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Optineurin is potentially associated with TDP-43 and involved in the pathogenesis of inclusion body myositis

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Aims: Increasing evidences suggest a similarity in the pathophysiological mechanisms of neuronal cell death in amyotrophic lateral sclerosis (ALS) and myofibre degeneration in sporadic inclusion body myositis (sIBM). The aim of this study is to elucidate the involvement of ALS-causing proteins in the pathophysiological mechanisms in sIBM. **Methods:** Skeletal muscle biopsy specimens of five patients with sIBM, two with oculopharyngeal muscular dystrophy (OPMD), three with polymyositis (PM), three with dermatomyositis (DM), three with neurogenic muscular atrophy, and three healthy control subjects were examined. We analysed the expression and localization of familial ALS-causing proteins, including transactive response DNA binding protein-43 (TDP-43), fused in sarcoma/translocated in liposarcoma (FUS/TLS), Cu/Zn superoxide dismutase (SOD1) and optineurin (OPTN) by immunohistochemistry. **Results:** TDP-43, OPTN and, to a lesser extent, FUS/TLS were more frequently accumulated in the cytoplasm in patients with sIBM and OPMD than in patients with PM, DM, neurogenic muscular atrophy, or healthy control subjects. SOD1 was accumulated in a small percentage of myofibres in patients with sIBM and OPMD, and to a very small extent in patients with PM and DM. Confocal microscopy imaging showed that TDP-43 proteins more often colocalized with OPTN than with FUS/TLS, p62 and phosphorylated Tau. **Conclusions:** These findings suggest that OPTN in cooperation with TDP-43 might be involved in the pathophysiological mechanisms of skeletal muscular degeneration in myopathy with rimmed vacuoles. Further investigation into these mechanisms is therefore warranted.

Keywords: amyotrophic lateral sclerosis, Cu/Zn superoxide dismutase, fused in sarcoma/translocated in liposarcoma, optineurin, sporadic inclusion body myositis, TAR-DNA-binding protein-43

Introduction

Sporadic inclusion body myositis (sIBM) is a progressive myopathy characterized by muscle weakness and atrophy and onset of symptoms after 50 years of age; a successful treatment for this disease is unavailable [1]. sIBM belongs to the category of inflammatory myopathies and can be considered a conformational disorder, because it is associated with abnormal intracellular accumulation of multiple unfolded/misfolded proteins, including amyloid-beta (Aβ), phosphorylated tau (p-tau) in the form of paired helical filaments, and others, with ubiquitin immunoreactivity [2].

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sIBM is known to mimic the clinical and electrophysiological features of motor neuron disease: some patients with a pathological diagnosis of sIBM could initially be misdiagnosed as having motor neuron disease or amyotrophic lateral sclerosis (ALS) [3]. Regarding the cellular abnormalities associated with sIBM muscle fibres, several proteins, including Aβ42 and its oligomers, and p-tau in the form of paired helical filaments, form aggregates within muscle fibres [1,2]. In addition, TAR DNA binding protein (TDP-43), which is known to accumulate in the anterior horn cells in ALS, was recently identified in normal myonuclei and in the sarcoplasm of sIBM muscle [4–8]. Moreover, a recent investigation using exome sequencing identified mutations in the valosin-containing protein (VCP) gene in families with autosomal dominant inherited ALS that were previously identified in families with inclusion body myopathy, Paget disease and frontotemporal dementia (IBMPFD) [9]. Thus, increasing evidence suggests a similarity in the pathophysiological mechanisms of neuronal cell death in ALS and myofibre degeneration in sIBM.

To elucidate the involvement of ALS-causing proteins in the pathophysiological mechanisms of myopathy with rimmed vacuoles, we examined the expression and localization of familial ALS-causing proteins such as TDP-43, fused in sarcoma/translocated in liposarcoma (FUS/TLS), SOD1, and optineurin (OPTN), by immunohistochemistry using skeletal muscle samples of patients with sIBM, oculopharyngeal muscular dystrophy (OPMD), polymyositis (PM), dermatomyositis (DM) and neurogenic muscular atrophy.

Materials and methods

Patients and muscle biopsies

In this study, we examined muscle biopsy samples of five patients with sIBM, two with OPMD, three with PM, three with DM, three with neurogenic atrophy, and three healthy control subjects. The clinical and histopathological diagnoses of sIBM, and other diseases were based on specific diagnostic criteria as described in previous reports [10–12]. All the sIBM biopsies showed Congo-red positivity with fluorescence. OPMD patients were genetically confirmed after informed consent [13]. Age at biopsy, age at onset, gender, and serum creatine kinase levels of all the patients are summarized in Table 1. In the case of all patients, muscle biopsy specimens were obtained for diagnostic purposes after obtaining informed consent. The study was approved by the Ethics Committee of the Kumamoto University Hospital. All samples had previously been examined by routine histochemical techniques. Fresh frozen samples were kept at −80°C until used.

Table 1. Clinical summary of the patients

Case no.	*Diagnosis*	*Age at biopsy (age at onset)*	*Gender*	*CK (U/l)*	*Biopsy*
1	sIBM	72 (67)	Male	375	Biceps brachii
2	sIBM	79 (77)	Male	563	Quadriceps
3	sIBM	84 (79)	Male	235	Biceps brachii
4	sIBM	86 (86)	Male	510	Quadriceps
5	sIBM	70 (68)	Male	364	Quadriceps
6	PM	58 (58)	Female	4716	Biceps brachii
7	PM	54 (54)	Female	3810	Quadriceps
8	PM	65 (65)	Female	1273	Biceps brachii
9	DM	52 (51)	Male	2521	Deltoid
10	DM	39 (39)	Female	316	Deltoid
11	DM	27 (27)	Female	4986	Biceps brachii
12	OPMD	69 (60)	Female	252	Quadriceps
13	OPMD	81 (81)	Female	341	SCM
14	SBMA	55 (52)	Male	1734	Biceps brachii
15	PMA	63 (58)	Male	1029	Biceps brachii
16	ALS	83 (79)	Female	384	Gastrocnemius

sIBM, sporadic inclusion body myositis; PM, polymyositis; DM, dermatomyositis; OPMD, oculopharyngeal muscular dystrophy; SBMA, spinobulbar muscular atrophy; PMA, progressive muscular atrophy; ALS, amyotrophic lateral sclerosis; CK, creatine kinase (normal range: 57–284 U/l); SCM, sternocleidomastoid.

Immunohistochemical analyses

The fresh frozen tissue sections were fixed with 4% paraformaldehyde, and blocked with 5% normal donkey serum/0.1% Triton-X in phosphate-buffered saline. The following primary antibodies were used: Rabbit anti-TDP-43 (1:500; ProteinTech Group, Chicago, IL, USA), rabbit anti-FUS/TLS (1:250; Sigma-Aldrich, St Louis, MO, USA), rabbit anti-OPTN (1:250; Cayman), and sheep anti-SOD1 (1:500; Calbiochem, San Diego, CA, USA). Immunolabeled proteins were visualized using the avidin-biotin-peroxidase complex (ABC) method with a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) or Cy3-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, West Grove, PA, USA). The number of immunostained myofibres was expressed as a proportion of the total number of fibres through observation of a total of 100 muscle fibres in each staining. One hundred myofibres were also counted in three randomly selected areas for a total of 300 myofibres per muscle sample.

For the double labelling, the combinations of mouse anti-TDP-43 (1:500; ProteinTech Group) and rabbit anti-FUS/TLS (1:250; Sigma-Aldrich, St Louis, MO, USA), or rabbit anti-OPTN (1:250; Cayman) were employed. The combinations of rabbit anti-TDP-43 (1:500; ProteinTech Group) and mouse anti-phospho TDP-43 (pS409/410) (1:3000; Cosmo Bio, Tokyo, Japan); mouse anti-p62/SQSTM1 (1:250; Medical & Biological Laboratories, Nagoya, Japan); or mouse anti-phospho Tau (Ser396) (1:500; Cell Signaling Technology, Beverly, MA, USA) were also used. Immunolabelling was visualized by fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin antibody (1:200; Jackson ImmunoResearch) and Cy3-conjugated anti-rabbit immunoglobulin antibody (1:200; Jackson ImmunoResearch). Sections were examined by using confocal microscopy (FV300, Olympus, Tokyo, Japan).

Statistics

All values are expressed as the mean ± SEM. Differences among means were analysed using one-way ANOVA. When ANOVA showed significant differences, pairwise comparisons were performed by Tukey post-hoc test.

Results

TDP-43, FUS/TLS, OPTN and SOD1 protein accumulation was higher in sIBM muscle tissue than in PM or DM muscles

The localization of familial ALS-causing proteins, such as FUS/TLS, OPTN, TDP-43 and SOD1, was examined in skeletal muscle biopsy specimens. We summarized the cytoplasmic expression patterns in each immunohistochemistry in Table 2. In healthy control subjects, FUS/TLS was localized in the nuclei of the muscle fibres (Figure 1**a**). No subsarcolemmal, diffuse cytoplasmic or cytoplasmic inclusion staining was seen. Immunohistochemical analysis of FUS/TLS revealed mainly diffuse cytoplasmic staining in 9.8 ± 1.0% of muscle fibres in sIBM, as well as the nuclear localization (Figure 1**b**,**c** and Figure 2**a**). FUS/TLS-immunoreactive cytoplasmic staining was detectable in 4.0 ± 0.6% and 1.7 ± 0.7% of myofibres with PM and DM, respectively (Figure 1**d**,**e** and Figure 2**a**).

In healthy control subjects, OPTN was faintly stained in the cytopalsm of the muscle fibres (Figure 1**f**). In sIBM patients, immunohistochemistry for OPTN showed exclusively cytoplasmic granular staining (Figure 1**g**,**h**). The percentage of abnormal accumulation of OPTN was 23.0 ± 3.0% of muscle fibres (Figure 2**b**). OPTN-immunoreactive myofibres were less frequent in PM and DM than in sIBM (8.0 ± 1.2% in PM; 4.3 ± 0.9% in DM) (Figure 1**i**,**j** and Figure 2**b**). The cytoplasmic OPTN staining seen in PM and DM was mainly diffuse (Table 2).

Table 2. Summary of the cytoplasmic expression patterns in each immunohistochemistry

	sIBM	*PM*	*DM*	*OPMD*	*Neurogenic atrophy*
TDP-43	Granular	Diffuse	Diffuse	Rimmed vacuoles	–
FUS/TLS	Diffuse	Diffuse	Diffuse	Rimmed vacuoles	–
OPTN	Granular	Diffuse	Diffuse	Rimmed vacuoles	–
SOD1	Granular	–	–	–	–

sIBM, sporadic inclusion body myositis; PM, polymyositis; DM, dermatomyositis; OPMD, oculopharyngeal muscular dystrophy.

