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SECONDARY IMPAIRMENT OF INTRACELLULAR CHOLESTEROL TRANSPORT IN CELLS WITH NIEMANN–PICK DISEASE TYPE C

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Abstract

Niemann–Pick disease type C (NPC) is an autosomal recessive lipidosis resulting from mutations of the *NPC1* or *NPC2* gene, clinically characterized by hepatosplenomegaly and progressive neurological symptoms including vertical supranuclear ophthalmoplegia, progressive ataxia, dystonia, and dementia. Neurodegeneration in NPC shows a number of pathological features similar to those observed in Alzheimer disease. Biochemically, this disease is featured by a defect in intracellular trafficking of exogenous cholesterol that leads to the lysosomal and late–endosomal accumulation of unesterified cholesterol. Some reports have shown disturbance of cholesterol efflux in cells with *NPC1*, or *NPC2* gene mutations, resulting in plasma lipid abnormalities including low levels of high–density lipoprotein (HDL) cholesterol as part of the phenotype in NPC. To elucidate the molecular basis for low HDL cholesterol in human plasma, mRNA expressions of 4 ATP–binding cassette (ABC) transporters related to lipid metabolism, ABCA1, ABCA3, ABCA7, and ABCG1, were analyzed in fibroblasts with NPC1 gene mutations by real–time RT–PCR using hybridization probes. These analyses were performed using two fibroblasts of NPC from a patient with two novel compound heterozygous *NPC1* mutations, c.1891A>G and c.581_592delinsG, and a patient with other two novel compound heterozygous *NPC1* mutations, c.2800C>T and c.3418G>A. Based on these analyses, the mRNA levels of ABCA1 and ABCG1 were significantly decreased in the fibroblasts. These findings suggest that secondary dysfunctions of ABCA1 and ABCG1 may cause impairment of cholesterol efflux in the peripheral cells, leading to low plasma levels of HDL cholesterol in NPC. Second, to clarify whether the secondary acid sphingomyelinase deficiency in NPC cells is related to the intracellular pathology of NPC, we investigated the effects of an acid sphingomyelinase inducer, butyrate, on the accumulation of unesterified cholesterol in NPC cells. The results demonstrated that correction of the secondary acid sphingomyelinase deficiency could ameliorate the extent of cholesterol accumulation.

Key words : Niemann–Pick disease type C, ABC transporters, Acid sphingomyelinase

Introduction

Niemann–Pick disease type C (NPC) is an autosomal recessive lipidosis resulting from mutations in the *NPC1* or *NPC2* gene[1,2]. This disease is clinically characterized by hepatosplenomegaly and progressive neurological symptoms including vertical supranuclear ophthalmoplegia, progressive ataxia, dystonia, and dementia. Neurodegeneration in NPC shows a number of pathological features similar to those observed in Alzheimer disease. Cholesterol metabolism impairment, endosome/lysosomal dysfunction, and tauopathies are observed in both diseases. Biochemically, NPC is characterized by a defect in intracellular trafficking of exogenous cholesterol that leads to the lysosomal and endosomal accumulation of unesterified (free) cholesterol[3,4]. Approximately 95%

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of patients have mutations in the *NPC1* gene that encodes a large membrane glycoprotein with 13 putative transmembrane (TM) domains and sterol-sensing domains (SSDs)[5-8]. The remaining patients have mutations in the NPC2 gene, encoding a small soluble lysosomal protein that binds cholesterol with high affinity. NPC1 has been extensively studied at the cellular and molecular levels. NPC1 plays important roles in moving cholesterol and other lipids out of endosomes by vesicular trafficking, but the precise function of NPC1 is not yet known.

Recent clinical observations demonstrated plasma lipid abnormalities including low high-density lipoprotein (HDL) cholesterol as part of the phenotype in NPC[9]. HDL cholesterol is thought to remove cholesterol from peripheral tissues and plays a critical role in protection against atherosclerotic vascular disease. Recently, some genes have been identified as regulators of HDL formation. The membrane transporter ATP-binding cassette transporter A1 (ABCA1) plays a key role in HDL particle formation, possibly facilitated by the actions of other membrane transporters[10]. Low HDL cholesterol is one of the major features in Tangier disease, in which affected cells are characterized by defective cholesterol efflux due to mutations in the *ABCA1* gene. At the cellular level, loss of ABCA1 function eliminates the efflux of cholesterol and phospholipids in response to stimulation with the major apolipoprotein of HDL, apolipoprotein A-I (apoA-I). Recently, it was shown that basal and cholesterol-stimulated expression of ABCA1 is diminished in cells from a patient with NPC, leading to impaired lipidation of apoA-I[11]. In NPC, secondary impairment of ABCA1 regulation may cause a low level of plasma HDL cholesterol.

