Materials and methods

Thirty-five FALS pedigrees, 218 SALS patients, and 271 controls, all of whom were from the Japanese population, were enrolled in this study. Of the 35 FALS pedigrees, 17 harbored causative mutations in other causative genes for FALS with the autosomal dominant mode of inheritance. The remaining 18 pedigrees consisted of 13 with the autosomal dominant mode of inheritance, two pedigrees with affected sibs with consanguinity, and three pedigrees with affected sibs without consanguinity. The 218 SALS patients, most of whom visited the University of Tokyo Hospital, included 33 from Yamagata Prefecture, on the northern part of Honshu island, and 15 from the Kii Peninsula, on the southern part of Honshu island. The mean age at onset of the SALS cohort was 58.9 years, and the male: female ratio was 3: 2. All of the genomic DNA samples were obtained from the participants of this study with their written informed consent, and this research was approved by the Institutional Review Board of the University of Tokyo.

Mutational analysis

Mutations in causative genes for FALS were analyzed employing a DNA microarray-based resequencing system as described elsewhere (24) or a direct nucleotide sequencing method conducted using a BigDye Terminator ver. 3.1 cycle sequencing kit on a 3100 ABI Prism Genetic Analyzer (Applied Biosystems). All the coding exons of *OPTN* (exons 4–16) were amplified by genomic PCR using specific primers for each exon recently reported (18) and further subjected to direct nucleotide sequence analysis.

Mutations in other causative genes for FALS, including *SOD1, ALS2, DCTN1, VAPB, CHMP2B, ANG*, and *TARDBP*, were firstly excluded employing a DNA microarray-based resequencing system. Secondary, mutational analysis of *FUS* employing a direct nucleotide sequencing method was performed. The remaining samples were subjected to mutational analysis of *OPTN* by direct nucleotide sequence analysis.

The variants identified by the mutational analysis were evaluated using databases of dbSNP (http://www.ncbi.nlm.nih.gov/SNP/index.html), 1000 Genomes Project (http://www.1000genomes.org/), and Exome Sequencing Project (https://esp.gs.washington.edu/). When novel non-synonymous variants not registered in these databases were identified, they were further screened in 271 controls by direct nucleotide sequence analysis. The effect of amino acid changes caused by identified novel variants was predicted using the PolyPhen-2 website (http://genetics.bwh.harvard.edu/pph2/).

Results

Of the 35 FALS pedigrees enrolled in this study, 17 harbored causative mutations in other causative genes for FALS including 14 *SOD1*, two *FUS*, and one *TARDBP*. The remaining 18 pedigrees were subjected to mutational analysis of *OPTN*. Five variants including four known SNPs and a novel synonymous variant in exon 16 were identified (Table I). We did not observe any causative mutations in *OPTN* in the FALS pedigrees in our cohort.

In the 218 SALS patients, seven variants including four known SNPs, two novel synonymous variants in exons 4 and 7, and one novel non-synonymous variant in exon 6 not registered in dbSNPs, 1000 Genomes Project, or Exome Sequencing Project were identified (Table II). Known causative mutations for ALS were not identified in the SALS patients. The novel heterozygous non-synonymous variant of c.481G >A in exon 6 substituting methionine for valine at amino acid position 161 (p.V161M) was identified in a SALS patient (Figure 1A, B). This novel variant of V161M was not present in 271 controls (542 chromosomes). Although the amino acid valine at position 161 was not necessarily highly conserved among species (Figure 1C), the PolyPhen-2 prediction was possibly damaging with a score of 0.913.

Interestingly, the patient with V161M mutation originated from the southernmost part of the Kii Peninsula, where the prevalence of ALS is high and patients with the ALS-parkinsonism-dementia

Table I. Summary of *OPTN* variants identified in 18 FALS patients.

Exon	SNP ID*	Base changes	Annotation	Amino acid changes	Number of pedigrees (Allele frequency)		Allele frequency (1000 Genomes)**
4	rs2234968	c.102G>A	Synonymous		3 homozygotes, 1 heterozygote#	(0.389)	0.182
5	rs11258194	c.293T>A	Non-synonymous	p.Met98Lys	1 heterozygote	(0.028)	0.110
10	rs523747	c.964A>G	Non-synonymous	p.Lys322Glu	18 homozygotes	(1.000)	1.000
16	rs75654767	c.1634G>A	Non-synonymous	p.Arg545Gln	2 heterozygotes#	(0.056)	0.028
16	Novel	c.1713C>T	Synonymous		1 heterozygote	(0.028)	0.000

*SNP ID is the single-nucleotide polymorphism identification obtained from dbSNP database.
**The allele frequencies in East Asian populations were obtained from 1000 Genomes Project (http://www.1000genomes.org/).
#One patient carried both the heterozygous c.102G>A variant and the heterozygous c.1634G>A variant.

Table II. Summary of *OPTN* variants identified in 218 SALS patients.

