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An Early-Onset Neuronopathic Form of Acid Sphingomyelinase Deficiency: A *SMPD1* p.C133Y Mutation in the Saposin Domain of Acid Sphingomyelinase

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Acid sphingomyelinase (ASM) is a lysosomal hydrolase that degrades sphingomyelin into ceramide and phosphocholine. Recent crystallographic studies revealed the functional role of the N-terminal ASM saposin domain. ASM deficiency due to mutations in the ASM-encoding *sphingomyelin phosphodiesterase 1 (SMPD1)* gene causes an autosomal recessive sphingolipid-storage disorder, known as Niemann-Pick disease Type A (NPA) or Type B (NPB). NPA is an early-onset neuronopathic disorder, while NPB is a late-onset non-neuronopathic disorder. A homozygous one-base substitution (c.398G>A) of the *SMPD1* gene was identified in an infant with NPA, diagnosed with complete loss of ASM activity in the patient's fibroblasts. This mutation is predicted to substitute tyrosine for cysteine at amino acid residue 133, abbreviated as p.C133Y. The patient showed developmental delay, hepatosplenomegaly and rapid neurological deterioration leading to death at the age of 3 years. To characterize p.C133Y, which may disrupt one of the three disulfide bonds of the N-terminal ASM saposin domain, we performed immunoblotting analysis to explore the expression of a mutant ASM protein in the patient's fibroblasts, showing that the protein was detected as a 70-kDa protein, similar to the wild-type ASM protein. Furthermore, transient expression of p.C133Y ASM protein in COS-7 cells indicated complete loss of ASM enzyme activity, despite that the p.C133Y ASM protein was properly localized to the lysosomes. These results suggest that the proper three-dimensional structure of saposin domain may be essential for ASM catalytic activity. Thus, p.C133Y is associated with complete loss of ASM activity even with stable protein expression and proper subcellular localization.

Keywords: acid sphingomyelinase deficiency; disulfide bond; Niemann-Pick disease type A; saposin domain; subcellular localization
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Introduction

Acid sphingomyelinase (ASM) is a lysosomal sphingolipid hydrolase that catalyzes the degradation of sphingomyelin into ceramide and phosphocholine. The 1,896-bp open reading frame of the full-length human ASM cDNA encodes a 631-residue polypeptide synthesized in the endoplasmic reticulum as a catalytically inactive 75-kDa pre-pro-polypeptide (Hurwitz et al. 1994). After signal peptide cleavage, the precursor is processed in the ER-Golgi complex to a minor non-glycosylated 57-kDa form, and a major catalytically active 70-kDa mature form localized within the lysosome. Human ASM contains five N-glycosylation sites and eight disulfide bonds that play an important role in protein folding and stability (Ferlinz et al. 1997; Lansmann et al. 2003).

ASM deficiency due to loss-of-function mutations in the ASM-encoding *sphingomyelin phosphodiesterase 1 (SMPD1)* gene causes an autosomal recessive sphingolipid-storage disorder of Niemann-Pick disease Type A (NPA) or Type B (NPB), characterized by lysosomal accumulation of sphingomyelin primarily in reticuloendothelial cells

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(Schuchman and Desnick 2017). NPA is an early-onset neuronopathic disorder clinically characterized by rapidly progressive neurodegeneration and hepatosplenomegaly. Patients with NPA die within the first 3 years of life. In contrast, NPB is a late-onset non-neuronopathic disorder characterized by no neurological symptoms, hepatosplenomegaly, and lung involvement. Most patients with NPB survive into adulthood. In addition, chronic neurovisceral ASM deficiency is an intermediate NPA/NPB phenotype that exhibits delayed symptom onset and slower neurological and visceral disease progression (Pavlů-Pereira et al. 2005). Generally, there is a correlation between the severities of enzyme deficiency and phenotypes in lysosomal enzyme deficiencies. NPA is characterized by complete or near-complete loss of ASM enzyme activity, whereas NPB is characterized by only 5-20% of normal ASM enzyme activity (Desnick et al. 2010). To date, more than 170 different *SMPD1* gene mutations have been reported. Correlation between the mutant genotype and phenotype has been analyzed through clinical findings in patients with ASM deficiency, and suggests involvement of the structural characteristics of ASM polypeptide (Zampieri et al. 2016).

