Table 2 A VLCAD activity assay[†]

Subject	Palmitoyl-CoA dehydrogenase activity (pmol/min/10^6 lymphocytes)
Patient	0.42
Control	25.1
Normal (n = 31)	54.5 ± 17.5

[†]Using lymphocytes and palmitoyl-CoA as a substrate. VLCAD, very-long-chain acyl-coenzyme A (CoA) dehydrogenase.

These data suggested that the newborn patient may have very-long-chain acyl-coenzyme A (CoA) dehydrogenase (VLCAD) deficiency; enzyme assay[1] was then performed. Palmitoyl-qCoA dehydrogenase activity in lymphocytes was reduced to approximately 1% that of the mean in normal control subjects (Table 2). The diagnosis of VLCAD deficiency was confirmed on these findings. Gene analysis identified a homozygote c.1332G> A mutation in the exon–intron junction of the acyl-CoA dehydrogenase, very-long-chain (ACADVL) gene (Fig. 1b), indicating a splicing abnormality. After confirming the diagnosis, the patient had normal development with long-term dietary therapy and supplementation of L-carnitine and medium-chain triglyceride (MCT) oil. After the neonatal period, echocardiography and ECG were normal. Vomiting and diarrhea were sometimes associated with metabolic acidosis, but the patient recovered quickly after rapid transfusion of glucose and electrolytes. The day before his death at 2 years old, he had cough and low-grade fever. He presented to the emergency department, which he often visited for regular treatment. He was diagnosed as having respiratory syncytial virus (RSV) infection according to the RSV detection kit. Given that his respiratory condition was satisfactory and blood gas analysis was normal, it was decided that hospitalization was not necessary. He was therefore returned his home with some medicine for cough and fever, but he had only half the usual quantity of MCT milk that night. He coughed and woke up early in the morning, and he was conscious until just before the attack. His breathing sounded normal, then he suddenly stood up and fell down and became unconscious. He seemed to be in cardiopulmonary arrest when he arrived at the emergency department. After efforts at resuscitation we confirmed his death at the hospital. A cardiogenic cause, particularly arrhythmia, was most likely the cause of the sudden death. The death was too sudden to be due to breathing problems or brain lesion. Because there was no sign of vomiting, no sign of abuse, no congestion due to suffocation, we speculated that his death was due to arrhythmia induced by VLCAD deficiency.

Discussion

VLCAD deficiency is an autosomal recessive disorder and the prevalence of VLCAD deficiency has been estimated to be 1 in 150 000. The phenotype of VLCAD deficiency is heterogeneous. It is classified into three clinical forms on the basis of the onset of symptoms: a severe form with neonatal onset; a milder form with childhood onset; and a late-onset form. The neonatal form is the most common, and patients present with cardiomyopathy, hepatopathy, and skeletal myopathy. This form has a higher mortality rate than the others.[2] VF and respiratory arrest have been reported in patients who develop VLCAD deficiency within 1 year of birth.[3] In the present case, the patient developed VF and was rescued by cardiopulmonary resuscitation, because the pediatrician was at his bedside during the development of VF. When the patient was transferred to NICU, metabolic acidosis was improved by glucose transfusion. First, we suspected mitochondrial disease and secondary cardiac disorder. MS/MS was very useful for the final diagnosis of VLCAD deficiency.

In Kumamoto, MS/MS analysis was initiated as a pilot study 5 years ago, and MS/MS was introduced for mass screening of newborns with approximately 100% agreement. Because clinical manifestations in the present case were observed 2 days after birth, the patient was not covered by standard screening. The abnormality was detected only when post-symptom high-risk screening was performed. Elevations in C14:1, C16, and C16+18/C2 were identified on MS/MS, and VLCAD deficiency was suspected. At this point, the patient was given MCT milk and carnitine. Next, we performed a fatty acid β-oxidation assay and found that the metabolism of C14 to C12 was abnormal. We also performed a VLCAD enzyme assay and ACADVL gene analysis.[1] Palmitoyl-CoA dehydrogenase activity in the present patient was found to be severely decreased. Molecular analysis of the ACADVL gene encoding VLCAD showed that the patient had a single base mutation, c,1332G>A, at the exon–intron junction. To the best of our knowledge, this case presents a novel mutation. We examined the sequences by calculating splicing score (http://www.fruitfly.org/seq_tools/splice.html). In the normal sequence (CTTCATGAAGGTACAGGACGGT), splice site was recognized with donor score 0.9. The false-positive (FP) rate and the correlation coefficient (CC) were 1.1% and 0.73. In the present patient's sequence (CTTCATGAAAGTACAGGACGGT), the splicing was not recognized. As a result of the mutation, abnormal splicing of the mRNA would occur. We assumed that it was a mutation causing exon-skipping or connection to a new junction.[4]

An inborn error in metabolism is one of the differential diagnoses of unknown cardiomyopathy or arrhythmia. In this case, MS/MS was insufficient for preclinical diagnosis because of the delayed time of sampling to detect early-onset VLCAD, but it was very useful for accurate diagnosis.[5] It is possible to prevent secondary complications of VLCAD with intake of MCT milk and carnitine supplementation and with diet therapy. In past reports, patients surviving the initial episode have nearly normal cardiac function by avoidance of fasting, and using a low-fat diet with frequent meals and vigilance during intercurrent illness.[6] We can expect normal development with careful follow up for most patients.[7] It is important to start a glucose infusion, not only in cases of gastroenteritis and starvation, but also in cases of general infection with the potential for exacerbation.

The prognosis for control of VLCAD deficiency is very challenging, even after successful resolution of several crises. Early institution of i.v. glucose treatment may be important to

reduce the frequency and severity of life-threatening episodes. In addition, active immunization with vaccine, such as palivizumab (anti-RSV mAb), might be necessary.

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Transverse myelitis and acute motor sensory axonal neuropathy due to *Legionella pneumophila*: A case report

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Abstract Guillain–Barré syndrome is a rapidly progressive symmetrical muscle weakness associated with acute inflammatory disease. Transverse myelitis (TM) is the inflammation of the spinal cord characterized by rapidly evolving muscle weakness in the lower extremities, defects in sensory level and sphincter dysfunction. Guillain–Barré syndrome, and TM association occurs very rarely in childhood. A 7-year-old girl presented with complaints of neck pain, spout-style vomiting, cough, shortness of breath, and acute paraparesis with sensory and sphincter disturbance. The patient was intubated because of increased respiratory distress. A positive direct fluorescein antigen test in bronchoalveolar lavage confirmed *Legionella pneumophila* infection. Imaging and neurophysiologic studies were diagnostic for TM with acute motor and sensory axonal neuropathy. She was treated with a combination of high-dose methylprednisolone and intravenous immunoglobulins, and we observed incomplete recovery. The presented case is the first child with concomitant TM and acute motor and sensory axonal neuropathy related to *L. pneumophila* infection.