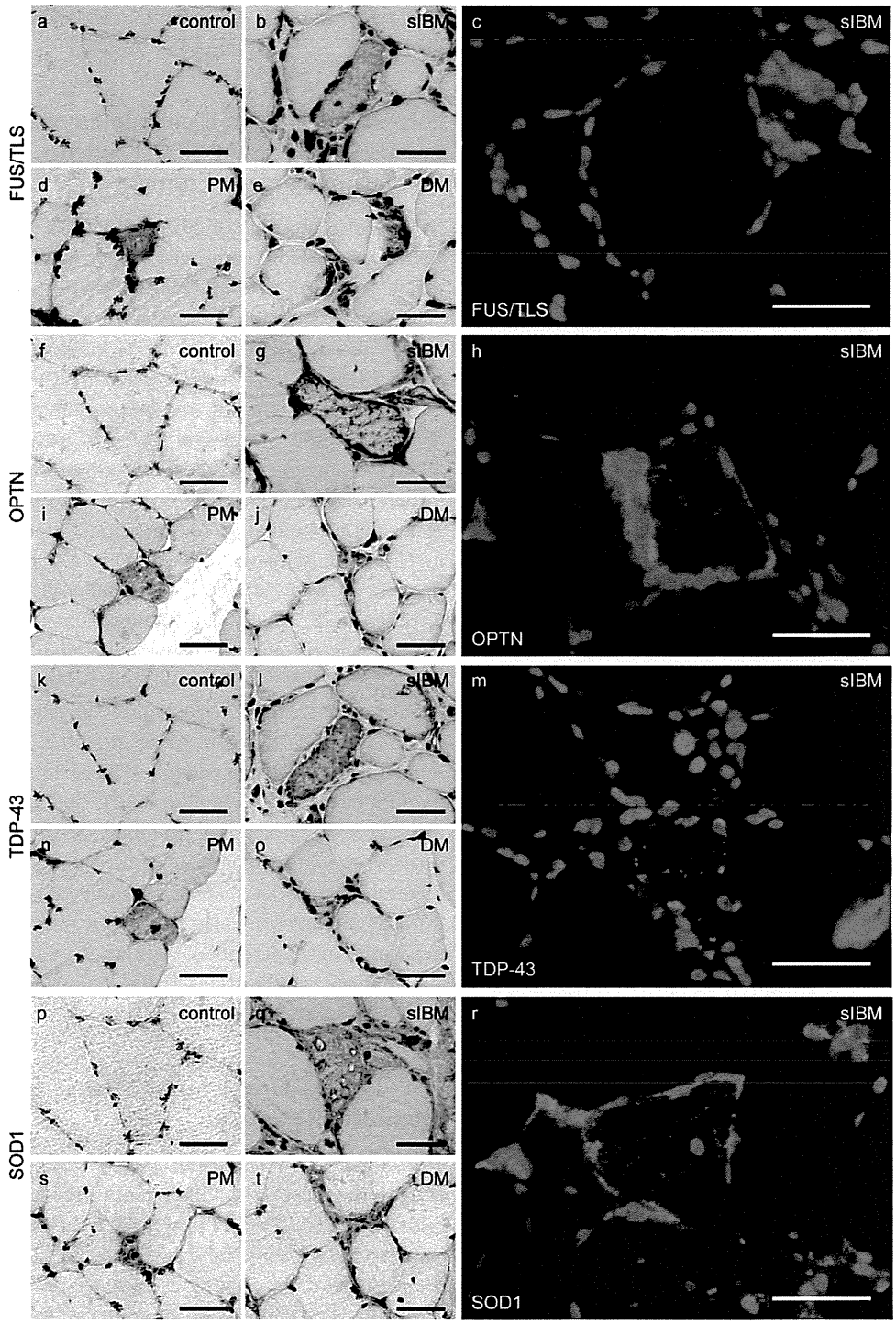

Figure 1. Representative immunohistochemical staining for FUS/TLS (**a**–**e**), OPTN (**f**–**j**), TDP-43 (**k**–**o**) and SOD1 (**p**–**t**) by using biopsy specimens of skeletal muscles of healthy control subjects (**a**, **f**, **k** and **p**) and patients with sIBM (**b**, **c**, **g**, **h**, **l**, **m**, **q** and **r**), PM (**d**, **i**, **n** and **s**), and DM (**e**, **j**, **o** and **t**). Nuclei were stained with haematoxylin (**a**, **b**, **d**, **e**, **f**, **g**, **i**, **j**, **k**, **l**, **n**, **o**, **p**, **q**, **s** and **t**) or 4′,6-diamidino-2-phenylindole (DAPI) (**c**, **h**, **m** and **r**). Scale bars, 50 μm.

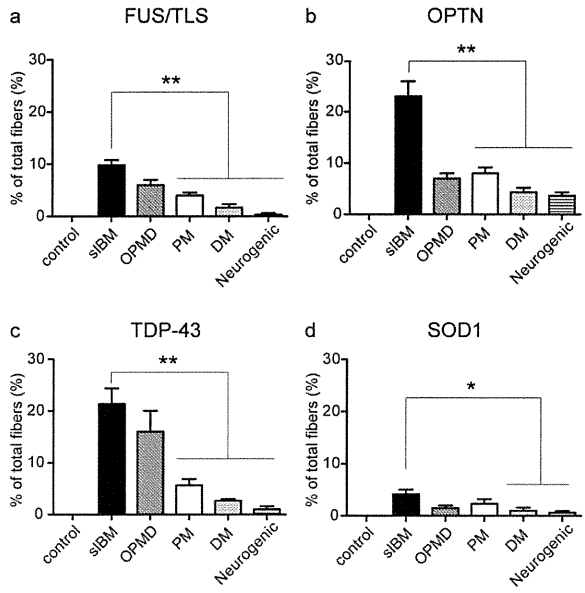

Figure 2. The percentage of myofibres with abnormal cytoplasmic staining for FUS/TLS (**a**), OPTN (**b**), TDP-43 (**c**) and SOD1 (**d**) in the total number of myofibres. $^{*}P < 0.05$; $^{**}P < 0.01$.

TDP-43 was localized in the nuclei of the muscle fibres in healthy control subjects (Figure 1**k**). In all patients with sIBM, TDP-43 accumulated in cytoplasmic granules within muscle fibres, which showed granular or dot-like patterns in addition to the nuclear localization (Figure 1**l**,**m**), as previously reported [4–8]. TDP-43 staining counterstained with nuclear marker, 4′,6-diamidino-2-phenylindole (DAPI), clearly excluded the possibility that the dot-like patterns of TDP-43 staining in sIBM aggregates were merely labelling of internalized nuclei (Figure 1**m**). Within myofibres having TDP-43-immunoreactive cytoplasmic aggregates, the nuclei were less immunoreactive for TDP-43 (Figure 1**m**). Abnormal accumulation of TDP-43 was detected in 21.4 ± 3.0% of total muscle fibres of the sIBM patients (Figure 2**c**). In the case of the PM and DM patients, TDP-43-immunoreactive cytoplasmic diffuse staining were observed in 5.7 ± 1.2% and 2.7 ± 0.3% of total muscle fibres, respectively, and in particular in necrotic fibres (Figure 1**n**,**o** and Figure 2**c**).

SOD1 was faintly observed in the cytoplasm of the muscle fibres in healthy control subjects (Figure 1**p**). Immunohistochemical analysis of SOD1 revealed mainly granular staining in 4.2 ± 0.9% of myofibres of sIBM patients (Figure 1**q**,**r** and Figure 2**d**), but they were rarely observed in the muscle fibres of patients with PM and DM (2.3 ± 0.9% in PM; 1.0 ± 0.6% in DM) (Figure 1**s**,**t** and Figure 2**d**).

FALS-related protein accumulation was detected in muscle tissue from OPMD patients, but not in those with neurogenic atrophy

Next, the localization of FUS/TLS, OPTN, TDP-43 and SOD1 was examined in muscles from patients with OPMD, which is a form of hereditary myopathy with rimmed vacuoles. FUS/TLS-immunoreactive aggregates were mainly detected in the vicinity of rimmed vacuoles in 6.0 ± 1.0% of total muscle fibres of OPMD patients (Figures 2**a** and 3**a**), but they were rarely (0.3 ± 0.3%) detected in the muscles of patients with neurogenic atrophy including patients with progressive muscular atrophy (PMA) (Figures 2**a** and 3**b**). The percentage of abnormal accumulation and staining patterns of OPTN in OPMD muscles was 7.0 ± 1.0% of total myofibres (Figures 2**b** and 3**c**). OPTN-immunopositive myofibres were seen in 3.7 ± 0.7% of total myofibres from patients with neurogenic atrophy (Figures 2**b** and 3**d**). In patients with OPMD, TDP-43 was abnormally accumulated in the vicinity of rimmed vacuoles (Figure 3**e**). Abnormal accumulation of TDP-43 was observed in 16.0 ± 4.0% of total muscle fibres in OPMD patients (Figure 2**c**). In contrast, immunohistochemical analysis of TDP-43 showed rarely (1.0 ± 0.6%) cytoplasmic granular staining except the nuclear staining in patients with neurogenic atrophy (Figures 2**c** and 3**f**). Unlike their presence in sIBM muscles, SOD1-immunopositive aggregates were rarely observed in muscles from OPMD patients or in those with neurogenic atrophy (1.5 ± 0.5% in OPMD; 0.7 ± 0.3% in neurogenic atrophy) (Figure 2**d** and Figure 3**g**,**h**).

TDP-43 colocalized with OPTN and to a lesser extent with FUS/TLS

To examine the relationship among FALS-causing proteins in the muscles of sIBM patients, the localization of FUS/TLS, OPTN, TDP-43 and SOD1 was analysed using serial sections from sIBM muscles. In the sIBM patients, the identical fibres showed a certain level of abnormal accumulations, which were immunoreactive for TDP-43, FUS/TLS, OPTN, and SOD1 (Figure 4).

