Molecular Genetic Characterization of Novel Sphingomyelin Phosphodiesterase 1 Mutations Causing Niemann–Pick Disease

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Abstract Niemann–Pick disease (NPD) types A and B are autosomal recessive disorders caused by acid sphingomyelinase (ASM) deficiency due to mutation in the sphingomyelin phosphodiesterase 1 gene (*SMPD1*). Although a number of *SMPD1* mutations were reported, expression studies were performed for only a small number of missense mutations. We evaluated three unrelated patients with clinical manifestations of NPD. Sequence analysis revealed two previously described (S248R and W391G) and two novel (G247D and F572L) missense mutations. To analyze the effects of the novel mutations on ASM function, cDNA was generated by site-directed mutagenesis and expressed in

COS-7 cells. In vitro biochemical assays revealed marked deficiency of ASM activity consistent with the disease phenotype in cells homoallelic for each mutation. We show that each mutation dramatically reduced half-life and catalytic activity of ASM with more pronounced decrease by the G247D mutation. These data suggest that impaired protein stability and decreased enzyme activity are responsible for the disease in sphingomyelinase-deficient patients carrying the G247D and F572L mutations.

Introduction

Niemann–Pick disease (NPD) types A (NPDA) and B (NPDB) are autosomal recessive lysosomal storage disorders caused by acid sphingomyelinase (ASM; E.C.3.1.4.12) deficiency due to mutations in the sphingomyelin phosphodiesterase 1 gene (*SMPD1*; Supplementary Fig. 1) (Da Veiga et al. 1991). NPDA (MIM #257200) is a severe neurodegenerative disease with early onset and rapidly progressing psychomotor retardation, and death by 2–3 years of age (Da Veiga et al. 1991; Schuchman and Desnick 2001). In contrast, NPDB (MIM #607616) is the non-neuropathic, slowly progressing visceral form with hepatosplenomegaly and pulmonary involvement, and survival to adolescence or adulthood (Dardis et al. 2005; Simonaro et al. 2002). Some NPDB patients may develop mild peripheral neuropathy and cherry-red spot and halo (Mihaylova et al. 2007). Recent studies on intermediate forms (NPDA/B) have somewhat questioned this classification, and suggested a continuous disease spectrum related to the amount of residual ASM activity rather than two well-defined extreme clusters (Harzer et al. 2003; Pavlu-Pereira et al. 2005; Schuchman 2007; Wasserstein et al. 2006).

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Although a number of *SMPD1* mutations have been identified, expression studies were performed for only a small number of missense mutations (Rodríguez-Pascau et al. 2009; Desnick et al. 2010). Here we report two novel missense mutations in *SMPD1* and present biochemical and molecular genetic data showing decreased ASM activity and reduced protein stability of the mutant proteins.

Materials and Methods

Patients

Written informed consent was obtained from the parents in accordance with the Declaration of Helsinki. All studies were approved by the Institutional Review Board of the University of Debrecen.

Two Hungarian and one Gypsy patients and their family members were studied (Table 1, Fig. 1). Diagnosis of NPDA and NPDB was made by analysis of clinical and genetic findings, and segregation of *SMPD1* mutations in the families. Patient M1 was born to non-consanguineous Hungarian parents, and there was no history of chronic illness in the family. Rapid deterioration of organ functions led to death at 2 years of age. Patients A1 and S1 have no neurological disease manifestations.

Molecular Genetics

Genomic DNA was isolated from leukocytes. Exons 1–6 of *SMPD1* and the flanking intronic regions were amplified using polymerase chain reaction (PCR) (Erdős et al. 2005; Jiao et al. 2008). Mutational analysis was performed using the BigDye Terminator Cycle sequencing kit v3.1 (Applied Biosystems, Foster City, CA), and an ABI 3130 DNA analyzer (Applied Biosystems) (Tóth et al. 2010). Sequence data were compared to the published sequence of *SMPD1* (GeneCards GC11P006368 and HGMD). The DNA mutation numbering was based on the cDNA sequence (Acc. No. NM_000543) and the first nucleotide is A in the translation initiation codon.