Key words acute motor and sensory axonal neuropathy, child, immune modulation, *Legionella pneumophila*, transverse myelitis.

Demyelinating disorders can affect any part of the nervous system. Transverse myelitis (TM), which is characterized by focal spinal cord inflammation, may be idiopathic, parainfectious or disease-associated. Diseases associated with TM include demyelinating conditions and connective tissue disorders. Apart from TM attributable to direct spinal cord infection, TM is autoimmune. Demyelination limited to the spine is known as TM, whereas radicular and peripheral nerve demyelination is recognized as the acute inflammatory demyelinating form of Guillain–Barré Syndrome (GBS).[1,2] Acute motor and sensory axonal neuropathy (AMSAN), a subtype of GBS, is an autoimmune and usually post-infectious disease characterized by endoneurial inflammation with both primary demyelination and axonal degeneration.[2]

To our knowledge, this is the first presentation of a child with simultaneous TM and AMSAN related to *Legionella pneumophila* infection in the English-language medical literature.

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p.E66Q mutation in the *GLA* gene is associated with a high risk of cerebral small-vessel occlusion in elderly Japanese males

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See editorial by Meschia on page 3.

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Background and purpose: *GLA* is the causative gene of Fabry disease, an X-linked lysosomal storage disorder resulting from α-galactosidase A (α-GAL) deficiency. Stroke is an important manifestation of Fabry disease, and recent epidemiological studies have indicated that up to 4.9% of young male cryptogenic stroke patients have *GLA* mutations. To determine the importance of *GLA* mutations in the general stroke population, the frequency of *GLA* mutations in Japanese male ischaemic stroke (IS) patients with various risk factors and ages was measured.

Methods: A total of 475 male IS patients (mean age 69.7 ± 12.5 years), were enrolled in this study. A blood sample was obtained to produce blood spots for measurement of α-GAL activity. Blood samples with decreased enzymatic activity were reassayed and the entire *GLA* gene was analyzed by direct DNA sequencing if α-Gal A activity was consistently low.

Results: α-Gal A activity was decreased in 10 men, five of whom (1.1%) had the *GLA* gene mutation, p.E66Q. All IS patients with p.E66Q mutation had substantial residual α-Gal A activity, in contrast to patients with classic-type Fabry disease. Clinically, all patients with p.E66Q mutation were > 50 years old and had multiple small-vessel occlusions (lacunar infarctions). Statistical analysis using Fisher's exact test showed the allele frequency of *GLA* p.E66Q in patients with small-vessel occlusion to be significantly higher than that in the general Japanese population [odds ratio (OR) = 3.34, $P = 0.025$).

Conclusions: *GLA* p.E66Q mutation is a genetic risk factor for cerebral small-vessel occlusion in elderly Japanese males.

Introduction

Fabry disease (MIM301500) is an X-linked lysosomal storage disorder resulting from deficiency of α-galactosidase A (α-Gal A) [1]. The enzymatic defect leads to progressive accumulation of globotriaosylceramide (GL-3) and related glycosphingolipids in the vascular endothelial lysosomes of the kidneys, heart, brain, and skin. Affected males who have little or no detectable α-Gal A activity exhibit the classic phenotype with onset of angiokeratoma, acroparesthesia, and hypohidrosis in childhood. With advancing age, the occurrence of renal failure, cardiac disease, and stroke lead to a decline in activities of daily living and premature death. Stroke is one of the major complications of classic Fabry disease [2] and has been described in 6.9–24.2% of patients [3–5]. Estimates of the prevalence of classic Fabry disease vary from 1 in 40 000 to 1 in 60 000 [1,6].

On the other hand, patients with substantial levels of residual α-Gal A activity have late-onset milder phenotypes, including renal [7] and cardiac [8] variants. Recent studies involving newborn screening for α-Gal A activity in Fabry disease showed surprisingly high incidences of mutations of 1 in 1250–3100 male infants [9,10]. Most mutations found in newborn screening were associated with the late-onset variant phenotype, suggesting that many patients with these

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GLA mutations are under-diagnosed. Screening for Fabry disease in high-risk populations identified previously undiagnosed Fabry patients in 0.2–1.2% of males undergoing hemodialysis [7,11,12] and 0.9–4% of males with left ventricular hypertrophy or hypertrophic cardiomyopathy [8,13,14]. Furthermore, the prevalence of unrecognized Fabry disease in young male patients with stroke was reported to be up to 4.9% [15–20]. However, most stroke patients are elderly and there has been only one population-based study in unselected patients with stroke [21]. It is likely that *GLA* mutation is itself a risk factor for accelerated atherosclerosis and cardiac and renal disease, which can lead to emboli and hypertension, and therefore unrecognized Fabry patients may be found amongst elderly stroke patients [22]. To determine the importance of *GLA* mutations in the general stroke population, the frequency of *GLA* mutations in Japanese male ischaemic stroke (IS) patients with various risk factors and ages was measured.

Methods

Patients

Fifteen clinical neurology departments in Nagano prefecture, Japan, participated in this prospective cross-sectional study. From August 2007 to December 2011, 475 male patients aged 20–91 years (mean ± SD, 69.7 ± 12.5 years), presenting consecutively at a participating neurology department with IS, were enrolled in this study. Patients who were unable to provide informed consent or who had already been diagnosed with Fabry disease were excluded from the study. This study was approved by the Ethical Committee of Shinshu University School of Medicine and the ethics committees of each of the participating clinical neurology centers, and written informed consent was obtained from each patient prior to enrollment. After informed consent was obtained, demographic data, cerebrovascular risk factors, presence of signs and symptoms of Fabry disease, and clinical and neuroimaging data were registered in a database using case report forms. Assessment of clinical symptoms and signs suggestive of Fabry disease was performed in all patients with *GLA* gene mutations. Screening for angiokeratoma was performed by routine clinical examination, and the presence of acroparesthesia and hypohidrosis was determined by anamnesis. In addition, cardiac function tests, including serum brain natriuretic peptide (BNP) and human atrial natriuretic peptide (hANP) concentrations, chest roentgenography, electrocardiography, and echocardiography, and renal function tests, including routine urine test and determination of serum creatinine and blood urea nitrogen (BUN) levels, were performed in all patients with *GLA* mutation.

