Movie S1 Representative transport of SNB-1::DsRed puncta (red) in a ventral motor neurons from the control worm.
(MPEG)

Movie S2 Representative transport of SNB-1::DsRed puncta (red) in a ventral motor neurons from the *dnc-1* KD worm.
(MPEG)

Movie S3 Representative transport of Lgg1::DsRed puncta (red) in a primary motor neuron from the control worm.
(MPEG)

Movie S4 Representative transport of Lgg1::DsRed puncta (red) in a primary motor neuron from the *dnc-1* KD worm.
(MPEG)

Movie S5 Representatie transport of Lgg1::DsRed puncta (red) in a ventral motor neurons from the control worm.
(MPEG)

Movie S6 Representatie transport of Lgg1::DsRed puncta (red) in a ventral motor neurons from the *dnc-1* KD worm.
(MPEG)

Materials and Methods S1 Detailed materials and methods for *C. elegans* and human protocols.
(DOCX)

Author Contributions

Conceived and designed the experiments: KI MK FT IM GS. Performed the experiments: KI K. Kawai ZH YI YJ K. Kobayashi TK MW. Analyzed the data: KI K. Kawai MK. Contributed reagents/materials/analysis tools: TK K. Kobayashi IM. Wrote the paper: KI MK FT GS.

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RESEARCH PAPER

Clinical features and a mutation with late onset of limb girdle muscular dystrophy 2B

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ABSTRACT

Objective and methods Dysferlin encoded by *DYSF* deficiency leads to two main phenotypes, limb girdle muscular dystrophy (LGMD) 2B and Miyoshi myopathy. To reveal in detail the mutational and clinical features of LGMD2B in Japan, we observed 40 Japanese patients in 36 families with LGMD2B in whom dysferlin mutations were confirmed.

Results and conclusions Three mutations (c.1566C>G, c.2997G>T and c.4497delT) were relatively more prevalent. The c.2997G>T mutation was associated with late onset, proximal dominant forms of dysferlinopathy, a high probability that muscle weakness started in an upper limb and lower serum creatine kinase (CK) levels. The clinical features of LGMD2B are as follows: (1) onset in the late teens or early adulthood, except patients homozygous for the c.2997G>T mutation; (2) lower limb weakness at onset; (3) distal change of lower limbs on muscle CT at an early stage; (4) impairment of lumbar erector spinal muscles on muscle CT at an early stage; (5) predominant involvement of proximal upper limbs; (6) preservation of function of the hands at late stage; (7) preservation of strength in neck muscles at late stage; (8) lack of facial weakness or dysphagia; (9) avoidance of scoliosis; (10) hyper-Ckaemia; (11) preservation of cardiac function; and (12) a tendency for respiratory function to decline with disease duration. It is important that the late onset phenotype is found with prevalent mutations.

INTRODUCTION

Dysferlinopathies are autosomal recessive muscular dystrophies caused by mutations in the dysferlin gene (*DYSF*, MIM# 603009). Dysferlin deficiency leads to two main phenotypes: limb girdle muscular dystrophy (LGMD) 2B and Miyoshi myopathy (MM).[1 2] Dysferlin is located on the plasma membrane of skeletal muscle and is deficient in patients with MM and LGMD2B.[3 4] However, atypical immunostaining in muscle from patients with dysferlin mutations occurs,[5 6] and dysferlin expression is not normal in sarcoglycanopathy, dystrophinopathy,[7] caveolinopathy[8 9] or calpainopathy[6] muscles. Therefore, the final diagnosis of dysferlinopathy requires identification of mutations in the dysferlin gene. We first reported dysferlin mutations in Japanese patients with MM[10] and in a patient from a non-European ethnic group with distal anterior compartment myopathy (DACM),[11] a relatively new phenotype of dysferlinopathy.[12] Furthermore, we revealed that, in MM, four mutations (c.1566C>G, c.2997G>T, c.3373delG and c.4497delT) were relatively more prevalent in the Japanese population and the c.2997G>T mutation was associated with late onset.[13] Although mutation analysis of the dysferlin gene is a time consuming task because of the large size of the gene,[14] large series of patients with dysferlin gene mutations have been studied.[6 15–24] However, few detailed analyses of the clinical features of LGMD2B, especially in relation to various types of mutations, have been reported. Here we report the clinical features of a series of 40 patients in 36 families with LGMD2B in whom dysferlin mutations were confirmed, and cardiac and respiratory functions were involved. In particular, we took into account the duration that had elapsed since onset when the clinical data were examined.

MATERIALS AND METHODS

We retrospectively observed 40 Japanese patients in 36 families with LGMD2B in whom dysferlin mutations were confirmed. LGMD was defined as symptomatic myopathy excluding MM and DACM at the first visit to a neurologist. Mutational analysis was performed by single strand conformation polymorphism analysis and sequencing on genomic DNA using our previously reported method,[13 14 25] with minor modifications (Ref Seq NM_003494.2), and with informed consent and approval of our local ethics committee. We retrospectively reassessed the history of onset and progression of the disease. The clinical examination included manual muscle testing using the Medical Research Council (MRC) Scale and assignment of scales for the proximal limb muscles, as proposed by Brooke *et al*.[26] Clinical examination was carried out by neurologists from the study groups for muscular dystrophy in Japan.

Most patients had undergone muscle CT scans at some stage of the disease. Serum creatine kinase (CK) activity was measured. Cardiac and respiratory functions were evaluated. We selected the first and last manual muscle testing data and the last data on the scales for the proximal limb muscles, CK activity, cardiac function and respiratory function. We used all muscle CT scans. Patients were divided into three groups according to whether they had the homozygous c.2997G>T (p.Trp999Cys) mutation, the heterozygous c.2997G>T (p.Trp999Cys) mutation or other mutations. Difference in age at onset among the groups was evaluated by the Kruskal–Wallis test. Multiple comparison of age at onset between each group was assessed by Scheffé's F test. Difference in the first symptom among the groups was evaluated by the χ^2 for independence test. Kaplan–Meier curves with log rank test were used to examine survival at each milestone of progression in the groups. Pearson's correlation coefficient test was used to identify significant associations in ejection fraction (EF) (n=21), atrial natriuretic peptide (n=9), brain natriuretic peptide (n=7), per cent vital capacity (%VC) (n=23), carbon dioxide partial pressure (pCO_2) (n=16) and oxygen partial pressure (pO_2) (n=16) with disease duration. Because supine and standing position chest x-rays were mingled, Pearson's correlation coefficient test was not used for the cardiothoracic ratio (n=22).

RESULTS

Mutations

We identified 17 different mutations in 36 families (table 1). Two mutations (c.2974T>C (p.Trp992Arg) and c.2997G>T (p.Trp999Cys)) were missense mutations and four mutations (c.342+1G>A, c.937+1G>A, c.2643+1G>A and c.4794 +1G>A) were splice site mutations. The others were nonsense mutations. The c.2997G>T (p.Trp999Cys) mutation was present in 26 alleles (36.1%). The c.1566C>G (p.Tyr522X) mutation and the c.4497delT mutation were both present in eight alleles (11.1%). We identified the c.3373delG mutation that has high frequency in Japanese patients with MM[13] in only one allele.

Clinical course

Mean age at onset of all patients was 26.6±9.9 years (range 14–58): the group homozygous for the c.2997G>T (p.Trp999Cys) mutation, 37.9±10.2 years (19–58); the group heterozygous for the c.2997G>T (p.Trp999Cys) mutation, 26.8 ±7.6 years (14–35); and the group without the c.2997G>T (p.Trp999Cys) mutation, 21.8±5.8 years (14–41). The difference between the group homozygous for the c.2997G>T (p.Trp999Cys) mutation and the group heterozygous for the c.2997G>T (p.Trp999Cys) mutation was significant ($p<0.05$). The difference between the group homozygous for the c.2997G>T (p.Trp999Cys) mutation and the group without the c.2997G>T (p.Trp999Cys) mutation was significant ($p<0.01$) (figure 1). The first symptom in most patients was lower limb weakness. Walking on tiptoe was the first sign in one patient. In three patients with the homozygous c.2997G>T (p.Trp999Cys) mutation, the first symptom was upper limb weakness. In two patients, one of them carrying the homozygous c.2997G>T (p.Trp999Cys) mutation, upper limb weakness was concomitant with lower limb weakness. There was a significant difference ($p<0.01$) in the probability that the first symptom was upper limb weakness among these three groups. The first symptom in one patient was left brachial pain and in another patient it was limitation of the elbow, hip, knee and spine.[27] In the early stage of the clinical course, hypertrophy of the calves was noticed in three patients. Winged scapula, rigid spine[27] and hollow foot were observed in one patient each. Lordosis was observed in two patients. Weakness of the face, dysphagia, scoliosis and kyphosis were not found. Choreic movements and pollakisuria were found in one patient.[28] Mean duration at difficulty in running was 1.3 years from disease onset (range 0–10), 3.1 years (0–14) for difficulty in climbing stairs, 5.5 years (0–16) for stumbling, 6.0 years (0–10) for difficulty in standing on tiptoe, 6.7 years (0–15) for rising from the floor, 8.7 years (0–25) for noticing weakness in a proximal upper limb, 12.5 years (3–23) for walking with a cane, 20.4 years (9–35) for noticing weakness in a distal upper limb, 21.6 years (13–29) for using a wheelchair and 31.0 years (16–45) for using an electric wheelchair. There was a significant difference ($p<0.05$) in the survival ratio only at the stage of difficulty in standing on tiptoe among these three groups (figure 2). There were no significant differences in survival at the stage of the other symptoms between these three groups. According to the scales for the proximal limb muscles, only two patients each reached the severest stage of arms and shoulders (cannot raise hands to mouth and have no useful function) and hips and legs (confined to bed).

Manual muscle testing

In the first decade of the disease, the muscles of the lower limbs were predominantly involved. In the upper limbs, the deltoid muscle was predominantly involved. In the second decade of the disease, the biceps and triceps brachii muscles became weak. Later on, the flexor and extensor of the hand became weak. Muscle weakness progressed and, in the fifth decade of the disease, the muscles of the lower limbs were very weak (1 on the MRC Scale). In the patient with 52 years of disease duration, although the muscles of the upper and the lower limbs were 0 on the MRC scale, the flexor and extensor of the neck were relatively preserved (2 on the MRC Scale).

