

Variable expression of microglial *DAP12* and *TREM2* genes in Nasu-Hakola disease

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Abstract Nasu-Hakola disease (NHD) is a form of presenile dementia associated with sclerosing leukoencephalopathy and polycystic lipomembranous osteodysplasia. This extremely rare inherited disease is caused by mutations in either *DAP12* or *TREM2*. The present study was designed to assess the relationship between *DAP12/TREM2* genotype, mRNA and protein expression levels by both Western blotting and immunohistochemistry, and the tissue distribution and pathomorphological phenotype of the microglia. Molecular genetic testing performed in three NHD cases confirmed that two cases had mutations in *DAP12* and that one case carried a mutation in *TREM2*. Protein levels were analyzed in four cases. Interestingly, significant DAP12 expression was found in numerous microglia in one NHD case with a homozygous

DAP12 single-base substitution, and both real-time PCR and Western blotting confirmed the finding. In contrast, levels of both *DAP12* and *TREM2*, respectively, were much lower in the other cases. Immunohistochemistry using established microglial markers revealed consistently mild activation of microglia in the cerebral white matter although there was no or only little expression of DAP12 in three of the NHD cases. The highly different expression of DAP12 represents the first description of such variable expressivity in NHD microglia. It raises important questions regarding the mechanisms underlying dementia and white matter damage in NHD.

Keywords DAP12 · Macrophage · Microglia · Nasu-Hakola disease · PLOSL · TREM2

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Introduction

Nasu-Hakola disease (NHD), also known as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL), was first reported separately by Nasu and Hakola in Japan and Finland, respectively, in the 1970s [1, 2]. The disease is distributed worldwide but is extremely rare. Approximately 160 cases have been reported globally [3], including more than 30 autopsy cases from Japan [4]. NHD is an autosomal recessively inherited disease characterized by multiple bone cysts and early-onset progressive frontal-type dementia. Neurologic symptoms sometimes precede the osseous ones and patients with NHD usually die during the fifth decade of life. The molecular causes of NHD are mutations in either one of two genes, *DAP12* (DNAX-activation protein 12), also known as TYROBP (TYRO protein tyrosine kinase-binding protein), and *TREM2* (triggering receptor expressed on myeloid cells 2). The disease phenotype appears to be identical irrespective of the affected

gene. At the level of individual brain cells, DAP12 is exclusively expressed in microglia both in mouse and in human tissue, and colocalization of DAP12/*TREM2* with microglia/macrophage markers has been previously reported in mouse brain [5, 6].

Microglia are the resident macrophages and main mediators of inflammation in the CNS, and they function as specialized scavengers that eliminate pathogens and supply neurons with trophic factors [7, 8]. On the other hand, microglial overactivation may lead to exacerbated generation of neurotoxic molecules, such as reactive oxygen species and proinflammatory cytokines [9]. At present, Iba1 (ionized calcium-binding adapter molecule 1) is the most versatile marker for mammalian microglia (it works on paraffin sections and across species, and a polyclonal antibody allows double labelings), but a single specific microglia marker that does not label other myeloid cell types in the body has not yet been identified [10]. Further microglia markers available for routine paraffin sections include antibodies GLUT5 (directed against glucose transporter 5), CD68 (a pan-macrophage marker that recognizes lysosomes), HLA-DR, CD163, and CD204 (scavenger receptor class A) [7, 11–13]. Microglial phagocytosis appears to be adapted to the brain environment for remodeling tasks such as engulfment of synapses (“synaptic stripping”), axonal and myelin debris, or clearance of proteins such as amyloid beta protein. Comparatively little is still known about the specific molecules involved in microglial phagocytosis although different types of receptors that enable microglia to recognize uptake targets include *TREM2* and *DAP12* [14]. Detailed comparative data on *DAP12* at the DNA, RNA, and protein levels in diseased human brains are currently not available.

Therefore, in order to obtain a more complete picture of microglia and macrophages in NHD and to characterize the relationship between *DAP12/TREM2* mutations and microglial pathology, we investigated the expression of several microglial activation markers as well as *DAP12* messenger RNA (mRNA) and protein levels in NHD brains and controls.

Materials and methods

Patient samples

We examined four Japanese autopsy cases of NHD. Clinical and pathological features of all NHD cases are summarized in Table 1. All NHD patients had died during the fifth decade of life. There was no family history in the NHD patients with the exception of NHD case 4 whose parents were first cousins and her elder sister had died of NHD at the age of 37 [15]. Clinically, all NHD cases showed neurological symptoms, including presenile dementia, and the duration of dementia was between 6 and 10 years. Osseous lesions, such as bone fracture

and/or bone cysts, as well as characteristic lipomembranous lesions of the bone and adipose tissues, were present in all of the cases. There was no evidence of sepsis in any of them.

Neuropathologically, all NHD cases showed severe brain atrophy (mean brain weight 882.5 g). There was diffuse loss of myelin and axons from the white matter with accentuation in the frontal lobe while subcortical arcuate fibers were relatively spared. A small number of fat granule cells were observed around blood vessels in all cases but within brain tissue (in the occipital lobe) only in NHD case 1. The cerebral neocortex was well preserved in NHD cases 2–4. In contrast, NHD case 1 showed moderate to mild neuronal loss in the frontal, temporal, and parietal lobes.

DAP12 gene mutations in two of the cases have been reported previously, i.e., a point mutation (T to C) in the start codon of exon 1 in case 1 and as a single-base deletion of exon 3 in case 2 [16].

Three control brains were included for Western blot analysis: control 1, female, aged 49 years at death, clinicopathological diagnosis of Crow-Fukase syndrome; control 2, female, aged 49 years at death, clinicopathological diagnosis of diabetic nephropathy; and control 3, male, aged 51 years at death, clinicopathological diagnosis of panperitonitis. For our immunohistochemical studies, two individuals without neurological disease (a 59-year-old female with liver cirrhosis and a 34-year-old male with sleep apnea syndrome) and two autopsy cases of adrenoleukodystrophy (a 58-year-old male and a 54-year-old male) served as normal and disease-specific controls, respectively. All human samples were obtained and accessed in accordance with institutional research ethics guidelines.