α-Gal A enzyme assay and mutation analysis

A blood sample was obtained for production of blood spots for measurement of α-Gal A activity. α-Gal A activity was determined using a fluorescent substrate as described previously [23]. Briefly, 40 μl of McIlvan buffer (0.1 M citrate, 0.2 M NaH_2PO_4, 36.8:63.2, pH 6.0) was added to each well of 96-microwell plates. Three-millimeter punch specimens of dried blood spots were added to the buffer and processed for extraction at room temperature for 2 h. Aliquots of 30 μl of blood extract were transferred to fresh 96-microwell plate. An aliquot of 100 μl of the reaction mixture (3.5 mM 4-MU galactosylpyranoside, 100 mM citrate, 200 mM phosphate, 100 mM *N*-acetylgalactosamine) was added to each well of the microwell plates and incubated at 37°C for 24 h. The reaction was terminated with 150 μl of termination solution (300 mM glycine, NaOH, pH 10.6) immediately after the reaction. Fluorescence intensity from the 4-methylumbelliferones in the wells was measured with a fluorescence plate reader (BIO-TEK, Winooski, VT, USA) at 450 nm. One unit (1 AgalU) of enzymatic activity was equal to 0.34 pmol of 4-methylumbelliferyl-D-galactopyranoside cleaved/h per disc. Blood samples with decreased enzymatic activity (< 17 AgalU) were reassayed.

If blood αGal A activity was consistently low, the entire *GLA* gene was analyzed. For DNA analysis, total genomic DNA was extracted from leukocytes of patients. All seven exons and the flanking intronic sequences of the *GLA* gene were amplified by PCR, and the amplification products were analyzed by direct sequencing (Fig. 1).

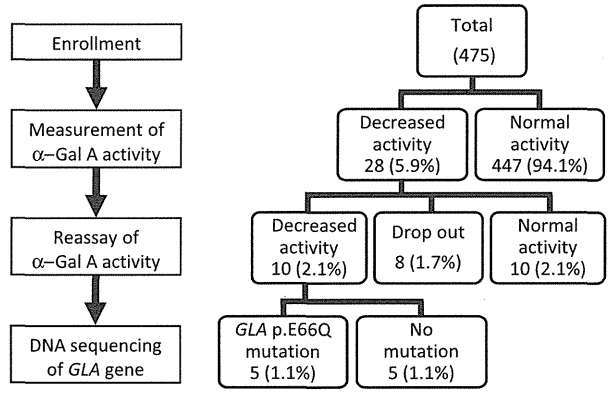

Figure 1 Flowchart for the present study.

Results

Clinical data of enrolled patients

Table 1 summarizes the demographic characteristics of the enrolled patients. The mean age (±SD) in the cohort of 475 patients participating in this study was 69.7 ± 12.5 years. Stroke etiology was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria [24] as large-artery atherosclerosis in 114 patients (24.0%), cardioembolism in 79 (16.6%), and small-vessel occlusion (lacunar infarction) in 240 (50.5%). Stroke of other determined etiology was present in 12 patients (2.5%): cervicocephalic arterial dissection ($n = 4$) and paraneoplastic coagulopathy ($n = 8$). Stroke of undetermined etiology was present in 61 patients (12.8%). Subtypes of IS overlapped in 31 patients, as they had histories of multiple ISs with different subtypes.

Diagnostic test results

The average α-Gal A activity of the study population was 27.7 ± 10.7 AgalU. The distribution of α-Gal A activity in the whole study population is shown in Fig. 2. Initial screening for α-Gal A activity in blood spots from 475 male patients with stroke detected 28 (5.9%) patients with enzyme level below the normal cut-off value of 17.0 AgalU. A repeat blood spot was obtained from 20 patients. When retested, 10 (2.1%) patients had α-Gal A activities < 17.0 AgalU, whereas the other 10 (2.1%) had normal enzyme activities (≥ 17 AgalU). DNA sequencing of *GLA* was performed in these 10 doubly screened-positive patients with low α-Gal A activity, and *GLA* gene mutation was identified in five (1.1%) patients. All five patients had the same missense mutation, a single base sequence change (c. 196G>C), causing substitution of a glutamate reside with glutamine at codon 66 (p.E66Q). The average α-Gal A activity of the stroke patients with the p.E66Q mutation was 11.3 ± 1.6 AgalU (range 9.8–13.5 AgalU), which was relatively high compared with patients with classic-type Fabry disease.

Clinical data of patients with *GLA* p.E66Q mutation

The clinical, biochemical, and molecular features of the patients with the *GLA* p.E66Q mutation are summarized in Table 2. All patients with the p.E66Q mutation were older than 50 years, with a mean age of 69.6 ± 12.5 years. All patients had multiple small-vessel occlusions (Fig. 3), which were accompanied by white matter lesions (leukoaraiosis) in three patients

Table 1 Demographic and characteristics of the patient population

		Subtypes of IS[a]				
	All IS	Large-artery atherosclerosis	Cardioembolism	Small-vessel occlusion	Stroke of other determined etiology	Stroke of undetermined etiology
Number of patients	475	114	79	240	12	61
Age (mean ± SD)	69.7 ± 12.5	70.4 ± 11.0	74.1 ± 12.9	68.5 ± 12.1	67.3 ± 17.2	73.6 ± 13.2

[a]Subtypes of IS overlapped in 31 patients, as they had episodes of multiple IS with different subtypes.

Figure 2 The distribution of α-galactosidase A (α-Gal A) activity in all the patients included in this study. The *x*-axis indicates age (years) and the *y*-axis indicates α-Gal A activity (AgalIU). The cut-off α-Gal A activity was 17 AgalIU. Filled diamonds, open triangles, open squares, and open circles indicate individuals with normal enzymatic activity in dried blood spot screening, low enzymatic activity without *GLA* gene mutations, low enzymatic activity without DNA analysis, and low enzymatic activity with *GLA* p.E66Q mutation, respectively.

Table 2 Clinical features of patients with *GLA* gene mutation

Patient	Age	Sex	α-Gal A activity (AGalU; normal > 17)	*GLA* gene mutation	Type of cerebral infarction		Cerebral hemorrhage	Risk factor for stroke	Other complications of Fabry disease
1	70	M	11.6	p.E66Q (c.196G>C)	Multiple	Lacunar	Thalamic hemorrhage	–	–
2	83	M	12.0	p.E66Q (c.196G>C)	Multiple	Lacunar/ atherothrombotic/ leukoaraiosis	Putaminal hemorrhage	Hypertension (good control)	–
3	76	M	13.5	p.E66Q (c.196G>C)	Multiple	Lacunar/leukoaraiosis	Multiple microbleeds	–	–
4	66	M	9.8	p.E66Q (c.196G>C)	Multiple	Lacunar/leukoaraiosis	Symptomatic hemorrhage[a]	–	–
5	50	M	9.8	p.E66Q (c.196G>C)	Multiple	Lacunar	–	Hypertension (good control)	–

α-Gal A, α-galactosidase A;
[a]Location of the hemorrhage was unknown.

