electrophoretic mobility, reflecting increased sialic acid content [21]. Among the three participants with decreased platelet counts, two had ITP, raising the possibility of a causal relationship between GNE mutations and ITP, although the underlying mechanisms are unclear and further studies would be necessary to address this issue. Of note, however, this information was obtained based on self-report by patients and/or their families. Thus, the accuracy of the diagnosis is unclear.

It is noteworthy that in some patients, initial symptoms were difficulty lifting heels, but not toes. It is conventionally thought that the initial symptom of GNE myopathy is "foot drop," as tibialis anterior muscles are strikingly affected. Our study suggests that patients whose symptoms start with calf muscle weakness may have GNE myopathy. It is also surprising that some patients had neck and finger weakness from disease onset, despite GNE myopathy being known as "distal myopathy." Thus, GNE myopathy appears to be associated with more phenotypes than expected. However, we are not confident that all patients who chose "difficulty lifting heels" exhibited prominent calf weakness in reality as well, i.e., they experienced greater calf weakness relative to tibialis anterior muscle weakness. This is one limitation of using medical histories and a registration system to collect patient data.

Although we previously reported respiratory dysfunction associated with GNE myopathy [15], 35% of participants in the present study were not examined for respiratory function, indicating that many physicians and neurologists are unaware of the clinical significance of respiratory function in the context of this disease. Although we did not observe any cases of cardiac dysfunction, it may occur in older patients or those with advanced disease. Supporting this is evidence from a study showing that 20% of GNE myopathy mice develop fibrosis in cardiac tissue after 30 weeks of age, with some exhibiting marked endomysial fibrosis, amyloid deposition, and occasionally rimmed vacuoles in cardiomyocytes [8]. This suggests that the risk of cardiopulmonary dysfunction in GNE myopathy should be considered.

In this study, four participants harbored single heterozygous mutations, although they exhibited clinicopathologically definite findings of GNE myopathy. The age at disease onset did not significantly differ between homozygotes and compound heterozygotes. Given that we limited our analysis to all exons and their flanking introns, it is possible that single heterozygotes who exhibited features of GNE myopathy may have mutations in other genomic regions of *GNE*. Yet, in the absence of data using disease-specific biomarkers, it is difficult to distinguish whether these participants had other myopathies and carried a single heterozygous mutation in the *GNE* gene.

Among registry participants, 46% were in the abnormal range of BMI, and the number of underweight participants was markedly higher in both men and women, compared to the normal population. None of the participants or patients of NCNP had dysphagia or other medical problems which might promote weight loss. The BMI of non-ambulant patients tended to be higher than ambulant patients, suggesting that muscle atrophy itself did not cause weight loss. Mechanisms underlying the weight changes may differ from those observed in muscular dystrophy such as DMD [22] or myotonic dystrophy [23], given that obesity was not an issue with most patients with GNE myopathy. It is not clear whether being underweight is beneficial relative to having normal weight in these patients. Prospective analyses will be needed to reveal the relationship between motor function prognosis and body weight.

This study has some limitations worth noting. First, we could not unify the method of grip power assessment. Second, we relied on descriptions of motor function as a crude benchmark for designing clinical trials. Finally, we could not address phenotype-genotype correlations in more depth than was previously reported [9], given the limited number of homozygote patients harboring mutations other than V572L. A larger cohort will be needed to address genotype-phenotype correlations. Similar to our collaborations involving the dystrophinopathy registry, we are currently in discussions to harmonize the international registry of GNE myopathy of the TREAT-NMD ALLIANCE [ClinicalTrials.gov Identifier NCT01784679, http://www.treat-nmd.eu/gne/patient-registries/international-registry/ *webcite*] (GNE-DMP), in hopes of gaining further insights into the disease. There are two major differences between GNE-DMP. First, as the Remudy aims to establish registration according to genetic diagnosis, inclusion criteria for genetics-based longitudinal natural history studies employing the Remudy-GNE registry require genetic diagnosis (including single heterozygote). Second, we are the only Japanese language registry system. Japan has one of the largest patient groups with GNE myopathy in the world [24]. It is important that patients with this disease receive information in their native language, and that domestic information is supplied for the purpose of Japanese patient accession. Harmonisation would be conducted in order to avoid duplication and double registration of GNE patients while providing the same benefits and opportunities to patients, regardless of where they live. Both registries are similar in their processes utilized for data collection as well as their fundamental ideas regarding the registries, and thus we hope to merge the two registries at some point. According to a tentative agreement, the Remudy-GNE will remain the primary entryway into the international registry as well as serve as the contact site for Japanese patients, and only anonymous data will be stored in the joint data set. Strategies for merging the two registries are currently under consideration.

Our Japanese registry and the TREAT-MND ALLIANCE registry work in close collaboration, and will serve as irreplaceable infrastructures that accelerate research, therapy development, and trial readiness, in addition to increasing opportunities for collaboration and improving global patient care.

5 Conclusion

The patient registry for GNE myopathy in Japan is useful for gaining a better understanding of the disease, and recruiting patients with genetically-confirmed GNE myopathy for upcoming clinical trials. Further advances and insights can be expected through a soon-to-be-launched international GNE myopathy registry.

Abbreviations

GNE: Glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase

NCNP: National Center of Neurology and Psychiatry

REMUDY: Registry of muscular dystrophies

DMRV: Distal myopathy with rimmed vacuoles

UDP-GlcNAc: Uridine diphosphate-*N*-acetylglucosamine

TREAT-MND ALLIANCE: Translational Research in Europe–Assessment and Treatment of Neuromuscular Diseases

SD: Standard deviation

ITP: Idiopathic thrombocytopenia

FVC: Forced vital capacity

NPPV: Non-invasive positive pressure ventilation

EF: Ejection fraction

FS: Fraction shortening

CK: Creatine kinase

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

MMY drafted/revised the manuscript, conceived of the study, participated in its design, performed data acquisition, data analysis and interpretation, and statistical analysis, and supervised the study. NY conceived of the study, participated in its design, and performed the statistical analysis. YKH drafted/revised the manuscript, performed genetic analysis, and supervised the study. IN conceived of the study, participated in its design, and carried out the genetic analysis. MM drafted /revised the manuscript and supervised the study. EK, HN, and ST conceived of the study and supervised the study. All authors read and approved the final manuscript.

Additional files

Additional file 1: Table S1.. Genotyping. ED = Glucosamine (UDP-N-acetyl)-2-epimerase domain, KD = N-acetylmannosamine kinase domain. There were more participants who were either homozygous for p.V572L or heterozygous for p.D176V/p.V572L compared to those with other mutations. Although p.D176V was the second most frequent mutation, there was only one participant in the registry who was homozygous for p.D176V.

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Additional file 2: Table S2.. Allelic frequency. p.V572L was the most frequent mutation.

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Additional file 3: Figure S1.. Muscle CT of 29 year-old GNE myopathy patient who reported difficulty lifting his heels as one of the first symptoms. Ankle plantar flexion (MMT 2) was prominently impaired (MMT5), and muscle CT revealed that fatty replacement and atrophy were far more prominent in the calf than the anterior part of the lower legs.

Format: TIFF Size: 17.3MB Download file

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RESEARCH Open Access

Immunoreactivity of valosin-containing protein in sporadic amyotrophic lateral sclerosis and in a case of its novel mutant

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Abstract

Background: Mutations in the *valosin-containing protein (VCP)* gene were first found to cause inclusion- body myopathy with early-onset Paget disease and frontotemporal dementia (IBMPFD). Mutations in the *VCP* gene were later reported to occur in familial amyotrophic lateral sclerosis (ALS). But the role of VCP in the neurodegenerative processes that occur in ALS remains unknown. The purpose of the present study was to elucidate the role of VCP in the neurodegeneration seen in sporadic and *VCP* mutant ALS.

Results: Immunohistochemistry demonstrated that the frequency of distinct VCP-positive nuclei of spinal motor neurons of patients with sporadic ALS (SALS) and the ALS with *VCP* novel mutation (ALS-VCP, M158V) was increased, compared with that of the control cases. No VCP-positive inclusion bodies were observed in SALS patients, a ALS-VCP patient or in control subjects. Neuropathologic examination of the ALS-VCP case showed loss of motor neurons, the presence of Bunina bodies, and degeneration of the corticospinal tracts. Bunina bodies detected in this case were confirmed to show immunohistochemical and ultrastructural features similar to those previously described. Furthermore, neuronal intracytoplasmic inclusions immunopositive for TAR DNA-binding protein 43 kDa (TDP-43), phosphorylated TDP-43, ubiquitin (Ub), p62, and optineurin were identified in the spinal and medullary motoneurons, but not in the neocortex. Gene analysis of this ALS-VCP patient confirmed the *de novo* mutation of M158V, which was not found in control cases; and bioinformatics using several *in silico* analyses showed possible damage to the structure of VCP. Immunocytochemical study of cultured cells showed increased cytoplasmic translocation of TDP-43 in cells transfected with several mutant *VCP* including our patient's compared with wild-type VCP.

Conclusion: These findings support the idea that VCP is associated with the pathomechanism of SALS and familial ALS with a *VCP* mutation, presumably acting through a dominant-negative mechanism.

