Molecular Genetics and Metabolism Reports 14 (2018) 68–72

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: www.elsevier.com/locate/ymgmr

Ten-year-long enzyme replacement therapy shows a poor effect in alleviating giant leg ulcers in a male with Fabry disease

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ARTICLE INFO

Keywords:
α-Galactosidase A
Enzyme replacement therapy
Leg ulcer
Zebra bodies

ABSTRACT

Fabry disease is an X-linked lysosomal storage disorder caused by a deficiency of α-galactosidase A (α-gal A), leading to the progressive accumulation of glycosphingolipids. Classical hemizygous males usually present symptoms, including pain and paresthesia in the extremities, angiokeratoma, hypo- or anhidrosis, abdominal pain, cornea verticillata, early stroke, tinnitus, and/or hearing loss, during early childhood or adolescence. Moreover, proteinuria, renal impairment, and cardiac hypertrophy can appear with age. Enzyme replacement is the most common therapy for Fabry disease at present which has been approved in Japan since 2004. We report a case involving a 27-year-old male with extreme terminal pain, anhidrosis, abdominal pain, tinnitus, hearing impairment, cornea verticillata, and recurrent huge ulcers in the lower extremities. At the age of 16 years, he was diagnosed with Fabry disease with a positive family history and very low α-gal A activity. He then received enzyme replacement therapy (ERT) with recombinant human agalsidase beta at 1 mg/kg every 2 weeks for 10 years. Throughout the course of ERT, his leg ulcers recurred, and massive excretion of urinary globotriaosylceramide and plasma globotriaosylsphingosine was observed. Electron microscopy of the venous tissue in the regions of the ulcer showed massive typical zebra bodies in the vascular wall smooth muscle cells.

1. Introduction

Fabry disease (OMIM #301500) is an X-linked disorder characterized by deficiency of the lysosomal hydrolase α-galactosidase A (α-gal A; E.C. 3.2.1.22) due to mutations in the *GLA* gene [1,2]. Patients with partial or complete deficiency of α-gal A are unable to effectively degrade glycosphingolipids, globotriaosylceramide (Gb3), and glycosphingolipids-related compounds, such as galabiosylceramide (Gb2) and globotriaosylsphingosine (Lyso-Gb3), which then accumulate in the body fluids and in the lysosomes of a variety of cell types, including capillary endothelial cells, renal cells (podocytes and tubular, glomerular endothelial, mesangial, and interstitial cells), cardiac cells (cardiomyocytes and fibroblasts), eye cells, and nerve cells [3,4]. In the electron microscopic images of these tissues, the existence of these accumulations is described as zebra bodies [2].

Since enzyme replacement therapy (ERT) was introduced in 2001, the current biweekly intravenous administration of recombinant human agalsidase alpha (Replagal®, Shire) or beta (Fabrazyme®, Sanofi-Genzyme) has played a major role in providing comfortable lives for patients with a pathologically missing or functionally impaired *GAL* gene, leading to an α-gal A deficit [5]. A recent study investigated the 10-year-long therapeutic effects of ERT on major organs, including those of the cardiac and renal systems [6,7].

Another therapeutic alternative is substrate reduction therapy, which is administered orally, and was also found to be effective in a recent study [8]. Chemical chaperone [9] and gene therapy are also currently being investigated as options [10].

We here present a case of Fabry disease treated with the recombinant enzyme agalsidase beta over 10 years. The patient showed clinical improvement with respect to pain and anhidrosis, and no progression in abdominal pain, hearing impairment, and cornea verticillata; however, there was massive Gb3 and lyso-Gb3 excretion in the urine and plasma, respectively, and zebra bodies were detected in the vascular smooth muscle cells while huge ulcers recurred in the lower

Abbreviations: α-gal A, α-galactosidase A; Gb3, globotriaosylceramide; Lyso-Gb3, globotriaosylsphingosine; ERT, enzyme replacement therapy

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https://doi.org/10.1016/j.ymgmr.2017.12.004
Received 19 September 2017; Received in revised form 7 December 2017; Accepted 7 December 2017
Available online 22 December 2017

extremities.