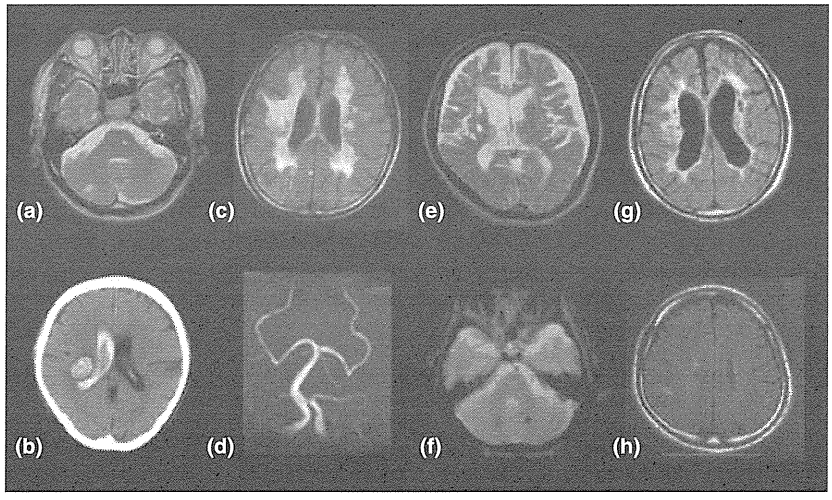

Figure 3 Brain MRI and CT findings of patients with *GLA* p.E66Q. (a, b) Patient 1. T2-weighted MRI showed small-vessel occlusions in the right cerebellar hemisphere and pons (a). Brain CT showed large left thalamic hematoma with rapture into the lateral ventricle (b). (c, d) Patients 2. Fluid-attenuated inversion recovery (FLAIR) MRI showed multiple small-vessel occlusions accompanied by marked leukoaraiosis (c). Magnetic resonance angiography (MRA) showed moderate dolichoectasia of the basilar and vertebral arteries (d). (e, f) Patient 3. T2- (e) and T2*-weighted (f) MRI showed multiple small-vessel occlusions and microbleeds in the cerebral white matter and basal ganglia. Microbleeds were also observed in the dentate nucleus. (g, h) FLAIR MRI of patient 4 (g) and patient 5 (h) showed multiple small-vessel occlusions in the cerebral white matter. Leukoaraiosis was also observed in patient 4 (g).

(Fig. 3c,e,g). Intracerebral hemorrhage was observed in four patients. Three patients had a history of symptomatic intracerebral hemorrhage (Fig. 3b) and the other patient had asymptomatic multiple microbleeds detected by T2*-weighted MRI (Fig. 3f). Two patients took low doses of aspirin when they developed cerebral hemorrhage. Vertebrobasilar dolichoectasia was observed in one patient (Fig. 3d). Increased signal intensity in the pulvinar region on T1-weighted MRI was not observed in any patients. Two patients had a history of hypertension; however, their blood pressures were well controlled by antihypertensive drugs. None of the patients had other common risk factors for stroke, including dyslipidemia, diabetes mellitus, hyperuricemia, and smoking. No patients with the *GLA* p.E66Q mutation showed characteristic symptoms of Fabry disease, such as renal dysfunction, cardiomyopathy, acroparesthesia, hypohidrosis, and angiokeratoma. None of the patients with the p.E66Q mutation had a family history of Fabry disease, although an uncle of patient 1 and mothers of patients 2 and 3 had histories of cerebral infarction, and a brother of patient 2 had a history of chronic renal failure (Figure S1).

Allele frequencies of the *GLA* p.E66Q mutation in the Japanese population and statistical analysis

To estimate the frequency in the general Japanese population of the *GLA* p.E66Q mutation found in the IS patient cohort, the data from newborn screening for Fabry disease performed in the Kumamoto prefecture from October 2009 to May 2010 were used. In this screening, 5051 consecutive male neonates (5051 alleles) were tested. This study was approved by the Kumamoto University Ethics Committee and written informed consent was obtained from each parent prior to enrollment. Genomic DNAs were isolated from whole blood, and exon 2 of the *GLA* gene was amplified by PCR. The amplification product was analyzed by direct sequencing. Thirty-two hemizygous male neonates were identified, and the allele frequency of the *GLA* p.E66Q in the Japanese population was thus determined to be 0.637%. Statistical analysis using Fisher's exact test indicated that the allele frequency of *GLA* p.E66Q in patients with small-vessel occlusion was significantly higher than that in the general Japanese population (OR = 3.34, $P = 0.025$; Table 3). However, in all IS, large-artery atherosclerosis, cardioembolism, and non-cardioembolism patients, the ORs were 1.67, 1.39, 0, and 2.01, respectively; the differences were not statistically significant (Table 3).

Discussion

Screening for Fabry disease in high-risk populations became an important concern when enzyme replacement therapy became available [25]. Studies performed in different settings indicated severe complications of Fabry disease, including left ventricular hypertrophy/hypertrophic cardiomyopathy [8,13,14], renal insufficiency [7,11,12], and stroke [15–21]. The prevalence of unrecognized Fabry disease in young male patients with stroke was first reported in 2005 [15]. Since then, several studies have estimated the prevalence of Fabry disease in young male patients with stroke as ranging from 0% to 4.9% [15–20]. Recently, Rolfs *et al.* [19] reported the results of the largest screening for Fabry disease in young patients with acute cerebrovascular disease. They enrolled 5023 patients from 15 European countries and found 27 patients (0.54%) with definite Fabry disease and 18 patients (0.36%) with probable Fabry disease. However, most stroke patients are elderly and there has been only one population-based study in unselected patients with stroke [21].