Exon	SNP ID*	Base changes	Annotation	Amino acid changes	Number of cases (Allele frequency)		Allele frequency (1000 Genomes)**
4	rs2234968	c.102G>A	Synonymous		3 homozygotes, 59 heterozygotes	(0.149)	0.182
4	Novel	c.147C>T	Synonymous		1 homozygote	(0.004)	0.000
5	rs11258194	c.293T>A	Non-synonymous	p.Met98Lys	17 heterozygotes	(0.039)	0.110
6	Novel	c.481G>A	Non-synonymous	p.Val161Met	1 heterozygote	(0.002)	0.000
7	Novel	c.630A>T	Synonymous		1 heterozygote	(0.002)	0.000
10	rs523747	c.964A>G	Non-synonymous	p.Lys322Glu	218 homozygotes	(1.000)	1.000
16	rs75654767	c.1634G>A	Non-synonymous	p.Arg545Gln	13 heterozygotes	(0.030)	0.028

*SNP ID is the single-nucleotide polymorphism identification obtained from dbSNP database.
**The allele frequencies in East Asian populations were obtained from 1000 Genomes Project (http://www.1000genomes.org/).

complex are clustered. We further conducted the mutational analysis of *OPTN* recruiting four additional patients with SALS in the same district. These patients, however, harbored neither the V161M mutation nor any other mutations in *OPTN*.

The clinical features of the patient with the V161M mutation are briefly presented as follows. The patient was a 35-year-old male at the time of diagnosis of ALS, who developed upper extremity weakness for one year. Weakness and atrophy predominantly in upper extremities gradually worsened. Neurological examination at the age of 39 years revealed tongue atrophy and fasciculation, attenuated tendon reflexes and muscle wasting in the upper extremities, and enhanced tendon reflexes in the lower extremities with bilateral extensor plantar reflexes. He became mechanical-ventilator-dependent at the age of 50 years. There was no evidence of parkinsonism or cognitive impairment at the age of 50 years. His medical history included unexplained vision loss of his right eye in his childhood. His father, who also originated from the southernmost part of the Kii Peninsula, was alive and did not show any symptoms indicative of motor neuron disease when the index patient was 35 years old. His mother, who originated from southeastern part of the Kii Peninsula, died of liver cirrhosis, but her age at death was not indicated.

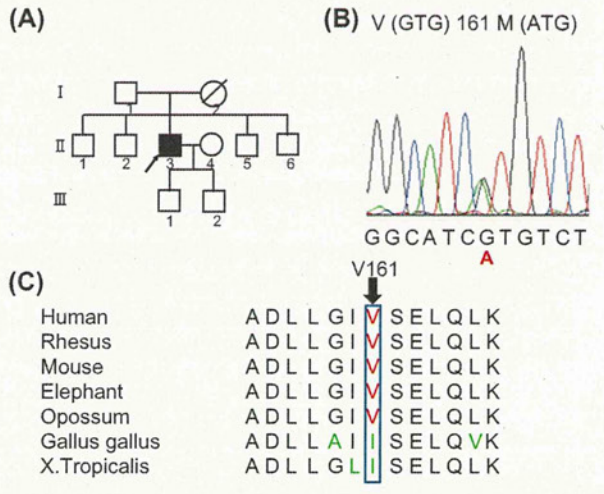

Figure 1. (A) Pedigree chart of patient with V161M variant in *OPTN*. Affected individuals are indicated by filled symbols. The proband is indicated by an arrow. Unaffected individuals are indicated by open symbols. Slashed symbols indicate deceased subjects. Ages at death are shown when information is available. Squares denote male family members and circles denote female family members. (B) Electropherogram of heterozygous *OPTN* c.481G>A (p.Val161Met) point mutation. (C) Conservation of *OPTN* amino acid sequences among different animal species. The valine residue at codon 161 is not necessarily highly conserved among different species (shown in red). Non-conserved amino acids are shown in green.

Discussion

In this study, we conducted a comprehensive mutational analysis of *OPTN* in a large cohort of Japanese FALS and SALS patients. Among our 35 FALS pedigrees, 17 families had mutations in other causative genes previously reported, as described in Results, and we did not find any causative mutations in *OPTN* in the remaining 18 pedigrees. On the other hand, among the 218 patients with SALS, we identified a patient carrying a novel non-synonymous mutation of *OPTN*.

Previous genetic studies on *OPTN* mutations in different cohorts have demonstrated that the frequencies of *OPTN* mutations are from 0% to 4.35% (pedigree frequency) in FALS (18–23) (Table IIIA). *OPTN* was initially identified as a causative gene for FALS in a consanguineous pedigree through homozygosity mapping followed by sequencing of candidate genes in the homozygous region. In our cohort, autosomal recessive inheritance was suggested in only five of the 35 FALS families, which may account for the fact that we did not identify any causative mutations in *OPTN* in the FALS families. Since the number of families enrolled in this study is limited, further extensive mutational analysis of larger cohorts of FALS will be necessary to establish the genetic epidemiology of FALS patients with *OPTN* mutations.

