

## Non-phosphorylated FTY720 Induces Apoptosis of Human Microglia by Activating SREBP2

Takashi Yoshino · Hiroko Tabunoki ·  
Shigeo Sugiyama · Keitaro Ishii · Seung U. Kim ·  
Jun-ichi Satoh

Received: 3 February 2011 / Accepted: 14 April 2011 / Published online: 26 April 2011  
© Springer Science+Business Media, LLC 2011

**Abstract** A synthetic analog of sphingosine named FTY720 (Fingolimod), phosphorylated by sphingosine kinase-2, interacts with sphingosine-1-phosphate (S1P) receptors expressed on various cells. FTY720 suppresses the disease activity of multiple sclerosis (MS) chiefly by inhibiting S1P-dependent egress of autoreactive T lymphocytes from secondary lymphoid organs, and possibly by exerting anti-inflammatory and neuroprotective effects directly on brain cells. However, at present, biological effects of FTY720 on human microglia are largely unknown. We studied FTY720-mediated apoptosis of a human microglia cell line HMO6. The exposure of HMO6 cells to non-phosphorylated FTY720 (FTY720-non-P) induced apoptosis in a dose-dependent manner with IC50 of  $10.6 \pm 2.0 \mu\text{M}$ , accompanied by the cleavage of

caspase-7 and caspase-3 but not of caspase-9. The apoptosis was inhibited by Z-DQMD-FMK, a caspase-3 inhibitor, but not by Pertussis toxin, a Gi protein inhibitor, suramin, a S1P3/S1P5 inhibitor, or W123, a S1P1 competitive antagonist, although HMO6 expressed S1P1, S1P2, and S1P3. Furthermore, both phosphorylated FTY720 (FTY720-P) and SEW2871, S1P1 selective agonists, did not induce apoptosis of HMO6. Genome-wide gene expression profiling and molecular network analysis indicated activation of transcriptional regulation by sterol regulatory element-binding protein (SREBP) in FTY720-non-P-treated HMO6 cells. Western blot verified activation of SREBP2 in these cells, and apoptosis was enhanced by pretreatment with simvastatin, an activator of SREBP2, and by overexpression of the N-terminal fragment of SREBP2. These observations suggest that FTY720-non-P-induced apoptosis of HMO6 human microglia is independent of S1P receptor binding, and positively regulated by the SREBP2-dependent proapoptotic signaling pathway.

**Electronic supplementary material** The online version of this article (doi:10.1007/s10571-011-9698-x) contains supplementary material, which is available to authorized users.

T. Yoshino · H. Tabunoki · J. Satoh (✉)  
Department of Bioinformatics and Molecular Neuropathology,  
Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose,  
Tokyo 204-8588, Japan  
e-mail: satoj@my-pharm.ac.jp

S. Sugiyama · K. Ishii  
Department of Chemistry of Functional Molecules, Meiji  
Pharmaceutical University, 2-522-1 Noshio, Kiyose,  
Tokyo 204-8588, Japan

S. U. Kim  
Division of Neurology, Department of Medicine, University  
of British Columbia Hospital, University of British Columbia,  
Vancouver, BC, Canada

S. U. Kim  
Medical Research Institute, Chung-Ang University  
College of Medicine, Seoul, Korea

**Keywords** Apoptosis · Cholesterol · FTY720 ·  
Microglia · S1P1 · SREBP2

### Abbreviations

CNS	Central nervous system
DAVID	Database for Annotation, Visualization, and Integrated Discovery
EDG	Endothelial differentiation gene
FTY720-non-P	Non-phosphorylated form of FTY720
FTY720-P	Phosphorylated form of FTY720
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G-protein-coupled receptor
INSIG1	Insulin-induced gene 1
LDLR	Low density lipoprotein receptor

MS	Multiple sclerosis
OPC	Oligodendroglial progenitor cell
S1P	Sphingosine-1-phosphate
SPHK2	Sphingosine kinase-2
SREBP	Sterol regulatory element-binding protein
PARP	Poly-ADP-ribose-polymerase
PTX	Pertussis toxin

## Introduction

FTY720 (Fingolimod) is a synthetic analog of sphingosine generated by chemical modification of myriocin, a natural product of the fungus *Isaria sinclairii*. FTY720, phosphorylated by endogenous sphingosine kinase-2 (SPHK2), is converted into the biologically active form FTY720-P that binds to sphingosine-1-phosphate (S1P) receptors expressed on various cells (Brinkmann et al. 2010). S1P receptors belong to the endothelial differentiation gene (EDG) receptor family of G-protein-coupled receptors (GPCRs). FTY720-P interacts with S1P1, S1P3, S1P4, and S1P5 but not with S1P2. S1P1, S1P2, and S1P3 are distributed widely in the immune system, cardiovascular system, and the central nervous system (CNS), and S1P4 expression is more restricted to the lung, spleen, and thymus, while S1P5 is located chiefly on the skin, spleen, and brain. FTY720-P not only serves as an agonist for S1P receptors, but also acts as a functional antagonist for S1P1 by downregulating the receptor expression via internalization, ubiquitination, and proteasomal degradation (Mullershausen et al. 2009). The latter induces unresponsiveness to endogenous S1P.

Recent clinical trials indicate that FTY720 has promising therapeutic effects on multiple sclerosis (MS), a human demyelinating disease affecting exclusively the CNS white matter (Brinkmann et al. 2010). Oral administration of FTY720 reduces the number of gadolinium-enhanced lesions on MRI and decreased annual relapse rate in the patients with relapsing-remitting MS (Kappos et al. 2006). Consequently, US Food and Drug Administration (FDA) approved FTY720 as the first oral medication for MS in September 2010. FTY720-mediated immunomodulatory effects on the disease activity of MS are chiefly attributable to inhibition of S1P-dependent egress of autoreactive T lymphocytes from secondary lymphoid organs (Brinkmann et al. 2010).

Increasing evidence indicates that FTY720, capable of passing the blood–brain barrier due to its lipophilicity, exerts anti-inflammatory and neuroprotective effects within the CNS by interacting with a battery of S1P receptors expressed on neural cells (Dev et al. 2008). Reactive

astrocytes in active MS lesions show a robust increase in S1P1 and S1P3 expression, where FTY720-P inhibits production of proinflammatory cytokines from astrocytes (Van Doorn et al. 2010). FTY720-P persistently downregulates S1P1 expression on astrocytes, and thereby attenuates the disease activity of experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Choi et al. 2011). FTY720-P induces rapid phosphorylation of ERK1/2 and activates the PI3-kinase/Akt pathway in rat oligodendrocyte progenitor cells (OPCs), and subsequently protects OPCs from apoptosis caused by proinflammatory mediators (Coelho et al. 2007). FTY720-P promotes process extension of human OPCs and enhances their survival (Miron et al. 2008).

Microglia, acting as antigen-presenting cells and proinflammatory effector cells in the CNS, play a central role in development of demyelinating lesions in MS (Jack et al. 2005). Therefore, it is possible that FTY720 acts directly on microglia at the site of inflammation in MS brains. S1P1-expressing cells positive for CD68, a marker of microglia/macrophages, are accumulated in MS lesions (Van Doorn et al. 2010). Rat microglial cells express mainly S1P1 and S1P3 (Dev et al. 2008). In mouse organotypic cerebellar cultures affected with lysolecithin-induced demyelination, FTY720-P induces proliferation of microglia (Miron et al. 2010), while FTY720 reduces the accumulation of reactive microglia/macrophages in the lesions of traumatic brain injury (Zhang et al. 2007). FTY720 reduces the lesion size of cerebral infarct in mice with middle cerebral artery (MCA) occlusion and improves neurological deficits, accompanied by a decrease in the number of activated microglia/macrophages and apoptotic neurons (Wei et al. 2011). FTY720-P does not affect the global cytokine production by cultured human microglia (Durafourt et al. 2011). However, at present, immunomodulatory effects of FTY720 on human microglia remain largely unknown. The aim of the present study is to investigate biological effects of FTY720 on a human microglial cell line HMO6.

## Methods

### Human Microglia Cell Line HMO6

The HMO6 cell line was established by immortalizing cultured microglia isolated from human embryonic telencephalon tissues with a retroviral vector PASK1.2 encoding v-myc oncogene (Nagai et al. 2001). HMO6 cells express the markers of the microglia/macrophage lineage cells, including CD11b, CD68, CD86, HLA-ABC, HLA-DR, and ricinus communis agglutinin lectin-1 (RCA), serving as a model of human microglia both in

vitro and in vivo (Narantuya et al. 2010). The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (feeding medium). Human neural and non-neural cell lines other than HMO6 were described elsewhere. LDH release from cultured cells was assessed by using a LDH cytotoxicity detection kit (Takara Bio, Shiga, Japan).

## Chemicals

Non-phosphorylated FTY720 (FTY720-non-P; Calbiochem, Darmstadt, Germany) and (*S*)-FTY720 phosphate (FTY720-P; Echelon Biosciences, Salt Lake City, UT, USA) were usually dissolved in dimethyl sulfoxide (DMSO), providing the stock solution at the concentration of 10 mM. For negative controls, the inclusion of DMSO at the concentration of 0.1% v/v (1:1000 dilution) was applied. We found that the solvent alone never induces apoptosis of HMO6 at any incubation time. Sphingosine 1-phosphate (S1P) was obtained from Sigma, St. Louis, MO, USA. SEW2871, a selective S1P1 agonist and W123, a competitive S1P1 antagonist were obtained from Cayman Chemical, Ann Arbor, MI, USA. Suramin, a S1P3/S1P5 inhibitor, Z-DQMD-FMK, a caspase-3 inhibitor, and simvastatin, a HMG-CoA reductase inhibitor were obtained from Calbiochem. Pertussis toxin (PTX), a Gi protein inhibitor, was obtained from Seikagaku Biobusiness, Tokyo, Japan.

## RT-PCR Analysis

Total cellular RNA was extracted by using TRIZOL (Invitrogen). RNA treated with DNase I was processed for cDNA synthesis using oligo(dT)<sub>20</sub> primers and SuperScript II reverse transcriptase (Invitrogen). Then, cDNA was amplified by PCR using HotStar Taq DNA polymerase (Qiagen, Valencia, CA, USA) and a panel of sense and antisense primer sets following: 5'aagcgtcttacttggtcgtgg3' and 5'tgatctccaccctcccagtgcat3' for an 189 bp product of S1P1; 5'ccacagacctgggtgatgtt3' and 5'tcccctaaatgctgctgcc3' for a 200 bp product of S1P2; 5'acttgggctccagagtcttc3' and 5'cattctacgcacagaaatgtagtg3' for an 193 bp product of S1P3; 5'gttcagctcttcggtggtgatgg3' and 5'ggtgacatgggaagccatttg3' for an 183 bp product of S1P4; 5'aggaaatgcctgcgcaag3' and 5'cttctatggctcccacctcactc3' for a 200 bp product of S1P5; and 5'ccatgttcgcatgggtgtaacca3' and 5'gccagtagaggcaggatgatgttc3' for a 251 bp product of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene.

For quantitative real-time RT-PCR (qPCR) analysis, cDNA was amplified by PCR in LightCycler ST300

(Roche Diagnostics, Tokyo, Japan) using SYBR Green I and a panel of sense and antisense primer sets with the following: 5'tgatcgtccagaagtggccttgg3' and 5'aactgtcgtcctatgtcccacc3' for an 186 bp product of insulin-induced gene 1 (INSIG1) and 5'ctgggggtcttctctatggaag3' and 5'cacgtcatcctccagactgacct3' for an 168 bp product of low density lipoprotein receptor (LDLR). The expression levels of target genes were standardized against the levels of G3PDH, an internal control, detected in corresponding cDNA samples. All the assays were performed in triplicate.

## Microarray Analysis

For microarray analysis, total cellular RNA was isolated by using the TRIZOL Plus RNA Purification kit (Invitrogen). The quality of total RNA was evaluated on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). One hundred ng of total RNA was processed for cRNA synthesis, fragmentation, and terminal labeling with the GeneChip Whole Transcript Sense Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA, USA). Then, it was processed for hybridization at 45°C for 17 h with Human Gene 1.0 ST Array that contains 28,869 genes (Affymetrix). The arrays were washed in the GeneChip Fluidic Station 450 (Affymetrix), and scanned by the GeneChip Scanner 3000 7G (Affymetrix). The raw data were expressed as CEL files and normalized by the robust multiarray average (RMA) method with the Expression Console software version 1.1 (Affymetrix). The annotation was studied by searching genes on the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (david.abcc.ncifcrf.gov) (da Huang et al. 2009).