Laboratory techniques

Sequencing of *DAP12* and *TREM2*

DAP12 and/or *TREM2* were analyzed in three cases of NHD patients (cases 1, 2, and 4), according to the methodology described in the previous papers [17, 18]. We were unable to perform genetic and protein analyses for NHD case 3, since no frozen brain tissue was available. Briefly, genomic DNA was extracted from frozen frontal lobe tissue using the Genra Puregene Blood Kit (Qiagen), and all exons of each gene were amplified by PCR and subjected to direct sequencing. For complementary DNA (cDNA) analysis, total RNA was extracted from autopsied brain tissues from the above three patients, and cDNA was synthesized using a Transcription First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) and amplified by PCR using gene-specific primers. The cDNA products were then subcloned into pTAC-2 vector (ByoDynamics Laboratory, Tokyo, Japan) and the purified plasmid DNA was sequenced with M13 forward and reverse primers.

Table 1 Summary of clinical, genetic, and pathological features of the NHD cases

| Case | Case 1 | | Case 2 | | Case 3 | | Case 4 | |
|--|-------------------------------|--------------------------------|---|--------------------------------|---|-----------------------------------|--|--|
| | Female | Male | Female | Male | Female | Male | Female | Male |
| Clinical features | | | | | | | | |
| Sex | Female | Male | Female | Male | Female | Male | Female | Male |
| Age at death (years) | 50 | 50 | 48 | 48 | 48 | 48 | 41 | 41 |
| Family history | – | – | – | – | – | – | + | + |
| Onset (age) | Personality change (38 years) | Bone fracture (17 years) | Bone cyst/pain (20 years) | Bone fracture (17 years) | Bone cyst/pain (20 years) | Bone fracture (17 years) | Loss of memory (35 years) | Loss of memory (35 years) |
| Initial neurological symptoms (age) | Personality change (38 years) | Epilepsy (39 years) | Personality change (32 years) | Epilepsy (39 years) | Personality change (32 years) | Epilepsy (39 years) | Loss of memory (35 years) | Loss of memory (35 years) |
| Other neurological findings | Dementia (40 years) | Dementia, epileptic seizure | Tremor, gait disturbance, dementia (44 years) | Dementia, epileptic seizure | Tremor, gait disturbance, dementia (44 years) | Dementia, epileptic seizure | Extrapyramidal sign, epilepsy, dementia (40 years) | Extrapyramidal sign, epilepsy, dementia (40 years) |
| Bone fracture | + | + | + | + | + | + | + | + |
| Mutation gene | <i>DAP12</i> c.2T>C | <i>DAP12</i> c.141Gdel | NE | <i>DAP12</i> c.141Gdel | NE | <i>DAP12</i> c.141Gdel | <i>TREM2</i> c.197C>T | <i>TREM2</i> c.197C>T |
| Pathological features | | | | | | | | |
| Lipomembranous lesions of the bone | Present (autopsy) | Present (biopsy) | Present (biopsy) | Present (biopsy) | Present (biopsy) | Present (biopsy) | Present (biopsy) | Present (biopsy) |
| Brain weight (g) | 910 | 920 | 840 | 920 | 840 | 920 | 860 | 860 |
| Type of brain lesions | SL | SL | SL | SL | SL | SL | SL | SL |
| Cerebral WM | | | | | | | | |
| Loss of myelinated fibers ^a | +++ | +++ | ++ | +++ | ++ | +++ | ++ | ++ |
| Axonal spheroid | + | +++ | ++ | +++ | ++ | +++ | ++ | ++ |
| Astrocytosis | +++ | +++ | ++ | +++ | ++ | +++ | +++ | +++ |
| Vascular change | ++ | + | +++ | + | +++ | +++ | +++ | +++ |
| Fat granule cell | ++ (occipital, perivascular) | ++ (few, perivascular) | + | ++ (few, perivascular) | + | ++ (few, perivascular) | + | ++ (few, perivascular) |
| Cerebral GM | | | | | | | | |
| Neuronal loss | ++ | + | + | + | + | + | + | + |
| Astrocytosis | +++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Basal ganglia/thalamus | | | | | | | | |
| Mineralization | +++ | +++ | +++ | +++ | +++ | +++ | – | – |
| Neuronal loss | +++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Cerebellum | Not prominent | Not prominent | Not prominent | Not prominent | Not prominent | Not prominent | Mild loss of myelin | Mild loss of myelin |
| Brain stem | Mild degeneration (tract) | Atrophy with mild astrocytosis | Neuronal loss and gliosis (nigra) | Atrophy with mild astrocytosis | Neuronal loss and gliosis (nigra) | Neuronal loss and gliosis (nigra) | Not prominent | Not prominent |

NE not examined, GM gray matter, WM white matter, + mild, ++ moderate, +++ severe, SL sclerosing leukoencephalopathy

^a Frontal and temporal lobe predominant

Real-time PCR

One microgram of total RNA was reverse-transcribed using a Transcription First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The levels of mRNA were quantified using fluorescent dye SYBR Green-based assay (Roche Diagnostics, Mannheim, Germany). Real-time PCR was performed in a final volume of 10 μ l with gene-specific primers (0.25 μ M each) for β -actin, *DAP12*, and *TREM2* (Takara Bio, Otsu, Japan) on a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions were 10 min at 95 °C, then 40 cycles each of 15 s at 95 °C and 1 min at 60 °C. RNA quantity was normalized to its respective beta-actin mRNA quantity.

