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ATP-BINDING CASSETTE (ABC) TRANSPORTER GENES IN FIBROBLASTS WITH TYPES A AND B NIEMANN-PICK DISEASE

Daishi Hirai, Ayuko Narita and Tsutomu Takahashi

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Department of Pediatrics, Akita University Graduate School of Medicine, Akita 010-8543, Japan

Abstract

Types A and B Niemann-Pick disease (NPD) comprise an autosomal recessive disorder caused by a deficiency of lysosomal acid sphingomyelinase (ASM), which leads to the intracellular accumulation of sphingomyelin and cholesterol and results in abnormalities of lipid metabolism. We report two patients with types A and B NPD showing low levels of high-density lipoprotein (HDL) cholesterol in their sera. Three novel mutations, c.567delT, c.575delC, and c.1481-9T>G, and a known mutation, c.691T>C, of the *ASM* gene were identified in the patients. The c.691T>C mutation is a unique genotype related to a very mild phenotype of type B NPD in the Japanese population. Next, the mRNA expressions of 4 ATP-binding cassette (ABC) transporters related to lipid metabolism, ABCA1, ABCA3, ABCA7, and ABCG1, were analyzed in fibroblasts with types A and B NPD by real-time RT-PCR using hybridization probes. In the analyses, the mRNA levels of ABCG1 were significantly decreased in the fibroblasts of types A and B NPD. The decreased protein level of ABCG1 was also confirmed by Western blot in a fibroblast of type B NPD. The results suggest that secondary dysfunction of ABCG1 may cause an impairment of cholesterol efflux in the peripheral cells, leading to the low plasma levels of HDL cholesterol in types A and B NPD. *ABCG1* is a liver-X-receptor (LXR) target gene involved in cholesterol efflux to HDL, therefore LXR agonist treatment resulted in the reduction of cholesterol and sphingomyelin storage in the fibroblasts of type B NPD.

Key words : Types A and B Niemann-Pick disease, ABC transporters, low HDL cholesterol

Introduction

Types A and B Niemann-Pick disease (NPD) comprise an autosomal recessive disorder caused by a deficiency of lysosomal acid sphingomyelinase (ASM), which results in the intracellular accumulation of sphingomyelin and secondary storaged cholesterol[1]. Type A NPD is a debilitating neurodegenerative disorder of infancy characterized by failure to thrive, hepatosplenomegaly, and a rapidly progressive neurologic course that leads to death by 2 to 3 years of age. In contrast, type B NPD is a non-neurodegenerative disorder showing hepatosplenomegaly with progressive hypersplenism and gradual deterioration in pulmonary function, with most patients surviving into adulthood. Recent clinical observations demonstrated plasma lipid abnormalities including low high-density lipoprotein (HDL) cholesterol as part of the phe-

Correspondence : Tsutomu Takahashi, M.D.
Department of Pediatrics, Akita University Graduate School of Medicine
1-1-1 Hondoh, Akita 010-8543, Japan
Tel : 81-18-884-6159
Fax : 81-18-836-2620
E-mail : tomy@med.akita-u.ac.jp

notype in types A and B NPD[2,3].

HDL cholesterol is thought to remove cholesterol from peripheral tissues and play a critical role in protection against atherosclerotic vascular disease. Some genes have been identified as regulators of HDL formation. The membrane transporter ATP-binding cassette (ABC) transporter A1 (ABCA1) plays a key role in HDL particle formation, possibly facilitated by the actions of other membrane transporters[4]. Low HDL cholesterol is one of the major characteristics of Tangier disease, in which affected cells are characterized by a defective cholesterol efflux due to mutations in the *ABCA1* gene[5,6]. At the cellular level, the loss of ABCA1 function inhibits the efflux of cholesterol and phospholipids in response to stimulation with the major apolipoprotein of HDL, apolipoprotein A-I (apoA-I). Recently, low levels of plasma HDL cholesterol have been observed in patients with Niemann-Pick disease type C (NPC), which is a neurovisceral disorder caused by mutations in the *NPC1* or *NPC2* gene and characterized by the accumulation of unesterified cholesterol in late endosomes and lysosomes of visceral cells[7]. It was shown that basal and cholesterol-stimulated expression of ABCA1 was diminished in cells from a patient with NPC, leading to impaired lipidation of apoA-I[7]. In NPC, secondary impairment of ABCA1 regulation may leads to a low level of plasma HDL cholesterol.