Recently, the crystallographic structure of human ASM was reported, which provided insights into the molecular structure of ASM deficiency (Gorelik et al. 2016; Xiong et al. 2016; Zhou et al. 2016). The ASM enzyme is organized into four major regions: N-terminal saposin domain, proline-rich connector, catalytic domain, and C-terminal subdomain. Lysosomal sphingolipid hydrolases ordinarily require an external saposin activator protein for complete activity. However, the ASM polypeptide comprises a built-in N-terminal saposin domain. The saposin domain is connected to the catalytic domain by a 30-residue proline-rich linker. The saposin domain comprises four helices that are stabilized by three disulfide bonds. It was also proposed that ASM exists in a monomer/dimer equilibrium in solution at pH5 in lysosome as a result of flexibility in the saposin domain and the monomer is the catalytically relevant form of the enzyme (Xiong et al. 2016).

In this report, we describe a missense mutation of the *SMPD1* gene, p.C133Y, identified from a Japanese infant with NPA. This mutation was predicted to disrupt one of the three disulfide bonds between two helices in the saposin domain. Therefore, we performed functional characterization of the p.C133Y ASM protein.

Materials and Methods

Blood and skin fibroblast samples were collected after obtaining written informed consent from the five participants; the patient's guardian, the mother, and three normal controls for blood analyses, and the patient's guardians and a normal control for fibroblasts analyses. Ethical approval for this study was obtained from the Ethics Committee of Akita University, Graduate School of Medicine in Akita, Japan.

Case report

The patient was a 4-month-old girl who showed clinical symptoms including developmental delay, hepatosplenomegaly, and low set ears. The patient was born to Japanese parents with birth weight of 2,052 g and height of 43.5 cm. The consanguinity of the parents could not be confirmed. The patient was clinically diagnosed with one of a group of Niemann-Pick diseases through clinical examinations, which revealed hepatic dysfunction, low serum levels of high-density lipoprotein, cherry-red spots in the ocular fundus, and foam cells in the bone marrow. The laboratory findings were as follows: aspartate aminotransferase, 754 IU/L (normal, < 30 IU/L); alanine aminotransferase, 419 IU/L (normal, < 40 IU/L); and high-density lipoprotein, 18 mg/dL (normal, > 40 mg/dL). Then, the patient was diagnosed with ASM deficiency because of complete loss of ASM enzyme activity in the fibroblasts. Thereafter the patient was prone to pneumonia and exhibited rapid deterioration of neurological functions. The patient died of liver and respiratory failures at the age of 3 years and 1 month.

Enzyme activities and immunoblotting analysis of fibroblasts

ASM enzyme activity was assayed using ^{14}C-sphingomyelin, following previously described methods (Takahashi et al. 2005). Immunoblotting analysis was performed to evaluate the protein expression levels. Briefly, protein lysates from patient and control fibroblasts were prepared using RIPA buffer (Nacalai Tesque, Kyoto, Japan). The protein extracts (20 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA). The membrane was blocked with 1% bovine serum albumin (Nacalai Tesque, Kyoto, Japan) at room temperature for 1 h, and then incubated overnight at room temperature with primary antibodies against human ASM (dilution 1:1,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (dilution 1:1,000; Cell Signaling Technology, Danvers. MA, USA). After incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000) at room temperature for 2 h, the protein bands were visualized using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher, Waltham, MA, USA).

