specialists and counseling.

For male positives, subsequent evaluation will focus on exclusion of non-ALD peroxisomal disorders and initiation of appropriate health surveillance due to the risk of adrenal and cerebral ALD [19]. For positive screens in females, follow-up testing will aim to differentiate peroxisomal biogenesis disorders and ALD carrier status in these patients, followed by appropriate investigations and counseling.

The primary intent of ALD newborn screening is the detection of affected males rather than detection of non-ALD peroxisomal disorders or female heterozygotes. We expect that only a portion of heterozygotes will be identified by newborn screening due to variation in 26:0-lyso-PC levels. ALD is not only by far the most common peroxisomal disorder, but also treatable, a main criteria for the inclusion of testing for this condition in a screening program. As a quality measure, screening programs will need to ascertain if any patients diagnosed with ALD later in life escaped detection by newborn screening (false negatives).

Due to the complexities of peroxisomal disorders (different disorders and phenotypes, changes in therapies and the multifaceted effects of the diagnosis on families), the introduction of ALD screening programs will require the guidance of biochemical geneticists, other involved specialists, genetic counselors, ethicists and policy makers in regards to feasibility, protocols, risks and benefits. A stepwise introduction of screening for extended populations utilizing further study protocols which track, monitor and review all aspects of the screening is advisable. Dissemination of information about XALD and ALD screening, the formation of relevant task forces and expert panels to publish consensus statements and develop XALD practice guidelines will be an essential part in the

establishment of XALD screening and will ensure the highest standard of care for patients and their families.

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Abbreviations

AMN	adrenomyeloneuropathy
LC MS/MS	liquid chromatography tandem mass spectrometry
Lyso-PC	lysophosphatidylcholine
MRI	Magnetic resonance imaging
NDBS	newborn dried blood spots
ALD	adrenoleukodystrophy

Highlights

- Newborn screening for X-linked Adrenoleukodystrophy identifies males at risk.
- We reliably measured 26:0 lysophosphatidylcholine in 4689 newborn blood spots.
- We establish that high throughput screening for Adrenoleukodystrophy is feasible.

Table 1

26:0-lyso-PC and 26:0-lyso-PC/20:0-lyso-PC ratio for study newborn bloodspots and newborn bloodspots from known ALD patients (non-study samples for comparison). Values for 26:0-lyso-PC expressed as pmoles per 1/8 inch bloodspot (Mean $\pm$ SD). Statistical differences of study population versus X-linked ALD newborn samples are calculated using Student's t-test with unequal variances.

Sample Type	26:0-lyso-PC	Range	N	Significance
Study population: Newborn Bloodspots	0.36 $\pm$ 0.14	0.08-1.18	4689	
XALD Newborn Bloodspots* (Non-study samples)	5.12 $\pm$ 0.91	3.80-7.37	16	P<0.001
	26:0/20:0-lyso-PC ratio	Range	N	
Study population: Newborn Bloodspots	0.62 $\pm$ 0.27	0.11-2.14	4689	
XALD Newborn Bloodspots* (Non-study samples)	7.27 $\pm$ 1.85	4.60-11.16	16	P<0.001

* Data partially published previously [18].