Expression Constructs and Site-Directed Mutagenesis

SMPD1 cDNA was reverse transcribed from human peripheral blood mononuclear cells polyA⁺mRNA and cloned into the pCR4-TOPO vector (Invitrogen Corp. Carlsbad, CA). The coding sequence of the *SMPD1* cDNA was amplified by PCR using primer pairs 5'-GCGATCGC-CATGCCCCGCTACGGAGCGTCACTCCGCC-3' and 5'-CGTACGCGTTTAAACCTTATCGTCGTCATCCTTG-TAATCGCAAAACAGTGGCCTTG-3' and cloned into pCMV6-Neo vector (Origene, Rockville, MD). Mutations,

Table 1 Demographics, clinical findings, and *SMPD1* mutations in patients and family members

Patients and family members (NPD type)	Sex	Age (year)	Clinical manifestations	Nucleotide change	Predicted amino acid change	Mutation published
M1, proband (NPDA)	M	2 (died)	Hepatosplenomegaly, lymphadenomegaly, failure to thrive, psychomotor retardation, neurodegeneration	c.740G>A/c.1716C>G	p.G247D/p.F572L	This report
M2, mother of M1	F	30	—	c.740G>A/wt	p.G247D	
M3, father of M1	M	35	—	c.1716C>G/wt	p.F572L	
M4, sister of M1	F	6	—	c.1716C>G/wt	p.F572L	
A1, proband (NPDB)	F	5	Abdominal distension, hepatosplenomegaly	c.742A>C/c.742A>C	p.S248R	(Sikora et al. 2003)
A2, mother of A1	F	32	—	c.742A>C/wt	p.S248R	
A3, father of A1	M	40	—	c.742A>C/wt	p.S248R	
A4, stepbrother of A1	M	10	—	c.742A>C/wt	p.S248R	
S1, proband (NPDB)	M	6	Abdominal distension, hepatosplenomegaly, cherry-red spot	c.1171T>G/c.1171T>G	p.W391G	(Ferlinz et al. 1997)
S2, mother of S1	F	25	—	c.1171T>G/wt	p.W391G	
S3, father of S1	M	35	—	c.1171T>G/wt	p.W391G	
S4, brother of S1	M	9	—	c.1171T>G/wt	p.W391G	

Patient M1 developed clinical manifestations of NPD at age 6 months, whereas Patients A1 and S1 presented with abdominal distension and hepatosplenomegaly at 18 months and 24 months, respectively.

NPD Niemann–Pick disease, M male, F female

Fig. 1 Pedigree and electropherograms of the patient with NPDA and his family members. **(a)** Filled square denotes the male patient (II/2), half-filled square (I/2) and circles (I/1 and II/1) show heterozygous family members. Bar indicates the patient died. **(b)** Electropherograms of G247D and F572L mutations indicating heterozygous (I/1, I/2, II/1) and compound heterozygous (II/2) mutations of *SMPD1*. **(c)** Restriction digestion analysis of the c.740G>A mutation of *SMPD1*. Agarose gel electrophoresis of *BsmFI* digests of the amplified exon 2 is shown

in patient, his family members, and a control. *M* molecular weight marker, *UP* untreated product of the PCR amplification, *P* patient, *S* sister of patient, *F* father of patient, *M* mother of patient, *C* control. In the mutant allele (P, M), *BsmFI* digestions yielded a 507 and a 88 bp restriction fragments. Such a digestion pattern was not found in the sister and the father, and the control that carried two wild-type alleles at this locus as shown in the figure

to obtain cDNAs coding for G247D or F572L *SMPD1*, were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the supplier's recommendation. All constructs were verified by resequencing with the BigDye Terminator method to ensure that no additional mutation had occurred during the mutagenesis procedure.

Cells and Transfection

COS-7 cells were maintained in tissue culture flasks containing Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO). Transfection was performed with different expression vectors containing the appropriate cDNA insert using FuGENE 6 (Roche, Indianapolis, IN).

