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Newborn Screening for Mucopolysaccharidoses: Results of a Pilot Study with 100000 Dried Blood Spots

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Abstract

Objective—To test, in a newborn screening (NBS) laboratory, the performance of liquid chromatography-tandem mass spectrometry (LC-MS/MS) to assay 5 enzymatic activities in dried blood spots (DBS) for NBS of 5 lysosomal storage diseases (mucopolysaccharidosis [MPS]-II, MPS-IIIB, MPS-IVA, MPS-VI, and MPS-VII).

Study design—Three mm punches from de-identified DBS were obtained from the Washington NBS laboratory and submitted to the 5-plex LC-MS/MS assay. Screen cut-offs were established by analyzing the enzymatic activity in patients confirmed to have the MPS disorder. DNA sequencing of the relevant gene was performed on a second DBS punch for all samples with enzyme activity below 10% of the mean daily activity.

Results—(1) For MPS-II, 18 below cut-off samples, 1 pathogenic genotype, and 2 “high risk” genotypes; (2) For MPS-IIIB, no below cut-off samples; (3) For MPS-IVA, (4) For MPS-VI, 4 below cut-off samples and no high-risk genotypes; (5) MPS-VII, 1 below cut-off sample confirmed by genotype and clinical report to be affected.

Conclusions—These results establish that the number of initial screen positive samples is low and manageable. Thus, population newborn screening for these conditions is feasible in a state newborn screening laboratory.

The clinical availability of recombinant enzymes for the treatment of lysosomal disorders has changed the natural history of these diseases. Enzyme replacement therapies have been shown to improve the natural history of the disorders.^{1,2} The therapy is most effective if administered early in the course of the disease, prior to organ damage. Unfortunately, the majority of affected children are not clinically recognized until organ damage has occurred.

To overcome the delay in diagnosis for treatable conditions, we have developed an assay for the simultaneous measurement of a set of mucopolysaccharidoses (MPS). The assay is based on the use of tandem mass spectrometry (MS/MS), which has emerged as a powerful analytical platform to quantify many analytes in dried blood spots (DBS) on newborn

screening cards in a single multiplex assay.^{3–5} MS/MS is also the leading technology in the US for newborn screening of MPS-I and Pompe diseases, lysosomal storage diseases that are now part of the Recommended Uniform Screening Panel in the US.⁶ The current study is focused on MPS-II, MPS-IVA, MPS-VI, and MPS-VII. Each of these disorders is treatable by Food and Drug Administration-approved recombinant enzymes. We have also included MPS-IIIB because efforts are underway to develop an approved therapy. MPS-I is not included in our current study because it was part of an earlier pilot study.⁷ We also did not include MPS-IIIA because at the beginning of this pilot study, we did not have a functional assay for the relevant enzyme. However, this problem has recently been solved, and efforts are underway to include this disorder in future pilot studies.⁸

We now report the results of a pilot study of MPS-II, MPS-IIIB, MPS-IVA, MPS-VI, and MPS-VII using ~100000 3-mm newborn screening (NBS) from the Washington State Newborn Screening Laboratory. We show that it is feasible to add a mixture of 5 substrates to a single buffer solution containing a single 3-mm punch from a DBS. After incubation and a simple sample processing protocol, the mixture is analyzed by combined liquid chromatography/mass spectrometry (LC-MS/MS) similar to our prior assay.³ All 5 products and internal standards are quantified, which allows determination of the specific activity of each enzyme (uMoles/hour/L). We established cut-off values for each enzyme, and for all samples with below-cut-off enzymatic activities, a second 3-mm DBS punch was submitted to whole genome amplification and Sanger sequencing of the relevant gene. We studied the feasibility of incorporating screening for these disorders in the newborn period.

Methods

The pilot study using de-identified DBS was approved by the Washington State Institutional Review Board. Over 100000 anonymous samples were screened for MPS-II, MPS-IIIB, MPS-IVA, MPS-VI, and MPS-VII. DBS were provided by the Washington State NBS laboratory after being stored for 30–60 days at ambient room temperature (~20°C).