2. Materials and methods

2.1. Clinical characteristics of the patient

The patient is a 27-year-old Japanese male who was a child of healthy non-consanguineous parents, with an uneventful birth and neonatal history. He had a red rash on his chest since early infancy, and the skin rash then extended gradually to the whole chest, back, and arms (Supplementary Fig. 1). These lesions were not typical angiokeratoma, but rather represented numerous macular telangiectases of < 10 mm in size with no mucosal involvement. At the age of 5 years, his mother noticed that he did not sweat at all. At 8 years of age, the patient began to suffer from extreme pain in the extremities and joints, and developed a fever reaching 38°C. When he was 15 years old, round ulcers measuring 10–20 mm in diameter appeared on his left lateral malleolus and right medial malleolus. A skin biopsy specimen did not show any characteristic features of vasculitis but did show chronic mixed cell infiltrates around the dilated capillary vessels and mild extravasation of red blood cells in the upper dermis. After carefully gathering the family history, the patient's mother stated that her 57-year-old brother was suffering from pain, acroparesthesia, hypo-hidrosis, bilateral deafness, left ventricular hypertrophy, and chronic renal failure. The patient's maternal grandmother had died from dilated cardiomyopathy (Fig. 1). Measurement of the patient's α-gal A activity in the blood lymphocytes showed marked diminishment, leading to a diagnosis of Fabry disease [11]. ERT was then started with recombinant human agalsidase beta (Fabrazyme®, Sanofi-Genzyme) at 1 mg/kg in 2006, when the patient was 16 years old.

During his disease course, he eventually experienced abdominal pain, tinnitus, hearing impairment, and cornea verticillata. No significant abnormalities were detected in his abdominal and cranial scans. The leg ulcers that appeared before the diagnosis of Fabry disease were characteristic, and neither swollen nor painful. However, once they became infected, the ulcers were swollen and painful. Owing to the varicose veins and painful swelling of the lower extremities, the patient's quality of daily life was moderately impaired.

After 10 years of ERT, the ulcers that appeared around the right medial malleolus and left lateral malleolus had extended widely, measuring 60–80 mm. Ultrasonography of the leg veins and right leg venography showed venous reflux and varices on the great and small saphenous veins (Fig. 2). His other symptoms, including pain and anhidrosis, improved, and there was no progression in abdominal pain, hearing impairment, or cornea verticillata. No renal, cardiac, or cerebral impairment was observed.

Treatment, including rest, pressure stockings, and varices operation, improved the ulcers (Fig. 2); however, they were not yet completed cured.

His mother experienced mild pain, acroparesthesia and mild left cardiac hypertrophy and sister with mild pain. Both of them were receiving the same ERT treatment since 2007 and 2016, at the age of 45 and 23 years, respectively, and living almost normal lives with this treatment.

2.2. Measurement of α-gal A activity using 4MUGal substrate

The level of α-gal A activity was measured as previously reported [12]. In short, lymphocytes were separated from whole blood cells, and the whole cell lysates were incubated with a mixture containing 700 mM 4-methylumbelliferyl-α-D-galactopyranoside (4 MUGal; Sigma), 0.5 M *N*-acetyl-D-galactosamine (GalNAc; Sigma), and 50 mM citrate-phosphate (pH 4.5) buffer in a water bath at 37 °C for 3 h. Reactions were stopped with 150 mM ethylenediaminetetraacetic acid (pH 11.5) buffer. Fluorescence (excitation at 355 nm/emission at 460 nm) was measured with a microplate reader. Enzyme activity was calculated as $nmol \cdot h^{-1} \cdot mg\ protein^{-1}$.

2.3. Urinary glycosphingolipid detection by high-performance thin layer chromatography (HPTLC)

Random urine was collected in April 2017, after 10 years of ERT. Urinary excretion of Gb3 was detected as described previously [13]. In short, total lipids were extracted in chloroform:methanol (2:1) from the urine, and then the mixture was separated by HPTLC (Silica gel 60, Merck, Germany) in chloroform:methanol:water, 70:30:4 (v/v/v). Gb3 was then detected with a 50% sulphuric acid solution.

2.4. Quantification of lyso-Gb3 by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Fresh plasma was separated from whole blood cells in April 2017.