Keywords: Amyotrophic lateral sclerosis, Paget disease of bone, Valosin-containing protein (VCP), Inclusion body myopathy with early-onset Paget disease and frontotemporal dementia (IBMPFD), TAR DNA-binding protein 43 kDa (TDP-43), Golgi apparatus fragmentation

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Introduction

Valosin-containing protein (VCP) is a ubiquitous member of the AAA-ATPase supergene family. VCP is known to play an important role in cellular activities including ubiquitin (Ub) -dependent protein degradation [1], chromatin-associated protein degradation [2], messenger ribonucleic acid (mRNA) metabolism [3], autophagy [4], anti-apoptotic function [5], and post-mitotic Golgi apparatus reassembly [6]. Mutations in the *VCP* gene were first found to cause inclusion-body myopathy with early-onset Paget disease and frontotemporal dementia (IBMPFD) [7,8]. IBMPFD is an autosomal dominantly inherited disorder with variable penetrance of 3 predominant phenotypic features, i.e., myopathy, Paget disease of bone, and frontotemporal dementia (FTD) [9]. The penetrance of the gene is 82% for myopathy, 49% for Paget's disease, and 30% for FTD [10]. IBMPFD patients with *VCP* mutations can develop disorders in other organ systems including sphincters [11], cardiac muscles [12], auditory system [13], and liver [14], as well as in visuoconstructive ability [14]. Neurodegenerative diseases associated with a *VCP* mutation encompass scapuloperoneal muscular dystrophy and dropped head syndrome [15], Parkinson's disease [16-18], hereditary spastic paraplegia [19], and cerebellar ataxia [20]. In addition to mutations in *VCP* [7,8], mutations in *Heterogeneous Nuclear Ribonucleoprotein A2B1* (*HNRNPA2B1*) and *Heterogeneous Nuclear Ribonucleoprotein A1* (*HNRNPA1*) [21] have been identified in families with IBMPFD. Given these observations, the name of multisystem proteinopathy (MSP) has been proposed, using the nomenclature of MSP1 for IBMPFD caused by a *VCP* mutation, MSP2 for IBMPFD related to an *HNRNPA2B1* mutation, MSP3 for IBMPFD related to an *HNRNPA1* mutation, and MSP4 for IBMPFD due to some unidentified gene [22]. Clinically, 37.7% patients of IBMPFD with a *VCP* mutation (MSP1) develop FTD [9]. FTD cases with a *VCP* mutation (MSP1) present with TAR DNA-binding protein 43 kDa (TDP-43) and ubiquitin-positive short dystrophic neurites and frequently lentiform neuronal intranuclear inclusions in their neocortex [23-25]. On the other hand, only rare VCP-positive neuronal intranuclear inclusions are detected, and those that are detected lack the characteristic lentiform morphology [23]. This finding suggests that TDP-43 and ubiquitin positive-inclusions do not contain VCP and supports the idea that *VCP* gene mutations in IBMPFD produce a dominant-negative loss of VCP function [23,25].

Mutations in the *VCP* gene were later reported to occur in familial amyotrophic lateral sclerosis (ALS) [26]. A recent study with a large data set of patients with *VCP* mutations showed that 8.9% of these patients developed ALS [27]. Features of ALS with *VCP* mutations (ALS-VCP) are similar to those of sporadic ALS (SALS), including bulbar signs, spasticity, hyperreflexia, fasciculations, and electrophysiological evidence of lower motor neuron involvement such as denervation and reinnervation [27]. The neuropathology of ALS-VCP has been briefly described in 2 cases to date [26,28]. Both reports described TDP-43-positive intracytoplasmic inclusions in the motor neurons. These facts suggest that the neuropathologic features of ALS-VCP could be similar to those of SALS and that SALS would share its pathogenic role with ALS-VCP cases through dysfunction of VCP. To confirm this hypothesis, it is of importance to elucidate detailed pathological features of ALS-VCP and to compare them with those of SALS. However, specific inclusion pathology including their immunohistochemical properties and distributions, as well as detailed cytopathology of motor and non-motor neurons and glial cells, remain to be explored.

VCP immunoreactivity has been observed in Lewy bodies in Parkinson's disease and dementia with Lewy bodies, in neuronal nuclear inclusions in polyglutamine diseases and intranuclear inclusion body disease, in Marinesco bodies [29], and in epidermal cells from patients with SALS [30]. VCP-positive nuclei have also been reported in neocortical neurons [31] and in muscle cells [20] from IBMPFD cases with a *VCP* mutation (MSP1). However, the distribution of VCP in ALS-VCP cases is unknown. Moreover, it remains to be investigated whether a *VCP* mutation leads to VCP-positive inclusions in the ALS phenotype, and whether VCP-positive structures are present in the motor neurons of SALS patients.

To elucidate the role of VCP in neurodegenerative processes in ALS, in the present study we examined the neuropathology of a patient with ALS and Paget disease of bone with a novel *VCP* mutation, as well as the immunohistochemical localization of VCP in SALS cases and in the ALS-VCP patient.

Materials and methods

VCP immunohistochemistry in ALS

We investigated specimens from lumbar spinal cord, hippocampus, and the motor cortex from 9 patients with pathologically confirmed SALS (age range, 54–82 years; mean, 63.2 years; 6 men and 3 women), 8 control subjects (age range, 50–86 years; mean, 69.5 years; 6 men and 2 women), and 1 patient with ALS associated with a novel *VCP* mutation. The clinical profiles of all of these cases are summarized in Table 1. Among SALS cases, there was no familial case and no history of IBM, Paget disease or FTD.

For VCP immunohistochemistry, formalin-fixed, paraffin-embedded 6-μm-thick sections were deparaffinized and immunostained for VCP. We applied 2 distinct primary antibodies for VCP(mouse monoclonal [Cat. No. ab11433], Abcam plc, Cambridge, UK; 1:500; and rabbit polyclonal [Cat. No. AP6920b], Abgent, San Diego, California, USA; 1:75). Bound antibodies were detected with the appropriate VECTASTAIN Elite ABC Kit (Vector Laboratories, Burlingame, California, USA).

Table 1 Clinical findings of patients with amyotrophic lateral sclerosis and of control subjects

Case no.	Age at death (years)	Gender	Diagnosis	Postmortem delay (hours)	Duration of illness (months)	Percentage of VCP-positive nuclei
Control cases						
1	75	M	Chronic lymphocitic leukemia	2.5	NA	0
2	75	M	Cerebral infarction	1	NA	0
3	86	M	Cerebral infarction	5	NA	0
4	75	M	Meningitis	3	NA	1.7
5	83	F	Intracerebral hemorrhage	12	NA	4
6	52	M	Cerebral infarction	12	NA	8.9
7	50	F	Myotonic Dystrophy	10	NA	0
8	60	M	Myasthenia Gravis	2.5	NA	0
ALS cases						
9	54	F	SALS	3.5	11	26.3
10	82	M	SALS	1.5	14	0
11	62	M	SALS	1.5	14	0
12	56	F	SALS	22.5	20	0
13	65	M	SALS	1.5	23	8.3
14	63	M	SALS	1.5	23	15
15	64	M	SALS	3	24	17.6
16	65	M	SALS	16	24	94.1
17	58	F	SALS	3.5	35	47.1
18	41	M	VCP-ALS	2	48	7.7

M male, *F* female, *VCP* Valosin-Containing Protein, *SALS* sporadic amyotrophic lateral sclerosis, *VCP-ALS* ALS with M158V VCP mutation.

We assessed staining specificity by replacing the primary antibodies with an appropriate amount of phosphate-buffered saline solution containing 3% bovine serum albumin. No deposits of reaction products were seen in the sections thus treated. Procedures involving the use of human material were performed in accordance with ethical guidelines set by Kyoto University.

For quantitative study of VCP-positive nuclei of the motor neurons, we obtained 3 distinct sections from each case, taken at an interval of 30 μm, to avoid counting the same nucleus twice. All observations were made by 2 examiners who had no information regarding the clinical history and condition of the individual providing the spinal specimen. Only anterior horn cells (AHCs) with a distinct nucleus and nucleolus were counted. The frequency of VCP-positive motor neurons in the lumbar cord was compared between control patients and SALS patients by the nonparametric Mann–Whitney U test using Prism software (GraphPad, La Jolla, USA).

Clinical features of the ALS patient with a *VCP* mutation

Our patient was a 41-year-old Japanese man without any family history of ALS, IBM, Paget disease or FTD (Figure 1a). He had developed right arm and leg weakness at age 36. Based on the results of elevated alkaline phosphatase up to 2,968 IU/l, bone radiographs, and electromyography, he was diagnosed at age 37 as having ALS and Paget disease of bone. He gradually developed weakness of arms and legs, dysarthria, dysphagia, and respiratory failure, which required percutaneous endoscopic gastrostomy and noninvasive positive-pressure ventilation at age 38. He was referred to our hospital at age 39. Examinations disclosed weakness in all extremities, muscle atrophy in the distal arms, and fasciculation in the right leg. Atrophy or fasciculation of the tongue was not evident. Patellar tendon reflexes were brisk, but Achilles tendon reflexes and those in the upper limbs were decreased. Neither pathologic reflexes nor clonus was observed in the extremities. Neuropsychological tests showed no evidence of FTD. A computed tomography (CT) scan showed systemic osteolytic change, but no atrophy of the brain. Needle electromyography (EMG) showed active and chronic denervation potential including spontaneous discharges and late recruitment in the left arm and leg. A frozen muscle specimen from the right quadriceps muscle at this time showed neurogenic changes without evidence of IBM. Base on the revised El Escorial criteria [32], the patient was diagnosed as clinically possible ALS.