In the present study, five patients were identified as having *GLA* mutation and all of them had the same missense mutation, c.196G>C (p.E66Q). All patients with the p.E66Q mutation showed a similar clinical picture, i.e. multiple small-vessel occlusions with a high frequency of intracerebral hemorrhage. Interestingly, most patients with this mutation lacked common risk factors for stroke. Only two patients had hypertension and their blood pressures were well controlled by antihypertensive drugs (Table 2). The p.E66Q mutation was first identified in a male patient with classic-type Fabry disease. However, he also had another *GLA* missense mutation, p.R112C, in the same allele, which was predicted to cause a large structural change in the α-Gal A protein that leads to classic-type Fabry disease [26]. Subsequently, 26 patients with *GLA* p.E66Q mutation who developed adult-onset left ventricular hypertrophy or renal insufficiency were identified [7,12,27–29], and this mutation was therefore considered to be pathogenic, causing late-onset variant Fabry disease. However, none of these studies provided histological evidence confirming the diagnosis of Fabry disease in such cases. Recently, subjects harboring the p.E66Q mutation in the *GLA* gene have been found at unexpectedly high frequencies amongst Korean [30] and Japanese [31] populations, which has raised interest in the possibility that p.E66Q is a disease-causing mutation or a functional polymorphism.

Table 3 Allele frequencies of the *GLA* p.E66Q mutation in the Japanese men

	Control (newborn screening)[a]	All IS patients[b]	Subtypes of IS			
			Large-artery atherosclerosis[b]	Cardioembolism[b]	Small-vessel occlusion[b]	Non-cardioembolism[b]
Number of subjects	5051	475	114	79	240	396
Number of subjects with p.E66Q	32	5	1	0	5	5
p.E66Q allele frequency (%)	0.64	1.05	0.88	0	2.08	1.41
Odds ratio (versus control)		1.67	1.39	0	3.34	2.01
P-value (versus control)		0.244	0.522	1	0.025	0.188

[a]Frequency of p.E66Q mutation was determined by DNA sequencing;
[b]frequency of p.E66Q mutation was determined by enzymatic screening followed by DNA sequencing.

Recent studies have shown that the structure of the α-Gal A protein with the p.E66Q amino acid substitution was less stable than that of the wild-type protein [31], and plasma and white blood cell α-Gal A activities in male subjects harboring the p.E66Q mutation were 13–26% and 19–65% of the normal mean α-Gal A activity, respectively [30,31], compatible with our dried blood spot enzymatic assay results (*c.* 40% of the normal mean; Fig. 2). The levels of these residual α-Gal A activities of the individuals with p.E66Q mutation were almost the same as those of male stroke patients with p.R118C and p.D313Y mutations identified in a Portuguese screen (PORTYSTROKE study) [17]. *GLA* p.D313Y mutation was found in 0.45% of normal X chromosomes in the Caucasian population [32]. In the PORTYSTROKE study [17], the allele frequency of p.D313Y in the stroke population was higher than that in normal controls, although the difference was not statistically significant. In this study, it was shown that the allele frequency of the *GLA* p.E66Q in patients with small-vessel occlusion was significantly higher than that in the general Japanese population (OR = 3.34, $P = 0.025$; Table 3), indicating that this mutation confers a high risk of small-vessel occlusion in Japanese males.

In this study, the frequency of the p.E66Q mutation in control subjects was determined by DNA sequencing, whilst that in stroke patients was determined by enzymatic screening followed by DNA sequencing. Therefore, the frequency of p.E66Q in stroke patients is likely to have been underestimated. Fujii *et al.* [29] analyzed the prevalence of Fabry disease in Japanese hemodialysis patients using dried blood spot screening followed by DNA sequencing, which was identical to the method used in the present study. They found only one patient with the p.E66Q mutation amongst 625 Japanese male hemodialysis patients (the prevalence of the p.E66Q mutation was 0.16%), which was much lower than that in our newborn DNA screen (Table 3), suggesting that a substantial number of patients with this mutation may have been missed in dried blood spot enzymatic screening. In addition, eight patients whose α-Gal A activities were below the cut-off value at the initial screening could not be followed up, because they moved to other hospitals or clinics. Therefore, there may have been additional patients with *GLA* mutations amongst those who dropped out, and the frequency of p.E66Q in stroke patients might thus have been underestimated in this study. Another point to be taken into consideration is patient selection bias, as patients were enrolled only from selected neurology departments, whilst a substantial number of stroke patients may be managed by neurosurgeons. In addition, informed consent could not be obtained from some of the severe stroke patients. These may explain why the proportion of small-vessel occlusion was more than 50% in this study. Although DNA sequencing of the *GLA* gene in all patients is necessary to determine the precise frequency of p.E66Q mutation in stroke patients, it is clear that p.E66Q mutation in the *GLA* gene is an important genetic risk factor for small-vessel occlusion in elderly Japanese males.

The precise pathomechanism by which *GLA* p.E66Q mutation increases the risk of lacunar infarction remains unknown. Recently, it was reported that cerebral small-vessel disease, which is known to be associated with lacunar infarction, white matter lesions (leukoaraiosis), and cerebral hemorrhage, rather than large-artery stroke, is frequently observed in Fabry disease [4,5,33,34]. These observations are compatible with clinical findings of our patients with p.E66Q mutation who developed multiple small-vessel occlusions and cerebral hemorrhage. Our findings suggest that *GLA* mutations associated with relatively high residual α-Gal A activity may add to the risk of cerebral small-vessel disease, possibly by contributing to the underlying multifactorial pathogenesis rather than through a classic Mendelian effect.

Enzyme replacement therapy (ERT) is currently the only approved therapy for Fabry disease. However, patients with *GLA* p.E66Q are not considered to be candidates for ERT, as p.E66Q is not a causative mutation for classic-type or variant-type Fabry disease [30,31]. On the other hand, Shimotori *et al.* [28] reported that 1-deoxygalactonojirimycin, an active site-specific pharmacological chaperone (ASSC), significantly increased α-Gal A activity of COS-7 cells with *GLA* p.E66Q mutation, suggesting that ASSC may be a potential therapeutic option for patients with this mutation.

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Disclosure of conflicts of interest

The authors declare no financial or other conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Pedigrees of patients with p.E66Q mutation.