In our SALS cohort, a novel heterozygous non-synonymous variant, V161M, was identified in a patient. Previous genetic studies on *OPTN* mutations in different cohorts have shown a number of heterozygous missense mutations in SALS patients (20,21) and that the frequencies of *OPTN* mutations are from 0% to 3.54% (case frequency) in SALS (18–23) (Table IIIB). When we assess the implication of the

Table IIIA. Summary of *OPTN* variants identified in FALS patients in previous and present studies.

Studies	Ethnicity	Variant	Number of pedigrees	Status
Maruyama H, et al.[18]	Japanese	exon 5 deletion	4	1 homozygote
		p.Q398X		1 homozygote
		p.E478G		2 heterozygotes
Belzil VV, et al.[19]	European	c.1242 + 1G>A_insA	2	1 heterozygote
		p.A481V		1 heterozygote
Del Bo R, et al.[20]	Italian	p.G23X	2	1 heterozygote
		p.K557T		1 heterozygote
Iida A, et al.[21]	Japanese	p.E478G	1	1 homozygote
Millecamps S, et al.[22]	Caucasian	p.R96L	1	1 heterozygote
Sugihara K, et al.[23]	Caucasian	None	0	
Present study	Japanese	None	0	

mutation identified in an isolated case without any family history, we need to carefully consider various possibilities including the possibilities of causative mutation with reduced penetrance and *de novo* mutation. Another possibility is that the variant might not necessarily be associated with a risk of ALS.

Hexanucleotide repeat expansion within the *C9ORF72* gene has very recently been reported to be frequent as a cause of ALS with wider European ancestry. Our recent study on the same cohort indicated that the frequency of the patients with the hexanucleotide repeat expansions is very low (16), suggesting that the result of our molecular epidemiology study of *OPTN* was not substantially affected by that of *C9ORF72* in our Japanese cohort.

Previous studies showed that the clinical phenotypes of patients with *OPTN* mutations are heterogeneous for both age of onset and disease duration, but are characterized by a relatively slow progression, lower-limb onset, and frequent upper motor neuron signs. The relatively slow progression after the onset and the presence of upper motor neuron signs observed in the patient with the V161M variant are consistent with the previous reports (18–23). However, this patient differed from those in previous reports to the extent that the onset site is the upper extremities. Further accumulation of clinical information is essential to delineate the phenotypic spectrum and to illustrate the genotype-phenotype correlations of ALS with *OPTN* mutations.

Of note, the patient originated from the southernmost part of the Kii Penisula including the Koza River, where the prevalence of ALS has been described to be higher than in other areas of Japan (25). Neither the causes of the high prevalence nor the genetic risk factors common to ALS patients in the region have been elucidated. Mutational analysis of four additional ALS patients residing in the same district (Koza River and its vicinity), however, revealed neither the V161M mutation nor other mutations. V161M does not appear to be very common among the patients with ALS in this district.

Acknowledgements

We thank all patients and their family members for participating in this study. This work was supported in part by KAKENHI (Grant-in-Aid for Scientific Research on Innovative Areas) and Global COE Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a Grant-in-Aid for Research on Intractable Diseases and Comprehensive Research on Disability Health and Welfare from the Ministry of Health, Welfare and Labor, Japan.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Table IIIB. Summary of *OPTN* variants identified in SALS patients in previous and present studies.

Studies	Ethnicity	Variant	Number of cases	Status
Maruyama H, et al.[18]	Japanese	p.Q398X	1	1 homozygote
Belzil VV, et al.[19]	European	None	0	
Del Bo R, et al.[20]	Italian	c.552 + 1delG	4	1 heterozygote
		p.T282P		1 heterozygote
		p.Q314L		1 heterozygote
		c.1401 + 4A>G		1 heterozygote
Iida A, et al.[21]	Japanese	p.A93P	2	1 heterozygote
		p.E478G		1 heterozygote
Sugihara K, et al.[23]	Caucasian	None	0	
Present study	Japanese	p.V161M	1	1 heterozygote

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Acta Neuropathol (2012) 124:149–151
DOI 10.1007/s00401-012-0999-z

CORRESPONDENCE

Ubiquilin immunoreactivity in cytoplasmic and nuclear inclusions in synucleinopathies, polyglutamine diseases and intranuclear inclusion body disease

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Received: 23 April 2012 / Revised: 22 May 2012 / Accepted: 25 May 2012 / Published online: 3 June 2012

Ubiquilin-1 (UBQLN1), a member of the ubiquitin-like protein family (UBQLN1-4), is associated with neurofibrillary tangles in Alzheimer's disease (AD) and with Lewy bodies (LBs) in Parkinson's disease (PD) [7]. Mutations in *UBQLN2* cause dominant X-linked amyotrophic lateral sclerosis (ALS) [4]. UBQLN2-immunoreactive neuronal cytoplasmic inclusions (NCIs) are found in the hippocampus and spinal cord in ALS with or without *UBQLN2* mutation. Moreover, a distinct pattern of UBQLN2 pathology is seen in cases of ALS and frontotemporal lobar degeneration with TDP-43-positive inclusions (FTLD-TDP) showing *C9ORF72*-hexanucleotide repeat expansion [2], which is the most common genetic abnormality in ALS/FTLD [3, 12]. Here we report that UBQLN2 immunoreactivity is present in cytoplasmic and nuclear inclusions in various neurodegenerative diseases.

