γ-glutamyl transpeptidase. Prothrombin activity, total protein and albumin were decreased. The mutation types were 851del4/IVS11 + 1G > A throughout most of late infancy, being more than 5 months of age in patients 27, 28, 29 and 30.

Histological findings

Histological findings of the 30 patients are shown in Table 3. The results of the fibrosis staging and inflammation grading are shown in Figure 1.

Fibrosis

Most specimens showed varying degrees of fibrosis; 35% of all specimens were classified as stage 0, while stages 1 and 2 together accounted for 50%. However, there was a wide spectrum of fibrosis: more advanced liver lesions with distorted lobular architecture (stage 3) (Fig. 2) and cirrhosis were observed in four and one specimens, respectively. One patient with cirrhosis developed hepatic failure. Therefore, this patient underwent a living-related liver transplant. One patient with cirrhosis developed at 10 months of age.[10]

Inflammatory reaction

The degree of inflammation varied with the specimens, where half showed moderate or severe inflammatory changes. Inflammatory cell infiltration in the portal tracts and piecemeal necrosis were observed (Fig. 3). Inflammatory cells present in the portal tracts were predominantly lymphocytes. Focal necrosis and acidophilic bodies in the parenchyma were seen in 23 (77%) and 12 (40%) specimens, respectively. The sinusoids showed the proliferation of mononuclear cells with scarce neutrophils and the activation of Kupffer cells.

Fat deposition in hepatocytes

Fat deposition in hepatocytes was observed in all specimens except one and severe fatty liver was noted for 20 (67%) specimens (Fig. 4a). Fat droplets deposited in the cytoplasm of hepatocytes varied in size, and fat-laden hepatocytes were classified as those with macrovesicular fat droplets, those with foamy, microvesicular fat droplets, and those with mixed macrovesicular and microvesicular fat droplets. Hepatocytes with microvesicular fat droplets had a centrally located nucleus. In 80% of 29 specimens with fat deposition including all 20 specimens which showed severe fatty livers, there was a mixture of macro- and microvesicular fat droplets (Fig. 4b,c). Macrovesicular and microvesicular fatty liver alone accounted for three (10%) and one (4%) specimens, respectively. A moderate and severe fatty liver with an inflammatory reaction and lipogranuloma were diagnosed as steatohepatitis, which accounted for 60% of the patients. The histopathological findings in this disease were different from those in non-alcoholic steatohepatitis. The clinical features of one patient who had no fat deposition in hepatocytes did not differ from that of other patients with such fat deposition.

Cholestasis

Cholestasis was observed in 77% of the specimens and was severe in 57%. The acinar arrangement of hepatocytes was prominent in specimens with severe cholestasis (Fig. 5) and multinucleated giant cell transformation was found in one case (Fig. 6).

Hemosiderin deposition

Hemosiderin deposition, mostly mild and localized in periportal hepatocytes and macrophages in portal areas (Fig. 4b), was observed in 57% of the specimens.

A combination of all five features, fatty liver, inflammatory cell infiltration, fibrosis, cholestasis and hemosiderin deposition was observed in the same liver biopsy specimen in 12 (40%) of the total specimens.

Relationship between the age and the histological findings

The mean score of each histological finding in each of groups A, B and C are summarized in Table 4. The degree of fibrosis, necroinflammatory reaction such as focal necrosis and acidophilic bodies, acinar arrangement of hepatocytes, cholestasis and steatohepatitis of infants more than 3 months old (groups B and C) were more accentuated than those of the early infants of group A. Conversely, hemosiderosis and extramedullary hematopoiesis in groups B and C were less pronounced than in group A. The staging score of fibrosis, grade of inflammation and steatohepatitis were significantly higher in the older than in the younger group in order of group A, B and C.

Histological findings of follow-up biopsy

Follow-up biopsies were conducted for patients 8, 9, 13 and 18, and the findings were as follows: patients 8, 9 and 13 showed histological deterioration of cholestasis and fatty change. Of note, patient 9 underwent a liver transplant at the age of 16 years because of hepatic failure. The findings for the explant liver were

Table 3 Histological findings of liver biopsy in the 30 patients with neonatal intrahepatic cholestasis caused by citrin deficiency

Patient no.	1	2	3	4	5	6	7	8	9	10
Stage of fibrosis	0	0	1	0	0	0	0	0	3	2
Grade of inflammation	1	2	2	1	1	1	2	1	1	1
Focal necrosis[a]	1	1	2	0	0	0	1	0	0	1
Acidophilic body[b]	0	1	0	2	0	1	0	1	0	0
Acinar arrangement[c]	0	1	3	3	0	1	0	1	2	1
Cholestasis[d]	0	3	3	3	1	0	1	2	3	1
Degree of fat deposit[e]	1	3	3	3	3	3	2	3	3	3
Type of fat deposit[f]	1	3	0	3	3	3	1	3	0	0
Steatohepatitis[g]	0	1	1	1	0	1	1	1	0	2
Hemosiderosis[h]	0	2	1	2	0	0	1	2	0	2
Extramedullary hematopoiesis[i]	0	2	0	3	2	1	0	2	0	0
Patient no.	11	12	13	14	15	16	17	18	19	20
Stage of fibrosis	0	2	2	1	0	0	3	2	1	1
Grade of inflammation	1	1	1	2	1	2	2	2	3	1
Focal necrosis	1	0	1	1	1	2	1	1	3	0
Acidophilic body	1	0	0	1	0	0	1	0	0	0
Acinar arrangement	2	0	0	2	2	1	1	1	2	1
Cholestasis	3	0	0	3	3	2	2	2	3	3
Degree of fat deposit	3	0	2	2	3	2	3	3	2	3
Type of fat deposit	3	0	2	3	3	3	3	3	3	3
Steatohepatitis	2	0	0	1	1	1	1	1	2	1
Hemosiderosis	2	0	1	0	2	1	1	0	2	1
Extramedullary hematopoiesis	0	0	0	3	2	0	1	0	0	0
Patient no.	21	22	23	24	25	26	27	28	29	30
Stage of fibrosis	2	2	0	2	2	3	1	3	3	4
Grade of inflammation	3	2	1	2	3	2	1	2	3	3
Focal necrosis	1	2	1	1	3	1	1	1	2	1
Acidophilic body	1	2	0	1	1	1	0	0	0	2
Acinar arrangement	3	2	0	2	2	1	2	1	3	2
Cholestasis	3	3	0	3	0	3	3	3	3	3
Degree of fat deposit	3	3	3	3	1	3	2	3	3	3
Type of fat deposit	3	3	3	3	1	3	3	3	3	3
Steatohepatitis	0	3	2	1	0	2	1	3	3	3
Hemosiderosis	3	1	1	1	0	1	1	0	0	0
Extramedullary hematopoiesis	1	0	1	1	2	0	0	0	1	0

[a]Focal necrosis was graded from 0–3 based on the number of counts per 10 fields at a magnification of ×40. A score of denotes 0 is none, 1 denotes 1–2; 2 denotes up to 4, and 3 denotes >4.
[b]Acidophilic bodies were counted and graded from 0–3, as similar to that for focal necrosis.
[c]The acinar arrangements of the hepatocytes were graded 0–3. A rating of 0 denotes none, 1 denotes involvement up to 30% of the hepatocytes, 2 denotes 30–60%, and 3 denotes >60%.
[d]Cholestasis was graded from 0–3. A score of 0 denotes none, 1 denotes cholestasis without a bile plug, 2 denotes scattered bile plugs, and 3 denotes frequent bile plugs.
[e]The degree of fat deposition in hepatocytes was graded from 0–3 based on the percentage of hepatocytes in the biopsy involved. A rating of 0 denotes none; 1 denotes up to 30%, 2 denotes 30–60%, and 3 denote >60%.
[f]The type of fat deposit was classified as being between 0–3. A score of 0 denotes no fatty change, 1 denotes predominantly macrovesicular fat droplets, 2 denotes predominantly microvesicular fat droplets, and 3 denotes mixed microvesicular and macrovesicular fat droplets.
[g]Steatohepatitis was graded from 0–3, where 0 denotes none, 1 denotes steatosis involving up to 60% and intra-acinar inflammation with no or mild portal inflammation, 2 denotes steatosis (>66%) with both acinar and portal inflammation, and 3 denotes panacinar steatosis with intra-acinar inflammation and piecemeal necrosis.
[h]Hepatocellular iron was graded between 0–3, where 0 denotes none, 1 denotes localized deposition in the portal and /or periportal area; 2 denotes iron deposition involving up to 60% of the parenchyma, and 3 denotes >60%.
[i]Extramedullary hematopoiesis was graded between 0–3, similar to that for focal necrosis.

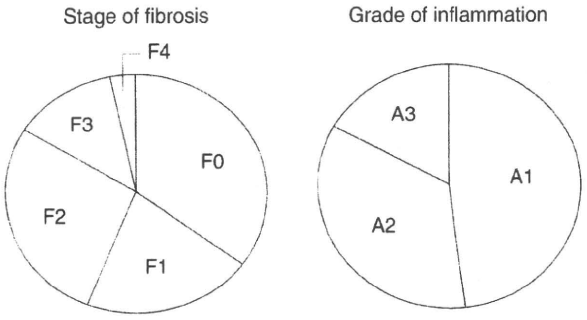

Figure 1 Results of fibrosis and the grade of necroinflammation.

more pronounced than those of the biopsy. Patient 8 showed progression of fibrosis from stage 1–3 and more pronounced portal inflammation. In contrast, patient 18 showed marked improvement of every

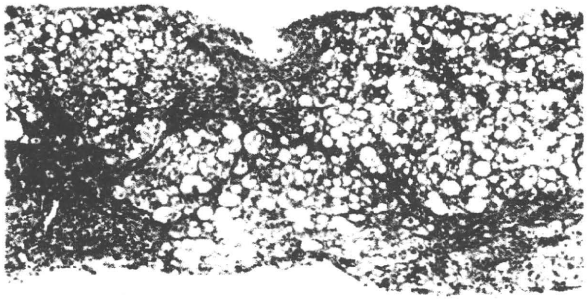

Figure 2 Severe fatty liver with distorted lobular architecture due to extensive fibrosis in stage 3 with portal inflammation (hematoxylin–eosin, original magnification ×50).