## Molecular Network Analysis

KeyMolnet is a comprehensive knowledgebase that contains the contents on 123,000 relationships among human genes and proteins, small molecules, diseases, pathways and drugs, regularly updated, and curated by expert biologists (Sato et al. 2009). By importing the list of Entrez Gene IDs derived from microarray data, KeyMolnet automatically provides corresponding molecules as a node on networks. Among various network-searching algorithms, the "neighboring" network-search algorithm selected one or more molecules as starting points to generate the network of all kinds of molecular interactions around starting molecules, including direct activation/inactivation, transcriptional activation/repression, and the complex formation within the designated number of paths from starting points. The generated network was compared side by side with 430 human canonical pathways of the KeyMolnet library. The algorithm counting the number of overlapping molecular relations between the extracted network and the

canonical pathway makes it possible to identify the canonical pathway showing the most significant contribution to the extracted network. The significance in the similarity between both is scored following the formula, where  $O$  = the number of overlapping molecular relations between the extracted network and the canonical pathway,  $V$  = the number of molecular relations located in the extracted network,  $C$  = the number of molecular relations located in the canonical pathway,  $T$  = the number of total molecular relations, and the  $X$  = the sigma variable that defines coincidence.

$$\text{Score} = -\log_2(\text{Score}(p)) \quad \text{Score}(p) = \sum_{x=0}^{\text{Min}(C,V)} f(x)$$

$$f(x) = {}_C C_x \cdot {}_{T-C} C_{V-x} / {}_T C_V$$

### Transient Expression of SREBP2

To transiently overexpress sterol regulatory element-binding protein-2 (SREBP2), the gene encoding the N-terminal fragment of SREBP2 spanning amino acid residues 1–484 was amplified by PCR using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) and a sense and antisense primer set of 5'gcatggacgacagcggcgagctg3' and 5'tcacagaagaatccg tgagcggctc3', and cloned in the expression vector pEF6 (Invitrogen). The vector was transfected into HMO6 cells by X-tremeGENE HP DNA transfection reagent (Roche Diagnostics). At 24 h after transfection, the cells were processed for western blot analysis. For the control, V5-tagged LacZ cloned in the pEF6 vector was transfected into sister cultures.

### Western Blot Analysis

To prepare total protein extract, the cells were homogenized in RIPA buffer supplemented with a cocktail of protease inhibitors (Sigma). The protein extract was centrifuged at 12,000 rpm for 5 min at room temperature (RT). The protein concentration was determined by a Bradford assay kit (BioRad Hercules, CA, USA). The mixture of the supernatant and a 2× Lammeli loading buffer was boiled and separated on SDS-PAGE gels ranging from 8 to 12%. After gel electrophoresis, the protein was transferred onto nitrocellulose membranes, and immunolabeled at RT overnight with rabbit anti-poly-ADP-ribose-polymerase (PARP) antibody (#11835238001; Roche Diagnostics), rabbit anti-cleaved caspase-3 (Asp175) antibody (#9661; Cell Signaling Technology, Danvers, MA, USA), mouse anti-caspase-7 antibody (#9494; Cell Signaling Technology), rabbit anti-caspase-9 antibody (#9502; Cell Signaling Technology), rabbit anti-S1P1 antibody (sc-25489, EDG-1, H-60; Santa Cruz Biotechnology, Santa Cruz, CA), or goat anti-SREBP2 antibody (sc-8151, N-19; Santa Cruz

Biotechnology). Then, the membranes were incubated at RT for 60 min with HRP-conjugated anti-mouse IgG, anti-rabbit IgG, or anti-goat IgG (Santa Cruz Biotechnology). The specific reaction was visualized by exposing the membranes to a chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA).

In some experiments, the antibodies were stripped by incubating the membranes at 50°C for 30 min in stripping buffer, composed of 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol. Then, the membranes were processed for relabeling with goat anti-heat shock protein HSP60 antibody (sc-1052, N-20; Santa Cruz Biotechnology) used for an internal control of protein loading, followed by incubation with HRP-conjugated anti-goat IgG.

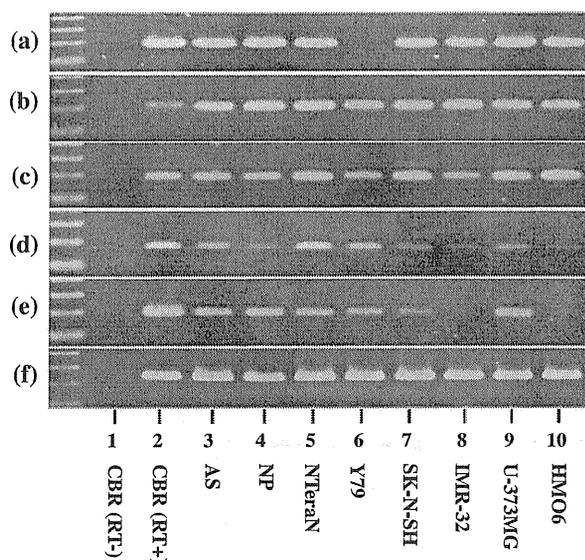
## Results

### S1P Receptor Expression on Human Microglia Cell Line HMO6

The expression of five S1P receptor mRNAs in a panel of human neural cells and tissues was determined by RT-PCR. All the cells and tissues examined, including the human cerebrum (CBR), fetal astrocytes (AS), neuronal progenitor (NP) cells, Ntera2 teratocarcinoma-derived neurons, SK-N-SH neuroblastoma, IMR-32 neuroblastoma, U-373MG astrogloma, and the microglia cell line HMO6, expressed varying levels of S1P1, S1P2, and S1P3 mRNAs, except for Y79 retinoblastoma that did not express S1P1 (Fig. 1a–c, lanes 2–10). In contrast, the levels of G3PDH, a housekeeping gene, were almost constant in the cells and tissues examined (Fig. 1f, lanes 2–10). Although discernible levels of S1P4 and S1P5 mRNAs were identified in the human cerebrum (CBR), both of these mRNAs were almost undetectable in HMO6 (Fig. 1d, e, lanes 2 and 10). No products were amplified when the reverse transcription step is omitted (Fig. 1a–f, lane 1). We verified S1P1 protein expression in HMO6 by western blot (not shown).

### Non-Phosphorylated FTY720 Induced Apoptosis of HMO6

A 6 h-exposure of non-phosphorylated FTY720 (FTY720-non-P) induced LDH release from HMO6 cells and cell death in a dose-dependent manner with IC50 of  $10.6 \pm 2.0 \mu\text{M}$  (Fig. 2a, c). It is worthy to note that the concentration of FTY720-non-P at lower than  $5 \mu\text{M}$  was completely ineffective in inducing cell death of HMO6 (Fig. 2a). Generally, LDH release did not discriminate apoptotic and necrotic cell death. The exposure of FTY720-non-P at a concentration of  $10 \mu\text{M}$  mediated the cleavage of PARP in the incubation time longer than 4 h,



**Fig. 1** S1P receptor expression in human neural cell lines. The expression of five S1P receptor mRNAs was studied by RT-PCR. **a** S1P1, **b** S1P2, **c** S1P3, **d** S1P4, **e** S1P5, and **f** G3PDH. The lanes (1–10) represent (1) the human frontal cerebral cortex (CBR) without inclusion of the reverse transcription (RT) step, (2) CBR with inclusion of the RT step, (3) cultured astrocytes (AS), (4) cultured neuronal progenitor (NP) cells, (5) NTera2 teratocarcinoma-derived neurons (NTera2N), (6) Y79 retinoblastoma, (7) SK-N-SH neuroblastoma, (8) IMR-32 neuroblastoma, (9) U-373MG astrocytoma, and (10) HMO6 microglia. The 100 bp ladder marker is shown on the left

indicating that FTY720-non-P induced cell death of HMO6 via apoptosis (Fig. 2d, lanes 7–10).

FTY720-non-P-induced apoptosis of HMO6 was accompanied by the cleavage of caspase-7 and caspase-3 (Fig. 3b, c, lane 2) but not of caspase-9 (Fig. 3e, lane 4), suggesting that the mitochondrial pathway of apoptosis that usually activates caspase-9 did not play a major role. Furthermore, Z-DQMD-FMK, a caspase-3-specific inhibitor, completely blocked FTY720-non-P-induced apoptosis of HMO6 (Fig. 3g, h, lane 10).

#### FTY720-Induced Apoptosis of HMO6 was Independent of S1P Receptor Binding

Because FTY720, when phosphorylated, binds to S1P1, S1P3, S1P4, and S1P5, all of which are G protein-coupled receptors (GPCR), we utilized Pertussis toxin (PTX), a Gi protein inhibitor, suramin, a S1P3/S1P5 inhibitor, and W123, a S1P1 competitive antagonist to block the ligand-receptor interaction. However, none of these receptor blockers could inhibit FTY720-induced apoptosis of HMO6 (Fig. 4a, lanes 4, 6, 8). Furthermore, SEW2871, a S1P1 selective agonist, and phosphorylated FTY720 (FTY720-P) at a concentration of 10  $\mu$ M each did not induce apoptosis of HMO6 during the incubation time of

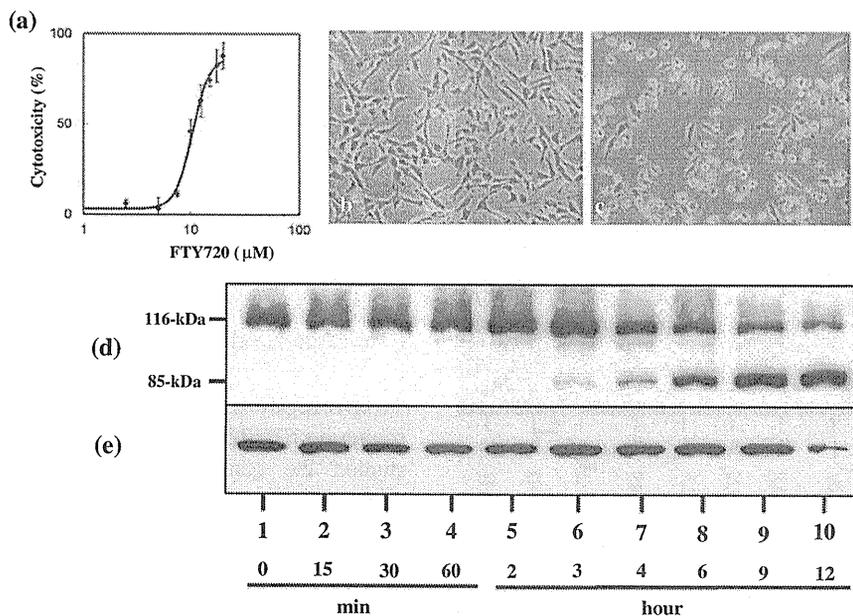
12 h (Fig. 4c, lanes 11 and 12). In addition, the combined administration of FTY720-P (10  $\mu$ M) and FTY720-non-P (10  $\mu$ M) did not inhibit apoptosis of HMO6, and treatment with sphingosine-1 phosphate (S1P) (10–50  $\mu$ M) did not induce apoptosis of HMO6 (data not shown). These results suggest that FTY720-non-P-induced apoptosis of HMO6 was independent of S1P receptor binding, and both FTY720-P and S1P were incapable of inducing apoptosis of HMO6.

#### FTY720 Induced SREBP-Responsive Genes

To investigate the molecular mechanism responsible for triggering FTY720-non-P-induced apoptosis of HMO6, we studied the genome-wide gene expression profile by microarray analysis. We identified 30 genes with an over 2-fold increase in HMO6 cells treated for 2 h with 10  $\mu$ M FTY720-non-P versus those exposed to the vehicle (DMSO) (Table 1). Among them, the DAVID program categorized seven genes as a group of the genes associated with steroid and/or sterol metabolism (Table 1). None of apoptosis initiator and executor genes were induced in HMO6 cells at 2 h after initiation of the treatment. Upregulated expression of INSIG1 and LDLR in FTY720-non-P-treated HMO6 cells was validated by qPCR analysis (Fig. 5a, b).

