Deficiency of the alkaline ceramidase ACER3 manifests in early childhood by progressive leukodystrophy

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ABSTRACT

Background/aims Leukodystrophies due to abnormal production of myelin cause extensive morbidity in early life; their genetic background is still largely unknown. We aimed at reaching a molecular diagnosis in Ashkenazi-Jewish patients who suffered from developmental regression at 6–13 months, leukodystrophy and peripheral neuropathy.

Methods Exome analysis, determination of alkaline ceramidase activity catalysing the conversion of C18:1- ceramide to sphingosine and D-ribo-C12-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD)-phytoceramide to NBD-C12-fatty acid using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and thin layer chromatography, respectively, and sphingolipid analysis in patients’ blood by LC-MS/MS.

Results The patients were homozygous for p.E33G in the ACER3, which encodes a C18:1-alkaline ceramidase and C20:1-alkaline ceramidase. The mutation abolished ACER3 catalytic activity in the patients’ cells and failed to restore alkaline ceramidase activity in yeast mutant strain. The levels of ACER3 substrates, C18:1- ceramides and dihydroceramides and C20:1- ceramides and dihydroceramides and other long-chain ceramides and dihydroceramides were markedly increased in the patients’ plasma, along with that of complex sphingolipids, including monohexosylceramides and lactosylceramides.

Conclusions Homozygosity for the p.E33G mutation in the ACER3 gene results in inactivation of ACER3, leading to the accumulation of various sphingolipids in blood and probably in brain, likely accounting for this new form of childhood leukodystrophy.

INTRODUCTION

Leukodystrophies are genetic disorders of myelin production or maintenance affecting the central nervous system. Their incidence is estimated at 1 in 7663 births. Signs and symptoms of leukodystrophies, though protean, reflect involvement of myelinated tracts and may include both pyramidal and extrapyramidal motor impairments, ataxia and late cognitive impairment. Diagnosis requires demonstration of abnormal white matter on brain imaging where several patterns may guide the clinician towards an informed genetic differential diagnosis. Despite the identification of a number of disease-causing genes, many patients with leukodystrophy still do not receive a firm molecular diagnosis. As part of our efforts to delineate the molecular basis of novel white matter disorders among consanguineous families using exome analysis, we now report the identification of a novel leukodystrophy gene in two siblings from a consanguineous Ashkenazi-Jewish family.

PATIENTS

Patient II-2, a female, and patient II-3, a male, are the second and third out of five children to remotely consanguineous parents of Ashkenazi-Jewish origin (figure 1A). Both children shared uneventful pregnancies, deliveries and perinatal course. Initial normal motor and language development was followed by stagnation and regression between 6 and 13 months of age, eventually rendering the children unable to communicate and lacking purposeful movement. The peak gross motor performance consisted of turning from prone to supine; expressive language peaked at 5–10 words. Examination was abnormal from their initial assessment at 9 months of age when truncal hypotonia, appendicular spasticity despite areflexia and dystonia were apparent. Other salient features were pale optic discs despite apparently normal vision and neurogenic bladder in both. Head circumference for individual II-2 was at the 50th centile at 10–17 months (44.5–46.2 cm) and at the 25th centile (47 cm) at 3 years 6 months. Head circumference for individual II-3 was at the 75th centile (47.2–48.5 cm) at 12–18 months. Currently aged 13 and 11 years, both patients are neurologically severely impaired with multiple contractures, late-onset relative macrocephaly (56.3 cm in both), short stature (140 and 118 cm, respectively), coarse facial features, sloping forehead, thick eye brows, low-set ears, prominent nose, relatively flat philtrum and prominent lower lip (figure 2A–C). Both require mechanical ventilation and feeding gastrostomies. Both parents are normoccephalic. Mother’s head circumference is 55 cm; father’s head circumference is 56 cm.

Patient II-2 underwent extensive investigation, which was positive for slightly increased plasma lactate level 3.1 mM (N<2.2). Repeated EEG disclosed generalised slowing but no epileptic activity. Brain MRI was normal at 1 and 2 years, but at 7 years disclosed diffuse abnormal white matter signal and severe atrophy (figure 3A–D). Brain magnetic resonance spectroscopy (MRS) revealed...
decreased n-acetyl aspartate (NAA) with normal choline and no excess of lactate. Sural nerve biopsy at 7 years showed a decrease in myelinated fibres. Chromosomal microarray analysis was performed on DNA from patient II-3 using the Affymetrix CytoScan 750K and was found to be normal [arr(1-22)x2,(XY)x1].