In ASM-deficient NPD macrophages, cholesterol efflux has been shown to be defective partly due to the sequestration of cholesterol by accumulated sphingomyelin, because sphingomyelin is a cholesterol-binding molecule that transiently exists with cholesterol in endosomes and lysosomes[8]. This may explain the low plasma levels of HDL cholesterol found in types A and B NPD. However, the secondary changes of transporters that control cholesterol and lipid metabolism, as described below, could have some effects on the levels of plasma lipids. ABC transporters are a superfamily of highly conserved member proteins that transport a wide variety of substrates including ions, amino acids, peptides, sugars, lipids, and sterols across cell membranes[9]. ABCA1, which functions to promote cellular phospholipid and cholesterol efflux to apoA-I and initiate the formation of HDL, belongs to the ABCA subfamily of ABC transporters. Recently, other ABC transporters have been reported as novel transporters mediating cellular lipid efflux. ABC transporter A7 (ABCA7), a member of the ABCA subfamily, is the closest homolog of ABCA1. ABCA7 was demonstrated to bind to apolipoproteins and promote the efflux of cellular phospholipids and cholesterol, suggesting a possible role of ABCA7 in the regulation of cellular lipid homeostasis[10]. ABC transporter A3 (ABCA3) is another member of the ABCA subfamily that is predominantly expressed at the limiting membrane of lamellar bodies in lung alveolar type II cells[11]. Mutations of the *ABCA3* gene cause fatal surfactant deficiency in the newborn[12]. A recent study indicated that ABCA3 plays an essential role in pulmonary surfactant lipid metabolism and lamellar body biogenesis, probably by transporting phosphatidylcholine and phosphatidylglycerol, which are abundant in normal surfactant, as substrates[13]. ABC transporter G1 (ABCG1), a member of the ABCG subfamily of ABC transporters, has been demonstrated to function as a transporter involved in lipid efflux in peripheral cells[14]. Expression of the *ABCG1* gene is up-regulated by liver-X-receptor (LXR) that functions as a cholesterol sensor responding to elevated intracellular levels of oxysterols[15].

In this report, we first determined molecular lesions in two patients with types A and B NPD. Both of these patients showed low plasma levels of HDL cholesterol at their initial examination in clinic. Similar to fibroblasts with NPC, two fibroblasts from the patients showed intracellular cholesterol storage demonstrated by filipin staining. The mRNA expression patterns of 4 ABC transporters related to lipid metabolism, ABCA1, ABCA3, ABCA7, and ABCG1, were analyzed in those two fibroblasts by real-time RT-PCR using hybridization probes. In addition, LXR agonist treatment was attempted to reduce the accumulated cholesterol and sphingomyelin in the fibroblasts of type B NPD.

Materials and Methods

1. Patients

The patient with type A NPD was a 3-year-old Japanese girl who initially presented with convulsion. On admission, she showed hepatomegaly, the liver was pal-

pable 10 cm below the right costal margin, but the spleen was not palpable. Developmental delay was recognized in this patient. She started walking at the age of 2 years, and then showed neurological deterioration. She could not speak at the age of 3 years. Laboratory data on admission included the following: white blood cell count, 10,800/mm^3; hemoglobin, 11.3 g/dl; platelet count, $14.9\times10^4/mm^3$; aspartate aminotransferase, 166 units/L; alanine aminotransferase, 115 units/L; serum total bilirubin, 1.4 mg/dL; total cholesterol, 282 mg/dL; triglycerides, 332 mg/dL; HDL cholestserol, 13 mg/dL; prothrombin time, 76.0%; thrombin time, 56.0%. ASM enzyme activity of the white blood cells was determined as 5% of the control cells.

The patient with type B NPD was a 28-year-old Japanese woman actively working in an office. She was admitted to the hospital because of abdominal pain and diagnosed with ovarian bleeding. Then, hepatosplenomegaly was detected, with the liver being palpable 3 cm below the right costal margin, and the spleen being palpable 5 cm below the left costal margin. She was clinically diagnosed with type B NPD, based on the existence of Niemann-Pick cells in bone marrow. Laboratory data on admission included the following: white blood cell count, 8,000/mm^3; hemoglobin, 10.5 g/dl; platelet count, $15.5\times10^4/mm^3$; aspartate aminotransferase, 58 units/L; alanine aminotransferase, 61 units/L; serum total bilirubin, 1.3 mg/dL; total cholesterol, 185 mg/dL; triglycerides, 186 mg/dL; HDL cholestserol, 13 mg/dL; prothrombin time, 56.8%; thrombin time, 31.8%. ASM enzyme activity of the white blood cells was determined as 30% of that in control cells.

2. Mutational analyses

To elucidate the molecular lesions in the two patients, we determined the whole sequence of *ASM* genomic DNA from each patient. We first designed primers (sequences available on request) encompassing each *ASM* coding exon and its splice site using the genomic contig NC_000011.8. The PCR products from genomic DNA were sequenced directly with an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, California, USA) using an automated sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystems, Carfornia, USA). The inheritance pathway of the identified mutations was determined among the family members of the patient with type B NPD. Blood samples were obtained from the family members after obtaining informed consent. To analyze the transcripts from the *ASM* gene of these patients, cDNA was synthesized using the *Total RNA Isolation System* (Promega, Wisconsin, USA) and SuperScript One-Step RT-PCR System (Life Technologies, California, USA) for sequencing.