The detailed procedure for performing the multiplex enzyme assay is given in the Appendix (available at www.jpeds.com). Here we give a brief description. A 3-mm punch of DBS was used for a single incubation in buffer containing 5 substrates and internal standards. After an overnight incubation to allow product formation, the reactions were quenched with methanol/ethyl acetate. Aqueous NaCl and ethyl acetate was added for liquid-liquid extraction. A portion of the ethyl acetate layer containing enzymatic products and internal standards was dried, and the residue resuspended in solvent for auto-injection for LC-MS/MS analysis. Product and internal standard peaks were detected by multiple reaction monitoring and used to obtain the enzymatic activity using the following formula:

Activity (uMoles/hour/L) = (product intensity/internal standard intensity) (uMoles of internal standard in assay)/incubation time (hour)/volume of blood (L).

Product and internal standard intensities are the multiple reaction monitoring MS/MS peak areas, determined using the QuanLynx software package from Waters Corporation, Milford,

MA. The volume of blood taken was 3.2 μL (from the 3-mm DBS punch). We assumed a relative response factor for the product and internal standards MS/MS signals of unity.

A second 3-mm DBS punch from each newborn was retained for DNA sequencing of all newborn samples that gave an enzymatic activity below the screen cut-off for each MPS disorder. Sanger DNA sequencing of all gene exons and intron/exon boundaries and sequence annotation were carried out as previously described.⁷

Results

Approximately 106 000 blood spots obtained from newborns were assayed for the enzymes responsible for MPS-II, MPS-IIIB, MPS-IVA, MPS-VI, and MPS-VII (iduronate-2-sulfatase, N-acetyl-alpha-D-glucosaminidase, galactosamine-6-sulfatase, arylsulfatase B, and beta-glucuronidase, respectively). This was performed with a single incubation in a 96-well plate with 5 substrates and 5 internal standards followed by a single LC-MS/MS run (5-plex). During method development, a cut-off value was established of less than 10% of the daily mean enzyme activity. The cut-off was chosen based on the measurement of the enzymatic activities in patients clinically diagnosed with the MPS disorder. Supplementary Table IV gives the patient enzymatic activities. In the Supplementary Figure, we provide the population distribution of the enzymatic activities along with the means and SDs. Assay precision data is provided in Supplementary Table IV along with the ratio of the average newborn assay response to the response for filter paper controls (no blood punch).

For this study, any sample with an enzymatic activity less than 10% of the daily mean (except as noted below) was subjected to DNA isolation from a residual 3-mm DBS punch and sequencing of the relevant gene.

MPS-II (Hunter Syndrome)

For 105 214 samples, the mean activity was $\mu\text{mol}/\text{hour}/\text{L}$ blood. There was a total of 18 samples with enzyme activity below 10%, with 7 of those samples having activity less than 5% of the daily mean (Table I). None of the 11 samples between 5% and 10% of the daily mean had a pathogenic genotype, with the majority having 1 of 2 previously reported pseudo-deficiency alleles; either *p.P284L* or *p.T500I*.⁹ Of the 7 samples with activity less than 5% of the daily mean, 1 was considered to be affected and 2 were “at risk” of developing clinical symptoms. The sample with the lowest enzyme activity ($-0.16 \mu\text{mol}/\text{hour}/\text{L}$ blood) had an amino acid variant of *p.R273W*. This genotype has previously been reported as being responsible for the Hunter phenotype.⁹ Two samples with activity less than 5% of the daily mean had been considered to place the infant “at risk” of developing clinical symptoms based on in-silico predictions only. These amino acid variants were *p.C53W* and *p.S470L* (Table I). Because MPS-II is X-linked, we estimate there were approximately 53 000 male samples from the total of 105 214 newborns samples analyzed for activity. These findings implicate a clinical frequency between 1 in 53 000 to 1 in 18000 male samples.

MPS-IIIB (Sanfilippo Syndrome Type B)

For the 103 001 samples screened, we found a mean enzymatic activity of $2.92 \mu\text{mol}/\text{hour}/\text{L}$ blood. None were found to have enzyme activity less than 10% of the daily mean.

