

Original Article

Optineurin immunoreactivity in neuronal and glial intranuclear inclusions in adult-onset neuronal intranuclear inclusion disease

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Received July 30, 2014; Accepted August 8, 2014; Epub September 6, 2014; Published September 15, 2014

Abstract: Optineurin (OPTN) is a multifunctional protein involved in cellular morphogenesis, vesicle trafficking, maintenance of the Golgi complex, and transcription activation through its interactions with the Rab8, myosin 6 (MYO6), huntingtin. Recently, OPTN immunoreactivity has been reported in intranuclear inclusions in patients with neuronal intranuclear inclusions disease (NIID). Other studies have shown that the RNA-binding protein, fused in sarcoma (FUS), is a component of intranuclear inclusions in NIID. We aimed to investigate the relationship between OPTN, its binding protein MYO6 and FUS in this study. In control subjects, OPTN (C-terminal) (OPTN-C) and MYO6 immunoreactivity was mainly demonstrated in the cytoplasm of neurons. In NIID patients, both neuronal intranuclear inclusions (NII) and glial intranuclear inclusions (GII) were immunopositive for MYO6 as well as OPTN-C. However, the intensity of OPTN-C immunostaining of the neuronal cytoplasm with and without NII was less than that of the control subjects. Double immunofluorescence staining for OPTN-C, ubiquitin (Ub), p62 and FUS revealed co-localization of these proteins within NII. Moreover, Ub positive inclusions were co-localized with MYO6. The percentage of co-localization of Ub with OPTN-C, FUS or MYO6 in NII was 100%, 52% and 92%, respectively. Ultrastructurally, the inclusions consisted of thin and thick filaments. Both filaments were immunopositive for Ub and OPTN-C. These findings suggest that OPTN plays a central role in the disease pathogenesis, and that OPTN may be a major component of NII.

Keywords: Optineurin, Myosin-6, neuronal intranuclear inclusion disease, FUS, intranuclear inclusion

Introduction

Neuronal intranuclear inclusion disease (NIID), also known as neuronal intranuclear hyaline inclusion disease, is a rare, slowly progressive and fatal neurodegenerative disorder characterized by the presence of eosinophilic hyaline intranuclear inclusions, together with varying degrees of neuronal loss [1]. The age at onset, clinical symptoms and pathological findings in patients are extremely varied. Sporadic and familial cases have been reported, but no causative gene mutations have been identified to date. To clarify the clinical picture, Fujigasaki has proposed classifying cases into three categories according to the age of onset: (I) an infantile form, with onset in infancy and a relatively short clinical course; (II) a juvenile form, with infantile or juvenile onset and a long clinical course of 10 years or more; and (III) an adult

form, with onset after the fifth decade [2]. The symptoms of infantile- and juvenile-onset cases appear to be related to distribution of multisystem degeneration. The principle clinical feature of adult onset cases is predominantly dementia with or without parkinsonism, cerebellar signs or autonomic dysfunction [3, 4].

Pathologically, neuronal intranuclear inclusions (NII) are usually observed in the neurons of the central and peripheral nervous systems. Glial intranuclear inclusions (GII) are also present, and these appear to be more common in adult cases. Immunohistochemically, the inclusions are immunopositive for ubiquitin (Ub) and ubiquitin-related proteins (SUMO-1 and p62), while inclusions are less often immunolabeled with anti-polyglutamine antibody 1C2 [5-10]. Recently, it has been reported that NII and GII are immunoreactive for fused in sarcoma (FUS) [11, 12].

Table 1. Clinical features of NIID cases

Case	Age at onset	Age at death	Sex	Family history	Dementia	Parkinsonism	Cerebellar signs	Autonomic dysfunction	Other
1	NA	NA	M	No	NA	NA	NA	NA	
2	78	84	M	No	+	-	+	-	Essential tremor
3	75	79	F	No	+	-	-	-	Polyradiculoneuropathy
4	46	56	M	Yes	+	-	+	-	

Abbreviations: NIID = neuronal intranuclear inclusion disease; M = male; F = female; NA = not available.

Optineurin (OPTN) is a cytoplasmic protein ubiquitously expressed in brain and other tissues. The OPTN gene codes for a 577 amino acid protein composed of multiple coiled-coil domains, a ubiquitin-binding domain, and a C-terminal zinc finger. OPTN is involved in cellular morphogenesis, vesicle trafficking, maintenance of the Golgi complex, and transcription activation through its interactions with the Rab8, myosin 6 (MYO6), huntingtin, and transcription factor IIIA proteins [13-15]. OPTN was recently identified as a negative regulator of NF- κ B signaling [16].