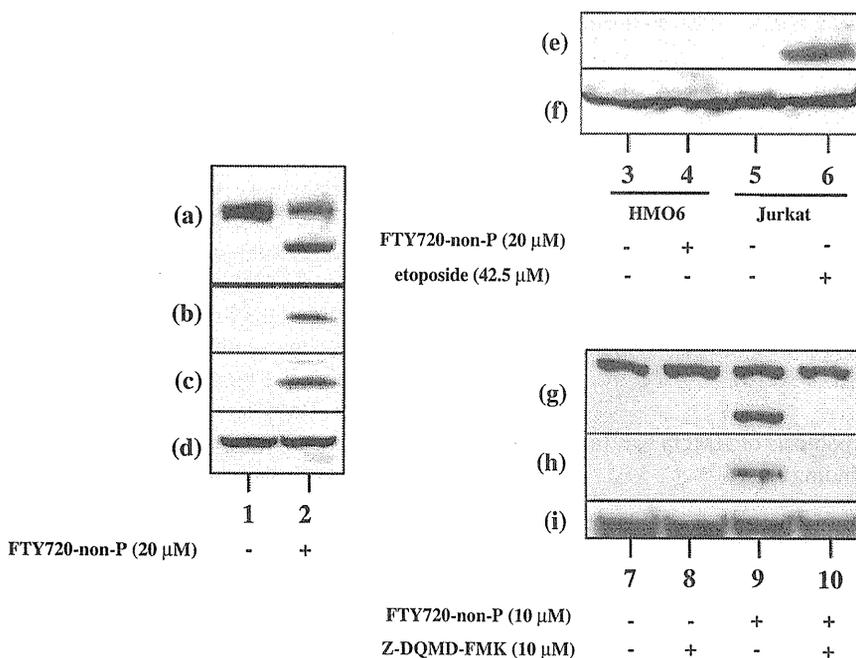
Next, we imported the list of Entrez Gene IDs of the 30 genes upregulated in FTY720-non-P-treated HMO6 cells into KeyMolnet, a tool for analyzing molecular interactions on the comprehensive knowledgebase. KeyMolnet generated the molecular network, presenting with the most significant relationship with transcriptional regulation by sterol regulatory element-binding protein (SREBP) (the score = 69.719 with the  $P$ -value = 1.029E–21) (Fig. 5c). These results suggest that in HMO6 cells, FTY720-non-P activates SREBP proteins, either SREBP1 or SREBP2, belonging to the bHLH-Zip transcription factor family that promotes the synthesis of enzymes involved in cholesterol and fatty acid biosynthesis. To exclude a direct effect of vehicle (DMSO), in which FTY720-non-P was dissolved, on gene expression, we performed an additional set of microarray experiment by exposing HMO6 cells to FTY720-non-P dissolved in ethanol. We again identified the similar gene expression profile composed of upregulation of key SREBP-target genes, regardless of the solvent (See Table 1 in Electronic Supplementary Material).

SREBP2 is primarily involved in cholesterol synthesis, while SREBP1 chiefly regulates fatty acid synthesis (Sato 2010). INSIG1 identified by microarray analysis encodes an ER protein that plays a pivotal role in regulating intracellular cholesterol levels by interacting with SREBP cleavage-activating protein (SCAP) having the sterol-sensing domain activated by reduced cellular cholesterol levels. Thereafter, we have focused on SREBP2 expression



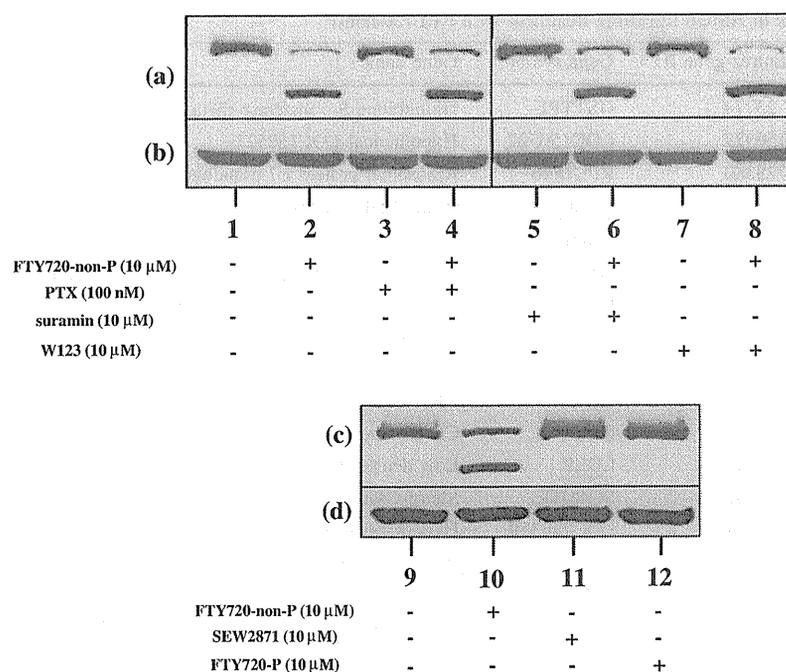
**Fig. 2** Non-phosphorylated FTY720 induced apoptosis of HMO6 cells. HMO6 cells were exposed for various time periods to varying concentrations of non-phosphorylated FTY720 (FTY720-non-P). **a** LDH release assay, **b** the phase contrast photomicrograph of the cells exposed for 6 h to vehicle (DMSO), **c** the phase contrast photomicrograph of the cells exposed for 6 h to 10 μM FTY720-non-P,

**d** western blot of PARP (an 116-kDa uncleaved form and an 85-kDa cleaved form), and **e** western blot of HSP60, an internal control of protein loading. The lanes (1–10) represent (1) untreated HMO6 cells, and HMO6 cells treated for (2) 15 min, (3) 30 min, (4) 1 h, (5) 2 h, (6) 3 h, (7) 4 h, (8) 6 h, (9) 9 h, and (10) 12 h with 10 μM FTY720-non-P



**Fig. 3** FTY720-induced apoptosis of HMO6 was accompanied by activation of caspases 3 and 7. HMO6 cells were exposed for various time periods to varying concentrations of FTY720-non-P. For the positive control of caspase-9 activation, Jurkat cells were exposed to etoposide, an apoptosis-inducing agent. **a–i** indicate western blot of **a**, **g** PARP, **b** caspase-7 (a 20-kDa cleaved form), **c**, **h** caspase-3 (a 19-kDa cleaved form), **e** caspase-9 (a 37-kDa cleaved form), and **d**, **f**,

**i** HSP60, an internal control of protein loading. The lanes (1–10) indicate HMO6 cells treated with (1, 3) vehicle (DMSO) and (2, 4) 20 μM FTY720-non-P for 9 h, and Jurkat cells treated with (5) vehicle (DMSO) and (6) 42.5 μM etoposide for 6 h, and (7) untreated HMO6 cells, and HMO6 cells treated with (8) 10 μM Z-DQMD-FMK, (9) 10 μM FTY720-non-P, and (10) a combination of 10 μM Z-DQMD-FMK and 10 μM FTY720-non-P for 12 h



**Fig. 4** FTY720-induced apoptosis of HMO6 was independent of S1P receptor binding. HMO6 cells were exposed for 12 h to 10  $\mu$ M FTY720-non-P with or without inclusion of various S1P receptor agonists and antagonists. Pretreatment started at 30 min before exposure to FTY720-non-P. **a–d** indicate western blot of **a**, **c** PARP and **b**, **d** HSP60, an internal control of protein loading. The lanes (1–12) indicate (1, 9) untreated HMO6 cells, and HMO6 cells treated with (2, 10) 10  $\mu$ M FTY720-non-P exposure alone, (3) 100 nM

pertussis toxin (PTX) pretreatment alone, (4) 100 nM PTX pretreatment and 10  $\mu$ M FTY720-non-P exposure, (5) 10  $\mu$ M suramin pretreatment alone, (6) 10  $\mu$ M suramin pretreatment and 10  $\mu$ M FTY720-non-P exposure, (7) 10  $\mu$ M W123 pretreatment alone, (8) 10  $\mu$ M W123 pretreatment and 10  $\mu$ M FTY720-non-P exposure, and HMO6 cells treated with a 12 h-exposure to (11) 10  $\mu$ M SEW2871 or (12) 10  $\mu$ M phosphorylated FTY720 (FTY720-P)

in HMO6 cells. The N-terminal fragment of SREBP2 is cleaved, dimerized, and translocated to the nucleus in response to the activating stimuli (Sato 2010). We identified the cleaved form of SREBP2 in HMO6 cells following an 1 h-exposure to FTY720-non-P ranging from 10 to 20  $\mu$ M or by treatment with 3  $\mu$ M simvastatin, a HMG-CoA reductase inhibitor capable of activating SREBP2 (Fig. 6a, lanes 2–4). Neither FTY720-non-P nor simvastatin alone at a concentration of 5  $\mu$ M each induced apoptosis of HMO6 (Fig. 2a; Fig. 6b, lanes 7, 8). In contrast, a 12 h-pretreatment with 5  $\mu$ M simvastatin enhanced FTY720-non-P-induced apoptosis of HMO6 cells, suggesting a proapoptotic effect mediated by SREBP2 activation following simvastatin treatment (Fig. 6b, lane 10).

A recent study showed that statins activate SREBP2, which positively controls the expression of caspase-7, resulting in induction of apoptosis of human gastric cancer cells (Gibot et al. 2009). When the N-terminal fragment of SREBP2 was overexpressed in HMO6 cells, the cleavage of PARP and caspase-3 was greatly enhanced, compared with the cells with overexpression of LacZ (Fig. 6c, f, lane 12), while the levels of procaspase-7 and cleaved caspase-7 were unaltered (Fig. 6g, lane 12).

## Discussion

The present study revealed that non-phosphorylated FTY720 (FTY720-non-P) induced apoptosis of human microglia HMO6 in a time- and dose-dependent manner with IC<sub>50</sub> of  $10.6 \pm 2.0$   $\mu$ M. The apoptosis was inhibited by Z-DQMD-FMK, a caspase-3 inhibitor, but not by Pertussis toxin, a Gi protein inhibitor, suramin, a S1P3/S1P5 inhibitor, or W123, a S1P1 competitive antagonist, although HMO6 expressed S1P1, S1P2, and S1P3. Furthermore, both phosphorylated FTY720 (FTY720-P) and SEW2871, S1P1 selective agonists, did not induce apoptosis of HMO6. These observations suggest that FTY720-non-P-induced apoptosis of HMO6 cells is independent of S1P receptor binding.