Muscle CT scans

The CT scans revealed low density changes in the gastrocnemius, especially in the medial heads, soleus, hamstrings and the erector spinal group, which was mainly affected in the lateral parts in the early disease stage. Low density abnormalities in the quadriceps femoris and the adductor magnus muscles were observed at close to 10 years after disease onset. The tibialis anterior, the peroneal group, the gluteal group, the quadrates lumborum and the deltoid muscles were involved at 10 years after onset. The gracilis and sarcorius muscles were preserved and had hypertrophied during the second decade of disease duration. Approximately 20 years after disease onset, low density changes in the dorsal muscles occurred in the neck region, particularly in the transversospinal group, except for the semispinalis capitis muscle. All muscles were replaced by low density tissue or were atrophied at 30 years after disease onset. The gluteal group, the psoas major muscle, the lateral abdominal group and the levator scapulae muscle were preserved in the late stage. There were exceptional cases. In one patient, low density changes in the dorsal muscles in the neck region occurred at an early stage. In another patient, the quadriceps femoris muscles were more severely damaged than the hamstrings at 14 years of disease duration.

Serum CK levels

Although serum CK levels were very high, there was a tendency for levels to decrease with disease duration (figure 3). There was a trend in the distribution of the levels by

Table 1 Summary of dysferlin gene mutations of patients in this study

Patient	Sex	Age (years)	Disease duration (years)	Exon	Nucleotide change	Protein change	State
Dys48-1	M	54	23	28	c.2997G>T	p.Trp999Cys	Homozygous
Dys58-1	F	51	8	28	c.2997G>T	p.Trp999Cys	Homozygous
Dys64-1	M	58	19	28	c.2997G>T	p.Trp999Cys	Homozygous
Dys93-1	F	57	17	28	c.2997G>T	p.Trp999Cys	Homozygous
Dys99-1	F	55	11	28	c.2997G>T	p.Trp999Cys	Homozygous
Dys106-1	F	22	3	28	c.2997G>T	p.Trp999Cys	Homozygous
Dys113-1	M	57	17	28	c.2997G>T	p.Trp999Cys	Homozygous
Dys123-1	F	59	25	28	c.2997G>T	p.Trp999Cys	Homozygous
Dys133-1	M	66	8	28	c.2997G>T	p.Trp999Cys	Homozygous
Dys170-1	F	38	7	28	c.2997G>T	p.Trp999Cys	Homozygous
Dys13-1	M	43	29	28 34	c.2997G>T c.3771G>A	p.Trp999Cys p.Trp1257X	Compound heterozygous
Dys55-1	F	47	22	28 37	c.2997G>T c.3959_3960insA	p.Trp999Cys p.Met1320IlefsX26	Compound heterozygous
Dys114-1	M	50	15	Intron 25 28	c.2643+1G>A c.2997G>T	Splice site p.Trp999Cys	Compound heterozygous
Dys120-1	M	55	25	18 28	c.1566C>G c.2997G>T	p.Tyr522X p.Trp999Cys	Compound heterozygous
Dys124-1	F	36	12	18 28	c.1566C>G c.2997G>T	p.Tyr522X p.Trp999Cys	Compound heterozygous
Dys127-1	F	56	23	18 28	c.1566C>G c.2997G>T	p.Tyr522X p.Trp999Cys	Compound heterozygous
4	M	75	49	Intron 25 41	c.2643+1G>A c.4497delT	Splice site p.Phe1499LeufsX4	Compound heterozygous
5	M	Died at 59	Died at 42	21	c.1958delG	p.Gly653ValfsX3	Homozygous
15	F	50	34	37	c.3959_3960insA	p.Met1320IlefsX26	Homozygous
39	F	74	52	Intron 25	c.2643+1G>A	Splice site	Homozygous
44	M	41	14	29 41	c.3112C>T c.4497delT	p.Arg1038X p.Phe1499LeufsX4	Compound heterozygous
47	M	55	37	37	c.3959_3960insA	p.Met1320IlefsX26	Homozygous
Dys37-1	M	60	40	Intron 10	c.937+1G>A	Splice site	Homozygous
Dys37-2	F	51	24	Sister of Dys37-1			
Dys43-1	F	36	19	18	c.1566C>G	p.Tyr522X	Homozygous
Dys43-2	F	35	21	Sister of Dys43-1			
Dys46-1	F	67	48	41	c.4497delT	p.Phe1499LeufsX4	Homozygous
Dys50-2	M	70	29	41	c.4497delT	p.Phe1499LeufsX4	Homozygous
Dys59-1	F	49	27	28	c.2974T>C	p.Trp992Arg	Homozygous
Dys61-1	F	43	21	41	c.4497delT	p.Phe1499LeufsX4	Homozygous
Dys78-1	F	54	30	Intron 10	c.937+1G>A	Splice site	Homozygous
Dys84-1	F	64	45	14	c.1321C>T	p.Gln441X	Homozygous
Dys84-2	M	52	32	Brother of Dys84-1			
Dys89-1	F	68	40	18 28	c.1566C>G c.2974T>C	p.Tyr522X p.Trp992Arg	Compound heterozygous
Dys117-1	M	32	9	18	c.1566C>G	p.Tyr522X	Homozygous
Dys117-2	M	30	16	Brother of Dys117-1			
Dys126-1	M	23	2	Intron 4 54	c.342+1G>A c.6135G>A	Splice site p.Trp2045X	Compound heterozygous
Dys145-1	F	27	10	6	c.610C>T	p.Arg204X	Homozygous
Dys146-1	F	58	34	31 Intron 43	c.3373delG c.4794+1G>A	p.Glu1125LysfsX9 splice site	Compound heterozygous
Dys163-1	M	36	11	6	c.493delC	p.Leu165SerfsX48	Homozygous

c.2997G>T (p.Trp999Cys) mutation. Levels in the group homozygous for the c.2997G>T (p.Trp999Cys) mutation were lower than those of the other two groups.

Cardiac function

Although supine and standing position chest x-rays were mingled, in half of the patients the cardiothoracic ratio was >50%. No significant correlation was observed between EF and disease duration. Except for two patients, levels of EF were >50%. In all the patients, concentrations of atrial natriuretic peptide were within the normal range. Except for one patient, concentrations of brain natriuretic peptide were within the normal range. No significant correlation was observed between these peptides and disease duration. Although 19 patients

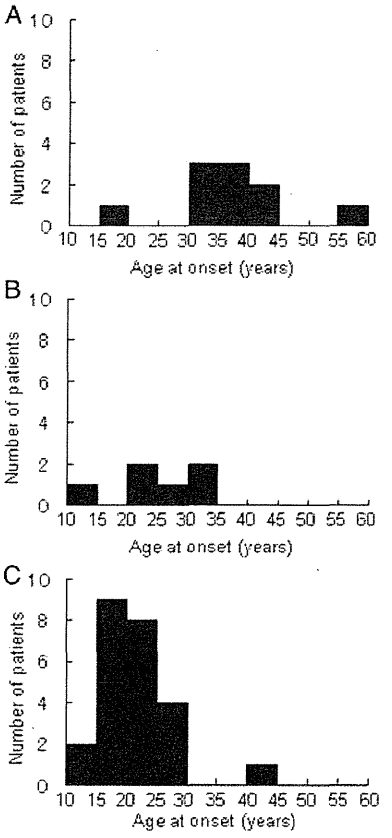

Figure 1 Histogram by age at onset. (A) Patients homozygous for the c.2997G>T mutation. (B) Patients heterozygous for the c.2997G>T mutation. (C) Patients without the c.2997G>T mutation.

exhibited normal findings on ECG, abnormalities were found in nine patients. The ECG showed premature ventricular contraction in one patient, one degree atrioventricular block in two patients, incomplete right bundle branch block in two patients, left axis deviation in two patients, left atrial hypertrophy in one patient, right ventricular hypertrophy in three patients, left ventricular hypertrophy in three patients, ST change in two patients and negative T in one patient.

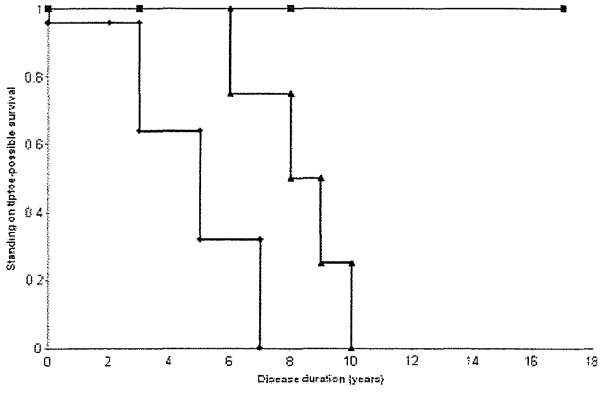

Figure 2 Standing on tiptoe—possible survival curve. Survival curve of patients homozygous for the c.2997G>T mutation (line with squares), those heterozygous for the c.2997G>T mutation (line with triangles) and those without the c.2997G>T mutations (line with diamonds).

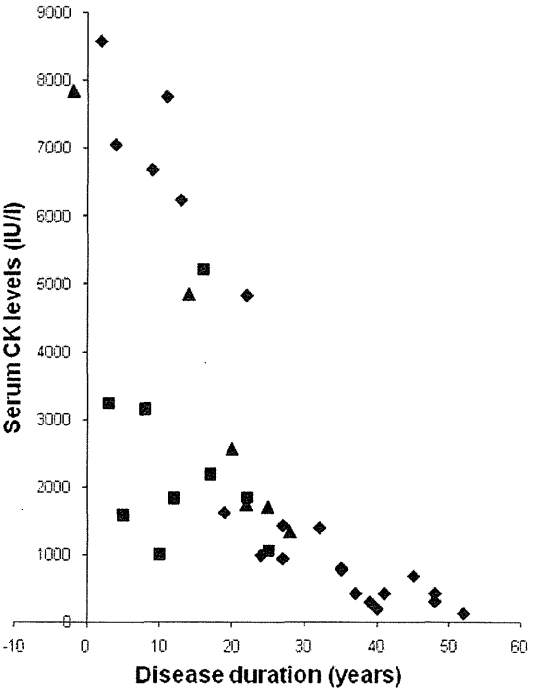

Figure 3 Serum creatine kinase (CK) levels during the disease course. Levels of serum CK in patients homozygous for the c.2997G>T mutation (squares), heterozygous for the c.2997G>T mutation (triangles) and those without the c.2997G>T mutations (diamonds).