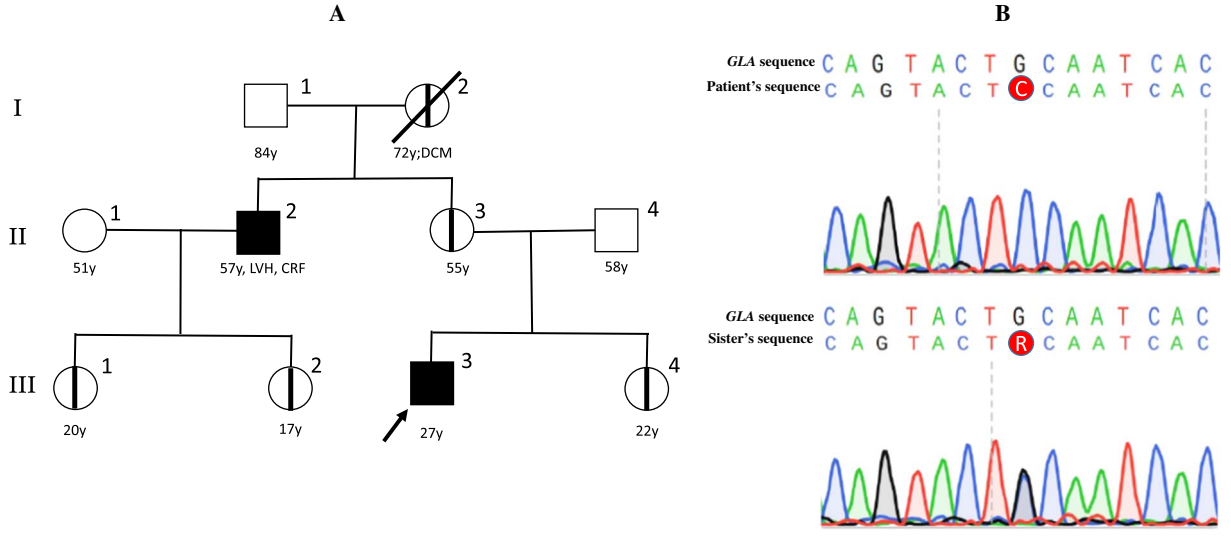

Fig. 1. Family pedigree of the index case (A). Two affected males, the index patient and his maternal uncle, shared similar phenotypes, including pain, hypo-hidrosis, and hearing impairments. The index case included additional manifestations of recurrent leg ulcers, abdominal pain, and cornea verticillata. DNA sequencing (B) revealed a single nucleotide substitution c.668G > C, which caused a novel missense mutation (p.C223S) in the hemizygous state for the affected male, which was in the heterozygous state for his sister. y, years; DCM, dilated cardiomyopathy; LVH, left ventricular hypertrophy; CRF, chronic renal failure.

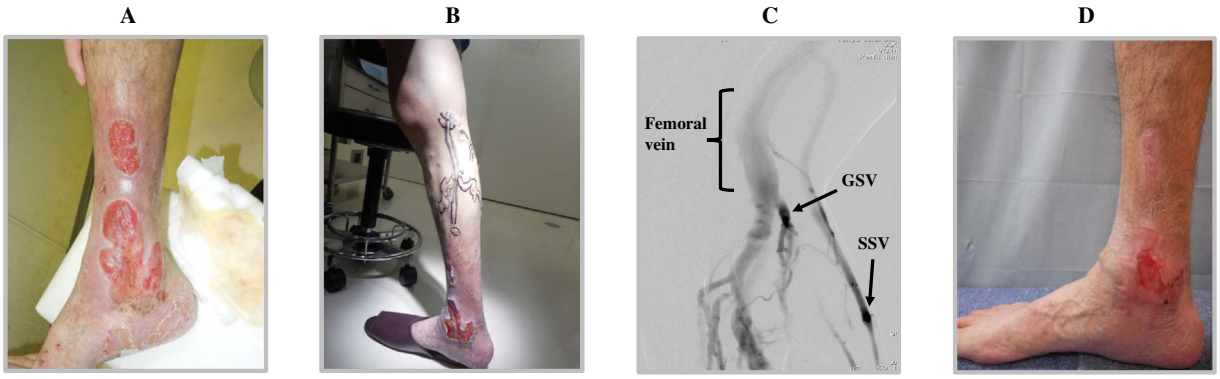

Fig. 2. Multiple huge ulcers were located at the lateral aspect of the left leg (A) and in the medial aspect of the right leg (B) in July 2016. Right leg venography (C) showed venous reflux and varices on the great saphenous vein and small saphenous vein. Three months after the varices operation (October 2016), the ulcers improved (D).