Supporting these observations, FTY720, serving as a potential anti-cancer agent, induces apoptosis of various human cancer cell lines derived from liver, kidney, pancreas, and breast, multiple myeloma and leukemia cells, which is often mediated by S1P receptor-independent mechanisms (Matsuoka et al. 2003; Lee et al. 2004; Liu et al. 2010; Nagaoka et al. 2008). The concentrations required to induce apoptosis of tumor cells in vitro are about

**Table 1** Upregulated genes in HMO6 following treatment with FTY720-non-P

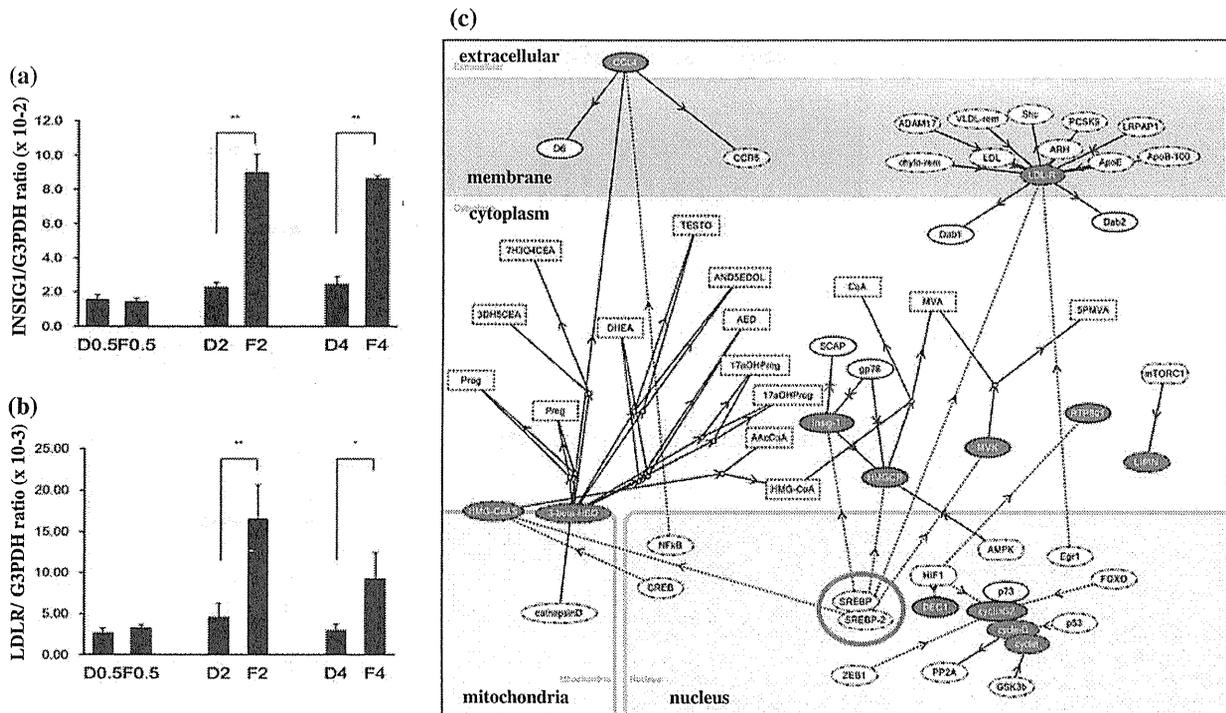
Rank	Fold change	Entrez gene ID	Gene symbol	Gene name
1	5.75	25774	GSTTP1	Glutathione S-transferase theta pseudogene 1
2	3.16	150527	LOC150527	Hypothetical LOC150527
3	2.78	728380	RPL7P26	Ribosomal protein L7 pseudogene 26
4	2.74	3638	<u>INSIG1</u>	Insulin induced gene 1
5	2.72	158160	HSD17B7P2	Hydroxysteroid (17-beta) dehydrogenase 7 pseudogene 2
6	2.70	3157	<u>HMGCS1</u>	3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (soluble)
7	2.68	346007	EYS	Eyes shut homolog (Drosophila)
8	2.48	26834	RNU4-2	RNA, U4 small nuclear 2
9	2.46	163720	CYP4Z2P	Cytochrome P450, family 4, subfamily Z, polypeptide 2 pseudogene
10	2.44	54541	DDIT4	DNA-damage-inducible transcript 4
11	2.41	6351	CCL4	Chemokine (C-C motif) ligand 4
12	2.39	3949	<u>LDLR</u>	Low density lipoprotein receptor
13	2.37	6307	<u>SC4MOL</u>	Sterol-C4-methyl oxidase-like
14	2.34	286359	LOC286359	Hypothetical LOC286359
15	2.30	391003	PRAMEF18	PRAME family member 18
16	2.29	23175	LPIN1	Lipin 1
17	2.25	54897	CASZ1	Castor zinc finger 1
18	2.16	3156	<u>HMGCR</u>	3-hydroxy-3-methylglutaryl-coenzyme A reductase
19	2.16	196335	OR56B4	Olfactory receptor, family 56, subfamily B, member 4
20	2.10	3283	<u>HSD3B1</u>	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
21	2.09	8553	BHLHE40	Basic helix-loop-helix family, member e40
22	2.07	10517	FBXW10	F-box and WD repeat domain containing 10
23	2.06	256892	OR51F1	Olfactory receptor, family 51, subfamily F, member 1
24	2.05	4598	<u>MVK</u>	Mevalonate kinase
25	2.04	196074	METT5D1	Methyltransferase 5 domain containing 1
26	2.04	901	CCNG2	Cyclin G2
27	2.03	439927	C1orf180	Chromosome 1 open reading frame 180
28	2.01	10551	AGR2	Anterior gradient homolog 2 ( <i>Xenopus laevis</i> )
29	2.01	91074	ANKRD30A	Ankyrin repeat domain 30A
30	2.00	1831	TSC22D3	TSC22 domain family, member 3

HMO6 cells were exposed to non-phosphorylated FTY720 (10  $\mu$ M) or vehicle (DMSO) for 2 h. The genome-wide transcriptome was studied on Human Gene 1.0 ST array. The genes with an over 2-fold increase in FTY720-non-P-treated HMO6 cells are listed. The genes associated with steroid and/or sterol metabolism annotated by the DAVID program are underlined

two orders of magnitude greater than the blood concentration in the clinical setting, i.e., 5.4 ng/ml in plasma (Brinkmann et al. 2001, 2010). FTY720 has a half-life of approximately 10 days in vivo, and is cleared predominantly by a metabolic pathway requiring cytochrome P450 4F2 (CYP4F2) (Jin et al. 2011). The enzymatic activity of CYP4F2 is inhibited by certain drugs like ketoconazole, and the gene encoding CYP4F2 has a variety of single nucleotide polymorphisms (SNPs) ([www.ncbi.nlm.nih.gov/snp](http://www.ncbi.nlm.nih.gov/snp)). Therefore, in poor metabolizers of FTY720 receiving a CYP4F2 inhibitor, if they exist, the blood concentration of FTY720 could increase up to the range of toxic levels.

FTY720-non-P goes through the plasma membrane without requirement of the receptor binding, and targets

directly key intracellular enzymes involved in sphingolipid metabolism, such as sphingosine kinases, phospholipase A2, and S1P lyase (Bandhuvula et al. 2005). FTY720 also inhibits ceramide synthases, resulting in a decrease in cellular levels of ceramide, dihydroceramide, sphingosine, and S1P, and an increase in dihydrosphingosine and dihydrosphingosine-1-phosphate, all of which alter the endogenous balance between survival and apoptotic signals (Berdyshev et al. 2009). FTY720-non-P promotes phosphorylation of 14-3-3zeta on Ser58 that disrupts 14-3-3 dimer formation, resulting in releasing proapoptotic mediators (Woodcock et al. 2010). FTY720, phosphorylated by SPHK2 located inside the plasma membrane, is transported outside the cells via the S1P transporter named spinstar



**Fig. 5** FTY720 Induced SREBP-Responsive Genes. HMO6 cells were exposed for 2 h to 10  $\mu$ M FTY720-non-P or vehicle (DMSO). Then, total RNA was processed for the genome-wide gene expression profiling on a microarray, followed by molecular network analysis by KeyMolnet and validation by qPCR. We identified 30 upregulated genes in FTY720-non-P-treated HMO6 cells (Table 1). a–c indicate qPCR of **a** INSIG1 and **b** LDLR, and **c** molecular network of FTY720-non-P-induced genes. Abbreviations: D, vehicle (DMSO); F, FTY720-non-P; 0.5, 30 min; 2, 2 h; and 4, 4 h. In c, red filled nodes

represent FTY720-non-P-induced genes, whereas white open nodes exhibit additional nodes extracted automatically from the core contents of KeyMolnet to establish molecular connections. The molecular relation is indicated by solid line with arrow (direct binding or activation), solid line with arrow and stop (direct inactivation), solid line without arrow (complex formation), dash line with arrow (transcriptional activation), and dash line with arrow and stop (transcriptional repression). The transcription factor SREBP (SREBP2) is highlighted by a red thick circle

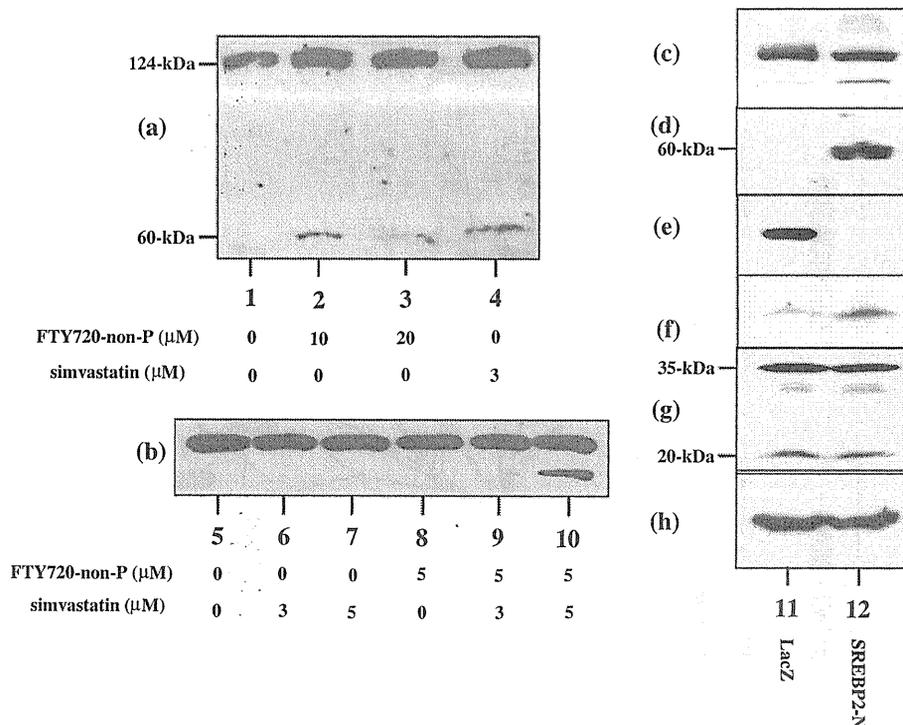
homolog 2 (SPNS2), and then the phosphorylated FTY720 binds to S1P receptors expressed on the surface of the plasma membrane (Hisano et al. 2011).

Being consistent with our observations, FTY720-non-P but not FTY720-P induces apoptosis of human breast and colon cancers (Nagaoka et al. 2008). FTY720 inhibits cytosolic phospholipase A2 (cPLA<sub>2</sub>) in a manner independent of S1P receptor binding (Payne et al. 2007). FTY720-non-P but not FTY720-P inhibits PKC activation, which is associated with cell-surface expression of S1P1 (Sensken and Gräler 2010). Furthermore, FTY720-P counteracts FTY720-non-P-induced apoptosis of human fibroblasts by activating Bcl-2 (Potteck et al. 2010).

Several previous studies showed that FTY720-induced apoptosis is often accompanied by activation of a series of caspases (Wang et al. 1999). We found activation of both caspase-3 and caspase-7 during FTY720-non-P-induced apoptosis of HMO6. Furthermore, FTY720-induced apoptosis also involves various mechanisms, such as dephosphorylation of protein kinase B (Akt) (Matsuoka et al. 2003; Lee et al. 2004), deregulation of mitogen-activated

protein kinases (MAPKs), focal adhesion kinase (FAK), and Rho-GTPase (Matsuda et al. 1999; Sonoda et al. 2001), and activation of protein phosphatase 2A (PP2A) (Liu et al. 2010). Here, we for the first time showed that FTY720-non-P-induced apoptosis of HMO6 cells is positively regulated by the SREBP2-dependent signaling pathway.