Recently, Maruyama et al. showed that mutations in the gene encoding for OPTN cause amyotrophic lateral sclerosis (ALS), and that TDP-43 positive cytoplasmic inclusions or SOD1-positive inclusions in sporadic ALS or familial ALS were immunostained for OPTN [17]. In addition, some groups reported that OPTN was present in cytoplasmic inclusions or intranuclear inclusions of various neurodegenerative diseases [18-22]. In the present study we investigated the relationship between OPTN, MYO6 and FUS with immunohistochemistry of hippocampus in NIID. We found that the number of intranuclear inclusions positive for OPTN was more frequent than those positive for MYO6 or FUS, suggesting that OPTN might play a significant role in pathomechanisms of NIID.

Materials and methods

4 NIID patients and 4 control patients without neurological disorder were investigated (**Table 1**). The age at death of NIID cases ranged from 56 to 84 years (average 73.0 years). The age at death of control cases ranged from 63 to 74 years (average 70.3 years). The clinical features of NIID cases are described below.

Case 2

At the age of 78, a male patient showed progressive gait disturbance and dementia. Neurological examination showed truncal ataxia and neuropsychological examinations show-

ed impaired cognitive function. He was diagnosed as having cerebellar degeneration and dementia. He died at the age of 84.

Case 3

At the age of 75, a female patient presented cognitive dysfunction. She also showed agitated behavior and visual hallucinations starting at the age of 78. Neurological examinations revealed confusion and disorientation, but without aphasia, as well as lower extremity weakness that was consistent with lumbar radiculoneuropathy. She died at the age of 79.

Case 4

At the age of 45, a male patient had gait disturbance and progressive dysarthria and dementia. One year later, he was diagnosed as having spinocerebellar degeneration. Neurological examination revealed limb and truncal ataxia as well as ataxic dysarthria, and neuropsychological examination showed impaired cognitive function. The brain MRI showed atrophy of frontal lobe, temporal lobe, cerebellum, and brainstem. The 99mTc-ethyl-cysteinate dimer (99mTc-ECD) SPECT study showed a decrease in cerebral blood flow in frontal and temporal lobes. CAG repeat lengths were determined for genes associated with familial spinocerebellar ataxias, spinocerebellar ataxia (SCA) 1, SCA2, SCA3, SCA6, and dentatorubropallidoluyisan atrophy (DRPLA), all within the normal ranges. He died of respiratory failure at the age of 56. After he died, his younger sister was diagnosed with dysarthria, ataxic gait and cognitive dysfunction in another hospital.

Neuropathology

The autopsied brain and spinal cord were fixed in 10% formalin. Samples of the main representative regions of the brain and spinal cord were embedded in paraffin, and then sliced into 5- μ m thick sections. The sections were stained with hematoxylin and eosin (H&E). Neuronal

loss and the frequency of the inclusions were assessed with a semiquantitative method. The degree of neuronal loss were scored as none (-), mild (+), moderate (++) and severe (+++). The presence of NII or GII were also scored as absent (-), low (+), moderate (++) and high (+++).

Immunohistochemistry

Paraffin-embedded blocks of hippocampus, where numerous NII are observed in all NIID patients, were cut at a thickness of 5- μ m. The sections were deparaffinized and then immunostained with rabbit polyclonal antibody against OPTN (C-terminal) (OPTN-C) antibody (#100000, 1:200, Cayman Chemical, Ann Arbor, MI, USA), rabbit polyclonal antibody against OPTN (Internal) (OPTN-INT) (#100002, 1:50, Cayman Chemical), rabbit polyclonal antibody against Myosin 6 (MYO6) (ab11096, 1:100, Abcam plc., Cambridge, UK), rabbit polyclonal antibody against FUS (HPA008784, 1:500, SIGMA, St. Louis, MO, USA), rabbit polyclonal antibody against Ub (U5379, 1:500, SIGMA), mouse monoclonal antibody against p62 (610832, 1:500, BD Transduction Laboratories, San Diego, CA, USA), mouse monoclonal antibody against SUMO-1 (18-2306, 1:200, Zymed Laboratories, South San Francisco, CA, USA), mouse monoclonal antibody against phospho-TDP-43 (pS409/410, 1:5000, Cosmo Bio Co., LTD. Tokyo, Japan), mouse monoclonal antibody against phospho-tau (AT8, 1:1000, Innogenetics, Gent, Belgium), rabbit polyclonal antibody against alpha-synuclein (AB5038, 1:1000, Chemicon International, Inc. Temecula, CA, USA), rabbit polyclonal antibody against amyloid-beta (A β) (AB1510, 1:1000, Chemicon International), mouse monoclonal antibody against phosphorylated neurofilament (pNF) (SMI31, 1:10 000, Sternberger-Meyer, Inc., Lutherville, MD, USA) and mouse monoclonal antibody against α -internexin (INA) (ab22039, 1:2000, Abcam plc.). Incubation was carried out overnight at 4°C. The bound primary antibody was visualized with the appropriate Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA), and 3, 3'-diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen.