Measurement of ASM Activity

COS-7 cells were resuspended in lysis buffer and sonicated for 2 min on ice. Protein concentrations were determined by Lowry method. Protein samples were incubated with 15 mM 2-*N*-hexadecanoylamino-4-nitrophenylphosphorylcholine (Calbiochem, Darmstadt, Germany) as substrate and 250 mM sodium acetate buffer at 37°C for 4 h.

A colorimetric method was used to determine the activity of ASM by measuring the absorbance at 410 nm.

Immunoblotting

Protein extracts from transfected and G418-resistant COS-7 cells (50 µg protein/lane) were subjected to sodium dodecyl sulfate denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore Corp, Billerica, MA). The blots were blocked in 5% nonfat milk in phosphate buffered saline (PBS) containing 0.2% Tween 20 (PBST) overnight. Subsequently, the membranes were probed with goat antihuman *SMPD1* antibody (R&D System, Minneapolis, MN) for 4 h in the presence of 1% nonfat milk in PBST. After incubation, the membranes were washed with PBST four times for 10 min. Rabbit anti-goat IgG-HRP (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used as secondary antibody. Images were obtained with the enhanced chemiluminescence system (Thermo Scientific, Rockford, IL). For detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), goat anti-GAPDH (Santa Cruz) antibody was used. Pre-stained molecular weight markers (Bio-Rad GmbH, Munich, Germany) were used as the molecular size standard.

Metabolic Labeling Study to Determine SMPD1 Half-Life

Transfected COS-7 cells expressing wild-type or mutant proteins were starved for methionine and cysteine for 1 h and then pulse-labeled for 3 h with Tran³⁵S-label (MP Biomedicals LLC, Santa Ana, CA). Cells were cultured in complete medium containing cycloheximide, and newly synthesized proteins were chased for various times. Cells were lysed, and the fate of radiolabeled ASM was analyzed by immunoprecipitation with the M2 anti-FLAG antibody (Sigma-Aldrich) and gel electrophoresis through a 4–15% SDS-PAGE from Bio-Rad (Hercules, CA). Gels were fixed and incubated with Amersham AmplifyTM Fluorographic Reagent (GE Healthcare Biosciences, Piscataway, NJ) before autoradiography. The density of each band was quantified.

Results and Discussion

Genetic Findings

The SMPD1 gene of three unrelated patients with typical clinical manifestations of NPD was analyzed. In two patients (Patients A1 and S1), we found previously described mutations in the *SMPD1* (Table 1). Genetic testing of patient M1 revealed compound heterozygosity for two missense mutations. Both c.740G>A in exon 2 and c.1716C>G in exon 6 were novel. The mutant alleles were confirmed by the analysis of two independent PCR products and were identified in the parents and the sister. The 740G>A mutation introduces a novel *Bsm*FI restriction enzyme recognition site into the DNA sequence. Restriction fragment length polymorphism (RFLP) was performed using PCR products carrying the c.740G>A sequence variant. The presence of the mutation was analyzed using the *Bsm*FI restriction enzyme. Exon 2 of the *SMPD1* gene was amplified in 595 bp products. In individuals with the c.740G>A mutation digestion with *Bsm*FI yielded 507 bp and 88 bp DNA fragments. However, *Bsm*FI did not digest wild-type exon 2 DNA of 595 bp. The novel sequence variants were not found in the control group of 50 healthy Hungarian individuals, representing 100 chromosomes by either RFLP analysis (c.740G>A) or gDNA sequencing (c.1716C>G). The co-segregation of the mutations with the disease in the family further suggested that these were disease-related.

The G247D novel mutation brings the total number of mutations affecting codon 247 to two. In previous studies, the G247S mutation was commonly reported in compound heterozygous patients with the NPDB (Simonaro et al. 2002). The mild course of the disease observed in those patients could be due to the second alleles with neuro-

protective mutations. However, when homoallelism was found for the G247S mutation, all patients presented with the clinical course of NPDA. The occurrence of G247D with F572L in our patient presented a classical NPDA phenotype with severe neurological involvement and fatal outcome suggesting that the novel mutation at amino acid position 572 may not exert neuroprotective effect.