Respiratory function

A statistically significant ($p<0.01$) correlation ($r=-0.545$) was observed between %VC and disease duration (figure 4). In 48% of patients, %VC was <80%. Although no significant correlation was observed between pCO_2 and disease duration, in 44% of patients, levels of pCO_2 were >45 mm Hg. In most patients, levels of pO_2 were >70 mm Hg. No significant correlation was observed between pO_2 and disease duration. Four patients had

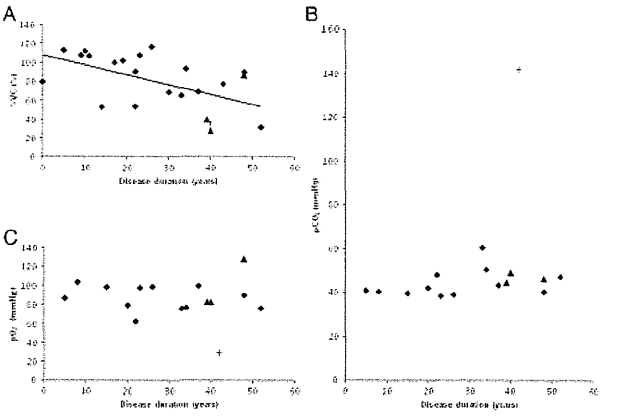

Figure 4 Respiratory function. (A) Per cent vital capacity (%VC) according to disease duration. The line is the regression line of %VC and disease duration. (B) Carbon dioxide partial pressure (pCO_2) according to disease duration. (C) Oxygen partial pressure (pO_2) according to disease duration. The triangles indicate levels in patients who had used non-invasive positive pressure ventilation. The cross (+) indicates the level in a patient who died after 42 years of disease duration of respiratory failure.

used non-invasive positive pressure ventilation (NIPPV). One patient who used NIPPV died after 42 years of disease duration from respiratory failure.

DISCUSSION

Three mutations (c.2997G>T (p.Trp999Cys), c.1566C>G (p.Tyr522X) and c.4497delT) were relatively more prevalent in the present Japanese patients with LGMD2B. Also, in Japanese patients with MM, these three mutations were relatively more prevalent in our previous study.[13] We identified the c.3373delG mutation that occurred with high frequency in Japanese patients with MM, as well as these three mutations,[13] in only one allele in Japanese patients with LGMD2B.

In MM, patients with the c.2997G>T (p.Trp999Cys) mutation had a significantly late onset.[13] Tagawa *et al*[6] reported that age of onset in patients homozygous for the c.2997G>T (p.Trp999Cys) mutation was later than the third decade of life. In this study, especially in patients homozygous for the c.2997G>T (p.Trp999Cys) mutation, onset was significantly late. Only 10% of patients homozygous for the c.2997G>T (p.Trp999Cys) mutation developed muscle symptoms by 30 years. In contrast, 90% of patients without this mutation developed muscle symptoms by the same age. However, it is difficult to explain why the c.2997G>T (p.Trp999Cys) mutation is related to late onset forms. In an immunohistochemical analysis by Tagawa *et al*,[6] although one patient with the homozygous c.2997G>T mutation (p.Trp999Cys) showed an 'abnormal' pattern, cytoplasmic accumulation of immunopositive material with deficiency of membrane staining or positive/negative mosaic membrane staining, three patients with the same mutation showed a negative pattern. Guglieri *et al*[21] reported that patients carrying two truncating mutations showed the first muscular symptoms earlier in life than subjects harbouring double missense substitutions. Indeed, the c.2997G>T mutation is theoretically deduced to be a missense mutation (p.Trp999Cys). Meanwhile, in this study, there were only two patients carrying missense mutations other than the c.2997G>T (p.Trp999Cys) mutation. Thus it is difficult to discuss statistically whether an association between the c.2997G>T (p.Trp999Cys) mutation and late onset forms is due to a missense mutation or other inherent characteristics. Nonetheless, it is important that the late onset phenotype is found with prevalent mutations.

Although patients homozygous for the c.2997G>T (p.Trp999Cys) mutation had late onset, no difference in progression was observed in those harbouring the c.2997G>T (p.Trp999Cys) mutation, except for difficulty in standing on tiptoe. This suggests that the c.2997G>T (p.Trp999Cys) mutation is related to late onset but not to the slow progression of the disease. Furthermore, the c.2997G>T (p.Trp999Cys) mutation may be relevant to the proximal dominant impairment in dysferlinopathy, diagnosed as the limb girdle type. Interestingly, there was only one patient homozygous for the c.2997G>T (p.Trp999Cys) mutation in 27 families with MM.[13] Moreover, patients homozygous for the c.2997G>T (p.Trp999Cys) mutation had a high probability that the muscle weakness started in the upper limbs. In addition, serum CK levels in patients homozygous for the c.2997G>T (p.Trp999Cys) mutation were lower than those in the other two groups.

We investigated the clinical features of 40 patients in 36 families with LGMD2B in whom dysferlin mutations were confirmed. Disease duration was long (26.6±9.9 years) in this study. The clinical features of LGMD2B in this study were as follows: (1) onset in the late teens or early adulthood except in patients homozygous for the c.2997G>T (p.Trp999Cys) mutation; (2) lower limb weakness at onset in most patients; (3) distal change of lower limbs on muscle CT in the early stage; (4) impairment of lumbar erector spinal muscles on muscle CT in the early stage; (5) predominant involvement of the proximal upper limbs; (6) preservation of function of the hands in late stage; (7) preservation of strength in the neck muscles in late stage; (8) lack of facial weakness or dysphagia; (9) avoidance of scoliosis; (10) hyper-Ckaemia; (11) preservation of cardiac function; and (12) tendency for respiratory function to decline with disease duration and occasional necessity for ventilatory assistance.

Age at onset was similar to that reported for various types of mutations.[18 19 21] However, patients with disease onset at 73 years[24 29] or congenital onset[30] have been reported. Clinicians may not have taken into account analyses of the dysferlin gene for such patients.

Nishida *et al* proposed the name 'distal limb girdle type muscular dystrophy' for patients with MM that develop proximal muscle involvement relatively early.[31 32] Nguyen *et al*[17] classified patients for whom it was not possible to distinguish between a distal phenotype of MM and a limb girdle phenotype, even when examined at onset, as a distinct 'proximodistal' phenotype group. Although patients with the typical features of MM[13 33] or asymptomatic hyper-CKaemia were excluded from the present study, variable patterns of weakness in the lower limbs were observed, as in previous studies.[17 19 20 22 24] However, the predominant muscle weakness was in proximal sites of the upper limbs in the present study.

The results of muscle CT scan in this study were similar to those of detailed imaging studies in LGMD2B at a relatively early stage.[23 34 35] In this study, we had only one case in whom a low density change in the dorsal muscle in the neck region occurred in the early stage. In contrast, two patients showed fatty muscle degeneration of the cervical elector spinae muscles after 9 or 10 years of disease duration, in a study performed using 3.0 T MRI.[35] Another exceptional finding was a case in which the quadriceps femoris muscles were more severely damaged than the hamstrings, which was reported in two of five patients.[34]

In the present study, only two patients showed levels of EF that were <50% and the patient who showed the lowest EF (32.7%) had no symptoms or signs of cardiomyopathy.[36] Guglieri *et al*[21] reported cardiac rhythm changes in three patients and left ventricular hypertrophy in four patients from a total of 22 LGMD2B patients. Wenzel *et al*[37] reported ECG abnormalities (repolarisation abnormalities or left ventricular hypertrophy) in four patients and pathological echocardiographic parameters in five of seven LGMD2B patients. Furthermore, two patients had symptoms and signs of dilated cardiomyopathy. Choi *et al*[38] reported left ventricular hypertrophy on ECG in two patients, no cardiac signs in one patient and mildly decreased EF (45%) in one patient among five MM patients. Therefore, we think that in most patients with LGMD2B, in spite of the existence of laboratorial abnormalities, cardiac function was clinically preserved.

Mahjneh *et al*[16] reported that patients with LGMD2B with disease durations of up to 7 years might show slight restrictive lung disease. Cagliani *et al*[39] reported that respiratory tests showed mild obstructive signs at the small airways in a 20-year-old man with LGMD2B. Illa *et al*[12] reported that pulmonary function tests revealed a mild reduction in VC in two

patients with DACM. In this study, many patients showed pathological levels of respiratory parameters and levels of %VC decreased with disease duration. Furthermore, some patients needed NIPPV and one patient died of respiratory failure. The change in muscles related to respiratory function worsens with disease duration. Therefore, it is important to pay attention to respiratory function in patients with dysferlinopathy.

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ORIGINAL ARTICLE

Exome sequencing identifies a novel *TTN* mutation in a family with hereditary myopathy with early respiratory failure

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Myofibrillar myopathy (MFM) is a group of chronic muscular disorders that show the focal dissolution of myofibrils and accumulation of degradation products. The major genetic basis of MFMs is unknown. In 1993, our group reported a Japanese family with dominantly inherited cytoplasmic body myopathy, which is now included in MFM, characterized by late-onset chronic progressive distal muscle weakness and early respiratory failure. In this study, we performed linkage analysis and exome sequencing on these patients and identified a novel c.90263G>T mutation in the *TTN* gene (NM_001256850). During the course of our study, another groups reported three mutations in *TTN* in patients with hereditary myopathy with early respiratory failure (HMERF, MIM #603689), which is characterized by overlapping pathologic findings with MFMs. Our patients were clinically compatible with HMERF. The mutation identified in this study and the three mutations in patients with HMERF were located on the A-band domain of titin, suggesting a strong relationship between mutations in the A-band domain of titin and HMERF. Mutation screening of *TTN* has been rarely carried out because of its huge size, consisting of 363 exons. It is possible that focused analysis of *TTN* may detect more mutations in patients with MFMs, especially in those with early respiratory failure.
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Keywords: A-band; cytoplasmic body; Fn3 domain; hereditary myopathy with early respiratory failure; HMERF; myofibrillar myopathy; titin; *TTN*

INTRODUCTION

Myofibrillar myopathies (MFMs) were proposed in 1996 as a group of chronic muscular disorders characterized by common morphologic features observed on muscle histology, which showed the focal dissolution of myofibrils followed by the accumulation of products of the degradative process.[1] The clinical phenotype of MFM is characterized by slowly progressive muscle weakness that can involve proximal or distal muscles, with onset in adulthood in most cases. However, other phenotypes are highly variable. Although 20% of patients with MFMs have been revealed to have mutations in *DES*, *CRYAB*, *MYOT*, *LDB* (*ZASP*), *FLNC* or *BAG3*, the major genetic basis of MFMs remains to be elucidated.