A recent study showed that statins induce apoptosis of human gastric cancer cells by activating SREBP1 and SREBP2, both of which transcriptionally upregulate caspase-7 (Gibot et al. 2009). Statin-dependent apoptosis is prevented by replenishment of mevalonate, the immediate product by the HMG-CoA reductase activity (Xia et al. 2001). A previous study showed that activation of caspase-3 releases SREBP proteins from ER membrane in a proteolytic reaction distinct from the sterol-regulated cleavage, resulting in nuclear transport of SREBP and transcriptional activation of sterol-regulatory genes (Higgins and Ioannou 2001). However, during FTY720-non-P-induced apoptosis of HMO6 cells, we identified activation of SREBP2 as early as at 1 h after initiation of the treatment, which is long before detection of the PARP cleavage, suggesting that



**Fig. 6** Activation of SREBP2 by FTY720-non-P in HMO6 cells. HMO6 cells were exposed to FTY720-non-P or simvastatin, or transfected with the vector expressing the N-terminal fragment of SREBP2 (SREBP2-N) or LacZ. **a–h** indicate western blot of **a**, **d** SREBP2, **b**, **c** PARP, **e** V5, **f** cleaved caspase-3, **g** caspase-7 (a 35-kDa proform and a 20-kDa cleaved form), and **h** HSP60. The lanes 1–12 represent (1, 5) untreated HMO6 cells, and HMO6 cells treated for 1 h with (2) 10  $\mu\text{M}$  FTY720-non-P, (3) 20  $\mu\text{M}$  FTY720-non-P, (4) 3  $\mu\text{M}$  simvastatin, and HMO6 cells pretreated for simvastatin

starting at 12 h before a 6 h-exposure to FTY720-non-P, whose conditions are composed of (6) 3  $\mu\text{M}$  simvastatin pretreatment alone, (7) 5  $\mu\text{M}$  simvastatin pretreatment alone, (8) no simvastatin pretreatment and 5  $\mu\text{M}$  FTY720-non-P exposure, (9) 3  $\mu\text{M}$  simvastatin pretreatment and 5  $\mu\text{M}$  FTY720-non-P exposure, and (10) 5  $\mu\text{M}$  simvastatin pretreatment and 5  $\mu\text{M}$  FTY720-non-P exposure, and HMO6 cells with overexpression of (11) V5-tagged LacZ or (12) SREBP2-N

SREBP activation is not a secondary phenomenon following caspase-3 activation. Furthermore, we found that activation of SREBP2 by overexpression of the N-terminal fragment of SREBP2 in HMO6 cells enhances the cleavage of PARP and caspase-3 in the absence of FTY720. Moreover, we found that pretreatment with simvastatin enhanced FTY720-non-P-induced apoptosis of HMO6 cells. Statins activate SREBP2 and induce apoptosis of various cells (Xia et al. 2001; Gibot et al. 2009). All of these observations suggest that the SREBP2-dependent signaling pathway is intrinsically proapoptotic, when it is aberrantly regulated.

A recent study showed that FTY720 inhibits intracellular transport of cholesterol to ER in human macrophages, being independent of S1P1 binding, indicating that FTY720-non-P certainly affects the cellular cholesterol processing (Blom et al. 2010). Importantly, cholesterol interacts specifically with sphingosine in human intestinal epithelial cells under physiological conditions (Garmy et al. 2005). S1P is intracellularly generated by sphingosine kinases SPHK1 and SPHK2 from sphingosine, a breakdown product of the cell membrane constituent sphingomyelin (Chi 2011). S1P and

its synthetic analog FTY720-P share S1P1, S1P3, S1P4, and S1P5 expressed on the plasma membrane. All of these observations propose a possible scenario that excessive amounts of intracellular FTY720-non-P disturb the complex metabolic network of cholesterol and sphingolipids, resulting in activation of the SREBP2-dependent proapoptotic signaling pathway.

**Acknowledgments** This work was supported by grants from the Research on Intractable Diseases, the Ministry of Health, Labour and Welfare, Japan (H22-Nanchi-Ippan-136; H21-Nanchi-Ippan-201; H21-Nanchi Ippan-217; H21-Kokoro-Ippan-018) and the High-Tech Research Center Project (S0801043) and the Grant-in-Aid (C22500322), the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The microarray data are available from Gene Expression Omnibus (GEO) under the accession number GSE28642.

## References

- Bandhuvula P, Tam YY, Oskouian B, Saba JD (2005) The immune modulator FTY720 inhibits sphingosine-1-phosphate lyase activity. *J Biol Chem* 280:33697–33700

- Berdyshev EV, Gorshkova I, Skobeleva A, Bittman R, Lu X, Dudek SM, Mirzapoiuzova T, Garcia JG, Natarajan V (2009) FTY720 inhibits ceramide synthases and up-regulates dihydrosphingosine 1-phosphate formation in human lung endothelial cells. *J Biol Chem* 284:5467–5477
- Blom T, Bäck N, Mutka AL, Bittman R, Li Z, de Lera A, Kovanen PT, Diczfalusy U, Ikonen E (2010) FTY720 stimulates 27-hydroxycholesterol production and confers atheroprotective effects in human primary macrophages. *Circ Res* 106:720–729
- Brinkmann V, Wilt C, Kristofic C, Nikolova Z, Hof RP, Chen S, Albert R, Cottens S (2001) FTY720: dissection of membrane receptor-operated, stereospecific effects on cell migration from receptor-independent antiproliferative and apoptotic effects. *Transplant Proc* 33:3078–3080
- Brinkmann V, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, Aradhye S, Burtin P (2010) Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat Rev Drug Discov* 9:883–897
- Chi H (2011) Sphingosine-1-phosphate and immune regulation: trafficking and beyond. *Trends Pharmacol Sci* 32:16–24
- Choi JW, Gardell SE, Herr DR, Rivera R, Lee CW, Noguchi K, Teo ST, Yung YC, Lu M, Kennedy G, Chun J (2011) FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 (S1P1) modulation. *Proc Natl Acad Sci USA* 108:751–756
- Coelho RP, Payne SG, Bittman R, Spiegel S, Sato-Bigbee C (2007) The immunomodulator FTY720 has a direct cytoprotective effect in oligodendrocyte progenitors. *J Pharmacol Exp Ther* 323:626–635
- da Huang W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4:44–57
- Dev KK, Mullershausen F, Mattes H, Kuhn RR, Bilbe G, Hoyer D, Mir A (2008) Brain sphingosine-1-phosphate receptors: implication for FTY720 in the treatment of multiple sclerosis. *Pharmacol Ther* 117:77–93
- Durafourt BA, Lambert C, Johnson TA, Blain M, Bar-Or A, Antel JP (2011) Differential responses of human microglia and blood-derived myeloid cells to FTY720. *J Neuroimmunol* 230:10–16
- Garay N, Taieb N, Yahi N, Fantini J (2005) Interaction of cholesterol with sphingosine: physicochemical characterization and impact on intestinal absorption. *J Lipid Res* 46:36–45
- Gibot L, Follet J, Metges JP, Auvray P, Simon B, Corcos L, Le Jossic-Corcos C (2009) Human caspase 7 is positively controlled by SREBP-1 and SREBP-2. *Biochem J* 420:473–483
- Higgins ME, Ioannou YA (2001) Apoptosis-induced release of mature sterol regulatory element-binding proteins activates sterol-responsive genes. *J Lipid Res* 42:1939–1946
- Hisano Y, Kobayashi N, Kawahara A, Yamaguchi A, Nishi T (2011) The sphingosine 1-phosphate transporter, SPNS2, functions as a transporter of the phosphorylated form of the immunomodulating agent FTY720. *J Biol Chem* 286:1758–1766
- Jack C, Ruffini F, Bar-Or A, Antel JP (2005) Microglia and multiple sclerosis. *J Neurosci Res* 81:363–373
- Jin Y, Zollinger M, Borell H, Zimmerlin A, Patten CJ (2011) CYP4F enzymes are responsible for the elimination of fingolimod (FTY720), a novel treatment of relapsing multiple sclerosis. *Drug Metab Dispos* 39:191–198
- Kappos L, Antel J, Comi G, Montalban X, O'Connor P, Polman CH, Haas T, Korn AA, Karlsson G, Radue EW, FTY720 D2201 Study Group (2006) Oral fingolimod (FTY720) for relapsing multiple sclerosis. *N Engl J Med* 355:1124–1140
- Lee TK, Man K, Ho JW, Sun CK, Ng KT, Wang XH, Wong YC, Ng IO, Xu R, Fan ST (2004) FTY720 induces apoptosis of human hepatoma cell lines through PI3-K-mediated Akt dephosphorylation. *Carcinogenesis* 25:2397–2405
- Liu Q, Alinari L, Chen CS, Yan F, Dalton JT, Lalapombella R, Zhang X, Mani R, Lin T, Byrd JC, Baiocchi RA, Muthusamy N (2010) FTY720 shows promising in vitro and in vivo preclinical activity by downmodulating cyclin D1 and phospho-Akt in mantle cell lymphoma. *Clin Cancer Res* 16:3182–3192
- Matsuda S, Minowa A, Suzuki S, Koyasu S (1999) Differential activation of c-Jun NH2-terminal kinase and p38 pathways during FTY720-induced apoptosis of T lymphocytes that is suppressed by the extracellular signal-regulated kinase pathway. *J Immunol* 162:3321–3326
- Matsuoka Y, Nagahara Y, Ikekita M, Shinomiya T (2003) A novel immunosuppressive agent FTY720 induced Akt dephosphorylation in leukemia cells. *Br J Pharmacol* 138:1303–1312
- Miron VE, Jung CG, Kim HJ, Kennedy TE, Soliven B, Antel JP (2008) FTY720 modulates human oligodendrocyte progenitor process extension and survival. *Ann Neurol* 63:61–71
- Miron VE, Ludwin SK, Darlington PJ, Jarjour AA, Soliven B, Kennedy TE, Antel JP (2010) Fingolimod (FTY720) enhances remyelination following demyelination of organotypic cerebellar slices. *Am J Pathol* 176:2682–2694
- Mullershausen F, Zecri F, Cetin C, Billich A, Guerini D, Seuwen K (2009) Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors. *Nat Chem Biol* 5:428–434
- Nagai A, Nakagawa E, Hatori K, Choi HB, McLarnon JG, Lee MA, Kim SU (2001) Generation and characterization of immortalized human microglial cell lines: expression of cytokines and chemokines. *Neurobiol Dis* 8:1057–1068
- Nagaoka Y, Otsuki K, Fujita T, Uesato S (2008) Effects of phosphorylation of immunomodulatory agent FTY720 (fingolimod) on antiproliferative activity against breast and colon cancer cells. *Biol Pharm Bull* 31:1177–1181
- Narantuya D, Nagai A, Sheikh AM, Masuda J, Kobayashi S, Yamaguchi S, Kim SU (2010) Human microglia transplanted in rat focal ischemia brain induce neuroprotection and behavioral improvement. *PLoS One* 5:e11746
- Payne SG, Oskeritzian CA, Griffiths R, Subramanian P, Barbour SE, Chalfant CE, Milstien S, Spiegel S (2007) The immunosuppressant drug FTY720 inhibits cytosolic phospholipase A2 independently of sphingosine-1-phosphate receptors. *Blood* 109:1077–1085
- Potteck H, Nieuwenhuis B, Lüth A, van der Giet M, Kleuser B (2010) Phosphorylation of the immunomodulator FTY720 inhibits programmed cell death of fibroblasts via the S1P3 receptor subtype and Bcl-2 activation. *Cell Physiol Biochem* 26:67–78
- Sato R (2010) Sterol metabolism and SREBP activation. *Arch Biochem Biophys* 501:177–181
- Satoh J, Tabunoki H, Arima K (2009) Molecular network analysis suggests aberrant CREB-mediated gene regulation in the Alzheimer disease hippocampus. *Dis Markers* 27:239–252
- Sensken SC, Gräler MH (2010) Down-regulation of S1P1 receptor surface expression by protein kinase C inhibition. *J Biol Chem* 285:6298–6307
- Sonoda Y, Yamamoto D, Sakurai S, Hasegawa M, Aizu-Yokota E, Momoi T, Kasahara T (2001) FTY720, a novel immunosuppressive agent, induces apoptosis in human glioma cells. *Biochem Biophys Res Commun* 281:282–288
- Van Doorn R, Van Horssen J, Verzijl D, Witte M, Ronken E, Van Het Hof B, Lakeman K, Dijkstra CD, Van Der Valk P, Reijerkerk A, Alewijnse AE, Peters SL, De Vries HE (2010) Sphingosine 1-phosphate receptor 1 and 3 are upregulated in multiple sclerosis lesions. *Glia* 58:1465–1476
- Wang JD, Takahara S, Nonomura N, Ichimaru N, Toki K, Azuma H, Matsumiya K, Okuyama A, Suzuki S (1999) Early induction of apoptosis in androgen-independent prostate cancer cell line by FTY720 requires caspase-3 activation. *Prostate* 40:50–55