Expression and Function of ASM Proteins

Western blot analysis showed that both wild-type and mutant proteins were expressed in COS-7 cells, meaning that the expression was stable for the plasmid constructs (Fig. 2A). We evaluated the functional effect of the G247D and F572L missense mutations on the activity of ASM. We found that ASM activity was severely decreased in cells expressing G247D protein when compared to COS-7 cells expressing wild-type *SMPD1* and reduced in cells transfected with the c.1716C>G mutant allele (Fig. 2B).

Fig. 2 (A) Detection of wild-type and mutant ASM proteins following transfection in COS-7 cells. Wild-type and mutant *SMPD1* cDNAs in pCMV6 were transfected in COS-7 cells and selected in the presence of 400 µg/ml G418. G418-resistant clones expressing the wild-type and mutant *SMPD1* cDNAs were expanded. Lanes contained 50 µg of cell extract of HL-60 (a), COS-7 cells expressing wild-type *SMPD1* (b), *SMPD1*-G247D (c), and *SMPD1*-F572L (d) mutant proteins, and COS-7 cells transfected by empty plasmid (e). (B) Activity of ASM translated from wild-type and mutant *SMPD1* alleles in COS-7 cells. The data are shown as mean ± SD of at least three different experiments, each performed in triplicate. Error bars correspond to standard deviation. WT wild-type, G247D and F572L, mutant ASM. (C) Autoradiography demonstrating half-life of [35S] methionine and [35S]cysteine-labeled ASM. Experiments were performed with wild-type and mutant enzymes for 12 h. At different time points (0, 2, 4, 8, and 12 h), excess cold amino acid was added to the culture medium. (D) Densitometry demonstrating half-life of ASM. The amount of wild-type and mutant proteins remaining at the indicated time points was also quantified by densitometry, and protein stability was plotted as percentage of the intensity of band compared to wild-type sample

Stability of ASM Proteins

To further evaluate the functional effect of the novel missense mutations on SMPD1 activity, we transfected the wild-type and mutant cDNAs cloned into pCMV6 vector into COS-7 cells. As a negative control, COS-7 cells were transfected with pCMV6 vector DNA (Fig. 2B). Cells were selected on 400 µg/ml G418 and G418 resistant cells were used to assay enzyme activity and immunoblotting. The mean wild-type SMPD1 activity was 97.6 ± 18.2 nmol/mg/h, 112-fold higher than the activity measured in COS-7 cells transfected with the empty vector. No SMPD1 protein was detected in the COS-7 cells transfected with pCMV6. Cells transfected with the construct coding for F572L proteins showed comparable level of expression to the wild-type protein. In contrast, cells expressing G247D showed marked reduction in mutant SMPD1 protein expression. To determine whether the SMPD1 mutations may lead to protein instability, we compared the half-life of wild-type and mutant proteins by measuring the protein levels in transfected cells after pulse chase and cycloheximide block of protein synthesis (Fig. 2C). According to the cycloheximide/immunoblot analysis, the wild-type SMPD1 protein has a half-life over 12 h. The half-life for the F572L protein was shorter, between 4 h and 8 h. The G247D mutation dramatically altered the stability of the mutant protein. The half-life of G247D SMPD1 protein was 2 h or less. The two substituted amino acids are highly conserved among human, mouse, marmoset, opossum, and *Caenorhabditis elegans*; therefore, it is not surprising that constructs bearing mutations of G247D and F572L expressed proteins severely compromised in function. Both mutations are in the metallophosphoesterase catalytic domain of SMPD1.

In summary, two novel mutations causing NPDA with severe neurological involvement and fatal outcome were identified and characterized. These data indicate that the decreased half-life of the G247D and F572L mutant proteins could be responsible for the low enzyme activity and the severe clinical phenotype in patient M1. This study provides further evidence to the genetic heterogeneity of *SMPD1* mutations causing NPD as well as a solid bases to predict the clinical phenotype in patients with the novel G247D/F572L heterozygous mutations.

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