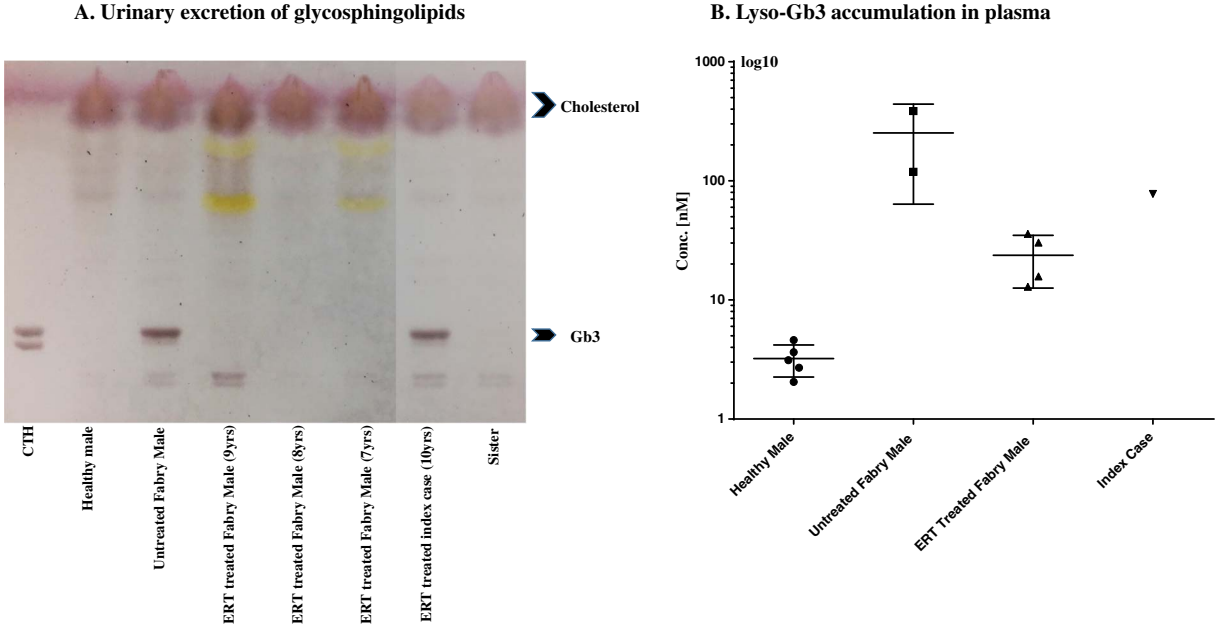

Fig. 3. Urinary excretion of glycosphingolipids (A). Total lipids were extracted and separated by high-performance thin layer chromatography. CTH, ceramide trihexoside (Gb3 marker). Lyso-Gb3 accumulation in plasma (B); the extracted plasma Lyso-Gb3 level was measured in nmol/L.

Eighty microlitres of the plasma was then placed into a 1.5-mL tube containing 720 μL 2-propanol (HPLC-grade, Kanto, Japan). After mixing for 2 min with a microtube mixer (Tommy, Japan), the extraction was centrifuged for 20 min at 12,000 *g*. The supernatant (640 μL) from the extraction was dried under air stream and reconstituted with 30 μL of methanol. The final solution was transferred to autosampler micro vials for measurement by the LCMS-8040 system (Shimadzu, Japan).

The calibration set consisted of LysGb3 (Matreya LLC, USA) standards with the same amount of *N*-glycinated LysoGb3 (internal standard, Matreya LLC, USA) in the methanol solvent. The calibrated amount of LysoGb3 was 0.0–0.8 ng (R^2 = 0.996), and one calibration set was performed for every 50 samples.

Five microliters of the solution was measured by high-performance liquid chromatography (HPLC) connected to a tandem mass spectrometer (LCMS-8040, Shimadzu, Japan). The mixture was eluted with mobile phase A (10 mM NH_4COOH, Wako, Japan) and phase B (10% 10 mM NH_4COOH/MeOH) through a C8 column (Inertsil C8–3, 3 μm, 2.1 × 50 mm, GL Sciences Inc., Japan) connected to an in-line filter unit (ACQUITY, Waters, USA). The multiple reaction monitoring (MRM) transitions monitored were 786.5 > 264.3 *m/z* for LysoGb3 and 843.5 > 264.3 m/z for *N*-glycinated LysoGb3. The quantification of LysoGb3 was performed by comparing the peak molecular target area with the internal standard area.

