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Detecting multiple lysosomal storage diseases by tandem mass spectrometry — A national newborn screening program in Taiwan

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ABSTRACT

Background: Interest in lysosomal storage diseases in newborn screening programs has increased in recent years. Two techniques, fluorescence (4-MU) and tandem mass spectrometry (MS/MS) methods are frequently used. We report a pilot study of large scale newborn screening for Fabry, Pompe, Gaucher, and MPS I diseases by using the MS/MS method in Taiwan and compared the performance of the MS/MS with 4-MU methods.
Methods: More than 100,000 dried blood spots (DBSs) were collected consecutively as part of the national Taiwan newborn screening programs. The enzyme activities were detected by the MS/MS method from a DBS punch. Mutation analysis was further performed for newborns with detected enzyme deficiency.
Results: The DNA sequence analysis for suspected cases revealed 64 newborns with confirmed Fabry mutations, 16 were classified as infantile or late-onset Pompe disease, and 1 was characterized as Gaucher disease. The positive predict value increased from 4.0% to 7.1% in the Pompe study, and from 61.0% to 95.5% in the Fabry study by the MS/MS method compared to 4-MU assay.
Conclusions: The MS/MS method has been validated as a more specific, powerful and efficient tool than the 4-MU assay. It also provided a multiplex solution of newborn screening for lysosomal storage diseases.

1. Introduction

Lysosomal storage diseases (LSDs) are a group of rare inherited metabolic disorders that result from deficiency of specific enzymes responsible for the degradation of substances present in lysosomes. Over 40 LSDs are known with a collective incidence of approximately 1 in 7000–8000 live births [1]. Enzyme deficiency causes progressive metabolite accumulation. The influence is usually systemic and causes multiple organ damage, such as cardiomyopathy, neuropathy, and renal disorders [2,3]. Due to the availability of treatment including enzyme replacement therapy (ERT) for some LSDs, early diagnosis is important. The US Food and Drug Administration has approved ERT products for six LSDs including Fabry, type I Gaucher, Pompe, MPS I, MPS II and MPS VI diseases [4,5]. ERT can stabilize the condition, prevent disease progression, and improve the outcome of these LSDs [6–9]. However, ERT for disorders with neurologic involvement, such as Tay–Sachs, type II Gaucher, and type A Niemann–Pick diseases, is not feasible because the intravenously administered enzymes cannot cross the blood–brain barrier [10–12]. Other therapeutic modalities such as hematopoietic stem cell transplantation, substrate reduction therapies, and chaperone therapy have been undertaken in many of the subtypes of LSDs [13–15]. Besides, clinical trials and recent reports have emphasized that early intervention for the treatable LSDs may prevent irreversible pathologic changes, avoid or significantly minimize disease manifestation, and improve long-term outcome [12]. For these reasons and technical advance in recent years, some LSDs have been incorporated into newborn screening panels [16].

For LSD screening, the fluorescence method using 4-methylumbelliferone (4-MU) for enzyme activity assays from dried blood spots (DBSs) has been successfully developed in the past decade. It has also been proved as a useful tool allowing for large scale and high-risk population screening [17]. However, quantifying enzymatic activity by the 4-MU method has limited capacity for multiplexing and higher false positive rates due to higher background signals and the presence of quenchers such as hemoglobin in DBS samples. Frequent false negative results also exist using the 4-MU assay for Niemann–Pick-A/B screening [18]. Besides

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the 4-MU method, other technologies are available to analyze DBS samples including immune-capture enzyme assays [19] and tandem mass spectrometry (MS/MS). The MS/MS technology was first described for Fabry, Gaucher, Krabbe, Niemann–Pick A/B, and Pompe diseases [20]. Using the MS/MS technology, several lysosomal enzyme activities can be determined with combined substrates and internal standards into a single buffer [21].

Newborn screening in Taiwan started as a pilot program in 1981. The coverage rate was 6.7% in the beginning, and is currently more than 99% [22]. The coordination of screening services is routine and centralized in three national newborn screening centers, including the Chinese Foundation of Health (CFOH). Each center is responsible for one third of the screening program (60,000–70,000 cases). Our group, CFOH, has started a large-scale newborn screening program for Fabry disease in 2006 using the 4-MU method. So far, we have screened more than 300,000 DBSs for Fabry and Pompe diseases, and have reported a high incidence of newborn males with the IVS4+919G>A mutation in Taiwan [23].

To establish a new technique platform in our center, we performed a small population study to determine enzyme activity for the associated enzymes by the MS/MS in 2009. Although several previous papers for single disease had been reported in Taiwan, CFOH was the first newborn screening center to use the MS/MS multiplex technology for large scale screening of LSDs since 2010 in Asia. Scott et al. recently also reported a successful pilot newborn screening pilot study of ~100,000 DBSs for Pompe, Fabry, and MPS-I diseases [24]. We report here a large sample size of newborn screening pilot study based on the MS/MS method for Fabry, Pompe, Gaucher and MPS I diseases. The performance and the incidence were also compared with the 4-MU method and with other centers.