The density of the OPTN-C immunoreactivity in the cytoplasm of pyramidal cells was assessed on a four point scale (3 = intense; 2 = moderate; 1 = mild; 0 = none). 5 random fields were chosen under 400 \times magnification. The mean cytoplasm OPTN-C score of each case was cal-

culated, and the score of both NIID and control groups was expressed as mean \pm standard deviation (SD).

In addition, we performed double immunofluorescence staining on hippocampal sections from NIID cases. We applied the following primary antibodies: rabbit polyclonal antibody against OPTN-C (#100000, 1:100, Cayman), rabbit polyclonal antibody against FUS (HPA008784, 1:200, SIGMA), rabbit polyclonal antibody against MYO6 (ab11096, 1:50, Abcam plc.), mouse monoclonal against Ub (MAB1510, 1:1000, Chemicon), mouse monoclonal antibody against p62 (610832, 1:100, BD Transduction Laboratories) and mouse monoclonal antibody against FUS (sc-47711, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). These primary antibodies were detected with Alexa Flour 488 goat anti-rabbit IgG (1:200, Molecular Probes, Eugene, OR, USA), Alexa Flour 546 goat anti-mouse IgG (1:200, Molecular Probes). The slides were observed on a LSM 510 META confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany).

The frequency of co-localization of the Ub with either OPTN-C, FUS or MYO6 was determined by analyzing 100 inclusions for quantitative analysis.

Electron microscopy

Small pieces of tissues from the hippocampus of formalin-fixed brain were processed for transmission electron microscopy (EM), and post-embedding immunogold EM (IEM) as previously described [23]. Antibodies for IEM were rabbit polyclonal antibody against Ub (1:20) [24] and rabbit polyclonal antibody against OPTN-C (#100000, 1:40, Cayman). Goat anti-rabbit IgG conjugated to 18 nm gold particles were from Jackson ImmunoResearch Laboratories (West Grove, PA). Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 208S electron microscope (FEI, Hillsbora, OR) fitted with a Gatan 831 Orius CCD digital camera (Gatan, Pleasanton, CA). The Digital images were processed using Photoshop software.

Results

Clinical feature

We had no detailed clinical feature of case 1. The main complaint of the other 3 cases used