DNA genomic sequencing and SMPD1 gene mutation nomenclature

Genomic DNA was extracted from the peripheral white blood cells of the patient, her mother, and a control. The six coding exons and the exon-intron boundaries of the *SMPD1* gene in the genomic DNA were amplified by PCR. The PCR products were directly sequenced in both directions using an ABI Prism BigDye Terminator Cycle

Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer, ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). In this study, *SMPD1* variants were analyzed and described based on reference sequence with GenBank Accession Number NM_000543.4.

Multiplex ligation-dependent probe amplification (MLPA) reaction

Three control DNA samples and one patient DNA sample were subjected to MLPA reaction using the MLPA kit P446-A1 GALC (MRC Holland, Amsterdam, the Netherlands), following the manufacturer's instructions. The MLPA reaction included 5 major steps: DNA denaturation, hybridization, ligation, PCR, and capillary electrophoresis to detect PCR products. The DNA samples were diluted to a concentration of 20 ng/μL using the elution buffers in the E.Z.N.A blood DNA mini kits (Omega Bio-tek, Inc., Norcross, GA, USA). The total amount of DNA (patient or three controls) used in the experiment was 100 ng (20 ng/μL, 5 μL). Coffalyser.Net software was used for data analysis to determine the relative size of fluorescent peaks and to calculate the final probe ratio, called the Dosage Quotient (DQ), through intra-sample and inter-sample normalization.

Site-directed mutagenesis and vector construction

A full-length cDNA encoding the wild-type ASM was custom-synthesized by FASMAC Co. Ltd. (Atsugi, Japan). An identified *SMPD1* gene variant was introduced into the wild-type ASM cDNA following a previously described site-directed mutagenesis method. The construct was re-sequenced with the BigDye Terminator method to ensure that no additional mutation had occurred during the mutagenesis procedure. The wild-type and mutant ASM cDNAs were respectively subcloned into the pCL-neo mammalian expression vector (Promega Corporation, Madison, WI, USA) to study the ASM enzyme activity. The wild-type and mutant ASM cDNAs were respectively subcloned into the pAcGFP-c1 vector (TaKaRa, Otsu, Japan) for a subcellular localization study of mutant ASM proteins, which can express GFP-fused to the N-terminus of ASM protein.

Cell transfection, ASM activity assay, and confocal microscopy

The wild-type and mutant constructs were transfected into COS-7 cells using the Lipofectamine 2000 reagent, following the manufacturer's instructions (Thermo Fisher, Waltham, MA, USA). ASM activity was assayed using ^{14}C-sphingomyelin, following previously described methods (Takahashi et al. 2005). COS-7 cells transfected with the pAcGFP-c1 vectors were examined under a Zeiss LSM 780 confocal microscope equipped with a UV laser. To visualize lysosomes, cells were incubated with 10 nM LysoTracker Red (Thermo Fisher, Waltham, MA, USA) for 30 min before observation.

Results

Enzyme activities and immunoblotting analysis of fibroblasts

ASM enzyme activity in the patient's fibroblasts was determined as 0.25 nmol/mg/h against 80.1 nmol/mg/h in