2. Methods

2.1. Study population

All studies were approved by the ethics committee of the China Medical University Hospital. Informed consent was obtained from subject parents whose children enrolled in the LSD study. Specimens from 1778 anonymous DBS samples, 3 confirmed Fabry patients, 4 Pompe patients, and 2 Gaucher patients were collected for the pilot study to generate newborn reference values in 2009 by both the 4-MU and MS/MS methods. Fabry and Pompe studies with the MS/MS method started from February, 2010 to January, 2013. Gaucher study started from September, 2011 to January, 2013. A total of 191,767, 191,786 and 101,134 newborns participated in Fabry, Pompe and Gaucher programs, respectively. Without informed consent, the data of MPS I pilot study was collected from April, 2012 to January, 2013, and 60,473 samples were conducted anonymously without recall DBSs and further genetic confirmation. Blood samples from newborns were collected and spotted on filter paper by age of 3 days. DBSs were analyzed once received every day and were put into plastic bags and stored at −20 °C for at least 3 years after analysis.

2.2. DBS test

For the 4-MU assay, enzyme activities in DBSs of acid α-galactosidase A (GLA), α-glucosidase (GAA), and β-glucocerebrosidase (ABG) were determined separately using 4-methylumbelliferyl-based high throughput methods according to previous reports [17,25,26]. For the multiplex MS/MS assay, 4 LSDs including the 3 mentioned LSDs and α-L-iduronidase (IDUA) were tested from one DBS simultaneously [20,27]. All methods regarding sample preparation and conditions of LC–MS/MS analysis were provided by the CDC (Atlanta, GA, USA).

2.3. Sample preparation and mass spectrometric analysis

One 3.2 mm diameter of spot from the DBSs (Whatman 903 filter paper) was punched into the well of 96-well PS plate (Greiner Bio-one, Germany) using BSD 700 puncher (BSD technologies, Australia). We then added 70 μL of extraction buffer (20 mM sodium acetate) to each well, mixed gently and sealed the plates with an aluminum plate sealer. Samples were extracted under 875 rpm shaking for 1 h at 37 °C. After centrifugation, 10 μL of extract was transferred to a new plate containing 15 μL of GLA, GAA, ABG and IDUA assay cocktail in each well. The plates were sealed and incubated for 20 h at 37 °C. The enzyme reactions were quenched by adding 100 μL of 1:1 ethylacetate:methanol to each well. After mixing well, samples were transferred to a 96 deep well plate. For liquid–liquid extraction, we added 400 μL ethylacetate and followed by 400 μL distilled water by liquid handler (Tecan Freedom EVO, Austria). Plates were shacked for 5 min at 500 rpm and then centrifuged at 4000 rpm for 5 min. After centrifugation, 300 μL of the upper layer was transferred into a new 96-well plate and dried under a steam of nitrogen. Samples were reconstituted in 100 μL of 19:1 ethylacetate: methanol, transferred into a 96-well filter plate containing silica gel to remove impurity substances, and washed four times with 400 μL of 19:1 ethylacetate: methanol. Samples and washes were collected into a 96 deep well plate and dried. Finally, samples were reconstituted with 300 μL of mobile phase (80% acetonitrile + 20% water with 0.2% formic acid). After shaking for 5 min, samples were analyzed with TSQ Vantage triple-quadruple MS/MS (Thermo Scientific, Waltham, USA).

2.4. Quality control, data processing and analysis

We used blank, low (enzyme activity below cutoff value), medium, and high (enzyme activity above cutoff value) controls from the CDC for each run. The blank sample was filter paper without spotted blood. To validate the coefficient of variation assay by the MS/MS method, 15 punches of each control were measured in one day for intra-assay data, and one punch of each control was collected on 15 different days for inter-assay data (Table 1). In the routine program, data were judged valid when the enzyme activities of CDC controls were in the range recommended by the manufacturer. All the MS/MS data were analyzed with LC QUAN software (version 2.6, Thermo Scientific). Data for statistical analyses were performed with SPSS (version 19). CIs were calculated by the Clopper–Person confidence method.

2.5. Screening procedure

The screening procedure is shown in Table 2. Enzyme activity from initial DBSs (first DBSs) was tested by the MS/MS method. Potentially enzyme-deficient samples were retested in duplicates from the same DBSs. Newborns with enzyme activity lower than the screening cutoff value but not inferior to the critical cutoff value were recalled for another DBSs (recall DBSs). Newborns with abnormal results of recall DBSs were requested for subsequent genetic mutation analyses. False positives are defined as samples with DBS enzymatic activity below the cutoff value but not confirmed with disease-causing mutations. The positive predictive values are defined as the percentages of disease-confirmed newborns among newborns referred to the hospital and who received mutation analysis.

2.6. Sequence analysis

Newborns with low enzyme activities were subjected to genetic analysis. DNA was isolated from whole blood using a DNA extraction kit (QIAamp DNA Mini Kit). Genetic mutation assays were performed by direct exon sequencing. Each exon was amplified by the polymerase chain reaction using appropriate primers [23,28,29]. Direct sequencing