Respiratory weakness is one of the symptoms of MFMs. The early or initial presentation of respiratory failure is not a common manifestation of MFMs as a whole, and there are limited reports regarding a fraction of patients with *DES*,[2] *MYOT*[3] or *CRYAB*[4] mutation. In 1993, our group reported a Japanese family with dominantly inherited cytoplasmic body (CB) myopathy,[5] which is now included in MFM. Currently, this family includes 20 patients in five successive generations who show almost homogeneous clinical features characterized by chronic progressive distal muscle weakness and early respiratory failure. However, the underlying genetic etiology in this family was unknown. The aim of this study was to determine the genetic cause in this family. To identify the responsible genetic mutation, we performed linkage analysis and whole-exome sequencing.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the Tohoku University School of Medicine, and all individuals gave their informed consent before their inclusion in the study.

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Clinical information on the family

This family includes 20 patients (13 males and 7 females) in five successive generations (Figure 1). The family is of Japanese ancestry, and no consanguineous or international mating was found. Of all patients, seven underwent a muscle biopsy, and two were autopsied. All of the histological findings were compatible with MFM (see clinical data).

The age of onset ranged from 27–45 years. The most common presenting symptom was foot drop. At the initial evaluations, muscle weakness was primarily distributed in the ankle dorsiflexors and finger extensors. The patients were generally built and showed no other extramuscular abnormalities. In addition to this chronic progressive distal muscle weakness, respiratory distress occurred between 0 and 7 years from the initial onset (average 3.8 years) in seven patients (IV-9, V-2, A, B, E, H, and J) with adequate clinical information. Two patients who had not had any respiratory care died of respiratory failure approximately a decade from the initial onset. The other patients have been alive for more than 10 years (maximum 18 years) but require nocturnal non-invasive positive pressure ventilation. They were 37–58 years of age as of 2012 and able to walk independently with or without a simple walking aid. Although the time at which patients recognized dysphagia or dysarthria varied between 1 to more than 10 years from the initial onset, decreased bulbar functions had been noted at the initial evaluation in most cases. Cardiac function was normally maintained in all patients of the family.

Clinical data

The level of serum creatine kinase was normal or mildly elevated. Electromyography of affected muscles showed a chronic myogenic pattern, and the nerve conduction study did not suggest any neuropathic involvement. Muscle imaging showed focal atrophy in the tibialis anterior, tibialis posterior, extensor hallucis and digitorum longus, peroneal and semitendinosus muscle on initial assessment (Figure 2A), and atrophy became clear in cervical muscles, shoulder girdles, intercostals and proximal limb muscles in the following several years. Upon muscle biopsy, the most common finding was numerous cytoplasmic bodies (CBs), which were found on 7.3% of myofibers in the tibialis anterior of individual E (Figure 2B (a–c)) and 50–80% of intercostals in other cases.[5] Other nonspecific findings were increased variability in the size of myofibers, central nuclei and rimmed vacuoles observed on a few fibers. No strong immunoreaction of desmin was seen in the CBs (Figure 2B (d, e)). An electron microscope examination showed that the regular sarcoplasmic pattern was replaced by abnormal fine filamentous structures, which seemed to attach to the Z-band. CBs were also found in almost all skeletal muscles and some smooth muscles in autopsied cases.[5] Cardiac myofibers also contained numerous CBs in one of the autopsied cases (V-2),[5] although the patient did not present any cardiac complication. The sequence analysis of the coding regions and flanking introns of *DES* and *MYOT* showed no pathogenic mutation in individual E. An array comparative genomic hybridization performed with the Agilent SurePrint G3 Human CGH 1M microarray format in individual A did not reveal any aberrations of genomic copy number.

Linkage analysis

DNA was extracted by standard methods. Linkage analysis was performed on nine family members (A–I in Figure 1; four of them were affected, and the others were unaffected) through genotyping using an Illumina Human Omni 2.5 BeadChip (Illumina, San Diego, CA, USA). We chose single-nucleotide polymorphisms (SNPs) that satisfied all of the following criteria: (1) autosomal SNPs whose allele frequencies were available from the HapMap project (http://hapmap.ncbi.nlm.nih.gov/), (2) SNPs that were not monomorphic among members and (3) SNPs that were not in strong linkage disequilibrium with neighboring SNPs (r2 values <0.9). Then, we selected the first five SNPs from each position of integer genetic distance from SNPs that met the above criteria for the initial analysis. The details were as follows; we chose a SNP closest to 0 cM and the neighboring four SNPs. If the genetic distance of a SNP was the same as that of the next SNP, we considered the genomic position to determine their order. We repeated this process at 1 cM, 2 cM and so on.

We performed a multipoint linkage analysis of the data set (17 613 SNPs) using MERLIN[6] 1.1.2 under the autosomal dominant mode with the following parameters: 0.0001 for disease allele frequency, 1.00 for individuals heterozygous and homozygous for the disease allele and 0.00 for individuals

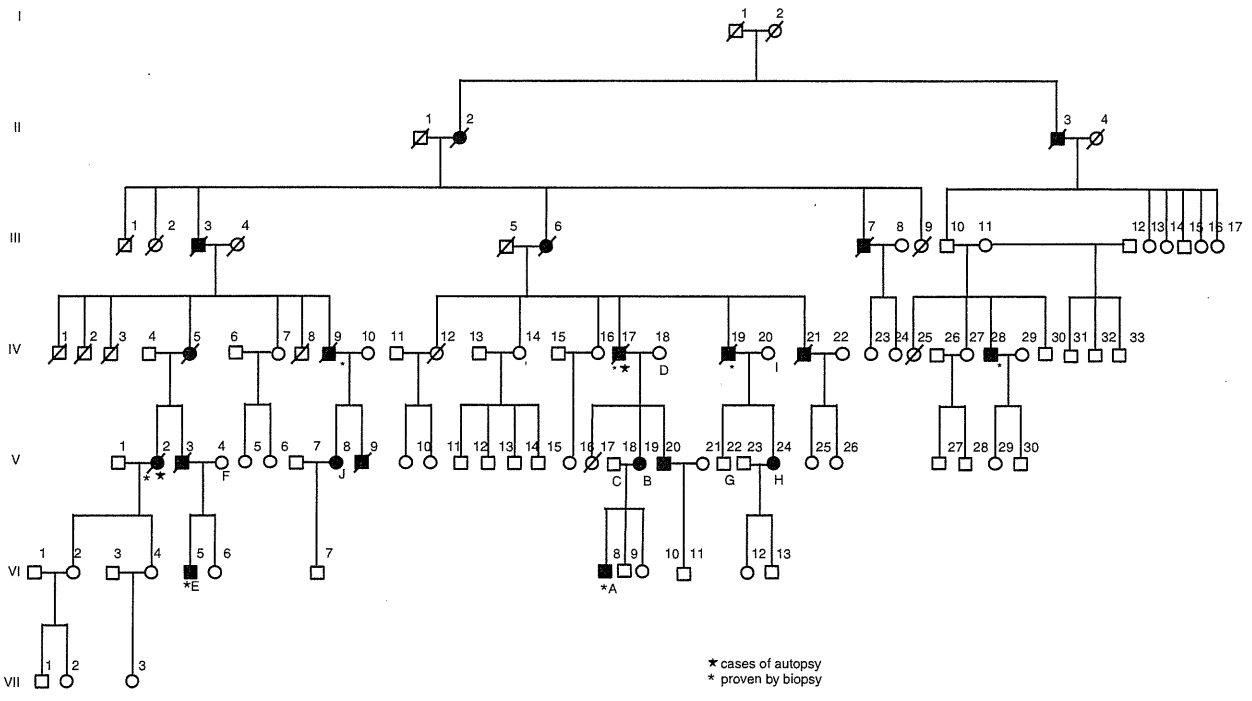


Figure 1 Family pedigree. Filled-in symbols indicate individuals with MFM. Empty symbols indicate unaffected individuals. A star and asterisk indicate autopsy-proven and muscle biopsy-proven cases, respectively. (A–J) indicates individuals whose DNA was used for this study.