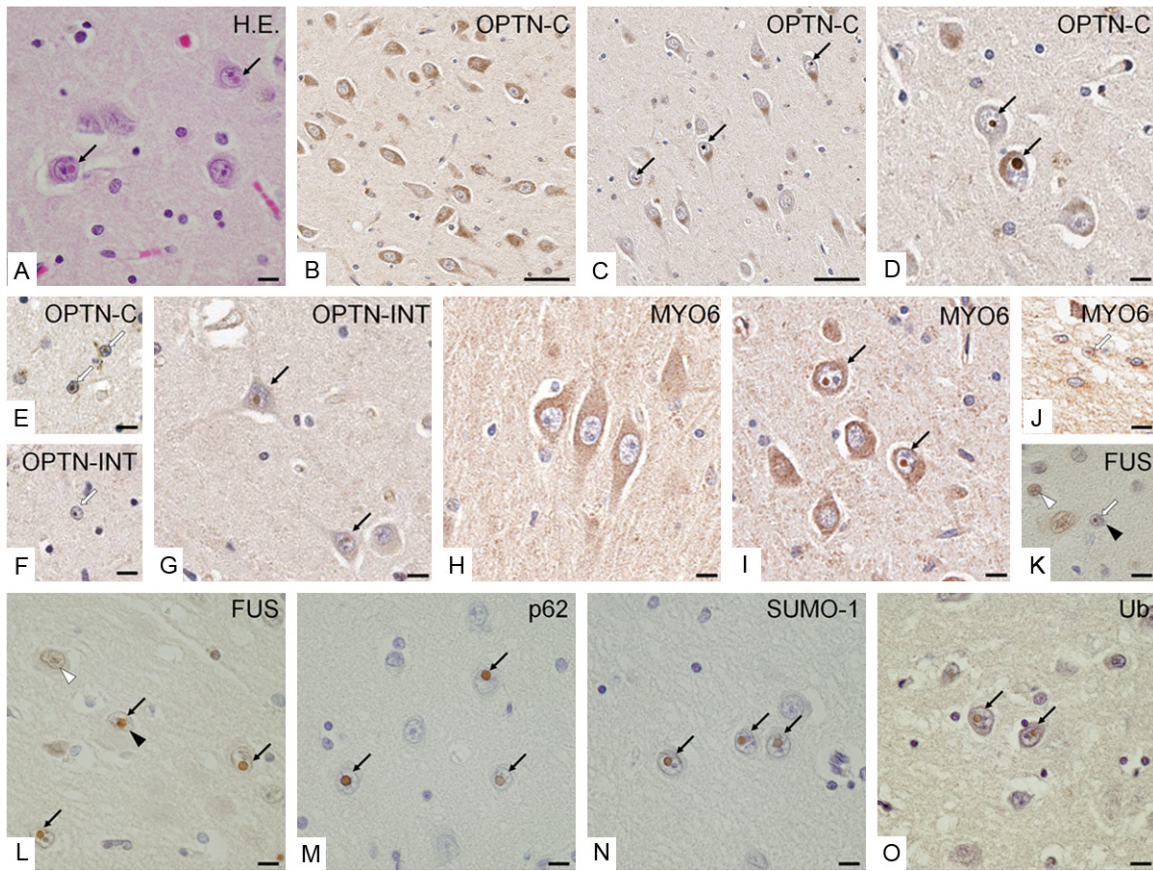


Figure 1. Hippocampal sections from control subjects (B, H) and from NIID (A, C-G, I-O). In NIID, H&E staining shows neuronal intranuclear inclusions (NII) (arrows) in the pyramidal cell layer of the hippocampus (A). In control cases, the cytoplasm of the neurons is immunopositive for OPTN-C, with the stain showing a granular appearance (B). MYO6 immunoreactivity is present in the cytoplasm of the neurons. In NIID, NII (arrows) and GII (white arrows) are immunopositive for OPTN-C (C-E). However, the OPTN-C staining density of the neuronal cytoplasm with and without NII appears to be less than that of controls (C). NII (arrows) and GII (white arrows) are also immunopositive for OPTN-C, MYO6, FUS, p62, SUMO-1 and Ub (C-E). Note that the intensity of FUS immunostaining of neuronal or glial nuclei containing intranuclear inclusions (arrowheads) is reduced when compared with those without inclusions (white arrowheads) (K, L). Scale bars = 50 µm (B, C) and 10 µm (A, D-O).

in this study was dementia, and was in accordance with the reports of previously reported adult onset cases. Two cases (Cases 2 and 4) showed cerebellar signs, including ataxic dysarthria and truncal ataxia.

Neuropathology

The calculated whole brain weights ranged from 850 g to 1100 g (average 1017 g). There was moderate atrophy of the cerebral hemisphere in all cases. The pons, the cerebellum and the spinal cord were also atrophic in Case 4. Microscopically, the features of all NIID cases were regional neuronal loss and intranuclear inclusions. Although intranuclear inclusions were widely distributed throughout the brain in Cases 1, 2 and 4, the inclusions were limited to the hippocampus in Case 3. The inclusions

were spherical, eosinophilic and 1-6 micrometers in diameter (**Figure 1A**). They usually were single, but occasionally two inclusions were present in a neuron. The frequency of NII, GII and the degree of neuronal loss in different regions of the brain are summarized in **Table 2**. Most NII and GII tended to be observed in brain regions with little or no neuronal loss.