ATP-binding cassette (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[12]. 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 members of ABC transporter have been reported as novel transporters mediating cellular lipid efflux. ATP-binding cassette transporter A7 (ABCA7), a member of the ABCA subfamily, is the closest homolog of ABCA1. ABCA7 was demonstrated to bind apolipoproteins and promote efflux of cellular phospholipids and cholesterol, suggesting a possible role of ABCA7 in the regulation of cellular lipid homeostasis[13]. ATP-binding cassette transporter A3 (ABCA3) is another member of the ABCA subfamily that was predominantly expressed at the limiting membrane of the lamellar bodies in lung alveolar type II cell[14]. Mutations of the *ABCA3* gene cause fatal surfactant deficiency in the newborn[15]. 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[16]. ATP-binding cassette 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[17]. 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[18].

NPC shows clinical similarities with types A and B Niemann-Pick disease, which result from the deficient activity of lysosomal acid sphingomyelinase (ASM), and are characterized by the cellular accumulation of sphingomyelin and cholesterol. ASM is responsible for the lysosomal hydrolysis of sphingomyelin to yield ceramide and phosphocholine and plays an important role in the intracellular relationship between cholesterol and sphingomyelin metabolism. Sphingomyelin has unique biological characteristics including an extremely high affinity for cholesterol, leading to an identical subcellular distribution of both lipids, which are concentrated in the plasma membrane[19]. An inhibition of human fibroblast sphingomyelinase by cholesterol was first shown in 1981[20]. In NPC fibroblasts, which have lysosomal accumulation of unesterified cholesterol, ASM activity is partially reduced by as much as 50% when the cells are grown in medium containing 10% fetal bovine serum or LDL cholesterol[3,21,22]. Removal of lysosomal cholesterol by growth in medium containing lipoprotein-deficient-serum (LPDS) restores ASM activity to a normal level[23]. This observation suggests that

ASM enzyme activity is influenced or regulated by the concentration of lysosomal cholesterol.

In this report, we attempted to characterize the expression of genes related to cholesterol homeostasis in NPC. We first determined the molecular lesions in two patients with NPC. Using fibroblasts from the patients, the mRNA expression patterns of 4 ABC transporters related with lipid metabolism, ABCA1, ABCA3, ABCA7, and ABCG1, were analyzed by real-time RT-PCR using hybridization probes. In addition, we investigated the effects of an acid sphingomyelinase inducer, butyrate, on secondary ASM deficiency and the accumulation of unesterified (free) cholesterol in NPC cells[24].

Materials and methods

1. Patients

Case 1 was a 5-month-old girl born to healthy Japanese parents by cesarean section at 34 weeks of pregnancy. At birth, she weighed 2,920 g and had severe hepatosplenomegaly and abnormal liver dysfunction tests. She showed mild dyspnea due to hepatosplenomegaly, but she was in stable condition. At the age of 15 days, bone marrow examination demonstrated the presence of numerous foam cells, leading to a diagnosis of Niemann-Pick disease. The patient was diagnosed with NPC disease, since cultured fibroblasts did not have deficient ASM activity, but showed positive filipin staining, demonstrating the storage of free cholesterol. She developed normally to the age of 5 months.

Case 2 was a 2-year-old girl born to healthy Japanese parents with an uneventful delivery at 36 weeks pregnancy. At birth, she weighed 1,995 g. She developed normally during infancy, but started to present with ataxic gait and upper limb trembling at the age of 1.4 years and 2.1 years, respectively. She was admitted to the hospital for examination of hepatosplenomegaly and neurological deterioration at the age of 2.5 years. Foam cells were detected in her bone marrow, suggesting a diagnosis of a lysosomal storage disease. Cultured fibroblasts did not demonstrate deficient ASM activity, but were stained strongly with filipin, leading to a diagnosis of NPC disease.

2. Cell culture and filipin staining

In NPC fibroblast cells, accumulation of intracellular unesterified cholesterol is demonstrated by staining with filipin. To diagnose these patients, we performed filipin staining in the fibroblasts[1]. Normal fibroblasts used in this study were obtained from control individuals. The cells were subsequently maintained in Eagle's minimal essential medium (E-MEM) supplemented with L-glutamine (2 mM), penicillin (100 IU/ml), streptomysin (100 μg/ml) and 10 % heat-inactivated fetal bovine serum (HI-FBS) (Dainippon Pharmaceutical, Osaka, Japan).

