immunoglobulin ($n = 33$), intravenous methylprednisolone plus therapy ($n = 32$), tacrolimus ($n = 22$), methotrexate ($n = 11$), azathioprine ($n = 11$), cyclosporine ($n = 9$), intravenous cyclophosphamide ($n = 3$), or plasma exchange ($n = 3$).

Clinical course

There were 3 types of clinical courses. In the first type, 26 (32 %) of 81patients responded well to immunotherapy without neurological deficits, although they required 2–3 months to respond to treatment. In the second type, 45 patients (56 %) were refractory to various immunotherapy regimens. The immunotherapy resulted in a decrease of the patients' creatine kinase levels, but the recovery of muscle weakness was incomplete. These patients required long-term immunotherapy and suffered from side effects. Finally, the remaining ten (12 %) patients showed progressive muscle weakness. Their response to immunotherapy was minimal.

Neurological outcome

The patients' neurological outcomes were assessed at 2 years after the initiation of immunotherapy using modified Rankin Scale. Of the 81 patients treated with immunotherapy, 22 (27 %) had difficulties in their daily living graded as modified Rankin Scale scores 3–5. We divided these 81 patients into 2 groups: those with a good outcome, defined as a modified Rankin Scale score of 0–2 ($n = 59$) and those with a poor outcome, defined as a modified Rankin Scale of 3–5 ($n = 22$). We compared the clinical features before immunotherapy between the 2 groups (Table 2).

We found that pediatric disease onset, severe limb weakness, dysphagia, muscle atrophy, absence of interstitial lung disease, and elevated C-reactive protein were associated with the poor outcome. Moreover, multivariate logistic analyses revealed that pediatric disease onset was the only independent factor associated with the poor outcome ($p = 0.003$, odds ratio: 28.4, 95 % confidential interval 2.82–845).

Based on the result of multivariate logistic analyses, the onset age was an important factor for anti-SRP myopathy. We compared clinical and laboratory characteristics between 13 patients with younger onset and 68 patients with older onset using the cutoff age of 30 years (Additional file 1). The younger onset was associated with chronic disease progression, severe limbs and neck weakness and muscle atrophy. The neurological outcome was more severe in the younger onset compared with the older onset.

Cause of death

Among 17 patients followed at Keio University Hospital for a longer follow-up, 7 patients died during the clinical course (age 74 ± 11 years). The side effects of the long-term use of corticosteroids may have been responsible for the death of 5 patients (cerebral infarction in 2 patients, ischemic heart diseases in 2, and bacterial pneumonia in 1 patient). The remaining 2 patients died of lung cancer and intestinal lung disease, respectively.

Table 2 Comparison of clinical features of 81 patients with good or poor outcomes

	Good outcome ($n = 59$)	Poor outcome ($n = 22$)	p
Females	35 (59 %)	16 (73 %)	0.3
Age at disease onset ≤ 15 years	1 (2 %)	6 (27 %)	0.0003
Disease progression > 12 months	12 (20 %)	8 (36 %)	0.1
Clinical characteristics			
Legs predominantly than arms	43 (73 %)	16 (73 %)	1.0
Severe limbs weakness	31 (53 %)	20 (91 %)	0.001
Neck weakness	37 (63 %)	17 (77 %)	0.2
Dysphagia	20 (34 %)	14 (63 %)	0.02
Muscle atrophy	32 (54 %)	20 (91 %)	0.002
Decreased deep tendon reflex	28 (47 %)	15 (68 %)	0.1
Myalgia	19 (32 %)	7 (32 %)	1.0
Interstitial lung disease	12 (20 %)	0 (0 %)	0.02
Laboratory findings			
Creatine kinase (IU/L)	6181 ± 4313	7079 ± 5263	0.5
Elevated C-reactive protein	7 (12 %)	8 (36 %)	0.01
Anti-SRP54 antibody index	1.4 ± 1.0	1.3 ± 1.1	0.8
Lymphocyte infiltration in histology	11 (19 %)	3 (14 %)	0.6

Discussion

The present case series of 100 patients with inflammatory myopathy with anti-SRP antibody provided the following findings: (i) patients of all ages were affected; (ii) neurological symptoms were characterized by severe limb, trunk, and bulbar muscle weakness with atrophy; (iii) histological diagnoses showed 84 patients had necrotizing myopathy; (iv) anti-SRP54 antibodies were undetectable in 18 serum samples containing autoantibodies to 7S RNA of SRP; and (v) pediatric disease onset was associated with the poor neurological outcome. Taken together, these results led us to conclude that anti-SRP antibodies can be used determine a distinct subset in inflammatory myopathy (anti-SRP myopathy).

The diagnosis of anti-SRP myopathy is based on both the detection of anti-SRP antibodies in patients' serum and the histological diagnosis of inflammatory myopathy, usually necrotizing myopathy. Ancillary tests and results including markedly elevated serum creatine kinase, electromyography, and muscle images support the diagnosis. Since the disease presentation and progression are variable, the clinical diagnosis is sometimes difficult. The chronic progression of anti-SRP myopathy accompanied by younger onset, severe muscle weakness and atrophy mimics muscular dystrophy. When a patient's muscle weakness appears to be progressing faster than expected, as in muscular dystrophy, it may be worth testing for anti-SRP antibodies. The broad clinical presentation of SRP autoimmunity requires an expanded consideration of the anti-SRP antibody detection test in patients of all ages.

Necrotizing myopathy is a heterogeneous pathological category including autoantibody-mediated, drug-induced, paraneoplastic and viral infections [7, 8]. The presence of anti-SRP antibody is the most frequent etiology. We recently reported that 34 (53 %) of 64 patients with necrotizing myopathy had anti-SRP antibodies [16]. Anti-HMGCR and anti-ARS antibodies are also possible candidates as serological markers of necrotizing myopathy [8]. Anti-HMGCR antibodies were first known as markers of statin-induced myotoxocicity; however, they were also found in patients without statin-exposure [15, 17]. In the present study, although 5 patients received statins at the disease onset, they all had anti-SRP antibodies but not anti-HMGCR antibodies. Moreover, anti-ARS antibodies were not detected in our 100 patients with anti-SRP antibodies. The strength of present study is that we screened these 3 autoantibodies using RNA immunoprecipitation and ELISAs. We estimate that 3 types of autoantibodies may be independently attributed to the immune-mediated pathogenesis of necrotizing myopathy.

We detected anti-SRP antibodies by 2 different methods: RNA immunoprecipitation and an SRP54 ELISA. Benveniste et al. developed an addressable laser bead immunoassay using SRP54, and they demonstrated that the levels of anit-SRP54 antibodies were associated with the clinical course [12]. However, epitopes of anti-SRP antibodies are not always located in SRP54. Valiyil et al. demonstrated that 3 of 8 patients with anti-SRP antibodies did not have the autoantibodies to SRP54 [18]. In the present study, we have shown that 18 serum samples with autoantibodies to 7S RNA of SRP were negative for anti-SRP54 antibodies. It should be emphasized that false-negative results on an SRP54-specific immunoassay can be misleading. In contrast, RNA immunoprecipitation can recognize the conformational epitopes of the SRP complex, although the procedure is technically difficult and requires some technical skills and cultured cell extracts.

The neurological outcome of the patients with anti-SRP myopathy was unsatisfactory regardless of the combination of immunosuppressive agents. Our analyses revealed that pediatric disease onset was the most significant factor associated with poor neurological outcomes, suggesting that the dysfunction of the regeneration process of muscle fibers may be important in the pathogenesis of anti-SRP antibodies. In addition, we observed there was no correlation between the interstitial lung disease and poor neurological outcome. The severity of interstitial lung disease seemed to be mild. We thought decreased capacity of respiratory function was due to respiratory or trunk muscle weakness. In contrast, the elevated C-reactive protein was associated with poor neurological outcome. Increased levels of C-reactive protein were probably due to the systemic inflammation of anti-SRP myopathy. However, severe neurological deficits in SRP-positive patients are not related to survival.

Other autoantibodies have been found to be closely associated with the lethal condition, including anti-melanoma differentiation-associated gene 5 antibodies with rapidly progressive interstitial lung disease, anti-transcription intermediary factor 1 antibodies with malignancy, and anti-mitochondrial antibodies with cardiac involvement [19–21].

Our study has some limitations. First, the entry of patients in the present study was based on the referrals from other institutions. The frequencies of anti-SRP antibodies were found to be 5 %–8 % of the clinical diagnoses of myositis. In contrast, the frequency was up to 20 % of patients using the strict criteria of histological diagnosis [16]. We suspect that the actual prevalence of anti-SRP antibodies depends on whether the population is defined by clinical or histological criteria. Second, we cannot determine the best regimens of immunotherapy for anti-SRP myopathy. However, we emphasized that our treatment experience of 100 patients with anti-SRP myopathy suggested the valuable information. The combination of oral corticosteroids and intravenous immunoglobulin

was effective as the first-line therapy. Additional immunosuppressive agents were also used for the purpose of reducing side effects of corticosteroids. The removal of anti-SRP antibodies by plasma exchange was considered as the second-line therapy.