3. Cell culture and filipin staining

In NPC fibroblasts, accumulation of intracellular unesterified cholesterol can be demonstrated by staining with filipin. To see whether the same accumulation of unesterified cholesterol as NPC is observed in types A and B NPD, we performed filipin staining of fibroblasts from our patients. Normal fibroblasts used in this study were obtained from control subjects. ASM-deficient fibroblasts were established from our two patients with types A and B NPD. The cells were subsequently maintained in Eagle's minimal essential medium (E-MEM) supplemented with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal bovine serum (HI-FBS) (Dainippon Pharmaceutical, Osaka, Japan). All cells were seeded overnight at the density of 2.5×10^4 cells/cm^2 before treatment. Cultures were maintained in a humidified 5% CO_2 incubator at 37°C.

For filipin staining, cells were incubated in medium supplemented with LDL cholesterol (50 μg/ml) (Sigma, St. Louis, USA) for 24 h, and then cells were stained with 300 μg/ml of filipin complex (Sigma, St. Louis, USA) in 1 X PBS for 30 min. Stained cells were examined using a Zeiss LSM 510 META confocal microscope equipped with a UV laser.

4. Hybridization probe assays

RNA was prepared from fibroblasts and purified using High Pure PCR Product Purification (Roche Diagnotics, Basel, Switzerland). For quantitative RT-PCR, 1 μg of RNA were first reverse-transcribed into cDNA for 10 min at 55°C. Real-time PCR for detecting target genes was performed using a LightCycler thermal cycler sys-

Table 1. Oligonucleotide primers and hybridization probes used in the LC-PCR assay

Primer or probe	Type	Sequence	Position
ABCA1F	Sense	5′-TAGTCCTCTTTCCCGCATTATC-3′	
ABCA1R	Antisense	5′-TGCCTTCCAGATCATGGAAC-3′	
ABCA1D	Donor Probe	5′-ATACACCTGACACTCCAGCCACAAGGCA-3′-Fluorescein	
ABCA1A	Acceptor Probe	5′-LCRed640-GTCATGGCTGAGGTGAACAAGACCTTCCA-3′-Phosphorylation	
ABCA3F	Sense	5′-CGCTGTCAAGCTCAACA-3′	
ABCA3R	Antisense	5′-CTCCGAGGAGTAGTTGATGG-3′	
ABCA3D	Donor Probe	5′-CAGCTGTATGCGGCCTTCTTCAGGAA-3′-Fluorescein	
ABCA3A	Acceptor Probe	5′-LCRed640-ATGGCCCAGAATTGCTGGCAGTGC-3′-Phosphorylation	
ABCA7F	Sense	5′-GCTACTCTTTGCACCAGATACAC-3′	
ABCA7R	Antisense	5′-AGGTCTGGGCTGCCTTCTT-3′	
ABCA7D	Donor Probe	5′-TCAGCAGGGTGAGCTCCTCGAAGGT-3′-Fluorescein	
ABCA7A	Acceptor Probe	5′-LCRed640-CGGTTGACCTGGGCCATGAGCTTC-3′-Phosphorylation	
ABCG1F	Sense	5′-GCCAAGAAGGTCTTGAGCAA-3′	
ABCG1R	Antisense	5′-GATCTGAAAGGGCACGTCTG -3′	
ABCG1D	Donor Probe	5′-ATGCCTACTGTTCTGACATTTCCCCTGGAG -3′-Fluorescein	
ABCG1A	Acceptor Probe	5′-LCRed640-TGGGAGTCTTTCTTCGGGAACACCTGAACT-3′-Phosphorylation	

tem according to the manufacturer's instructions. Primers and hybridization probes for *ABCA1*, *ABCA3*, *ABCA7*, and *ABCG1* cDNAs are shown in Table 1. The first hybridization probes were labeled with fluorescein as the donor fluorophore at its 3′ end. The second hybridization probes were labeled with the acceptor fluorophore LightCycler Red 640 (LCRed640) at its 5′ end and blocked from extending at its 3′ end by phosphorylation. The amplification started with an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 62°C for 15 s, and 72°C for 8 s with a temperature transition rate of 20°C/s. At the end of the 40 cycles, a cooling step of 40°C for 30 s was performed. The *GAPDH* mRNA was amplified as a housekeeping gene using the same method. The mRNA levels of each gene were standardized for *GAPDH* levels. LightCycler software version 3.3 (Roche Diagnostics, Basel, Switzerland) was used to analyze PCR kinetics and calculate quantitative data. A standard curve was generated by each run including one sample of a known concentration. The results from 3 separately performed experiments are presented as the mean ± standard deviation.