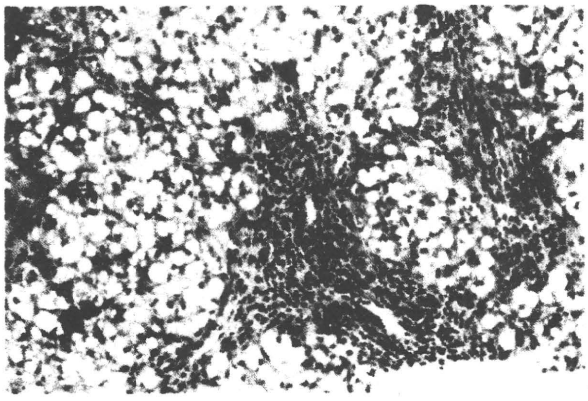

Figure 3 Fatty liver with moderate inflammatory cell infiltration in the portal tract and parenchyma, which is indicative of steatohepatitis (hematoxylin–eosin, original magnification ×100).

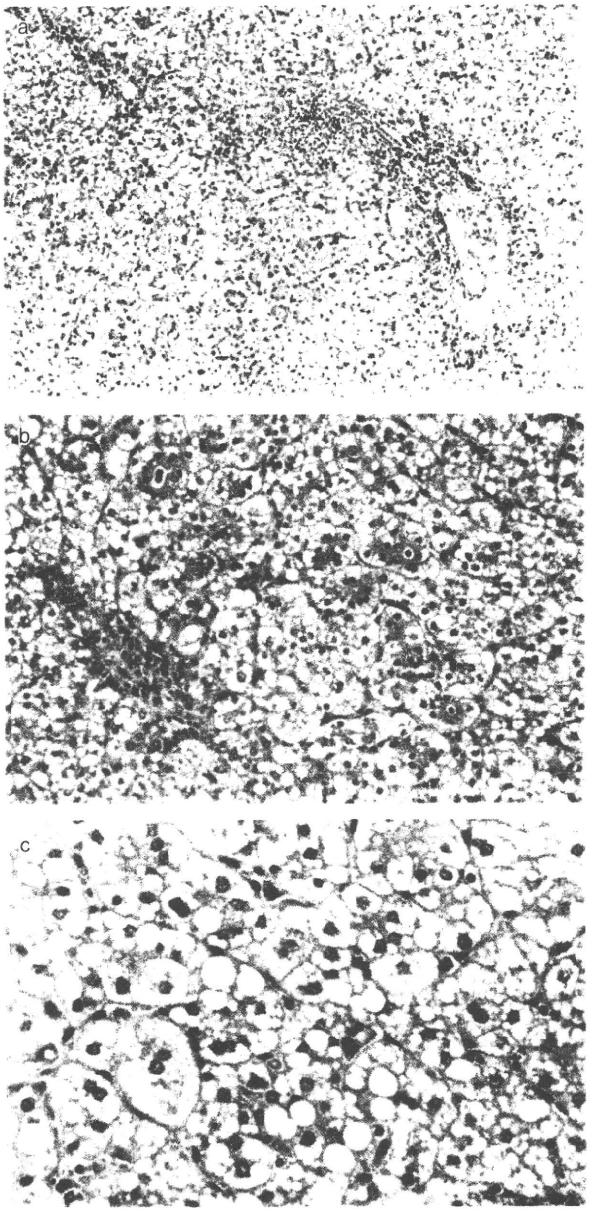

Figure 4 (a) Severe fatty liver with cholestasis. The portal tracts show mild inflammatory cell infiltration (hematoxylin–eosin [HE], original magnification ×50). (b) Pseudo-acinor transformation with bile plugs is observed. Hemosiderin-laden macrophages are present in a portal tract (HE, original magnification ×100). (c) Macro- and microvesicular-type fatty droplets. Some of the swollen hepatocytes have a foamy appearance and their cytoplasm packed with micro-fat droplets. Kupffer cells are activated (HE, original magnification ×200).

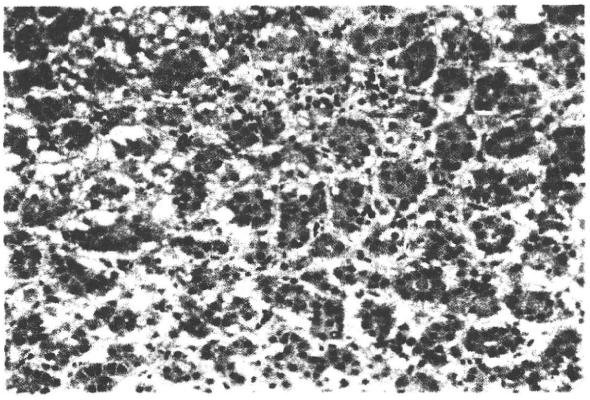

Figure 5 Striking pseudo-acinor transformation of the hepatic cords containing bile plugs. Small fatty droplets are present at the periphery of hepatocytes arranged in an acinar fashion (hematoxylin–eosin, original magnification ×100).

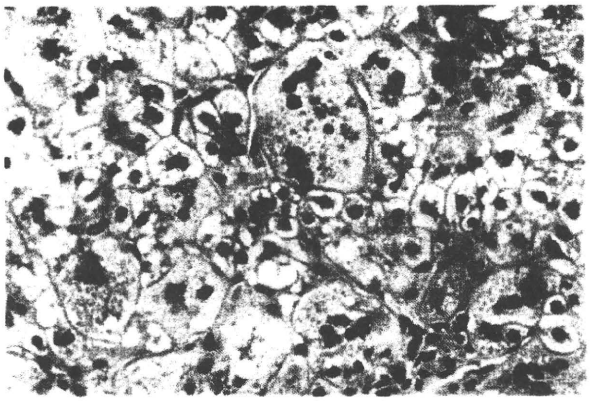

Figure 6 Giant cell hepatitis and cholestasis. Multinucleate giant cells contain several nuclei (hematoxylin–eosin, original magnification ×200).

histological finding, including decreased portal fibrosis and inflammation.

DISCUSSION

THE CAUSE OF liver dysfunctions such as fatty liver, hypoglycemia and galactosemia in this disease is as follows.[15] Citrin deficiency blocks the malate aspartate shuttle, which may increase the ratio of cytosolic nicotinamide adenine dinucleotide (NADH) to oxidized nicotinamide adenine dinucleotide ($NADH/NAD^+$), which in turn is associated with the inhibition of glycolysis and makes reduced alcohol metabolism. This may be why CTLN2 patients dislike carbohydrates and cannot drink alcohol, and why alcohol consumption often results in psychiatric symptoms. An increased $NADH/NAD^+$ ratio is also characteristic of the inhibition of gluconeogenesis involving reduced substrates.[19] This, together with the reduction in alanine metabolization to urea and glucose due to citrin deficiency may cause hypoglycemia in NICCD patients. Although NICCD patients suffer from galactosemia, which sometimes even leads to the development of cataracts, no abnormalities in the enzymes involved in galactose metabolism have been found.[20] Because uridine diphosphate-glucose epimerase which requires NAD as a cofactor is strongly inhibited by NADH,[21] galactosemia in NICCD may represent a high NADH level in the cytosol of the liver.

From the biochemical data of this study, 50% of the high level of total bilirubin was associated with direct bilirubin, but it was not always dominant. The levels of AST were increased to more than twice the levels of ALT. Low levels of total protein, albumin and prothrombin

Table 4 Relationship between age and histological changes

Pathological findings	Group A ($n = 16$) <2 months	Group B ($n = 9$) 3–4 months	Group C ($n = 5$) >5 months	*P*-value
Stage of fibrosis	0.69 ± 1.01	1.67 ± 0.87	2.80 ± 1.10	$P = 0.001$
Grade of inflammation	1.31 ± 0.48	2.11 ± 0.78	2.20 ± 0.84	$P = 0.004$
Focal necrosis	0.75 ± 0.68	1.44 ± 1.01	1.20 ± 0.45	$P = 0.063$
Acidophilic body	0.44 ± 0.63	0.67 ± 0.71	0.60 ± 0.89	$P = 0.523$
Acinar arrangement	1.19 ± 1.05	1.56 ± 0.88	1.80 ± 0.84	$P = 0.172$
Cholestasis	1.75 ± 1.29	2.11 ± 1.27	3.00 ± 0.00	$P = 0.059$
Degree of fat deposit	2.44 ± 0.89	2.67 ± 0.71	2.80 ± 0.45	$P = 0.333$
Steatohepatitis	0.81 ± 0.66	1.22 ± 0.97	2.40 ± 0.89	$P = 0.008$
Hemosiderosis	1.00 ± 0.89	1.11 ± 0.93	0.40 ± 0.55	$P = 0.356$
Extramedullary hematopoiesis	0.94 ± 1.18	0.67 ± 0.71	0.20 ± 0.45	$P = 0.297$

The data are expressed as means ± standard deviation.
P-values are by the Mantel–Haenszel linear trend test.

activity and high levels of citrulline, α-fetoprotein, ferritin and PSTI were observed as previously described in NICCD patients.[6–13] However, 11 patients showed high levels of ferritin, which were not observed in previous reports on NICCD patients. Therefore, the pediatric hepatologist should suspect NICCD when a neonatal cholestatic patient has high levels of AST of more than twice the ALT value, citrulline, α-fetoprotein and ferritin, and low levels of total protein and prothrombin activity.