Conclusion

Anti-SRP antibodies are associated with different clinical courses and histological presentations. Further studies should clarify the pathogenesis of anti-SRP antibodies.

Additional file

Additional file 1: Comparison of clinical features between younger and older onset. Clinical and laboratory characteristic between 13 patients with younger onset and 68 patients with older onset using the cutoff age of 30 years were compared.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SS, MK, and IN were involved in study concept and design. SS drafted the manuscript. SS, AN, HN, YW and IN were involved in clinical assessment and acquisition of the data. MK, JN and YH contributed to interpretation of the data and statistical analysis. MK and YH contributed to critical revision of the manuscript for important intellectual content. NS and IN were involved in study supervision. SS, NS and IN contributed to obtain the funding. All authors have read and approved the final version of the manuscript.

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RESPIRATORY AND CARDIAC FUNCTION IN JAPANESE PATIENTS WITH DYSFERLINOPATHY

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ABSTRACT: *Introduction:* We retrospectively reviewed respiratory and cardiac function in patients with dysferlinopathy, including 2 autopsy cases with respiratory dysfunction. *Methods:* Subjects included 48 patients who underwent respiratory evaluation (n = 47), electrocardiography (n = 46), and echocardiography (n = 23). *Results:* Of the 47 patients, 10 had reduced percent forced vital capacity (%FVC), and 4 required noninvasive positive pressure ventilation. %FVC was significantly correlated with disease duration, and mean %FVC was significantly lower in non-ambulatory patients, as well as in those aged ≥65 years with normal creatine kinase levels. On electrocardiography, QRS complex duration was prolonged in 19 patients, although no significant association with age, disease duration, or respiratory function was found. Echocardiography indicated no left ventricular dysfunction in any patient. Histopathology of autopsied cases revealed mild cardiomyopathy and moderate diaphragm involvement. *Conclusion:* Patients with dysferlinopathy may develop severe respiratory failure and latent cardiac dysfunction. Both respiratory and cardiac function should be monitored diligently.

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Additional Supporting Information may be found in the online version of this article.

Abbreviations: CK, creatine kinase; CPF, cough peak flow; DCM, dilated cardiomyopathy; DYSF, dysferlin; ECG, electrocardiogram; EF, ejection fraction; FVC, forced vital capacity; LGMD2B, limb-girdle muscular dystrophy type 2B; MM, Miyoshi myopathy; NADH-TR, nicotinamide adenine dinucleotide–tetrazolium reductase; NCNP, National Center of Neurology and Psychiatry; NIV, noninvasive positive pressure ventilation

Key words: autopsy; cardiac function; dysferlinopathy; LGMD2B; Miyoshi myopathy; respiratory function

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Dysferlinopathy is an autosomal recessive muscular dystrophy caused by dysferlin gene mutations (*DYSF*; MIM # 603009). The *DYSF* gene is located on chromosome 2p13 and codes for dysferlin, which is mainly localized in the sarcolemma and is associated with Ca^{2+}-dependent defective membrane repair and T-tubule system development. The 2 major phenotypes of dysferlinopathy are proximal dominant limb-girdle muscular dystrophy type 2B (LGMD2B) and distal dominant Miyoshi myopathy (MM). In patients with dysferlinopathy, dysferlin in the sarcolemma is absent or markedly reduced. Immunohistochemical staining (IHC), immunoblotting (IB), and gene analysis are useful for diagnosis.

Cardiac dysfunction (e.g., dilated cardiomyopathy, cardiac conduction defect) is variably present in patients with certain muscular dystrophies, including dystrophinopathy and sarcoglycanopathy, and respiratory failure is a major complication in many neuromuscular disorders. However, these are not part of the usual manifestations of dysferlinopathy[1]; only a limited number of studies have reported respiratory and cardiac function in patients with dysferlinopathy.[2–11] The majority of patients, including those with long disease duration, reportedly exhibited no respiratory problems, although some non-ambulatory, advanced-stage patients (but not necessarily with long disease duration) showed reduced vital capacity.[2–6] Moreover, dilated cardiomyopathy on echocardiography, and/or abnormalities on electrocardiogram (e.g., ST–T change), were observed in both advanced and non-advanced stage patients.[9–11] However,

Table 1. Patient characteristics.

	Mean	SD	Median	Range	Interquartile range (25th–75th percentile)
Age (years)	42.5	14.7	39.5	20–79	32.8–50.5
Age at onset (years)	22.5	7.5	20.5	10–46	16.0–29.0
Duration from disease onset (years)	20.0	12.3	17.5	3–46	11.0–26.0
Age at gait disturbance	43.8	10.6	45.5	22–60	36.0–50.0
CK (IU/L)	3,596	2,978	3,160	94–14,591	1,249–4,884
BMI	21.5	3.7	21.3	15.3–35.0	18.9–23.0
%VC (%)	92.6	28.3	101.8	23.1–129.0	86.8–112.8
%FVC (%)	92.7	28.9	100.6	20.1–131.5	84.7–112.5
EF (%)	68.0	8.3	66.0	48–84	65.0–73.5
QRS duration (ms)	97.5	12.7	94.0	78–138	88.0–108.0

CK, creatine kinase; BMI, body mass index; VC, vital capacity; FVC, forced vital capacity; EF, ejection fraction.

there are no clinical reports with details regarding respiratory dysfunction associated with dysferlinopathy, and only a few reports have assessed histological changes in respiratory and cardiac muscles in dysferlinopathy patients.[7,8]

Patients with neuromuscular disorders who present with respiratory dysfunction undergo respiratory training using the air stacking technique to increase thoracic capacity and to use assisted cough peak flow (CPF) from an early stage.[12] In addition, medication for cardiomyopathy, such as angiotensin-converting enzyme inhibitors and/or β-blockers, can be effective in delaying progression.[13,14]

If dysferlinopathy patients commonly develop respiratory and cardiac dysfunction, physicians should diligently monitor respiratory and cardiac function to detect early signs to enable respiratory training and prescription of cardiac medication from an early stage. In light of this background, we aimed to analyze factors that contribute to respiratory and cardiac dysfunction in dysferlinopathy.

METHODS

Study Population. We retrospectively reviewed medical charts of 48 patients with dysferlinopathy who visited the National Center Hospital of the National Center of Neurology and Psychiatry (NCNP) from July 1979 to September 2013. Patient diagnoses were confirmed by genetic analysis and/or IHC together with IB,[15] for those who satisfied 1 or more of the following diagnostic criteria: (1) known homozygous or compound heterozygous *DYSF* gene mutations; (2) 1 reported and 1 unreported, but causing aberrant splicing mutations; and (3) absence of dysferlin in the sarcolemma by IHC. All patients who had no or only 1 mutation in a single allele were verified to have absent dysferlin. This study was approved by the ethics committee of the NCNP.

Measurements and Analysis. We collected data on age at the time of examination, age at onset, disease duration, ambulation status, serum creatine kinase (CK) levels, disease phenotype, respiratory function (%FVC), and cardiac function (electrocardiogram and echocardiogram) for analysis. As percent vital capacity changed in parallel with %FVC, we only used %FVC in respiratory assessments. Decrement in %FVC and ejection fraction (EF) were defined as $<80\%$[16] and 55%,[17] respectively. Prolongation in QRS complex duration was defined as >100 ms.[18]

A *t*-test was used for group comparisons of continuous data, and the Fisher exact test was used for binary data. Correlation of data was assessed using the Spearman rank correlation coefficient, which is appropriate for both continuous and discrete variables, including ordinal variables. Fisher exact tests were used to analyze factors that potentially contribute to respiratory dysfunction: age $\geq$65 years (the age at which one is defined as elderly in Japan)[19]; age at onset $\geq$20 years; gender; ambulation status; serum CK levels; and disease phenotype. All analyses were performed using SPSS for Macintosh, version 18 (SPSS, Inc., Chicago, Illinois).

RESULTS

General Characteristics. The analysis included a total of 48 Japanese patients (25 men, 23 women). Age at onset was 22.5 ± 7.4 years, and mean age at the time of data collection was 42.5 ± 14.7 years (Table 1). Of these, 22 and 26 patients were diagnosed with LGMD2B and MM, respectively, 36 were still ambulatory, and 12 were completely wheelchair-bound. Among non-ambulatory patients, mean disease duration at loss of ambulation was 19.4 ± 5.9 years. Mean serum CK was 3,596 ± 2,978 IU/L. More men were included in the MM group, and more women were in the LGMD2B group; age at disease onset was significantly older in the LGMD2B group (see Table S1 in Supplementary Material, available online), although other parameters did not show a statistically significant difference.

Table 2. Genotypes of patients with dysferlinopathy.