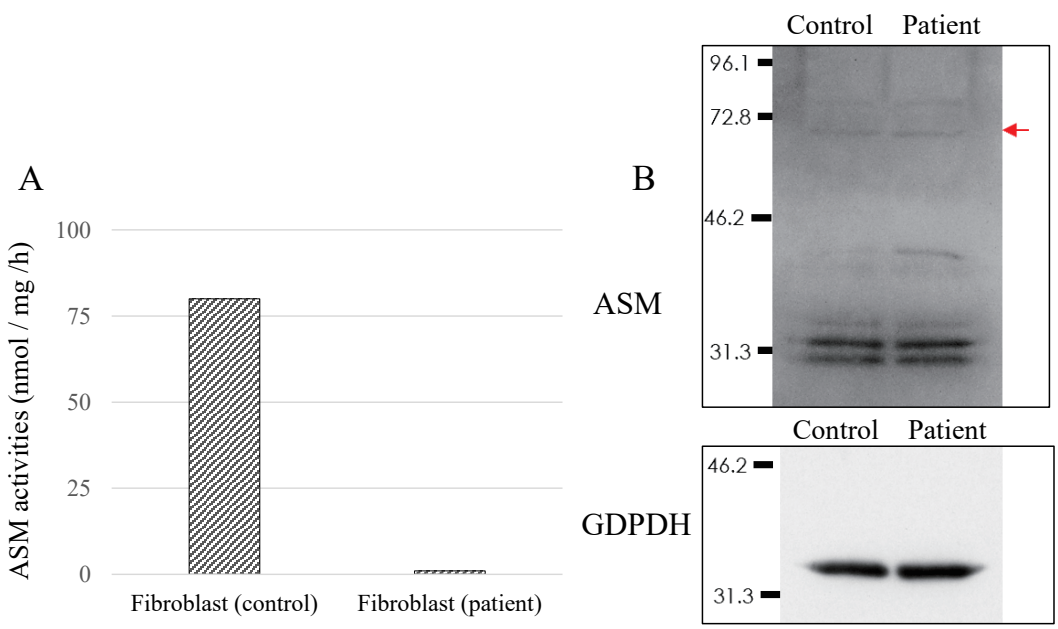

Fig. 1. Enzyme activities and immunoblotting analyses of acid sphingomyelinase (ASM) in normal and patient fibroblasts. (A) ASM enzyme activity of patient fibroblasts was determined as 0.25 nmol/mg/h against 80.1 nmol/mg/h in control fibroblasts. (B) Levels of endogenous ASM protein in control and patient fibroblasts were assessed by immunoblotting. An arrow shows the positive staining bands, 50 kDa, in control and patient samples. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was immunoblotted as inner protein contents of the fibroblasts.

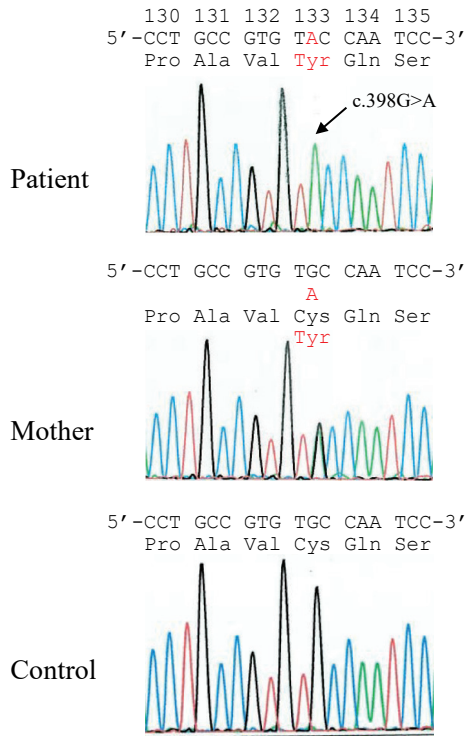

Fig. 2. Genomic exon 2 sequences of *SMPD1* gene in a patient with acid sphingomyelinase deficiency, her mother, and a control.
A homozygous one base substitution, designated as c.398G>A, was identified in the patient. The mother was heterozygous for c.398G>A against the control sequence. The c.398G>A substitution is predicted to substitute tyrosine for cysteine at amino acid residue 133.

the control skin fibroblasts (Fig. 1A). ASM protein levels were investigated in the control and patient fibroblasts by immunoblotting analysis. The ASM proteins were similarly detected as 70 kDa protein bands in the control and patient fibroblasts (Fig. 1B). Several higher and lower molecular weight bands were also observed in the results, which probably corresponded to a combination of immature and degraded ASM protein and non-specific binding of the antibody.