Post-mortem cases of sporadic ALS ($n = 5$), FTLD-TDP type B ($n = 4$), PD ($n = 5$), neocortical-type DLB ($n = 5$), multiple system atrophy (MSA; $n = 5$), AD ($n = 5$), Pick's disease ($n = 4$), progressive supranuclear palsy ($n = 4$), corticobasal degeneration ($n = 4$), argyrophilic grain disease ($n = 4$), Huntington's disease (HD; $n = 3$), dentatorubral-pallidoluysian atrophy (DRPLA; $n = 5$), spinal and bulbar muscular atrophy (SBMA; $n = 3$), spinocerebellar ataxia type 1 (SCA1; $n = 3$), SCA2 ($n = 1$), SCA3 ($n = 5$), intranuclear inclusion body disease (INIBD; $n = 5$) and controls ($n = 5$) were utilized. Immunohistochemistry was performed as described previously [10] with the following antibodies: UBQLN2, UBQLN1, phosphorylated α-synuclein, phosphorylated tau, ubiquitin, polyglutamine and TDP-43 (Online Resource). The total number of inclusions immunostained with each antibody was counted in contiguous sections.

In controls, neuronal nuclei were weakly immunolabeled with anti-UBQLN2 (Fig. 1a). UBQLN2-immunoreactive NCIs were found in the temporal cortex in FTLD-TDP (25 % relative to TDP-43-positive inclusions) as well as in the spinal cord in ALS (14 %) (Fig. 1b). In PD/DLB, both brainstem type and cortical LBs were intensely stained (Fig. 1c, d). Contiguous sections stained with anti-UBQLN2 and anti-α-synuclein revealed that 21 % of brainstem-type LBs and 48 % of cortical LBs were positive for UBQLN2. In MSA, 82 % of glial cytoplasmic inclusions (GCIs) were positive for UBQLN2 (Fig. 1e). In HD, DRPLA, SBMA, SCA1-3 and INIBD, more than 95 % of neuronal nuclear inclusions (NNIs) were strongly immunolabeled (Fig. 1f–l). In addition, Marinesco bodies (MBs)

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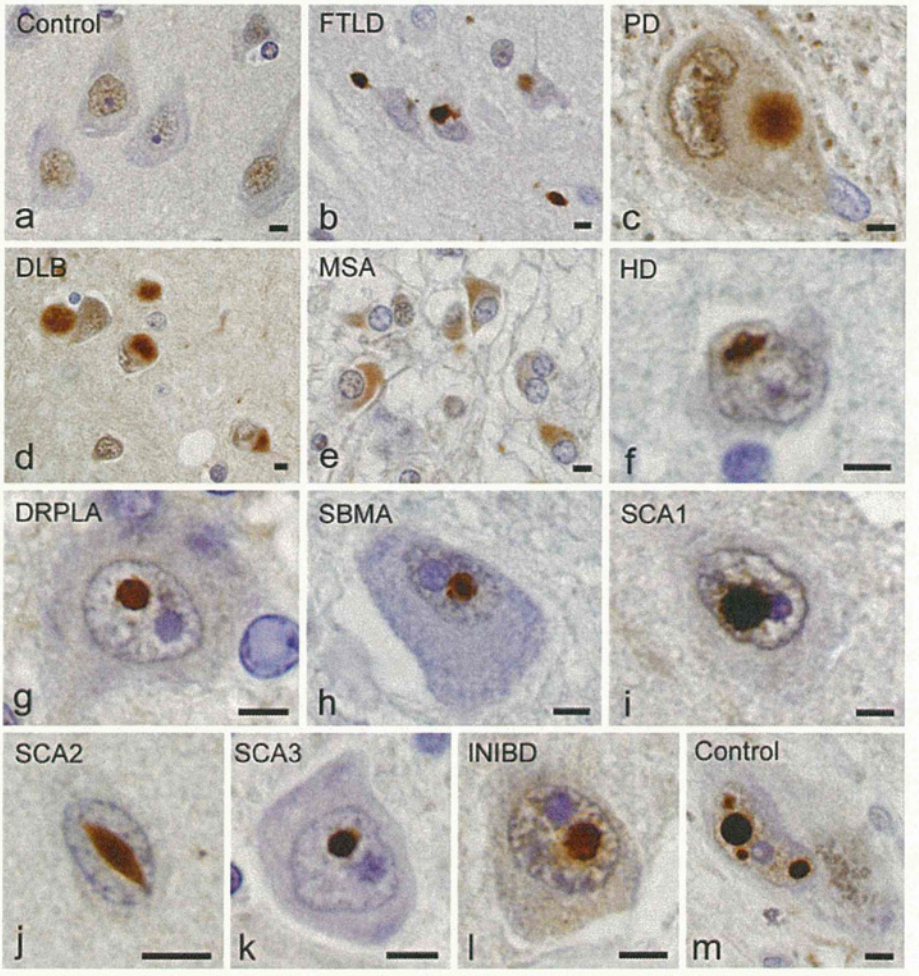