Homozygous	
c.493delC	1
c.663+1 G>C	1
c.855+4 T>C	1
c.1284+2 T>C	1
c.1852 G>A	1
c.2233–2235delAAC*	1
c.2551 C>T*	1
c.2997 G>T	2
c.4934 T>A*	1
c.4497delT	3
c.5077 C>T	2
c.5509 G>A	1
c.6135 G>A	1
Compound heterozygous	
c.342+1 G>A / c.6135 G>A	1
c.565 C>G / c.855+4 T>C	1
c.855+4 T>C / c.4200 delC	1
c.937+1 G>A / c.2643+1 G>A	1
c.1004 G>A* / c.4497 delT	1
c.1353+1G>A / c.5526-1 G>A	1
c.1566 C>G / c.2997 G>T	1
c.1556 C>G / c.5509 G>A	1
c.1566 C>G / c. 5698_5699 delAG	1
c2644-2 A>G / c.2997 G>C	1
c.2746_2747dupGG* / c.5309 T>C*	1
c.2997 G>T / c.3373 delG	2
c.2997G>T / c.3827 delT*	2
c.2997G>T / c.4497 delT	1
c.2997 G>T / c.4886+2 T>A*	1
c.2997 G>T / c.6135 G>A	2
c.3373 delG / c.4748_4750 delACAinsT	1
c.4720G>T / c.5036 G>A	1
Heterozygous	
c.2997 G>T	4
Not tested	6
Total	48

*Unreported.

Immunohistochemistry and Gene Analysis. IHC and IB were performed in 34 (71%) and 25 (52%) patients, respectively, revealing completely absent sarcolemmal dysferlin in all patients. *DYSF* gene analysis was performed in 42 (88%) patients. Of these patients, 17 were homozygous and 21 were compound heterozygous, including 7 with 8 unreported mutations (Table 2, and Table S2 online). Gene analysis and IHC were performed in 28 (58%) patients. Although the pathogenicity of these unreported mutations is unclear, they were not detected in 100 healthy Japanese people, and 6 patients with unreported mutations, with the exception of 1, showed abnormalities on IHC. One patient without muscle biopsy had c.2997 G>T and c.4886+2 T>A compound heterozygote. As c.2997 G>T is a well-known causative mutation and c.4886+2 T>A causes aberrant splicing, we considered these mutations to be pathogenic. Only 1 point mutation in a single allele was detected in 4 patients whose diagnosis was confirmed by IHC and IB. In these patients, another mutation may exist in non-translated or promoter regions. The most common mutation was c.2997 G>T.

Respiratory Function. None of the patients had lung or thoracic diseases that could affect their respiratory function. Mean %FVC was 92.7 ± 28.9 [median 100.6, interquartile range (25th–75th percentiles) 84.7–112.5, range 20.1–131.5], and no significant difference was found between the LGMD2B and MM groups (see Table S1 online).

Patients with Respiratory Dysfunction. Age and disease duration were significantly correlated with a reduced %FVC (Fig. 1). Significant factors associated with %FVC <80 identified by Fisher exact tests included age ≥65 years, non-ambulatory status, and CK levels (Table 3).

Decreased %FVC of <80 (range 20.1–71.2) was seen in 21.3% (10 of 47) of patients (see Table S3 online), including 9 patients who were completely wheelchair-bound and 4 who had serum CK levels within the normal range. Their mean age was significantly older (58.4 ± 16.9 vs. 38.7 ± 11.0, $P < 0.001$) than in patients with normal respiratory function. Patients aged <40 years or those who were still ambulatory also had a decreased %FVC. Among these patients, 5 had episodes of respiratory failure, such as hypoventilation coma during respiratory infection requiring nocturnal, or occasional noninvasive positive pressure ventilation

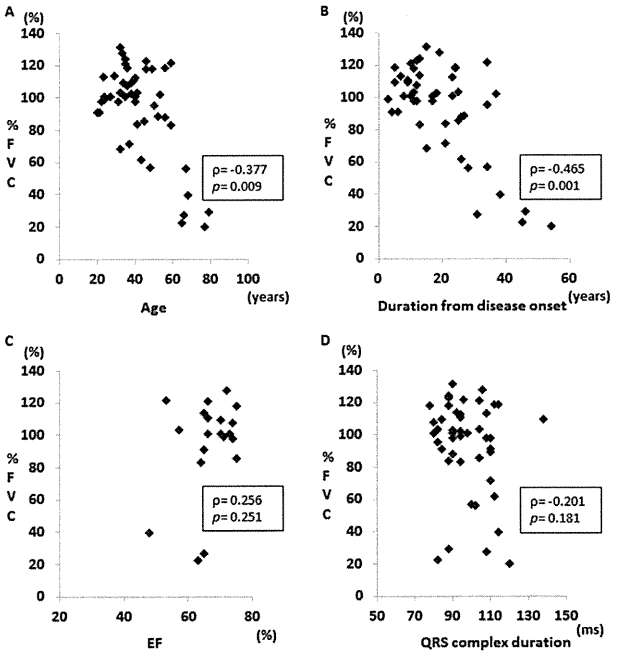

FIGURE 1. Scatterplots of percent forced vital capacity (FVC) and age **(A)**, disease duration from onset **(B)**, ejection fraction **(C)**, and QRS complex duration **(D)**. A significant correlation was found between disease duration and %FVC (ρ = 0.465, P = 0.001).

Table 3. Fisher exact tests for the influence of respiratory factors studied.

	Respiratory function		
	FVC ≥80%	FVC <80%	*P*
Age (years)			<0.001
≥65	0	6	
<65	37	4	
Age at onset (years)			1.00
≥20	21	6	
<20	16	4	
Gender			0.072
Men	16	8	
Women	21	2	
Ambulation status			<0.001
Ambulatory	34	1	
Non-ambulatory	3	9	
CK			0.001
Normal	0	4	
Elevated	37	6	
Phenotype			0.734
MM	21	5	
LGMD2B	16	5	

CK, creatine kinase; MM, Miyoshi myopathy; LGMD2B, limb-girdle muscular dystrophy type 2B; FVC, forced vital capacity.

(NIV); however, they did not share the same mutations, except c.2997G>T. Respiratory function was monitored for ≥10 years in 3 patients (Fig. 2).

Cardiac Function. A history of myocardial infarction was noted in 1 patient and hypertension in another. No other patients had a disease history that could affect their cardiac function. Electrocardiography and echocardiography were performed in 46 and 23 patients, respectively (Tables 4 and 5). Electrocardiographic abnormalities were observed

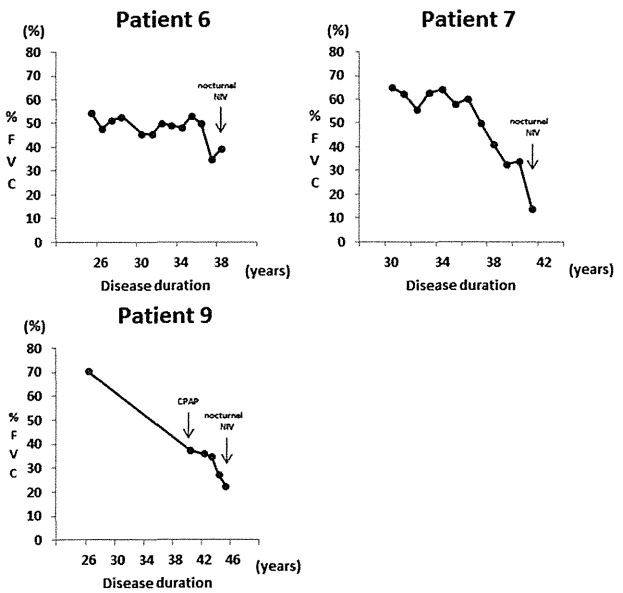

FIGURE 2. Progress of respiratory dysfunction in 3 patients.

Table 4. Electrocardiography findings (*n* = 46).

		%FVC			
QRS duration	Total	≥80%	<80%	Unknown	
<100 ms	Normal	22	21	0	1
	Abnormal	5*	3	2	0
≥100 ms	Non-specific ICD	15	10	5	0
	IRBBB	2	1	1	0
	CRBBB	1	1	0	0
	LAH	1	0	1	0

ICD, intraventricular conduction delay; IRBBB, incomplete right bundle-branch block, CRBBB, complete right bundle branch block; LAH, left anterior hemiblock; FVC, forced vital capacity.

**Premature atrial contraction (PAC) / non-specific ST–T abnormality and left axis deviation (LAD) / flattened T wave / PAC / premature ventricular contraction, negative T wave, LAD, atrial fibrillation.*

in 24 patients, including 9 patients with %FVC <80. Intraventricular conduction delay (i.e., QRS duration prolongation) was detected in 19 patients, but there was no significant association with age (P = 0.911), disease duration (P = 0.932), or %FVC (P = 0.181). Echocardiographic abnormalities were observed in 7 patients, but they were not clinically significant. Of these, 5 patients had mild valvular regurgitation, including 1 with mild left atrial dilation. Mean EF was 68.0 ± 8.3 (median 66.0, interquartile range 65–73.5, range 48–84), but there was no significant difference between patients with %FVC ≥80% and <80% (69.6 ± 7.5 vs. 58.7 ± 9.3, P = 0.156).