His extremities gradually became weaker, resulting in a bedridden condition. Shortly after he developed a fracture of the right femoral bone, he died of respiratory failure at the age of 41. Throughout the clinical course he did not

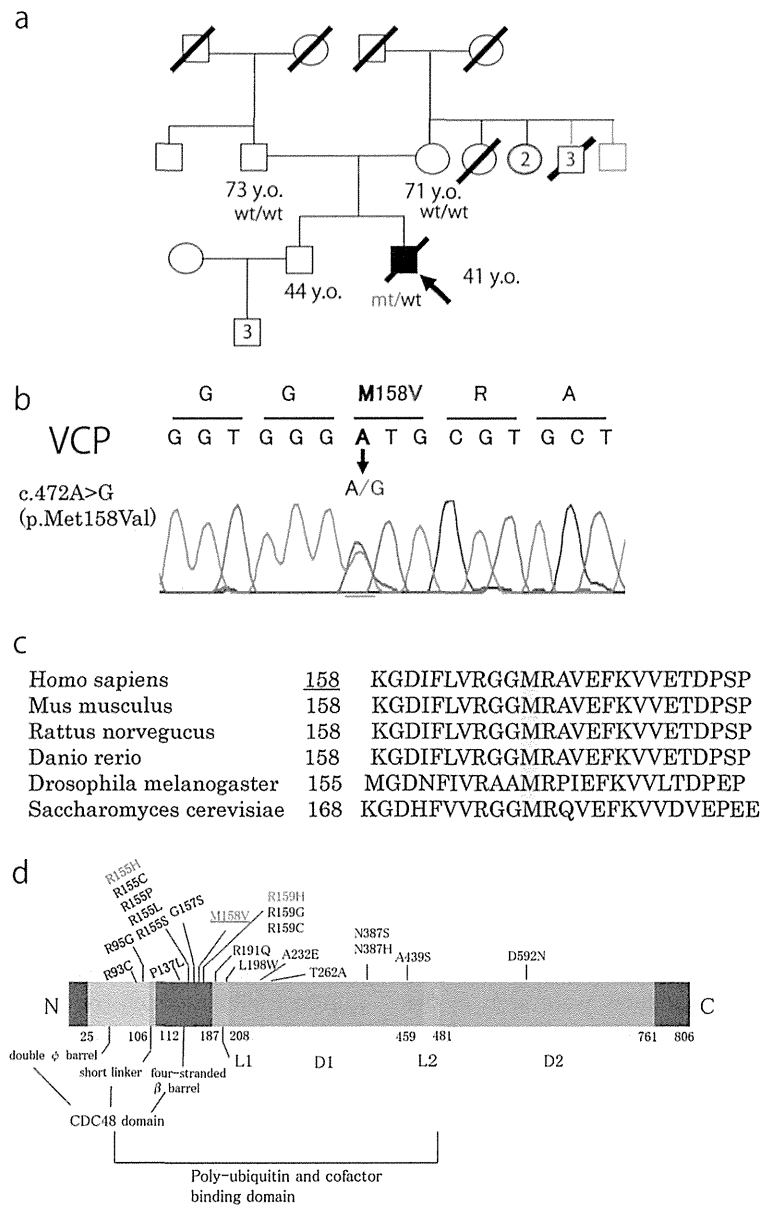

Figure 1 Genetic information. (a) The presented case had no family history of ALS, dementia, myopathy or Paget disease. Family members with diagonal black lines had already died. The ages indicated are those at the time of the proband's death. **(b)** The proband carried the missense mutation of the *VCP* gene (c. 472A > G, p.M158V). Neither the father nor the mother carried the *VCP* mutation identified in the proband. **(c)** Sequence alignment for amino acids revealed the mutation site to be conserved across different species. **(d)** Domain structure of VCP protein and mutation site in this case and in reported mutations (Mutations with autopsy are shown in red type) associated with ALS and IBMPFD.

develop any cognitive decline or mood disorder, Parkinsonism, spastic paraplegia, cerebellar ataxia or decubitus ulcer. Also, the patient did not have any abnormalities in cardiac, hepatic, visual, auditory, sensory or autonomic systems. No frozen brain tissue was available.

Genetic analysis

Genetic analysis of *VCP, Cu/Zn superoxide dismutase 1 (SOD1), TDP-43, fused in sarcoma (FUS), Charged multivesicular body protein 2b (CHMP2B), angiogenin, Sequestosome 1 (SQSTM1), and chromosome 9 open reading frame 72 (C9ORF72)* was approved by either the Kyoto University Graduate School of Medicine Ethical Committee or Hiroshima University Ethical Committee, and written informed consent was obtained from the above patient prior to his demise. DNA was extracted from a blood sample; and by using previously reported primers, we amplified the exons of each gene and sequenced them on anABI310 sequencer (Applied Biosystem, Foster City, California, USA). To detect *C9ORF72*

expansion, we performed a repeat-primed polymerase chain reaction, as reported previously [33]. As a result we identified a novel heterozygous M158V mutation in the *VCP* gene (Figure 1b). This mutation was not present in the 1000 Genomes project (http://www.1000genomes.org/), dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) or in the Human Genetic Variation Browser (includes genetic variations determined by exome sequencing of 1,208 Japanese individuals and genotyping data of common variations obtained from a cohort of 3,248 individuals [http://www.genome.med.kyoto-u.ac.jp/SnpDB/]). The other genes were normal. Conservation analysis of the mutated amino acid by using National Center for Biotechnology (NCBI) HomoloGene demonstrated conservation among species at the mutation site (Figure 1c). The mutation site in this case (amino acid 158 in VCP) was located near the reported mutation sites (amino acids 155 [26] and 159 [28] in VCP) within the same domain (Figure 1d). In Figure 1d, mutation sites in *VCP* gene associated with ALS and IBMPFD are also listed from the literature [8,9,13-15,26,28,31,34-39].

His parents were still alive and not affected with ALS or any other neurodegenerative diseases. His father was 73 years of age and had been diagnosed with prostate cancer, whereas his mother was 71 years old and healthy. Neither parent carried the *VCP* mutation identified in the proband. Haplotype analysis using 15 short tandem repeat markers supported their genetic kinship, confirming that the mutation occurred *de novo* in the present case. No sample from the patient's brother was available.

Neuropathologic examinations

For neuropathologic examinations, formalin-fixed, paraffin-embedded 6-μm-thick sections were deparaffinized and stained with hematoxylin and eosin (H&E) or used for Klüver-Barrera (KB) staining. For immunohistochemistry, after antigen retrieval by heat/autoclaving (10 min at 121°C in 10 mM anhydrous citric acid buffer, pH 6.0), the sections were immunostained as described above.

The antigens recognized by the primary antibodies used in this study were the following: TDP-43(rabbit polyclonal [Cat No. 10782-2-AP], ProteinTec Group Inc., Chicago, Illinois, USA; 1:200), phosphorylated TDP-43 (mouse monoclonal [Cat No. TIP-PDT-M01], Cosmo Bio Co., Ltd., Tokyo, Japan; 1:2000), trans-Golgi-network (sheep polyclonal [Cat No. 610832], Novus Biologicals, Inc, Littleton, Colorado, USA; 1:250), VCP(mouse monoclonal [Cat No. ab11433], Abcam plc, Cambridge, UK; 1:500; rabbit polyclonal [Cat No. AP6920b], Abgent, San Diego, California, USA; 1:75), FUS(rabbit polyclonal [Cat No. HPA008784], Sigma-Aldrich Inc., St. Louis, Missouri, USA; 1:100), CD68 (mouse monoclonal [Cat No. M0814], DAKO, Glostrup, Denmark; 1:100), p62(mouse monoclonal [Cat No. 610832], Becton Dickinson and Company, Franklin Lakes, New Jersey, USA; 1:700), ubiquitin(rabbit polyclonal [Cat No. U5379], Sigma-Aldrich Inc., St. Louis, Missouri, USA; 1:100), and optineurin (rabbit polyclonal [Cat No. 100000], Cayman Chemical Company, Ann Arbor, Michigan, USA; 1:200).

Electron microscopy

In the case with the *VCP* mutation, the sample from formalin-fixed medulla was fixed in 2% glutaraldehyde with phosphate buffer (pH 7.4). After fixation, sections were cut into pieces about 1 mm thick, postfixed with 1% osmium tetroxide for 2 h, dehydrated, and embedded in epoxy resin. Each block was then cut into semithin sections about 1 μm in thickness and stained with toluidine blue. Appropriate regions were subsequently cut into ultrathin sections and stained with uranyl acetate and lead citrate for electron microscopy.

Proteomics analysis

The effect of the newly detected missense mutation (M158V) and previously reported mutations of 2 autopsied ALS-VCP cases (R155H and R159H) was analyzed with Mutation Taster (http://www.mutationtaster.org), Sorting Intolerant From Tolerant (SIFT, http://sift.bii.a-star.edu.sg/), and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph/).

Vectors for *in vitro* analysis

To assess the functional importance of the novel mutation identified in this study, we created vectors (pcDNA3) expressing wild-type (WT) *VCP* and mutant *VCPs* (R155H, M158V, R159H and A232E) tagged with FLAG at their N-terminus.

Cultured cells

SH-SY5Y and human embryonic kidney 293 T (HEK293T) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 1% penicillin-streptomycin-L glutamine sodium.

Plasmids

To generate FLAG-tagged VCP, we obtained WT VCP complementary deoxyribonucleic acid (cDNA) from a cDNA library and generated sequence variants, including R155H (464G > A), M158V (472A > G), R159H (476 G > A) and A232E (695C > A). WT and mutant VCPs were generated by PCR with primer pairs used to change the nucleotides and then inserted into the Not I/BamH I cloning site of the pcDNA3 vector. All constructs were verified by using the 3130xl Genetic Analyzer (Life Technologies, Carlsbad, California, USA. Cultured cells were transfected with the vector by using a FuGene HD transfection kit (Roche, Basel, Switzerland) according to the manufacturer's protocol.

Immunoblot analysis

Cells were lysed in lysis buffer containing 10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate (Na-DOC), 0.1% sodium dodecyl sulfate (SDS), and a protease inhibitor cocktail (Complete EDTA-free protease inhibitor; Nacalai Tesque). Nuclei and membrane fractions were removed by centrifugation. Lysates were separated by SDS-poly-acrylamide gel electrophoresis (SDS-PAGE), and proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with the appropriate primary antibody, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology Inc, Dallas, Texas, USA) secondary antibody. Immunoreactive proteins were visualized by using the Pierce ECL Western Blotting Substrate (Thermo Scientific Pierce, Kanagawa, Japan) and an LAS3000 scanning system (Fuji Film, Tokyo, Japan). The following primary antibodies were used in the immunoblot analysis: anti-VCP (rabbit polyclonal [Cat No. AP6920b], Abgent, San Diego, California, USA; 1:200) and anti-β-actin (mouse monoclonal [Cat No. A5441], Sigma-Aldrich Inc., St. Louis, Missouri, USA; 1:2,000).