To assess the possible synergism between FALS-causing proteins, the colocalization between TDP-43 and FUS/TLS was compared in muscles from sIBM and OPMD patients

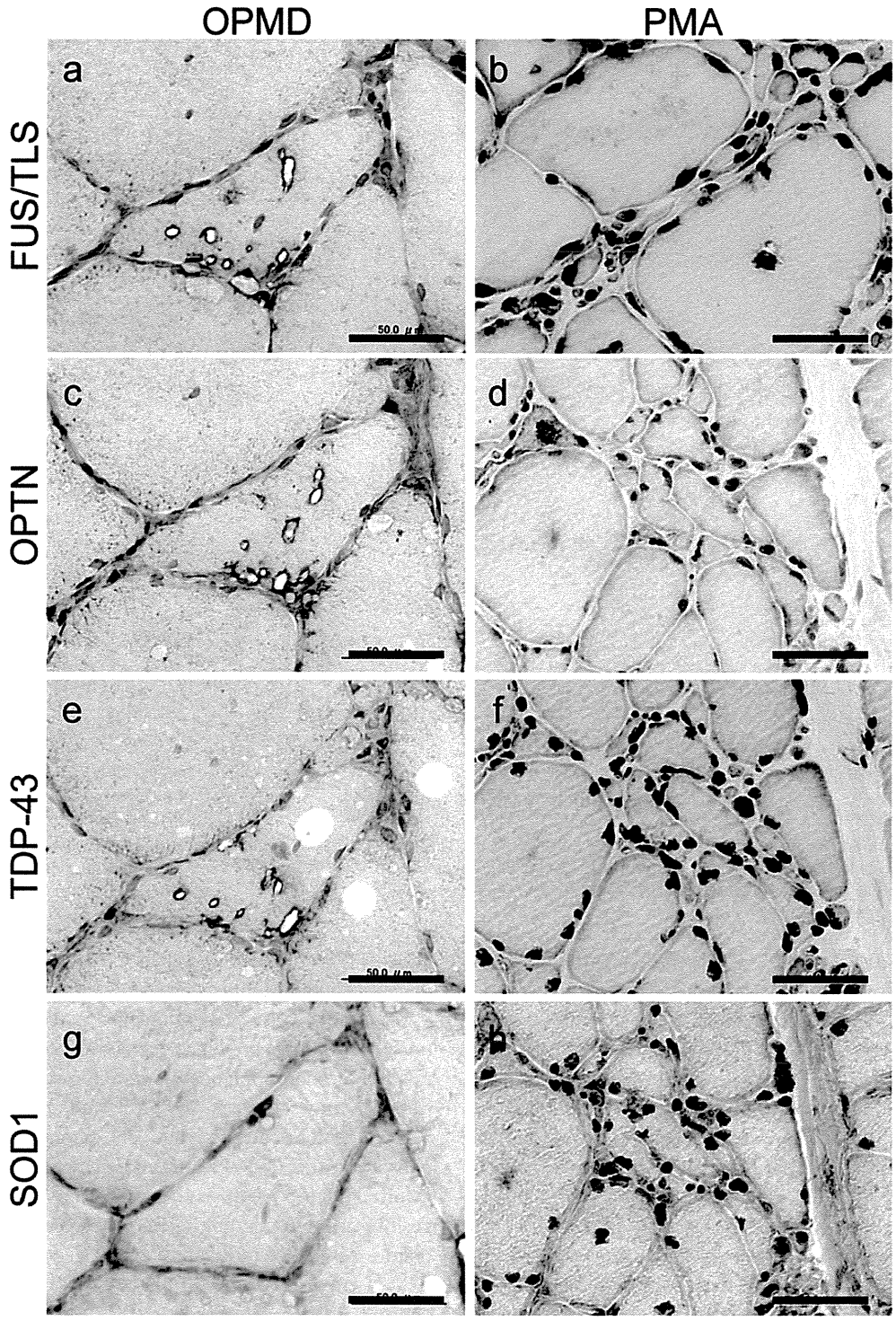

Figure 3. Representative immunohistochemical staining for FUS/TLS (**a**, **b**), OPTN (**c**, **d**), TDP-43 (**e**, **f**) and SOD1 (**g**, **h**) by using biopsy specimens of skeletal muscles of patients with OPMD (**a**, **c**, **e** and **g**) and progressive muscular atrophy (PMA) (**b**, **d**, **f** and **h**). Nuclei were stained with haematoxylin. Scale bars, 50 μm.

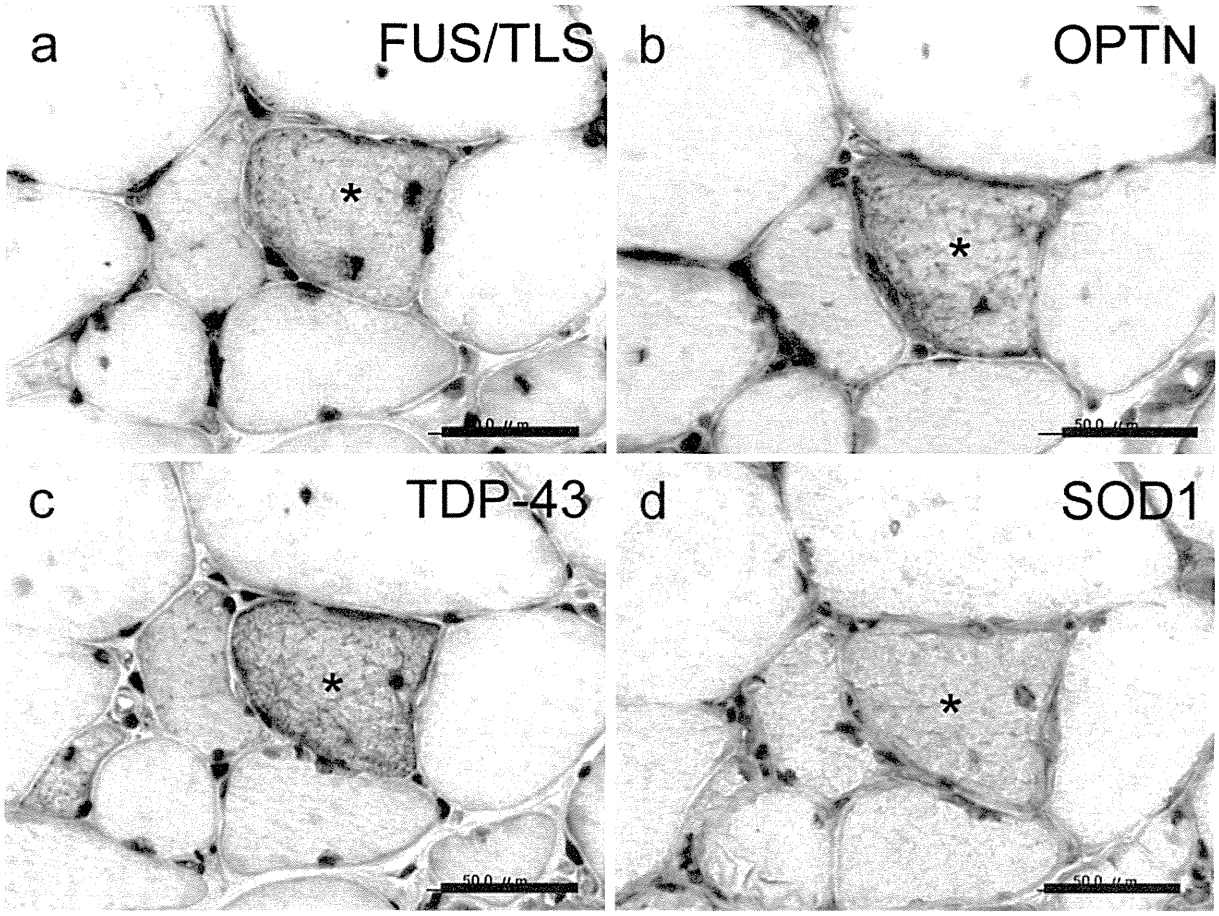

Figure 4. Immunohistochemical staining for FUS/TLS (**a**), OPTN (**b**), TDP-43 (**c**) and SOD1 (**d**) by using serial sections of sIBM muscles. Asterisks indicate identical muscle fibres. Nuclei were stained with haematoxylin. The same muscle fibres showed abnormal aggregates that were immunoreactive for FUS/TLS, OPTN, TDP-43 and SOD1. Scale bars, 50 μm.

(Figure 5). TDP-43 or FUS/TLS-immunoreactive granules were observed in sIBM muscles as described above (Figure 5**a**,**f**). However, TDP-43-positive granules did not colocalize with FUS/TLS (Figure 5**a**–**d**). Similarly, FUS/TLS-positive cytoplasmic aggregates did not colocalize with TDP-43 (Figure 5**e**–**h**). Thus, neither myofibres with TDP-43-positive granules nor fibres with FUS/TLS-positive granules contained colocalized signals between TDP-43 and FUS/TLS except nuclear staining. In OPMD muscles, TDP-43 and FUS/TLS were not colocalized despite their accumulation in identical muscle fibres (Figure 5**i**–**l**). Assessment of the colocalization of TDP-43 and OPTN in muscles from sIBM and OPMD patients by confocal microscopy (Figure 6) revealed that TDP-43-immunoreactive areas were frequently colocalized with OPTN in cytoplasmic granules in the muscles of sIBM patients (Figure 6**a**–**h**) as well as in the muscles of OPMD patients (Figure 6**i**–**l**).

We next assessed the relationship between TDP-43 and non-FALS-relating proteins which have been accumulated in sIBM myofibres, including p62 and p-tau. The colocalization study between TDP-43 and phosphorylated TDP-43 demonstrated that both phosphorylated and non-phosphorylated TDP-43-immunoreactive granules were involved (Figure 7**a**–**d**), as shown previously [7]. TDP-43-positive granules did not necessarily colocalize with p62 (Figure 7**e**–**h**) or p-tau (Figure 7**i**–**l**).