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ORIGINAL ARTICLE

Newborn screening for Fabry disease in Japan: prevalence and genotypes of Fabry disease in a pilot study

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Fabry disease (FD) is an X-linked lysosomal storage disorder caused by a deficiency of α-galactosidase A (GLA) activity. Enzyme replacement therapy (ERT) for FD is available, and newborn mass screening for FD is being implemented. Here, we undertook a pilot study of newborn mass screening for FD in Japan. GLA activity in dried blood spots was measured using a fluorescence assay and confirmed by measurement of GLA activity in white blood cells (WBCs) in infants with abnormally low GLA activity. This was followed up by genetic testing. A total of 21 170 neonates were enrolled in the study. Of these, seven (five boys, two girls) had low GLA activities, which were verified by the WBC GLA activity assay. Thus, the initial fluorescence assay was suitable for newborn mass screening for FD. Pathogenic mutations of the *GLA* gene, that is, V199M and IVS4 + 919G > A, were found in two boys and one boy, respectively. Functional mutations, E66Q and c. −10C > T: g.1170C > T, were found in two boys and one girl, respectively. The prevalence of test-positive newborns was 1/3024, while that of those with a pathogenic mutation was 1/7057. The numbers are higher than those previously anticipated. Standardized management for FD found during newborn mass screening, including an ERT regimen, remains to be established.
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Keywords: enzyme replacement therapy; Fabry disease; genetic counseling; mass screening; metabolic disorder; newborn screening; X-linked recessive lysosomal storage disorder

INTRODUCTION

Fabry disease (FD: MIM 301500) is an X-linked inherited lysosomal storage disorder caused by a deficiency in α-galactosidase A (GLA) activity.[1] This deficiency leads to the accumulation of globotriaosylceramide in different tissues and can cause progressive malfunctions in systemic organs, such as the skin, eyes, kidneys, ears, lungs, heart and brain.[1–3]

Male patients with classic early-onset FD usually have very low GLA activity and are generally asymptomatic in early childhood (onset symptoms are reported at a mean age of 9 years).[4,5] The major clinical symptoms of classical early-onset FD include pain in the peripheral extremities, angiokeratoma, hypohidrosis, corneal opacity, and renal, cardiac and cerebrovascular diseases.[1] In contrast, patients with late-onset FD exhibit residual GLA activity and milder clinical manifestations than those with classical early-onset FD.[1] Heterozygous females with FD have wide clinical manifestation spectra, ranging from asymptomatic to severely affected.[6]

In 2001, in the USA and EU, enzyme replacement therapy (ERT) was approved for the treatment of FD; Japan began using ERT in 2004. In all three of these regions, ERT has been shown to be effective in alleviating many of the signs and symptoms of the disease and in slowing or even reversing disease progression.[7–9] Several studies have demonstrated that ERT must be administered before the occurrence of renal or cardiac failure in order to achieve optimal results.[10–12] As the importance of early treatment is now generally recognized, newborn mass screening for FD is being implemented in several countries. Such newborn mass screening, however, had not yet been undertaken in Japan; hence, the prevalence and genotypes of FD in association with GLA activity have not been studied in the Japanese population.

In this work, we present the results of a pilot study for newborn mass screening for FD in Fukuoka City and its vicinity in Japan.

SUBJECTS AND METHODS

Subjects

This study was conducted from April 2007 to April 2010 in Fukuoka City and its vicinity in Japan. Among newborns who took the conventional newborn mass screening during the study period, only newborns whose parent gave their written consent to participate were enrolled in the study. Conventional newborn mass screening has taken place as a local administrative service nationwide in Japan to screen six disorders: cretinism, congenital adrenal hypertrophy, galactosemia, phenylketonuria, homocystinemia and maple syrup

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urine disease. Virtually all newborns born in Japan take this conventional newborn screening. The dried blood spots that remained after completion of the conventional newborn mass screening were used for this study. The sex of newborns enrolled in the study was judged according to information written on the filter papers; this information was not available for some of the enrolled newborns because of incomplete descriptions.

Methods

Venous blood was collected from neonates on days 4–6 after birth, transferred to filter paper, and dried at room temperature for the conventional newborn screening and this study. A small circle of 3 mm in diameter was punched out of the dried blood spot and used for mass screening for FD.[3,13,14]

In the mass-screening study, GLA activity was determined using a fluorescent substrate, as described previously.[3,13,14] In brief, 40 μl McIlvan buffer (0.1 M citrate: 0.2 M NaH_2PO_4, 36.8:63.2 v/v, pH 6.0) was added to each well of 96-microwell plates. Punched dried blood spots were added to the buffer and processed for extraction at room temperature for 2 h. Aliquots of 30 μl of blood extract were transferred to new 96-microwell plates. An aliquot of 100 μl of the reaction mixture (3.5 mM 4-methylumbelliferone (4-MU) galactosylpyranoside, 100 mM citrate, 200 mM phosphate and 100 mM *N*-acetylgalactosamine, pH 4.4) was added to each well of the 96-microwell plates and incubated at 37 °C for 24 h. The reaction was terminated with 150 μl termination solution (300 mM glycine, NaOH, pH 10.6) immediately after the reaction. The fluorescence intensity from 4-MUs in the wells was measured with a fluorescence plate reader (Bio-Tek, Winooski, VT, USA) at 450 nm. One unit (AgalU) of enzymatic activity was equal to 0.34 pmol of 4-methylumbelliferyl-D-galactopyranoside cleaved per hour per disc. When GLA activity was identified as being abnormally low (i.e., less than the cutoff value of 20 AgalU), a second measurement was taken 2 or 3 weeks later to verify the initial measurement.[14] Newborns whose GLA activity had been verified as <20 AgalU were brought to the Department of Pediatrics of Fukuoka University or Kyushu University.

Cases in which GLA activity was found to be <20 AgalU in the mass screening, white blood cell (WBC) GLA activity was measured by a fluorometric enzyme assay. Briefly, whole blood was collected from the neonates and immediately treated with EDTA-2Na. The assay mix included 50 μl leukocyte lysate (WBC pellet prepared from 5 ml blood treated with EDTA-2Na was lysed by sonication in 1.0 ml water) and 50 μl of the reaction mixture (8.0 mM 4-methylumbelliferyl-α-D-galactopyranoside, 100 mM citrate phosphate buffer and 200 mM *N*-acetylgalactosamine, pH 4.5). This was incubated for 1 h at 37 °C. The reaction was terminated with 1.5 ml of 200 mM glycine buffer (pH 10.7) immediately after the reaction was completed. The fluorescence intensity from 4-MUs was measured with a fluorescent plate reader (Jasco Co, Tokyo, Japan) at 365 and 450 nm.

For genetic analysis, total genomic DNA was extracted from leukocytes of patients. All seven exons of the *GLA* gene were amplified by PCR, and the amplification products were analyzed by direct sequencing.[14] Detailed information on the PCR protocol is available upon request.

Before testing, counseling was provided as to the nature of the disease, its future medical management and risk of recurrence. Informed consent from each child's parent(s) for newborn mass screening for FD was at birth and that for WBC GLA activity assay and genetic analysis were obtained at their first visit to our hospitals.

All studies were approved by the Ethical Committees of Kumamoto University and Fukuoka University.