Immunocytochemistry

SH-SY5Y and HEK293T cells transfected with FLAG-conjugated WT or mutant *VCP* were fixed and immunostained with anti-TDP-43 or anti-FLAG, and stained with DAPI, at 48 h post-transfection. For immunofluorescence analysis of cell cultures, cultured cells were fixed with 4% PFA for 20 min, washed 3 times with PBS (2 min each), and then rendered permeable and blocked with 0.2% Triton X-100/5% goat serum in PBS for 15 min. The transfected cells were incubated with primary antibodies (anti-TDP-43 antibody, rabbit polyclonal, ProteinTech, 1:1000 and anti-FLAG M2, mouse monoclonal, Sigma-Aldrich Inc., St. Louis, Missouri, USA; 1:500, diluted in PBS containing 0.2% Triton X-100/5% goat serum) overnight and washed 3 times with PBS (5 min each). After the final wash, the cells were incubated with secondary antibodies (Alexa Fluor 488 donkey anti-rabbit IgG (H + L) or Alexa Fluor 546 donkey anti-mouse IgG (H + L), diluted in PBS containing 0.2% Triton X-100/5% goat serum) for 1 h, washed 3 times with PBS (5 min each), and mounted with Vectashield plus DAPI (Vector Laboratories, Burlingame, California, USA). Digital imaging was performed with an OLYMPUS FV-100 IX microscope (Olympus, Tokyo, Japan) using FV-10-ASW 3.1 software (Olympus). A blinded examiner counted more than 100 FLAG-tagged VCP-expressing cells from separate cultures for the presence of cytoplasmic TDP-43-positive cells. Results were analyzed by One-way ANOVA of Bonferroni's multiple-comparison test using Prism software (GraphPad, La Jolla, USA).

Results

Immunohistochemistry for VCP in control subjects, SALS patients, and the *VCP* mutant case

Two distinct anti-VCP antibodies applied in the present study gave the same results.

In the 8 controls, VCP-positive nuclei or cytoplasm was rarely observed in the AHCs investigated (Figure 2a); only a few nuclei of AHCs showed faint immunoreactivity against VCP (Figure 2a inset). Glial nuclei were virtually not stained in these subjects (Figure 2b). In contrast, AHCs from 9 SALS cases frequently showed positive VCP immunoreactivity in the cytoplasm and in the nucleus (Figure 2c). The staining intensity of neuronal nuclei of the SALS cases was much greater than that in the control subjects. Within the cytoplasm, the deposits of immunoreaction product were diffusely distributed, but this antibody recognized no intranuclear inclusion, skein-like inclusion, round hyaline inclusion or Bunina body. Moreover, the glial nuclei were also stained in the cases with SALS (Figure 2d). However, VCP-positive glial cytoplasmic inclusions (GCIs) were not observed.

We also identified some neuronal (Figure 2e) and glial (Figure 2f) nuclei that were immunoreactive for VCP in the ALS case with the heterozygous M158V *VCP* mutation (case 18 in the Table). In the ALS-VCP case, VCP immunoreactivity in the AHCs was less robust than that in the SALS cases. No VCP-positive inclusions were detected in any of the cases investigated.

In the motor cortex and hippocampus, there was no significant immunoreactivity for VCP except for faint immunoreactivity of glial nuclei.

The frequency of VCP-positive nuclei in the lumbar cord of patients with SALS (mean ± standard deviation, 26.1 ± 31.5) was significantly higher ($p < 0.05$, Mann–Whitney U test) than that for the control subjects (1.8 ± 3.2%; Figure 2g). In the ALS-VCP case 7.7% of the AHC nuclei were VCP positive. On serial section analysis, there was no apparent relationship between VCP immunoreactivity and intracytoplasmic TDP-43 accumulation.

Neuropathologic examinations of the patient with M158V ALS-VCP

Frozen muscle specimens from the right quadriceps muscle at age 39 showed neurogenic changes without evidence of IBM (Figure 3a). In the immunohistochemical investigation, TDP-43 was normally detected in the muscle nuclei, which showed no abnormal inclusions (Figure 3b). Immunostaining for neither VCP nor OPTN revealed any abnormal inclusions.

At autopsy of the M158V ALS-VCP patient, pneumonia, fatty liver, and atheromatous plaques of the aorta were recognized. Microscopic examination of the vertebra revealed mixed osteoclastic-osteoblastic activity and a chaotic picture of trabecular bone ("mosaic" pattern)

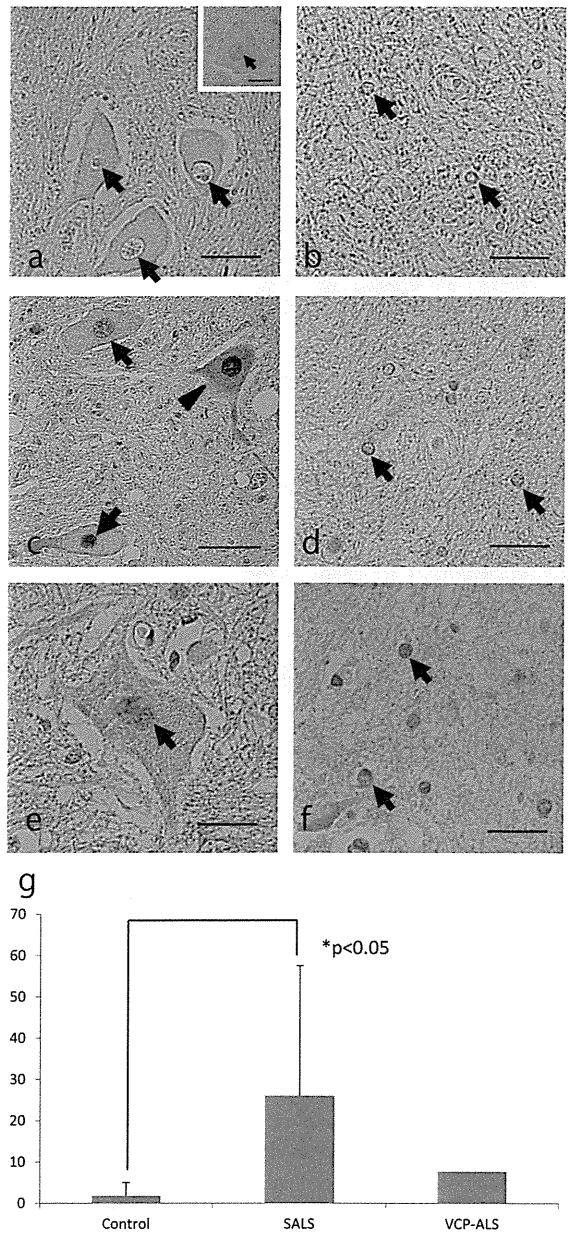

Figure 2 Immunohistochemistry of VCP in the spinal cords. (a,b) In the control case, neuronal (**a**, *arrows*) and glial (**b**, *arrows*) nuclei are devoid of immunoreactivity. The nucleus of this AHC (**a**, inset, arrow) in the control case was stained faintly with the anti-VCP antibody. **(c,d)** In the ALS cases the same antibody distinctly immunolabels nuclei of the AHCs (**c**, *arrows*) and those of the glial cells (**d**, *arrows*). The cytoplasm of the indicated AHC (**c**, *arrowhead*) is also stained diffusely. **(e,f)** In the ALS-VCP patient a nucleus of an AHC (**e**, *arrow*) and glial nuclei (**f**, *arrows*) give positive staining with the anti-VCP antibody. **(g)** The frequency of VCP-positive neuronal nuclei in the lumbar cord of the patients with SALS (mean ± standard deviation, 26.1 ± 31.5%) was significantly higher than that of the controls (1.8 ± 3.2%). Values are the means of the percentage of VCP-positive neuronal nuclei in each case; and error bars represent the standard deviation (*$p < 0.05$, Mann–Whitney U test). Immunohistochemistry was performed with the rabbit polyclonal antibody against VCP. *Scale bars* = 25 μm.

that was compatible with Paget disease of bone (Figure 3c).

Neuropathologically, the formalin-fixed brain weighed 1,435 g. Macroscopic examination showed no evidence of cerebral atrophy (Figure 3d, e). In H&E- and KB-stained sections, the spinal anterior horns and the corticospinal tracts showed evidence of degeneration, especially in the lumbar cord (Figure 3f). Cervical and lumbar cord showed cell loss with gliosis in the anterior horns (Figure 3g). Onuf's nucleus and Clarke's column were preserved. H&E-stained sections revealed the presence of Bunina bodies (Figure 3h) and spheroids in the anterior horns of the cervical and lumbar cords.

Immunohistochemical investigation demonstrated TDP-43-positive intracytoplasmic inclusions in the AHCs (Figure 4a). GCIs positive for TDP-43 were sparsely scattered in the spinal cord (Figure 4b). The TDP-43-positive intracytoplasmic inclusions were also reactive with the anti-ubiquitin antibody (Figure 4c,d). The nucleus of these inclusion-bearing neurons was invariably immunonegative for TDP-43 (Figure 4c). Analysis of consecutive sections revealed that these TDP-43-positive inclusions were also reactive with anti-p62 antibody (Figure 4e,f) and anti-OPTN antibody (Figure 4g,h). However, TDP-43-positive inclusions (Figure 4i) were indiscernible on the VCP-immunostained sections (Figure 4j). Careful examination of 164 AHCs in 8 VCP-stained sections from the cervical, thoracic, and lumbar spinal cords revealed no immunopositive inclusions with any of the VCP antibodies. Analysis of serial sections disclosed no apparent correlation between the presence of TDP-43-positive inclusions and nuclear and cytoplasmic distribution of VCP immunoreactivity in AHCs. In the superior frontal cortex, temporal cortex, motor cortex, and hippocampus, there was no significant immunoreactivity with VCP antibody.

Immunohistochemical investigation of the Golgi apparatus revealed characteristic fragmentation of it in the AHCs (Figure 4k), whereas non-motor neurons in the posterior horn had preserved Golgi apparatuses (Figure 4l). Using the same method reported previously [39], we found that in our case 61.5% (16/26) of the AHCs from 3 distinct spinal cord segments had a fragmented Golgi apparatus. In the corticospinal tracts, immunostaining for CD68 showed infiltration of CD68-positive microglia.

In the hypoglossal nuclei, the motoneurons were depleted in number. Within the cytoplasm of the residual neurons Bunina bodies and TDP-43- and p62-positive inclusions were identified. The Bunina bodies were immunopositive for cystatin C (Figure 4m). Electron microscopy demonstrated Bunina bodies consisting of irregularly shaped electron-dense material containing vesicles (Figure 5a-c).