The histological findings in this study such as a fatty liver, cholestasis, necroinflammatory reaction and iron deposition are not pathognomonic findings because they occur in various liver diseases.[22] However, the combination of mixed macrovesicular and microvesicular fatty hepatocytes and these histological findings are almost never observed in other liver diseases in infants and adults. Microvesicular fatty deposition was found in NICCD, this type of fatty change is a characteristic feature of Reye syndrome[23] and other rare conditions.[22] However, the histogenesis of the microvesicular fatty deposition in NICCD is unclear. It might reflect the acute impairment of β-oxidation of fatty acid due to mitochondrial dysfunction as in Reye syndrome.

Although our series of NICCD patients shared common liver histological findings as described above, there seemed a tendency that late infants of group C had more advanced fibrosis and more accentuated inflammation than those of early infants of group A. The duration of illness may be an aggravating factor of the progression of the disease in some cases. There was no difference between the liver histological findings and mutation type. Interestingly, however, the mutation type of patients with severe fibrosis who were 6 and 7 months of age was 851del4/IVS11 + 1G > A. Because evidence for this relationship between the mutation type and the progression of fibrosis is not clear, further investigation is needed. Moreover, in the follow-up liver biopsy patients, we observed improvements in their liver histological findings as the liver dysfunction was ameliorated. Therefore, we speculate that the correlations between the stage of the liver histological findings and the biochemical test data exist.

This study found that NICCD is a disease with characteristic hepatopathological features. If NICCD is suspected in the presence of cholestasis during infancy, a liver biopsy should be performed to screen for liver diseases. We believe that a liver biopsy is of high diagnostic value for NICCD, and is useful for accurately assessing inflammation and the degree of the progression of fibrosis.

Although we were not able to elucidate the natural history of the disease, we previously found that despite a benign course in the majority of the patients, it leads to the development of liver cirrhosis in some patients with CTLN2.[5,10] This suggests that it involves the risk of progressive fibrosis and eventually leads to the development of cirrhosis. This possibility is suggested by the above histopathological findings characteristic of NICCD in the patients who progressed to stage 3 chronic hepatitis and cirrhosis. Although the process responsible for the progression of liver lesions is not clear, some patients with steatohepatitis including non-alcoholic steatohepatitis (NASH) progress to cirrhosis.[24] In this study, steatohepatitis was found in 60% of the specimens. It is likely that, in NICCD, steatohepatitis repeatedly deteriorates, persists and progresses.

In conclusion, if NICCD is suspected in the presence of cholestasis during infancy, a liver biopsy should be performed to screen for other liver diseases. NICCD is a disease with characteristic hepatopathological features, such as the combination of mixed macrovesicular and microvesicular fatty hepatocytes, cholestasis, necroinflammatory reaction and iron deposition. Therefore, it is possible to diagnose NICCD based on histological liver findings in most cases. However, when cirrhosis with fat deposition is detected in hepatocytes in liver specimens, the patient should be carefully observed, because the prognosis of NICCD patients is not always fair, with some developing progressive liver failure by 1 year of age. Finally, we could not infer the development of CTLN2 from the histological findings of the patients with NICCD who were examined in this study.

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Improved assay for differential diagnosis between Pompe disease and acid α-glucosidase pseudodeficiency on dried blood spots

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ABSTRACT

The high frequency (3.3–3.9%) of acid α-glucosidase pseudodeficiency, c.[1726G>A; 2065G>A] homozygote (AA homozygote), in Asian populations complicates newborn screening for Pompe disease (glycogen storage disease type II or acid maltase deficiency) on dried blood spots, since AA homozygotes have a considerably low enzyme activity. We observed that hemoglobin in the enzyme reaction solution strongly interferes with the fluorescence of 4-methylumbelliferone released from 4-methylumbelliferyl α-D-glucopyranoside (4MU-αGlc) by acid α-glucosidase. Therefore, we have searched for a method to effectively eliminate hemoglobin in the reaction solution. Hemoglobin precipitation with barium hydroxide and zinc sulfate (Ba/Zn method) carried out after the enzyme reaction considerably enhances the fluorescence intensity while it does not reduce the intensity to any extent as can occur with conventional deproteinization agents like trichloroacetic acid. The Ba/Zn method greatly improved the separation between 18 Japanese patients with Pompe disease and 70 unaffected AA homozygotes in a population of Japanese newborns in the assay with 4MU-αGlc on dried blood spots. No overlap was observed between both groups. We further examined acid α-glucosidase activity in fibroblasts from 11 Japanese patients and 57 Japanese unaffected individuals including 31 c.[1726G; 2065G] homozygotes, 18 c.[1726G; 2065G]/[1726A; 2065A] heterozygotes and 8 AA homozygotes to confirm that fibroblasts can be used for definitive diagnosis. The patients were reliably distinguished from three control groups. These data provide advanced information for the development of a simple and reliable newborn screening program with dried blood spots for Pompe disease in Asian populations.

1. Introduction

Early diagnosis is a critical issue for effective enzyme replacement therapy in lysosomal storage diseases. To this end, efforts have been made to develop methods for newborn screening. Most methods are based on the direct measurement of lysosomal enzyme activities in dried blood spots (DBSs) [1–6]. Other procedures include antibodies to increase the specificity of the assay, or to determine the amount of enzyme protein rather than activity, or to probe lysosomal disease markers [7–10]. Multiplex assays with the parallel measurement of several lysosomal enzyme activities are aimed to improve the cost effectiveness of newborn screening [11–14].

Using DBSs a first large scale newborn screening program in Taiwan was shown to improve clinical outcomes for patients with Pompe disease [4], also known as glycogen storage disease type II or acid maltase deficiency (OMIM No. 232300). Pompe disease is an autosomal recessive disorder of glycogen metabolism resulting from a generalized deficiency of the lysosomal enzyme acid α-glucosidase (AαGlu; EC 3.2.1.20/3). The enzyme deficiency causes intralysosomal glycogen storage in numerous tissues, but predominantly in muscle. The disorder exhibits a broad clinical spectrum with regard to age of onset, cardiac involvement and progression of skeletal muscle dysfunction. Since 1999, several clinical trials have shown that patients with Pompe disease can benefit from enzyme replacement

Abbreviations: AαGlu, acid α-glucosidase; AA homozygote, c.[1726A 2065A] homozygote acid α-glucosidase pseudodeficiency; GG/AA heterozygote, c.[1726G 2065G]/c.[1726A 2065A] heterozygote; GG homozygote, c.[1726G 2065G] homozygote; 4MU, 4-methylumbelliferone; 4MU-αGlc, 4-methylumbelliferyl α-D-glucopyranoside; DBS, dried blood spot; TCA, trichloroacetic acid; Ba/Zn method, barium hydroxide and zinc sulfate method.

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therapy [15–20]. The effect of enzyme therapy in severely affected infants is readily recognized by regression of the cardiomegaly, prolonged survival and acquirement of motor skills. Beneficial effects of enzyme replacement therapy in children, adolescents and adults with Pompe disease also have been reported and are promising, but the crucial outcome of long term treatment has still to be awaited [18–22]. Further, it appears that infants with rather well preserved muscle morphology respond better to therapy than those who are diagnosed late and have severe muscle damage at start of treatment. Early diagnosis seems a must in Pompe disease to optimize any form of therapeutic intervention [23].

Previously, we examined AαGlu activity in 715 apparently healthy Japanese newborns with DBSs and showed that the distribution of the activity was bimodal. The median activity of the minor group (31 individuals, 4.3% of the samples) was 6.5 times lower than that of major group [6]. Genetic analysis revealed that 28 individuals of the minor group were homozygous for c.[1726G>A; 2065G>A], also known as pseudodeficiency (AA homozygote). Four of the AA homozygotes had activities in the patients' range (the AαGlu activity range of the AA homozygotes is 1.4–10.1 pmol/h/disk; the activity range of the Pompe patients is 0–2.8 pmol/h/disk). The AαGlu pseudodeficiency allele has a much higher frequency in the Asian compared to the Caucasian populations [6,24]. Substitution p.E689K caused by c.2065G>A characterizes the "GAA4" allozyme, which is found in Chinese and Japanese populations with frequencies of 0.27–0.28 and 0.27–0.31, respectively, and reduces the AαGlu activity by 50–60% of average normal [25–27; JSNP, http://snp.ims.u-tokyo.ac.jp/]. On the contrary, substitution p.G576S caused by c.1726G>A reduces the AαGlu activity to such extent that it may overlap with the patient range [24]. Thus, to achieve reliable newborn screening for Pompe disease in Asian populations, sensitivity and selectivity of the method should be improved to distinguish pseudodeficiency from pathologic deficiency. We were informed that hemoglobin precipitation with trichloroacetic acid (TCA) improves 4MU-based diagnostic assays for lysosomal storage disease in DBSs because it eliminates quenching of the fluorescence signal [28].

In this study, we have looked for the most effective method to eliminate hemoglobin from the reaction solution in order to maximize the separation between newborns with Pompe disease and AA homozygotes in Asian populations. We here describe that hemoglobin precipitation with barium hydroxide and zinc sulfate after the enzyme reaction considerably improves the 4MU fluorescence intensity and circumvents the potential problem of signal reduction by TCA precipitation.

2. Subjects, materials and methods

2.1. Subjects and DBS collection

DBSs from 252 Japanese newborns (second to fifth day postpartum) and 18 Japanese patients with Pompe disease were used in this study. The patient group included one child with classic infantile Pompe disease, 6 juveniles, 10 adults, and one patient with unknown phenotype. The DBSs on filter paper were obtained with the standard heel-stick for collecting newborn screening samples, or prepared by drop-wise application of EDTA-blood samples on the filter paper (filter paper #510AD01, Advantec, Tokyo, Japan) that is routinely used for newborn screening in Japan. DBSs were dried at room temperature for at least 3 h but no more than 16 h, and were subsequently stored at −20 °C in sealed plastic bags until use. Written informed consent was obtained from all subjects, and all samples from these subjects were prepared and analyzed in accordance with the protocols approved by the Ethics Committee for Gene Analysis and Genome Research of Kumamoto University.