Western blot analysis

Proteins were extracted from frozen tissue of the cerebral cortex (occipital lobe) of three patients with NHD (cases 1, 2, and 4) as previously described [19]. Frozen brain tissues were homogenized in buffer (10 mmol Tris-HCl, pH 7.5, 1 mmol EGTA, 1 mmol dithiothreitol, 10 % sucrose, 1 mmol Na₃VO₄, 5 mmol NaF with protease inhibitor cocktail) and centrifuged at 25,000 \times g for 30 min at 4 °C. The resulting pellets were subsequently extracted in homogenization buffer containing 1 % Triton X-100 and 0.5 % SDS. After centrifugation at 180,000 \times g for 30 min at 4 °C, the soluble fractions were subjected to Tris-Tricine SDS-polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting. A rabbit polyclonal anti-DAP12 antibody that recognizes amino acids 1–113 of human DAP12 (FL113, Santa Cruz Biotechnology) was used for the detection of DAP12. Actin was visualized using an anti-actin antibody (I-19, Santa Cruz Biotechnology) as the loading control.

Immunohistochemistry (IHC)

After formalin fixation, brains were sectioned, processed routinely, and embedded in paraffin. Immunohistochemistry was performed on 5- μ m-thick section taken from the frontal lobe (containing the middle frontal gyrus), temporal tip, hippocampus, precentral gyrus (motor cortex), occipital lobe (containing the striate area), thalamus, lenticular nuclei, cerebellum, medulla oblongata, and spinal cord using the biotin-streptavidin (B-SA) immunoperoxidase method (Nichirei, Tokyo, Japan) with the following antibodies (abs): Iba1 (rabbit polyclonal ab; 1:500 dilution, Wako, Japan), Glut5 (rabbit polyclonal ab against glucose transporter 5; 1:100, IBL, Japan), CR3/43 (mouse monoclonal ab against human MHC class II antigen; 1:50, Dako, Denmark), PG.M1 (mouse monoclonal ab against CD68, 1:100, Dako, Denmark), SRA-E5 (mouse monoclonal ab against CD204, 1:25,

TransGenic Inc., Japan), 10D6 (monoclonal ab against CD163; 1:100, Novocastra, UK), and GFAP (rabbit polyclonal ab against glial fibrillary acidic protein; our own, 1:5000). For staining of paraffin sections with the antibodies except anti-GFAP and anti-MHC class II, antigen retrieval was performed by autoclaving (121 °C, 10 min, citrate buffer). The sections were incubated with each of the primary antibodies overnight at 4 °C, incubated with the appropriate secondary antibody at room temperature (RT) for 30 min, and then reacted with peroxidase-labeled streptavidin at RT for 30 min. The immunoreaction was visualized with diaminobenzidine (DAB) and briefly counterstained with hematoxylin.

For the primary ab against DAP12 of human origin, we used a rabbit polyclonal antibody against amino acid 1–113 full-length DAP12 (FL113, sc-20783, 1:500, Santa Cruz Biotechnology, CA, USA). Immunoreactivity was visualized with EnVision™ FLEX (Link) (Dako). Antigen retrieval for *DAP12* was performed by autoclaving (121 °C, 5 min, citrate buffer, pH 6.0).

For immunofluorescence, sections of a control case (a 59-year-old female without neurological disease) were double-labeled with DAP12 and CD68 abs. Briefly, the sections were incubated in 0.01 M citrate buffer, pH 6.0, at 90 °C for 15 min for antigen retrieval. Subsequently, the sections were incubated with DAP12 ab followed by incubation with biotinylated anti-rabbit IgG (Nichirei) and streptavidin-Cy3 conjugate (Sigma-Aldrich, St. Louis, MO, USA). This was followed by incubation with CD68 antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (H + L) (Thermo Fisher, Rockford, IL, USA).

Quantification of immunoreactivity

For the evaluation of microglial activation, both quantification of Iba1 immunoreactivity and semiquantitative analysis of the other activation markers were performed. Morphometric analysis of microglial cells was carried out on Iba1-immunostained sections. Images were obtained using a Nikon or Olympus microscope with a \times 20 objective connected to a color video camera (Fujix, 3CCD, HC-2500) and analyzed using the computer-based image analysis system WinROOF (version 6.4, Mitani Corporation, Fukui, Japan) or Image Scope (Aperio, Vista, CA, USA). We determined the cross-sectional tissue area taken by cell processes and somata of Iba1-labeled microglial cells across the entire thickness of the cerebral cortex and the adjacent white matter. Manual editing was used to exclude preparation artifacts. For determination of the relative tissue area occupied by Iba1-positive microglia/macrophages, all stained cellular profiles were counted and high-contrast pseudocoloring was generated (green for the measurements). Statistical analysis was performed using the Mann-Whitney *U* test. Differences demonstrating a *p* value <0.05 were considered significant.