Betz cells were not apparently depleted. Hippocampal sclerosis was not found. In the primary motor, superior frontal, and temporal cortices, putamen, thalamus,

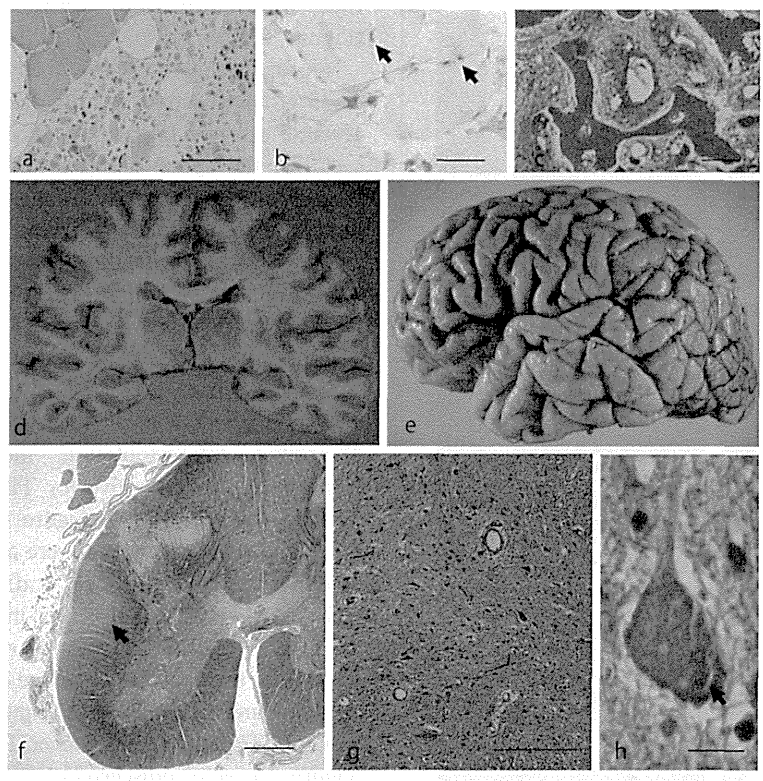

Figure 3 Photographs and photomicrographs of general pathology and neuropathology of the ALS patient with the *VCP* mutation. (a,b) In this muscle specimen from the right quadriceps muscle, large-group atrophy indicating neurogenic changes is demonstrated, whereas no evidence of IBM is observed **(a)**. The muscle nuclei are normally immunopositive with the anti-TDP-43 antibody (**b**, *arrows*), but abnormal inclusions are not detected. **(c)** H&E staining of a vertebra demonstrates irregular broad trabeculae with disorganized cement lines appearing in a mosaic pattern. **(d,e)** Macroscopically, the brain shows no apparent atrophy. **(f)** A KB-stained lumbar spinal cord reveals degeneration of the corticospinal tract (**f**, *arrow*). **(g)** A section of cervical cord stained by H&E shows neuronal loss with gliosis in the anterior horn. **(h)** Typical Bunina bodies (*arrow*) are evident in the cytoplasm of this cervical AHC. *Scale bars* = 250 μm **(a)**, 200 μm **(b)**, 50 μm **(c)**, 1 mm **(f)**, 100 μm **(g)**, and 12.5 μm **(h)**.

cerebellum, and hippocampus, there were no neuronal nuclear or intracytoplasmic inclusions or GCIs found by any of the staining procedures, including p62 immunohistochemistry.

By amyloid β and AT8-immunohistochemistry, this case showed no senile plaque, but only a few isolated neurofibrillary tangles in the hippocampus and amygdala (CERAD 0, Braak-Braak stage II). Immunostaining for α-synuclein and FUS revealed no pathologies.

Bioinformatics and *in vitro* studies

The effect of the newly detected missense mutation on VCP protein structure was analyzed by *in silico* analysis using different software. In Mutation Taster (http://www.mutationtaster.org), M158V was estimated to be disease causing (probability > 0.9999), and no Single Nucleotide Polymorphism (SNP) was found in the site of mutation. The known disease-causing VCP mutations R155H and R159H were also diagnosed as disease causing (probability > 0.9999) in Mutation Taster.

In SIFT (http://sift.bii.a-star.edu.sg/) the M158V mutation, as well as the known mutation R155H, was not tolerated (p = 0.00), meaning that the mutation is deleterious. On the other hand, the known mutation R159H [28,34,38] was tolerated (p = 0.07, in tolerant threshold <0.05), although the p-value was near the threshold. Polyphen-2 (http://genetics.bwh.harvard.edu/pph/) analysis showed possible damage to the structure by M158V, as indicated by a Position-Specific Independent Counts (PSIC) value of 0.896 (sensitivity: 0.82; specificity: 0.94), R155H was analyzed to be possibly damaging with a score with PSIC value 0.849 (sensitivity: 0.83; specificity: 0.93); although R159H ,which is indeed a disease-causing mutation, was predicted to be benign with a score of PSIC value 0.1 (sensitivity: 0.93; specificity: 0.85). Taken together, *in silico* analysis strongly suggested that out novel mutation M158V is pathogenic.

To assess the functional importance of the novel mutation identified in our patient, we created mammalian expressing vectors for WT *VCP* and mutant *VCPs* (R155H, M158V, R159H, and A232E) tagged with FLAG at their N-terminus. Immunoblotting detected overexpression of WT and mutant VCP in the transfected SH-SY5Y cells (Figure 6a) and HEK293T (Additional file 1: Figure S1a),

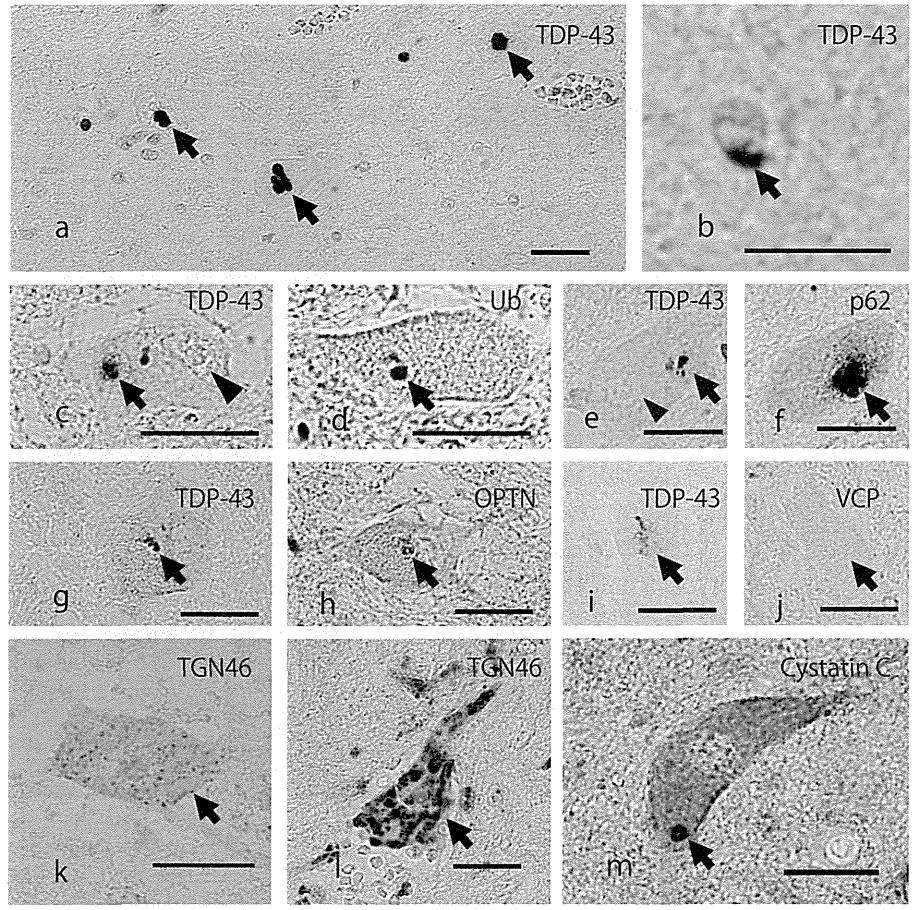

Figure 4 Representative photomicrographs of the lumbar anterior horn (a-l) and the hypoglossal nucleus (m). Intracytoplasmic inclusion bodies immunoreactive for TDP-43 are identifiable in the lumbar AHCs (**a**, *arrows*). A glial cytoplasmic inclusion (GCI) is evident with the anti-TDP-43 antibody (**b,** *arrow*). Analyses of consecutive sections indicated that TDP-43-positive inclusions in the AHCs (**c**, **e**, **g**, **i**, *arrows*) are also positive for ubiquitin (Ub; **d**, *arrow*), for p62 (**f**, *arrow*), and for OPTN (**h**, *arrow*), but devoid of immunoreactivity for VCP (**j**, *arrow*). The nucleus of the inclusion-bearing neuron lacks immunolabeling for TDP-43 (**c**, **e** *arrowhead*). Fragmentation of the Golgi apparatus is apparent in the AHCs immunostained with TGN-46, a marker protein of the Golgi, in comparison with the preserved Golgi apparatus in the non-motor neuron of the posterior horn (**l,** *arrow*). A Bunina body in the hypoglossal nucleus is immunopositive for cystatin C **(m)**. *Scale bars* = 25 μm **(a, c-m)**, 12.5 μm **(b)**.

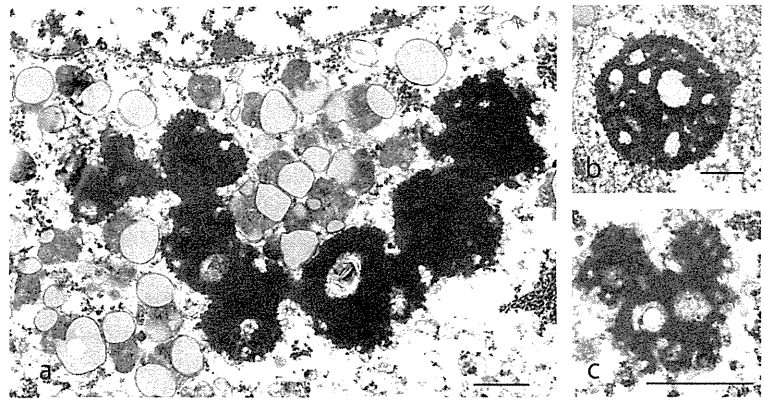

Figure 5 Electron microscopy of Bunina bodies. Three instances of Bunina bodies with irregularly shaped, amorphous, electron-dense material including vesicles and cisternae in the motor neurons of hypoglossal nuclei are depicted. *Scale bars* = 1 μm **(a-c)**.