Immunohistochemistry

In control cases, OPTN-C immunoreactivity was mainly identified within the cytoplasm of the neurons (**Figure 1B**). The cytoplasmic labeling by OPTN-C appeared to be granular. The nuclei of a small number of neurons were also immunolabeled by OPTN-C. OPTN-INT immunoreactivity was not observed in the nucleus or the cytoplasm of the neurons and glial cells. MYO6

Table 2. Pathological findings in NIID cases

Brain weight (grams)	Case 1			Case 2			Case 3			Case 4		
	NA			1100			1100			850		
	NL	NII	GII	NL	NII	GII	NL	NII	GII	NL	NII	GII
Neocortex	-	+	++	-	+	+	-	-	-	-	+	-
Hippocampus	-	+++	+	+++	+++	+++	-	++	-	-	+++	+
Striatum	-	+	+	-	++	+	-	-	-	-	+	-
Globus pallidus	-	-	+	-	+	++	-	-	-	-	+	-
Basal nucleus of Meynert				-	-	++	+++	-	-			
Thalamus				-	-	+				-	+	-
Subthalamic nucleus				-	-	-	-	-	-	-	+	-
Substantia nigra	-	+	+	+	-	+	+			+++	+	-
Locus ceruleus	+	++	++	-	-	-	+	-	-			
Inferior olivary nucleus	++	-	+							+	+	-
Purkinje neurons	-	-	+	+	-	+	-	-	-	+	+	-
Cerebellar granular neurons	-	-	+	-	-	-	-	-	-	+	-	-
Dentate nucleus	-	-	+	-	-	++	-	-	-	++	++	-
Anterior horn cell	-	-	++							-	+	-

Abbreviations: NIID = neuronal intranuclear inclusion disease; NL = neuronal loss; NII = neuronal intranuclear inclusion; GII = glial intranuclear inclusion; NA = not available. The degree of neuronal loss: - = none; + = mild; ++ = moderate; +++ = severe. The frequency of NII or GII: - = none; + = sparse; ++ = moderate; +++ = frequent.

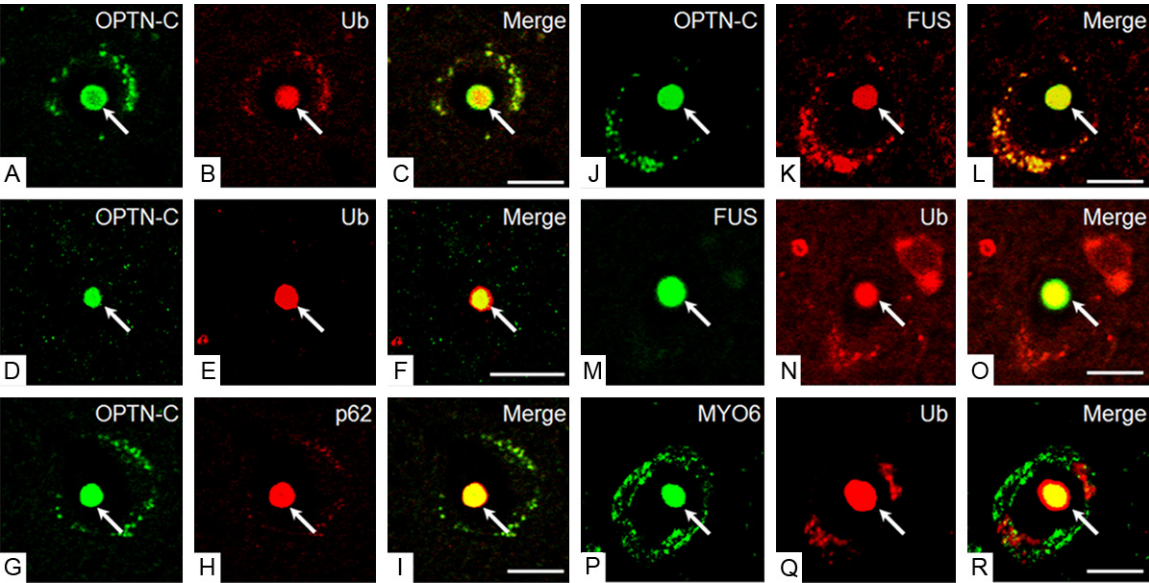


Figure 2. Double immunofluorescence labeling for OPTN-C (A, D; green), either Ub (B, E; red), p62 (H; red) or FUS (K; red) and merged image (C, L), and that for either FUS (M; green) or MYO6 (P; green), Ub (N, Q; red), and merged images (O, R) in sections of hippocampus from NIID. OPTN-C is co-localized with Ub in a neuronal intranuclear inclusion (NII) and in a glial intranuclear inclusion (cf., arrows in A-C, D-F). OPTN-C is co-localized with p62 and FUS in a NII. (cf., arrows in G-L). FUS and MYO6 are also co-localized with Ub in a NII (cf., arrows in M-R). Scale bars = 10 µm.

immunoreactivity was recognizable in the cytoplasm of neurons (**Figure 1H**).