-IVA (Morquio Syndrome)

For the 106106 samples screened for MPS-IVA, the mean enzymatic activity was 2.10 $\mu\text{mol}/\text{hour}/\text{L}$ blood. Eight samples had enzymatic activities below 10% of the daily mean, with none falling below 5% of the daily mean (Table II). One sample was heterozygous for *p.F167V*, a previously reported pathogenic variant. An additional sample was homozygous for *p.A231G*. This later variant has a population frequency of 0.05 and is not considered pathogenic. Four of the samples with activity below 10% of the daily mean gave poor DNA amplification, and genotypes could not be obtained.

MPS-VI (Maroteaux-Lamy Syndrome)

For the 103 259 samples screened, we found a mean enzymatic activity of 14.63 $\mu\text{mol}/\text{hour}/\text{L}$ blood. Four samples had activity below 10% of the daily mean, and one of those samples was less than 5% of the daily mean (Table II). None of the 3 samples above 5% of the daily mean had nucleotide variants that were considered pathogenic. The single sample less than 5% of the daily mean had enzyme activity of 0.32 $\mu\text{mol}/\text{hour}/\text{L}$ blood and the variants *p.V358M* and *p.V376M*. These variants are common in the population (0.20–0.50) and are not pathogenic.

MPS-VII (Sly Syndrome)

For the 94 931 samples screened for MPS-VII, we found a mean enzymatic activity of 43.10 $\mu\text{mol}/\text{hour}/\text{L}$ blood. A single sample had enzymatic activity less than 10% of the daily mean with an activity of 0.11 $\mu\text{mol}/\text{hour}/\text{L}$ blood (<5% of the daily mean Table II). Genotyping revealed 2 pathogenic variants, *p.P67L* and *p.A442V*. This sample was interpreted as being affected. At the same time as the pilot assay was performed, an infant was locally recognized in a clinical center as having MPS-VII with the identical genotype as described above.

Discussion

We have previously demonstrated that MS/MS can simultaneously measure 2 or more enzymes if appropriate substrates are available. The ability to multiplex a core group of enzymes that are responsible for early onset genetic disease has been accepted by the NBS community. This has led to the introduction of NBS for treatable lysosomal storage diseases such as Pompe disease and Hurler disease.

To further expand the detection of treatable disorders, we have investigated the possibility of identifying 5 additional lysosomal diseases for which there are therapies available or in clinical development to prevent or delay the most significant clinical features. These disorders are Hunter syndrome (MPS-II), Sanfilippo B syndrome (MPS-IIIB), Morquio syndrome (MPS-IVA), Maroteaux-Lamy syndrome (MPS-VI), and Sly syndrome (MPS-VII). For each of these disorders, unique substrates and internal standards were synthesized and combined into a single cocktail to assay each of the responsible enzymes. Detection and quantification of the enzymatic products and internal standards proved to be satisfactory for routine screening of a newborn population.

The assay led to the identification of a total of 31 below cut-off newborns who required further evaluation by DNA sequencing (MPS-II, -IVA, VI, and VII). The assay did not identify any samples for MPS-IIIB that would require further evaluation. Presumably this was because of low population frequency for this disorders because 3-mm blood spots from known affected patients were confirmed to have low enzyme activity by this assay. Based on dried blood spots obtained from patients with a clinical diagnosis, there were only 2 samples from the alleged clinical cases that had enzymatic activity greater than 5% of the population mean activity. Most affected patients actually had activity of 2% or less of the daily mean (Supplementary Table IV). The need for further evaluation of the below cut-off samples for MPS-IVA is not known from this study because of the inability to obtain DNA sequence from the DBS punch.

We choose DNA sequencing analysis as our second-tier test. It remains to be seen whether analysis of accumulated glycosaminoglycans in DBS is a more powerful approach to explore the likelihood of a disease-positive newborn. Genotypes can be inconclusive because of the presence of variants of unknown pathogenic significance and because disease severity prediction is problematic with combinations of partially penetrant mutations. There is a lack of sufficient data on glycosaminoglycan levels in newborn DBS to know if these markers are always elevated at birth in patients who will develop an MPS disorder. It is likely that both DNA analysis and glycosaminoglycan assay will be carried out together in a live NBS program.