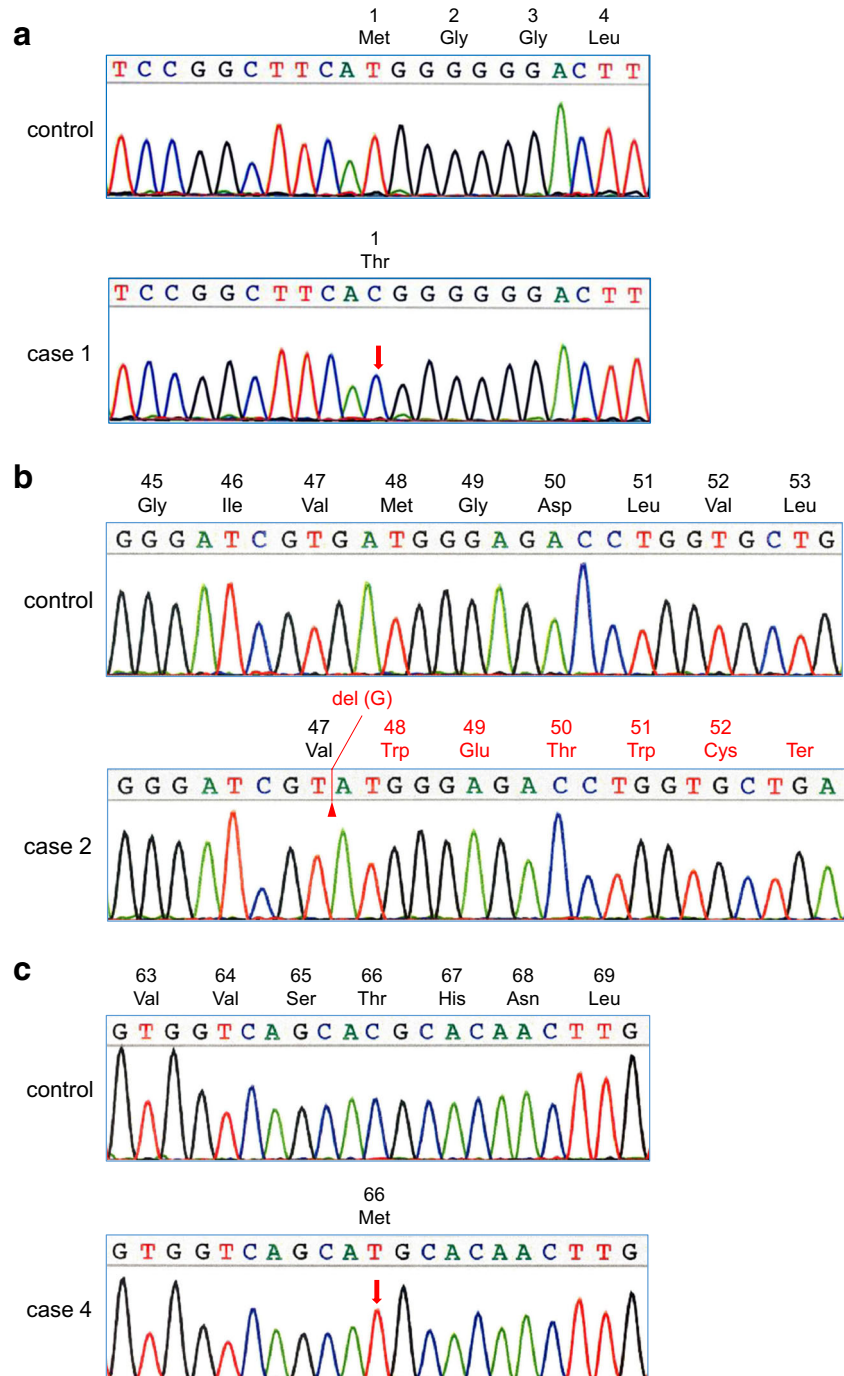
Results

Analyses of *DAP12* and *TREM2* genes

We reconfirmed that NHD case 1 had a homozygous single-base substitution (c.2T>C) in the start codon of *DAP12* exon 1 (Fig. 1a), as previously reported [16]. Amplification by RT-PCR using various primer sets specific for *DAP12* exons clearly generated products of the predicted sizes, each of which corresponded to the *DAP12* sequence (Fig. 2).

Sequencing of 21 cDNA clones, however, showed several alternatively spliced transcripts. All the transcripts had a c.2T>C substitution at the original translation initiation site. Eleven clones had this point mutation only, but eight clones lacked the last 5 nucleotides of exon 1 (AAGTG) and the first 2 nucleotides of exon 2 (GT), in addition to the c.2T>C substitution. In NHD case 2, a homozygous single-base deletion (c.141delG) was found in exon 3 of *DAP12* (Fig. 1b), presumably resulting in the appearance of the early termination codon (p.M48WfxX6). NHD case 4 did not carry a *DAP12* mutation,

Fig. 1 Sequencing electropherograms of amplified genomic DNA from NHD patients. **a** Homozygous single-base substitution (c.2T>C) of *DAP12* exon 1 in NHD case 1, **b** homozygous single-base deletion (c.141delG) of *DAP12* exon 3 in NHD case 2, and **c** homozygous mutation (c.197C>T) in *TREM2* in NHD case 4. The predicted amino acid sequences followed by a stop codon are shown in red



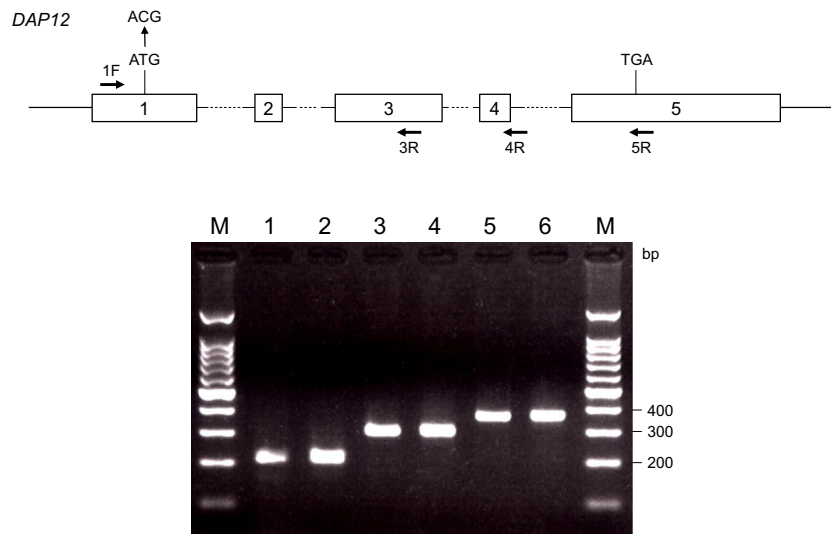
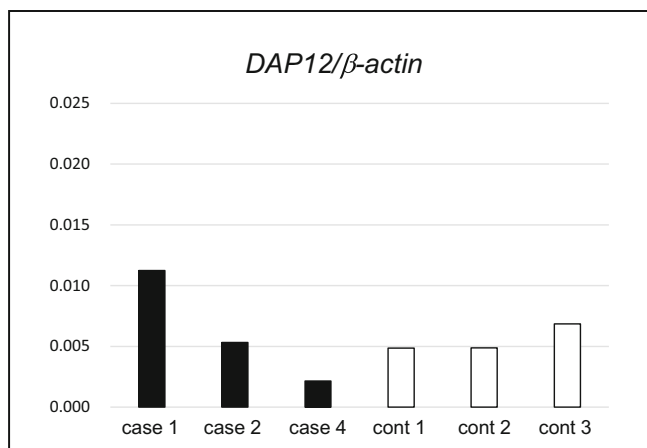