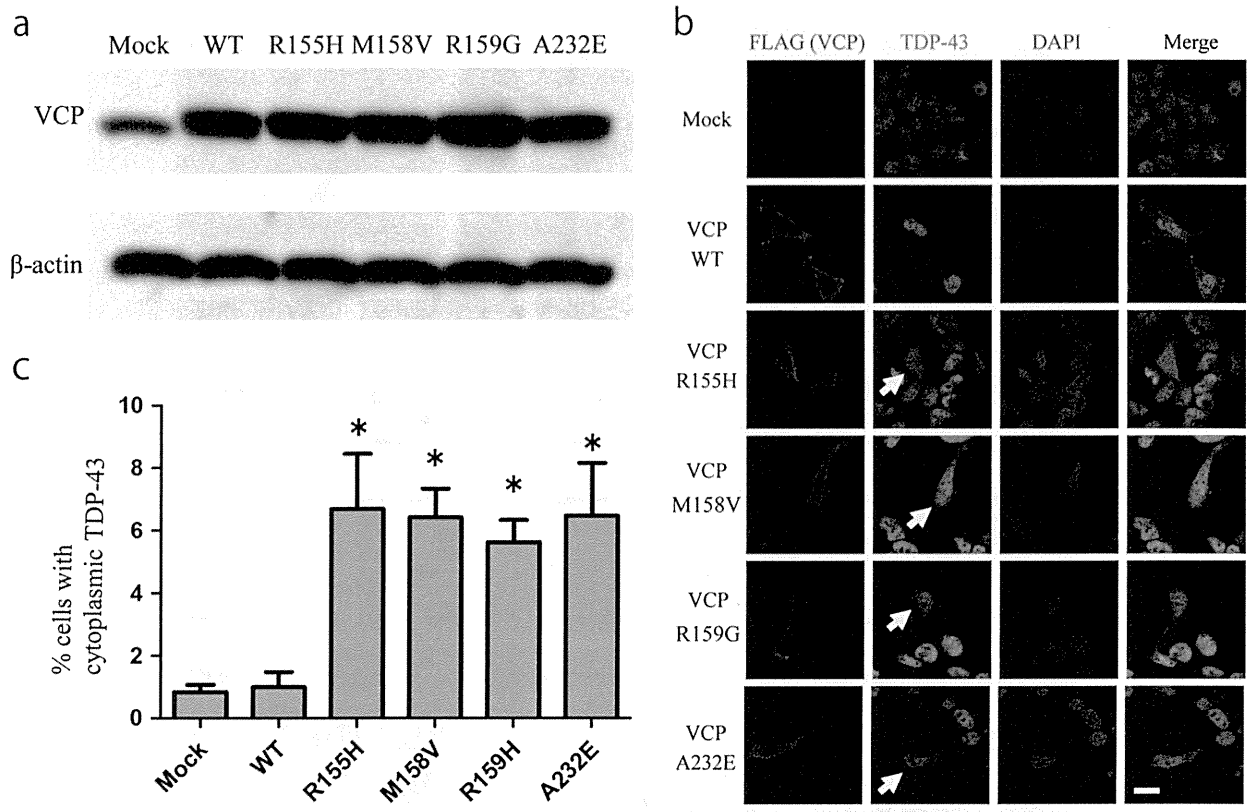

Figure 6 *In vitro* studies (SH-SY5Y cells). Immunoblotting detects overexpression of wild-type (WT) and mutant *VCPs* in transfected SH-SY5Y cells at 48 hours after transfection **(a)**. Expression of any mutant VCP (R155H, M158V, R159H or A232E) is associated with significantly more cytoplasmic TDP-43 **(b, c)**. Values are the means from 5 trials; error bars represent standard deviation; *$p < 0.05$, One-way ANOVA of Bonferroni's multiple-comparison, compared with mock and WT. The arrows in "**b**" indicate cytoplasmic TDP-43-positive cells.

compared with the expression in the mock-transfected cells, at 48 hours after transfection. TDP-43 is a predominantly a nuclear protein, and translocation of it from the nucleus to the cytoplasm and aggregation therein are features of previously reported types of mutant *VCP*-related ALS [26,28]. Mutations in VCP were also reported to cause abnormal TDP-43 translocation in cultured cells including SH-SY5Y [40] and HEK293T [41]. So we investigated whether our novel mutation (M158V) caused the translocation of TDP-43 from the nucleus to the cytoplasm. Immunocytochemistry showed TDP-43 to be located in the nuclei of mock-transfected cells and in those transfected with WT-VCP (Figure 6b). In the mutant VCP-expressing cells, immunoreactivity of TDP-43 was often observed in the cytoplasm by 48 hours after transfection (Figure 6b). However, no evident aggregation of TDP-43 or VCP was observed in the transfected cells. Quantitative evaluation of cytoplasmic TDP-43-positive cells demonstrated that the expression of mutant VCP was significantly associated with the presence of cytoplasmic TDP-43 (Figure 6c). There was no statistical difference in the number of cells with cytoplasmic TDP-43 between the cultured cells transfected with different *VCP* mutations. In the study using HEK293T, an increase in cytoplasmic TDP-43 translocation was also observed in cells transfected with mutant VCP (Additional file 1: Figure S1b, c).

Discussion

In the present study, we provided detailed neuropathology of an ALS case with the novel M158V *VCP* mutation, focusing on the similarity and difference to sporadic ALS. In addition, for the first time we demonstrated that the frequency of AHCs with VCP-immunoreactive cytoplasm and nucleus was increased in SALS patients as well as in this mutant case as compared with that in the controls.

Besides concomitant Paget disease of bone, the neurological manifestations and neuropathologic findings of our novel ALS-VCP patient were indistinguishable from those of the SALS patients. To date, 2 autopsied ALS-VCP cases with distinct mutations have been reported [26,28]. Clinically, both cases were diagnosed as ALS without symptoms of FTD. The initial patient was associated with an R155H *VCP* mutation. Because of the limitation of specimen availability, neuropathologic examinations of this case were confined to brainstem and spinal cord motor neurons,

revealing the loss of these neurons and the presence of Bunina bodies and TDP-43-positive cytoplasmic inclusions with concomitant loss of nuclear staining in the surviving motor neurons [26]. The disease of the second autopsied ALS-VCP patient was associated with an R159H *VCP* mutation. Neuropathologic description of this case was also limited, literally describing the presence of p62-, ubiquitin-, and TDP-43-positive inclusions. Additionally, they indicated a few p62- and ubiquitin-positive inclusions in the neurons of the hippocampal granular layer and frontotemporal lobes [28]. Although neocortical inclusion pathology was not apparent in our novel ALS-VCP case, the patient exhibited otherwise similar neuropathologic findings as reported in the previous 2 cases. Furthermore, we demonstrated that in our mutant case TDP-43 was co-localized with ubiquitin, p62, and optineurin, but not with VCP, in the cytoplasmic inclusions in the AHCs. TDP-43-positive GCIs were obviously present. The lack of VCP immunoreactivity in the TDP-43-positive inclusions may imply that the M158V mutant VCP would have lost ability to associate with aggregated TDP-43. In addition, we disclosed that the Bunina bodies in our ALS-VCP patient showed immunohistochemical and ultrastructural properties indistinguishable from those observed in SALS patients. Furthermore, we identified fragmented Golgi apparatuses in our mutant case. This finding is noteworthy, because several reports have described that the Golgi apparatus is frequently fragmented in the AHCs of patients with SALS [42-44]. It is plausible that VCP dysfunction led to vulnerability of the Golgi apparatus in the present case, because VCP plays an important role in assembly of the Golgi apparatus membrane [45]. Taken together, our observations on the neuropathology of the present ALS-VCP case resembled considerably those on that of SALS, supporting the idea that VCP would underlie the pathomechanism of SALS.

Patients with FTD in IBMPFD associated with a *VCP* mutation are known to show lentiform intranuclear inclusions in the neurons of their neocortex [23-25]; however, the present case showed no such inclusions there. One possibility for this discrepancy would be the presence of modifier genes. Kimonis et al. evaluated modifier genes in a database of 231 members of 15 original families with IBMPFD and suggested a potential link between the APOE 4 genotype and FTD [10]. Alternatively, the mutation site might influence the phenotype. Recently, a study using a large data set of patients with *VCP* mutations suggested that several mutations (R155C, A232E) are correlated with a severe phenotype and reduced survival; although the precise genotypic correlation with respect to *VCP* mutation has not been elucidated yet, because some groups of mutations were very small in number [27].

The mutation site of our case (M158V) was very close to the reported sites of mutation leading to the ALS phenotype (R155H [26] and R159H [28]). In protein feature analysis with Mutation Taster, these mutations were shown to be disease causing. (Although only the R159H mutation was analyzed as moderately damaging in SIFT and Polyphen-2, this mutation in ALS and IBMPFD was reported to occur in various cases [28,34,38]) All 3 of these sites are located within the N-terminal domain, which contains ubiquitin and cofactor-binding domains (Figure 1d) [12]. The accumulation of p62 and OPTN in our case suggests that the autophagy system was defective in this ALS-VCP patient, because VCP [46-48], p62 [49], and OPTN [50] play important roles in the autophagy-dependent protein clearance system. Our results and previous reports [40,41] revealed that cells transfected with mutant *VCP* showed abnormal TDP-43 translocation. These results support the notion that neurodegeneration in *VCP* mutant cases would be attributable to dysfunction of protein clearance systems, including ubiquitin-dependent protein degradation and autophagy.