- Wei Y, Yemisci M, Kim HH, Yung LM, Shin HK, Hwang SK, Guo S, Qin T, Alsharif N, Brinkmann V, Liao JK, Lo EH, Waeber C (2011) Fingolimod provides long-term protection in rodent models of cerebral ischemia. *Ann Neurol* 69:119–129
- Woodcock JM, Ma Y, Coolen C, Pham D, Jones C, Lopez AF, Pitson SM (2010) Sphingosine and FTY720 directly bind pro-survival 14-3-3 proteins to regulate their function. *Cell Signal* 22:1291–1299
- Xia Z, Tan MM, Wong WW, Dimitroulakos J, Minden MD, Penn LZ (2001) Blocking protein geranylgeranylation is essential for lovastatin-induced apoptosis of human acute myeloid leukemia cells. *Leukemia* 15:1398–1407
- Zhang Z, Zhang Z, Fauser U, Artelt M, Burnet M, Schluesener HJ (2007) FTY720 attenuates accumulation of EMAP-II+ and MHC-II+ monocytes in early lesions of rat traumatic brain injury. *J Cell Mol Med* 11:307–314

## SHORT COMMUNICATION

## Nasu–Hakola disease with a splicing mutation of TREM2 in a Japanese family

Y. Numasawa<sup>a</sup>, C. Yamaura<sup>a</sup>, S. Ishihara<sup>a</sup>, S. Shintani<sup>a</sup>, M. Yamazaki<sup>b</sup>, H. Tabunoki<sup>c</sup> and J.-I. Satoh<sup>c</sup><sup>a</sup>Department of Neurology, Toride Kyodo General Hospital, Toride City, Ibaraki; <sup>b</sup>The 2nd Department of Internal Medicine, Nippon Medical School, Bunkyo-ku, Tokyo; and <sup>c</sup>Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University, Kiyose, Tokyo, Japan**Keywords:**

exon 3 skipping, Nasu–Hakola disease, PLOSL, transcriptome, TREM2 mutation

Received 28 September 2010

Accepted 18 November 2010

**Background:** Nasu–Hakola disease (NHD) is a rare autosomal recessive disorder, characterized by a combination of progressive presenile dementia and formation of multifocal bone cysts, caused by genetic mutations of DAP12 and TREM2, which constitute a receptor/adaptor signaling complex expressed on osteoclasts, dendritic cells, macrophages, and microglia. No Japanese patients with TREM2 mutations have been reported previously.**Methods:** We reported three siblings affected with NHD in a Japanese family. Amongst them, two died of NHD during the fourth decade of life. The analysis of genomic DNA, cDNA cloning, and western blot of lymphocyte proteins was performed on samples of the living patient. The transcriptome was studied in the autopsied brain of one patient.**Results:** We identified a homozygous conversion of a single nucleotide T to C at the second position of intron 3 in the splice-donor consensus site (c.482+2T>C) of the TREM2 gene, resulting in exon 3 skipping and aberrant expression of truncated proteins. We identified 136 upregulated genes involved in inflammatory response and immune cell trafficking and 188 downregulated genes including a battery of GABA receptor subunits and synaptic proteins in the patient's brain.**Conclusions:** This is the first report of a Japanese NHD family caused by a splicing mutation of TREM2 that induces both neuroinflammation and neurodegeneration.**Introduction**

Nasu–Hakola disease (NHD; OMIM 221770), also designated polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL), is a rare autosomal recessive disorder, characterized by a combination of progressive presenile dementia and formation of multifocal bone cysts [1]. The clinical course of NHD is divided into four stages: (i) the latent stage with normal early development, (ii) the osseous stage beginning at the third decade of life, characterized by pathological bone fractures, (iii) the early neuropsychiatric stage occurring at the fourth decade of life, presenting with a frontal lobe syndrome, and

(iv) the late neuropsychiatric stage, characterized by profound dementia, and death usually by age 50 years [1].

Nasu–Hakola disease is caused by a structural defect in one of the two genes, DAP12 (TYROBP) or TREM2, both of which induce a clinicopathologically identical phenotype [2,3]. DAP12, expressed as a disulfide-bonded homodimer on NK cells, monocytes/macrophages, dendritic cells, osteoclasts, and brain microglia, constitutes a transmembrane adaptor that non-covalently associates with several cell-surface receptors, including TREM2, TREM1, NCR2 (NKp44), SIRPB1, and MDL1, and transmits activating or inhibitory signals depending on the avidity of their ligands. The TREM2/DAP12 complex regulates key signaling events involved in immune responses, differentiation of dendritic cells and osteoclasts, and phagocytic activity of microglia. Until present, 17 different NHD-causing mutations have been identified in

Correspondence: J.-I. Satoh, MD, PhD, Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan (tel.: +81 42 495 8678; fax: +81 42 495 8678; e-mail: satoj@my-pharm.ac.jp).

either DAP12 or TREM2 [2,3]. DAP12 mutations are predominant in Finnish and Japanese, whilst TREM2 mutations are distributed widely. However, no NHD patients with TREM2 mutations have been previously found in Japan. In this study, we describe the first case of a Japanese NHD family caused by a splicing mutation of TREM2.

## Methods

### Mutation analysis

After written informed consent was obtained, high molecular weight genomic DNA was extracted from whole peripheral blood. The Ethics Committee of MPU approved this study. All five exons and 5' and 3' flanking regions of the TREM2 gene were amplified by PCR using primer sets following: exon 1, 5'caccgcttcataattcacc3' and 5'gactcctcctcccctctgtc3'; exon 2, 5'agtggtggttctgcacac3' and 5'gctcctcagggcaggattt3'; exon 3, 5'gctctagtgcttgaattgtagt3' and 5'agtgaatgacctgatccacatagga3'; and exons 4 and 5, 5'tcttcctcacgtgtctctcagcc3' and 5'aagcccatcccaggatggtct3'. The purified PCR products were processed for direct sequencing on the 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). cDNA and protein extract were prepared from peripheral blood mononuclear cells (PBMC). The junctional sequence of exons 3 and 4 or the sequence spanning the entire open-reading frame (ORF) and 5' and 3' flanking regions amplified by PCR of cDNA was cloned in the pcDNA4/HisMax-TOPO vector (Invitrogen, Carlsbad, CA, USA) and processed for sequencing analysis.

### DNA microarray and molecular network analysis

Total RNA was isolated from a small piece derived from the fragmented bulk of autopsied frozen frontal lobe tissues of the patient 1. In parallel, we studied gene expression profile of the normal human frontal lobe RNA (636563; Clontech, Mountain View, CA, USA), pooled from unspecified regions isolated from four male/female Caucasians of ages 32–61 died of sudden death, serving as a control. The RNA integrity number (RIN) of total RNA on 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) was 6.1 for the control and 6.7 for the patient's sample, respectively. One hundred ng of total RNA was processed for microarray analysis with Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) containing 28 869 genes. The CEL file data were normalized by the robust multiarray average (RMA) method. When compared with gene expression levels in the control, the genes showing upregulation greater than fourfold or down-

regulation smaller than 0.1-fold were defined as differentially expressed genes. To identify biologically relevant molecular pathways, we utilized Ingenuity Pathways Analysis (IPA) tool (Ingenuity Systems). By uploading the list of Gene IDs and expression values, the network-generation algorithm identified focused genes integrated in a global molecular network with the score *P*-value reflecting the statistical significance of association between the genes and the networks by the Fisher's exact test.

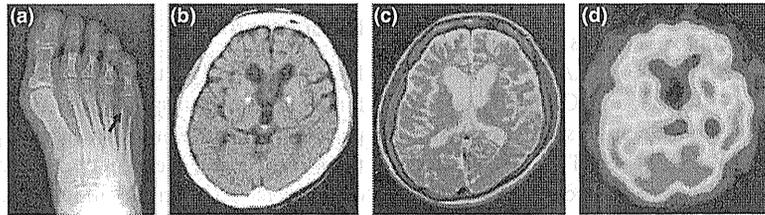
## Results

### The Japanese NHD family

A 36-year-old woman (the patient 3; the index) was admitted to the hospital because of epileptic seizure. Her parents are consanguineous. The patient has three siblings. The eldest brother (the patient 1) developed intractable epileptic seizures at age 33 was diagnosed as having NHD, became bedridden, and died at age 39 because of decubital infection in a different hospital, where the autopsy was performed. The second elder brother (the patient 2) was also clinically diagnosed as NHD in another hospital, and lead to sudden death of an unknown cause at age 31. The elder sister at age 44 is currently healthy.

The patient 3 has been normal until age 34, when she gradually began to show personality change, characterized by laziness, lack of concentration and insight, and loss of judgment and social inhibitions. At age 36, she showed a sign of apathy and memory disturbance and was affected with generalized tonic-clonic seizures. On admission, she was postictal but neurologically normal except for diffuse hyperactive deep-tendon reflexes and occasional myoclonic jerks. She showed a profound decrease in the score (seven of 30) on examination of a revised version of Hasegawa's Dementia Scale.

X-ray examinations showed multifocal radiolucent lesions distributed in distal ends of humerus, radius, femur, tibia, and metatarsal bone (Fig. 1a). Brain CT revealed bilateral calcification of the basal ganglia (Fig. 1b). Brain MRI showed moderate atrophy of the frontotemporal cortex and marked dilatation of the lateral ventricle on a T2-weighted image (Fig. 1c). 99mTc-ECD SPECT showed moderate hypoperfusion in the frontotemporal cortex with preservation of the cerebral blood flow in the basal ganglia (Fig. 1d). An EEG displayed sporadic spike waves in diffuse slow activities. The routine laboratory examination of blood and cerebrospinal fluid was normal. All of neurological and radiological findings described earlier supported a clinical diagnosis of NHD.



**Figure 1** Neuroimaging features of the patient 3. (a) X-ray film. A cystic lesion in the fifth metatarsal bone (arrow). (b) Brain CT. High density lesions in the bilateral basal ganglia. (c) Brain MRI. Moderate atrophy of the frontotemporal cortex and marked dilatation of the lateral ventricle on an axial T2-weighted image (TR: 4500 ms, TE: 98 ms). (d) 99mTc-ECD SPECT. Moderate hypoperfusion in the frontotemporal cortex with preservation of the cerebral blood flow in the basal ganglia.

### Identification of a splicing mutation in intron 3 of the TREM2 gene

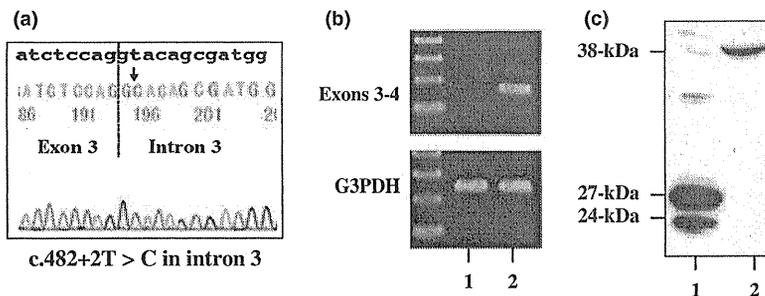
In the patient 3, we identified a homozygous conversion of a single nucleotide T to C at the second position of intron 3 in the splice-donor consensus site (c.482+2T>C) of the TREM2 gene (Fig. 2a). No mutations were found in the DAP12 gene. The junctional sequence between exon 3 and exon 4 was not detected by PCR of cDNA of the patient 3 (Fig. 2b, lane 1). By western blot, a 38-kDa full-length TREM2 protein was identified in PBMC of a normal subject (Fig. 2c, lane 2), whereas 27-kDa and 24-kDa truncated TREM2 proteins were detected in the patient 3 (Fig. 2c, lane 1).

The cloning and sequencing of the TREM2 ORF verified exon 3 skipping in combination with deletion of exon 2 and/or exon 4, followed by emergence of the premature or original stop codons (Fig. S1, panels a–d). The exon 3-skipped genes are predicted to encode four distinct truncated proteins, composed of 157 amino acids (35-kDa), 135 amino acids (28-kDa), amino acids 40 (11-kDa), or 18 amino acids (0.5-kDa), tentatively named as variant 1, 2, 3, or 4, respectively. The wild-

type full-length TREM2 protein consists of 230 amino acids, composed of a 13-amino acid signal peptide, followed by an 154-amino acid extracellular immunoglobulin superfamily (Ig-SF) domain pivotal for binding to the ligand, a 33-amino acid transmembrane (TM) domain essential for binding to DAP12, and a 30-amino acid cytoplasmic domain. InterProScan (<http://www.ebi.ac.uk/Tools/InterProScan>) search indicated that variants 1 and 2 have the Ig-SF domain but do not have the TM domain, whilst variants 3 and 4 lack both Ig-SF and TM domains, suggesting that all of these variants are non-functional. It remains unknown whether these variants expressed as truncated proteins produce a dominant negative effect on TREM2/DAP12 signaling.