As clinical trials establish the beneficial effects of new commercial products for therapy of MPS disorders there will be a need for screening methods that can identify children prior to the development of significant clinical involvement. The method described in this study could be readily integrated into newborn screening laboratories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

DBS	Dried blood spots
LC-MS/MS	Liquid chromatography-MS/MS
MPS	Mucopolysaccharidosis
MS/MS	Tandem mass spectrometry
NBS	Newborn screening

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Table I.

Hunter syndrome (MPS-II) screened samples (n = 105 214)

	Activity for iduronide sulfatase ($\mu\text{mol/h/L}$ blood)	% Of daily mean	cDNA (NM_000202)	Protein	Interpretation
1	1.15	9.90%	poor DNA quality	—	—
2	1.44	9.59%	c.1499C>T	<i>p.T500I/</i>	Benign
3	1.03	8.59%	c.1090C>T	<i>p.P364S/</i>	Benign
4	1.46	8.57%	c.851C>T	<i>p.P284L/</i>	Benign
5	0.83	8.37%	c.851C>T		Benign
6	1.3	7.68%	c.1090C>T	<i>p.P364S/wt</i>	Benign (female)
7	1.17	6.64%	c.851C>T	<i>p.P284L/</i>	Benign
8	0.69	6.19%	wt/	wt/	Benign
9	1.14	5.36%	c.851C>T	<i>p.P284L/</i>	Benign
10	0.57	5.33%	wt/	wt/	Benign
11	0.57	5.16%	wt/	wt/	Benign
12	0.69	4.45%	c.1409C>T		At risk
13	0.6	4.22%	c.684A>G/c.851C>T	<i>p.P228P + p.P284L/</i>	Benign
14	0.58	4.16%	c.851C>T	<i>p.P284L/</i>	Benign
15	0.97	3.98%	c.159T>C	<i>p.C53W/</i>	At risk
16	0.65	3.93%	c.851C>T	<i>p.P284L/</i>	Benign
17	0.55	3.30%	c.851C>T	<i>p.P284L/</i>	Benign
18	-0.16	-1.09%	c.817C>T	<i>p.R273W/</i>	Affected

Table II.

Screening Results for MPS-IIIB, MPS-IVA, MPS-VI, and MPS-VII.

Sanfilippo syndrome B (MPS-IIIB) Samples screened n = 103 001					
Activity for N-acetyl- α -D-glucosaminidase (μ mol/hr/L blood)	% of daily mean	cDNA (NM_000263)	Protein	Interpretation	
No positive screens					
Morquio syndrome (MPS-IVA) Samples Screened n = 106 106					
Activity for Galactose amino-6-sulfatase (μ mol/hr/L blood)	% of daily mean	cDNA (NM_000512)	Protein	Interpretation	
1	0.24	c.499T>G/wt	p.F167V/wt	Carrier	
2	0.17	—	wt/wt		
3	0.21	c.692C>G/c.692C>G	p.A231G/p.A231G	Benign	
4	0.2	—	wt/wt		
5	0.17		unable to sequence		
6	0.13		unable to sequence		
7	0.11		unable to sequence		
8	0.16		unable to sequence		
Maroteaux-Lamy syndrome (MPS-VI) Samples screened n = 103 259					
Activity for arylsulfatase B (μ mol/h/L blood)	% of daily mean	cDNA (NM_000046)	protein	Interpretation	
1	1.47	c.1072G>A/wt	p.V358M/wt	Benign	
2	1.07	—	wt/wt	Benign	
3	0.7	c.1126G>A/c.1191G>A	p.V376M/p.P397P	Benign	
4	0.32	c.1072G>A/c.1126G>A	p.V358M/p.V376M	Benign	
Sly syndrome (MPS-VII) Screened samples n = 94 931					
Activity for β -glucuronidase (μ mol/h/L blood)	% of daily mean	cDNA (NM_000181)	Protein	Interpretation	
1	0.11	c.200C>T/c.1325C>T	p.P67L/p.A442V	Affected	