5. Immunoblotting experiments

To detect ABCG1 protein by Western blotting, 15 μg of total protein from cell lysates of the patient firbrobasts obtained by mixing cells with NuPAGE LDS sample buffer (Invitrogen Technologies, California, USA) was denatured by heating at 70°C for 10 min, and electrophoresed on 4-12% NuPAGE Bis-Tris Mini Gels (Invitrogen Technologies, California, USA) using NuPAGE SDS running buffer (Invitrogen Technologies, California, USA). Transfer to a nitrocellulose membrane was performed by using iBlot® Western Detection kit (Invitrogen Technologies, California, USA). The nitrocellulose membranes were blocked with non-fat dry milk (1%) for 1 h, and were subsequently incubated with primary antibodies for 1 h. For the detection of ABCG1, a rabbit monoclonal anti-human ABCG1 antibody (1 : 2,000) (Abcam, Cambridge, England) was used. Human β-actin protein was detected on the same membrane to quantify protein loading. A rabbit polyclonal antibody against β-actin (1 : 2,000) (Abcam, Cambridge, England) served as a loading control. The primary antibody was detected by anti-rabit secondary antibody (1 : 2,000) with chemiluminescence reagent (Bio-Rad, California, USA) for 3 min and exposed to X-ray film for 15 s. To examine an effect of LXR agonist,

TO-901317, on the expression levels of ABCG1 protein, the mediums were supplemented with 5 μM TO-901317 for 24 h.

6. Immunofluorescence staining of human fibroblasts by lysenin

Skin fibroblasts from a control subject and a patient with type B NPD were cultured and maintained in Eagle's minimal essential medium (E-MEM) supplemented with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal bovine serum (HI-FBS) as described before[16]. To see an effect of LXR agonist, TO-901317, on intracellular levels of cholesterol and sphingomyelin, normal and type B NPD fibroblasts were incubated in the medium in the absence or presence of 5 μM TO-901317 (Sigma, St. Louis, USA) plus HDL cholesterol (Sigma, St. Louis, USA) for 24 h, and stained with filipin or lysenin. TO-901317 was demonstrated to reduce intracellular level of accumulated cholesterol in the fibroblasts of NPC[17]. This reduction was induced by normalization of the impaired cholesterol trafficking through the correction of secondary decreases of ABCA1 and ABCG1 gene expressions in the NPC cells.

Intracellular cholesterol and sphingomyelin of the cells were visually quantified using confocal microscope. For lysenin staining, after washing with PBS, the cells were fixed with 3.7% (w/v) formaldehyde in PBS, washed, and permeabilized with digitonin (50 μg/ml) for 10 min, blocked with 2% BSA-PBS for 15 min. The cells were incubated with lysenin (1 μg/ml in 2% BSA-PBS) for 2 h, washed with PBS, and incubated with anti-lysenin antiserum diluted 1/1,000 with 2% BSA-PBS for 1 h, followed by incubation with 10 μg/ml tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG (Chemicon International Inc., California, USA) on 2% BSA-PBS for 1 h. Fluorescence microscopy was performed using a Zeiss LSM 510 META confocal microscope equipped with a UV laser.