We did not detect VCP-positive inclusion bodies in our case with M158V ALS-VCP. The absence of VCP inclusions in our patient is in consistent with previous findings obtained from FTD patients with *VCP* mutations [14,23,25]. The lack of VCP-positive inclusion bodies makes a toxic gain-of-function mechanism implausible. On the other hand, since normal VCP [1] and mutant VCP [46] are reported to form heteromeric complexes, a mutant VCP could conceivably impair the formation of properly functioning hexamers, thus having a dominant-negative effect [25].

Immunohistochemical investigations on the SALS cases revealed, similar to those on the ALS-VCP patient, increased frequencies of VCP-immunoreactive structures such as neuronal cytoplasm, neuronal nuclei, and glial nuclei. These findings suggest that SALS could share its pathomechanism with ALS-VCP through dysfunctional VCP. The cytoplasm of AHCs in SALS was moderately and diffusely immunoreactive for VCP. Endoplasmic reticulum (ER) stress due to accumulation of misfolded proteins [51,52] and activated autophagy [53] in the cytoplasm have been reported to occur in SALS cases. Considering that VCP governs protein degradation processes in both ER-associated ubiquitin-dependent protein degradation and autophagy [1,4], the increase in cytoplasmic VCP in SALS may reflect a process that modifies these protein degradation systems. Neuronal and glial nuclei were more frequently immunopositive for VCP in SALS cases than in the controls. VCP-positive nuclei have also been detected in the neocortical neurons [31] and muscle cells [20] in IBMPFD cases with a VCP mutation (MSP1). On the other hand, in our study the frequency of VCP-positive neuronal nuclei in the ALS-VCP case was lower than that of these nuclei in the SALS cases, presumably because of the functional loss of the M158V mutant VCP to recognize intranuclear ubiquitinated proteins. Recently,

nuclear VCP has emerged as an essential regulator of genome stability through the degradation of chromatin-associated proteins [2]. VCP is recruited at nuclear sites of damaged DNA and facilitates degradation of ubiquitinated chromatin-associated proteins to regulate DNA repair and transcription [54-56]. Notably, VCP regulates splicing pattern [3] and chromatin-associated proteins including RNA polymerase II subunit 1 (Rpb1) [56], which plays an important role in producing heterogeneous nuclear RNA (hnRNA). It is noteworthy that the alternative splicing of hnRNA is regulated by hnRNP A2/B1 and hnRNP A1 [57], which are the products of causative genes of MSP2 and MSP3 [21,22]. The dysfunction of these proteins, including VCP, hnRNP A1, and hnRNP A2/B1, could play an IBMPFD (MSP) pathogenic role through disruption of RNA metabolism and transcription processes. The increase in nuclear VCP in the ALS cases observed in the present study could be associated with the process by which VCP is recruited for intranuclear protein degradation to maintain the RNA metabolism and the transcription process. Alternatively, increased intranuclear VCP accumulation in ALS implicates loss-of-function mechanism of VCP as a pathogenesis of this disorder. Yang H et al. proposed that aggregtes of polyQ-expanded Atx3 sequester VCP into protein inclusions, and leads to neurodegeneration. It could be possible that VCP is entrapped by other intranuclear protein aggregation [58].

Our neuropathological investigations support the idea that VCP was associated with the pathomechanism of SALS and FALS with a *VCP* mutation. For determination of the pathological importance of VCP in these disorders, further studies with additional cases are warranted.

Additional file

Additional file 1: Figure S1. *In vitro* studies (HEK 293 T cells). Immunoblotting shows overexpression of wild-type (WT) and mutant *VCPs* of transfected HEK 293 T cells at 48 hours after transfection (a). Cells expressing any of the mutant VCPs show an increased number of expressing cytoplasmic TDP-43 compared with the cells transfected with mock or WT VCP by 48 hours after transfection (b, c). Values are the means from 3 trials; error bars represent standard deviation; *$p < 0.05$, One-way ANOVA of Bonferroni's multiple-comparison, compared with mock and WT. The arrows in "c" indicate cytoplasmic TDP-43-positive cells.

Competing interests
The authors declare that they have no competing interests.

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症例報告

筋症状のみを呈した慢性ミオパチー型筋サルコイドーシスの1例

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要旨：症例は69歳女性である．3年で進行する四肢の筋力低下を主訴に入院した．針筋電図では安静時の自発放電をともなう筋原性変化をみとめた．左上腕二頭筋の筋生検所見では，視野の大部分が結合織で占められ，筋線維は全視野で数本しかみとめなかったが，一部血管周囲にリンパ球が集積していた．数十枚の連続切片を追加し検索したところ，非乾酪性類上皮細胞肉芽腫と多核巨細胞が確認され，慢性ミオパチー型筋サルコイドーシスと診断した．全身検索をおこなったが，他臓器症状はみとめなかった．筋症状のみを呈した慢性ミオパチー型筋サルコイドーシスの臨床病理の報告はまれであり，貴重な症例と考えられた．

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Key words：筋サルコイドーシス，慢性ミオパチー型，筋ジストロフィー

はじめに

全身性サルコイドーシスの中では，症状の有無を問わずその50～80％に類上皮細胞肉芽種をみとめる筋病変を有するといわれている[1)]．無症候性であることがほとんどで，症状を有するのは全身性サルコイドーシスの0.5～2.3％と推定されている[2)]．症状を有するばあい，腫瘤型，急性・亜急性筋炎型，慢性ミオパチー型に分類されるが，中でも慢性ミオパチー型は頻度が低く，臨床症状が非特異的で，他臓器にサルコイドーシス病変がみられないばあい診断が困難である．筋病理でも肉芽種がみられないこともあり，病理学的に証明された報告は少ない．われわれは今回，他臓器病変をみとめず筋病理より診断にいたった慢性ミオパチー型筋サルコイドーシスの1例を経験した．筋サルコイドーシスに関する文献的考察を加えて報告する．

症　例

症例：69歳，女性
主訴：四肢の筋力低下
既往歴：骨粗鬆症，腰部脊柱管狭窄症．
家族歴：近親婚なし，家系内に筋疾患の発症者はいない．
現病歴：出生，生育歴に問題はなかった．2008年下肢の筋力低下が出現し平地では躓いたりせず問題がなかったが，階段昇降が困難となった．2009年には転倒しやすくなり，徐々に外出が減り，家の中でも動くことが少なくなった．2011年に上肢の脱力を自覚し，ボタンはめができなくなり，同時期より重いものが持ちあげられなくなった．2年で5 kgの体重減少があり，2011年10月に入院した．

入院時現症：身長148 cm，体重30 kg，体温36.7°C，血圧167/104 mmHg，脈拍76回/分・整．胸腹部に異常をみとめず，皮疹や表在リンパ節腫脹はみとめなかった．顔貌に異常はなく，四肢の関節拘縮はなかった．神経学的には，意識清明，認知機能に異常をみとめず，構音障害はなく，脳神経系に異常をみとめなかった．四肢・体幹，とくに下肢近位筋や傍脊柱筋で著明な筋萎縮がみられ，翼状肩甲をみとめた．徒手筋力検査（右/左）では頸部前屈3，三角筋4-/4-，上腕二頭筋3/3，上腕三頭筋で3/3，手掌背屈3/3，手掌底屈4-/4，腸腰筋2/2，大腿四頭筋2/2，前脛骨筋3/3，腓腹筋4+/4+と下肢近位筋優位に四肢の筋力低下をみとめた．腱反射は上肢で低下，下肢で消失していた．病的反射はみとめなかった．運動失調はみとめず，感覚系と自律神経系の異常はみとめなかった．起立・歩行は介助がなければ困難であった．

検査所見：血算では異常をみとめなかった．血清CK 250 IU/l（基準値46～168），LDH 300 IU/lと高値，TP 8.5 g/dlと高値，Alb 3.8 mg/dlと軽度低値であった．血清Ca 8.9 mg/dlと正常，尿中Caは測定しなかった．免疫学的検査では抗核抗体640倍（基準値40未満，Homogeneous型，Speckled型）と高値，抗TPO抗体0.4 U/ml（0.3未満），Tg抗体0.8 U/ml（0.3未満）と軽度高値，IgG 2,964 mg/dl（680～1,620），IgG4 153 mg/dl（5.3～116），IgA 511 mg/dl（84～438），IgE 689 IU/ml（250未満）と高値であった．IgMは正常範囲であった．C3 69.9 mg/dlと低値，C4，CH50は正常範囲であった．リウマチ因子，PR3-ANCA，MPO-ANCA，Jo-1抗体は陰性であった．可溶性IL-2受容体は1,350 U/mlと高値であった．心電図，

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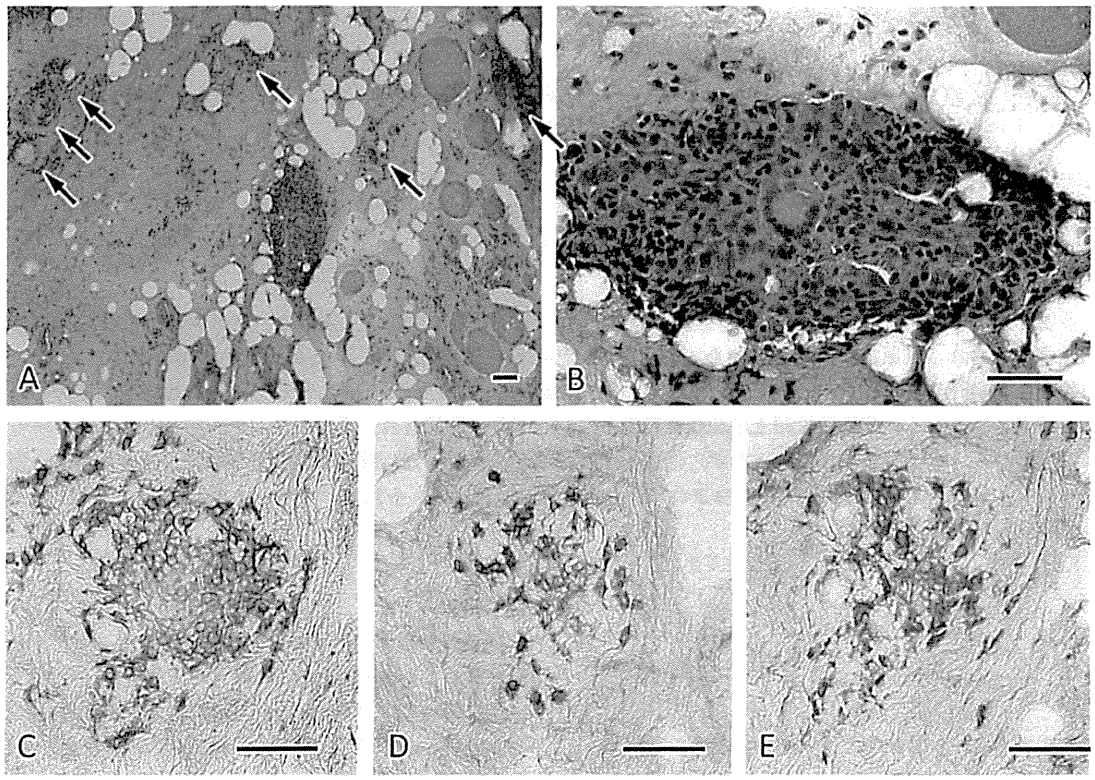