Autopsy. Patient 6 was found to have asymptomatic nocturnal hypoxemia and was started on nocturnal NIV at age 68. An electrocardiogram at age 69 showed non-specific intraventricular conduction delay (QRS duration 114 ms) and left axis deviation. He died from peritonitis at age 70. Patient 7 required NIV temporarily at age 79, with %FVC of 28.9%. An electrocardiogram at age 79 revealed atrial fibrillation, premature atrial contraction, and non-specific ST–T abnormality, but no prolonged QRS duration. He died from multiple-organ failure

Table 5. Echocardiography findings (*n* = 23).

		%FVC		
	Total	≥80%	<80%	Unknown
Normal	15	13	1	1
Abnormal				
Mild TR	2	2	0	0
Mild TR + PR	1	1	0	0
Mild MR	1	1	0	0
Mild AR	1	0	1	0
Mild LA dilation	1	1	0	0
EF < 55%	2	1 (53%)	1 (48%)	0

TR, tricuspid regurgitation; PR, pulmonary regurgitation; MR, mitral regurgitation; AR, aortic regurgitation; LA, left atrium; EF, ejection fraction; FVC, forced vital capacity.

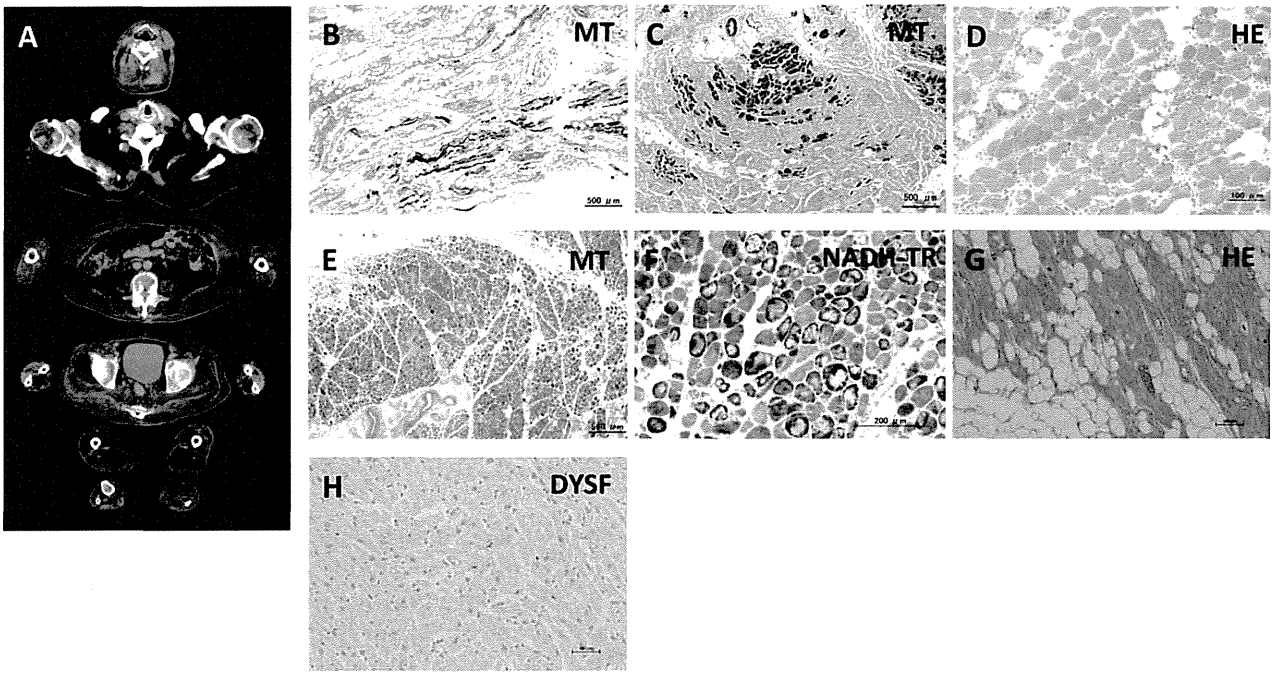

FIGURE 3. Pathological specimens and skeletal muscle computed tomography (CT) from patient 6. Skeletal muscle CT at age 61 years shows marked muscle atrophy and adipose infiltration **(A)**. Almost all fibers were replaced by adipose tissue and fibrous tissue in biceps brachii **(B)** and iliopsoas **(C)**. Flexor digitorum superficialis was relatively preserved **(D)**. The diaphragm exhibited moderate to severe variation in fiber size, adipose tissue infiltration, and endomysial fibrosis **(E, F)**. Cardiac muscle was preserved compared with diaphragm. Minimal variation in fiber size and scattered adipose tissue infiltration was observed **(G)**. Dysferlin was completely deficient in the sarcolemma of cardiac muscle **(H)**. MT, Masson trichrome; HE, hematoxylin and eosin; NADH-TR, nicotinamide adenine dinucleotide–tetrazolium reductase, DYSF, dysferlin.

at age 79. Both patients had complications of diabetes mellitus.

Histopathologically, both patients showed similar findings. Almost all fibers were replaced by adipose tissue and fibrous tissue in limb muscles (Figs. 3B and C and 4A–F). The diaphragm showed moderate to severe fiber size variation, endomysial fibrosis, and adipose tissue infiltration (Figs. 3E and F and 4H–J). The cardiac muscle showed minimal to mild variation in fiber size, endomysial fibrosis, and scattered adipose tissue infiltration (Figs. 3G and H and 4K). Dysferlin was completely absent in cardiac muscle sarcolemma (Figs. 3K and 4L).

DISCUSSION

Respiratory function in patients with dysferlinopathy has attracted little attention so far. Although some reports have described respiratory symptoms or abnormal findings in patients with dysferlinopathy,[2–5,7] there are insufficient data to allow prediction of which patients are susceptible to respiratory dysfunction, or when the signs of respiratory dysfunction may appear. In a previous autopsy case, degeneration of the diaphragm was noted, but no detailed description regarding the degree of severity in each muscle (e.g., limbs, diaphragm) was provided.[7] As appropriate induction of respiratory rehabilitation and NIV can improve outcome and quality of life in patients, an effort should be made to promote early detection of respiratory compromise. Our study showed that age, disease duration, ambulation status, and serum CK levels were associated with respiratory function (Fig. 1). Moreover, patients <40 years of age and those who were still ambulatory also had respiratory insufficiency.

In previous studies, patients with LGMD2B[2,4] or proximodistal dysferlinopathy[3] were reported to have respiratory dysfunction. Because diaphragm and respiratory muscle weakness can cause respiratory dysfunction, we predicted that a proximal-dominant phenotype, LGMD2B, may be associated with an early onset of respiratory failure relative to a distal-dominant type, MM. As average %FVC did not significantly differ between the MM and LGMD2B groups, it may be difficult to predict respiratory dysfunction on the basis of clinical phenotype. However, among the patients with %FVC <80, the decrease in %FVC was greater in LGMD2B patients (see Table S3 online). LGMD2B patients with respiratory dysfunction were older and had longer disease duration compared with MM patients, and thus it is difficult to determine