Fig. 2 Reverse transcription-PCR for *DAP12* in NHD case 1 using three different primer sets. The genomic structure of *DAP12* and locations of primers for cDNA amplification are schematically illustrated (upper panel). The products of expected sizes (primers 1F and 3R, 206 bp; 1F and 4R, 298 bp; 1F and 5R, 360 bp) were obtained in NHD case 1 (lanes

2, 4, and 6), as well as in the control (lanes 1, 3, and 5) (lower panel). Lanes 1 and 2: amplification with primers 1F and 3R; lanes 3 and 4: amplification with primers 1F and 4R; lanes 5 and 6: amplification with primers 1F and 5R; lane M: 100 bp DNA Ladder

Cerebral white matter



Cerebral cortex

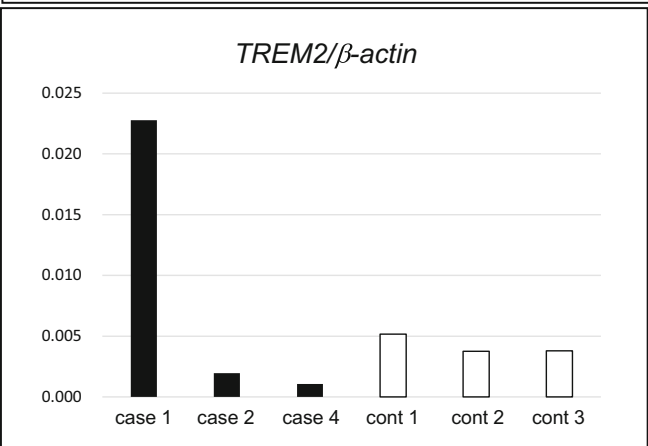
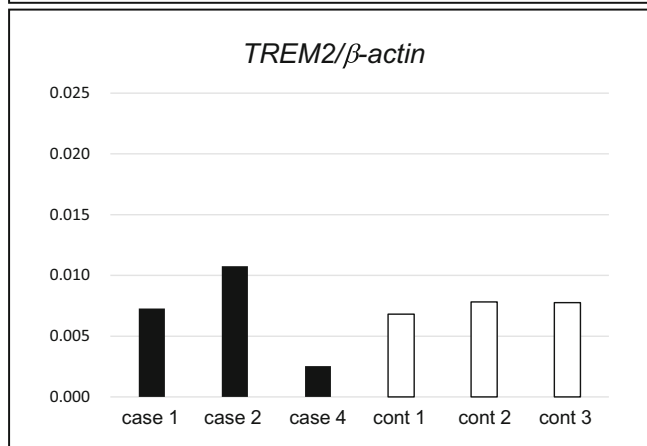
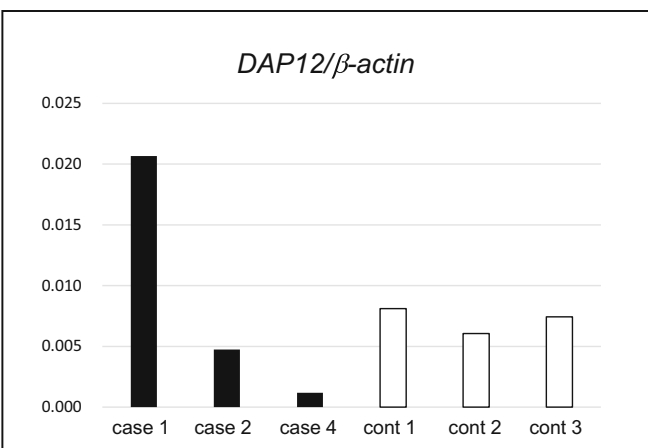


Fig. 3 Determination of *DAP12* and *TREM2* mRNA levels by real-time PCR. *DAP12* and *TREM2* mRNA levels relative to *beta-actin* mRNA are indicated. Both *DAP12* and *TREM2* mRNA levels were increased markedly in the cerebral cortex of NHD case 1. They were significantly

decreased both in the cerebral cortex and white matter in NHD case 4 compared with normal controls (cont 1–3). Cases 1, 2, and 4 correspond to NHD cases 1, 2, and 4, respectively

but showed a homozygous mutation in *TREM2*, c.197C>T (p.T66M) (Fig. 1c).

Real-time PCR and Western blot

As revealed by real-time quantitative PCR analysis, levels of both *DAP12* and *TREM2* mRNA in the cerebral cortex were considerably higher in NHD case 1 than in the controls and slightly and considerably lower in NHD cases 2 and 4, respectively, than in the controls (Fig. 3). There was no significant difference of β -actin expression between NHD and control cases (data not shown).

We examined the protein expression of DAP12 using brain homogenates from NHD cases 1, 2, and 4. Western blot analysis using the anti-DAP12 antibody (FL113) revealed a band migrating at ~12 kDa in the brains from control subjects (Fig. 4). As expected, no DAP12 expression was observed in case 2 with a frameshift mutation of *DAP12*, which is predicted to cause nonsense-mediated mRNA decay. Notably, increased expression of DAP12 was observed in case 1 with the p.M1T mutation of *DAP12* by Western blot analysis (Fig. 4), although this mutation occurring at the initiation codon would be predicted to abolish the translation of DAP12.