Fig. 1 UBQLN2 immunoreactivity in controls and neurodegenerative diseases. Diffuse neuronal nuclear staining in the frontal cortex (**a**). Neuronal cytoplasmic inclusions in the temporal cortex (**b**). Lewy bodies in the substantia nigra (**c**) and temporal cortex (**d**). Glial cytoplasmic inclusions in the pons (**e**). Neuronal intranuclear inclusions in the caudate nucleus (**f**, **j**), pontine nucleus (**g**, **k**), spinal anterior horn (**h**) and frontal cortex (**i**, **l**). Marinesco bodies in the substantia nigra (**m**). *Scale bars* 5 μm

were also positive for UBQLN2 (Fig. 1m). No UBQLN2 immunoreactivity was found in a variety of neuronal and glial inclusions in tauopathies.

Since the anti-UBQLN2 antibody used here is likely to detect both UBQLN2 and UBQLN1 [2], we further examined the above specimens using anti-UBQLN1-specific antibody that does not cross react with UBQLN2 [2]. The antibody immunolabeled NCIs in ALS/FTLD, LBs in PD/DLB, NNIs in polyglutamine diseases and INIBD, and MBs (Fig. 2).

In this study, we demonstrated for the first time that UBQLN2 immunoreactivity is present in LBs in PD/DLB, GCIs in MSA, and NNIs in polyglutamine diseases and INIBD, as well as in MBs in aged control subjects. However, the most striking finding was that almost all of the NNIs in six polyglutamine diseases (HD, DRPLA, SBMA, SCA1-3) were intensely immunolabeled with anti-UBQLN2. This is in line with the finding that both UBQLN1 and UBQLN2 interact with proteins containing polyglutamine expansions [5]. Interestingly, ubiquitin- or p62-positive, TDP-43-negative inclusions are found in the neuronal cytoplasm and nucleus in the cerebellar granular layer and hippocampus in cases of ALS and FTLD-TDP with *C9ORF72* expansion [1]. TDP-43-positive NNIs are one of the features of FTLD-TDP with *C9ORF72* expansion [11]. These findings suggest that the neuronal nucleus is a target in the disease process of ALS/FTLD with *C9ORF72* expansion.

We further demonstrated that NNIs in INIBD were immunoreactive for both UBQLN1 and UBQLN2. Although the major component of intranuclear inclusions in this disease is uncertain, they are immunoreactive for FUS [9]. Mutations in the gene encoding the FUS protein are now known to be the cause of familial ALS [6]. Recently, we have shown that NNIs in polyglutamine diseases and INIBD, and also MBs, are positive for optineurin [10]. Mutations of the optineurin gene are associated with familial and sporadic ALS [8]. These findings suggest that several proteins associated with familial ALS may contribute to the formation or degradation of NNIs in certain neurodegenerative conditions.