Fig. 1　Cryostat muscle sections from the patient.
A, B: H & E staining; C–E: serial sections, immunohistochemistry for CD4(C), CD8(D), CD68(E). Extensive connective tissue is shown with few residual muscle fibers (A). A number of inflammatory cells are around the vessels (A; arrows). The section shows a glanuloma with a multinucleated giant cell (B). A cell cluster predominantly consists of CD4+ cells. Some CD8+ cells are present inside and outside of the granuloma. Many CD68+ cells are also seen (C–E). Bar = 50 μm.

胸部単純レントゲンでは異常をみとめず，呼吸器機能は異常なかった．ツベルクリン反応，気管支肺胞洗浄はおこなわなかった．骨格筋CTでは近位筋優位に全身の筋にまだら状の脂肪化をともなう筋萎縮をみとめたが，とくに腰椎レベル内側の傍脊柱筋や大腿部背側に顕著であった．針筋電図は右上腕二頭筋と右前脛骨筋で，持続時間の短い低振幅多相性の運動単位電位（motor unit potential; MUP）を多数みとめ，また安静時に線維自発放電と陽性棘波をみとめた．左上腕二頭筋からの筋生検所見では，視野の大部分が結合織で占められ，全視野で筋線維は数本しかみとめず，血管周囲の炎症細胞浸潤をみとめた．40～50枚の連続切片を追加し検索したところ，非乾酪性類上皮細胞肉芽腫が確認できた（Fig. 1A, B）．免疫組織化学的染色では，CD4陽性細胞が優位にみられた（Fig. 1C～E）．筋サルコイドーシスをうたがい検査を追加したところ，血清ACE値32.3 IU/l（7.7～29.4），血清リゾチーム値22.0 μg/ml（4.2～11.5）と軽度高値であった．全身Gaシンチグラフィ，全身PET-CTでは集積像はみとめなかった．

臨床経過：筋生検結果より慢性ミオパチー型筋サルコイドーシスと診断し，プレドニゾロン（prednisolone; PSL）30 mg/日の内服を開始した．治療により四肢の筋力低下はごく緩徐に改善し，全身の筋力はMMT（manual muscle testing）で1段階程度改善をみとめたが，筋萎縮は残存した．ステロイドは漸減したが，投与開始120日目，PSL 20 mg/日にして，筋症状の悪化はみとめなかった．その後，白内障手術のため入院時におこなった心電図で尖鋭増高P波を指摘されたが，経過観察でおこなった心エコーにて心機能に異常をみとめなかった．

考　察

本症例は3年以上という慢性の経過で四肢体幹の著明な筋萎縮と筋力低下が進行し，筋症状の他臨床症状をみとめず，当初は肢帯型筋ジストロフィーをうたがった．筋生検をおこなったところ，筋線維はほとんどみられず，病理診断は困難と思われたが，血管周囲にリンパ球の集簇がみとめられたため，切片をさらに追加して検索をおこなったところ，巨細胞をともなう非乾酪壊死性肉芽腫が確認できた．採血検査ではACE（angiotensin converting enzyme）が上昇しており，他の原因となる筋疾患は否定的であることより，慢性ミオパチー型筋サルコイドーシスと診断した．

一般にサルコイドーシスの診断は，非乾酪性類上皮細胞肉芽種が存在する多臓器病変と全身反応を示す検査所見によるが，筋症状のみの症例があることに注意が必要であることが，診断基準に記されている[3]．筋内に類上皮肉芽腫をみとめる疾患としては，粟粒結核，第三期梅毒，トキソプラズマ症，重症筋無力症，胸腺腫をともなう筋炎，リウマチ様関節炎，強皮症，癌などが鑑別としてあげられる[4]．また，皮膚筋炎，オーバーラップ症候群，重症筋無力症などはサルコイドーシスに合併しうる．これらは感染徴候や起因菌の証明，全身病変から診断可能であることが多いが，他臓器病変をみとめず筋病変のみのばあい，鑑別はほとんど不可能である．本症例の病歴は慢性で，感染症状はなく，他の臓器病変はみられず，原因不明の肉芽腫性病変といえる．臨床所見・検査所見より代謝性ミオパチー，血管炎や感染性ミオパチー，自己免疫疾患重複症候群などは否定的，筋病理所見からは筋ジストロフィーや皮膚筋炎・多発性筋炎も否定的であった．日本神経学会による筋サルコイドーシスの診断基準によると，①サルコイドーシスの神経・筋病変を示唆する臨床所見がある，②組織診断にて神経・筋組織内にサルコイドーシスに一致する所見をみとめる，③上記所見をともなった他の可能性ある疾患を除外できる，以上3点は満たしていたが，サルコイドーシスの診断基準の全身反応を示す検査所見は（1）肺門リンパ節腫脹，（2）血清ACE活性高値，（3）ツベルクリン反応陰性，（4）Gallium-67 citrateシンチグラフィーにおける著明な集積所見，（5）気管支肺胞洗浄検査でリンパ球増加またはCD4/CD8比高値，（6）血清あるいは尿中カルシウム高値，のうち（2）の1項目しか確認できなかった．臨床症状，検査所見よりただし書きにあるisolated neurosarcoidosisの症例と考えられた．ただし，今後の合併症については注意深い経過観察が必要である．

生検筋で組織学的に診断された筋サルコイドーシスは無症候性が大半を占め，症候性はごく一部で，0.5～2.3％と報告されている[1)2)]．症候性のものはさらに腫瘤型，急性・亜急性筋炎型，慢性ミオパチー型の3群に分類される．なかでも慢性ミオパチー型はまれである．腫瘤型はしばしば外表から腫瘤を触れ，特徴的な画像所見を示し，生検での診断も比較的容易であるが，急性･亜急性ミオパチー型，慢性ミオパチー型のばあい，画像所見は非特異的で，他の臓器に病変がないばあい，診断が困難である．本症例でも，四肢の著明な筋力低下・筋萎縮・血清CK値上昇をみとめたが，その他に臨床所見・画像所見をみとめなかった．

過去の報告より慢性ミオパチー型は，閉経後の女性に多く[1)4)~6)8)~11)]，緩徐進行性の筋力低下・筋萎縮を呈し，CK上昇はないか，あっても軽度にとどまる．1960年代までは他の臓器病変をみとめないpure myopathic formが27例中11例という報告もみられたが[6)]，その後，1970年代以降の報告では詳細に検索すれば他の臓器に病変が高率にみとめられるとされる[5)9)~11)]．本邦の近年の報告でも，筋病理で肉芽種が証明されず，他の臓器障害より筋サルコイドーシスと診断した報告が散見される[12)]．本症例では高齢女性に近位筋優位の筋力低下・筋萎縮がみられ，CK上昇は軽度であった．筋サルコイドーシスの治療の第一選択はステロイド療法であるが，無作為化対照試験はなく，投与量・方法は一定していない．過去の報告では慢性ミオパチー型筋サルコイドーシスのステロイドに対する反応は様々だが[1)5)6)9)~11)]，本症例では軽度ではあるがステロイドに反応がみられた．

サルコイドーシスの筋症状発症の機序として，肉芽種による機械的圧迫や微小血管病変の関与などの仮説があるが，腫瘤型においては，近年の熊本らの筋病理の免疫組織化学的な検討をおこなった報告では，肉芽腫性炎症による直接浸潤が考えられている．その証拠としてCD4陽性細胞が肉芽種の中心にあるのに対し，常にCD8陽性細胞が肉芽種の周辺に位置し，まず筋線維に小孔を開ける役割を担っていると推定されている[13)]．本症例ではCD8陽性細胞は肉芽種の周辺だけでなく，内部にも散在していた．このリンパ球の分布の違いについては，経過が長く，浸潤するべき筋線維がすでに存在しないステージであったことによる影響かもしれないし，あるいは，病型の違いによる可能性も考えられる．また，腫瘤型がミオパチー型に進展することはほとんどなく，ミオパチー型は肉芽腫形成にくらべ筋線維の崩壊・脱落が高度であることから，全身性の自己免疫・代謝性の機序の可能性も示唆されている[13)]．本症例では抗核抗体が陽性であるが，皮膚症状などなく，膠原病の合併は現時点では否定的である．本邦のサルコイドーシス患者61名全例で抗核抗体を測定した報告[14)]では，抗核抗体陽性患者は30名と，49％を占めたが，臨床的な自己免疫疾患合併例は全体で7人であった．筋サルコイドーシスにおける抗核抗体はそれ自体自己免疫性の機序が発症に関与していることを表しているのかもしれない．慢性ミオパチー型は筋病理の報告がまれであり，病態の違いの解明には，免疫組織化学的検索もふくめた症例の蓄積が必要である．

本報告の要旨は，第96回日本神経学会近畿地方会で発表し，会長推薦演題に選ばれた．

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※本論文に関連し，開示すべきCOI状態にある企業，組織，団体はいずれも有りません．

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