Results

Mutational analyses

Two heterozygous *ASM* gene mutations were identi-

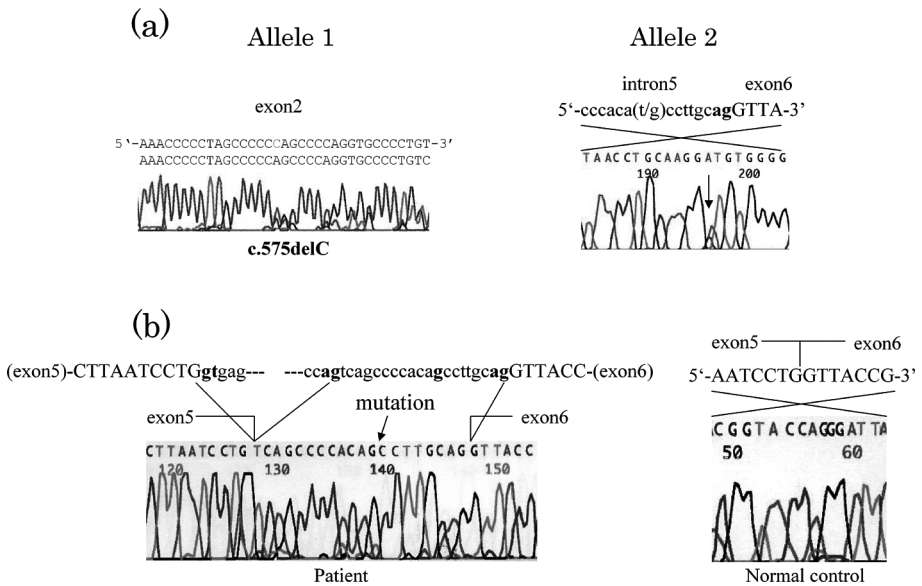

Fig. 1. Nucleotide sequences of the ASM mutations identified in the patient with type A NPD. (a) Two heterozygous *ASM* gene mutations were identified in the patient with type A NPD. One of the *ASM* alleles in this patient contained a one-base deletion (designated as c.575delC) in exon 2, which caused the premature termination codon (TGA) at *ASM* codon 261. The other *ASM* allele had a T-to-G transversion mutation (c.1481-9T>G) at the consensus acceptor splice site of intron 5. (b) The *ASM* cDNA had an insertion sequence, 20 bases of the acceptor splice site of intron 5, between exons 5 and 6, compared to the wild-type sequence.

fied in the patient with type A NPD (Fig. 1a). One of the *ASM* alleles in this patient contained a one-base deletion (designated as c.575delC) in exon 2, which caused the premature termination codon (TGA) at *ASM* codon 261. The other *ASM* allele had a T-to-G transversion mutation (c.1481-9T>G) at the consensus acceptor splice site of intron 5. This second mutation can be considered one of the consensus sequences for mRNA splic-

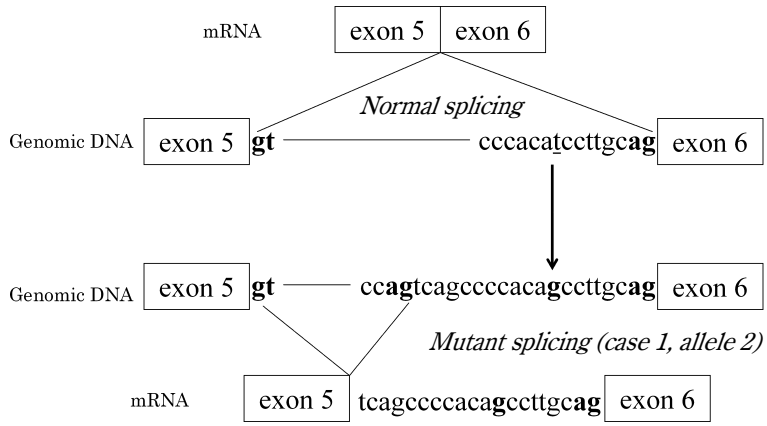

Fig. 2. Identification of abnormal splicing in a patient with type A NPD. The mutation is positioned in the consensus sequence in the acceptor splice site of intron 5 of the genomic DNA. The *ASM* mRNA of the patient had an insertion sequence, 20 bases of the acceptor splice site of intron 5, between exons 5 and 6. The creation of another acceptor site due to the mutation of the consensus sequence might cause this aberrant insertion. In this sequence, contrary to expectation, there was no other allelic sequence found, suggesting that the other allelic mutation, a premature stop codon mutation (c.575delC), may make the mutant transcript unstable, resulting in nonsense-mediated delay of the mutant transcripts.

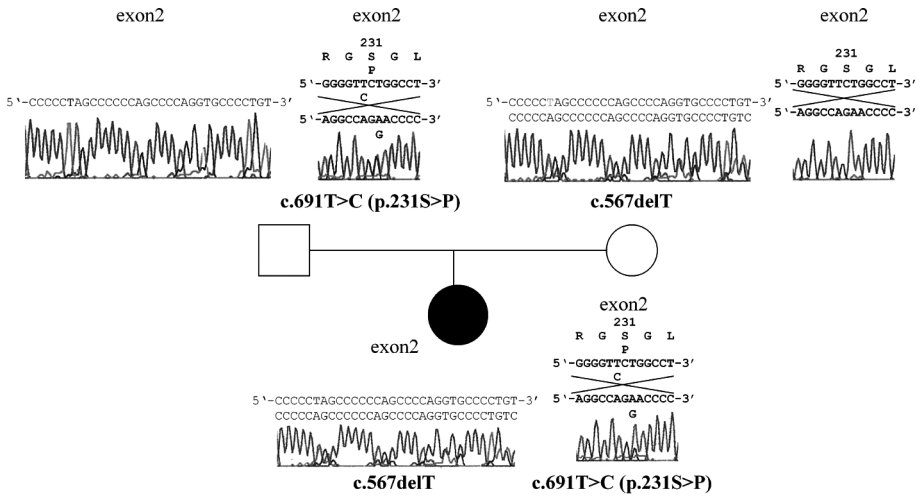

Fig. 3. Nucleotide sequences of the ASM mutations identified in the patient with type B NPD. There were two heterogeneous mutations in the *ASM* gene. One was a one-base deletion (designated as c.567delT) in exon 2, which caused the premature termination codon (TGA) at *ASM* codon 261. The other allele contained a T-to-C transversion in exon 2, which predicted a serine (TCT) to proline (CCT) change at codon 231 (designated as c.691T>C, or p.S231P).