Fibroblasts from 57 Japanese unaffected individuals (controls) and 11 patients with Pompe disease were used for this study. Fibroblasts were cultured under standard conditions in Dulbecco's modified Eagle's medium with 10% fetal calf serum and antibiotics (50 kU/L penicillin, 50 mg/L streptomycin). After reaching confluency, the fibroblasts were harvested and washed with phosphate-buffered saline. The cell pellets were stored at −40 °C until use. The pellets ($2–4 \times 10^6$ cells) were homogenized in 500 μL water by sonicating on ice for two times 10 s, using a UP50 ultrasonic processor (Hielscher, Teltow, Germany) with a 2 mm diameter tip size, set at 100 μm amplitude. The protein concentration of the cell homogenates was measured using the Pierce BCA protein assay reagent kit (Rockford, IL) with bovine serum albumin as a calibrator. The protein concentration of the homogenates was adjusted to 0.6–1.2 mg/mL unless otherwise indicated.

2.2. Chemicals, reagents and instrument

4-Methylumbelliferyl α-D-glucopyranoside (4MU-αGlc), glycogen (type III, from rabbit liver) and glucose were purchased from Sigma-Aldrich (St. Louis, MO). Acarbose and 4-methylumbelliferone (4MU) were from Toronto Research Chemicals (North York, Canada) and Nacalai Tesque (Kyoto, Japan), respectively. The chromogen, 10-N-methylcarbamoyl-3,7-bis(dimethylamino)phenothiazine, for highly sensitive detection of glucose from glycogen were provided from Kyowa medix (Tokyo, Japan). Other chemicals were of reagent grade and from Sigma-Aldrich or Nacalai Tesque. The fluorescence intensity of 4MU liberated from 4MU-AαGlu by AαGlu was measured with the CORONA spectrofluorometer (MTP-800AFC, Colona Electric, Hitachinaka, Japan) at excitation and emission wavelengths of 360 nm and 450 nm, respectively. COBAS MIRA automatic analyzer (Roche, Basel, Switzerland) was used to measure AαGlu activity with glycogen as a substrate.

2.3. Effect of hemoglobin elimination on 4MU detection

A solution of 60 μmol/L 4-methylumbelliferone in a buffer consisting of 0.2 mol/L citrate/0.4 mol/L potassium-phosphate at pH 4.0 with different concentrations of hemoglobin added to it (0, 225, 450, 900 and 1800 mg/L in the final reaction mixture) was used to compare two different methods for hemoglobin elimination. For the hemoglobin precipitation with TCA (TCA method), 60 μL of the sample solution in a 1.5 ml reaction tube was incubated at 37 °C for 120 min, and then 20 μL of 16% chilled TCA was added. After vortex mixing and incubation at 4 °C for 10 min, the sample solution was centrifuged at 10,000 g at 4 °C for 5 min. 60 μL of the supernatant was transferred to a 96-well black microwell-plate (PerkinElmer, Boston, MA), and then 190 μL of 0.5 mol/L sodium-carbonate/sodium-bicarbonate buffer at pH 10.7 containing 0.1% Triton X-100 was added for measurement of fluorescence intensity. For the hemoglobin precipitation with barium hydroxide and zinc sulfate (Ba/Zn method), 60 μL of the sample solution in a 1.5 mL reaction tube was treated with the Ba/Zn method as described below and the fluorescence intensity was measured. As a control, 60 μL of the sample solution was treated as in the TCA method except that distilled water was used instead of 16% TCA.

2.4. Measurement of AαGlu activity in DBSs

We assayed AαGlu activity in DBSs with two different methods. One method was without hemoglobin elimination, as previously published (previous method) [6]. In the other we used the Ba/Zn method to eliminate hemoglobin. For the previous method, a 3.2-mm diameter disk punched from the DBSs was incubated in a well of a 96-well clear microwell-plate (Corning, New York, NY) with 100 μL distilled water for 1 h at room temperature while mixing gently. A 20 μL aliquot of the water extract was then added to 40 μL of 2.0 mmol/L 4MU-AαGlc in 0.2 mol/L citrate/0.4 mol/L potassium-phosphate buffer at pH 4.0 containing 4.5 μmol/L acarbose (3.0 μmol/L in final concentration) in a 96-well black microwell-plate

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(PerkinElmer). The reaction mixture was incubated at 37 °C for 24 h, and the reaction was stopped by addition of 190 μL of 0.2 mol/L glycine/NaOH buffer at pH 10.7 containing 0.1% Triton X100 to measure fluorescence intensity. For the Ba/Zn method, a similar 3.2-mm diameter disk punched from the DBSs was placed in a 1.5 mL reaction tube and gently mixed for 10 min at room temperature in 60 μL of 0.2 mol/L citrate/0.4 mol/L potassium-phosphate buffer at pH 4.0 containing 2.0 mmol/L 4MU-AαGlc and 3.0 μmol/L acarbose. The reaction mixture was then incubated at 37 °C for 24 h. After this period, 30 μL of 0.15 mol/L barium hydroxide was added and, after vortex mixing, the reaction tube was left at room temperature for 5 min. Thereafter, 30 μL of 0.15 mol/L zinc sulfate was added and again, after vortex mixing, the tube was left for 10 min at room temperature. The tube was then centrifuged for 5 min at 10,000 *g* and 4 °C. Finally, 90 μL of the supernatant was transferred to a 96-well black microwell-plate (PerkinElmer), and 160 μL of 0.4 mol/L glycine/NaOH buffer at pH 10.7 containing 0.1% Triton X-100 was added to measure fluorescence intensity. We used stock solutions of 0, 6.25, 12.5, 25, 50 and 100 μmol/L 4MU in 20 mmol/L sodium-phosphate buffer at pH 7.0 to calibrate the measurement of liberated 4MU. The enzyme activity was expressed as picomoles 4MU released per hour per 3.2 mm diameter disk (pmol/h/disk). Each assay was performed in duplicate.

2.5. Measurement of AαGlu activity in fibroblasts

AαGlu activity in fibroblasts was measured with 4MU-αGlc as substrate as described [29] with minor changes. Briefly, 10 μL of the cell homogenate was added to 40 μL of the substrate solution containing 2.0 mmol/L of 4MU-αGlc in 0.2 mol/L citrate/0.4 mol/L potassium-phosphate buffer at pH 4.0 with 3.75 μmol/L acarbose (3.0 μmol/L in the final concentration) in a well of a 96-well black microwell-plate (PerkinElmer). The reaction mixture was incubated at 37 °C for 1 h, and the reaction was stopped by addition of 200 μL of 0.2 mol/L glycine/NaOH buffer at pH 10.7 containing 0.1% Triton X100 to measure fluorescence intensity, and corrected for substrate blank. We used a stock solution of 250 μmol/L 4-methylumbelliferone in 20 mmol/L sodium-phosphate buffer at pH 7.0 to calibrate the measurement of liberated 4MU. Each assay was performed in duplicate. The enzyme activity was expressed as nanomoles 4MU released per hour per milligram cellular protein (nmol/h/mg protein).

AαGlu activity in fibroblasts was also measured with glycogen as substrate followed by an enzymatic determination of liberated glucose. Briefly, 12 μL of the cell homogenates was added to 48 μL of the substrate solution containing 62.5 mg/mL glycogen in 0.1 mol/L citrate/0.2 mol/L sodium-phosphate buffer at pH 4.0 with 3.75 μmol/L Acarbose (3.0 μmol/L in the final concentration) and incubated for 1 h at 37 °C in a 1.5 mL reaction tube. The reaction was terminated by heating at 95 °C for 5 min. Then the reaction tube was immediately cooled on ice and centrifuged at 10,000 *g* for 3 min. An aliquot of the supernatant was subjected to quantitative analysis for liberated glucose with two reagents on COBAS MIRA automatic analyzer. Reagent 1 consisted of 0.15 mmol/L of 10-N-methylcarbamoyl-3,7-bis(dimethylamino)phenothiazine, 0.38 kU/L mutarotase (from porcine kidney, Wako, Osaka, Japan), 0.77 mmol/L triethylenetetraminehexaacetic acid, 0.2% Triton X100 and 0.23 mol/L Tris/0.36 mol/L sodium-phosphate buffer at pH 7.0. Reagent 2 consisted of 86 kU/L of glucose oxidase (from *Aspergilles niger*, Sigma-Aldrich), 3.8 kU/L peroxidase (from horseradish, TOYOBO, Tokyo, Japan), 70 μmol/L potassium ferrocyanide and 0.23 mol/L Tris/0.36 mol/L sodium-phosphate buffer at pH 7.0. The analytical conditions on the COBAS MIRA automatic analyzer were as follows: sampling volume, 15 μL (washing distilled water volume, 35 μL); the reagent 1 volume, 130 μL; the reagent 2 volume, 70 μL; wavelength, 660 nm; temperature, 37 °C; and calculation mode, endpoint assay with a reagent blank. Timing (25 s per one cycle) for sample and reagent additions was: sample and the reagent 1, cycle 1; the reagent 2, cycle 5. Timing for readings was: first, cycle 4; and last, cycle 30. The reaction time after the addition of the reagent 2 was 10 min 50 s. The enzyme activity was expressed as nanomoles glucose released per hour per milligram cellular protein (nmol/h/mg protein). To avoid erroneous results attributable to turbidity of the glycogen solution and the free cellular glucose, we also performed the assay without adding the cell homogenate (glycogen blank) and without adding the glycogen (sample blank). Each assay was performed in duplicate. The activity was calculated after correcting for glycogen and sample blanks. As a calibrator for the measurement of glucose, we used 400 μmol/L glucose dissolved in distilled water.