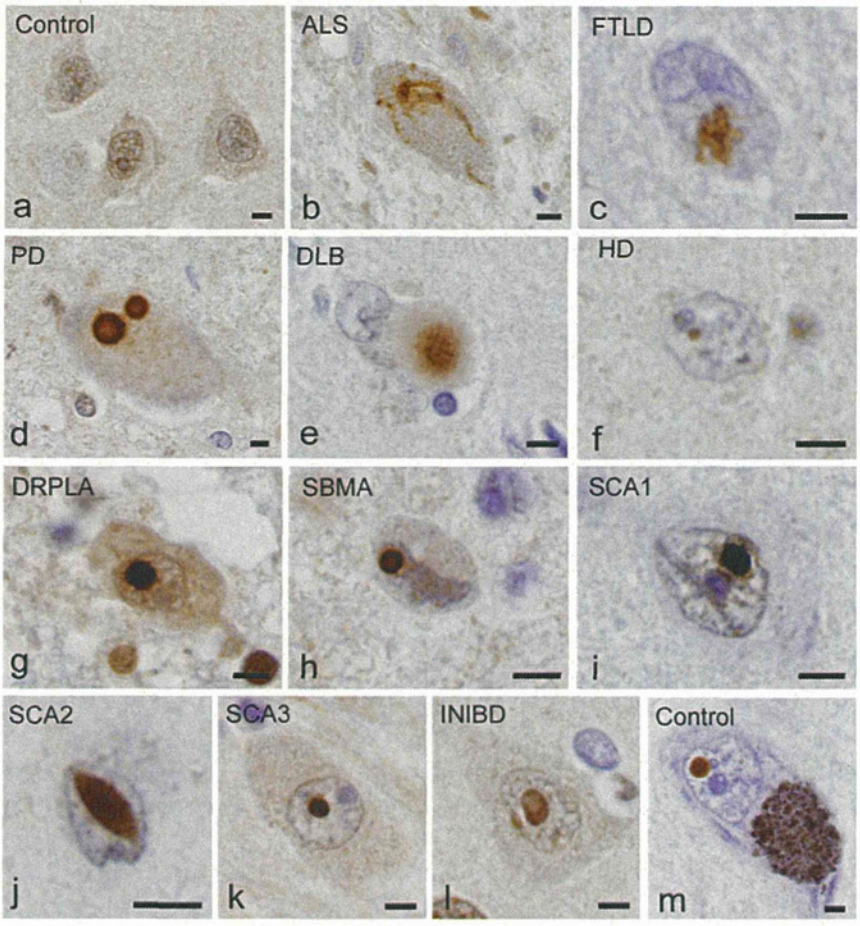

Fig. 2 UBQLN1 immunoreactivity in controls and neurodegenerative diseases. Weak neuronal cytoplasmic and nuclear staining in the frontal cortex (**a**). Skein-like inclusions in the spinal cord (**b**). Neuronal cytoplasmic inclusions in the temporal cortex (**c**). Lewy bodies in the locus coeruleus (**d**) and temporal cortex (**e**). Neuronal intranuclear inclusions in the caudate nucleus (**f**, **j**), pontine nucleus (**g**, **k**), spinal anterior horn (**h**) and frontal cortex (**i**, **l**). Marinesco bodies in the substantia nigra (**m**). *Scale bars* 5 μm

Acknowledgments This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, a Grant for Hirosaki University Institutional Research, the Collaborative Research Project (2012-2209) of the Brain Research Institute, Niigata University, Grants-in Aid from the Research Committee for Ataxic Disease, the Ministry of Health, Labour and Welfare, Japan, and an Intramural Research Grant (24-5) for Neurological and Psychiatric Disorders of NCNP.

Conflict of interest The authors report no conflicts of interest.

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Acta Neuropathol (2012) 123:747–749
DOI 10.1007/s00401-012-0956-x

CORRESPONDENCE

Optineurin immunoreactivity in neuronal nuclear inclusions of polyglutamine diseases (Huntington's, DRPLA, SCA2, SCA3) and intranuclear inclusion body disease

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Received: 28 December 2011 / Revised: 31 January 2012 / Accepted: 1 February 2012 / Published online: 9 February 2012

Optineurin (OPTN) is a ubiquitous cytoplasmic protein with various functions including membrane trafficking and signal transduction [9]. *OPTN* mutations are associated with adult-onset glaucoma and familial and sporadic amyotrophic lateral sclerosis (ALS) [4]. OPTN immunoreactivity has been reported in a variety of ubiquitinated inclusions including ALS-associated neuronal cytoplasmic inclusions, neurofibrillary tangles in Alzheimer's disease, Pick bodies in Pick disease, Lewy bodies in Parkinson's disease and glial cytoplasmic inclusions in multiple system atrophy [3, 6]. With respect to polyglutamine diseases, neuronal nuclear inclusions (NNIs) in Huntington's disease (HD) and spinocerebellar ataxia type 3 (SCA3) are reported to be immunonegative for OPTN [2]. However, Schwab et al. [7] have contended that NNIs in HD are immunopositive for OPTN. We report that OPTN immunoreactivity is present in NNIs in various polyglutamine diseases and intranuclear inclusion body disease (INIBD).

A total of 30 post-mortem cases were utilized in the present study; these included cases of HD ($n = 3$), dentatorubral-pallidoluysian atrophy (DRPLA; $n = 5$), SCA2 ($n = 1$), SCA3 ($n = 5$), SCA6 ($n = 2$), SCA17 ($n = 1$), spinal and bulbar muscular atrophy (SBMA; $n = 3$), INIBD ($n = 5$) and normal control subjects ($n = 5$). The diagnoses of polyglutamine diseases were confirmed genetically and histopathologically. Immunohistochemical analysis was carried out using formalin-fixed, paraffin-embedded sections from the basal ganglia in HD, SCA2 and SCA17, the pons in DRPLA and SCA3, the cerebellum in SCA6, the spinal cord in SBMA, the frontal lobe in INIBD, and the cortical and subcortical regions in control subjects. Anti-ubiquitin (1B3; MBL, Nagoya, Japan), anti-polyglutamine (1C2; Chemicon, Temecula, CA, USA) and anti-human OPTN C terminus (#100000; Cayman CHEMICAL, Ann Arbor, MI, USA) [2, 7, 8] were used as primary antibodies.

In controls, anti-OPTN antibody weakly immunolabeled the neuronal cytoplasm in a diffuse granular pattern, and neuronal nuclei were negative for OPTN (Fig. 1a). OPTN-immunoreactive NNIs were found in all the cases of HD, DRPLA, SCA2 and SCA3 (Fig. 1b–e). Intranuclear inclusions in neurons, but not in glial cells, in INIBD were also immunopositive for OPTN (Fig. 1f). Double immunofluorescence analyses revealed that OPTN immunoreactivity was found in 69% of NNIs in HD, 46% in DRPLA, 17% in SCA2, 55% in SCA3 and 50% in INIBD (Fig. 2). Although ubiquitinated NNIs were seen in SCA17 and SBMA, these inclusions were