### Gene expression analysis of autopsied brain tissues

We again identified the c.482+2T>C mutation in the genomic DNA isolated from the brain of the patient 1. The transcriptome analysis of the brain identified totally 136 upregulated genes (Table S1) and 188 downregulated genes (Table S2). Macrophage/microglia marker genes, such as CD163, MSR1, and CD68,



**Figure 2** TREM2 mutation of the patient 3. (a) Direct sequencing. PCR and sequencing of all five exons and exon–intron boundaries of the TREM2 gene identified a homozygous conversion of a single nucleotide T to C in the splice-donor consensus site at the second position of intron 3 (c.482+2T>C). (b) The junctional sequence of exons 3 and 4. The junctional sequence of exons 3 and 4 of TREM2 (upper panel) and G3PDH (lower panel) amplified by PCR from cDNA of PBMC. The lanes (1, 2) represent (1) the patient and (2) normal subject. (c) TREM2 protein expression. Western blot of PBMC protein with anti-TREM2 antibody (HPA010917, Sigma). The lanes (1, 2) represent (1) the patient and (2) normal subject.

were greatly upregulated, whereas the genes encoding nine different GABA receptor subunits, such as GABRG2, GABRA1, GABRB2, GABRA2, GABRA4, GABRA3, GABRA5, GABRB3, and GABRB1, along with various synaptic components, such as SYT1, SNAP25, SV2B, SYNPR, SYT4, SYT13, SYN2, and SYT5, were coordinately downregulated. Top 10 genes, either upregulated or downregulated, are listed in Table 1. Upregulation of RGS1 and CD163 and downregulation of GABRG2 and SYT1 were validated by qRT-PCR analysis (Fig. S2). With respect to neuronal and glial marker genes, the fold change of gene expression levels in the patient's brain vs. the control is NEFH (neurons), 0.30; PVALB (GABA neurons), 0.18; GFAP (astrocytes), 2.57; MBP (oligodendrocytes), 1.60; and G3PDH (housekeeping gene), 0.63, suggesting marked loss of neurons with enhanced astrogliosis in the NHD brain at a terminal stage.

By IPA, the set of 136 upregulated genes constituted a highly complex network of 64 focused genes showing the most significant relationship with inflammatory response, cellular movement, and immune cell trafficking ( $P = 1.00E-116$ ) (Fig. S3a), whilst 188 downregulated genes formed the network of 91 focused genes showing the most significant relationship with cell-to-cell signaling and interaction, nervous system development and function, and genetic disorder ( $P = 1.00E-168$ ) (Fig. S3b).

## Discussion

Here, we described the first report of a Japanese NHD family caused by a splicing mutation of TREM2. Three patients showed clinical features typical of NHD, and the patient 1 and the patient 3 exhibited a homozygous c.482+2T>C mutation inducing exon 3 skipping of the TREM2 gene. The identical mutation was reported previously in two patients of an Italian family [2–4]. Until present, 20 patients with 11 different TREM2 mutations have been reported worldwide, including one patient with G40T (Q14stop), four with C97T (Q33stop), one with G132A (W44stop), two with G233A (W78stop), one with 267delG (frameshift followed by premature termination), one with 313delG (frameshift followed by premature termination), two with T377G (V126G), one with A401G (D134G), two with G558T (K86N), two with c.482+2T>C (splicing mutation), or three with c.40+3delAGG at the third position of intron 1 in the splice-donor consensus site (splicing mutation) [2–6].

TREM2 is expressed exclusively in osteoclasts, dendritic cells, tissue-infiltrating macrophages, and a subpopulation of microglia, located in the Golgi complex and exocytic vesicles, on cell-surface membranes, and in part released extracellularly [7]. TREM2 expressed on mouse microglia plays a key role in tissue debris clearance and resolution of inflammation [8]. We

**Table 1** Top 10 upregulated and downregulated genes in the brain of a Nasu–Hakola disease patient with TREM2 mutation

Rank	Fold change	Gene ID	Gene symbol	Gene description
<b>Upregulated genes</b>				
1	40.2	5996	RGS1	Regulator of G-protein signaling 1
2	13.3	9103	FCGR2C	Fc fragment of IgG, low affinity IIc, receptor for (CD32)
3	13.2	9332	CD163	CD163 molecule
4	12.9	312	ANXA13	Annexin A13
5	12.1	4640	MYO1A	Myosin IA
6	11.6	7852	CXCR4	Chemokine (C-X-C motif) receptor 4
7	11.5	118932	ANKRD22	Ankyrin repeat domain 22
8	10.7	84689	MS4A14	Membrane-spanning 4-domains, subfamily A, member 14
9	10.7	1610	DAO	D-amino acid oxidase
10	10.4	3606	IL18	Interleukin 18 (interferon-gamma-inducing factor)
<b>Downregulated genes</b>				
1	0.008	7447	VSNL1	Visinin-like 1
2	0.010	2566	GABRG2	Gamma-aminobutyric acid (GABA) A receptor, gamma 2
3	0.010	2554	GABRA1	Gamma-aminobutyric acid (GABA) A receptor, alpha 1
4	0.011	5999	RGS4	Regulator of G-protein signaling 4
5	0.011	6857	SYT1	Synaptotagmin I
6	0.012	6616	SNAP25	Synaptosomal-associated protein, 25kDa
7	0.014	8507	ENC1	Ectodermal-neural cortex (with BTB-like domain)
8	0.016	114569	MAL2	Mal, T-cell differentiation protein 2
9	0.018	4753	NELL2	NEL-like 2 (chicken)
10	0.018	9899	SV2B	Synaptic vesicle glycoprotein 2B

The transcriptome of frontal lobe tissues of the patient 1 was studied using Human Gene 1.0 ST array (Affymetrix). When compared with the gene expression levels in the control, top 10 upregulated and downregulated genes are listed with fold change, Entrez Gene ID, gene symbol, and description. See Tables S1 and S2 for the entire list.

for the first time characterized the genome-wide transcriptome of the NHD brain, although the case, the control, and the region of the brain, possibly affecting gene expression profiles, are fairly limited because of restricted availability of the samples. We identified a panel of upregulated genes closely associated with inflammatory response and immune cell function, and downregulated genes related to the nervous system function, indicating a concurrence of neuroinflammatory and neurodegenerative events in NHD brains.

### Acknowledgements

This work was supported by grants to J-IS from Research on Intractable Diseases, the Ministry of Health, Labour and Welfare, Japan (H22-Nanchi-Ippan-136), and the High-Tech Research Center Project (S0801043) and the Grant-in-Aid (C22500322), the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The gene sequence data are registered in GenBank with the accession number AB601768, and the microarray data are available from Gene Expression Omnibus (GEO) under the accession number GSE25496.

### Disclosure of conflict of interest

The authors declare no financial or other conflict of interests.

### References

1. Bianchin MM, Capella HM, Chaves DL, *et al.* Nasu-Hakola disease (polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy-PLOSL): a dementia associated with bone cystic lesions. From clinical to genetic and molecular aspects. *Cell Mol Neurobiol* 2004; **24**: 1–24.
2. Klünemann HH, Ridha BH, Magy L, *et al.* The genetic causes of basal ganglia calcification, dementia, and bone cysts: DAP12 and TREM2. *Neurology* 2005; **64**: 1502–1507.
3. Paloneva J, Manninen T, Christman G, *et al.* Mutations in two genes encoding different subunits of a receptor signaling complex result in an identical disease phenotype. *Am J Hum Genet* 2002; **71**: 656–662.
4. Salmaggi A, Maccagnano E, Musso A, Di Lena L, Paloneva J, Boiardi A. An Italian family with Nasu-Hakola disease. *J Neurol* 2003; **250**: 878–880.
5. Soragna D, Papi L, Ratti MT, Sestini R, Tupler R, Montalbetti L. An Italian family affected by Nasu-Hakola disease with a novel genetic mutation in the TREM2 gene. *J Neurol Neurosurg Psychiatry* 2003; **74**: 825–826.
6. Chouery E, Delague V, Bergougnoux A, Koussa S, Serre JL, Mégarbané A. Mutations in TREM2 lead to pure early-onset dementia without bone cysts. *Hum Mutat* 2008; **29**: E194–E204.
7. Sessa G, Podini P, Mariani M, *et al.* Distribution and signaling of TREM2/DAP12, the receptor system mutated in human polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy dementia. *Eur J Neurosci* 2004; **20**: 2617–2628.
8. Takahashi K, Rochford CD, Neumann H. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *J Exp Med* 2005; **201**: 647–657.

### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** The c.482 + 2T > C mutation induces exon 3 skipping. The ORF of TREM2 amplified by PCR was cloned in the vector. Four distinct clones were processed for sequencing analysis. The panels (a–d) represent (a) variant 1 with deletion of exon 3, composed of 157 amino acids, (b) variant 2 with deletion of exons 3 and 4, composed of 135 amino acids, (c) variant 3 with deletion of exons 2 and 3, composed of 40 amino acids, and (d) variant 4 with deletion of exons 2, 3, and 4, composed of 18 amino acids. The stop codon is underlined in variants 2 and 4.

**Figure S2.** Validation of microarray data by qRT-PCR. The microarray data was validated by quantitative RT-PCR on LightCycler ST300 (Roche Diagnostics). The panels represent (a) RGS1, (b) CD163, (c) GABRG2, and (d) SYT1. The expression levels of each gene were standardized against those of G3PDH. Frontal lobe cDNA of NC (the normal control), NHD (the patient 1), ALS1 and ALS2 (two patients with amyotrophic lateral sclerosis), and AD1 and AD2 (two patients with Alzheimer disease) were analyzed.

**Figure S3.** Molecular network of 136 upregulated and 188 downregulated genes in the brain of a NHD patient with TREM2 mutation. By Ingenuity Pathways Analysis of brain transcriptome data of the patient 1, 136 upregulated genes constitute the network of 64 focused genes (red) that has the most significant relationship with inflammatory response, cellular movement, and immune cell trafficking ( $P = 1.00E-116$ ) (panel a), whilst 188 downregulated genes constitute the network of 91 focused genes (green) that has the most significant relationship with cell-to-cell signaling and interaction, nervous system development and function, and genetic disorder ( $P = 1.00E-168$ ) (panel b).

**Table S1.** The list of 136 upregulated genes in the brain of a NHD patient with TREM2 mutation.

**Table S2.** The list of 188 downregulated genes in the brain of a NHD patient with TREM2 mutation.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

## A Japanese Case with Nasu-Hakola Disease of DAP12 Gene Mutation Exhibiting Precuneus Hypoperfusion

Kiyotaka Nakamagoe<sup>1</sup>, Ayako Shioya<sup>1</sup>, Tetsuto Yamaguchi<sup>1</sup>, Hiroyuki Takahashi<sup>1</sup>,  
Reiko Koide<sup>1</sup>, Tatsuya Monzen<sup>2</sup>, Jun-ichi Satoh<sup>3</sup> and Akira Tamaoka<sup>1</sup>

### Abstract

A 38-year-old Japanese man with Nasu-Hakola disease (NHD) had repeated pathological fractures and frontal lobe symptoms which developed when he was 18 and 26 years old, respectively. Neuropsychological testing showed memory impairment, and in particular, visuo-spatial memory at the age of 35. Furthermore, single-photon emission computed tomography revealed precuneus hypoperfusion. The patient later suffered prolonged convulsive seizures, which left him in a persistent vegetative state. Genetic testing confirmed a heterozygous mutation in the DAP12 gene (a single-base deletion of 141 G in exon 3) specific to NHD. Precuneus dysfunction might contribute to characteristic memory impairment of NHD.