For filipin staining, cells were incubated in medium supplemented with low-density lipoprotein (LDL) cholesterol (50 μg/ml) (Sigma, St. Louis, MO, USA) for 24 hrs, 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 UV laser.

3. Mutational Analysis

To determine the whole sequence of the *NPC1* genomic DNA from the patients, we designed primers (sequences available on request) encompassing each *NPC1* coding exon and its splice site using the genomic contig NT_000018. The PCR products from genomic DNA were sequenced directly with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, California, USA) using an automated sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystems, California, USA). The inheritances of the identified mutations were determined among the family members. Blood samples were obtained from family members after obtaining informed consent.

4. Hybridization probe assays

RNA was prepared from fibroblast cells and purified using High Pure PCR Product Purification (Roche Diagnotics, Basel, Switzerland). For quantitative RT-PCR, 1 μg of RNA was 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 system according to the manufacture's instructions. Primers and hybridization probes of *ABCA1*, *ABCA3*,

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	

ABCA7, and *ABCG1* cDNAs were shown in Table 1. The first hybridization probes were labeled with fluorescein as the donor fluorophore on its 3′ end. The second hybridization probes were labeled with the acceptor fluorophore LightCycler Red 640 (LCRed640) at its 5′ end and is blocked from extension at its 3′ end by phosphorylation. Amplification started with an initial denaturation step at 95℃ for 10 min, followed by 40 cycles of 95℃ for 10 s, 62℃ for 15 s, and 72℃ for 8 s with a temperature transition rate of 20℃/s. At the end of the 40 cycles, a cooling step at 40℃ 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 during each run including one sample of known concentration. The results from 3 separately performed experiments are presented as the mean±standard deviation.

5. Acid sphingomyelinase inducer

Lymphoblastic cells were established from a normal control and a patient with NPC. The cells were subsequently maintained in RPMI 1640 (Sigma, St. Louis, USA) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS). In the ASM inducer study, the cells were treated with butyrate at various concentrations (0 mM, 1 mM, 5 mM, 10 mM) for 24 h and the enzyme activity of ASM was determined in the cellular lysates by the method described previously[25]. The concentrations of total cholesterol and free cholesterol were also determined in the cellular lysates using Amplex® Red Cholesterol Assay Kit (Invitrogen, Paisley, UK). Statistical analysis was performed by *t* test using StatView-J4.5 (Abacus Concepts, Berkeley, CA, USA). Unless otherwise indicated, results are presented as means±SD of three separate experiments.

Results

Filipin Staining

Compared to normal fibroblasts, cultured fibroblasts from each patient demonstrated intracellular distributions of cholesterol with filipin staining in a characteristic distribution near the nucleus. Positive staining for filipin demonstrated a granular pattern in case 1, but filipin

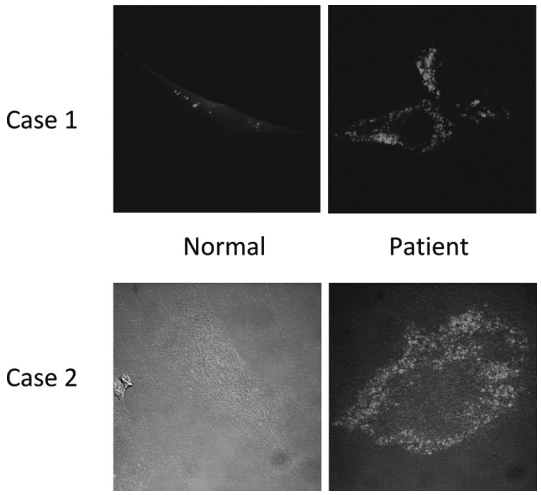

Fig. 1. Filipin staining of fibroblast cells. Fibroblasts from a normal control and those from two patients with NPC were stained with filipin. The patients showed intracellular distributions of cholesterol with filipin staining in a characteristic distribution near the nucleus. Positive staining of filipin demonstrated a granular pattern in case 1, but filipin was diffusely stained in case 2.

was diffusely stained in case 2 (Fig. 1).