RESULTS

During the 37-month-period from April 2007 to April 2010, a total of 39 224 newborns took the conventional newborn mass screening. Among these, 21 170 (54.0%) newborns were enrolled in this pilot study after written informed consent was provided by the parent(s). The enrollees included 10 827 boys, 10 343 girls. Among the 21 170 newborns, seven (five boys and two girls) showed GLA activity <20 AgalU in the mass-screening test. Six newborns (four boys and two girls) were referred to Fukuoka University Hospital, while one boy (Case 5) was referred to Kyushu University for further examinations. The parents of one girl (Case 7) refused additional medical examinations; therefore, this child was not included in further analyses (Figure 1). All newborns had been delivered without incident at term. Cases 1 and 2 were brothers.

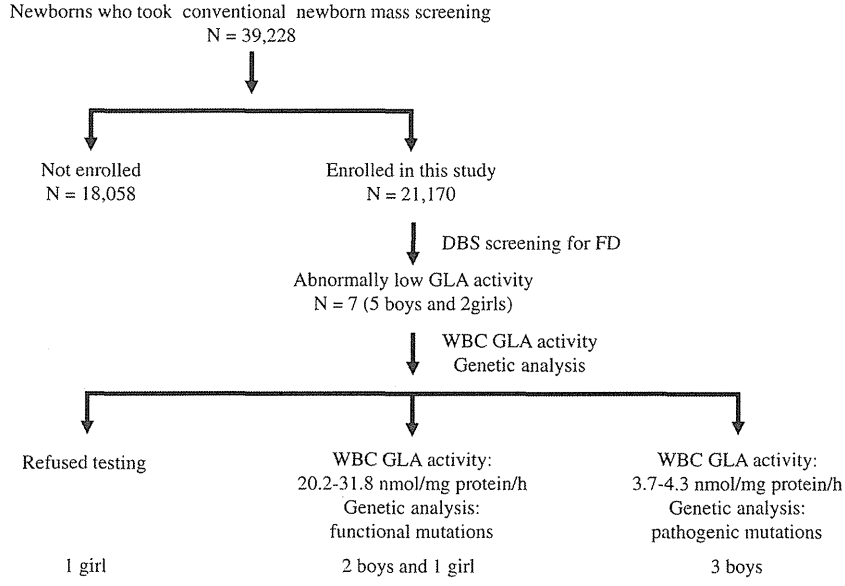

Figure 1 Flow chart of newborn screening for Fabry disease. During a 37-month period from April 2007 to April 2010, 39 224 newborns took the conventional newborn mass screening test in Fukuoka City and its vicinity; this test is given to all newborns in Japan. Among these, 21 170 (54.0%) newborns were enrolled in this pilot study with written informed consent. Seven newborns (five boys and two girls) showed an α-galactosidase A activity <20 AgalU (cutoff value) in the mass screening. The parents of one girl (Case 7) refused additional medical examinations. Four newborns had their white blood cell GLA activities measured, while all six underwent genetic testing. Pathogenic mutations, that is, V199M and IVS4+919G>A, were found in two boys and one boy, respectively. Functional mutations or polymorphisms (see text), that is, E66Q and heterozygous c.−10C>T: g.1170C>T, were found in two boys and one girl, respectively.

One boy (Case 5) presented with neonatal hyperbilirubinemia, high-pitched crying, a dysmorphic face (low-set ears and small mouth), brachydactyly (left shorter fifth finger) and hypotonia. Karyotyping revealed that this boy had a chromosomal abnormality, 46,XY,der(3)t(3;4)(p26;p14). However, we believe that this condition was unrelated to the presence of FD because the *GLA* gene locus was not involved in his chromosomal abnormality (Table 1).

The mother of one boy (Case 3) had a history of familial renal disease, but the other six subjects had no histories that would suggest a risk of FD. Additional details of the characteristics of familial renal disease in the mother of Case 3 were not available. Except for the dysmorphic features observed in Case 5, none of other six children presented with abnormalities at 1 month of age. The newborns' parents and other family members with GLA deficiency were counseled and offered medical evaluations and medical follow-up.

WBC GLA activity was very low (3.7 and 4.3 nmol mg^{-1} protein per hour) in two boys (Cases 1 and 3). In contrast, in one boy and one girl (Cases 4 and 6, respectively), WBC GLA activity was markedly higher, at 31.8 and 20.2 nmol mg^{-1} protein per hour, respectively, but still well below the normal range of 49.8–116.4 nmol mg^{-1} protein per hour as determined from 48 healthy volunteers. WBC GLA activity was not measured for two boys (Cases 2 and 5) (Table 1).

Genetic analyses found V199M and IVS4 + 919G > A mutations in two brothers (Cases 1 and 2) and one boy (Case 3), respectively. V199M is considered pathogenic and is thought to cause the classical phenotype of FD,[15] while IVS4 + 919G > A is also considered pathogenic, but is thought to cause the late-onset phenotype.[16,17] E66Q and heterozygous c. −10C > T: g.1170C > T mutations were found in two boys (Cases 4 and 5) and one girl (Case 6), respectively. These are both functional mutations and are not thought to cause FD, that is, they are considered polymorphisms, but were subjected to further investigations[18–22] (Table 1).

Cases 1–4 were last followed up when the patients were 2–3 years old, by then none of them showed any symptoms of FD.

DISCUSSION

This pilot study of newborn mass screening for FD comprised a large cohort of 21 170 newborns, most of them were of Japanese ethnicity. The screening used a fluorescent GLA activity measurement from a dried blood spot. A total of seven newborns tested positive with respect to low GLA activity (cutoff value: <20 AgalU) and four of these newborns were verified to have low GLA activity by a WBC GLA activity measurement. Thus, the prevalence of positive testing in this screening was 1/3024. Subsequent genetic analysis revealed that six newborns harbored different mutations in the *GLA* gene. Given that only V199M and IVS4 + 919G > A are thought to be pathogenic, the prevalence of individuals with pathogenic mutations in our cohort was 1/7057, as three newborns had such mutations. These findings support the contention that the mass-screening system used in this study was legitimate and hence useful for the early diagnosis of FD. At the same time, however, these findings raised concerns about the timing of ERT.

The prevalence of FD in this study (1/7057) suggests that the disease may be far more prevalent than previously thought and much more common than the 1/40 000 males estimated by Desnick *et al.*[1] Nevertheless, this number is lower than those reported in newborn screenings in other countries: 1/1100 boys in Italy (with a 11:1 ratio of late-onset to classic phenotype);[23] 1/3859 boys and girls in Austria (no instances of classic phenotype, but one case in which the phenotype could not be determined)[24] and 1/1250 boys in Taiwan (86% with the late-onset mutation IVS4 + 919G > A).[17] These differences may have to do with the genetic backgrounds of the newborns examined in each study.