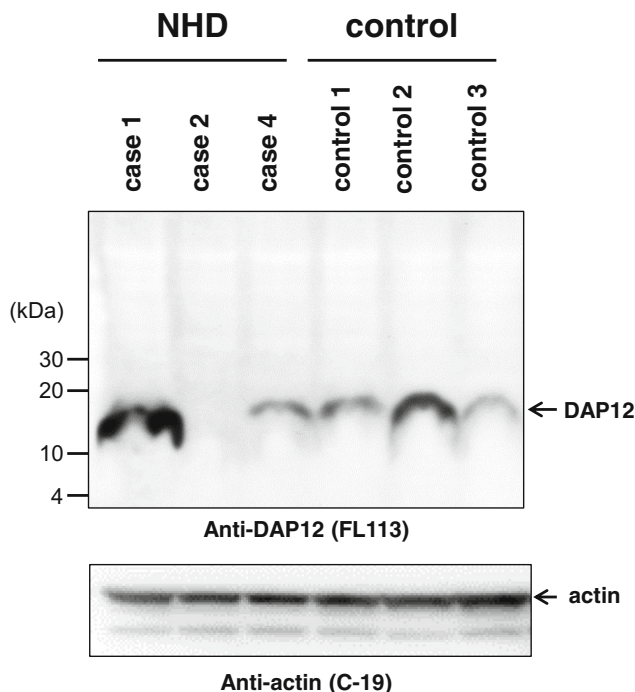


Fig. 4 Western blotting. Immunoblot analysis of DAP12 using anti-DAP12 antibody (FL113) in detergent-soluble fraction from occipital cortex of three autopsied cases and three control subjects without neurological disorders. The immunoreactive band migrating at ~12 kDa is visualized in the brains from control subjects. Note that no DAP12 expression is observed in case 2 with the frameshift mutation of *DAP12* and that increased expression of DAP12 is detected in case 1 with the p.M1T missense mutation of *DAP12*. Molecular weights (kDa) are indicated on the left. Equivalency of protein loading is shown in the actin blot (bottom)

The expression of DAP12 in case 4 with the *TREM2* mutation was comparable to that of the control subjects.

DAP12 immunohistochemistry on brain tissue

DAP12 immunolabeling demonstrated that microglial cells were positive at various intensities in the control cases (data not shown). The DAP12 antigen was diffusely distributed in the cytoplasm of resting/activated microglia and macrophages. Both astrocytes and oligodendroglia were negative, and positivity was observed rarely in occasional neurons. We confirmed the expression of DAP12 by microglia using double immunofluorescence (data not shown). In the brains of the NHD cases, one (case 1) exhibited intense positivity for DAP12 in microglial cells in both the cerebral cortex and the white matter (Fig. 5a–c). Two cases (cases 2 and 3) and one case (case 4) showed no or little immunoreactivity for DAP12, respectively (Fig. 5d–f).

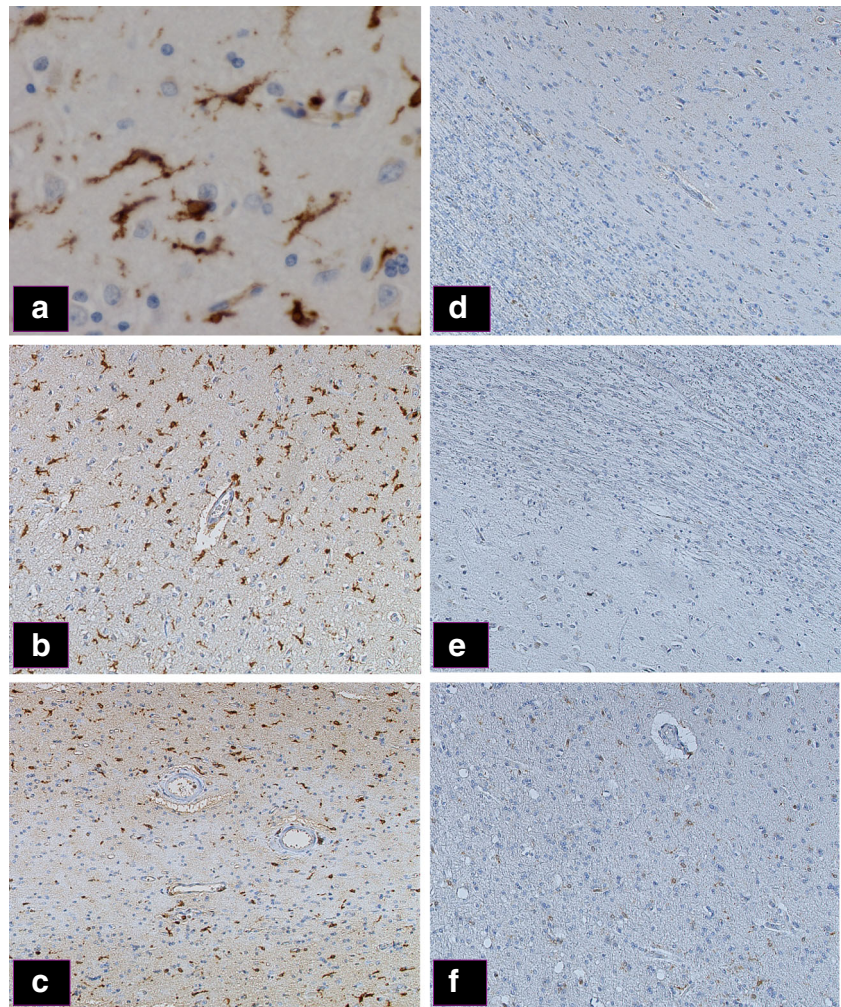
Microglial morphology and microglial marker expression in NHD cases

Microglial cells and macrophages that were labeled with antibodies against Iba1, MHC class II, CD68, CD204, and CD163 were observed in the cerebral white matter of all NHD cases (Supplementary Fig. 1). The cell density of labeled (activated) microglial cells was not high, except in the perivascular area. In case 1, a cluster of lipid-laden macrophages was seen only in the occipital white matter.

With respect to gray matter, Iba1-positive microglial cells with activated morphology were observed in the thalamus (all four cases) and basal ganglia (three of four cases). In the cortex, most of the Iba1-positive and Glut5-positive cells were morphologically compatible with resting ramified microglia and some with activated microglia with short stout processes (Fig. 6). The reactivity of activation markers (MHC class II, CD68, CD204, CD163) was variable (Supplementary Table 1). NHD case 2 demonstrated rod cells with intense reactivity to Iba1, Glut5, and MHC class II (Fig. 6). In the morphometric analysis of Iba1-immunostained sections of the frontal lobes in four NHD cases and two nonneurological control cases, the percentage tissue area occupied by microglial cell bodies and processes in the white matter was not significantly different between NHD cases and controls, and the percentages of three NHD cases (cases 1, 3, and 4) were slightly lower than those of the control cases (2.723 % in NHD cases; 3.22 % in control cases). By contrast, the percentage tissue area occupied by microglial cell bodies and processes in the frontal cortex was significantly higher ($p < 0.0001$) in two cases (cases 1 and 2) of NHD (8.42 %) than in the control cases (2.145 %) (Supplementary Fig. 2).