**MATERIALS AND METHODS**

**Whole exome analysis**

Exonic sequences were enriched in the DNA sample of patient II-3 (detailed in the online supplementary file). Parental consent was given for DNA studies.

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**Figure 1**  Family pedigree and the c.A98G, p.Glu33Gly mutation in the ACER3 gene. (A) Family pedigree and mutation genotype; filled symbols represent affected individuals. (B) The mutation (arrow) in patient II-2 (upper), the mother I-1 (middle) and the healthy sister II-6 (lower chromatogram). (C) Conservation of the Glu33 residue throughout evolution and among alkaline ceramidase proteins.

**Figure 2** Dysmorphic features of patients with ACER3 deficiency. Coarse facial features, sloping forehead, thick eye brows, prominent nose and prominent lower lip are shown in patient II-2 (A) and II-3 (B) as well as lower limb contractures (C).
Carrier rate determination was performed among 934 anonymous Ashkenazi-Jewish adults using TaqMan RT-PCR (detailed in the online supplementary file).

Site-directed mutagenesis
The codon of Glu33 (E33) in the ACER3 open reading frame (ORF) in the yeast expression plasmid pYES2-ACER3 was switched to Gly codon using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technology; Danbury, Connecticut, USA) (detailed in the online supplementary file).

Protein expression in yeast cells
Cells of the *Saccharomyces cerevisiae* mutant strain Δypc1Δydc1, in which both yeast alkaline ceramidase genes *YPC1* and *YDC1* were deleted, were transformed with pYES2-ACER3, pYES2-ACER3\(^{E33G}\), and the empty vector (pYES2-EV), respectively. The resulting transformants were grown in 2% galactose medium to induce ACER3 expression (detailed in the online supplementary file). Total membranes were prepared from yeast cells, and the expression of ACER3 or ACER3\(^{E33G}\) mutant was determined by western blot analyses using anti-FLAG antibody as described.

Alkaline ceramidase activity assays
Alkaline ceramidase activity was determined using D\(\_\)ribo-C12-NBD-phytoceramide (NBD-C12-PHC) as a substrate and quantitation of the product NBD-C12-fatty acid (NBD-C12-FA) by thin layer chromatography method or using regular ceramide (C\(_{18:1}\)-ceramide) as a substrate and quantitation of sphingosine (SPH) as a product by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (detailed in the online supplementary file).

Sphingolipid analysis by LC-MS/MS
Blood samples were collected from the two patients and from an unrelated healthy control and lipids were extracted using ethyl acetate/isopropanol extraction buffer (detailed in the online supplementary file). Sphingolipids were determined by LC-MS/MS performed on a TSQ 7000 triple quadruple mass spectrometer (Thermo Finnigan; Ringoes, New Jersey, USA) as described in. The amount of sphingolipids in each sample was normalised to phosphates.

RESULTS
The exome analyses of the DNA of patient II-3 yielded 58.8 million confidently mapped reads (mean coverage X81). Following variant filtering, four homozygous variants remained (filtering detailed in the online supplementary file and supplementary table S1); however, only one of them, Chr11:76572118 A>G, NM_001300953:c.A98G, p.Glu33Gly (E33G) in the ACER3 gene segregated with the disease in the family (figure 1A, B). The variant was not carried by any of the ∼9000 individuals, whose exome analyses included this gene (ExAC, accessed May 2015). Of 934 anonymous Ashkenazi-Jewish adults, only one carried the mutation.