In all NIID cases, not only neuronal cytoplasm was immunopositive, but also NII and GII were immunopositive for OPTN-C (**Figure 1C-E**).

However, the OPTN-C staining density of the neuronal cytoplasm with and without NII appeared to be less than that of the control subjects. Using a semi-quantitative scale, NIID cases had an average intensity of 1.01 ± 0.06 compared to 2.38 ± 0.59 in the control cases.

NII demonstrated an intense immunostaining pattern for OPTN-C throughout the inclusion. Although both antibodies against OPTN-C and OPTN-INT immunolabeled intranuclear inclusions, the former was more sensitive than the latter (**Figure 1C-G**). Furthermore, these intranuclear inclusions were also immunopositive for MYO6 (**Figure 1I, 1J**).

As previously reported, NII and GII in NIID cases were immunolabeled with FUS, p62, SUMO-1 and Ub (**Figure 1K-O**). The number of inclusions positive for FUS was fewer than those positive for p62 or Ub. Neither neuronal nor glial nuclei with intranuclear inclusions showed FUS immunostaining, and that staining was confined to the inclusions. However, nuclear FUS immunoreactivity was preserved in neurons and glial cells without inclusions (**Figure 1K, 1L**). The intranuclear inclusions were immunonegative for phospho-TDP-43, phospho-tau, alpha-synuclein, A β , pNF, and INA.

Double immunofluorescence for OPTN-C and for either Ub, p62, or FUS revealed co-localization of OPTN-C immunoreactivity with Ub, p62, or FUS within intranuclear inclusions (**Figure 2A-L**). FUS and MYO6 were also co-localized with Ub in NII (**Figure 2M-R**). The percentage of co-localization of Ub with either OPTN-C, FUS or MYO6 in NII was 100%, 52% and 92%, respectively.

Electron microscopy

Neurons in the CA 4 region of hippocampus contained NII of various sizes and shapes. **Figures 3** and **4** showed prominent NII containing two separate but adjacent aggregates of filaments. One type of filaments was thicker and denser than the other type. Both were labeled with Ub and OPTN-C antibodies. Other NII contained either thin or thick filaments only. Sometimes, dense material was found inside the NII composed of thin filaments.

Discussion

In this study, we examined 3 sporadic and 1 familial adult-onset NIID. Dementia was the principle clinical feature of our cases. A sporadic case (Case 2) and one familial case (Case 4) had similar clinical course and both were diagnosed as having cerebellar ataxia. These clinical features are in accordance with previously reported adult onset cases of NIID.

Neuropathological findings revealed regional neuronal loss and intranuclear inclusions. In childhood onset NIID cases, intranuclear inclusions are usually observed in neurons, while in the adult onset cases, they are more frequently found in glial cells than in neurons [3, 4, 6, 9]. In Cases 3 and 4, intranuclear inclusions were mainly observed in neurons, rather than glial cells, in contrast to previously reported adult onset cases.

In the present study, we demonstrated that OPTN-C was co-localized with Ub and p62 within NII and GII in the hippocampus of NIID. Furthermore, we found for the first time that NII and GII were immunopositive for MYO6. Very recently, Mori et al. reported that intranuclear inclusion in neocortical neurons, but not in glial cells, in frontal lobe of NIID cases were immunopositive for OPTN and that OPTN immunoreactivity was found in 50% of NII [20]. In our study, we demonstrated that OPTN immunoreactivity was present in all NII as well as a large proportion of GII. Our findings were different from the previous investigation, possibly because of the regional differences (frontal cortex versus hippocampus). These findings suggest that the characteristics of inclusions in NIID may be regionally different.

The presence of OPTN and MYO6 immunoreactivity in intranuclear inclusions of NIID is the most conspicuous finding of the present study; however, the underlying pathomechanisms are currently uncertain. MYO6 is a multifunctional motor protein associated with different intracellular compartments. It is the only myosin that moves toward the minus end of actin filaments. MYO6 plays an important role in both endocytic and secretory membrane trafficking pathways [25]. Furthermore, MYO6 is recruited to the Golgi apparatus by OPTN and involved in the maintenance of Golgi morphology [15]. The loss of OPTN leads to not only Golgi fragmentation, but also MYO6 mislocalization [15]. In our study, we observed that the cytoplasm staining intensity for OPTN-C of affected neurons was reduced, when compared with neurons of controls. This finding suggests the possibility that the function of Golgi could be impaired by the loss of OPTN within cytoplasm in NIID cases.