Concerning anatomic correlation with microglial activation within the same NHD patients (cases 1 and 2), the percentage

Fig. 5 DAP12 immunohistochemistry. Note the strong expression of DAP12 in microglial cells in the cortex (**a, b**) and white (**c**) matter of NHD case 1, but no expression in two NHD cases (cases 2 and 3) (**d, e**) and weak expression in one NHD case (case 4) (**f**). Primary magnification— $\times 40$ (**a**), $\times 10$ (**b–f**)



tissue area occupied by Iba1-positive microglia did not show any significant difference in the white matter, while the percentage cortical tissue area taken up by microglia labeled with Iba1 was highest in the frontal lobe (data not shown).

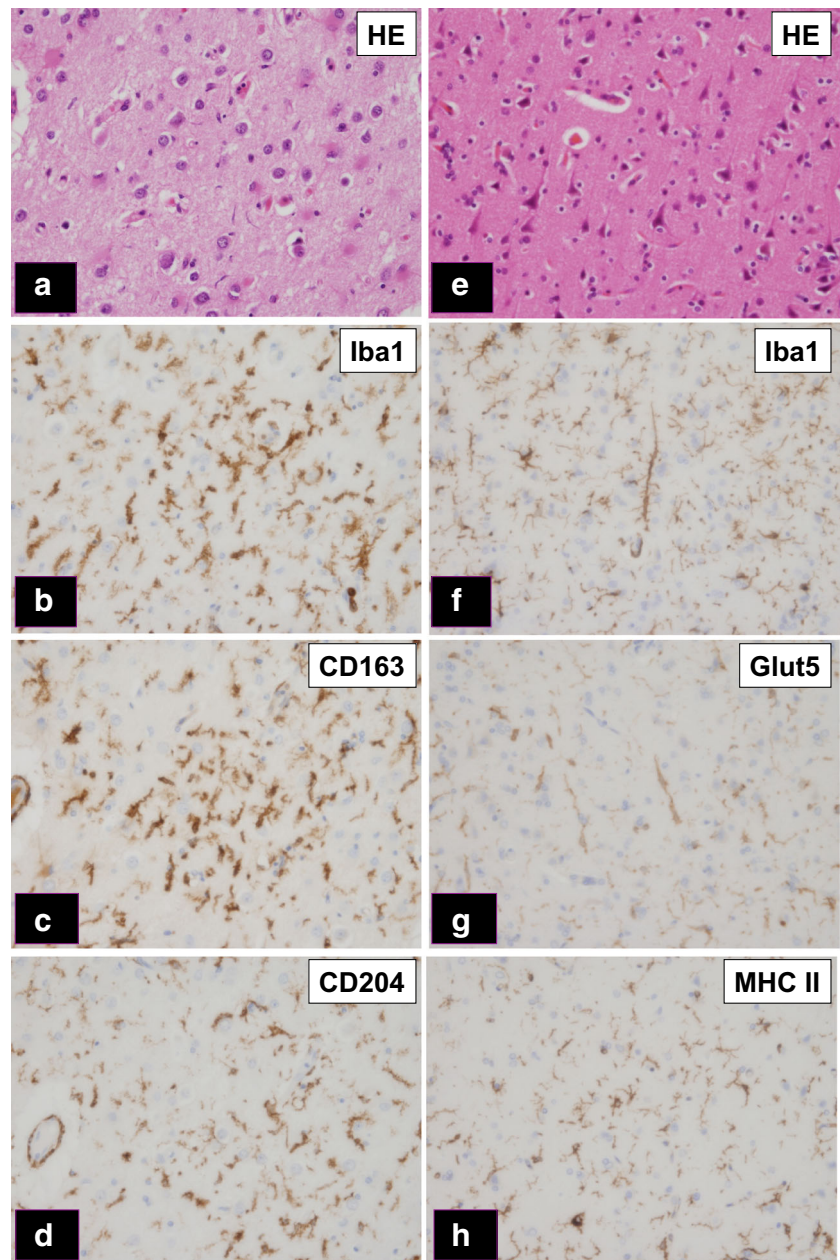
Discussion

The adaptor protein of 12 kDa known as DAP12 is a member of a transmembrane adaptor protein family containing immunoreceptor tyrosine-based activation motifs (ITAMs) as docking sites for protein kinases [20, 21]. DAP12 plays an important role in myeloid cells and natural killer (NK) cell activation and thus in triggering and amplifying inflammatory responses [22]. In the murine CNS, *DAP12* expression is localized in microglia [5, 6, 23, 24]. *DAP12* mutant mice show dysfunction of osteoclasts and microglia, as well as bone and brain abnormalities [25]. In the human CNS, *DAP12* is expressed in microglia as confirmed by this study.

Previous reports on *DAP12* gene alterations in NHD demonstrated five Japanese patients with 141delG, including a

case of the present study (NHD case 2), and two Japanese patients with a c.2T>C transition in exon 1, including our NHD case 1 [4, 16]. Surprisingly, Kondo et al. did not detect a band corresponding to DAP12 in brain tissue lysates of our NHD case 1 by Western blot analysis, suggesting that this mutation results in a lack of DAP12 protein [16]. However, in our study, this case (NHD case 1) showed numerous activated microglia labeled with the DAP12 antibody, and RT-PCR and Western blot analyses confirmed significant expression of DAP12 mRNA and protein, respectively. The reason for the negative result of Kondo et al. is unclear, but it has been shown that an ACG codon is able to initiate translation although with lower efficiency [26]. It is further noteworthy that a T-C transition polymorphism at the translation initiation codon of the human vitamin D receptor gene appears to modulate bone mineral density in Japanese women [27]. Thus, our findings illustrate that some *DAP12* mutations can lead to overproduction of *DAP12* transcript and protein in microglia. While clinically similar to the other NHD cases, NHD case 1 also showed marked microglial activation in the cortical gray matter and high expression of *TREM2* mRNA.