Mutational analysis

A homozygous one-base substitution (c.398G>A) was identified in the patient by PCR-mediated direct sequencing of the *SMPD1* gene. This mutation, which was abbreviated as p.C133Y, was predicted to substitute tyrosine for cysteine at amino acid residue 133. The mother was heterozygous for c.398G>A, whereas the father's genotype was not known because sequencing was not performed in the father (Fig. 2). Therefore, MLPA was performed for the relative quantification of copy number for all exons on *SMPD1* gene in the patient. MLPA analysis revealed that the copy numbers of all the exons in the patient were the same as those in the normal controls (data not shown). This confirmed that the patient was homozygous for the c.398G>A variant in exon 2 on the *SMPD1* gene.

Expression of p.C133Y ASM cDNA in COS-7 cells

To confirm that the p.C133Y mutation affects ASM enzyme activities, we used site-directed mutagenesis to study the expression of mutant ASM enzyme in COS-7 cells. The mutant p.C133Y ASM protein was transiently expressed in COS-7 cells and the ASM activity of COS-7

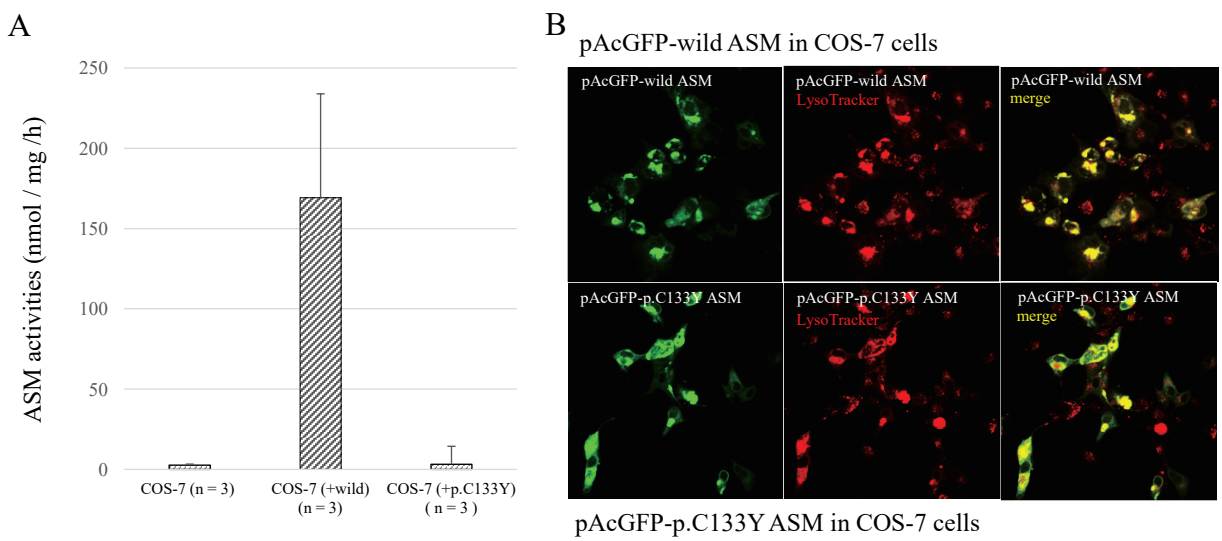

Fig. 3. Transient expression and subcellular localization studies of wild-type and p.C133Y mutant ASMs in COS-7 cells.
(A) ASM activity in COS-7 cells without transfection, with transient expression of wild-type ASM protein, and transient expression of p.C133Y ASM protein are shown. The data are expressed as the mean ± standard deviation (SD). (B) COS-7 cells transfected with pAcGFP-wild-type ASM and pAcGFP-p.C133Y ASM fusion protein vectors were analyzed using confocal microscopy. Colocalization of the ASM proteins with lysosomes is indicated as yellow color, which was obtained by the merging of pAcGFP, indicated as green color, and LysoTracker Red (a fluorescence probe for lysosome), indicated as red color.

cells was determined. The ASM activity in COS-7 cells expressing the mutant p.C133Y ASM was significantly lower than that in COS-7 cells expressing the wild-type ASM (Fig. 3A). This indicated that the p.C133Y mutant affects ASM enzyme activity, resulting in no residual ASM enzyme activity in the patient.