NII and GII were immunopositive for Ub and SUMO-1. Ultrastructurally, the inclusions were composed two types of filaments. The filaments

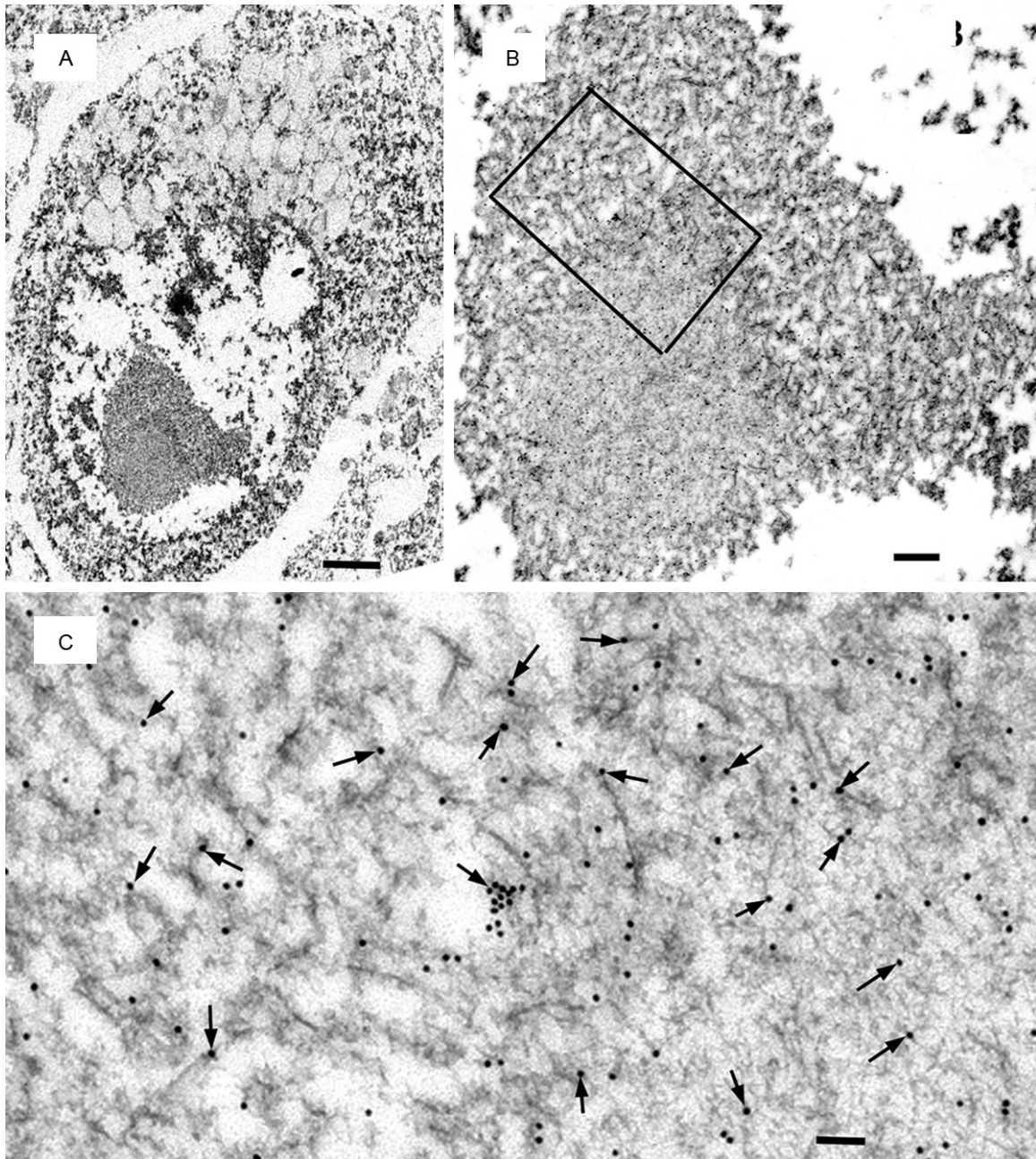


Figure 3. Immunogold labeling of Ub. (A) A prominent neuronal intranuclear inclusion (NII) in a CA 4 neuron. Note the presence of lipofuscin. (B) Enlargement of NII shows two separate but adjacent aggregates of filaments. (C) Further enlargement of boxed area in (B) shows one aggregate containing thick dense filaments (left) while the other contains thin pale filaments (right). Both types of filaments are heavily labeled (arrows point to some of the gold particles). Scale Bars = 2 μ m (A), 0.4 μ m (B), 0.1 μ m (C).