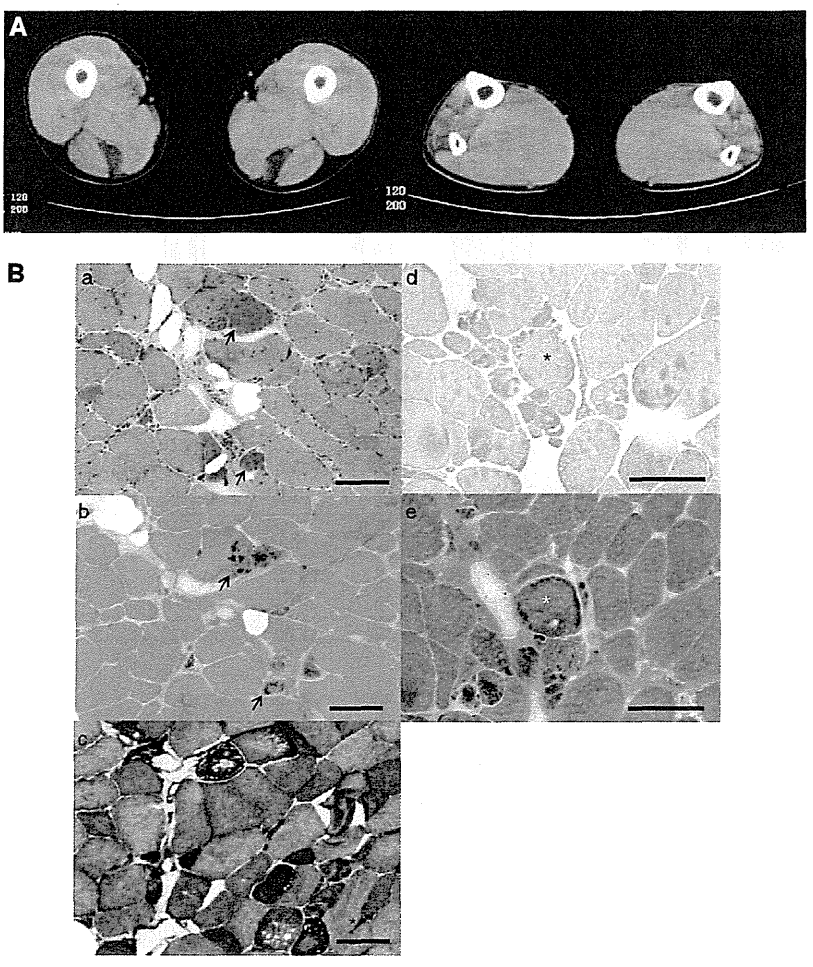


Figure 2 Family clinical data. (**A**) Muscle computed tomography of affected lower extremity. The imaging in the initial assessment of individual A showed symmetrical atrophy and fatty replacement of the semitendinosus in the proximal lower extremities (left) and the tibialis anterior, tibialis posterior, extensor hallucis and digitorum longus, and peroneal muscle in the distal (right) lower extremities. (**B**) Pathology of muscle biopsy. Hematoxylin-eosin (a), Gomori-trichrome (b) and NADH (nicotinamide adenine dinucleotide)-tetrazolium reductase (c) staining of the muscle biopsy sample from the tibialis anterior of individual E are shown. CBs are indicated by arrows. CBs were round or oval, 5–10 μm in diameter and predominantly located in the periphery of type 1 fibers, which stained eosinophilic with hematoxylin-eosin and blue–purple with Gomori-trichrome. NADH-tetrazolium reductase staining showed disorganization of the myofibrillar network. Immunostaining for desmin (d) and Gomori-trichrome staining (e) are serial sections of the muscle biopsy from individual E. Stars indicate corresponding fibers. No strong immunoreaction of desmin was seen in the CBs. Scale bars = 100 μm

homozygous for the alternative allele. After this first analysis, a second analysis was performed with all SNPs fulfilling the above criteria around the peaks identified in the first analysis.

Exome sequencing

Exome sequencing was performed on seven family members in three generations (A–E, H and I in Figure 1), four of whom were affected. Exon capture was performed with the SureSelect Human All Exon kit v2 (individuals E, H and I) or v4 (A–D) (Agilent Technologies, Santa Clara, CA, USA). Exon libraries were sequenced with the Illumina Hiseq 2000 platform according to the manufacturer's instructions (Illumina). Paired 101-base pair reads were aligned to the reference human genome (UCSChg19) using the Burrows-Wheeler Alignment tool.[7] Likely PCR duplicates were removed with the Picard program (http://picard.sourceforge.net/). Single-nucleotide variants and indels were identified using the Genome Analysis Tool Kit (GATK) v1.5 software.[8] SNVs and indels were annotated against the RefSeq database and dbSNP135 with the ANNOVAR program.[9] We used the PolyPhen2 polymorphism phenotyping software tool[10] to predict the functional effects of mutations.

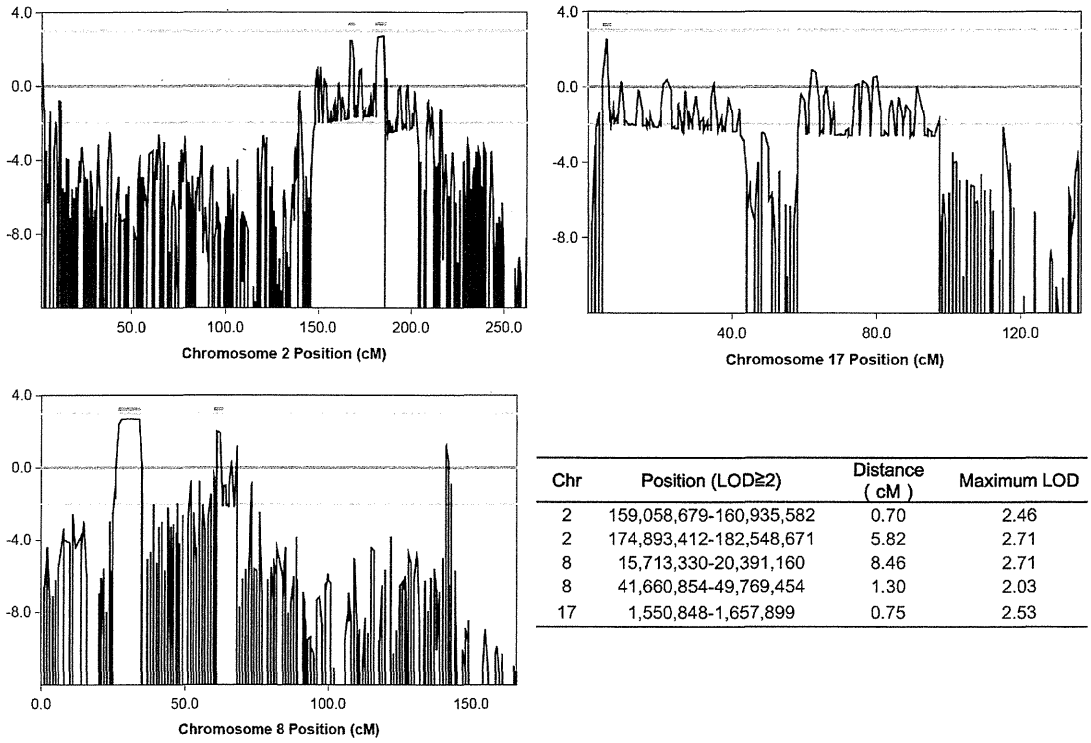
Sanger sequencing

To confirm that mutations identified by exome sequencing segregated with the disease, we performed direct sequencing. PCR was performed with the primers shown in Supplementary Table 1. PCR products were purified with a MultiScreen PCR plate (Millipore, Billerica, MA, USA) and sequenced using BigDye terminator v1.1 and a 3500xL genetic analyzer (Applied Biosystems, Carlsbad, CA, USA).

RESULTS

Linkage analysis

The first linkage analysis identified five regions across autosomes with a logarithm of odds (LOD) score greater than 2 (Figure 3). Of the five regions, two were on chromosome 2 (from 167 cM to 168 cM, with a maximum LOD score of 2.46 and from 182 cM to 185 cM, with a maximum LOD score of 2.71), the other two were on chromosome 8 (from 27 cM to 34 cM, with a maximum LOD score of 2.71 and at 61 cM, with a maximum LOD score of 2.03), and one was on



Chr	Position (LOD≧2)	Distance (cM)	Maximum LOD
2	159,058,679-160,935,582	0.70	2.46
2	174,893,412-182,548,671	5.82	2.71
8	15,713,330-20,391,160	8.46	2.71
8	41,660,854-49,769,454	1.30	2.03
17	1,550,848-1,657,899	0.75	2.53

Figure 3 Linkage analysis. Linkage analysis was performed on nine family members (four of them were affected, the others were unaffected) using an Illumina Human Omni 2.5 BeadChip. Five regions with an LOD score greater than 2 (indicated by bar) were identified. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 1 Summary of detected variants by exome sequencing

Individual *Morbidity*	*A* *Affected*	*B* *Affected*	*C* *Unaffected*	*D* *Unaffected*	*E* *Affected*	*H* *Affected*	*I* *Unaffected*	*Segregated in seven family members*
Exonic, splicing	10089	10064	10079	10065	10230	10194	10216	64
Nonsynonymous, splicing, indel, nonsense	4987	5020	5055	5038	5143	5234	5200	32
Allele frequency not available	577	600	536	555	671	794	786	2

chromosome 17 (at 5 cM, with a maximum LOD score of 2.53). In the second detailed linkage analysis, these peaks were determined to range from 167.49 cM at rs4233674 at position 159 058 679 to 168.19 cM at rs7598162 at position 160 935 582, and from 181.23 cM at rs4402725 at position 174 893 412 to 187.05 cM at rs7420169 at position 182 548 671 on chromosome 2; from 26.42 cM at rs2736043 at position 15 713 330 to 34.88 cM at rs9325871 at position 20 391 160, and from 61.02 cM at rs6999814 at position 41 660 854 to 62.32 cM at rs10957281 at position 49 769 454 on chromosome 8; and from 4.7 cM at rs11078552 at position 1 550 848 to 5.45 cM at rs1057355 at position 1 657 899 on chromosome 17. Haplotypes shared by affected individuals in these regions were confirmed by visual inspection. There were a few incompatible SNPs in these regions, presumably due to genotyping error.