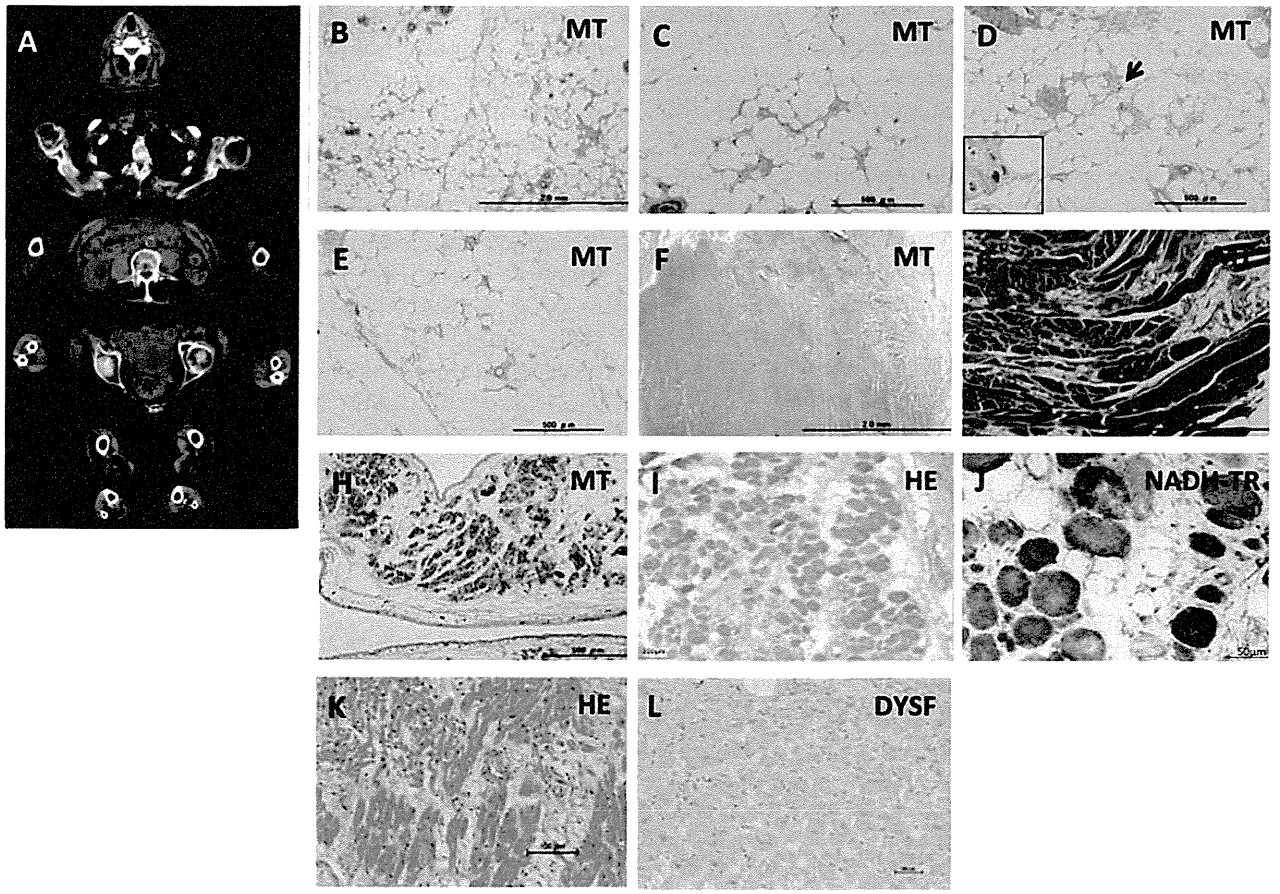

FIGURE 4. Pathological specimens and skeletal muscle computed tomography (CT) from patient 7. Skeletal muscle CT at age 73 shows proximal dominant marked muscle atrophy and adipose infiltration **(A)**. Almost all fibers were replaced by adipose tissue and fibrous tissue in biceps brachii **(B)**, iliopsoas **(C)**, tibialis anterior **(D)**, and rectus femoris **(E, F)**, and only a few muscle fibers remained [**(D)** arrow]. The tongue was well preserved **(G)**. The diaphragm exhibited moderate to severe variation in fiber size, adipose tissue infiltration, and endomysial fibrosis **(H–J)**. Cardiac muscle was preserved compared with diaphragm. Mild endomysial fibrosis and scattered adipose tissue infiltration with muscular degeneration was observed **(K)**. Dysferlin was completely deficient in cardiac muscle sarcolemma **(L)**. MT, Masson trichrome; HE, hematoxylin and eosin; NADH-TR, nicotinamide adenine dinucleotide–tetrazolium reductase; DYSF, dysferlin.

whether the difference in %FVC was attributable to the phenotypic difference. However, it is possible that patients with LGMD2B develop respiratory failure in earlier stages.

Almost all reports of patients with dysferlinopathy who have severe respiratory dysfunction are from Japan. Therefore, we considered the possibility that the severity of the condition is influenced by genetic factors. In this study, however, we found different *DYSF* gene mutations in every patient (except for siblings), and no mutations other than the heterozygous c.2997G>T mutation were common in patients with reduced %FVC. The c.2997G>T mutation has been reported very frequently among Japanese patients,[20] and most of the patients with the c.2997G>T mutation in our study had normal respiratory function; therefore, no specific relationship was suggested between gene mutations and respiratory dysfunction. Because background conditions, such as age and disease duration, vary from patient to patient, the number of patients from a single institution was too small to consider the relationship between gene mutations and disease severity. Multicenter trials and long-term follow-up are necessary to reach a conclusion.

Only a few studies have evaluated cardiac function in patients with dysferlinopathy.[7,11] In our study, electrocardiographic abnormalities were more frequently observed in patients with %FVC <80. As the mean age of patients with %FVC <80 was higher, and electrocardiogram abnormalities such as premature beats or right bundle-branch block reportedly increase with age,[21] there may be some association between abnormal electrocardiogram findings and aging. On the other hand, QRS complex duration was prolonged in some patients regardless of age, disease duration, or %FVC. Prolonged QRS duration is caused by conduction delays at various sites within the ventricles.

Intraventricular conduction delay may be caused by structural changes or by the functional properties of the cardiac conduction system. Shinmura *et al.* reported that the QRS complex duration did not change significantly with age in healthy elderly people.[22] Ilkhanoff *et al.* reported that QRS complex duration >100 ms was significantly associated with incident heart failure.[23] Although follow-up is necessary to determine whether cardiac dysfunction appears in the future in patients with prolonged QRS duration, this may suggest potential cardiac dysfunction and could be used as an indicator for detecting early-stage cardiomyopathy. Moreover, patients with prolonged QRS complex duration were distributed from early to advanced stages in this study and, according to Kuru *et al.*[8] and Wenzel *et al.*,[9] patients with dilated cardiomyopathy (DCM) who maintained walking ability with assistance had high serum CK levels. Therefore, cardiac dysfunction may occur irrespective of stage of disease progression. On echocardiography, valvular regurgitation was observed in some patients with or without respiratory dysfunction, but the degree of regurgitation was mild in all patients. Given that trivial to mild valvular regurgitation on echocardiography has been reported in many healthy people with normal cardiac function,[24] the abnormal findings seen in our study are likely nonpathogenic.

Only a few histopathological assessments have been performed on respiratory and cardiac muscles in patients with dysferlinopathy,[7–9] In our autopsy cases, limb muscles were most severely affected. The diaphragm was also damaged, but to a milder degree compared with limb muscles. As limb muscle weakness tends to be more prominent than respiratory dysfunction, respiratory problems in patients with dysferlinopathy may have been underestimated. Cardiac muscle showed mild changes. Suzuki *et al.*[7] reported that, in an autopsy case with respiratory and cardiac involvement, the diaphragm was more severely affected than the cardiac muscle, suggesting that the myocardium tends to be preserved better than the diaphragm. In our patients, old age, diabetic complications, and/or severe arteriosclerosis may have affected myocardial changes, including cardiac muscle degeneration. However, our findings (i.e., variation in fiber size, endomysial fibrosis, and complete dysferlin deficiency in the sarcolemma) are consistent with those reported previously in patients with DCM,[9] and may be attributed to dysferlinopathy.

We are aware that recruiting patients from the NCNP, an institution highly specialized in muscle disease, is a potential source of selection bias, because these patients may be more severely affected than the general patient population. Therefore, our study may not accurately reflect the general patient population. Investigations of small populations may also underestimate the statistical significance. A broader investigation, such as one that uses an international registry and clinical outcome studies, will be needed in the future.

In conclusion, as patients with dysferlinopathy are prone to respiratory dysfunction, respiratory function should be evaluated regularly, especially in older, advanced-stage patients. Furthermore, as QRS complex duration prolongation on the electrocardiogram could also occur, irrespective of age, disease duration, and %FVC, it is preferable to evaluate cardiac function regularly.

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Original Article

Phenotypic Variability in Childhood of Skeletal Muscle Sodium Channelopathies

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ABSTRACT

BACKGROUND: Mutations of the *SCN4A* gene cause several skeletal muscle channelopathies and overlapping forms of these disorders. However, the variability of the clinical presentation in childhood is confusing and not fully understood among pediatric neurologists. **PATIENTS:** We found three different mutations (p.V445M, p.I693L, and a novel mutation, p.V1149L) in *SCN4A* but not in the *CLCN1* gene. The patient with p.V445M showed the clinical phenotype of sodium channel myotonia, but her clear symptoms did not appear until 11 years of age. Her younger sister and mother, who have the same mutation, displayed marked intrafamilial phenotypic heterogeneity from mild to severe painful myotonia with persistent weakness. The patient with p.I693L exhibited various symptoms that evolved with age, including apneic episodes, tonic muscular contractions during sleep, fluctuating severe episodic myotonia, and finally episodic paralyses. The patient with the novel p.V1149L mutation exhibited episodic paralyses starting at 3 years of age, and myotonic discharges were detected at 11 years of age for the first time. **CONCLUSION:** The present cohort reveals the complexity, variability, and overlapping nature of the clinical features of skeletal muscle sodium channelopathies. These are basically treatable disorders, so it is essential to consider genetic testing before the full development of a patient's condition.