Mutational analyses

In case 1, a heterozygous c.1891A>G mutation, which predicts a methionine (ATG) to valine (GTG) change at codon 631 (designated as p.M631V), was identified as one mutational allele and the other allele contained a 12-base (TCAATAAGGACA) deletion followed by a one-base (G) insertion (designated as c.581_592delinsG), which caused a frameshift beginning with codon 194, leading to the occurrence of a premature termination at codon 224 (Fig. 2). Case 2 also had two heterozygous NPC1 mutations. One of the NPC1 alleles in this patient contained a c.2800C>T mutation, which changed an arginine codon (CGA) to a termination (TGA) at codon 934 (designated as p.R934X). The other NPC1 allele in case 2 showed a c.3418G>A mutation, which predicts a glycine (GGA) to arginine (AGA) change at codon 1140 (designated as p.G1140R) (Fig. 3).

Hybridization probe assays

The mRNA levels of 4 ABC transporters, ABCA1, ABCA3, ABCA7, and ABCG1, were analyzed in one fibroblast from a normal control and two fibroblasts from two

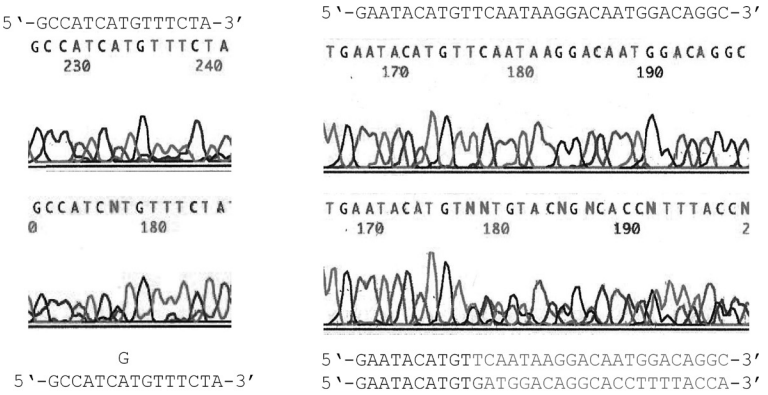

Fig. 2. Nucleotide sequences of the NPC1 mutations identified in a patient with NPC. In case 1, a heterozygous c.1891A>G mutation, which predicts a methionine (ATG) to valine (GTG) change at codon 631 (designated as p.M631V), was identified as one mutational allele and the other allele contained a 12-base (TCAATAAGGACA) deletion followed by a one-base (G) insertion (designated as c.581_592delinsG), which caused a frameshift beginning with codon 194, leading to the occurrence of a premature termination at codon 224.

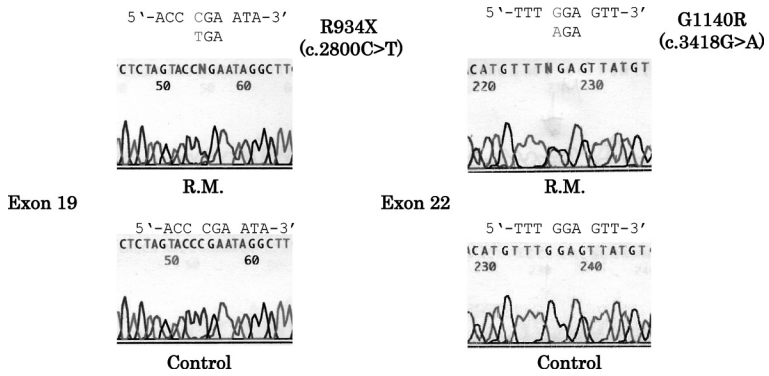

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Table 2. Expression pattern of ABCA genes in the fibroblast cells with *NPC1* mutations

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}
	Case 1	$2.47(\pm0.19)\times10^{3}$	$1.76(\pm0.07)\times10^{6}$	$1.41\times10^{-3*}$
	Case 2	$9.62(\pm1.19)\times10^{3}$	$1.85(\pm0.06)\times10^{6}$	$5.21\times10^{-3*}$
ABCA3	Normal	$6.08(\pm1.04)\times10^{2}$	$2.39(\pm0.10)\times10^{6}$	2.54×10^{-4}
	Case 1	$5.99(\pm0.11)\times10^{2}$	$1.76(\pm0.07)\times10^{6}$	3.41×10^{-4}
	Case 2	$1.91(\pm0.16)\times10^{2}$	$1.85(\pm0.06)\times10^{6}$	1.04×10^{-4}
ABCA7	Normal	$4.20(\pm0.18)\times10^{2}$	$2.39(\pm0.10)\times10^{6}$	1.75×10^{-4}
	Case 1	$1.29(\pm0.07)\times10^{2}$	$1.76(\pm0.07)\times10^{6}$	$0.73\times10^{-4*}$
	Case 2	$2.18(\pm0.04)\times10^{2}$	$1.85(\pm0.06)\times10^{6}$	$1.18\times10^{-4**}$
ABCG1	Normal	$4.80(\pm0.59)\times10^{2}$	$2.39(\pm0.10)\times10^{6}$	2.01×10^{-4}
	Case 1	$3.29(\pm0.52)\times10^{0}$	$1.76(\pm0.07)\times10^{6}$	$1.87\times10^{-6**}$
	Case 2	$5.11(\pm0.45)\times10^{0}$	$1.85(\pm0.06)\times10^{6}$	$2.77\times10^{-6**}$