Confocal microscopy

pAcGFP was fused to the N-terminus of the wild-type or p.C133Y ASM protein and the fused product was transiently expressed in COS-7 cells. The GFP signals of both wild-type and p.C133Y mutant ASM proteins were detected to be co-localized with the lysosome fluorescent probe, Lysotracker Red, in COS-7 cells (Fig. 3B).

Discussion

A mutation of the *SMPD1* gene, p.C133Y, was identified in a patient with NPA, who was homozygous for the mutation. The ASM enzyme assay revealed no residual activity in the fibroblasts, suggesting that the mutation may totally affect ASM catalytic activity. The ASM enzyme comprises four major domains: The N-terminal saposin domain, proline-rich connector, catalytic domain, and C-terminal subdomain (Gorelik et al. 2016; Xiong et al. 2016; Zhou et al. 2016). The N-terminal saposin domain is made up of 83 amino-acid residues and consists of four α-helices (H1, H2, H3, and H4) along with six structurally critical cysteine residues. The six cysteine residues form three pairs of disulfide bonds to stabilize the domain structure. The two disulfide bonds connect the N and C termini of the saposin domain, whereas the third disulfide bond connects the H2 and H3 helices. As C133 is the fourth cysteine residue in the saposin domain, the p.C133Y mutation was predicted to disrupt the third disulfide bond between the H2 and H3 helices in the saposin domain (Fig. 4)

To date, seven missense mutations (p.C91H, p.C94W, p.L105P, p.V114M, p.V132A, p.L139P, and p.C159R) have been reported in the ASM saposin domain in patients with ASM deficiency. This suggested the functional importance of the saposin domain for ASM catalytic activity (Simonaro et al. 2002; Pittis et al. 2004; Dardis et al. 2005; Hollak et al. 2012; Gucev et al. 2013). Among them, p.C91H, p.L105P, and p.L139P were reported to be genotypes associated with an intermediate or non-neurological type of ASM deficiency. A patient with homozygous C91H mutation was reported to have an intermediate A/B type of ASM deficiency (Hollak et al. 2012). A patient with compound heterozygous mutations of p.L105P and p.T544fsX70 was also reported to show intermediate A/B type of ASM deficiency (Pittis et al. 2004). A patient with homozygous L139P exhibited a mild B type of ASM deficiency (Simonaro et al. 2002).

Among the ASM saposin domain mutations, three mutations (p.C91H, p.C94W, and p.C159R) were positioned at cysteine residues that form the disulfide bonds in the domain. Interestingly, these three mutations cause ASM deficiency in patients, but their phenotypes are milder than those of an early-onset neuronopathic form. This suggests that only one disruption of two disulfide bonds connecting the saposin domain N and C termini does not cause complete loss of ASM function. However, the p.C133Y mutation that disrupts the third disulfide bond between H2 and H3 helices of the saposin domain causes the most severe phenotype, which is an early-onset neuropathic form of ASM deficiency. Therefore, the third disulfide bond between H2 and H3 helices of the saposin domain is suggested to be critical for ASM function.