ing. Therefore, to discover whether aberrant splicing of *ASM* mRNA was occurring in the patient, we analyzed the mRNA of the *ASM* gene. In this patient, the *ASM* cDNA had an insertion sequence, 20 bases of the acceptor splice site of intron 5, between exons 5 and 6, compared to the wild-type sequence (Fig. 1b). The creation of another acceptor site due to the mutation of the consensus sequence might have caused this aberrant insertion (Fig. 2). In this sequence, contrary to expectation, there was no other allelic sequence found, suggesting that another mutational allele, premature stop codon mutation (c.575delC), may make the mutant transcript unstable, resulting in a nonsense-mediated decay of the mutant transcripts. In the patient with type B NPD, there were two heterogeneous mutations in the *ASM* gene (Fig. 3). One was a one-base deletion (designated as c.567delT) in exon 2 which caused the premature termination codon (TGA) at *ASM* codon 261. The other allele contained a T-to-C transversion at the *ASM* cDNA, which predicted a serine (TCT) to proline (CCT) change at codon 231 (designated as c.691T>C, or p.S231P).

Filipin staining

Compared to normal fibroblasts, cultured fibroblasts from the patients showed intracellular distributions of cholesterol with filipin staining in a characteristic distribution pattern near the nucleus, showing that fibroblasts exhibited intracellular storage of cholesterol after incubation with LDL cholesterol (Fig. 4). This observation suggested that ASM deficiency causes intracellular cholesterol storage in fibroblasts similar to that in cells with NPC.

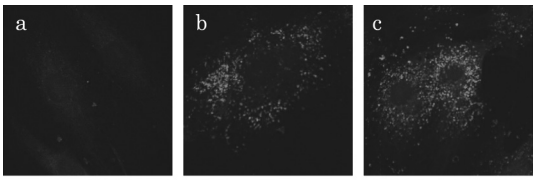

Fig. 4. Filipin staining of normal and type A and B NPD fibroblasts. Accumulations of unesterified cholesterol were visualized by confocal microscopy. Three different fibroblast types were examined in this study : normal cells (a), type A NPD cells (b), and type B NPD cells (c). Fibroblasts in (b)-(c) were filipin-positive and had cholesterol-laden lysosomes.

Hybridization probe assays

The mRNA levels of 4 ABC transporters, ABCA1, ABCA3, ABCA7, and ABCG1, were analyzed in one fibroblast from a control subject and two fibroblasts from the two patients with types A and B NPD by real-time RT-PCR using hybridization probes (Table 2). After being standardized for *GAPDH* levels, the mRNA levels of ABCA1 and ABCG1 were significantly decreased in the

Table 2. Expression pattern of ABCA genes in the fibroblast cells with types A and B Niemann-Pick disease

Genes	Samples	Sample Copy number (n=3, average±S.D)	GAPDH Copy number (n=3, average±S.D.)	Corrected Copy number
ABCA1	Normal	$4.24(\pm0.18)\times10^{4}$	$2.39(\pm0.10)\times10^{6}$	1.77×10^{-2}
	type A NPD	$2.46(\pm0.04)\times10^{3}$	$3.90(\pm0.21)\times10^{6}$	$6.31\times10^{-3*}$
	type B NPD	$1.62(\pm0.13)\times10^{3}$	$1.93(\pm0.04)\times10^{6}$	$8.41\times10^{-3*}$
ABCA3	Normal	$6.08(\pm1.04)\times10^{2}$	$2.39(\pm0.10)\times10^{6}$	2.54×10^{-4}
	type A NPD	$5.37(\pm0.44)\times10^{2}$	$3.90(\pm0.21)\times10^{6}$	1.38×10^{-4}
	type B NPD	$3.95(\pm0.29)\times10^{2}$	$1.93(\pm0.04)\times10^{6}$	2.05×10^{-4}
ABCA7	Normal	$4.20(\pm0.18)\times10^{2}$	$2.39(\pm0.10)\times10^{6}$	1.75×10^{-4}
	type A NPD	$1.34(\pm0.09)\times10^{2}$	$3.90(\pm0.21)\times10^{6}$	3.44×10^{-5}
	type B NPD	$5.18(\pm0.22)\times10^{2}$	$1.93(\pm0.04)\times10^{6}$	2.69×10^{-4}
ABCG1	Normal	$4.80(\pm0.59)\times10^{2}$	$2.39(\pm0.10)\times10^{6}$	2.01×10^{-4}
	type A NPD	$1.16(\pm0.07)\times10^{0}$	$3.90(\pm0.21)\times10^{6}$	$2.97\times10^{-5**}$
	type B NPD	$2.36(\pm0.12)\times10^{0}$	$1.93(\pm0.04)\times10^{6}$	$1.22\times10^{-5***}$