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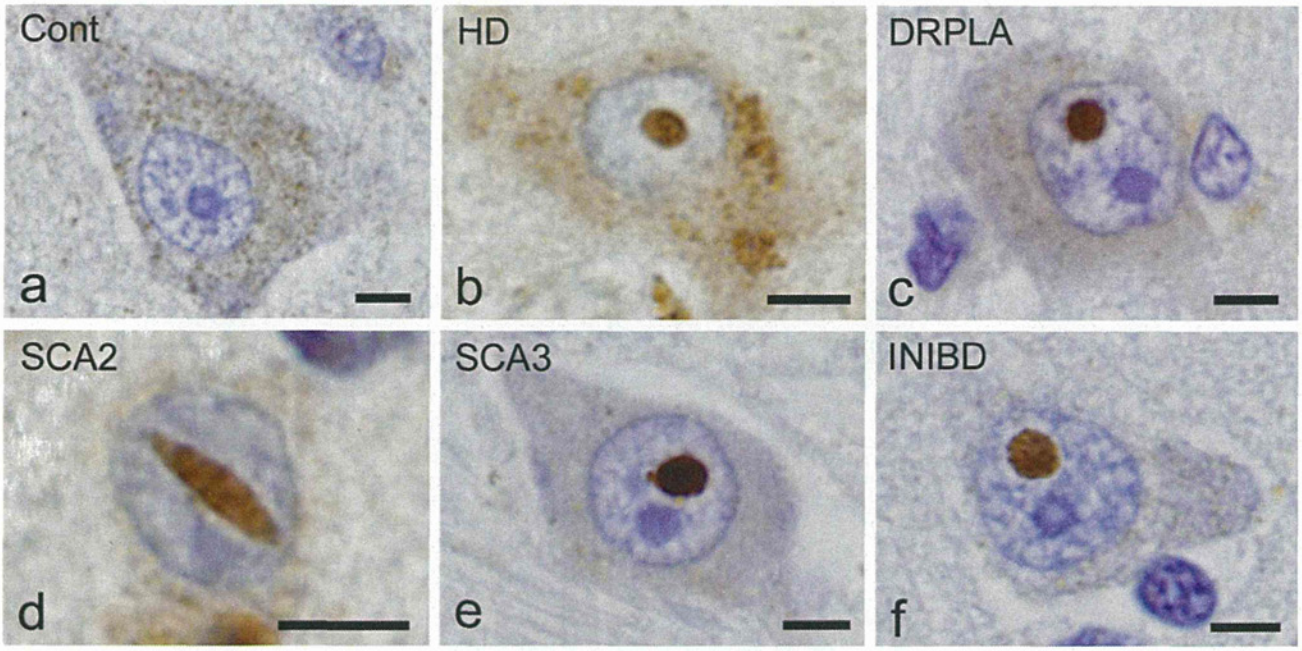

Fig. 1 Optineurin-immunoreactive structures in controls (**a**), polyglutamine diseases (**b–e**) and intranuclear inclusion body disease (**f**). Diffuse neuronal cytoplasmic staining in the frontal cortex (**a**). Neuronal intranuclear inclusions in the caudate nucleus in Huntington's disease (**b**) and spinocerebellar ataxia type 2 (SCA2) (**d**), the pontine nucleus in dentatorubral-pallidoluysian atrophy (**c**) and SCA3 (**e**), and the frontal cortex in intranuclear inclusion body disease (**f**). *Bars* = 5 μm

negative for OPTN. No OPTN-immunoreactive intranuclear or cytoplasmic inclusions were noted in SCA6. Consistent with a previous study [8], Marinesco bodies in the substantia nigra were positive for OPTN in patients with and without neurodegenerative diseases (data not shown).

Polyglutamine diseases include nine entities: HD, DRPLA, SBMA, SCA1, SCA2, SCA3, SCA6, SCA7 and SCA17, in which expanded polyglutamine forms inclusions, mainly in the nucleus. The inclusions could affect nuclear function and recruit other proteins, which might result in loss of physiological function of recruiting proteins followed by dysfunctions in neurons [10]. However, inclusion formation in polyglutamine diseases may be protective for the polyglutamine toxicity. Similar mechanisms may exist in the pathogenesis of INIBD, although the major component of nuclear inclusions in this disease is uncertain. In the present study, we demonstrated that OPTN immunoreactivity is present in a significant proportion of NNIs in four polyglutamine diseases (HD, DRPLA, SCA2 and SCA3) as well as in INIBD. In contrast, NNIs in SCA17 and SBMA were negative for OPTN, suggesting that there are some differences in the mechanism of inclusion body formation between these two disorders and the other NNI-containing diseases.

OPTN is one of the proteins interacting with huntingtin, a disease protein of HD. It is noteworthy that OPTN is abundantly expressed in interneurons, and low levels of OPTN are observed in medium-sized projecting neurons in the striatum, suggesting that the distribution of OPTN protein shows an inverse relationship to the pattern of neuronal loss in HD [5]. In response to an apoptotic stimulus, OPTN changes its subcellular localization from the Golgi to the nucleus [1]. It is possible that OPTN translocates from the cytoplasm to the nucleus to protect cells from cytotoxic events. Considering that OPTN is localized with cytoplasmic inclusions in tauopathies, synucleinopathies and TDP-43 proteinopathies, as well as in NNIs in polyglutamine diseases and INIBD, OPTN may be involved in the formation of cytoplasmic and nuclear inclusions in many neurodegenerative diseases or may represent a secondary event with or without pathological effects.