Discussion

In the present study, TDP-43, OPTN and, to a lesser extent, FUS/TLS accumulated more frequently in the cytoplasm of

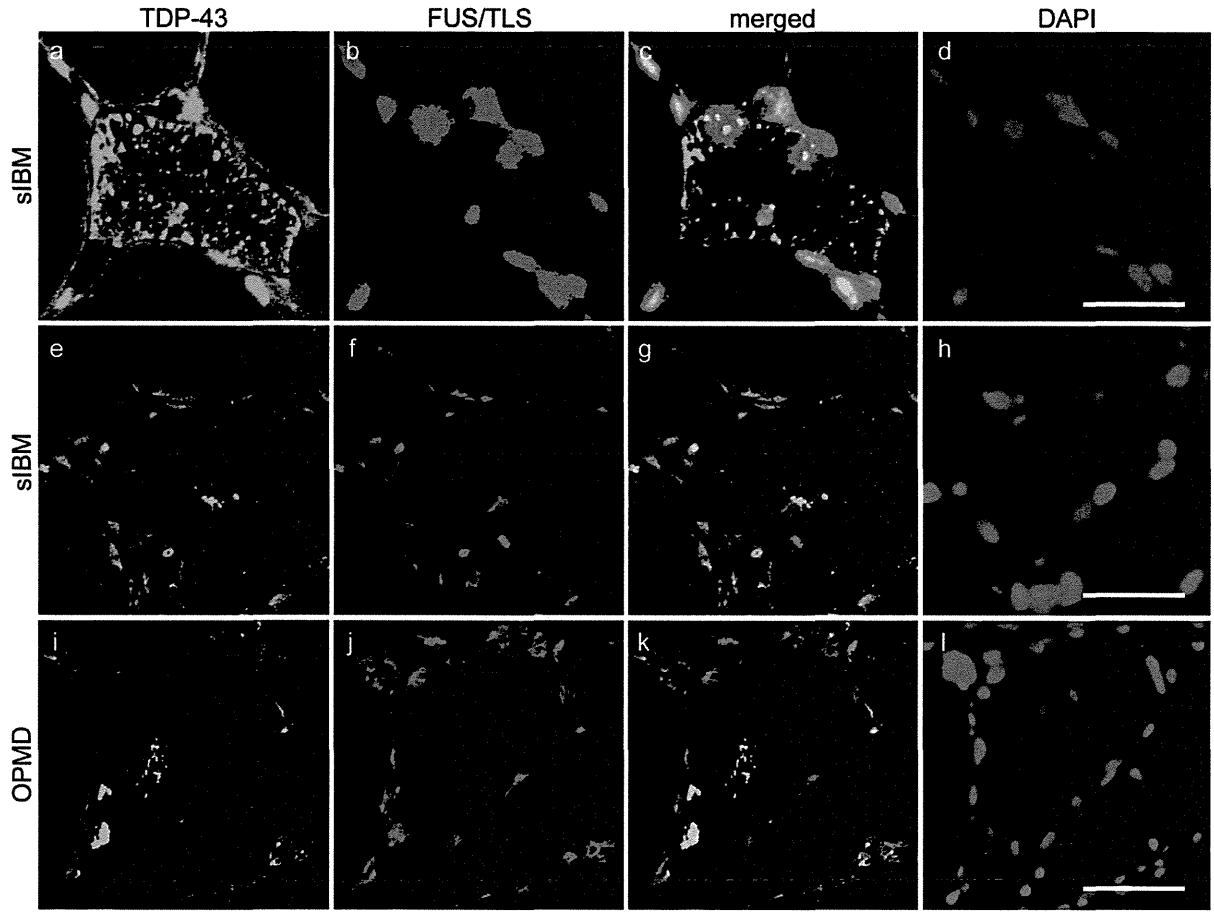

Figure 5. Confocal microscopic analysis of the localization of TDP-43 (**a**, **e** and **i**) and FUS/TLS (**b**, **f** and **j**) in muscles of patients with sIBM (**a–h**) and OPMD (**i–l**). Merged images are presented in **c**, **g** and **k**. Nuclei were stained with DAPI (blue; **d**, **h** and **l**). TDP-43-positive granules (green) were not necessarily colocalized with FUS/TLS signals (red). Scale bars, 20 μm.

patients with sIBM and OPMD than in the cytoplasm of patients with PM, DM, or neurogenic muscular atrophy. SOD1 accumulated in a small percentage of myofibres of patients with sIBM, and to a very small extent in the myofibres of patients with PM and DM. Interestingly, confocal microscopy revealed that TDP-43 proteins colocalized more often with OPTN than with FUS/TLS, p62, and p-tau.

Since Weihl *et al.* [5] first reported TDP-43-immunoreactive cytoplasmic inclusions within sIBM muscle fibres, several studies have shown the abnormal accumulation of TDP-43 in skeletal muscles of patients with sIBM [4–8]. In these studies, phosphorylated as well as non-phosphorylated TDP-43-immunoreactive granules were present in sIBM muscle fibres, as shown in motor neurons of patients with ALS. In contrast, TDP-43-positive granules were observed not only in sIBM but also in other vacuolar myopathies, including OPMD, IBMPFD and DMRV. Thus, TDP-43-positive aggregates appear to be a generic phenomenon among myopathies associated with rimmed vacuoles and a common endpoint of muscle cell degeneration [8,14].

Maruyama *et al.* [15] recently revealed that OPTN could be a causative gene in familial ALS. In their study, TDP-43-positive inclusions in sporadic ALS showed positive immunolabeling with anti-OPTN antibodies, suggesting that OPTN is involved in the pathogenesis of ALS. Northern blot analysis for human OPTN revealed a more prominent expression of the OPTN transcripts in skeletal muscles than in the heart, brain, placenta, liver,

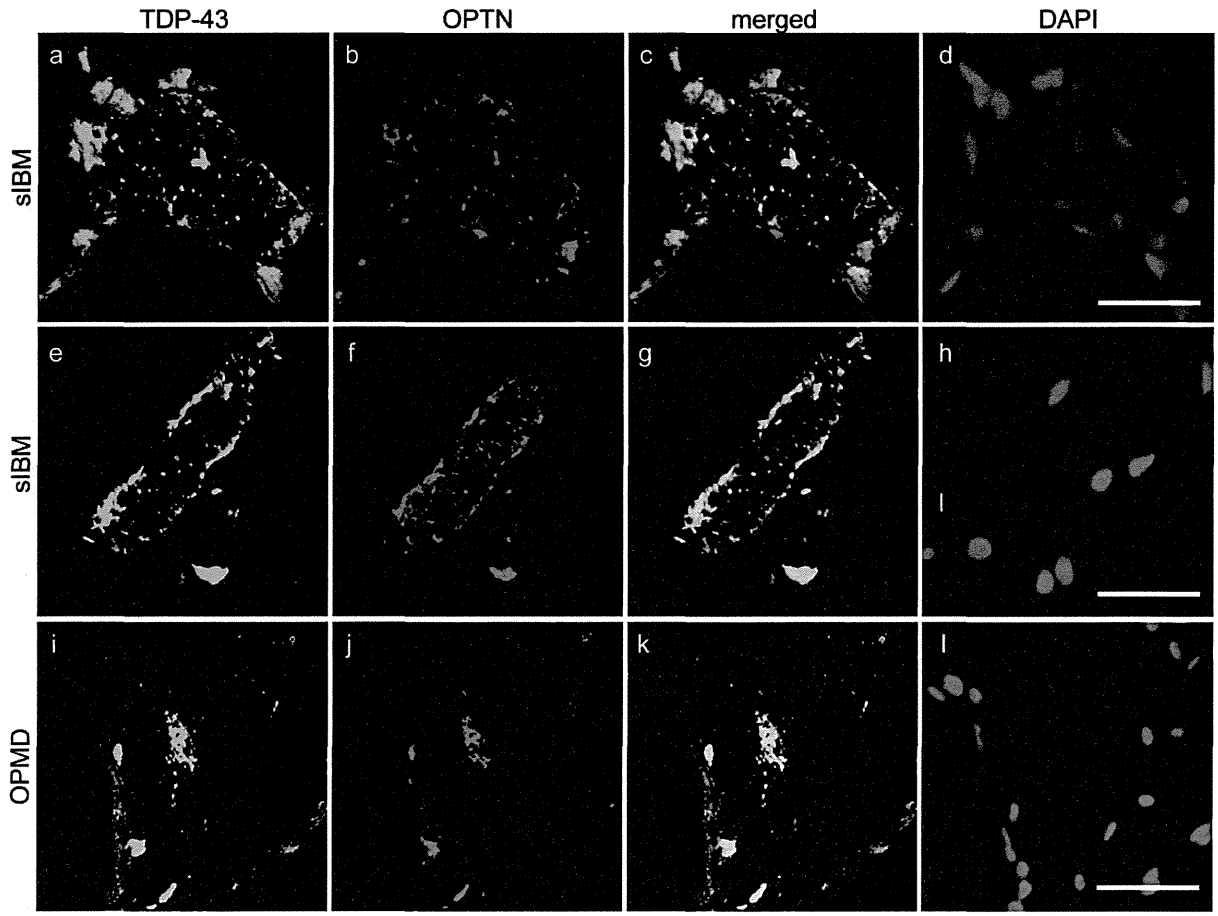

Figure 6. Confocal microscopical analysis of the localization of TDP-43 (**a**, **e** and **i**) and OPTN (**b**, **f** and **j**) in muscles of patients with sIBM (**a–h**) and OPMD (**i–l**). Merged images are presented in **c**, **g** and **k**. Nuclei were stained with DAPI (blue; **d**, **h** and **l**). TDP-43-immunoreactive accumulations (green) were frequently colocalized with OPTN (red) in cytoplasmic granular or subsarcolemmal inclusions. Scale bars, 20 μm.

kidney, and pancreas [16]. Yeast two-hybrid screens and protein interaction studies have identified OPTN as a binding partner for myosin VI at the Golgi complex [17]. OPTN knockdown resulted in the loss of myosin VI from the Golgi complex, accompanied by the fragmentation of the Golgi. OPTN is therefore considered a link between myosin VI and the Golgi complex and thought to play a central role in the maintenance of Golgi morphology. VCP, a causative agent of IBMPFD, associates with many protein adaptors to perform a variety of cellular processes, including Golgi assembly/disassembly [18]. Our study is the first to show OPTN accumulation in sIBM muscle fibres. Whether the accumulation of OPTN in sIBM muscles serves as a trigger for Golgi dysfunction, or a consequence of Golgi disruption due to muscle fibre degeneration remains to be elucidated. However, the observation that OPTN accumulation associated with TDP-43 may indicate that the abnormal granules of OPTN is an essential event in the pathophysiology of myopathies with rimmed vacuoles.