Exome sequencing and segregation analysis

In exome sequencing, an average of 215 million reads enriched by SureSelect v4 (SSv4) and 319 million reads enriched by SureSelect v2 (SSv2) were generated, and 99% of reads were mapped to the reference genome by Burrows-Wheeler Alignment tool. An average of 57% (SSv4) and 61% (SSv2) of those reads were duplicated and removed, and an average of 80% (SSv4) and 66% (SSv2) of mapped reads without duplicates were in target regions. The average coverage of each exome was 163-fold (SSv4) and 130-fold (SSv2). An average of 85% (SSv4) and 69% (SSv2) of target regions were covered at least 50-fold (Supplementary Table 2). On average, 10 133 SNVs or indels, which are located within coding exons or splice sites, were identified per individual (Table 1). A total of 64 variants were common among patients and not present in unaffected individuals, and 32 of those were left after excluding synonymous SNVs. In these variants, only the heterozygous mutation c.90263G>T (NM_001256850) at position 179 410 777 of chromosome 2, which was predicted to p.W30088L in *TTN*, was novel (that is, not present in dbSNP v135 or 1000 genomes). Polyphen2 predicted this mutation as probably damaging. This mutation was located in a candidate region suggested by the linkage analysis in the present study. The other variants were registered with dbSNP135, and the allele frequencies, except for one SNV, rs138183879, in *IKBKB*, ranged from 0.0023 to 0.62.

a

Gene	Chr	Position	Variant type	Reference	Variant	dbSNP v135	Coding change	Prediction	Segregaion analysis
TTN	2	179 410777	Missense	G	T	Not present	W30088L	Probably damaging	Segregating
IKBKB	8	42 188 457	Missense	C	T	rs138183879	T742M	Benign	Non-segregating

b

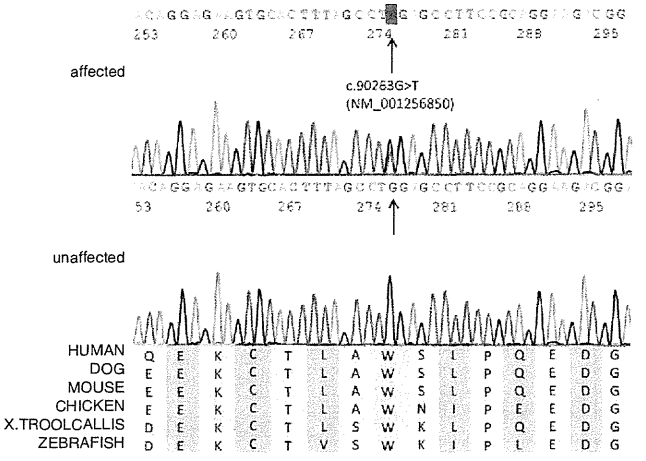


Figure 4 Identified mutations by exome sequencing. (**a**) We performed segregation analysis of two candidates. (**b**) The identified *TTN* mutation and its conservation among species. Sanger sequencing confirmed the heterozygous G to T substitution (indicated by the arrow) at the position chr2;179 410 777, which corresponds to c.90263G>T in exon 293 (NM_001256850.1). The substitution leads to p.W30088L (NP_001243779.1), and this amino acid is conserved among species.

These values were not compatible with the assumption that MFM was a rare disease and showed complete penetrance in this family. The allele frequency of rs138183879 was not available in dbSNP135, and this SNV was in the candidate region on chromosome 8 based on linkage analysis.

We then performed a segregation analysis on the two candidates, the novel mutation c.90263G>T in *TTN* and rs138183879 in *IKBKB*, through Sanger sequencing in 10 family members (A–J in Figure 1; Figure 4a). The rs138183879 SNP was not found in individual J, that is, it was not segregated with the disease in this family. In contrast, the novel mutation c.90263G>T in *TTN* was detected in all patients ($n=5$) and not detected in any of the unaffected family members ($n=5$) or 191 ethnically matched control subjects (382 chromosomes). These results suggested that this rare mutation in *TTN* segregated with the disease in this family.

DISCUSSION

In this study, we found that a novel missense mutation in *TTN* segregated with MFM in a large Japanese family. The identified c.90263G>T mutation in *TTN* (NM_001256850) was considered to be the genetic cause of MFM in our family, because (1) exome sequencing revealed that this was the best candidate mutation after filtering SNPs and indels, (2) this mutation is located in a region on chromosome 2 shared by affected family members, (3) the segregation with MFM was confirmed by Sanger sequencing, (4) this mutation was not detected in 191 control individuals, (5) this mutation was predicted to alter highly conserved amino acids (Figure 4b) and (6) *TTN* encodes a Z-disc-binding molecule called titin, which is similar to all of the previously identified causative genes for MFMs, which also encode Z-disc-associated molecules.

Recently, three mutations in *TTN* have been reported as the causes of hereditary myopathy with early respiratory failure (HMERF, MIM #603689),[11–16] which has similar muscle pathology to MFMs. The identified novel missense mutation c.90263G>T in our study was located on the same exon as recently reported HMERF mutations: c.90272C>T in a Portuguese family[16] and c.90315T>C in Swedish and English families[14,15] (Table 2). This finding suggests the possibility that our family can be recognized as having HMERF from a clinical aspect.

Compared with symptoms described in the past three reports on HMERF (also see Table 2), our patients have common features, such as autosomal dominant inheritance, early respiratory failure, the absence of clinically apparent cardiomyopathy, normal to mild elevation of serum CK and histological findings compatible with MFM. Early involvement of the tibialis anterior is also common, except for the Portuguese family, who reported isolated respiratory insufficiency and a milder presentation of HMERF. Thus, our family shares major clinical manifestations with patients with HMERF, suggesting that the identified mutation is novel for MFM and HMERF.

To date, mutations in *TTN* have been identified in skeletal myopathy and cardiomyopathy.[17,18] The relationship between the variant positions on *TTN* and phenotypes accompanied by skeletal or respiratory muscle involvement is summarized in Table 2. Titin is a large protein (4.20 MDa) that extends from the Z-disk to the M-line within the sarcomere, and it is composed of four major domains: Z-disc, I-band, A-band and M-line (Figure 5). All four HMERF mutations detected by other groups and our study were consistently located in the A-band domain, while mutations in tibial muscular dystrophy (TMD) (MIM #600334),[19–24] limb-girdle muscular dystrophy type 2J (LGMD2J) (#608807)[19,25] and early-onset myopathy with fatal cardiomyopathy (#611705)[26] were located in the M-line domain. HMERF and TMD have some common clinical characteristics, such as autosomal dominant inheritance with onset in adulthood and strong involvement of the tibialis anterior muscle.

Table 2 Previously reported TTN mutations with skeletal and/or respiratory muscle involvement

Phenotype	*LGMD*	*HMERF*	*Our family*	*HMERF*	*HMERF*	*TMD*	*TMD*	*LGMD2J*	*TMD*	*TMD*	*TMD*	*TMD*	*TMD*	*Early-onset myopathy with fatal cardiomyopathy*	*Early-onset myopathy with fatal cardiomyopathy*
Reported by	Vasli *et al.*[16]	Ohlsson *et al.*,[14] Pfeffer *et al.*[15]	Abe *et al.*[5]	Vasli *et al.*[16]	Edstrom *et al.*,[12] Nicolao, *et al.*[11] Lang e *et al.*[13]	Hackman *et al.*[23]	Udd *et al.*,[20] Hackman *et al.*[19]	Udd *et al.*,[25] Hackman *et al.*[19]	Pollazzon *et al.*[24]	Van den Bergh *et al.*[22]	Seze *et al.*,[21] Hackman *et al.*[19]	Hackman *et al.*[23]	Hackman *et al.*[23]	Carmignac *et al.*[26]	Carmignac *et al.*[26]
Mutation identified in Nucleotide (NM_001256850.1)	2012 c.3100G>A, c.52024G>A	2012 c.90315T>C	2012 c.90263G>T	2012 c.90272C>T	2005 c.97348C>T	2008 c.102724delT	2002 102857_ 102867 del11ins11	2002 102857_ 102867 del11ins11	2010 c.102914A>C	2003 c.102917T>A	2002 c.102944T>C	2008 c.102966delA	2008 c.102967C>T	2007 g.289385del ACCAAGTG	2007 g.291297delA
Protein (NP_001243779.1)	p.V1034M, p.A17342T	p.C30071R	p.W30088L	p.P30091L	p.R32450W				p.H34305P	p.I34306N	p.L34315P		p.Q34323X		
Domain	I-band, A-band	A-band (Fn3)	A-band (Fn3)	A-band (Fn3)	A-band (kinase)	M-line	M-line	M-line	M-line	M-line	M-line	M-line	M-line	M-line	M-line
Population	French	Swedish English	Japanese	Portuguese	Swedish	French	Finnish	Finnish	Italian	Belgian	French	Spanish	French	Sudanese	Moroccan
Inheritance	AR	AD	AD	AD	AD	AD	AD	AR	AD	AD	AD	AD	AD	Consanguineous siblings	Consanguineous siblings
Onset	35	33–71	27–45	46	20–50s	20–30s	35–55	20–30s	50–60s	47	45	40–50s	30s	Neonatal	Infant-early childhood
Skeletal muscles															
Major	Proximal UL and LL	TA, PL, EDL, ST	TA, ST	No	TA, neck flexor, proximals	TA, GA,HAM, pelvic	TA	All proximals	TA	TA	TA	TA	TA, HAM, pelvic	General muscle weakness and hypotonia	Psoas, TA, GA, peroneus
Minor		Neck flexor	Cervical, shoulder girdles, intercostals, proximal limb			QF				EDL, peroneal, TP	GA, femoral, scapular	HAM, GA	GA, distal UL		QF, proximal UL, neck, facial, trunk flexor
Spared			Facial			Proximal UL	Facial, UL, proximals	Facial		UL, proximal LL	Facial	UL	Proximal UL, QF		
Cardiac muscles	ND	No	No	ND	ND	ND	No	No	ND	ND	ND	ND	ND	DCM, onset; in the first decade	DCM, onset; 5–12 years old
Respiratory failure	ND	Yes, within 5–8 years	Yes, within 7 years	Isolated respiratory failure	Yes, as first presentation	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Muscle pathologic features	ND	Inclusion bodies (major) and RVs (minor)	Cytoplasmic bodies (major) and RVs (minor)	Cytoplasmic bodies	Cytoplasmic bodies, positive for rhodamine-conjugated phalloidin	Dystrophic pattern without vacuoles	Nonspecific dystrophic change	Nonspecific dystrophic change, loss of calpain-3	Dystrophic pattern with RVs	Nonspecific, RV	Nonspecific	Dystrophic pattern with RVs	Nonspecific	Minicore-like lesions and abundant central nuclei	Minicore-like lesions and abundant central nuclei

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; DCM, dilated cardiomyopathy; EDL, extensor digitorum longus; GA, gastrocnemius; HAM, hamstrings; LL, lower limb; ND, not described; no, no involvement; PL, peroneus longus; QF, quadriceps femoris; RV, rimmed vacuole;
ST, semitendinosus; TA, tibialis anterior; TMD, tibial muscular dystrophy; TP, tibialis posterior; UL, upper limb.