3. Results and discussion

The high frequency of c.[1726G>A;2065G>A] homozygotes (3.3–3.9%; AA homozygotes) with a very low AαGlu activity (AαGlu pseudodeficiency) critically complicates newborn screening for Pompe disease in Asian populations [6,24,30,31]. Complete separation between affected infants with hardly any residual AαGlu activity and AA homozygotes demands a very sensitive assay. We have followed up on the observation that elimination of hemoglobin by TCA precipitation greatly improves the measurement of the AαGlu activity with 4MU-αGlc substrate [28]. To this end we have compared two different methods to precipitate hemoglobin from the reaction mixture. Fig. 1 shows the results obtained with either TCA or barium hydroxide/zinc sulfate precipitation. While hemoglobin greatly decreases the fluorescence intensity in a dose-dependent manner, both precipitation methods significantly restore the loss of fluorescence intensity. However, we noticed an important difference between the two methods: over the whole range of hemoglobin concentrations the actual fluorescence intensities obtained with the TCA method were 28–34% lower than that with Ba/Zn method. This counter effect of TCA on the free 4MU fluorescence intensity proved highly dependent on the precise analytical conditions (*e.g.*, wavelength and band-pass) and type of the spectrofluorometer (data not shown). Based on these results, we chose the hemoglobin precipitation method with barium hydroxide/zinc sulfate to measure the AαGlu activity in DBSs from 18 Pompe patients, 70 AA homozygotes, 70 c.[1726G; 2065G]/c.[1726A; 2065A] heterozygotes (GG/AA heterozygotes) and 112 c.[1726G; 2065G] homozygotes (GG homozygotes). Comparison of Figs. 2A and B shows that the separation between the patient group and the control groups is greatly improved by application of the Ba/Zn method. The overlap between 11 of the 70 AA homozygotes (15.7%) and the patient group (Fig. 2A + inset) in our previously used procedure was virtually resolved using the Ba/Zn method (Fig. 2B + inset). These data suggest that newborn screening in Asian populations can be improved by applying Ba/Zn precipitation of hemoglobin.

With regard to the definitive diagnosis, it is true that the activities of some AA homozygotes remained very close to the patient range despite the Ba/Zn method. Hence, if large numbers of newborns are subjected to the screening program, some AA homozygotes will still be scored false positive. Fig. 3 illustrates that measuring the activity of AαGlu in cultured fibroblasts using 4MU-αGlc and glycogen as substrates can make the final diagnosis. Others have used a lymphocyte assay for this purpose [31]. Examining 11 Pompe patients and 57 unaffected individuals with three different genotypes including 8 AA homozygotes, 18 GG/AA heterozygotes and 31 GG homozygotes, we found that the three subgroups of unaffected individuals were completely separated from the patient group in both assay methods using either 4MU-αGlc or glycogen as substrate (Fig. 3).

Any attempt to compare the results of existing methods for measuring the activity of AαGlu in DBS using 4MU-αGlc as substrate is complicated by the fact that each laboratory uses its preferred DBS extraction methodology, substrate concentration, incubation time, measuring procedure and activity units. However, *a priori* it is evident

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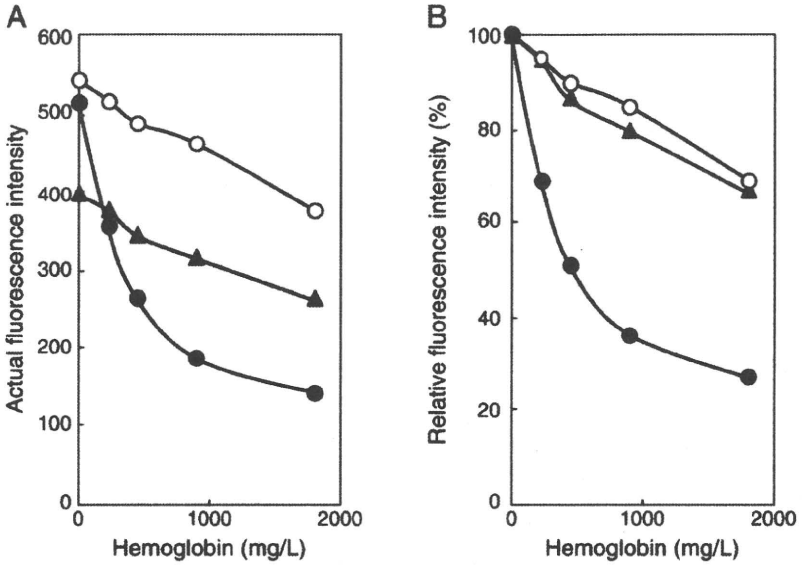

Fig. 1. Effect of hemoglobin elimination on the fluorescence intensity of 4MU. 4MU solution in the presence of different concentrations of hemoglobin (0–1800 mg/L in final reaction mixture) was subjected to two different treatments for hemoglobin elimination, and the fluorescence intensity was assayed as described in the subjects, materials and methods. The symbols represent as follows; open circles, treatment with barium hydroxide and zinc sulfate; closed triangles, treatment with TCA; closed circles, without treatment. The detected fluorescence intensity was expressed as actual reading value (A) and % of those without hemoglobin (B).

that the highest 4MU fluorescence intensities will be measured and the best separation between affected and unaffected individuals will be obtained using the most concentrated DBS samples with nevertheless the lowest hemoglobin concentration. A substrate concentration of at least 2 times the Km of AαGlu for 4MU-αGlc (>2 mmol/L) will further optimize the separation between affected and unaffected individuals. Our new method approaches these ideal conditions since the DBS is not extracted in water like in other procedures and the AαGlu extract is not diluted prior to the incubation with substrate. Instead, the DBS as a whole is immediately immersed in the substrate solution containing a final 4MU-αGlc concentration of 2 mmol/L. All other published procedures use lower substrate concentrations ranging from 0.7 to 1.47 mmol/L. The Ba/Zn method eliminates the negative effect of the relatively high hemoglobin concentration in the DBS extract obtained with our procedure. In other procedures the negative effect of hemoglobin is partially eliminated by the water extraction and sample dilution, but that action leads to reduction of the final output signal in terms of fluorescence units and negatively affects the separation between the activity ranges of affected and unaffected newborns.

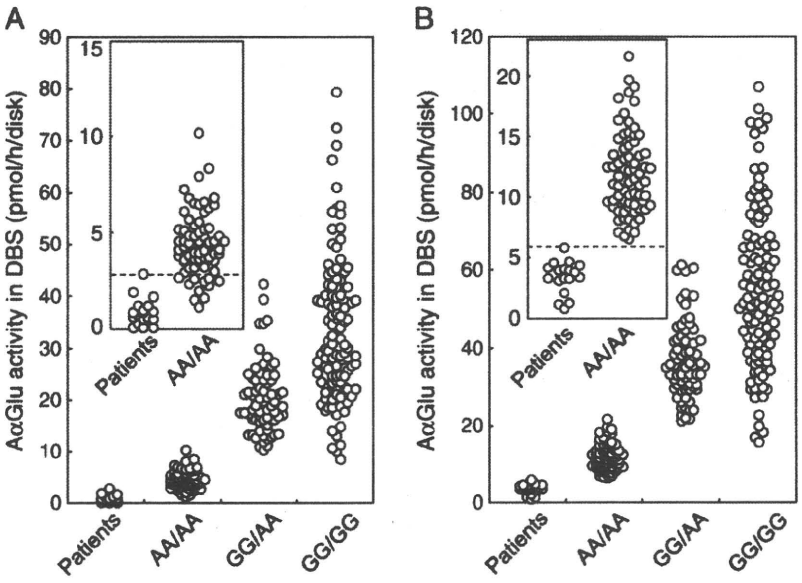

Fig. 2. AαGlu activity in DBSs with the previous and Ba/Zn methods. The AαGlu activity was measured with the previous (A) and Ba/Zn (B) methods with DBSs from 18 Japanese Pompe patients and 252 Japanese healthy newborns [112 GG homozygotes (GG/GG), 70 GG/AA heterozygotes (GG/AA), 70 AA homozygotes (AA/AA)]. The enzyme activities (mean ± SD, pmol/h/disk) with the previous method were 33.3 ± 13.7 for the GG/GG, 20.1 ± 6.7 for the GG/AA, 4.5 ± 1.7 for the AA/AA and 0.77 ± 0.75 for the patients, and the range of the activities were 8.5–79.3 for the GG/GG, 10.2–42.3 for the GG/AA, 1.2–10.2 for the AA/AA and 0–2.8 for the patients. The enzyme activities with the Ba/Zn method were 55.1 ± 20.3 for the GG/GG, 36.6 ± 9.8 for the GG/AA, 11.8 ± 3.4 for the AA/AA and 3.4 ± 1.3 for the patients, and the range of the activities were 15.4–106.9 for the GG/GG, 20.9–61.1 for the GG/AA, 6.4–21.4 for the AA/AA and 0.9–5.9 for the patients. The measurement was performed as described in the subjects, materials and methods, and the data were expressed as an average of duplicate determinations. The inset shows the enlarged distribution of the activities for the patients and AA homozygotes.