2.5. Sequencing analysis for the GLA gene

After obtaining informed consent, genomic DNA from the patient, his mother, and his sister was extracted from whole blood cells in April 2016. Polymerase chain reaction amplification and direct DNA sequencing of the *GLA* gene were conducted as described previously [13].

3. Results

3.1. Activity of α-gal A

The whole cell lysate from lymphocytes was used to detect α-gal A activity. Zero activity was found for the patient (normal range 10–12 nmol·h^{-1}·mg protein^{-1}), whereas his mother and sister showed intermediate activity (7 and 8 nmol·h^{-1}·mg protein^{-1}, respectively).

3.2. Urinary glycosphingolipid detection

For urinary glycosphingolipid detection, 10 mL of urine was

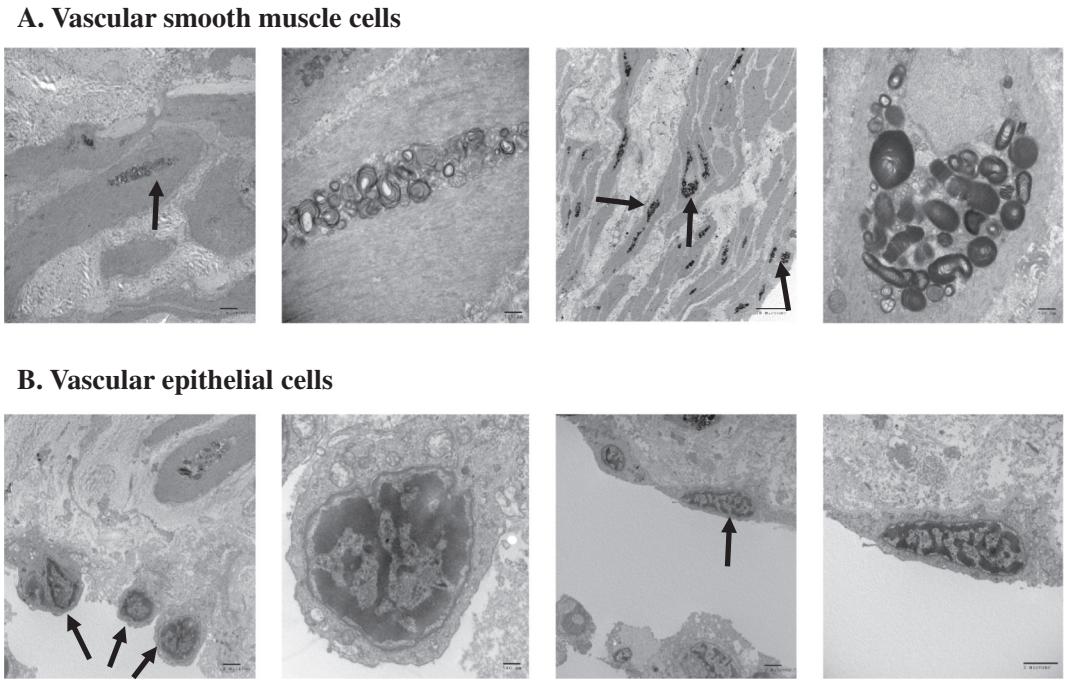

Fig. 4. Electron micrograph of a venous biopsy showing lysosomal deposition of glycosphingolipid as a zebra body in vascular smooth muscle cells (A) and few accumulations were noted in the vascular endothelium (B).

collected from the patient, his sister, a healthy male, and ERT-treated and untreated males, dried, and then the lipid were separated by HPTLC. Strong bands of Gb3 were detected for the patient and untreated male, although the former band was stronger than the latter. No Gb3 bands were detected from the samples of the patient's sister and other treated males (Fig. 3A).

3.3. Quantification of lyso-Gb3

Fresh plasma was collected from the patient, and from other ERT-treated and untreated males, and lyso-Gb3 was extracted in methanol. The amount of lyso-Gb3 for the patient was found to be very high, similar to that of untreated males (Fig. 3B).