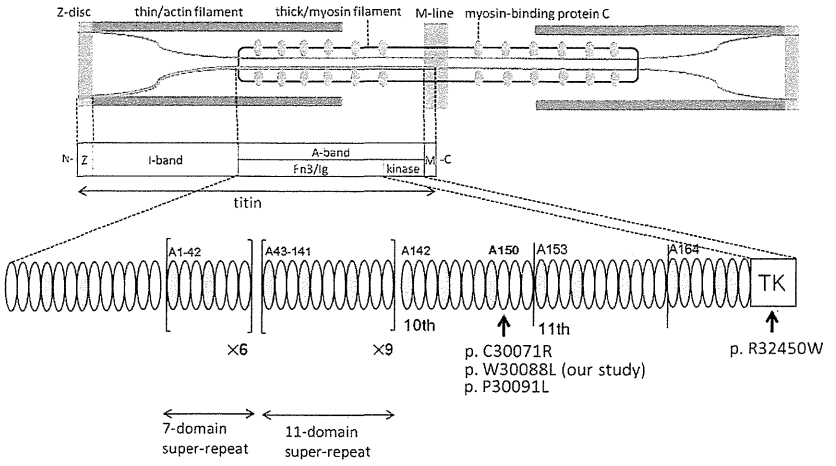


Figure 5 Structure of titin and mutation distribution in the A-band domain. Human *TTN* was mapped to 2q31.2. *TTN* is 294 kb and is composed of 363 exons that code for a maximum of 38 138 amino-acid residues and a 4.20-MDa protein[32] called titin. Titin is expressed in the cardiac and skeletal muscles and spans half the sarcomere, with its N-terminal at the Z-disc and the C-terminal at the M-line.[33] Titin is composed of four major domains: Z-disc, I-band, A-band and M-line. I-band regions of titin are thought to make elastic connections between the thick filament (that is, myosin filament) and the Z-disc within the sarcomere, whereas the A-band domain of titin seems to be bound to the thick filament, where it may regulate filament length and assembly.[34] The gray and white ellipses indicate an Ig-like domain and fibronectin type 3 domain, respectively. Our mutation (p.W30088L) and the neighboring two mutations (that is, p.C30071R and p.P30091L) were all located in the 6th Fn3 domain in the 10th domain of large super-repeats. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

In contrast, one of the distinctive features of TMD is that early respiratory failure has not been observed in patients with TMD. Histological findings of TMD usually do not include CBs but show nonspecific dystrophic change. The underlying pathogenic processes explaining why mutations on these neighboring domains share some similarities but also some differences are unknown.

Three of four HMERF mutations in the A-band domain are located in the fibronectin type 3 and Ig-like (Fn3/Ig) domain, and one of four HMERF mutations is located in the kinase domain (Table 2, also see Figure 5). The missense mutation c.97348C>T in the kinase domain was the first reported HMERF mutation. It has been shown that the kinase domain has an important role in controlling muscle gene expression and protein turnover via the neighbor of BRCA1 gene-1-muscle-specific RING finger protein-serum response transcription factor pathway.[13] Moreover, the Fn3/Ig domain is composed of two types of super-repeats: six consecutive copies of 7-domain super-repeat at the N-terminus and 11 consecutive copies of 11-domain super-repeat at the C-terminus.[27–29] These super-repeats are highly conserved among species and muscles. Our identified mutation (c.90263G>T) and the neighboring two mutations (that is, c.90272C>T and c.90315T>C shown in Table 2) were all located on the 6th Fn3 domain in the 10th copy of 11-domain super-repeat (that is, A150 domain[30]) (Figure 5). Although some Fn3 domains are proposed to be the putative binding site for myosin,[31] the role with the majority of Fn3 domains, how it supports the structure of each repeat architecture, and the identity of its binding partner have not been fully elucidated. Our findings suggested that the Fn3 domain, in which mutations clustered, has critical roles in the pathogenesis of HMERF, although detailed mechanisms of pathogenesis remain unknown.

In conclusion, we have identified a novel disease-causing mutation in *TTN* in a family with MFM that was clinically compatible with HMERF. Because of its large size, global mutation screening of *TTN* has been difficult. Mutations in *TTN* may be detected by massively parallel sequencing in more patients with MFMs, especially in patients with early respiratory failure. Further studies are needed to understand the genotype–phenotype correlations in patients with mutations in *TTN* and the molecular function of titin.

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Short communication

Comparison of REM sleep behaviour disorder variables between patients with progressive supranuclear palsy and those with Parkinson's disease

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ABSTRACT

Purpose: Rapid eye movement (REM) sleep behaviour disorder (RBD) is an important indicator of underlying synucleinopathies. However, the frequency of RBD in tauopathies such as progressive supranuclear palsy (PSP) remains unclear. In this study, we compared RBD-related symptoms and polysomnographic (PSG) findings between patients with PSP and those with Parkinson's disease (PD).
Methods: We conducted clinical interviews of 20 patients with PSP, 93 patients with PD and their caregivers regarding RBD-related symptoms, and conducted PSG recordings on all the subject patients. We then compared the clinical backgrounds, PSG parameters, and frequency of RBD-related symptoms between the two groups.
Results: PSP patients had more severe symptoms of Parkinsonism and cognitive impairment, and took lower doses of dopaminergic agents compared with PD patients. The PSP group had lower values for both estimated total sleep time and sleep efficiency on PSG compared with the PD group ($p = 0.002$, $p = 0.021$, respectively). The PSP group also included a significantly smaller number of patients having REM sleep without atonia (RWA) compared with the PD group ($n = 5$, 20.0% vs. $n = 56$, 60.2%, $p = 0.003$). None of the PSP patients were experiencing RBD-related symptoms at the time of the investigation, while 30 PD patients (32.3%) had RBD-related symptoms.
Discussion: The existence of RWA as well as RBD-related symptoms was less frequent in patients with PSP versus patients with PD. Differences in brain stem pathology and/or disease course between the two disorders might influence this difference.

1. Introduction

Recently, several reports have revealed that rapid eye movement (REM) sleep behaviour disorder (RBD) precedes or follows the onset of synucleinopathies such as Parkinson's disease (PD) and dementia with Lewy bodies (DLB), but only a few reports have investigated the prevalence of RBD among patients with tauopathies [1]. It is believed that RBD is rarely co-morbid with tauopathies such as progressive supranuclear palsy (PSP), cortico-basal degeneration or Alzheimer's disease. This belief comes from the idea that RBD is a specific preclinical symptom of synucleinopathies. However, a recent report showed a similar rate of REM sleep without atonia (RWA), an important physiological basis for RBD occurrence [1], in patients with PSP and those with PD [2,3]. Most patients with PSP have degeneration of cholinergic neurons at the level of the pedunculopontine tegmentum (PPT), which plays a primary role in the regulation of REM sleep [4]. Given this, it is possible that patients with PSP are susceptible to RBD. However, there have been no studies on this issue in Japanese patients with PSP. Therefore, we compared the results of polysomnographic (PSG) examination and clinical interviews regarding RBD symptoms between patients with PSP and those with PD.

2. Subjects and methods

This study was approved by the ethics committees of Tottori University, and all subjects gave informed consent to take part in the study. We examined patients with PSP or PD who were consecutively hospitalised at the Department of Neurology in Tottori University Hospital from December 2004 to March 2011. Twenty patients (15 male and five female) with PSP (mean age of 75 ± 7 years) and 93 patients (39 male and 54 female) with PD (mean age 73.4 ± 7.9 years old) were targeted. The diagnoses of the disorders were made according to the standard criteria for PD[5] and PSP [6]. The PSP group consisted of 13 patients with probable PSP and seven patients with possible PSP based on information about the period between the onset of vertical supranuclear palsy and prominent postural instability accompanying falls [6]. Moreover, we divided patients with PSP into six sub-types according to the clinical

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features reported by Barsottini et al. [7], that is, Richardson's syndrome (RS), PSP-parkinsonism (PSP-P), pure akinesia with gait freezing (PAGF), PSP-progressive non-fluent aphasia, PSP-cerebellar syndrome and PSP-corticobasal syndrome.

We compared descriptive variables including age at the time of investigation, gender, dose of dopaminergic agents as levodopa dose equivalents (LDEs) [5], length of morbidity, Hohen and Yahr grade and scores on the Mini-Mental State Examination (MMSE) between the patients with PD and those with PSP. We also recorded details related to medications, especially central nervous system-acting drugs that may have influenced PSG measures (including RBD-related variables) in patients with both disorders [8].

Overnight PSG recordings together with video monitoring were performed by standardised methods [5]. During REM stage sleep, submental phasic electromyographic (EMG) activity (defined as 3-s mini-epochs containing phasic twitches that are at least four times higher than the background EMG activity) or submental tonic EMG activity with durations of more than half of a 30-s epoch, were scored as RWA [5].

Systematic interviews were made on the subjects and their caregivers regarding sleep problems, with special focus on dream enactment sleep talking and abnormal motor behaviours while dreaming that occurred within 1 month prior to the investigation. The interviews were conducted by physicians specialising in sleep disorders. Based on the PSG findings and the results of the interviews, we evaluated the existence of RWA on PSG as well as its relationship to nocturnal RBD-related symptoms. We diagnosed a patient as having violent RBD if there were both RWA on PSG and violent dream-enactment behaviour according to the 2nd edition of the International Classification of Sleep Disorders [1]. We also diagnosed non-violent RBD when a patient had RWA and sleep talking without having clear violent behaviour while dreaming, and defined both violent RBD and non-violent RBD as RBD-related symptoms [5].

For statistical analyses between the two groups, we used chi-square tests for categorical variables and Mann–Whitney U tests for continuous variables. Data were expressed as mean ± standard deviation (SD). A p-value <0.05 was considered to be statistically significant. The statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS, ver. 15.0J, 2006; SPSS, Tokyo, Japan).