With regard to the relationship between FUS/TLS and sIBM, a study showed the presence of abnormal TDP-43 and FUS/TLS protein fragments in sIBM muscles [19]. This study showed that a decrease in the levels of full-length (73 kDa) FUS/TLS in sIBM was correlated with the increase in the levels of a 140 kDa band, which was assumed to correspond to FUS/TLS dimers or other

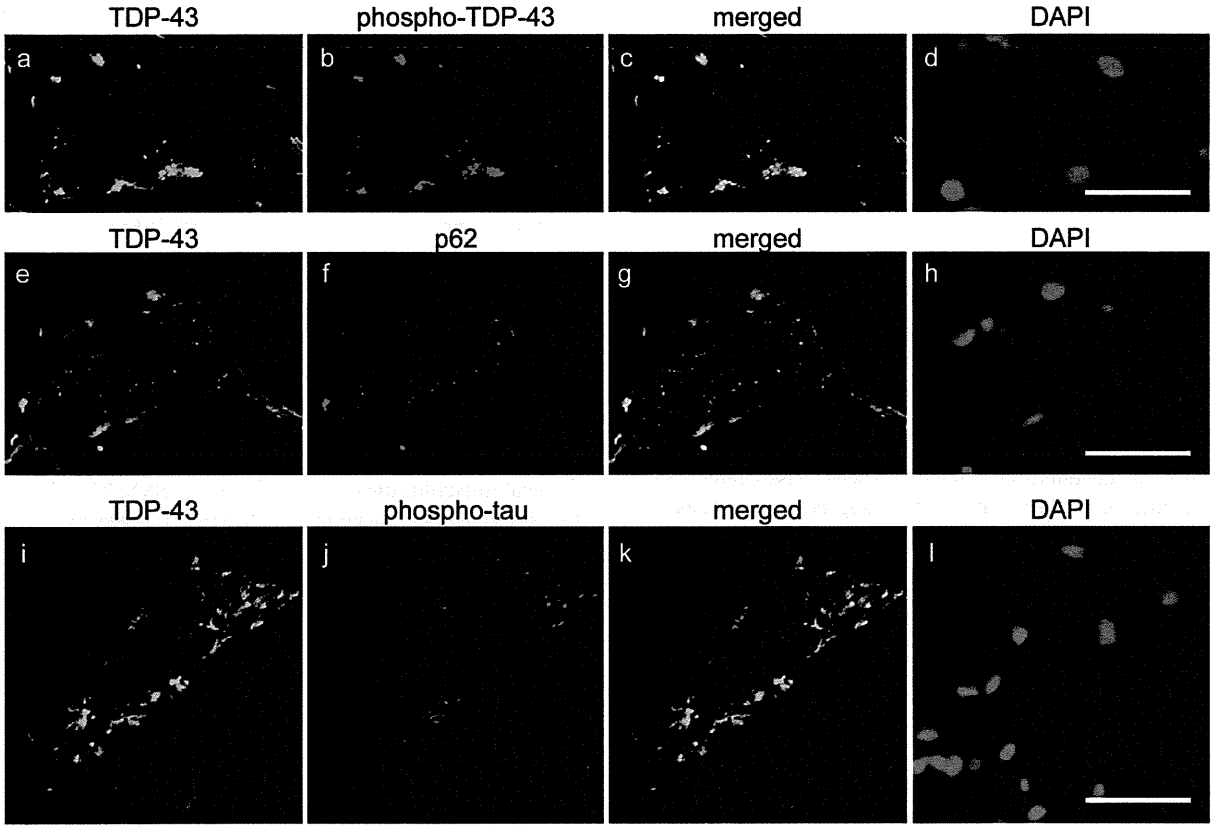

Figure 7. (**a–d**) Confocal microscopic analysis of the localization of pan-TDP-43 (**a**) and phosphorylated TDP-43 (**b**) in myofibre of sIBM patients. Merged images are presented in **c**. Nuclei were stained with DAPI (**d**). (**e–h**) Confocal microscopic analysis of the localization of TDP-43 (**e**) and p62 (**f**) in myofibre of sIBM patients. Merged images are presented in **g**. Nuclei were stained with DAPI (**h**). (**i–l**) Confocal microscopic analysis of the localization of TDP-43 (**i**) and p-tau (**j**) in myofiber of sIBM patients. Merged images are presented in k. Nuclei were stained with DAPI (**l**). TDP-43-positive granules did not necessarily colocalize with p62 or p-tau. Scale bars, 20 μm.

conglomerates. The authors of these studies did not find alterations in the localization of FUS/TLS in sIBM muscles, which was inconsistent with our results. This inconsistency between the two studies may be attributable to differences in the techniques and antibodies used or to differences in the disease stage of the patients included in each study.

Our observations suggest that OPTN in cooperation with TDP-43, are involved in the pathophysiological mechanisms of skeletal muscular degeneration in myopathies with rimmed vacuoles. Impairment in the protein degradation machinery, including the ubiquitin-proteasome system and autophagy, could be associated with myofibre degeneration in the pathogenesis of sIBM, as in the motor neuron death of ALS. Further investigation is required to clarify the association between these proteins and the pathogenesis of sIBM.

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Conceived and designed the experiments: S.Y., E.K., Y.M. and M.U. Performed the experiments: S.Y., E.K., N.T. H.S., T.N. and Y.M. Analysed the data: S.Y., E.K., Y.M., T.H., M.U. and Y.A. Contributed reagents/materials/analysis tools:

S.Y., E.K., N.T., H.S., T.N., Y.M. and Y.A. Wrote the paper: S.Y., E.K., T.H. and M.U.

Conflict of interest

The authors declare that they have no conflict of interest.

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Respiratory dysfunction in patients severely affected by GNE myopathy (distal myopathy with rimmed vacuoles)

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Abstract

GNE myopathy is a rare and mildly progressive autosomal recessive myopathy caused by *GNE* mutations. Respiratory dysfunction has not been reported in GNE myopathy patients. In this study, we retrospectively reviewed the respiratory function of 39 severely affected GNE myopathy patients (13 men, 26 women) from medical records, and compared these parameters with various other patient characteristics (e.g., *GNE* mutations, age at onset, creatine kinase levels, and being wheelchair-bound) for correlations. The mean % forced vital capacity [FVC] was 92 (26) (range, 16–128). In 12/39 (31%) patients, %FVC was <80%. Of these 12 patients, 11 (92%) were entirely wheelchair-dependent. These patients exhibited significantly earlier onset (20 [4] vs. 30 [8] years, $p < 0.001$) and lower creatine kinase levels (56 [71] vs. 279 [185] IU/L) than patients with normal respiratory function. Two patients exhibited severe respiratory failure and required non-invasive positive pressure ventilation. Patients with a homozygous mutation in the *N*-acetylmannosamine kinase domain exhibited lower %FVC, while only one compound heterozygous patient with separate mutations in the uridinediphosphate-*N*-acetylglucosamine 2-epimerase and the *N*-acetylmannosamine kinase domains had respiratory dysfunction. Our results collectively suggest that GNE myopathy can cause severe respiratory failure. Respiratory dysfunction should be carefully monitored in patients with advanced GNE myopathy characterized by early onset and homozygous homozygous mutations in the *N*-acetylmannosamine kinase domain.

Keywords: GNE myopathy; Distal myopathy with rimmed vacuoles (DMRV); Hereditary inclusion body myopathy; Respiratory dysfunction; Uridinediphosphate-*N*-acetylglucosamine (UDP-GlcNAc) 2-epimerase domain; *N*-acetylmannosamine kinase domain

1. Introduction

GNE myopathy, also known as distal myopathy with rimmed vacuoles (DMRV), Nonaka myopathy, or hereditary inclusion body myopathy (hIBM), is an early adult-onset, slowly progressive myopathy that preferentially affects the tibialis anterior muscle but relatively spares the quadriceps femoris muscles [1,2]. Respiratory dysfunction has not been reported in GNE myopathy [3]. Nonaka et al. reported that respiratory muscles were rarely involved even in bed-ridden patients, but no data were presented [1]. However, we had noticed that a few patients with GNE myopathy exhibited mild but progressive respiratory loss, with some experiencing recurrent pneumonia due to reduced airway clearance. Recent recommendations suggest training patients with neuromuscular disease with respiratory dysfunction using the air stacking technique to increase their thorax capacity and assisted cough peak flow (CPF) from an early stage to maintain lung compliance and chest mobility, and to clean the airways [4]. If respiratory dysfunction is not rare in patients with GNE

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myopathy, then, physicians should punctually monitor their respiratory function with pulmonary function tests to look for early signs of respiratory dysfunction, perform respiratory training, coup with airway infection using a mechanical in-exsufflator (MI-E), and induce mechanical ventilation if required, as they do for patients with neuromuscular disease who exhibit respiratory failure.

The aim of this study is to evaluate past and present clinical respiratory function test parameters of GNE myopathy patients, and analyze factors that correlate with disease severity.

2. Patients and methods

2.1. Study population

Medical records of all genetically confirmed GNE myopathy patients who underwent pulmonary function tests at the National Center Hospital, National Center of Neurology and Psychiatry, were retrospectively reviewed. We collected data on genetic diagnosis, respiratory function (% vital capacity [%VC], % force vital capacity [FVC], cough peak flow [CPF]), creatine kinase (CK), chest X-ray and/or CT scan and body mass index (BMI) for analysis.

2.2. Data handling and analysis

Data were summarized using descriptive statistics, and each variable was compared against age, sex, respiratory dysfunction (whether their %FVC was up to or over 80%), and domain mutation (i.e., within the UDP-GlcNAc 2-epimerase domain: ED or *N*-acetylmannosamine kinase domain: KD). The *t*-test was used to compare the means of each group. Data for the two study populations were calculated using chi-square contingency table analysis. Multivariate regression analysis was performed with %FVC as the dependent variable. Explanatory variables included age at disease onset, CK and BMI. We found that the variables age, duration from onset to present, age upon wheelchair use, age at loss of ambulation, were highly correlated (over 0.5) with age at disease onset. As such, we eliminated these three due to multicolinearity in the multivariate regression analysis. When past %FVC data were available, the present data were compared with serial changes in respiratory function during the preceding 5–7 years, and changes in %FVC over time were determined by calculating the difference between past and present data. All analyses were performed using SPSS for Macintosh (Version 18; SPSS Inc., Chicago, IL).

3. Results

3.1. General characteristics

A total of 39 Japanese patients (13 men, 26 women) were recruited. The mean age at the time of data collection was 43.1 (11.3) years (mean [standard deviation, SD]) (Table 1). The mean age at first appearance of symptoms was 26.8 (9.0) years (range, 15–58 years; median, 25 years). Present age, age at disease onset, age at wheelchair use, and present ambulation status were not significantly different between men and women; 20.5% (8/39) had symptom onset before age 20. Of the 39 patients, 51.3% (20/39) could walk but needed assistance, and 69.2% (27/39) were wheelchair-bound (8/27 and 19/27 were partially and totally wheelchair-bound, respectively). Age at first use of a wheelchair was 33.3 (10.8) years (range, 18–59 years; median, 31.5 years) and that for loss of ambulation was 36.9 (11.9) years (Table 1).

3.2. GNE mutations

Of the 39 patients, 30.7% (12/39) carried homozygous mutations, while 69.2% (27/39) harbored compound heterozygous mutations (Supplementary Table 1). Among the homozygous patients, 66.7% (8/12) harbored the p.V572L mutation. Among the compound heterozygous patients, 25.9% (7/27) exhibited the p.D176V/p.V572L genotype, while the other patients each had a different mutation. With respect to the location of the mutation (i.e., protein domain), 28.2% (11/39) homozygous patients carried mutations only in ED (ED/ED), 46.2% patients (18/39) were compound heterozygotes with 1 mutation each in the ED and KD (ED/KD), and 25.6% patients (10/39) had a mutation in the KD of both genes (KD/KD) (Table 2). The allelic frequencies of p.V572L, p.D165V, p.C13S, and p.R129Q were 35.9% (28/78), 28.2% (22/78), 11.5% (9/78), and 2.6% (2/78), respectively, while all other mutations had only 1 allele each (Supplementary Table 1).