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

patients with NPC by real-time RT-PCR using hybridization probes (Table 2). The mRNA levels of ABCA1, ABCA7, and ABCG1 were significantly decreased as the corrected copy number in fibroblasts from patients, compared with those in normal control. For ABCG1, the mRNA expression levels were determined to be about one-hundredth of the mRNA level in the normal control, suggesting significant suppression of ABCG1 mRNA expression in fibroblasts with NPC.

Acid sphingomyelinase inducer

After incubating normal lymphoblastic cells with butyrate for 24 h, the ASM activity was increased in a dose-dependent manner (Fig. 4). The ASM activity of the NPC cells was significantly suppressed, but incubation of NPC cells in the 10 mM butyrate increased the ASM activity by ～4-fold. The concentrations of total and free cholesterol were determined in the NPC cells with or without incubation with 10 mM butyrate (Fig. 5). Incubation with 10 mM butyrate led to a decrease in free

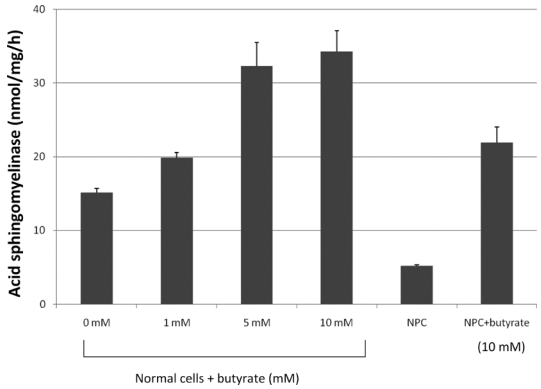

Fig. 4. ASM activities of lymphoblastic cells incubated in butyrate. After incubating normal lymphoblastic cells with butyrate for 24 h, the ASM activity was increased in a dose-dependent manner. The ASM activity of NPC cells was significantly suppressed, but incubation of NPC cells in 10 mM butyrate increased the ASM activity by ～ 4-fold.

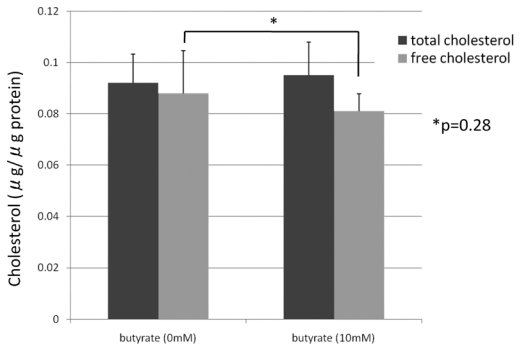

Fig. 5. Quantitative determination of cholesterol in lymphoblastic cells of NPC. The concentrations of total and free cholesterol were determined in the NPC cells with or without incubation with 10 mM butyrate. Incubation with 10 mM butyrate led to a decrease in free cholesterol and reversely increased the total cholesterol.

cholesterol and reversely increased the total cholesterol, but these changes were not statistically significant.