3. Results

At the time of investigation, there was no significant difference in age between the patients with PD and those with PSP. However, there was a significantly larger ratio of male patients in the PSP group (M:F ratio = 15:5) compared with the PD group (M:F ratio = 39:54) ($p = 0.007$). Demographic variables and disease-related information on all the patients, including the dose of dopaminergic agents taken expressed as LDEs [5], were examined. The length of PD morbidity was 7.3 ± 6.9 years, their LDEs at the time of the investigation were 314 ± 219 mg day^{-1} and their Hohen and Yahr grade was 2.8 ± 0.8. The MMSE score in patients with PD was 25.9 ± 3.7. The numbers of patients with PD taking clonazepam, antipsychotics and donepezil were 7, 2 and 2 (respectively). The number of patients taking clonazepam and antipsychotics, donepezil and antipsychotics, donepezil and clonazepam, antipsychotics and Yi Gan San and antipsychotics and benzodiazepines other than clonazepam were 2, 1, 1, 1 and 1 (respectively). In comparison, the PSP patients were taking dopaminergic drugs with LDEs of 202 ± 168 mg day^{-1}, which was significantly smaller than the doses used in the PD patients ($p = 0.026$). The length of PSP morbidity was 5.4 ± 3.3 years, which was roughly similar to the length of morbidity of PD patients. The Hohen and Yahr grade in patients with PSP (3.9 ± 0.9, $p = 0.001$) was higher than that observed in PD patients, and the MMSE score in patients with PSP was lower than that seen in the PD group (PSP: 20.3 ± 8.1, $p = 0.001$) (Table 1). The number of patients with PSP taking benzodiazepines other than clonazepam, clonazepam and both clonazepam and other benzodiazepines were 2, 1 and 1 (respectively). Sixteen patients with PSP did not take any of these kinds of drugs. There were no patients taking antidepressants in either group at the investigation.

Comparison of the PSG parameters between the two groups showed that the patients with PSP had lower values for estimated total sleep time and sleep efficiency compared with those with PD ($p = 0.002$, $p = 0.021$, respectively, Table 2). There were no significant differences in the percentages of stage 1, stage 2, stage 3/4 or REM stage sleep between the two disorder groups. However, the number of patients having RWA in the PSP group was significantly smaller compared with the PD group (5/20 vs. 56/93, $p = 0.004$). There was also a significant difference in the proportion of RWA/(RWA + REM) between the two patient groups ($p = 0.021$).

Table 1
Comparison of descriptive variables between patients with PSP and PD.

	PSP ($n = 20$)	PD ($n = 93$)	p-value
Age (years)	75 ± 7	73.4 ± 7.9	n.s.
Gender (Male/Female)	15/5	39/54	0.007
Length of morbidity (years)	5.4 ± 3.3	7.3 ± 6.9	n.s.
Hohen &Yahr grade	3.9 ± 0.9	2.8 ± 0.8	0.001
Levodopa dose equivalents (mg/day)	202 ± 168	314 ± 219	0.026
Mini Mental State Examination (scores)	20.3 ± 8.1	25.9 ± 3.7	0.001

Levodopa dose equivalents = Levodopa + Selegiline × 0.75 + Prampexole × 67 + Pergolide × 0.1 + Bromocriptine × 10 + Talipexole × 37.5.
The values are expressed as mean ± SD except for gender.
n.s. = not significant, Mann–Whitney U test.

With regard to PSP sub-types, nine patients were categorised as RS, eight patients as PSP-P and three patients as PAGF. Among these, one patient with RS, two patients with PSP-P and two patients with PAGF had RWA on PSG. There were no significant differences in the proportion of patients having RWA among the three sub-types.

Thirty patients with PD (32.3%) had RBD-related symptoms. Among these, seven patients had violent RBD symptoms and the other remaining 23 patients had non-violent RBD symptoms. Out of 93 PD cases, 26 PD patients with RWA (28.0%) had no experiences of possible RBD-related symptoms. As for the relationship between the clinical course of PD and RBD-related symptoms, 15 patients (15.6%) out of 93 PD cases reported having symptoms from the period before the onset of PD to the time of the investigation. Out of these 15 patients, two patients reported that the RBD symptoms changed from violent symptoms before the onset of PD to non-violent symptoms after the onset of PD. One patient (1.1%) having RBD-related symptoms before the onset of PD reported that the symptoms completely disappeared after the onset of PD. In comparison, 14 patients (14.6%) reported that the symptoms appeared after the onset of PD.

By contrast, none of the PSP patients had symptoms of sleep talking or violent nocturnal behaviour while dreaming. As a result, there was a significant difference between the two disorder groups in terms of the number of patients with RBD-related symptoms ($p = 0.012$).

4. Discussion

Previously, Montplaisir et al. reported that patients with PSP had a significantly shorter total sleep time and reduced sleep efficiency compared with controls [9]. Sixel-Doring et al. also reported that

Table 2
Comparison of PSG findings between patients with PSP and PD.

	PSP ($n = 20$)	PD ($n = 93$)	P value
Total sleep time (min)	244 ± 84	312 ± 92	0.002
Sleep efficiency (%)	46.6 ± 16.0	56.3 ± 17.4	0.021
Stage 1 (%)	17.7 ± 7.9	18.2 ± 7.6	n.s.
Stage 2 (%)	27.2 ± 13.9	33.6 ± 14.7	n.s.
Stage 3 + 4 (%)	1.4 ± 2.8	2.9 ± 5.7	n.s.
Total stage REM (RWA + REM) (%)	8.9 ± 4.9	11.0 ± 8.9	n.s.
Proportion of patients having RWA (%)	20 (5/20)	60.2 (56/93)	0.003
RWA/(RWA + REM) (%)	10.5 ± 28.2	13.1 ± 22.1	0.021

RWA:REM sleep without atonia. Sleep stage variables are expressed as absolute values and the proportions. A p-value <0.05 was considered statistically significant.
n.s. = not significant. Mann–Whitney U test.

polysomnographically recorded sleep was more severely impaired in patients with PSP versus patients with PD [3]. In the present study, patients with PSP had lower values of estimated total sleep time and sleep efficiency versus those with PD. Taking these findings together, PSP is accompanied by disturbances in the initiation and/or maintenance of nocturnal sleep.

When diagnosing RBD, it is necessary to confirm both the existence of RWA on PSG and violent dream-enactment behaviour [1]. In this study, we conducted thorough clinical interviews on all the subject patients and their caregivers regarding RBD-related symptoms. In this regard, only two previous studies examined RWA together with RBD-related symptoms in patients with PSP [2,3], and this study was the third to systematically investigate both PSG findings and clinical RBD symptoms among patients with PSP. The number of our PSP patients was the same as that in the report by Sixel-Doring et al [3].

The percentage of PSP patients with RWA in this study (5/20, 25%) was quite similar to the rate reported by Arnulf et al. (4/15, 27%). In that report, they stated that the percentage of patients with RWA was not different between the PD group and the PSP group. However, the percentage of PD patients with RWA in Arnulf's report (4/15, 27%) [2] was clearly lower than that observed in our study (56/93, 60.2%), which is quite consistent with reported rates of RWA positivity in PD [10]. Comparatively, not only the number of PSP patients with RWA (17/20, 85%) but also the number of PD patients with RWA (19/20, 95%) in Sixel-Doring's reports [3] were strikingly higher than both the present results and Arnulf's reports [2]. The reason for the difference among the studies is unclear. However, in contrast to the study by Montplasir et al., in which none of the PSP patients had RWA on PSG [8], recent studies including the present study demonstrate that a significant number of PSP patients have RWA. We speculate that while a certain number of patients with PSP could have RWA, the rate of PSP patients having RWA is lower relative to RWA occurrence in PD. In addition, our results raise the possibility that even in RWA-positive PSP patients the amount of RWA is lower than that in RWA-positive PD patients.

It has been hypothesised that RWA occurs based on the dysfunction of the locus coeruleus (LC) and the pedunculopontine nucleus (PPN), both of which regulate REM sleep. Warren et al. indicated that cholinergic nerves in PPN are degenerated even in the early stages of PSP [4]. Also, Mann et al. pointed out that neuronal loss in LC is clearly more severe in cases with PD than in cases with PSP [10]. Given these studies, we speculate that a combined degeneration of PPN and LC is important for the formation of RWA, and that the difference in the frequency of RBD between PD and PSP comes from differences in the severity of LC pathology.

Generally, the percentage of PD patients having RBD symptoms ranges from 15% to 34% [11]. Lavault et al. showed that the length of PD morbidity does not correlate with the occurrence of RBD [12]. Of note, none of the patients with PSP in our study had experienced RBD-related symptoms, while the rate of PD patients having symptoms was 32.3%, which is quite consistent with previously reported rates [11]. In addition, no clear relationship between the clinical course of PD and the occurrence of RBD was observed, consistent with the aforementioned report by Lavault et al. [12] In Sixel-Doring's report, the percentage of PSP patients having clinical RBD symptoms (35.0%) was clearly lower than the percentage of PD patients with these symptoms (68.4%) [3]. Thus, it is possible that RBD-related symptoms are less likely to occur in PSP compared with PD even in RWA-positive cases. The reason for this phenomenon is unclear. However, one possible cause is that a higher severity of motor symptoms and/or cognitive impairment, both of which are known to progress more rapidly in PSP versus PD [6], might act to mask the RBD symptoms. As a second possible cause, PSP patients are less likely to have terrifying and uncomfortable dreams, the rate of which is considerably higher in PD populations [13].

The present study has several limitations. First, we confirmed RBD-related symptoms by retrospective interviews with patients and their caregivers. For this reason, it is possible that we might have overlooked the existence of mild RBD-related symptoms. Second, some patients were medicated with drugs that can impact RBD-related symptoms and PSG findings. Third, our study sample included patients from only one institute, and it is possible that a sampling bias existed.

However, conclusively, RBD was commonly observed in PD patients, but the rate of PSP patients having RWA was lower and none of the PSP patients had RBD symptoms. To draw more definitive conclusions about the existence of RWA and RBD-related symptoms in patients with PSP and its relation to PSP sub-types, it would be necessary to conduct a prospective study including neuropathological examination on a large number of patients with the disorder.

Conflict of interest

The authors have no conflicts of interest pertaining to this study.

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