ACER3 encodes an alkaline ceramidase, which catalyses the hydrolysis of several unsaturated long chain (ULC), D-e-C\(_{18:1}\), C\(_{20:1}\)-ceramides or C\(_{20:4}\)-ceramides (ULC-cer) and is as efficient towards ULC dihydroceramides or phytoceramides as towards their ceramide counterparts. To determine if the p.E33G
mutation affected ACER3 enzymatic activity, we measured alkaline ceramidase activity on the ceramide analogue NBD-C₁₂-PHC, a preferred synthetic substrate of ACER3, and on the ULC-cer D-ε-C₁₈:₁-ceramide (18:1-Cer), a preferred natural substrate of ACER3, in skin fibroblasts and lymphoblasts of the patients and two unrelated healthy controls. We found that alkaline ceramidase activity on both NBD-C₁₂-PHC and C₁₈:₁-cer was readily detected in fibroblasts and lymphoblasts of the patients and two unrelated healthy controls. We found that the p.E33G mutation inactivates the catalytic function of ACER3.

For further verification, we determined alkaline ceramidase activity following overexpression of WT-ACER3 and E33G-ACER3 in the yeast mutant strain (Δypc1Δydc1) in which the endogenous alkaline ceramidase was completely absent. Overexpression of WT-ACER3, but not E33G-ACER3, increased alkaline ceramidase activity on NBD-C₁₂-PHC (figure 5A, B) or C₁₈:₁-cer (figure 5C) although their expression levels were similar (figure 5D), confirming that the p.E33G mutation inactivates the catalytic function of ACER3.

To investigate how the p.E33G mutation affected ceramide metabolism (figure 6) in the patients, we determined sphingolipid levels in blood samples from the patients and from five healthy individuals. As expected, the two patients had higher levels of the ULC-cer metabolite (C₁₈:₁-ceramide and C₂₀:₁-ceramide) (figure 7A) and ULC dihydroceramides (C₁₈:₁-dihydroceramide and C₂₀:₁-ceramides) (figure 7B) in the blood compared with the healthy individual, because these ceramide and dihydroceramide species

Figure 4  ACER3 activity is abolished in patients’ cells by the p.E33G mutation (A–F). Total membranes were isolated from skin fibroblasts (A, B and E) and lymphoblasts (C, D and F) from healthy individuals (Control 1, Control 2, Control 3 and Control 4) or from the patients (Patient 1 and Patient 2) and were subjected to alkaline ceramidase activity assays using either NBD-C₁₂-phytoceramide (NBD-C₁₂-PHC) (A, B, C and D) or C₁₈:₁-ceramide (E and F) as a substrate. The release of the fluorescent product NBD-C₁₂-fatty acid (NBD-C₁₂-FA) from the substrate NBD-C₁₂-PHC was detected by thin layer chromatography (A and C) and quantified by densitometry (B and D). The release of sphingosine from C₁₈:₁ (E and F) was determined by HPLC/MS. Data represent mean values ±SD, n=3. *p<0.05.
are preferred substrates of ACER3. Unexpectedly, the patients also had higher levels of other ceramide (Figure 7A) and dihydroceramide species (Figure 7B) although these lipids do not appear to serve as substrates of ACER3. Consistent with the increase in the levels of ceramides, complex sphingolipids, including monohexosylceramides (Figure 7C, G) and lactosylceramides (Figure 7D, G), were also increased in the patients. However, there was no significant difference in the levels of most sphingomyelin species and the total amount of sphingomyelins between healthy controls and the patients (Figure 7E). Finally, the patients had higher levels of the sphingoid bases, SPH and dihydrosphingosine (DHS) (Figure 7F) and their phosphates, sphingosine-1-phosphate (S1P) and dihydrosphingosine-1-phosphate (DHS1P) (Figure 7F) although the free sphingoid bases are ACER3’s products that were expected to be decreased by the ACER3 inactivation. Taken together, these results suggest that ACER3 inactivation increases the level of various sphingolipids in the blood of the patients.

DISCUSSION
In two patients who suffered from leukodystrophy manifesting by neurological regression at 6–13 months of age, truncal hypotonia, appendicular spasticity, dystonia, optic disc pallor, peripheral neuropathy and neurogenic bladder, we identified a homozygous missense mutation, p.E33G in the ACER3 gene. It may be argued whether the disorder described herein is a true leukodystrophy according to the consensus statement published recently but as the clinical features and white matter changes on MRI were consistent with leukodystrophy we choose to classify the disease as such.