GLA activities measured by the fluorescence assay were in accordance with GLA activities measured from WBC samples, an accepted method for GLA measurement in the diagnosis of FD. This again supports the notion that our screening system, which utilizes a fluorescence assay to measure GLA activity, is reliable. WBC GLA activities were very low, that is, 3.7 and 4.3 nmol mg^{-1} protein per hour, in Cases 1 and 3, respectively. The mutations identified in these newborns were V199M and IVS4 + 919G > A in the *GLA* gene, which are considered pathogenic mutations and are thought to cause classical and late-onset phenotypes of FD, respectively. In contrast, WBC GLA activity was markedly higher, at 31.8 and 20.2 nmol mg^{-1} protein per hour, in Cases 4 and 6, respectively, but still well below the normal range of 49.8–116.4 nmol mg^{-1} protein per hour. The mutations identified in these two cases are functional mutations and are considered polymorphisms that lead to low GLA activity but may not evolve into FD; however, whether they are totally benign

Table 1 Case summary

		GLA activity			*GLA mutation*		
Case	*Sex*	*First measurement (AgalU)*	*Second measurement (AgalU)*	*WBC measure GLA activity[a] (nmol mg^{-1} protein per hour)*	*Location*	*Mutation*	*Deduced phenotype*
1	M	4.4	5.2	3.7	Exon4	V199 M	Classic
2	M	5.0	ND	ND	Exon4	V199 M	Classic
3	M	8.2	9.2	4.3	Intron4	IVS4 + 919 G > A	Late-onset
4	M	13.6	16.3	31.8	Exon2	E66Q	Normal
5[b]	M	12.0	14.8[c]	ND	Exon2	E66Q	Normal
6	F	13.1	16.4	20.2	5′UTR	g.1170C > T (c. −10C > T)	Normal
7	F	15.1	17.9	ND	ND		

Abbreviations: GLA, α-galactosidase A; ND, not determined; UTR, untranslated region; WBC, white blood cell.
Seven newborns tested positive in a pilot newborn mass screening for FD. Of these, The parents of one girl (Case 7) refused additional medical examinations; four underwent WBC GLA activity measurements (Cases 1, 3, 4 and 6); and all six underwent genetic testing for mutations in the *GLA* gene. Cases 1 and 2 were brothers. Case 5 had a chromosomal abnormality and underwent GLA measurements four times.
[a]Normal range of GLA activity in WBCs was 49.8–116.4 nmol mg^{-1} protein per hour (n = 48 healthy volunteers).
[b]46,XY,der(3)t(3;4)(p26;p14) chromosomal abnormality was identified, but was considered unrelated to FD because the *GLA* gene locus was not affected by the chromosomal abnormality.
[c]Third measurement: 22.9, fourth measurement (1 year later): 10.7.

polymorphisms is still controversial.[18–22] Overall, the GLA activity measurements in this study seemed to reflect the nature of the identified *GLA* gene mutations.

Given that newborn mass screening for FD is conducted to allow for early diagnosis and, in turn, early treatment with ERT, this early diagnosis raises a number of concerns. First, the timing of ERT is still controversial, and there is uncertainty as to when ERT should be initiated in neonates (many of whom will be asymptomatic). Several studies have demonstrated that ERT must be administered before instances of renal or cardiac failure in order to achieve optimal results.[10–12] However, Ross[25] insists that 'premature treatment may cause more harm than good' as a result of 'side-effects' and 'medicalizing of a normal childhood.' Others, instead, indicate that there is no evidence that early ERT is ineffective or that it harms the patient.[26–30] Indeed, the findings that are currently available on the efficacy and side effects of ERT are based on a small sampling and are the result of very few ERT follow-up reports for young children. Therefore, the implementation of mass screening for FD, which has only recently been initiated in very limited regions of the world, should provide significant insights into these controversies. In addition, it will be necessary to accumulate enough long-term follow-up data on the efficacy and safety of ERT to conclusively determine the effectiveness of ERT in newborns diagnosed with FD during newborn mass screening; as the prevalence of FD is very low, this process may take many years.

Second, in relation to the first concern raised above, neither GLA activity nor genotypes necessarily correlate with phenotypes in terms of severity and onset of the disease. This is particularly the case in heterozygous females who may have significantly reduced enzyme activity but no symptoms. Of course, positive results in newborn screening followed by further examinations, including WBC GLA measurement and genetic analyses, enable ERT to be initiated at first onset of the condition and alert the child's female relatives to the necessity of future medical examinations. Serum or urinary globotriaosylceramide or serum globotriaosyl spingosine levels may give us better clues for anticipating the severity and onset of the disease identified in newborn mass screening, although they were not investigated in the present study.

Third, there are also ethical issues associated with early screening for FD. Newborns who tested positive in newborn mass screening may be given a presymptomatic diagnosis by genetic testing. According to the Japanese Association of Medical Science, presymptomatic diagnosis by genetic testing should only be performed after the examinee has sufficiently understood the available preventive measures and therapeutic strategies.[31] Obviously, imparting such information to a neonate is impossible, but providing a parent with sufficient counseling to making an informed decision is essential. Thus, careful genetic counseling is required, preferentially from a knowledgeable geneticist.

Likewise, adequate education on the symptoms and disease progression for FD should also be provided to parents and their relatives. On their first visit to our hospitals, parents were unfamiliar with FD, and, to ease their concerns, it was necessary to provide them detailed information about its nature, future medical management and risk of recurrence. Furthermore, as FD is an X-linked inherited disease, inadequate counseling may create a burden to mothers and even affect family relationships, which may lead to serious consequences, such as divorce. From our experience with the seven cases reported in this study, some parents, for whatever reason, were surprised that FD might be serious, while others were overly alarmed by the disease.

To overcome all concerns raised, certain guidelines for management of FD, including when ERT is appropriate, should be established based on evidence collected as a result of accumulating experiences with FD identified in newborn mass screening. Although FD is quite rare, it is feasible to establish such guidelines, as guidelines are available for similar rare diseases, that is, Gaucher disease[32] and Pompe disease.[33] These worldwide guidelines have proven to be extremely useful.[32,33] In addition, these guidelines could aid in the collection of global data that would assist in determining not only the appropriate age for ERT, but also the nature and duration of follow-up care and genetic counseling, providing, in short, a means of coordinating both research and treatment.

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新しい新生児代謝スクリーニング時代に適応した先天代謝異常症の診断基準作成と治療ガイドラインの作成および新たな薬剤開発に向けた調査研究班

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