Keywords: sodium channelopathy, *SCN4A*, mutation, myotonia, periodic paralysis, electromyography, genetic testing
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Introduction

Mutations of the *SCN4A* gene, which encodes the skeletal muscle voltage-gated sodium channel $Na_v1.4$, cause various skeletal muscle disorders. Paramyotonia congenita, sodium channel myotonia (SCM), hyperkalemic periodic paralysis (hyperPP), and hypokalemic periodic paralysis are representatives of these disorders. To date, more than 60 mutations of *SCN4A* have been reported.[1,2] However, these disorders are not described adequately in the field of pediatric neurology because of phenotypic heterogeneity[3] and the complicated pathophysiology of ion channels.[1,2] Recently, electrophysiological protocols for the diagnosis of muscle channelopathies have became prevalent and prompted the application of genetic testing.[4,5]

Over the past decade, we encountered five patients with three underlying genetic bases for their skeletal muscle sodium channelopathies. They exhibited various phenotypes because of the different heterozygous point mutations. The diagnostic process for each patient was difficult because of the

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phenotypic variability in childhood. These patients provide some clues that will yield a better understanding and diagnosis of skeletal muscle sodium channelopathies.

Methods

DNA analysis

We analyzed the nucleotide sequences of *SCN4A* and the skeletal muscle chloride channel *CLCN1* genes of the patients and their parents with the Sanger method. A *CLCN1* mutation causes myotonia congenita, the phenotype that is often similar to SCM. Written informed consent was obtained from the parents for the mutation screening. This study was approved by the ethics committee of Kagoshima University Graduate School of Medical and Dental Sciences.

Electrophysiological analysis

We performed needle electromyography to check for myotonic discharges. Then we analyzed the compound muscle action potential (CMAP) amplitude in response to a short (10-12 seconds) exercise test with/without muscle cooling and a long (5-minute) exercise test, following the protocols proposed by Fournier et al.[4,5]

Patient Descriptions

Clinical and electrophysiological features of all patients are summarized in Table 1.

Patient I-1

The proband was a 13-year-old girl who visited us in 2011 with myotonic symptoms. She was delivered by Cesarean section and showed intellectual disability with speech delay at 1 year of age. By 3 years of age, she had developed walking difficulties and was diagnosed with paroxysmal kinesigenic choreoathetosis. Carbamazepine failed to relieve her symptoms. She developed grip and eyelid myotonia at 11 years of age. Then, molecular genetic analysis revealed no expansion of the repeat length at the *DM1* locus. Her symptoms were induced by exercise, but she never showed paradoxical myotonia. Her leg stiffness occurred during the initial 20 m of a 100-m run. At 12 years of age, she experienced a respiratory problem following a difficult extubation after surgery with general anesthesia and was referred to us for further examination. She was short in height, with a short neck and scoliosis. Her IQ was 57. Percussion of her tongue and palms easily elicited her myotonia. She exhibited dysarthria because of her myotonia. Her myotonia occurred not only immediately after movement initiation, but also with a delayed onset after a brief rest following long exercise. Her myotonia showed warm-up phenomenon and cold insensitivity. Cold temperature was a precipitating factor for her myotonia, along with fatigue, lack of sleep, and emotional stress. Her muscle consistency was increased, and her extremities and buttocks were hypertrophic. Serum creatine kinase and electrolytes were normal. Needle electromyography revealed myotonic discharges. CMAP amplitude did not change during short and long exercise tests. DNA sequencing revealed a c.1333G>A (p.V445M) mutation in *SCN4A* but not in the *CLCN1* gene. Her younger sister (patient I-2) and her mother (patient I-3) also showed myotonia, the severity of which was remarkably different. An identical mutation was found in both the mother and sister. All three patients in this family were diagnosed with SCM.

Patient I-2

This is the younger sister of the proband. This patient has autism, and her myotonia was the mildest of the three

TABLE 1.
Clinical and Electrophysiological Features of the Five Patients

Patient	I-1	I-2	I-3	II	III
SCN4A mutation	p.V445 M	p.V445 M	p.V445 M	p.I693 L	p.V1149 L
Diagnosis	SCM	SCM	SCM	SCM with PP	HyperPP with myotonia
Gender	F	F	F	M	M
Age at onset	3 years	2 years	3 years	7 days	3 years
Age at diagnosis	14 years	12 years	41 years	7 years	12 years
Clinical myotonia	+	+	+	+	-
Severity	Moderate	Mild	Severe	Mild to severe, fluctuating	/
Warm-up	+	-	+	-	/
Paradoxical myotonia	-	-	-	-	/
Cold sensitivity	-	-	-	-	/
Delayed myotonia	+	-	+	-	/
Painful myotonia	-	-	+	+	/
Episodic weakness	-	-	-	+	+
Persistent weakness	-	-	+	+	-
Muscle hypertrophy	+	-	+	+	-
Muscle atrophy	-	-	+	+	-
Potassium sensitivity	/	/	/	/	-
Myotonic discharge	+	/	/	+	+
Short exercise test	No change	/	/	No change	No change
Long exercise test	No change	/	/	No change	Initial increase Delayed decrease
Fournier pattern I-V	III	/	/	III	Unclassified

Abbreviations:
/ = Not examined, or not applicable
HyperPP = Hyperkalemic periodic paralysis
PP = Periodic paralysis
SCM = Sodium channel myotonia

affected family members and she sometimes would go full days without any myotonia. Electrophysiological analysis was impossible because of her intellectual disability. Myotonia occurred just after initiation of movement, and dysarthria was only observed upon speech initiation. Myotonia could be induced by percussion of tongue and thumbs. Cold and sleep deprivation were precipitating factors. Mexiletine was partially effective for her myotonia.

Patient I-3

This is the mother of proband. She suffered from severe myotonia with pain and was the most severe case in her family. External ocular muscles and swallowing muscles were involved. During her pregnancies, she used a wheelchair because of exacerbation of myotonia. She gradually developed muscle atrophy in her distal extremities. Severe pain was reported in the left hand and right soleus. Manual muscle testing revealed weakness in all tested muscles. Various drugs, including mexiletine, acetazolamide, and phenytoin, failed to ameliorate her symptoms.

Patient II

This is a 13-year-old boy harboring the SCN4A mutation c.2077A>C (p.I693L) whom we reported in 2012.[6] Seven days after birth, he experienced apneic episodes with generalized muscle stiffness while crying. He often exhibited tonic muscle contraction of the extremities during sleep starting at 11 months of age. At 2 years of age, severe episodic myotonia with daily fluctuation began. After age 7, he began to suffer from paralytic episodes several times per year. Episodic weakness lasted from hours to several days with loss of muscle consistency in both thighs. He also had several physical characteristics resembling Schwartz-Jampel syndrome.[7] But causative *HSPG2* gene analysis and immunohistochemical staining of perlecan in biopsied muscle, which is encoded by *HSPG2*, were normal. Functional analyses of p.I693L mutated Nav1.4 heterologously expressed in vitro revealed enhanced activation and disruption of slow inactivation, which were consistent with an overlapping form of SCM and periodic paralysis. Acetazolamide, mexiletine, and phenytoin had some beneficial effects on his severe episodic myotonia.

Patient III

This 14-year-old boy was 9 years old when he was referred to us for paralytic episodes. His mother had had two episodes of sudden paralysis of the extremities accompanied by elevated serum creatine kinase during her pregnancies. His perinatal and developmental history was normal. At 3 years of age, paralysis of extremities suddenly appeared on the morning of the day following a febrile episode. Then, elevated serum creatine kinase and normal potassium levels suggested normokalemic periodic paralysis. His paralytic episode resolved within a week without residual muscle weakness. At age 9, he suffered from sudden, painful muscle paralysis of the extremities after a soccer game. His creatine kinase level was transiently elevated to 3685 U/L. At a later date, physical and neurological examinations, including electrophysiological tests, glucose plus insulin or potassium loading tests, were all normal. At age 11, reexamination via needle electromyography revealed weak myotonic discharges. Therefore, we retried all the examinations. The short exercise test revealed no change in CMAP amplitude. However, during the long exercise test, an early increase (22.7%) and delayed decrease in CMAP amplitude after 5 minutes of isometric exercise were observed. DNA sequence analysis revealed a novel heterozygous mutation of c.3445G>T (p.V1149L) in *SCN4A*. This mutation was also detected in his mother. His final diagnosis was hyperPP with myotonia. His paralytic symptoms disappeared completely upon administration of carbamazepine.