**Key words:** Nasu-Hakola disease, DAP12, TREM2, SPECT, MRI, precuneus

(Intern Med 50: 2839-2844, 2011)

(DOI: 10.2169/internalmedicine.50.5891)

### Introduction

Nasu-Hakola disease (NHD), an extremely rare autosomal recessive inherited disease also referred to as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOS), occurs most commonly among the Japanese and Finnish populations. Our PubMed literature search identified approximately 50 reported cases of NHD.

The cardinal symptoms include multiple bone cysts accompanied by pathological fractures and early-onset dementia induced by leukoencephalopathy. The clinical course of NHD follows four stages: latent, osseous, early neurological, and late neurological stages (1-5). The latent stage generally takes place before the patient reaches the age of 20. The osseous stage, which begins after the patient is 20, is characterized by the development of multiple bone cysts, which occur most frequently at the epiphyses of long tubular bones, accompanied by bone pain and repeated pathological fractures. The early neurological stage starts after the patient is 30, with the manifestation of frontal lobe symptoms, including disinhibition, euphoria, and personality disorder, as well as the onset of epileptic seizures. The late neurological

stage sets in after the patient is 40, and the patient develops a rapidly progressive, profound dementia, and eventually becomes bedridden. Most patients die in their 30s or 40s.

According to previous reports, the homozygous deletion or point mutation of DAP12 (TYRO protein tyrosine kinase-binding protein, TYROBP) or the triggering receptor expressed on myeloid cells 2 (TREM2) is the typical genetic defect observed in NHD (3, 4, 6, 7). Furthermore, Kuroda et al reported a case in which a compound heterozygote of a defective DAP12 gene was detected (8). Genetic mutations of DAP12 and TREM2 lead to losses of function expressed in microglia in the brain and osteoclasts in the bone, which cause symptoms to appear in the central nervous system and bone.

NHD can be diagnosed with relative ease when it is accompanied by bone lesions, in which case the presence of polycystic lipomembranous osteodysplasia, which is specific to NHD, in the bone biopsy specimen confirms the diagnosis. However, without such bone lesions, diagnosis can be very difficult. According to Chouery et al, some patients in whom the mutation of the TREM2 gene was present developed early-onset dementia without exhibiting bone lesions (9). In such patients, genetic testing may be needed to

<sup>1</sup>Department of Neurology, Institute of Clinical Medicine, University of Tsukuba, Japan. <sup>2</sup>Department of Neurology, Ushiku Aiwa General Hospital, Japan and <sup>3</sup>Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University, Japan

Received for publication May 25, 2011; Accepted for publication August 15, 2011

Correspondence to Dr. Kiyotaka Nakamagoe, Nakamagoek@md.tsukuba.ac.jp

Table 1. Summary of Neuropsychological Tests

Neuropsychological Tests	Results
Mini-Mental State Examination (MMSE)	28/30 (errors on calculation and memory)
Wechsler Memory Scale-Revised (WMS-R; Index scores less than 85 indicate abnormal decline.)	Verbal Memory Index: 82 Visual Memory Index: <50 General Memory Index: 65 Attention/Concentration: 73 Delayed Memory: <50
Alzheimer's Disease Assessment Scale-cognitive component-Japanese version (ADAS-J cog.)	9/70
Clock Drawing Test	Error in standard version. Improvement in copied version.
Verbal Fluency Test	Difficulty in recalling words with initial Japanese letter, <i>ka</i> .

The testing was performed when the patient was 35 years old, before the onset of convulsive seizures. The profile shows the impairment in memory dysfunction (especially, visuospatial memory) and frontal dysfunction, although overall cognitive function (by MMSE) was shown to be still intact

arrive at a diagnosis of NHD.

Recent research has revealed an immune abnormality induced by genetic mutations associated with NHD (5, 10). In this paper, we report a case of NHD exhibiting a defective DAPI2 gene with a focus on the relationship between clinical symptoms and image findings.

### Case Report

The present case is a 38-year-old man. In his family history, his brother, who had multiple bone cysts and was suspected of having NHD, died in a traffic accident at the age of 19. The present patient had previously been healthy. At the age of 18, he experienced pain in the joints of both legs and had repeated pathological fractures. His X-rays showed multiple cystic radiolucent areas at the epiphyses of both legs and thickening of the trabecular bone. A bone biopsy, performed following autologous transplantation of the bones of both ankles, revealed polycystic lipomembranous osteodysplasia. Signs of disinhibition, personality changes, and behavioral abnormalities became evident when the patient was 26. Particularly abnormal behaviors, such as stealing and the reckless borrowing of money, escalated over time. T2-enhanced brain MRI images taken when the patient was 34 years old revealed diffuse high-intensity areas in the white matter as well as hippocampal atrophy. Neuropsychological tests performed when the patient was 35 showed higher-order cognitive dysfunction, including memory impairment (Table 1). Nine months after the testing, the patient had generalized seizures and subsequently received the antiepileptic agent phenytoin. Later, hypobulia became evident, and the patient became withdrawn. However, he was still able to perform daily activities, such as eating and walking. At the age of 36, he was admitted to our hospital immediately after having generalized tonic-clonic seizures, which resulted in prolonged convulsive seizures.

Physical findings on admission: The patient exhibited profoundly impaired consciousness with a score of 4 (E1, V1, M3) on the Glasgow Coma Scale. The size of both pupils was 2 mm and the light reflexes were absent from both eyes. The deep tendon reflexes were exaggerated in all

limbs. Foot and knee cloni were positive bilaterally. Both Babinski and Chaddock reflexes were positive bilaterally. The palmomental reflex was also positive. Furthermore, the patient had double incontinence.

Disease progression after admission: The patient was put on a mechanical ventilator. Intravenous phenytoin and continuous intravenous midazolam infusion were initiated to treat prolonged generalized tonic-clonic seizures. Shortly afterwards, the generalized seizures disappeared and never returned. The patient had only transient episodes of partial seizures of the left upper limb. As the patient's condition improved, he was weaned from the mechanical ventilator on the 11th day of hospitalization. Around that time, the patient showed decerebrate rigidity. The convulsions were controlled by administration of 500 mg/day phenytoin via a gastric tube. Since that time the patient remained in a persistent vegetative state for over one year with no sign of improvement in the level of consciousness.

Neuropsychological testing was administered when the patient was 35 years old, prior to the first episode of convulsive seizures (Table 1). The patient scored 28 on the Mini-Mental State Examination (MMSE), indicating normal overall cognitive function. He made some errors on the items related to calculation and memory. On the Wechsler Memory Scale-Revised (WMS-R), the patient disclosed memory impairment in all indexes (less than 85), among which the scores were extremely low in visual memory and delayed memory (less than 50). On the Clock Drawing Test (CDT), the patient could not image the clock face exactly and hesitated to draw by organizing the parts of the clock; however, he could copy the clock face when he was shown a clock drawn by the tester, indicating that his error in CDT was not due to constructive apraxia but frontal executive dysfunction and memory dysfunction. There was also evidence of frontal lobe dysfunction, i.e., difficulty in verbal fluency task (recalling words with Japanese *ka* as the initial letter). With these results, the neuropsychological profile of this case resembles those often observed in patients with mild cognitive impairment (MCI), i.e., normal overall cognitive function with an abnormal decline in memory and frontal function; however, there is an additional striking feature

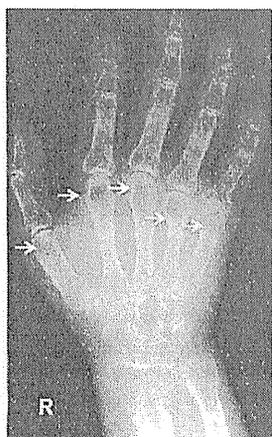


Figure 1. Plain X-ray images of hands and fingers. Multiple bone cysts were found at the epiphyses (arrow).

in this case, namely, that extreme dysfunction which was observed in visuo-spatial memory in the WMS-R visual memory index as well as in the errors on the CDT, which seemed to be inconsequential to the normal MMSE score obtained.

#### Imaging findings:

Plain X-ray images of hands and fingers revealed multiple bone cysts at the epiphyses (Fig. 1). T2-enhanced brain MRI images revealed diffuse high-intensity areas in the white matter as well as low-intensity areas in the basal ganglia (Fig. 2). FLAIR images showed progressive brain atrophy, with particularly significant bilateral atrophy of the hippocampus. The atrophy of the brain progressed much more rapidly after the patient suffered prolonged convulsive seizures compared to before the seizures.

A statistical analysis of the brain tomographic images obtained via N-isopropyl-p[<sup>123</sup>I] iodoamphetamine single-photon emission computed tomography (<sup>123</sup>I-IMP SPECT) was performed (Fig. 3). The spatial distribution of abnormal regional cerebral blood flow (rCBF) was evaluated using the Neurological Statistical Image Analysis Software (3), and the Z score was calculated (11). Data on normal controls aged 30 to 39 years were derived from a normal database (ChibaDB\_ver2) developed by Chiba University Hospital for age-group analysis. The result showed a decrease in blood flow to the frontal lobe after the convulsive seizures compared to that before the seizures. Diminished blood flow was also observed in the posterior parietal area, including the precuneus.

Genetic analysis: After informed consent was obtained, high-molecular-weight genomic DNA was extracted from whole peripheral blood using the GenomicPreP blood DNA isolation kit (GE Healthcare UK, Ltd., Buckinghamshire, England). All five exons and 5' and 3' flanking regions of the *DAP12* gene (*TYROBP*; GenBank accession No. AF019563) were amplified by PCR using the primer sets specific for individual exons (3). The purified PCR products

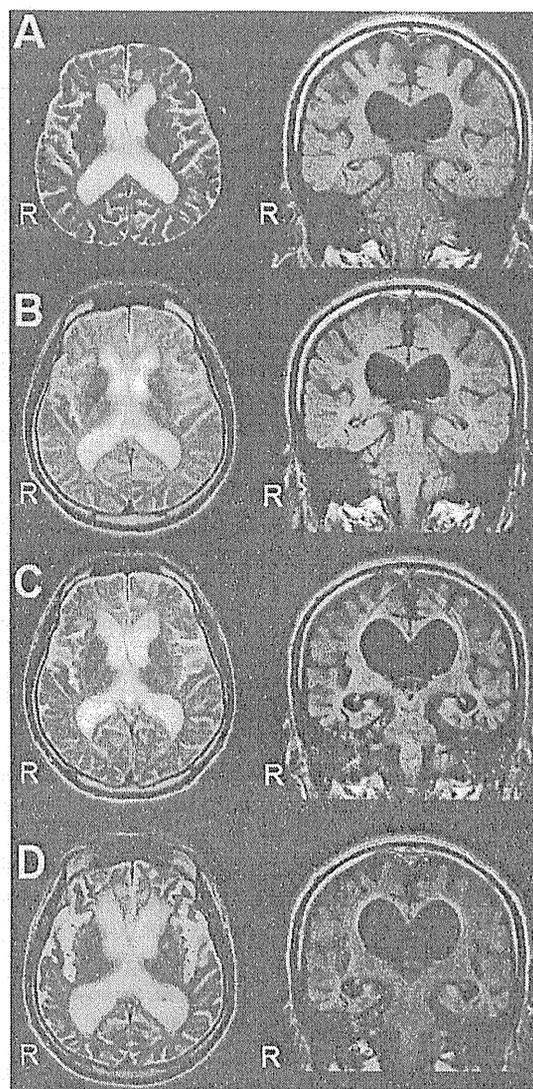


Figure 2. Brain MRI. A, B, C, and D show T2-enhanced images and FLAIR images taken when the patient was 34 and 35 years old, immediately after the onset of convulsive seizures when the patient was 36 years old, and one month after the convulsive seizures, respectively. A comparison of A and B, which represent images taken before the onset of prolonged convulsive seizures, reveals only a mild progression of brain atrophy. In contrast, rapid progression of brain atrophy is evident when comparing C, which represents images taken after the convulsive seizures, and B. D, which represents images taken one month after the convulsive seizures, shows even greater progression of brain atrophy.

were processed for direct sequencing by the dideoxynucleotide chain termination method on the 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). A single-base deletion of 141 G (141delG) was found in exon 3 of the *DAP12* gene (Fig. 4). Furthermore, this genetic mutation was found to be a homozygous point mutation. These findings confirmed the diagnosis of NHD on the basis of a dem-