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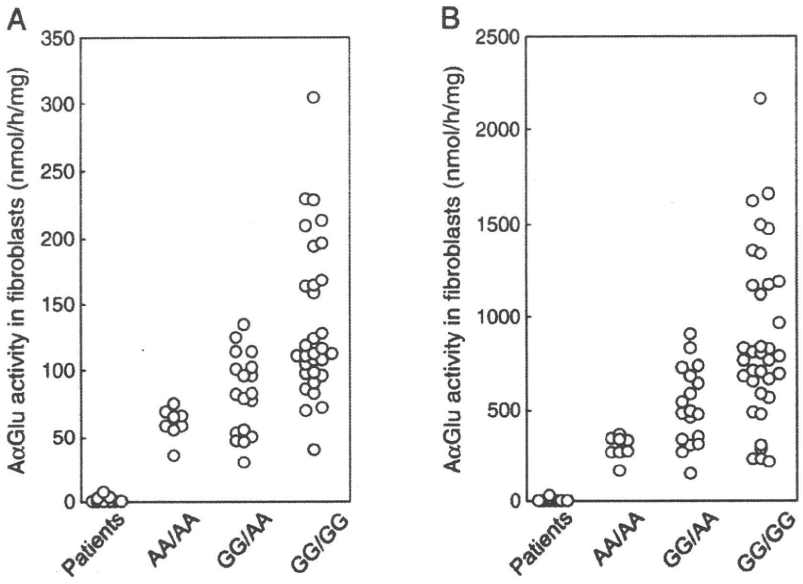

Fig. 3. AαGlu activity in fibroblasts with 4MU-αGlc and glycogen as substrate. The AαGlu activity was measured with 4MU-αGlc (A) and glycogen (B) as substrate in fibroblasts from 11 Japanese Pompe patients and 57 Japanese unaffected individuals (31 GG/GG, 18 GG/AA, 8 AA/AA). The enzyme activities (mean ± SD, nmol/h/mg protein) with 4MU-αGlc were 136 ± 58 for the GG/GG, 82 ± 30 for the GG/AA, 60 ± 12 for the AA/AA and 1.2 ± 2.0 for the patients, and the range of the activities were 40–304 for the GG/GG, 30–134 for the GG/AA, 35–75 for the AA/AA and 0.1–6.6 for the patients. The enzyme activities with glycogen were 954 ± 419 for the GG/GG, 515 ± 207 for the GG/AA, 294 ± 65 for the AA/AA and 3.5 ± 8.4 for the patients, and the range of the activities were 282–2163 for the GG/GG, 146–900 for the GG/AA, 164–364 for the AA/AA and 0–26 for the patients. The measurement was performed as described in the subjects, materials and methods, and the data were expressed as an average of duplicate determinations.

4. Conclusion

We have demonstrated that the elimination of hemoglobin with barium hydroxide/zinc sulfate greatly improves the enzymatic diagnosis of Pompe disease in DBSs. This new method provides the solution for the critical issue of newborn screening for Pompe disease in Asian populations due to high incidence of AA homozygotes with a very low AαGlu activity.

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ARTICLE

Newborn Screening for Lysosomal Storage Disorders

KIMITOSHI NAKAMURA,* KIYOKO HATTORI, AND FUMIO ENDO

Lysosomes are intracellular organelles containing acid hydrolases that degrade biological macromolecules. Lysosomal storage disorders (LSDs) are caused by absent activity of one or more of these enzymes due to mutations of genes encoding lysosomal hydrolases or enzymes that process, target, and transport these enzymes. The specific signs and symptoms of each LSD derive from the type of material accumulated within the lysosome, the site (organ) of accumulation and the response of the body (sometimes in the form of an inflammatory or immune response) to the accumulated material. Interest for inclusion of these disorders in newborn screening programs derives from the availability of effective therapy in the form of enzyme replacement or substrate reduction therapy and bone marrow transplant that may improve long-term outcome especially if started prior to irreversible organ damage. Based on the availability of therapy and suitable screening methods, Gaucher disease, Fabry disease, Pompe disease, mucopolysaccharidosis I and II, Niemann–Pick disease, and Krabbe disease are candidates for newborn screening. Pilot newborn screening projects have been performed for some of these conditions that indicate the feasibility of this approach. This review will provide insight into these screening strategies and discuss their advantages and limitations.

KEY WORDS: tandem mass spectrometry; multiplex assays; mucopolysaccharidosis; Fabry disease; Pompe disease,

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INTRODUCTION

A lysosome is an intracellular organelle containing acid hydrolases that degrade proteins, glycoproteins, proteoglycans, lipids, and other complex macromolecules from phagocytosis, endocytosis, and autophagy [Futerman and van Meer, 2004; Fletcher, 2006; Eckhardt, 2010]. These macromolecules are degraded to smaller molecules through the action of various acid hydrolases. The resulting small molecules are then catabolized or recycled by the cell after export to the cytoplasm by passive diffusion or through the use of transporters. For some pathways, these recycled metabolites play a major role in the synthesis pathway. For example, almost 90% of sphingolipids are synthesized in this recycled pathway in many cells [Fredman, 1998; Gillard et al., 1998]. Lysosomal hydrolases are transported from the endoplasmic reticulum to the lysosome by a vesicular transporter. This vectorial transport is dependent on the presence of mannose 6-phosphate residues on their oligosaccharide chains attached to the lysosomal enzyme by a Golgi-localized phosphotransferase complex [Kollmann et al., 2010]. Mannose-6-phosphate receptors capture these processed enzymes into transport vesicles of the *trans*-Golgi network and deliver them to the lysosome. These enzymes can be endocytosed again by neighboring cells and delivered to the lysosome. This latter pathway plays a key role in allowing enzyme replacement therapy (ERT) to reach the lysosome of target cells.

More than 40 LSD are known and have a total estimated incidence of 1:7,000–1:9,000 [Meikle et al., 1999; Fletcher, 2006]. Symptom severity and disease onset of most LSD vary. This heterogeneity can be explained to some extent by the difference in organs affected and, in part, by the type of mutation. In general, mutations leaving very low residual enzyme activity cause the most severe early onset forms of the diseases. In contrast, higher residual enzyme activity delays disease onset [Kolter and Sandhoff, 1999]. Disease severity and onset are remarkably different in the late-onset forms of LSD and can vary even between siblings with identical mutations [Clarke et al., 1989; Wenger et al., 2000; Zhao and Grabowski, 2002]. The major lysosomal storage disorders (LSDs) for which a therapy is

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available and newborn screening is at different stages of development will be briefly described.

SELECTED LYSOSOMAL STORAGE DISORDERS

Fabry Disease

Fabry disease is an X-linked LSD that was initially described in 1898 [Anderson, 1898; Fabry, 1898]. Women can also have symptoms, but onset is generally later than for men and life expectancy is reported better. Fabry disease is caused by α-galactosidase A (Gal A) deficiency [Desnick et al., 2001]. The enzymatic defect leads to progressive accumulation of glycosphingolipids such as globotriaosylceramide (GL-3), especially in the brain, heart, kidney, eye, and skin. The classic disease phenotype consists of angiokeratomas, acroparesthesias, hypohidrosis, and corneal opacities during childhood. Accumulation of GL-3 in the vascular endothelium leads to renal and cardiac failure and cerebrovascular disease. Late-onset cardiac and renal variants with residual Gal A activity have been identified in individuals lacking some or all of the early classic manifestations mentioned above. Patients with the cardiac variant present with left ventricular hypertrophy (LVH), arrhythmia, and/or cardiomyopathy [Nakao et al., 1995], whereas patients with the renal variant develop proteinuria and end-stage renal disease (ESRD) [Kotanko et al., 2004] after 50 years of age. In addition, some patients with acute strokes after adolescence were found to have previously undiagnosed Fabry disease, 30% of whom had, retrospectively, classic manifestations. Fabry disease is diagnosed by measuring enzyme activity in white cells or plasma in males. Females can have normal enzyme activity and DNA testing is necessary to confirm or exclude the diagnosis in them.

ERT for Fabry disease was approved in Eng et al. [2001] and clinical trials are ongoing for pharmacologic enzyme enhancement therapy [Desnick and Schuchman, 2002]. The estimated incidence of classic Fabry disease is 1 in 50,000 males. Screening of males in hemodialysis, cardiac, and stroke clinics by determination of plasma Gal A activities detected previously undiagnosed Fabry disease in 0.25–1% of males undergoing hemodialysis, in 3–4% of males with LVH or hypertrophic cardiomyopathy, and in 5% of males with acute cryptogenic strokes [Brouns et al., 2010].

Newborn screening using a fluorometric enzyme assay in 37,104 males in Italy with follow-up mutation analysis identified 1 in 3,100 patients with Fabry disease. The mutations identified in this cohort predicted later-onset rather than classic Fabry disease with an 11:1 ratio [Spada et al., 2006]. In Japan, a newborn screening pilot program for Fabry disease has been carried out by Nakamura et al. (submitted for publication) using the fluorometric enzyme assay and subsequent mutation analysis. The incidence of the disease was approximately 1 in 4,700 males, with 88% of mutations being associated with a later-onset phenotype. In Taiwan [Sands and Davidson, 2006], a newborn screening pilot program for Fabry disease using the fluorometric enzyme assay found an incidence of approximately 1 in 1,250 males [Hwu et al., 2009; Lin et al., 2009]. All these studies suggest that Fabry disease may be underdiagnosed, especially the late-onset variants.

Mucopolysaccharidoses

Mucopolysaccharidoses (MPS) are LSDs that are characterized by the accumulation of glycosaminoglycans (GAGs) in urine, plasma, and various tissues. Primary treatment options for MPS include hematopoietic stem cell transplantation (HSCT) and ERT. ERT is now available for MPS I, MPS II, and MPS VI [Kollmann et al., 2010]. ERT reduces GAG accumulation, improves the clinical status and quality of life. Clinical trials of ERT for other types of MPS are underway.

Newborn screening for these conditions can be accomplished by measuring urinary GAG or directly by measuring enzyme activity in blood spots. Methods have been proposed for the quantification and qualitative evaluation of GAGs in urine by LC-MS/MS. This method can screen for MPS I, II, and VI by quantifying dermatan sulfate (DS) and heparan sulfate (HS) in urine.

In blood spots, eight lysosomal enzymes (α-L-iduronidase, iduronate sulfatase, arylsulfatase B, β-D-glucuronidase, β-D-galactosidase, α-D-mannosidase, α-L-fucosidase, and β-hexosaminidase), including those involved in selected MPSs, can be assayed. This can screen for MPS I, MPS II, MPS VI, MPS VII, GM1 gangliosidosis, galactosialidosis, MPS IV B, α-mannosidosis, fucosidosis, Sandhoff disease, and mucolipidosis II and III [Chamoles et al., 2001a,b, 2004]. Unfortunately, there are still no methods described for multiplexing these assays.