Discussion

Myotonia is the cardinal symptom in paramyotonia congenita, SCM, and the overlapping forms of paramyotonia congenita and hyperPP (paramyotonia congenita/hyperPP).[8] But myotonia is limited or absent in hyperPP and essentially absent in hypokalemic periodic paralysis.[7] The overlapping forms of sodium channelopathies are not unheard of, whereas paramyotonia congenita/hyperPP is relatively common.[9,10] As for normokalemic periodic paralysis, which was initially suggested in patient III, it has been argued that normokalemic periodic paralysis is identical to hyperPP, because the underlying mutations of normokalemic periodic paralysis are the same as for hyperPP.[7,11] Moreover, hypotonia, stridor, and laryngospasm among neonates were found to be the symptoms of sodium channelopathies by genetic testing.[12-14] Skeletal muscle sodium channelopathies are basically treatable disorders, but can be fatal, as in patient II, who experienced a life-threatening episode at 7 days after birth. Thus, early diagnosis may improve the outcome of affected neonates. Clinical and electrophysiological characteristics of skeletal muscle sodium and chloride channel myotonia are summarized in Table 2.[1,2,8-10,15]

In our experience, the difficulty with the clinical diagnosis of skeletal muscle sodium channelopathy lies in the phenotypic variability during a patient's development. It took quite some time to arrive at a definitive diagnosis in patients I-1 and III because clear symptoms did not appear until 11 years of age. The grip and eyelid myotonias of patient I-1 appeared at 11 years of age, which easily suggested myotonic disorders. The subclinical myotonia of patient III was suddenly detected as myotonic discharges at age 11, which strongly suggested hyperPP.[7] Patient II was an uncommon case with resemblance to Schwartz-Jampel syndrome. We originally excluded Schwartz-Jampel syndrome by immunohistochemical and genetic analyses; thereafter, the exploration of pathogenesis was directed to sodium channel myotonias.

The electrophysiological protocols published by Fournier et al. are useful.[4,5] These authors investigated the relationship between electromyographic findings and specific channel protein mutations and identified five different electromyographic patterns that correspond to the subgroups of mutations and clinical categories of muscle channelopathy.

Electrophysiological analysis of patients I-1, II, and III, and all patients showed myotonic discharges. Flat patterns

TABLE 2.
Clinical and Electrophysiological Characteristics of Skeletal Muscle Sodium and Chloride Channel Myotonias

Disorder	RMC	DMC	SCM	PMC	HyperPP/PMC	HyperPP
Gene mutation	CLCN1	CLCN1	SCN4A	SCN4A	SCN4A	SCN4A
Age at onset (years)	<10, late	<10, early	<10	<10, early	<10	<10
Clinical myotonia	+	+	+	+	+	+/-
Severity	Moderate to severe	Mild to moderate	Mild to severe	Mild to moderate	Mild to severe	Mild
Warm up	+	+	+/-	-	-	+
Paradoxical myotonia	-	-	-	+	+	+/-
Cold sensitivity	+/-	+/-	+/-	+	+	+/-
Delayed myotonia	-	-	+/-	-	-	-
Painful myotonia	+/-	-	+/-	-	-	-
Episodic weakness	+/-	-	-	+	+	+
Persistent weakness	+/-	-	-	-	+/-	+/-
Muscle hypertrophy	+	+/-	+/-	+/-	+/-	-
Muscle atrophy	+/-	+/-	-	-	-	-
Potassium sensitivity	-	-	+	+/-	+	+
Myotonic discharge	+	+	+	+	+	+/-
Short exercise test	Initial decrease	No change or initial decrease	No change	Decrease	Increase*	Increase
Long exercise test	No change or initial decrease	No change or initial decrease	No change	Decrease	Changes similar to PMC or SCM or hyperPP†	Initial increase Delayed decrease
Fournier pattern I-V	II	II/III	III	I	/	IV

Abbreviations:
+/− = Plus or minus
DMC = Dominant myotonia congenita (Thomsen disease)
HyperPP = Hyperkalemic periodic paralysis
HyperPP/PMC = Overlapping form of hyperkalemic periodic paralysis and paramyotonia congenita
PMC = Paramyotonia congenita
RMC = Recessive myotonia congenita (Becker disease)
SCM = Sodium channel myotonia
* Data from reference 14.
† Data from reference 15.

of CMAP amplitude in both short and long exercise tests were observed in patients I-1 and II, which corresponded to Fournier pattern III and suggested SCM for both patients. Although patient III was not classified into any Fournier patterns because of the conflict of the flat pattern in the short exercise test and the initial increase and delayed decrease pattern in the long exercise test. However, SCM or hyperPP was suggested for this patient. In addition to delayed myotonia, patient I-1 had myotonia immediately after movement initiation, which showed warm-up phenomenon and cold insensitivity. This type of myotonia is usually observed after rest in chloride channel myotonia (i.e., myotonia congenita caused by *CLCN1*). For this reason, *CLCN1* should be considered another candidate gene for mutation analysis in such a case. Nevertheless, this electrophysiological analysis can only be performed on children who are willing to be cooperative during the examination.

We also illustrated the location of the mutations associated with skeletal muscle sodium channelopathies and the three mutations of our patients on a schematic diagram of the α-subunit of $Na_v1.4$ (Figure).[1,2] From this diagram, it is clear that mutations are abundant at the inner side of the sarcoplasmic membrane, and several hotspots were observed that corresponded to the disorders.

In a previous report,[16] the patients with p.V445M exhibited severe painful myotonias, especially in the chest. The region of pain was different from that in the present cohort: patient I-3 had pain in the extremities, whereas the myotonias of patient I-1 and I-2 were painless. Patient I-3's muscle atrophy and persistent weakness are signs that are rarely observed in SCM. It is unclear whether her muscle weakness resulted from severe myotonia or age-dependent myopathy. However, the family of patient I exhibited significant intrafamilial phenotypic heterogeneity and provided a key to understand the complexity of sodium channelopathy.

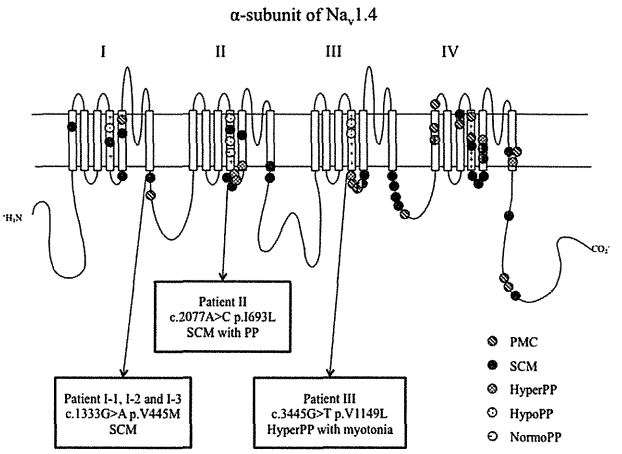

FIGURE.
Schematic representation of the α-subunit of $Na_v1.4$ showing the location of the mutations associated with paramyotonia congenita, sodium channel myotonia, hyperkalemic periodic paralysis, hypokalemic periodic paralysis, and normokalemic periodic paralysis[1,2] in addition to p.V445M, p.I693L, and p.V1149L of our patients. HyperPP = hyperkalemic periodic paralysis; hypoPP = hypokalemic periodic paralysis; normoPP = normokalemic periodic paralysis; PMC = paramyotonia congenita; PP = periodic paralysis; SCM = sodium channel myotonia.

Concerning p.I693L of patient II, another substitution at the same site (p.I693T) has already been reported and could generate various phenotypes, including cold-induced weakness without stiffness,[17] muscle stiffness and episodic weakness,[4] neonatal hypotonia,[12] paramyotonia congenita,[18] and hyperPP.[11] Patient II was diagnosed with SCM with periodic paralysis.

Although p.V1149L in patient III was a novel mutation, it was located in a highly conserved mutation hotspot region. This mutation was absent in the Single Nucleotide Polymorphism database or 1000 Genomes Project database and was not found in 200 control DNA samples. Moreover, it was predicted to be possibly damaging and deleterious in PolyPhen2 and SIFT, respectively. In addition, this patient's mother, who harbors the same mutation, also experienced prolonged paralytic attacks twice during her pregnancies. Taken together, this mutation was considered to be causative in this family.

Our experience highlights the complexity, variability, and overlapping nature of the clinical features of several skeletal muscle sodium channelopathies. A definite diagnosis is necessary if physicians are to alleviate symptoms or even to save lives. A genetic study based on careful clinical examination and accurate electromyography tests is recommended. Special attention should also be paid to the evolution of clinical phenotypes. We hope our experiences will help pediatric neurologists better understand this group of disorders.

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Case report

A missense mutation in domain III in *HSPG2* in Schwartz–Jampel syndrome compromises secretion of perlecan into the extracellular space

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Abstract

Schwartz–Jampel syndrome (SJS) type 1 is characterized by short stature, myotonia, and chondrodysplasia, and is caused by partial loss-of-function mutations in *HSPG2* encoding perlecan. Six missense mutations have been reported in SJS to date and only one has been characterized using a recombinant protein. We report an 11-year-old Japanese boy with SJS, who shows "rigid" walking with less flexion of knees/ankles and protruded mouth. His intelligence is normal. We identified by whole genome resequencing a heterozygous missense p.Leu1088Pro in domain III-2 and a heterozygous nonsense p.Gln3061Ter in domain IV of perlecan. Expression studies revealed that p.Leu1088Pro markedly reduces the cellular expression of domain III-2 and almost nullifies its secretion into the culture medium. As five of the seven missense mutations in SJS affect domain III of perlecan, domain III is likely to be essential for secretion of perlecan into the extracellular space.