3.3. Respiratory function

None of the patients had lung and/or thoracic diseases that could affect their respiratory function in chest X-ray and/or chest computed tomography. The %VC and %FVC in patients with GNE myopathy were 91.9 (26.9) (range, 18.2–126.3; median, 100.3) and 92.0 (25.8) (range, 16.4–128.5; median, 100.5; Table 1), respectively.

3.4. Patients with respiratory dysfunction

In 30.7% of patients (12/39), %FVC was <80. Of these 12 patients, 91.6% (11/12) were wheelchair-dependent and 83.3% (10/12) had already lost ambulation. Their onset was significantly earlier (19.3 [4.4] vs. 30.3 [8.4], $p < 0.001$) and mean CK level was significantly lower (55.8 [71.6] vs. 279.0 [184.7], $p = 0.004$) than those of patients with normal respiratory function. Four patients exhibited advanced respiratory dysfunction (%FVC < 50% and cough peak flow [CPF] < 160 L/min) (Table 2). All 4 patients had experienced recurrent pneumonia, and 2 patients required nocturnal NPPV. They were all early onset (before 20 years old) and non-ambu-

Table 1
Patient characteristics by respiratory function.

	Total	%FVC ⩾ 80%	%FVC < 80%	
n	*39*	27	12	*p*
Age (years)	43.0 ± 11.3	44.3 ± 11.7	39.9 ± 10.3	0.267
Age at onset (years)	26.8 ± 9.0	30.2 ± 8.4	19.2 ± 4.4	<0.001
GNE/GNE	10 (25.6%)	7 (70.0%)	3 (30.0%)	0.640
GNE/MNK	18 (46.2%)	16 (88.9%)	2 (11.1%)	0.018
MNK/MNK	11 (28.2%)	4 (36.4%)	7 (63.6%)	0.009
Duration from onset of disease to present	16.2 ± 8.4	14.1 ± 7.8	20.8 ± 8.2	0.021
Wheelchair use (%)	27 (69.2%)	16 (59.3%)	11 (40.7%)	0.141
Wheelchair use since (years)	33.3 ± 10.8	37.9 ± 11.3	26.6 ± 5.1	0.002
Lost ambulation	19 (48.7%)	8 (42.1%)	11 (57.9%)	0.014
Age at lost ambulation (years)	36.9 ± 11.9	41.2 ± 11.7	28.2 ± 6.4	0.018
CK (IU/L)	201.3 ± 187.5	279.0 ± 184.7	55.8 ± 71.6	0.004
BMI	21.1 ± 4.2	20.8 ± 3.2	21.9 ± 5.8	0.457
FVC (%)	91.9 ± 26.9	106.9 ± 12.5	58.2 ± 18.7	<0.001
VC (%)	92.0 ± 25.8	106.4 ± 11.6	59.5 ± 17.6	<0.001
CPF (L/min)	334.2 ± 139.5	378.0 ± 105.7	250.2 ± 161.5	0.008

Most patients with reduced respiratory function had already lost ambulation and were entirely wheelchair-dependent. Their onset was significantly earlier and CK levels significantly lower than those of patients with normal respiratory function. FVC: forced vital capacity, VC: vital capacity, CPF: cough peak flow, BMI: body mass index, CK: creatine kinase.

Table 2
Patients with FVC < 50% and CPF < 160 L/min.

Case	Age	Sex	Mutation	Mutant domain	Ambulation status	Disease onset	Disease duration	Age at lost ambulation	%VC	%FVC	CPF (L/min)	Reccurent pneumonea	NPPV	CK (IU/L)	BMI
1	51	Man	p.C13S homozygote	ED/ED	Non-ambulant	17	34	25	18.2	16.4	48.0	Yes	Nocturnal	13	18.6
2	42	Woman	p.V572L homozygote	KD/KD	Non-ambulant	16	26	23	37.6	34.4	141.6	Yes	Nocturnal	13	22.2
3	45	Woman	p.V572L homozygote	KD/KD	Non-ambulant	17	28	31	49.0	48.3	147.6	Yes	No	8	31.6
4	37	Woman	p.V572L homozygote	KD/KD	Non-ambulant	16	21	24	53.7	48.6	118.8	Yes	No	No data	20.4

Table 3
Multivariate regression analysis of predictive factors for respiratory dysfunction.

	Regression coefficient	*p*	Lower limit of 95% confidence interval	Upper limit of 95% CI
Age at onset	0.949	0.042	0.038	1.86
CK	0.068	0.008	0.02	0.115
BMI	−1.8	0.09	−3.811	0.302

Multivariate linear regression analysis was performed to evaluate the relationship between %FVC and other clinical parameters. Age at onset and CK were significantly correlated with %FVC.

lant. The majority (7/12) of patients had KD/KD mutations, whereas significantly fewer patients with respiratory dysfunction had ED/KD mutations.

In order to identify predictive factors for respiratory dysfunction in GNE myopathy, we performed multivariate analysis to determine the relationship with %FVC. This revealed age at onset ($p = 0.042$) and CK ($p = 0.008$) as significantly correlated to %FVC (Table 3, Fig. 1).

Past (5–7 years ago) data were available for 9 patients. The %FVC decrements in 5 patients with respiratory dysfunction were significantly greater than those of patients without dysfunction (20.9 [6.0] vs. 0.8 [9.7], $p = 0.004$; Supplementary Table 2).

4. Discussion

To our knowledge, we are the first to report respiratory dysfunction in GNE myopathy. Our study demonstrates that (1) certain GNE myopathy patients in Japan exhibit respiratory dysfunction, and (2) early onset and lower CK levels resulting from severe muscle atrophy and weakness, and KD/KD mutations can be risk factors for respiratory dysfunction.

Malicdan et al. reported that pathological changes in the diaphragms of the GNE (−/−) hGNED176V-Tg model mice were variable and ranged from almost normal to the presence of marked fibrosis and rimmed vacuoles. On the other hand, the gastrocnemius muscles of all mice exhibited myopathic features [5]. The features in these mice correspond to individual differences observed in the patients of our study. The fact that not all cases in our study exhibited respiratory dysfunction as observed in the GNE (−/−)

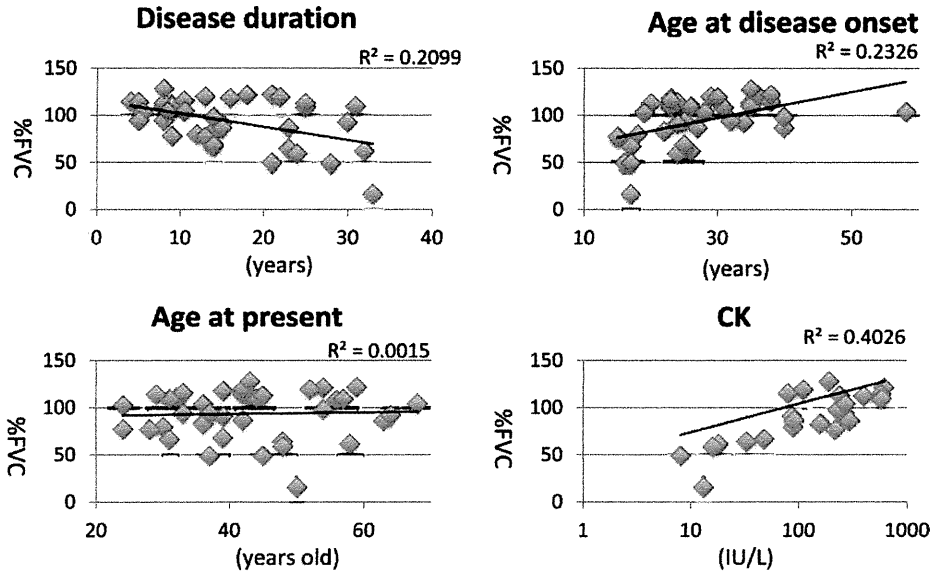

Fig. 1. Scatterplots of %FVC as functions of age, age at disease onset, disease duration, and creatine kinase (CK) level. Age at disease onset, disease duration, and CK level were correlated with %FVC.

hGNED176V-Tg mice indicates that severe respiratory muscle involvement is not a constant feature of GNE myopathy. Yet, since about 30% of patients had decreased %FVC and severe respiratory dysfunction was overlooked by neurologists or physicians, clinicians should be made more aware of the possibility of respiratory dysfunction, particularly in patients with advanced GNE myopathy. If %VC decreases to 70%, patients should be taught air stacking as with other neuromuscular disorders [4,6]. CPF should be routinely measured in patients with GNE myopathy, given that its decrement was associated with recurrent pneumonia in our study. Early induction of assisted CPF and/or MI-E is required if patients with reduced CPF have an airway infection. Serial data suggest that %FVC decreased from the normal range to %FVC < 80, indicating that continuous monitoring is required even in patients with normal respiratory function. Moreover, respiratory function parameters may provide quantitatively useful data for clinical trials, particularly those directed to non-ambulant patients.

All 4 patients with severe respiratory dysfunction exhibited early onset, homozygous mutations, and advanced muscle weakness. However, not all early onset, homozygous, or non-ambulant patients exhibited severe respiratory dysfunction. Although the underlying reasons are unclear, we also found that ED/KD mutations were less associated with decreased respiratory function, while many patients with KD/KD mutations showed respiratory dysfunction. A large scale, cross-sectional study could better identify key factors responsible for respiratory dysfunction and genotype-phenotype correlations.

We are aware that the recruitment of patients from NCNP, highly specialized for muscle disease, is a potential source of selection bias, because they may be particularly more severely affected than the general patient population. Therefore, our study may not correctly reflect the general patient population. Investigations of small populations may underestimate the statistical significance as well. However, our previous GNE myopathy questionnaire study revealed a similar correlation between genotypes and phenotypes [7]. We are currently in the process of establishing a Japanese national GNE myopathy patient registry called Registration of Muscular Dystrophy (REMUDY, http://www.remudy.jp) to perform a broader epidemic investigation of associated conditions, including respiratory dysfunction. To clarify the relationship between respiratory dysfunction and other clinical/laboratory factors, we have initiated a prospective observational study on GNE myopathy.

Three of 4 patients with severe respiratory dysfunction had homozygous p.V572L mutations. Given the frequency of the p.V572L mutation in the Japanese population, it will be interesting to determine whether non-Japanese individuals harboring this mutation also exhibit respiratory dysfunction.

In conclusion, advanced GNE myopathy patients are at risk for respiratory dysfunction. The KD/KD genotype, early onset, loss of ambulation/wheelchair use, and low CK level resulted in advanced muscle atrophy may be associated with respiratory dysfunction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nmd.2012.09.007.