More recently, specific substrates have been developed to allow the use of MS/MS [Duffey et al., 2010a,b]. The advantage of this approach is that it allows multiplexing with simultaneous assays for MPS I, MPS II, MPS IIIA, and MPS VI.

Pompe Disease

Pompe disease, also known as glycogen storage disease type II, is an autosomal recessive disorder caused by deficiency

of the enzyme α-glucosidase (GAA), resulting in the accumulation of lysosomal glycogen in the skeletal muscles and heart [Kishnani et al., 2006]. This disorder causes a steady accumulation of glycogen substrate that leads to progressive muscle damage and organ failure. The rates of substrate accumulation and tissue damage are variable and reflect the residual enzyme activity and immune response to the accumulated material. In 2006, alglucosidase alfa was approved as the ERT for Pompe disease. A pilot program for Pompe disease newborn screening was started in Taiwan in 2005 that measures GAA activity using a fluorometric assay [Chien et al., 2009]. A thorough examination was performed to screen positive newborns. A diagnosis of Pompe disease was made clinically after the onset of symptoms. Screening revealed five severely affected infants with an incidence of approximately 1 in 41,000 screened newborns. ERT for Pompe disease was started in the five severely affected infants. In unscreened infants, the clinical diagnosis of Pompe disease was made later, at an average of 4 months of age. Initiation of earlier treatment of infants after newborn screening resulted in normal cardiac function and growth and acquisition of age appropriate milestones.

Krabbe Disease

Krabbe disease (globoid cell leukodystrophy) is an autosomal recessive disorder caused by deficiency of the lysosomal enzyme galactosylceramide β-galactosidase (GALC). This results in the accumulation of galactosylceramide and psychosine that in most cases cause abnormalities of the brain white matter. Most patients present early in life with an early infantile or "classic" phenotype. Symptoms usually appear before 6 months of age and death occurs before 2 years of age. Other patients can present later in life with an attenuated phenotype. HSCT is the only available treatment for infants with early infantile Krabbe disease and must be performed prior to neurodegeneration. Newborn screening has been performed for Krabbe disease [Duffner et al., 2009]. Newborns treated with HSCT can have progressive central myelination and continued gains in developmental skills and cognitive function, whereas children who undergo transplantation after symptom onset experience minimal neurologic improvement. Transplantation is not effective in all cases of Krabbe disease and some transplanted patients have experienced developmental delays. Screening involves GALC activity detection by a fluorescent assay and subsequent DNA mutation analysis. Molecular analysis of the GALC gene is used for diagnostic confirmation.

THERAPEUTIC ADVANCES FOR LYSOSOMAL STORAGE DISORDERS

Hematopoietic Stem Cell Transplantation

Allogenic HSCT was one of the first therapies attempted in LSDs to introduce metabolic cross-correction. Therapy may also be useful for neurodegenerative LSDs because microglia cells are derived from hematopoietic stem cells [Asheuer et al., 2004; Boelens, 2006]. Clinical trials of HSCT have suggested that cells migrate across the blood–brain barrier. In animal models, it has been shown that donor cells produce the defective enzyme and that donor macrophages replace microglial cells in the brain [Kennedy and Abkowitz, 1997; Malatack et al., 2003]. Repopulation of transplanted cells in the brain is relatively slow because of the long lifespan of microglia [Kennedy and Abkowitz, 1997].

HSCT has shown efficacy in presymptomatic or mildly affected patients with some LSDs. It has been used in patients with MPS I, II, and VI; Gaucher disease; Wolman disease; metachromatic leukodystrophy; and Krabbe disease. Each LSD responds differently to HSCT, and transplantation timing relative to symptom onset seems critical for some disorders. HSCT is not effective for the patients with Fabry disease because secreted α-galactosidase lacks mannose-6-phosphate residues and the enzyme is seldom taken up by cells with the enzyme defect. Complications after HSCT are common and limit the usefulness of this treatment. These include graft versus host disease, toxicity of the conditioning regimen, and graft failure.

In addition to HSCT, transplantation of neural stem cells to the brain has been performed in an animal model for LSDs. This was first demonstrated in an MPS VII mouse model by injection of neural stem cells overexpressing β-glucuronidase into the ventricles of newborn mice [Snyder et al., 1995]. Clinical improvement has been observed after neural stem cell transplantation in animal models [Lee et al., 2007; Strazza et al., 2009]. There are no human data for this type of therapy.

Enzyme Replacement Therapy

Marked progress has been made in the treatment of LSDs over the past few decades [Brady et al., 1974; Achord et al., 1978; Brady, 2006]. Recombinant DNA techniques have allowed production of lysosomal enzymes in vitro. The recombinant enzymes are transported via the mannose-6 receptor pathway in Fabry disease, MPS I, II, and VI; and Pompe disease. In contrast,

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they are transported by macrophage mannose receptors in Gaucher disease. The exogenous enzymes are internalized by somatic cells and transferred to the lysosome where they degrade accumulated substrate and diminish the burden of the disease. ERT has been approved by regulatory agencies for Gaucher, Fabry, and Pompe disease in addition to MPS I, II, and VI. Before the introduction of ERT, no specific therapy was available for LSD patients, and supportive care and treatment were used only to manage complications. ERT consists in the regular administration of recombinant enzyme intravenously and since its success in patients with Gaucher disease, was extended to other LSDs. Clinical trials have demonstrated the clinical benefit of ERT in Fabry disease [Eng et al., 2001]; MPS I [Kakkis et al., 2001], II [Muenzer et al., 2006], and VI [Harmatz et al., 2005]; and in Pompe disease [Amalfitano et al., 2001].

The usefulness of ERT is limited because the enzyme is not always effective for all clinical symptoms. Clinical studies have shown that many symptoms of LSDs are irreversible in advanced cases despite the use of long-term ERT. Therefore, early diagnosis and treatment is important. In addition, recombinant proteins cannot cross the blood–brain barrier, and ERT has little or no effect on central nervous system (CNS) manifestations. Current clinical trials are assessing the effect of intrathecal enzyme replacement in MPS I and II.

Substrate Reduction Therapy

Substrate reduction therapy partially inhibits the biosynthesis of the accumulated product to reduce substrate influx into the catabolically compromised lysosome. A small-molecule oral substrate reduction therapy, miglustat, is available for Gaucher disease. The efficacy of substrate reduction therapy was evaluated in patients with Gaucher disease [Cox et al., 2000]. Adult Gaucher patients not treated with ERT were treated with *N*-butyldeoxynojirimycin for 12 months. Mean liver and spleen volumes were significantly decreased, and hematological parameters showed slight improvement. The most frequent adverse effect was diarrhea. In the extension study, statistically significant improvement was achieved in all major efficacy end points, indicating that treatment with *N*-butyldeoxynojirimycin was increasingly effective with time [Elstein et al., 2004]. The use of *N*-butyldeoxynojirimycin, known as miglustat (Zavesca), has been approved for Gaucher disease and is considered safe for adult patients, with mild or moderate symptoms, who are unwilling or unable to receive or to continue ERT or for patients with persistent signs of disabling disease activity despite maximal enzyme dosing. The drug may be applied in combination with ERT in these patients.

N-Butyldeoxynojirimycin is also considered an option for patients with Sandhoff disease, Tay–Sachs disease, or Niemann–Pick disease type C (NPC) because the drug is a small enough to cross the blood–brain barrier [Lachmann et al., 2004]. The drug is usually given at higher doses than in Gaucher disease to allow increased entry into the brain. A randomized clinical trial in patients with NPC demonstrated that miglustat improves or stabilizes horizontal saccadic eye movement velocity, a clinically relevant markers of NPC, with improvement in swallowing capacity, stable auditory acuity, and a slower deterioration in ambulatory index [Patterson et al., 2007; Wraith et al., 2010]. An open-label extension confirmed the persistence of clinical benefit that is more marked in patients with milder forms of the disease A lower dose of this drug was not effective in late-onset Tay Sachs [Shapiro et al., 2009]. Nevertheless, further developments in this area have the potential of developing effective an treatment for this condition.

Chemical Chaperons

Chemical chaperones can enhance the residual activity of the defective lysosomal enzyme. Imino sugars, such as deoxynojirimycin can act as both enzyme inhibitors and chaperones, which control the quality of newly synthesized proteins [Sawkar et al., 2002; Fan, 2008]. Under physiological conditions, chaperones help restore the native conformation of misfolded proteins. Chaperone therapy by using small molecules to stabilize and target a misfolded enzyme to the lysosome is in clinical trial for Gaucher, Fabry, and Pompe diseases caused by mutated but catalytically active enzymes. In animal models, these small molecules cross the blood–brain barrier and may be effective for CNS manifestations of LSDs. *N*-(*n*-nonyl)deoxynojirimycin for Gaucher disease and 1-deoxygalactonojirimycin for Fabry disease are good examples of chemical chaperones that show satisfactory response in vitro [Sawkar et al., 2002; Yam et al., 2005]. A similar effect was observed in fibroblasts from adult patients with Tay–Sachs disease and Sandhoff disease [Tropak et al., 2004]. Chemical chaperones may be therapeutically useful for treatment of various LSDs, although they are currently experimental and none is approved for the treatment of any LSD.