Discussion

Four novel mutations of the *NPC1* gene, c.1891A>G (p.M631V), c.581_592delinsG, c.2800C>T (p.R934X), and c.3418G>A (p.G1140R), were identified in 2 Japanese patients with NPC (Fig. 6). The *NPC1* gene predicts a protein of 1,278 amino acids with a molecular weight of 142 kDa[1]. The NPC1 protein is an integral membrane protein with 13 putative transmembrane domains and sterol-sensing domains (SSD) that have the same orientation as those of HMG-CoA reductase and SCAP (sterol regulatory element binding protein [SREBP] cleavage activation protein). The SSD is thought to transmit information regarding the sterol content of the membrane. The c.1891A>G (p.M631V) mutation identified from case 1 is located in the SSD. To date, more than 20 mutations have been identified in the SSD from patients with NPC and the majority of mutations in the SSD were associated with severe phenotype[1,5-8]. Because the other heterozygous mutation (c.581_592delinsG) caused a frameshift leading to the occurrence of a premature termination, the phenotype of case 1 is expected to be severe. The c.2800C>T (p.R934X) mutation is located in the luminal cysteine-rich loop, which spans amino acids 855 through 1098. This cysteine-rich loop domain resembles the RING-finger motif regulatory domain of protein kinase C and is expected to be functionally essential in the NPC1 membrane protein. Finally, the c.3418G>A (p.G1140R) mutation is located in the 10th transmembrane domain.

In fibroblasts from NPC, the mRNA expressions of *ABCA1*, *ABCA7*, and *ABCG1* genes were significantly decreased compared with normal fibroblasts. In particular, the *ABCG1* gene was markedly suppressed at one-hundredth of the normal control cells. In contrast, there were no significant differences between NPC and the normal control regarding mRNA expression of the *ABCA3* gene. 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[9]. 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[11]. However, our results showed that the *ABCG1* gene was more intensely suppressed than the *ABCA1*. Several reports presented evidence that ABCG1 is a mediator of cholesterol efflux to HDL cholesterol. While ABCA1 functions to promote cellular phospholipid and cholesterol efflux to apoA-I and

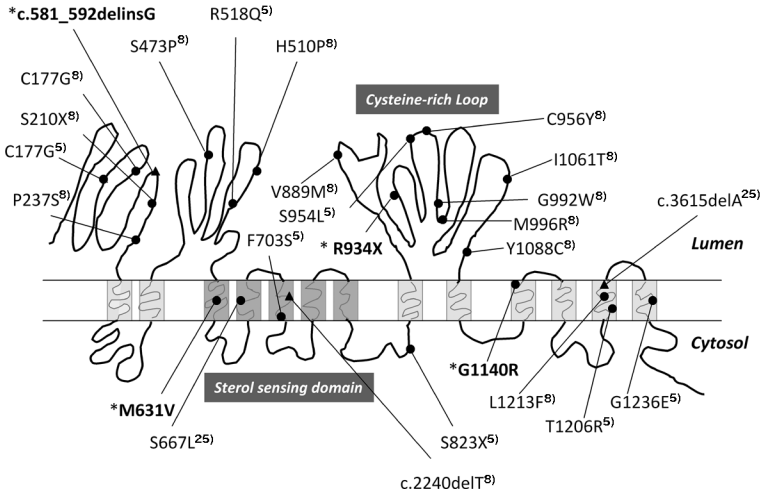

Fig. 6. Structure of NPC1 and *NPC1* gene mutations identified from Japanese patients with NPC. *NPC1* gene mutations identified to date in Japanese patients with NPC are demonstrated on the NPC1 protein structure. The four mutations described in this report are indicated by asterisks.

initiate the formation of HDL, ABCG1 functions as a transporter involved in lipid efflux to HDL but not to lipid-free apoA-I. ABCA1 and ABCG1 function cooperatively to remove phospholipids and cholesterol from cells. Our findings suggest that secondary dysfunction of ABCG1 may also cause the impairment of cholesterol efflux from peripheral cells, leading to low plasma levels of HDL cholesterol in NPC. Briefly, expressions of genes related to cholesterol efflux were extensively influenced by disturbances of the intracellular cholesterol transport caused by *NPC1* gene mutations.

Biochemically, NPC is characterized by a defect in intracellular trafficking of exogenous cholesterol that leads to the lysosomal and endosomal accumulation of unesterified (free) cholesterol[1,3]. This accumulation of free cholesterol causes the secondary ASM deficiency in the NPC cell[26]. In our study, the secondary ASM deficiency was corrected by incubation with 10 mM butyrate in the NPC cells. The molecular basis for secondary ASM deficiency remains to be elucidated, but the ASM inducer, butyrate, could correct even the accumulation of free cholesterol in the NPC cells, suggesting that secondary ASM deficiency plays an important role in the disturbance of intracellular cholesterol trafficking in NPC cells.

In summary, the expressions of genes related to cholesterol efflux and ASM activity are influenced by the *NPC* gene mutations or the consequent cholesterol accumulation, and these secondary changes could exacerbate the disturbance of intracellular cholesterol trafficking in NPC.

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