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Heterozygous UDP-GlcNAc 2-epimerase and *N*-acetylmannosamine kinase domain mutations in the *GNE* gene result in a less severe GNE myopathy phenotype compared to homozygous *N*-acetylmannosamine kinase domain mutations

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Questionnaire
Natural history

ABSTRACT

Background: Glucosamine (UDP-N-acetyl)-2-epimerase/*N*-acetylmannosamine kinase (GNE) myopathy, also called distal myopathy with rimmed vacuoles (DMRV) or hereditary inclusion body myopathy (HIBM), is a rare, progressive autosomal recessive disorder caused by mutations in the *GNE* gene. Here, we examined the relationship between genotype and clinical phenotype in participants with GNE myopathy.
Methods: Participants with GNE myopathy were asked to complete a questionnaire regarding medical history and current symptoms.
Results: A total of 71 participants with genetically confirmed GNE myopathy (27 males and 44 females; mean age, 43.1 ± 13.0 (mean ± SD) years) completed the questionnaire. Initial symptoms (*e.g.*, foot drop and lower limb weakness) appeared at a mean age of 24.8 ± 8.3 years. Among the 71 participants, 11 (15.5%) had the ability to walk, with a median time to loss of ambulation of 17.0 ± 2.1 years after disease onset. Participants with a homozygous mutation (p.V572L) in the *N*-acetylmannosamine kinase domain (KD/KD participants) had an earlier disease onset compared to compound heterozygous participants with mutations in the uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) 2-epimerase and *N*-acetylmannosamine kinase domains (ED/KD participants; 26.3 ± 7.3 vs. 21.2 ± 11.1 years, respectively). KD/KD participants were more frequently non-ambulatory compared to ED/KD participants at the time of survey (80% vs. 50%). Data were verified using medical records available from 17 outpatient participants.
Conclusions: Homozygous KD/KD participants exhibited a more severe phenotype compared to heterozygous ED/KD participants.

1. Introduction

Glucosamine (UDP-N-acetyl)-2-epimerase/*N*-acetylmannosamine kinase (GNE) myopathy, also known as distal myopathy with rimmed vacuoles (DMRV), Nonaka myopathy (MIM: 605820) or hereditary inclusion body myopathy (HIBM; MIM: 600737), is an early adult-onset, progressive myopathy that affects the tibialis anterior muscle, but spares quadriceps femoris muscles [1,2]. The disease is caused by a mutation in the *GNE* gene, which encodes a bifunctional enzyme [uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) 2-epimerase (GNE) and *N*-acetylmannosamine kinase (MNK)] known to catalyze two rate-limiting reactions involved in cytosolic sialic acid synthesis [3–7]. Mutations in the *GNE* gene result in decreased enzymatic activity *in vitro* by 30–90% [7–10]. Therefore, hyposialylation is thought to

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contribute to the pathogenesis of GNE myopathy. This is supported by the myopathic phenotype associated with a mouse model expressing the human D176V mutant GNE protein (GNE−/−hGNED176V-Tg) [11]. Muscle atrophy and weakness are prevented by oral treatment with sialic acid metabolites in this mouse model [12].

A phase I clinical trial using oral sialic acid therapy has recently been performed in Japan for the treatment of GNE myopathy (ClinicalTrials.gov; NCT01236898). A similar phase I study is currently underway in the United States (ClinicalTrials.gov; NCT01359319). Natural history and genotype–phenotype correlations need to be established for a successful phase II clinical trial for the treatment of GNE myopathy. However, only a small number of studies have been conducted that review the natural course of this disease. In addition, the presence of genotype–phenotype correlations is controversial in GNE myopathy, with most reports denying significant correlations [7]. In fact, substantial heterogeneity is observed among participants who have the same mutations. For example, few subjects with p.D176V and p.M712T mutations exhibited a normal or very mild phenotype, with disease onset after the age of 60 [3,13]. Furthermore, only a limited number of studies that analyze compound heterozygous patients are available. Nonetheless, such studies report a variable degree of severity [14–17].

To clarify the potential relationship between genotype and clinical phenotype (*i.e.*, age at onset, disease course, and current symptoms) of GNE myopathy, we performed a questionnaire-based survey of participants with confirmed GNE myopathy.

2. Participants and methods

2.1. Study population

We obtained approval for this study from the Medical Ethics Committee of the National Center of Neurology and Psychiatry (NCNP). Seventy-eight participants with known GNE myopathy were seen at 8 hospitals specializing in muscle disorders in Japan and 83 participants (not all genetically diagnosed) from the Participants Association for Distal Myopathies (PADM) were recruited. Participants provided written informed consent prior to completing the questionnaire.

A total of 75 participants completed and returned the questionnaire. Of the 75 participants analyzed, 4 were found to have only one heterozygous mutation. Because single heterozygous mutations have not been confirmed to cause GNE myopathy, these 4 participants were excluded from this study.

2.2. Study design

The present study is a retrospective and cross-sectional analysis, which includes 71 participants with genetically confirmed GNE myopathy. Clinical information was collected from participants using a questionnaire and genetic information was acquired from available medical records.

2.3. Questionnaire

Participants completed a self-reporting questionnaire regarding 1) developmental and past symptoms, 2) past and present ambulatory status, and 3) information about diagnosis and medical services (Supplementary material, original version in Japanese).

To determine developmental history, we collected the following information: 1) trouble before and/or during delivery, 2) body weight and height at birth, 3) age at first gait, 4) exercise performance during nursery, kindergarten, or school, and 5) age at onset and signs of first symptoms. Participants were also asked about the onset of 1) gait disturbance, 2) walking with assistance (*i.e.*, cane and/or orthotics and/or handrails), 3) wheelchair use, 4) loss of ambulation, and 5) current gait performance. With regard to medical history, participants were asked about 1) age at the time of first hospital visit, 2) whether or not they had symptoms at the time of visit, 3) age at the time of final diagnosis, 4) how many hospitals/clinics were visited before final diagnosis, and 5) whether a biopsy was performed.

2.4. Medical record examination

To verify the accuracy of the information provided by each participant, available medical records from 17 participants (23.9%) seen at outpatient clinics at NCNP were examined (9 males and 8 females).

2.5. Data handling and analysis

All variables were summarized using descriptive statistics, which included mean, standard deviation (SD), median, range, frequency, and percentage. Each variable was compared against age, sex, genotype, and domain mutation (*i.e.*, within the UDP-GlcNAc 2-epimerase domain: ED or *N*-acetylmannosamine kinase domain: KD). Student's *t* test was used to compare the means for each participant group (ED/ED, ED/KD and KD/KD participants). Data from the two participant groups were calculated using chi-square contingency table analysis. The time from disease onset to walking with assistance, time from disease onset to wheelchair use, and time from disease onset to loss of ambulation were evaluated using the Kaplan–Meier method with log-rank analysis. Questionnaire reliability was tested using intraclass correlation coefficients (ICCs), and two-sided 95% confidence intervals (CIs) were calculated using a one-way random effects analysis of variance model for inter-rater reliability. All analyses were performed using SPSS for Macintosh (version 18, SPSS Inc., Chicago, IL).

3. Results

3.1. General characteristics

A total of 71 Japanese individuals (27 males and 44 females) participated in the study. The mean age at data collection was 43.1 ± 10.7 years. None of the participants showed developmental abnormalities during infancy or early childhood.

3.2. GNE mutations

Forty-one percent of study participants (n = 29/71) had homozygous mutations, while 59% (n = 42/71) had compound heterozygous mutations (Table 1). Among homozygous participants, 86.2% (n = 25/29) harbored the p.V572L mutation, while the remaining participants had other mutations. No homozygous participants for the p.D176V mutation were identified. Among compound heterozygous participants, 28.5% (n = 12/42) had p.D176V/p.V572L mutations, while the remaining participants had other mutations. With respect to allelic frequency, 50.0% (71/142) were p.V572L, 20.4% (29/142) p.D176V, 3.5% (5/142) p.C13S, 2.8% (4/142) p.M712T, and 2.1% (3/142) p.A630T. All other mutations accounted for 2%. A total of 18.3% (n = 13/71) of participants were homozygous with a mutation in the GNE domain (ED/ED), 39.4% (n = 28/71) of participants were compound heterozygous with a mutation in the GNE domain and one in the MNK domain (ED/KD), and 42.3% (n = 30/71) of participants had a mutation in the MNK domain in both alleles (KD/KD).

3.3. Past and present symptoms

Mean participant age at symptom onset was 25.2 ± 9.2 years (range, 12–58 years; median, 24.5 years). There was no significant difference between males and females for current age, age at disease

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Table 1
Genotypes of the GNE myopathy patient population.

		Questionnaire	Outpatients
ED/ED	Total	13	4
	Homozygote	1	0
	p.C13S homozygote	1	
	Compound heterozygote	12	4
	p.C13S/p.M29T	1	1
	p.C13S/p.A631	1	1
	p.D176V/p.F233S	1	1
	p.D176V/p.R306Q	2	
	p.R129Q/p.D176V	1	
	p.R129Q/p.R277C	1	
	p.D27L/p.D176V	1	1
	p.B89S/p.D176V	1	
	p.D176V/p.R246W	1	
	p.D176V/p.R321C	1	
	p.D176V/p.V331A	1	
ED/KD	Total	28	8
	Compound heterozygote	28	8
	p.D176V/p.V572L	12	3
	p.C13S/p.V572L	1	1
	p.D176V/p.I472T	1	1
	p.D176V/p.L603F	1	1
	p.R177C/p.V572L	1	1
	383insT/p.V572L	1	1
	p.D176V/p.G708S	2	
	p.D187G/p.V572L	2	
	p.R8X/p.V572L	1	
	p.D176V/p.G568S	1	
	p.D176V/p.H626R	1	
	p.D176V/p.A630T	1	
	p.I276T/p.V572L	1	
	p.G295D/p.A631V	1	
	p.A600E/p.D176V	1	
KD/KD	Total	30	5
	Homozygote	28	5
	p.V572L homozygote	25	4
	p.M712T homozygote	2	
	p.A630T homozygote	1	
	Compound heterozygote	2	0
	p.V572L/p.R420X	1	1
	1756Gdel (stop)/p.V572L	1	

onset, age at walking with assistance, age at wheelchair use, and current ambulatory status. Initial symptoms included gait disturbance (66.2%, n=47/71), other lower limb symptoms (26.8%, n=19/71), easily fatigued (23.9%, n=17/71), and weakness of hands and fingers (8.5%, n=6/71). In addition, 21.1% (n=15/71) had onset of symptoms before the age of 20. When specifically asked, 47.8% (n=34/71) described themselves as slow runners during childhood, and 42.5% reported having had difficulty with physical exercise during school years.