Acknowledgments This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, a Grant for Hirosaki University Institutional Research (K.W.), the Collaborative Research Project (2011–2209) of the Brain Research Institute, Niigata University (F.M.), Grants-in Aid from the Research Committee for Ataxic Disease, the Ministry of Health, Labour and Welfare, Japan (K.W.), and the Intramural Research Grant (21B-4) for Neurological and Psychiatric Disorders of NCNP (K.W.).

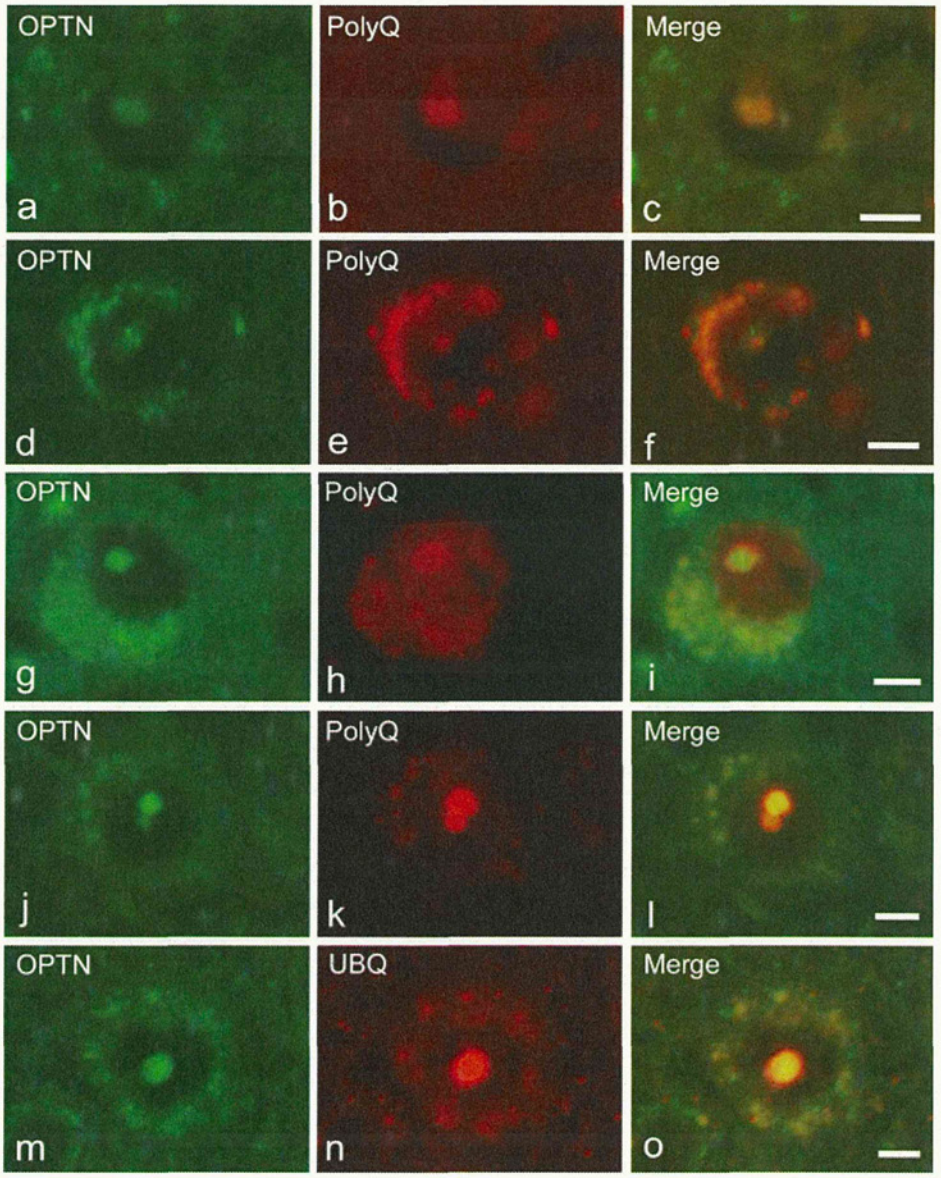

Fig. 2 Double-labeling immunofluorescence demonstrating co-localization of optineurin (OPTN) and polyglutamine (PolyQ) or ubiquitin (UBQ) in neuronal nuclear inclusions in the caudate nucleus in Huntington's disease (**a–c**) and spinocerebellar ataxia type 2 (SCA2) (**d–f**), the pontine nucleus in dentatorubral-pallidoluysian atrophy (**g–i**) and SCA3 (**j–l**), and the frontal cortex in intranuclear inclusion body disease (**m–o**). OPTN appears *green* and PolyQ or UBQ appears *red*. The overlap of OPTN and PolyQ or UBQ appears *yellow* (merge). *Bars* = 5 μm

Conflict of interest The authors report no conflicts of interest.

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