S.D., Standard Deviation, *p<0.0001, **p=0.0003, ***p=0.0002

fibroblasts from the patients compared with that in the normal control. In ABCG1, mRNA expression was determined to be about one-tenth of the mRNA expressed by the normal control, suggesting significant suppression of ABCG1 mRNA expression in fibroblasts with types A and B NPD. There was no significant difference in mRNA levels of the *ABCA3* and *ABCA7* genes.

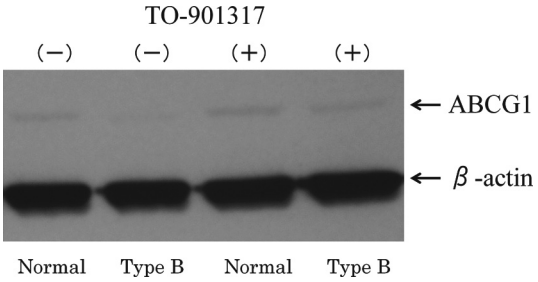

Fig. 5. Immunoblotting of ABCG1 proteins from the cell lysates of normal and type B NPD. ABCG1 proteins was detected using 15 μg each of the cell lysates. Compared to that in normal cells, a weaker band of ABCG1 protein was observed in the cell lysate of type B NPD, but the band was intensified in the presence of TO-091317. Human β-actin protein was detected on the same membrane to quantify protein loading.

Immunoblotting experiments

ABCG1 protein was detected using 15 μg of cell lysates from normal cells and from type B NPD (Fig. 5). Compared to that from normal cells, a weaker ABCG1 bands was observed in the cell lysates of type B NPD, but the band was intensified in the presence of TO-091317.

Immunofluorescence staining of human fibroblasts by lysenin

In normal fibroblasts, there was no positive staining of filipin or lysenin in either the absence or presence of TO-091317 plus HDL cholesterol (Fig. 6). Positive stainings of filipin and lysenin were observed in fibroblasts of type B NPD, but stainings were reduced in the presence of TO-091317 plus HDL cholesterol.

Discussion

Two novel mutations, c.575delC and c.1481-9T>G, were identified in a Japanese patient with type A NPD (Fig. 7). The c.1481-9T>G mutation is the third splice-site mutation of the *ASM* gene to be identified in patients with types A and B NPD. This mutation is positioned in the consensus sequence in the acceptor splice site of in-

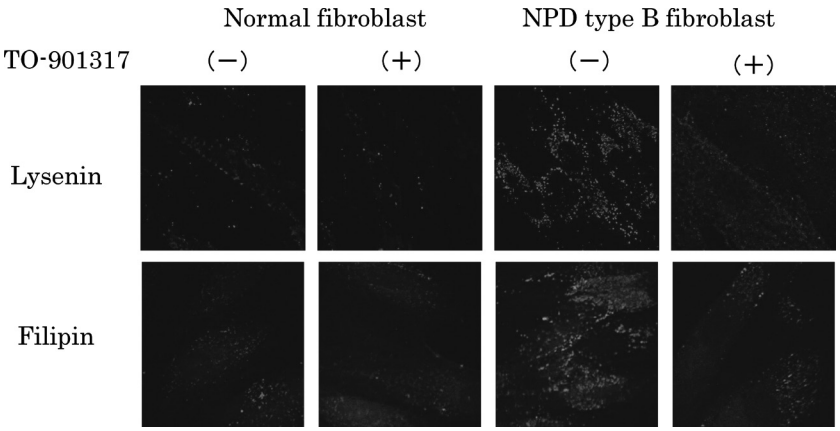

Fig. 6. Immunofluorescence staining of human fibroblasts by lysenin and filipin. To determine the effect of LXR agonist, TO-901317, on intracellular levels of cholesterol and sphingomyelin, normal and type B NPD fibroblasts were incubated in the medium in the absence or presence of 5 μM TO-901317 plus HDL cholesterol (Sigma) for 24 h, and stained with filipin or lysenin. In normal fibroblasts, there was no positive staining of filipin or lysenin in either the absence or presence of TO-091317 plus HDL cholesterol. Positive stainings of filipin and lysenin were observed in the fibroblasts of type B NPD, and these staining were reduced in the presence of TO-091317 plus HDL cholesterol.