3.4. Diagnosis

Mean participant age at diagnosis was 33.9±12.6 years (median, 29.5 years; range 17 to 67 years). Mean participant age at first physician visit was 29.6±10.4 years (median, 27 years; range, 12–62 years), and mean time between first visit and diagnosis was 4.4±8.3 years.

3.5. Walking with assistance and wheelchair use

At the time of the survey, 52.0% (n=37/71) were ambulant (41.3±12.8 years); however, only 15.5% (n=11/71, 40.0±13.6 years) could walk without assistance, with the remaining 35.2% requiring assistance (n=25/71, 41.8±12.7 years). Only 7.0% of these participants (n=5/71) could walk up stairs, while 49.3% (n=35/71) were non-ambulant. Wheelchairs were used by 63.6% (23.9% partially bound and 43.7% totally bound) and an electric wheelchair was used by 41.9% (n=31/71). Mean participant age of wheelchair users was 34.9±11.7 years (range, 18–70 years). Wheelchairs were not used by 32.4% (n=26/71) of participants. Current age of wheelchair-free participants was 39.4±12.3 years (range, 21–61 years; median, 34 years) and that of wheelchair-bound participants was 42.8±12.6 years (range, 21–71; median, 42 years).

Kaplan–Meier analysis revealed a median proportional age at walking with assistance of 30.0±1.4 years. Median proportional age of wheelchair users was 36.0±2.7 years, and that for loss of ambulation was 45.0±4.2 years. The time from disease onset to walking with assistance was 7.0±0.4 years, time from disease onset to wheelchair use was 11.5±1.2 years, and time from disease onset to loss of ambulation was 17.0±2.1 years.

3.6. Correlation between disease genotype and phenotype

To determine if a correlation between genotype and phenotype existed, we compared domain mutations (ED/KD, or both) available from medical reports to questionnaire answers (Table 2). Participants with KD/KD mutations (both homozygous and heterozygous) were younger and more severely affected compared to participants with ED/KD or ED/ED mutations. No significant difference in current age or age at disease onset between ED/ED and ED/KD participants was identified. Kaplan–Meier analyses revealed that the proportional time from disease onset to wheelchair use and from disease onset to loss of ambulation was significantly shorter in KD/KD compared to ED/KD participants. ED/ED participants exhibited a shorter time of disease onset to wheelchair use compared to ED/KD participants (Table 3, Fig. 1).

3.7. Comparison between p.V572L homozygous and p.D176V/p.V572L compound heterozygous participants

To compare clinical features in patients with the same mutations, we specifically analyzed data from those with p.V572L (n=25/71, 35.2%) and p.D176V/p.V572L (n=12/71, 16.9%) mutations, as these two were the most frequent mutations in our study population (Table 2). Age at disease onset of homozygous participants (p.V572L) was 21.3±5.7 years (range, 12–32 years) and time from disease onset to wheelchair use was 11.3±5.4 years (range, 3–21 years). Only 16.0% (n=4/25) of these homozygous participants reported that they were not currently using a wheelchair. In contrast, the mean age at disease onset of heterozygous participants (p.D176V/p.V572L) was 35.5±14.1 years (range, 13.5–57 years) and time from disease onset to wheelchair use was 17.9±7.0 years (range, 11–28 years). A total of 66.7% of these compound heterozygous participants (n=8/12) reported that they were not using a wheelchair.

3.8. Questionnaire response compared to medical records

Questionnaires from 17 participants (NCNP outpatient participants) were compared to available medical records (Table 2). Age at disease onset, age at onset of gait disturbance, age at walking with assistance, and age at loss of ambulation were assessed for inter-rater reliability. Age at disease onset, age at onset of gait disturbance, age at walking with assistance, and age at loss of ambulation were assessed for inter-rater reliability. ICC values were 0.979 (95% CI 0.941–0.992) for age at disease onset, 0.917 (95% CI 0.752–0.972) for age at onset of gait disturbances, 0.985 (95% CI 0.949–0.995) for age at walking with assistance, and 0.967 (95% CI 0.855–0.993) for age at loss of ambulation.

4. Discussion

The present study provides a detailed overview of disease severity and progression in 71 Japanese participants with genetically confirmed GNE myopathy. Questionnaire-based surveys have been used to study

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Table 2
Comparison of disease course among genotypes.

		Total	ED/ED	ED/LD	KD/KD
Questionnaire	*n*	71	13	28	30
	Age (years old)	43.1 ± 10.7	44.2 ± 11.2	45.3 ± 13.4	40.6 ± 13.0
	Age at onset (years old)	25.5 ± 9.2	26.3 ± 7.3^{+}	29.8 ± 11.0*	21.2 ± 5.5$^{*,+}$
	Age at walking with assistance	31.8 ± 10.0	34.0 ± 11.1	35.6 ± 10.9*	27.8 ± 6.8*
	Duration from onset to walking with assistance	8.4 ± 6.5	7.5 ± 7.3	9.2 ± 6.5	8.0 ± 6.6
	Wheelchair user (%)	48 (67.8)	10(76.9)	14 (50.0)*	24 (80.0)*
	Wheelchair use since (age)	37.6 ± 8.6	36.4 ± 12.0	43.0 ± 8.7*	31.2 ± 9.3*
	Number of patients with lost ambulation	35 (49.8)	6(46.2)	8 (28.6)*	21 (70.0)*
	Age at lost ambulation	33.6 ± 9.2	31.2 ± 6.0	39.7 ± 9.5	32.1 ± 9.3
	Duration from onset to loss of ambulation	12.2 ± 5.2	9.8 ± 3.5	13.8 ± 6.4	12.4 ± 5.1
NCNP outpatients	*n*	17	4	8	5
	Age (years old)	43.9 ± 14.1	53.5 ± 8.9^{+}	44.3 ± 16.3	35.6 ± 9.2^{+}
	Age at onset (years old)	25.8 ± 9.2	33.4 ± 9.2^{+}	29.6 ± 13.5	19.6 ± 4.2^{+}
	Duration from onset to walking with assistance	7.5 ± 4.2	8.9 ± 5.1	8.1 ± 4.7	5.2 ± 1.5
	Wheelchair user (%)	12 (70.6)	3 (75.0)	4 (50.0)	4 (100)
	Wheelchair use since (age)	33.3 ± 12.6	47.5 ± 17.7	35.2 ± 12.4	25.8 ± 6.3
	Number of patients with lost ambulation	9 (52.9)	3 (75.0)	3 (28.6)*	5 (100)*
	Age at lost ambulation	33.8 ± 9.3	40.0 ± 0.0	39.0 ± 16.5	31.0 ± 8.2
	Duration from onset to loss of ambulation	10.7 ± 4.2	11.2 ± 5.6	11.1 ± 7.8	6.2 ± 2.6

In the questionnaire group, age at onset and age at walking with assistance were significantly younger in KD/KD patients than in ED/KD patients. The number of wheelchair users and patients with loss of ambulation was significantly higher in the KD/KD group than in the ED/KD group. In contrast, with the exception of age at onset, there were no significant differences between ED/ED and ED/KD or KD/KD patients in these clinical parameters. The ED/ED patients were older than the others, and KD/KD patients tended to show the fastest progression.

* $p<0.05$ between ED/KD and KD/KD.

$^{+}$ $p<0.05$ between ED/ED and KD/KD.

the natural disease course of other rare neuromuscular disorders, such as Pompe disease [18] and spinal muscular atrophy type-1 [19]. It is difficult to establish the natural history of such rare disorders using medical records only because patients are typically seen in many different hospitals. In the present study, we used a self-reporting questionnaire and support its use for complementing medical records because it provides a more complete disease overview and establishes specific clinical trends or correlations. Indeed, our questionnaire demonstrates excellent inter-rater reliability against medical records and yields several findings regarding differences in disease progression among genetically distinct, GNE myopathy participants.

Only 15.5% of participants could walk and 7.0% could walk up stairs without assistance, which reflects the fact that GNE myopathy patients often require canes and/or leg braces at an early disease stage. This indicates that traditional six-minute walk or four-step walking tests often used to evaluate muscular dystrophies or myopathies can only be applied in a very limited number of cases, such as natural disease course studies or clinical trials. Therefore, alternate evaluation tools are required, which should include functional measurements that can be completed without canes or braces. For example, the Gross Motor Function Measure is a useful tool for evaluating mildly and severely affected patients [20].

The male to female ratio in our study population (27 males and 44 females) was skewed from the expected ratio for autosomal recessive inheritance. However, the male to female ratio of the 17 NCNP outpatient participants was 9:8. One possible explanation for the observed sex ratio in our study population is that female participants tend to be more enthusiastic toward questionnaire-based and/or PADM activities. There was no significant difference in age at survey and age at disease onset between male and female participants. However, in a mouse model of GNE myopathy, weight loss and muscle atrophy were more pronounced and occurred earlier in females compared to males [11].

We showed that KD/KD mutations are associated with a more severe phenotype compared to ED/KD mutations. Indeed, KD/KD participants had an earlier disease onset, a more rapid and progressive disease course, and a shorter time from disease onset to loss of ambulation. This was also observed in the 17 NCNP outpatient participants analyzed in our study. In contrast, ED/ED participants did not show significant differences across disease course parameters analyzed except for an earlier and later age at disease onset compared to ED/KD and KD/KD participants, respectively. Thus, ED/ED participants appear to have a disease severity intermediate between ED/KD and KD/KD participants. One possible explanation is that the major mutation, p.V572L, may be associated with a more severe phenotype. In general, the reasons for this earlier onset and disease progression remain unknown. Jewish GNE myopathy patients with homozygous p.M712T mutations have a milder phenotype compared to Japanese patients, as most of their quadriceps are spared and they usually become wheelchair-bound 15 years or more after disease onset [13,21]. Our study population included two women with homozygous p.M712T mutations: a 38 year-old ambulant and a 35 year-old non-ambulant participant. Although the two participants had a slightly later disease onset (ages 23 and 27 years, respectively) compared to KD/KD participants, the difference was not significant.

An asymptomatic patient with a p.D176V homozygous mutation was previously reported [3]. The study suggested that p.D176V homozygous patients may show a mild or late disease onset phenotype. The results presented here may support this observation as no p.D176V homozygous participants were present in our study

Table 3
Inter-rater reliability of the questionnaire.

	Onset	Age of gait disturbance	Age of gait with help	Age at loss of ambulant
Number of patients	17	17	13	9
ICC (95% CI)	0.979 (0.941–0.992)	0.917 (0.752–0.972)	0.985 (0.949–0.995)	0.967 (0.855–0.993)
p	0.000	0.000	0.000	0.000

Age at onset, age at onset of gait disturbances, age at walking with assistance, and age at loss of ambulation were assessed in a subgroup of 17 outpatients to evaluate the inter-rater reliability of the questionnaire.

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