3.4. Electron microscopy

A Venus biopsy was taken from the patient in July 2016, after 10 years of ERT, and prepared for visualization by electron microscopy. Diffuse sphingolipid accumulation was found as typical zebra bodies in the vascular wall smooth muscle cells and few accumulations were detected in the vascular endothelium (Fig. 4).

3.5. IgG antibody titre against agalsidase beta

The IgG antibody titre of the patient was measured against agalsidase beta (Sanofi Genzyme), and was found to be very high at 3200–6400 ×.

3.6. Sequencing analysis

Amplified DNA was purified, and direct sequencing was performed for all seven exons and exon-intron boundaries for the patient. A hemizygous novel point mutation c.668G > C (p.C223S) was detected in exon 5 of the *GLA* gene (Fig. 1). The same mutation was detected for his mother and sister as a heterozygous state. This mutation has not been reported previously and is not recorded in the Human Gene Mutation or SNP databases.

4. Discussion

Clinical manifestations in classically affected homozygotes with Fabry disease include onset of symptoms during childhood or adolescence, such as pain and paresthesia in the extremities, vessel ectasia (angiokeratoma) in the skin and mucous membranes, hypo- or anhidrosis, abdominal pain, hearing impairment, and cornea verticillata. With aging, renal and cardiac impairment may appear [1]. Our patient, who presented symptoms at a very early age, had anhidrosis as an initial sign of this disease in early childhood. Later, he developed extreme terminal pain, abdominal pain, tinnitus, hearing impairment, cornea verticillata, and recurrent huge ulcers in the lower extremities, which became his predominant presentation throughout his disease course.

Although ERT limits the severity of the disease and irreversible organ damage, it is accompanied by antibody-mediated side effects; indeed, patients with high antibody titres tend to show significantly higher levels of Gb3 in urinary samples than patients without antibody production [14]. Tanaka and her colleagues [15] also reported that ERT in a patient with Fabry disease increased the antibody titre for agalsidase beta greater than that for agalsidase alpha. Our patient was receiving agalsidase beta for 10 years as ERT, and showed massive excretion of urinary Gb3 and a high level of plasma lyso-Gb3 compared to other patients that received treatment for different durations (Fig. 3). This difference is probably due to the patient's high antibody titre against the enzyme ranging from 3200 × to 6400 ×.

Sakuraba et al. [16] reported a difference in the amount of enzyme uptake by different organs, and Benichou et al. [17] reported that patients with a high antibody titre showed a higher amount of Gb3 deposition in dermal capillary endothelial cells. Furthermore, it is known that high-antibody serum inhibits the uptake of enzymes due to masking of mannose-6-phosphate receptor [18]. Our case showed massive typical zebra bodies (Gb3 deposition) in vascular wall smooth muscle cells (Fig. 4) possibly due to poor uptake by those cells. The poor enzyme uptake and excessive accumulation of the substrate would lead to venous reflux, varices on the blood vessels (Fig. 2), and hypoxia to the adjacent tissues, ultimately causing recurrent ulcerations.

Several studies have been conducted to attempt to overcome the

poor clinical response of ERT to Fabry disease. Eng et al. [19] reported that a higher dose of enzyme is more effective to clear Gb3 deposition than the currently applied smaller dose (1 mg/kg every 2 weeks). In addition, immunomodulation therapy against the enzyme might improve the efficacy of ERT [20].

5. Conclusion

In Fabry disease, lower leg ulcers are not necessarily distinctive. The long-term therapeutic effects of ERT on major organs, including those of the cardiac and renal system, have been reported; however, very few cases have been described concerning skin and vessel tissues. Therefore, further studies on the long-term effect of ERT on refractory ulcers due to massive zebra bodies accumulation in vascular wall smooth muscle cells and venous reflux, the predominant presentation of our case, can help to improve the diagnosis of Fabry disease and develop appropriate treatment strategies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2017.12.004.

Acknowledgment

This work was supported by grants from the Ministry of Education, Culture, Science, Sports and Technology Type C (H26-28) and Ministry of Health, Labour and Welfare of Japan Intractable disease- Lysosomal disease (H26-H28).

Conflict of interest

Nothing to be declared.

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