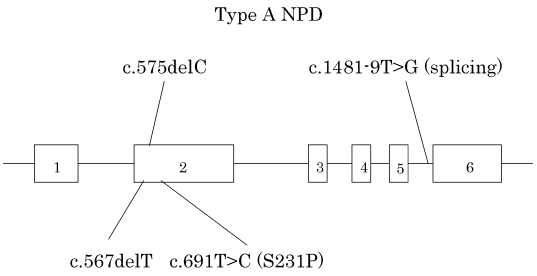

Fig. 7. Four mutations identified from two Japanese patients with types A and B NPD. Two novel genetic variants, c.575delC and c.1481-9T>G, were identified as compound heterozygous mutations in a Japanese patient with type A Niemann-Pick disease. Two mutations, c.567delT and c.691T>C, were also identified in a patient with type B NPD. The former, c.567delT, is a novel mutation, but we previously reported c.691T>C (S231P) as a homozygous mutation in an unrelated Japanese patient with type B NPD, suggesting that the c.567delT could be related to a mild phenotype of types A and B NPD.

tron 5, which creates another acceptor site, resulting in aberrant insertion. Two heterozygous mutations, c.567delT and c.691T>C, were identified in the *ASM* gene from a Japanese patient with type B NPD (Fig. 7). This patient showed a mild clinical course, actively working as an office clerk, with normal delivery of a healthy baby girl. The c.567delT mutation is a one-base deletion mutation causing a frameshift, which is expected to lead to a severe phenotype of type A NPD. Thus, the c.691T>C mutation could be related to the mild phenotype in this patient. In our previous report, homozygous mutation of c.691T>C was noted in a Japanese patient with type B NPD[18]. This patient was a 39-year-old man actively working as a fireman, unrelated to the present case. Mild liver dysfunction was detected at 27 years of age, and he was admitted to our hospital for examination of hepatic dysfunction at 39 years of age. The liver was palpable 5 cm below the right costal margin and the spleen 3 cm below the left costal margin. Laboratory data evaluating hepatic function and the coagulation system were not remarkable. ASM enzyme activity of cultured skin fibroblasts from the patient showed 19% of the activity of normal controls. Thus, we suggest that c.691T>C is a unique genotype related to a very mild phenotype of type B NPD in the Japanese population.

In fibroblasts from types A and B NPD, the mRNA expressions of *ABCA1* and *ABCG1* genes were significantly decreased compared with normal fibroblasts. In particular, the *ABCG1* gene was markedly suppressed to about one-tenth of that in normal control cells. In contrast, there was no significant difference between types A and B NPD and the normal control regarding mRNA expressions of *ABCA3* and *ABCA7* genes. Low plasma levels of HDL cholesterol were observed in most patients with NPC, and apoA-I-mediated lipid efflux was shown to be impaired in fibroblasts with NPC[7]. It was recently demonstrated that basal and cholesterol-stimulated expression of ABCA1 is reduced in fibroblasts from patients with NPC, leading to impaired lipidation of apoA-I[7]. Because the mRNA levels of ABCA1 were about half those of the normal control in our study, the mechanism involving impairment of ABCA1 function may partly contribute to the decreased cholesterol efflux, leading to low plasma levels of HDL cholesterol in types A and B NPD. However, impairment of ABCG1 function could be the main molecular reason for this HDL cholesterol pattern in types A and B NPD, because the *ABCG1* gene was more intensely suppressed than the ABCA1 in our study. There are several reports describing evidence that ABCG1 is a mediator of cholesterol efflux to HDL cholesterol. Macrophages lacking ABCG1 expression show impaired cholesterol efflux to HDL but not to lipid-free apoA-I *in vitro*[14,19]. ABCA1 and ABCG1 have been shown to function cooperatively to remove cholesterol from cells *in vitro*. In a previous study, low HDL-cholesterol levels were reported in two family members with the ASM deficiency type B NPD[20]. In the fibroblasts of these type B NPD cases, however, apoA-I-dependent cholesterol mobilization was reported to be normal[19] ApoA-I-dependent cholesterol efflux was not determined in our study, but ABCA1 may not be a key mediator having a negative effect on cholesterol efflux in cells with types A and B NPD. We suggest that a secondary dysfunction of ABCG1 may cause the impairment of cholesterol efflux in peripheral cells, leading to low plasma levels of HDL cholesterol in types A and B NPD. This result was supported by the LXR agonist experiment, in

which accumulated cholesterol and sphingomyelin were both reduced in fibroblasts of type B NPD following treatment with LXR agonist and HDL cholesterol. This finding also suggested the potential of using LXR agonist to treat types A and B NPD. Further studies will be needed to investigate this possibility.

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