Immunoblotting analysis of the patient's fibroblast

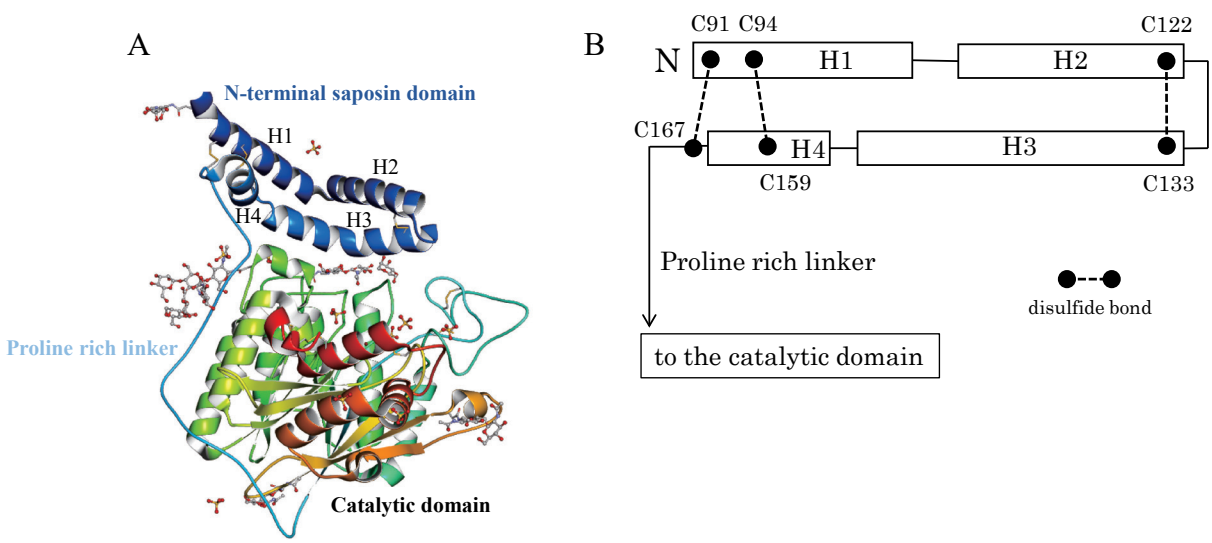

Fig. 4. Structure of acid sphingomyelinase (ASM) and saposin domain.
(A) Structure of ASM modified from ©Protein Data Bank Japan (PDBj) licensed under CC-BY-4.0 International PDBID:5i81. (B) The ASM saposin domain contains six structurally critical cysteine residues: C91, C94, C122, C133, C159, and C167. The six cysteine residues form three pairs of disulfide bonds to stabilize the structure of the domain.

extracts revealed that the mutant p.C133Y ASM protein was immunologically stained as a 70-kDa band with the antibody, similar to the wild type ASM protein in control fibroblasts. To the best of our knowledge, only one article reported the immunoblotting analyses of mutant ASM proteins including H243Y, L304P, R498L, and ΔR610, using a patient's fibroblasts (Jones et al. 2008). Their results also showed predominant bands of 70-kDa ASM protein from the fibroblasts lysates of ASM deficiency patients as observed in our patient. Additionally, subcellular localization analysis of the mutant p.C133Y ASM revealed that the mutant protein was intracellularly stained and colocalized with the lysosomal marker. This suggested that the mutant protein might be regularly localized in the lysosomes. Thus, loss of the saposin critical structure might cause loss of catalytic activity even with stable protein expression and proper subcellular localization.

Lysosomal sphingolipid hydrolases generally require an external saposin activator protein for complete enzyme activity. Saposins generated via proteolysis of a precursor prosaposin, are a set of four small glycoproteins that act as enzymatic activators during lysosomal sphingolipid degradation (Kishimoto et al. 1992). Contrastingly, the ASM polypeptide, a lysosomal sphingolipid hydrolase, comprises a built-in N-terminal saposin. A recent study proposed a model where ASM exists in equilibrium between open and closed forms of the saposin domain (Gorelik et al. 2016; Xiong et al. 2016; Zhou et al. 2016). In this proposed model, the open form of saposin domain serves as an interface at the lysosomal membrane, where the catalytic domain activates ASM for sphingomyelin hydrolysis. The C133Y mutation is predicted to disrupt one of the three disulfide bonds in the saposin domain, resulting in complete loss of ASM enzyme activity. The results of this study suggest the functional importance of the disulfide bond between C122 and C133 for ASM enzyme activity.

In conclusion, the p.C133Y mutation may disrupt the critical saposin three-dimensional structure that is essential for catalytic activity, which leads to complete loss of ASM activity, consistent with the phenotype in the patient. p. C133Y is thus a genotype for an early-onset neuronopathic form of ASM deficiency.

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Conflict of Interest

The authors declare no conflict of interest.

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