were immunopositive for Ub and OPTN-C. These findings indicate intranuclear inclusions of NIID might be formed by the dysfunction of the intranuclear protein degradation system or the accumulation of abnormal proteins in the nuclei of cells. The turnover of endogenous OPTN involves mainly the ubiquitin-proteasome sys-

tem, but when up-regulated or mutant, autophagy comes in to play [26]. Although OPTN is localized to the Golgi complex, OPTN translocates from the Golgi to the nucleus in a Rab8 dependent manner on apoptotic stimulation, resulting in increases in its own transcription and thereby preventing cell death [27]. In addi-

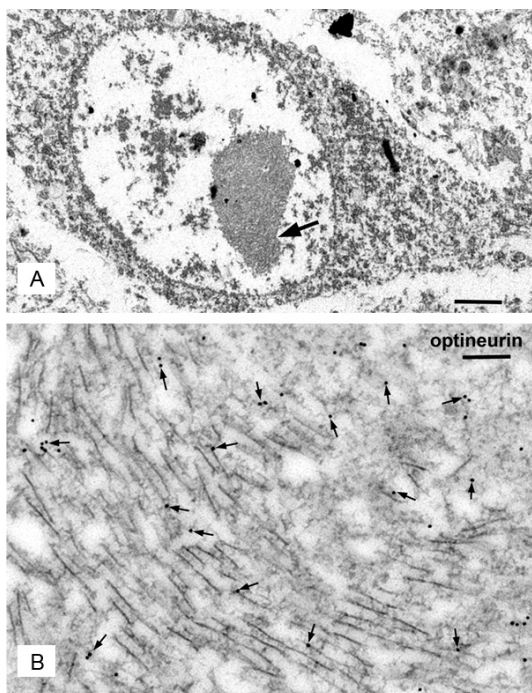


Figure 4. Immunogold labeling of OPTN-C. (A) NII in a CA 4 neuron also contains two adjacent aggregates whose border is indicated by arrow and enlarged in (B). The two types of filaments, similar to those in **Figure 3** are labeled (gold particles indicated by arrows). Scale Bars = 2 μ m (A), 0.2 μ m (B).

tion, OPTN is prone to oligomerization and aggregation when overexpressed or mutant [28]. As a response to aging stress, OPTN concentration may be increased to promote cell survival signals in the nucleus. Unlike the cytoplasm where two major protein degradation systems (ubiquitin-proteasome system and autophagy) work in parallel, only the ubiquitin-proteasome system seems to operate in the nucleus. The excessive mislocalization of OPTN might overload the capacity of the ubiquitin proteasome degradation system of nucleus. As a result, OPTN might form internuclear aggregates.

Since not all NII were immunopositive for FUS, FUS might be sequestered as secondary factor and not be a major component of the inclusions. However, we identified the loss of FUS immunoreactivity in neuronal and glial nuclei of inclusion-bearing cells. FUS is a multifunctional protein involved in transcription, RNA processing, and RNA transport [29, 30]. Nuclear MYO6 enhances RNA polymerase II-dependent transcription [31]. Therefore, it is plausible that the sequestration of FUS and MYO6 within NII may

interfere with the RNA metabolism, causing cell dysfunction and neurodegeneration.

NF- κ B regulates genes that function in diverse cellular processes like inflammation, immunity and cell survival and is involved in the pathogenesis of various diseases [32]. It was reported that activation of NF- κ B in neurons promotes their survival by inducing the expression of anti-apoptotic genes, whereas activation of NF- κ B in glial cells mediates the production of proinflammatory cytokines [33]. OPTN negatively regulates NF- κ B activation [34], but FUS acts as a co-activator of NF- κ B [35]. Aberrant regulation of NF- κ B by sequestration of both OPTN and FUS could be involved in the pathomechanisms of NIID.

In summary, we have demonstrated abnormal accumulation of OPTN and its binding partner MYO6 in intranuclear inclusions of NIID. Quantitative analysis by double immunofluorescence and electron microscopy suggest that OPTN accumulation is more likely to play a central role in the disease pathogenesis than FUS. Our findings implicate an important step in the pathomechanisms of NIID.

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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