The mutation was associated with undetectable ACER3 catalytic activity towards natural and synthetic ACER3-specific substrates in the patients’ cells and could not complement a mutant yeast strain which lacked alkaline ceramidase activity, indicating that Glu33 is an essential residue for ACER3 activity. Ceramidases catalyse the hydrolysis of ceramides to form free fatty acids and SPH and as such they are intermediates of complex sphingolipid synthesis (Figure 6) which in turn play an
important role in the integrity and function of cell membranes. Ceramides also act as bioactive molecules to mediate cellular responses, including cell growth arrest, differentiation and apoptosis. According to their pH optima for in vitro activity, ceramidases are classified into acid, neutral and alkaline types and differ also by their cellular localisation and substrate specificities.

In humans, there are three alkaline ceramidases: ACER1 is an endoplasmic reticulum (ER) ceramidase that is predominantly expressed in the skin and uses only very long-chain ceramides (≥C22) as substrates, ACER2 is a Golgi ceramidase that uses both long-chain and very long-chain ceramides as substrates and ACER3 is localised to both the Golgi complex and the ER, and as mentioned above, it catalyses the hydrolysis of ULC ceramides. Expectedly, the sphingolipid profiling in our patients’ blood disclosed increased levels of the ACER3 substrates, C18:1- and C20:1-ceramides and C18:1- and C20:1-dihydroceramides, and their complex sphingolipid derivatives (sphingomyelins, monohexosylceramides and lactosylceramides), further supporting the functional significance of the p.E33G mutation. The abnormally increased levels of ceramide species, which are not ACER3 substrates, are rather unexpected. Similarly, the increased levels of ACER3 products (sphingoid bases and their phosphates) are puzzling. Whether these aberrations are the result of compensatory mechanisms that augment the de novo biosynthesis of sphingolipids or the activity of other ceramidases (Figure 6) is a matter of conjecture. Compensatory induction of ACER2 was shown upon knocking down ACER3 activity in HeLa cells.

Either way, the aberrant levels of the saturated and ULC ceramides and dihydroceramides and their complex sphingolipid derivatives were previously shown to exert a devastating effect; ACER3 deficiency in HeLa cells impaired cell proliferation and modulated apoptosis and was associated with progressive neurological disease in ACER3 knockout mice. The selective involvement of the brain in both humans and mice is likely underlined by the fact that complex sphingolipids are the major lipid components of myelin, and most defects of sphingolipid metabolism manifest solely by aberrant central myelination. Of note, ACER3 activity is relatively low in a 2-month-old mouse brain where it is only one-tenth of ACER3 activity in the animal liver and is associated with C20:1-ceramide and C18:1-ceramide levels, which are 20-fold and 100-fold more abundant in the brain than in liver. These data suggest that early in life the brain ACER3 activity is a priori relatively low, rendering the brain more vulnerable to the consequences of ACER3 deficiency. Another point of interest is that all the mutant animals developed urinary retention at ~6 months and some died of enlarged urinary bladder with distended abdomen by 10–12 months of age (CM—unpublished observation), recapitulating the neurogenic bladder in our patients.

In conclusion, our data suggest that Glu33 in ACER3 is crucial for the alkaline ceramidase activity of ACER3 and that p.E33G mutation inactivates the catalytic function of ACER3, leading to accumulation of various sphingolipids in the blood and probably in the brain of the patients. Heretofore, only acid ceramidase deficiency was reported to associate with disease in
humans. Recessive mutations in ASAH1, encoding acid ceramidase, manifest by Farber lipogranulomatosis\textsuperscript{14} and by spinal muscular atrophy and progressive myoclonic epilepsy.\textsuperscript{15} The present report is the first to delineate the clinical phenotype associated with an alkaline ceramidase deficiency.

Figure 7  Sphingolipid profile in blood. Blood samples were collected from the five healthy individuals (Control) and the two patients (Patient). Each of the blood samples (100 μL) was subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the levels of individual ceramides (A), individual dihydroceramides (B), individual monohexosylceramides (C), individual lactosylceramides (D), individual sphingomyelins (E), sphingoid bases and their phosphates (F) and total amount of each type of sphingolipids (G). Data represent mean values±SD, n=2 (patients) or 5 (controls). *p<0.05.

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