Fig. 6 Microglial morphology and expression of microglial markers in the frontal cortex of NHD brains. Case 1 showed mild neuronal loss histologically and an increased number of activated microglia labeled with antibodies against Iba1 (**b**), CD163 (**c**), and CD204 (**d**), immunohistochemically. NHD case 2 revealed rod cells with intense reactivity for Iba1 (**f**), Glut5 (**g**), and MHC class II (**h**). **a, f** HE stain. Primary magnification— $\times 20$



DAP12 mutations as the cause of NHD predominate in Finland and Japan, while *TREM2* mutations are more widely distributed. At the time of this study, NHD patients with 11 different *TREM2* mutations and with 7 different *DAP12* mutations had been reported worldwide [18]. NHD case 4 of the present study is the second Japanese NHD case with a *TREM2* mutation, and the mutation is novel. There have been three reported cases of *TREM2* mutations in patients who manifested symptoms of dementia without bone lesions [28, 29]. In the present study, the NHD case with the *TREM2* mutation did however show clinicopathological findings, including bone lesion, similar to the other NHD cases. In addition, in the present study, microglial activation was mild and *DAP12* expression was low in the case with the *TREM2* mutation at both

mRNA and protein levels. Further studies are needed to examine whether this pattern of microglial activation is characteristic of NHD caused by a *TREM2* mutation.

The NHD cases analyzed in this study were very similar with regard to their clinical and gross neuropathological findings, which is consistent with previous reports [1, 2, 30–32]. Neuropathological examinations of all of our cases revealed generalized cerebral atrophy, particularly in frontal areas, and advanced sclerosing leukoencephalopathy with marked gliosis, loss of myelin, and axonal spheroids in the white matter. Our histopathological findings, showing microglia/macrophages labeled with many microglial activation markers, such as Iba1, Glut5, CD68, MHC class II, CD163, and CD204, suggest that microglia/macrophages are not only

well-preserved but immunophenotypically and morphologically activated in NHD brains. This fits with previous reports [33] and the observation that macrophages from DAP12-deficient mice express wild-type levels of most common markers of differentiation [34].

We were also able to confirm the presence of activated microglia in white matter lesions of NHD brains [31, 33]. However, our morphometric study using Iba1 immunohistochemistry suggests that the number of microglial cells in white matter may decrease at late stages of the disease.

Interestingly, in the present study, two of the four NHD cases showed clear microglial activation in the frontal cortex. The overall frequency of microglial activation in NHD cortex is unclear since no systematic analysis of microglial activation in that brain region has been performed in previous studies. Axonal destruction in the white matter could be among the possible causes of cortical microglial activation in NHD, but some activated microglia also exhibited a rod-like shape. Cortical rod cells are typically found in acutely dementing processes, e.g., subacute sclerosing panencephalitis or quaternary syphilis (general paralysis of the insane). They can align with dendrites and have been shown to interfere with their normal synaptic covering [35]. Such rod cells have not been previously reported in the literature on NHD. Furthermore, Aoki et al. [36] have shown that the gray matter is commonly affected by neuronal loss and gliosis in NHD, particularly in the thalamus, striatum, and substantia nigra. In this study, neuronal loss and microglial activation were also observed in the thalamus and basal ganglia. Thus, gray matter pathology may well be responsible for some of the clinical manifestations of the disease, including dementia.

The results of the present study do not exclude the possibility that activated microglia, in spite of expressing various activation markers in NHD, could still be dysfunctional due to lack of the normal DAP12/TREM2 signaling pathway. However, at present, very little is known about the specific molecules involved in DAP12/TREM2-dependent microglial phagocytosis as the ligand of TREM2 remains elusive. It is of interest to note that TREM2 is a risk factor for the most common dementias, Alzheimer's disease (AD), and frontotemporal dementia [37]. For instance, heterozygous carriers of a *TREM2* polymorphism are known to be at increased risk for late-onset AD [38]. TREM2 and DAP12 protein levels are significantly elevated in AD [39]. Neurodegeneration in both NHD and AD could be driven by dysfunction in microglial phagocytosis [40]. Phagocytosis is not only performed by amoeboid, activated microglia, but also by ramified, resting microglia [34, 41] including synaptic material.

Transgenic mice lacking DAP12 have enhanced pairing-induced hippocampal long-term potentiation [5]. In addition, DAP12-deficient mice show a degenerated synapse and accumulated synaptic vesicles [42]. Microglia serve important physiologic functions in learning and memory by producing

brain-derived neurotrophic factor and other mediators that affect synaptic function [43]. In the two different mice models of neuronal ceroid lipofuscinosis, which is the most frequent autosomal-recessive neurodegenerative disease of childhood, synapses and axons are important early pathological targets, and modified expression levels of two distinct proteins, voltage-dependent anion-selective channel 1 (VDAC1) and Pttg1, occur during the pre-/early-symptomatic stages of the disease [44]. Interestingly, VDAC1 is one of 21 altered proteins in proteomic analysis of lymphoblastoid cells from NHD patients [45]. Thus, it is tempting to speculate that microglial dysfunction in synaptic regulation (plasticity) is a primary disease-causing mechanism in NHD. This is not too surprising as there are other known disease-causing mutations affecting microglia, which result in behavioral disturbances [46] and leukoencephalopathy [47].

In conclusion, NHD is a primary microgliopathy, the exact pathogenesis of which deserves further scrutiny. The present study highlights the need for more neuropathological examinations in NHD that include molecular analyses of *DAP12* and *TREM2* at the DNA, mRNA, and protein levels.

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Ethical approval This study was conducted with the approval of the Ethical Committees of Niigata University and Saitama Medical University.

Conflict of interest The authors declare that they have no conflicts of interest.

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