Gene Therapy

Many LSDs respond to HSCT and are excellent candidates for gene transfer therapy [Sands and Davidson, 2006], since they are generally well-characterized single gene disorders, the enzymes defective are usually not subject to complex regulation mechanisms, and enzyme activity even only a little higher than normal should be clinically sufficient. In vivo and ex vivo gene therapy techniques have been developed to administer the gene to defective organs in LSD animal models via the bloodstream or directly to the brain. Gene therapy using adenoassociated viral (AAV) or lentiviral vectors has been tested in small animal models of LSDs and resulted in normalized enzyme activity [Cachon-Gonzalez et al., 2006; Broekman et al., 2007]. However, gene therapy was initiated before the appearance of clinical symptoms in these studies. Testing in large animal models of LSDs is under current study [Haskins, 2009]. After intracerebral injection of

AAV-encoding human arylsulfatase A (ASA) into nonhuman primates. ASA expression could be detected [Colle et al., 2010]. The wide distribution of enzyme expression appears to be mediated by axonal transport and secretion by transduced neurons. At present, gene therapy in humans with their much larger brains has yet to be initiated.

SCREENING FOR DISEASES

Newborn Screening

Newborn screening for metabolic disorders started with Robert Guthrie's study of phenylketonuria (PKU) in the early 1960s. After demonstration that early diagnosis and therapy could prevent mental retardation in PKU, neonatal screening has become routine practice in developed countries as part of a public health program [Guthrie and Susi, 1963; Scriver and Kaufman, 2001]. Newborn screening identifies a high-risk group of patients from normal infants and then thoroughly investigate this group. Initial tests screened for one disorder at a time. The introduction of screening by tandem mass spectrometry permits the measurement of multiple analytes at the same time, allowing the detection of multiple classes of metabolic disorders.

The potential use of MS/MS for newborn screening was first suggested in 1990 [Millington et al., 1990], and early studies soon demonstrated its practicality [Chace et al., 1993; Rashed et al., 1995; Ziadeh et al., 1995]. MS/MS could simultaneously detect a number of disorders, making it possible to screen for some disorders that might otherwise have seemed too rare. Many compounds are initially separated by mass to charge ratio in MS/MS. Each compound is then fragmented for identification. The process requires roughly 2 min per sample and can detect 30 or more inborn errors of metabolism just screening for amino acids and acylcarnitines. At the present time, expanded screening is used to detect disorders of amino acid, organic acid, and fatty acid metabolism. However, the technology can be applied to a much wider range of compounds, and the field appears ready to expand. Table I summarizes the enzymes defective in several LSDs and those for which newborn screening assays have been developed.

Advances in Newborn Screening Technologies for LSD

Enzymatic assays. The initial system to diagnose LSD was the measurement of enzyme activity using a fluorescent artificial substrate [Meikle et al., 2006]. Diagnosis of MPS I is performed on leukocyte or cultured fibroblast homogenates to assay α-L-iduronidase activity by using 4-methyhylumbeliferyl-L-iduronide. For newborn screening, the standard method was adapted to measure α-L-iduronidase activity in dried blood spotted on filter paper [Chamoles et al., 2001a]. A 3-mm-diameter punchout of a blood spot on filter paper is added to elution buffer containing 4-methyhylumbeliferyl-α-L-iduronide as the substrate. Fluorescence of the enzyme product 4-methylumbelliferone is then measured. Methods for detection of other LSD, including MPS II, Pompe, Fabry, Sandhoff, Gaucher, Niemann–Pick (type A/B, not C), and Tay–Sachs diseases have been reported using the revised enzymatic assay of dried blood spot samples [Chamoles et al., 2001b, 2004]. The limitation of these approaches is that each assay uses 4-methylumbelliferone as an indicator of enzyme activity. In these assays, multiplexing is not possible because all assays (for MPS I, MPS II, Pompe, Fabry, Sandhoff, Gaucher, Niemann–Pick, and Tay–Sachs diseases) yield the same product (4-methylumbelliferone) as the fluorescent product of the enzyme reaction.

A variation of this approach includes the use of antibodies to enrich for the enzyme to be tested. In the case of Pompe disease, antibodies against GAA are used to coat microtiter plates. The endogenous GAA from the dried blood spots is eluted, attaches to the antibodies and is assayed for enzyme activity using fluorescent substrate [Umapathysivam et al., 2000]. Hypothetically, microtiter plates could be coated with several different primary antibodies to capture different endogenous enzymes. However, if all of the substrates produce the same fluorescent enzyme product (4-methylumbelliferone), then multiplexing is not possible. These limitations would work against practical newborn screening using this method.

Functional Detection of Enzymatic Products by Using MS/MS

The second advancement in LSD screening technology involves analyzing the activity of endogenous lysosomal enzymes with electrospray ionization-MS/MS [Gerber et al., 2001; Li et al., 2004]. This method, modified from the one for cell lysates for use with dried blood spots, was used in Krabbe disease to detect galactocerebroside β-galactosidase (GALC) activity. The substrate β-Gal-C8-Cer is broken down by GALC to C8-Cer by the enzyme eluted from the dried blood spots. Both C8-Cer and C10-Cer, which is used as an internal standard, are quantified using MS/MS to detect GALC activity. The GALC enzyme on the dried blood spots is stable, allowing for sample transportation. A pilot program for Krabbe disease screening using MS/MS was started in 2006 [Orsini et al., 2009]. Out of 555,000 newborns, 10 were identified at risk for Krabbe disease. MS/MS has the advantage of being able to detect products of different mass to change ratio enabling the analysis of the results of different enzyme reactions. In theory, multiplexed assays can be developed for multiple diseases, including Pompe, Fabry, Gaucher, Niemann–Pick types A/B (NP A/B), Krabbe disease, and MPS-I [Zhang et al., 2008] and for five of them a multiplex assay has been proposed [Gelb et al., 2006]. In reality, the amount of activity measurable in a single blood spot is still limited. The assay for Pompe, Fabry and MPS-I can already be performed on the same blood spot [Duffey et al., 2010a]. MS/MS assays for blood spots have also been reported for MPS-VI [Duffey et al.,

TABLE I. Lysosomal Storage Disorders Amenable to Newborn Screening

Disease	Protein defect	Availability of screening strategies	Chromosomal localization	OMIM
Defects in glycosaminoglycan degradation (mucopolysaccharidoses)				
MPS I (Hurler, Scheie)	α-Iduronidase	Fluorometric, immune-quantification, multiplex	4p16.3	607015
MPS II (Hunter)	Iduronate sulfatase	Fluorometric, immune-quantification, multiplex	Xq28	309900
MPS IIIA (Sanfflippo A)	Heparan *N*-sulfatase	Immune-quantification, multiplex	17q25.3	252900
MPS IIIB (Sanfflippo B)	*N*-Acetylglucosaminidase	None	17q21	252910
MPS IIIC (Sanfflippo C)	Acetyl-CoA transferase	None	8p11.1	252930
MPS IIID (Sanfflippo D)	*N*-Acetylglucosamine-6-sulfatase	None	12q14	252940
MPS IVA (Morquio A)	*N*-Acetylgalactosamine-6-sulfatase	None	16q24.3	253000
MPS IVB (Morquio B9)	β-Galactosidase	None	3p21.33	230500
MPS VI (Maroteaux–Lamy)	*N*-Acetylgalactosamine-4-sulfatase	Fluorometric, MS/MS, immune-quantification, multiplex	5q11–13	253200
MPS IX	Hyaluronidase	None	3p21.3	601492
Defects in glycoprotein degradation (oligosaccharidoses)				
α-Mannosidosis	α-Mannosidase	None	19q12	248500
β-Mannosidosis	β-Mannosidase	None	4q22	248510
α-Fucosidosis	α-Fucosidase	None	1q34	230000
Sialidosis	α-Sialidase	None	6p21.3	608272
Galactosialidosis	Cathepsin A	None	20q13.1	256540
Aspartylglucosaminuria	Aspartylglucosaminidase	None	4q32	208400
Schindler disease, Kanzaki disease	α-Acetylglucosaminidase	None	22q13.1	104170
Others				
GM1-gangliosidosis	β-Galactosidase	None	3p21.33	230500
GM2-gangliosidosis (Tay–Sachs)	α-Subunit of β-hexosaminidase	Fluorometric	15q23	606869
GM2-gangliosidosis (Sandhoff)	β-Subunit of β-hexosaminidase	Fluorometric	5q13	606873
GM2-gangliosidosis (variant AB)	GM2 activator protein	None	5q31	272750
Gaucher disease	β-Glucocerebrosidase	Fluorometric, MS/MS, immune-quantification, multiplex	1q21	606463
Fabry disease	α-Galactosidase	Fluorometric, MS/MS, immune-quantification, multiplex	Xq22.1	301500
Pompe disease	Acid α-glucosidase	Fluorometric, MS/MS, immune-quantification, multiplex	17q25.2–q25.3	232300
Niemann–Pick type A and B	Sphingomyelinase	Fluorometric, MS/MS, immune-quantification, multiplex	11p15.2	607808
Krabbe disease	Galactosylceramidase	Fluorometric, MS/MS, immune-quantification, multiplex	14q31	245200

2010b] and Gaucher disease [Legini et al., 2011]. One issue with newborn screening is the identification of patients whose phenotype is not clear. For example, most patients identified by screening for Fabry disease have late-onset variants [Spada et al., 2006] and it is unclear whether they would have had clinical symptoms without treatment.

SUMMARY

Newborn screening is a major public health achievement that has improved the morbidity and mortality of inborn

errors of metabolism. The introduction of newborn screening for LSDs presents new challenges. The first is to be able to design a multiplex assay for multiple enzymes applicable to the limited amount of enzyme present in blood spots. These new assays must be validated in large numbers of newborns to confirm sensitivity and specificity. The second challenge is to have a better understanding of which forms of these diseases need treatment. This will allow us to determine if and when to start therapeutic interventions. In the absence of a family history, presymptomatic detection of an LSD can be achieved only through a newborn screening program. The efficacy and cost of the currently available therapies and the detection in newborns of diseases with later onset, often in adulthood, may raise ethical issues. The advancement of therapeutic options for treatment of LSD, especially in the field of small molecules, capable of entering he brain offers new hopes to affected patients in whom a timely diagnosis will become even more essential.

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