Keywords: Schwartz–Jampel syndrome; Perlecan; Whole genome resequencing analysis

1. Introduction

Schwartz–Jampel syndrome (SJS) type 1, also known as chondrodystrophic myotonia, is a rare autosomal recessive disorder characterized by short stature, neuromyotonia, chondrodysplasia, joint contractures, blepharophimosis, myopia, and pigeon chest [1]. SJS is caused by loss-of-function mutations in *HSPG2* encoding perlecan [2–4]. Dyssegmental dysplasia, Silverman–Handmaker type (DDSH), which is a lethal autosomal recessive form of dwarfism with characteristic anisospondylic micromelia, is an allelic disorder of SJS and is also caused by mutations in *HSPG2* [5,6]. *Hspg2*-deficient mice exhibit a similar phenotype as DDSH [7,8]. HSPG2 is a large gene composed of 97 exons spanning 115 kb with a coding region of 13,173 bp, which encodes 4391 amino acids with a predicted molecular weight of the core protein of 469 kDa. Perlecan is a heparan sulfate proteoglycan carrying three glycosaminoglycan chains at the N-terminus and five distinct domains with a molecular weight of ~800 kDa (Fig. 1). The N-terminal heparan sulfate-binding SEA domain (domain I) is unique to perlecan, whereas the other four domains have similarities with domains of the other molecules: the LDL receptor type A (domain II); laminin type 4 and laminin EGF-like (domain III); immunoglobulin superfamily important for assembly with extracellular matrix proteins (domain IV); and laminin type G and EGF-like repeats (domain V) [9]. These domains bind to a wide range of molecules including growth factors and other ECM components.

A patient-derived missense mutation (p.Cys1532Tyr) in *Hspg2* in mice causes continuous neuromyotonic discharges in skeletal muscles [10], which are due to dysmyelination of the preterminal Schwann cells and persistent axonal depolarization [11]. The neuromyotonia is also accounted for by partial deficiency of endplate acetylcholinesterase [12], because perlecan is essential for anchoring collagen Q, which makes a macromolecular complex with acetylcholinesterase, to the synaptic basal lamina [13,14].

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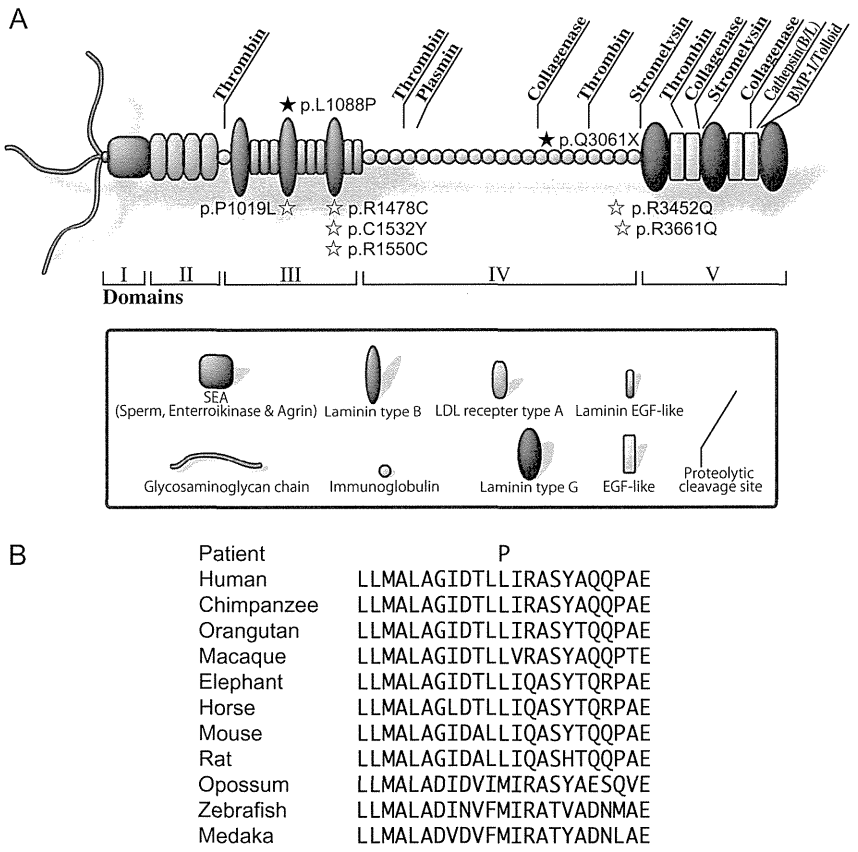

B

Patient	P
Human	LLMALAGIDTLLIRASYAQQPAE
Chimpanzee	LLMALAGIDTLLIRASYAQQPAE
Orangutan	LLMALAGIDTLLIRASYTQQPAE
Macaque	LLMALAGIDTLLVRASYAQQPTE
Elephant	LLMALAGIDTLLIQASYTQRPAE
Horse	LLMALAGLDTLLIQASYTQRPAE
Mouse	LLMALAGIDALLIQASYTQQPAE
Rat	LLMALAGIDALLIQASHTQQPAE
Opossum	LLMALADIDVIMIRASYAESQVE
Zebrafish	LLMALADINVFMIRATVADNMAE
Medaka	LLMALADVDVFMIRATYADNLAE

Fig. 1. (A) Domain structure of perlecan. Previously reported missense mutations are indicated by open stars. Currently identified missense and nonsense mutations are indicated by closed stars. Note that five of the seven missense mutations are located in domain III. (B) Alignment of codons 1077–1099 of perlecan in various species. Domain III-2 is comprised of codons 985–1112. Leu1088 is conserved across mammals except for opossum.

Here we functionally characterized a missense mutation in domain III-2 in perlecan that we identified in a Japanese patient with SJS. Expression of perlecan domain III-2 harboring the missense mutation in HEK293 cells demonstrated that the mutation facilitated cellular degradation of domain III-2 and nullified its secretion into the culture medium.

2. Patient and methods

2.1. Patient

Human studies were performed under the approval of the ethical review committees of Kanagawa Children's Medical Center, Juntendo University, and Nagoya University. The patient participated in the study after signing an appropriate informed consent form.

2.2. Whole genome resequencing analysis

Genomic DNA was isolated from the patient's and parents' blood with QIAamp Blood Mini Kit (Qiagen). We sequenced 150 bp of genomic fragments of the patient's DNA in paired-end directions with HiSeq X Ten (Illumina). The sequencing fragments were mapped to the human genome hg19/GRCh37 using BWA 0.7.5a [15] and BLAT v35 at http://genome.ucsc.edu/. SNVs/indels in *HSPG2* were called by Isaac (Illumina), Avadis NGS 1.5.0 (Strand) and VarScan 2.3 [16]. We directly sequenced candidate SNVs in *HSPG2* using 3730xl DNA analyzer (Applied Biosystems). We also searched for copy number variations (CNVs) by the CytoScan HD array (Affymetrix).

2.3. Expression of perlecan domains III-2 and 3 in HEK293 cells

Domain III-2 of human perlecan is comprised of codons 934–1125. To completely cover domain III-2, we cloned human *HSPG2* cDNA spanning codons 872–1270 into a cytomegalovirus-based mammalian expression vector AP-tag5 (Genhunter). The vector carried the Ig κ-chain secretion signal peptide at the 5′ end, and myc- and 6xHis-tags at the 3′ end. We deleted the alkaline phosphatase (AP) cDNA from the vector. The p.Leu1088Pro mutation was introduced into *HSPG2* domain III-2 using the QuikChange site-directed mutagenesis kit (Stratagene). HEK293 cells were grown in DMEM (Invitrogen) with 10% FBS (Thermo Scientific). HEK293 cells were transfected with pAP-Tag5-*HSPG2*-III-2 using the FuGENE6 transfection reagent (Promega). At 16 hrs after transfection, the medium was changed to DMEM without FBS

and with or without 20 μM MG-132 (Sigma). At 40 hrs after transfection, the medium and the cells were harvested.

2.4. Protein extraction and Western blotting

Whole cell extracts were prepared in a RIPA buffer (Thermo Fisher Scientific) with PhosStop (Roche Applied Science) and protease inhibitors of 3 μg/ml leupeptin (Sigma Aldrich), 10 μg/ml aprotinin (Sigma Aldrich), and 3 μg/ml pepstatin (Sigma Aldrich). The lysate was centrifuged at $13,000 \times g$, 4 °C for 10 min and the supernatant was obtained. For isolating secreted proteins, the medium was centrifuged at $4000 \times g$ at 4 °C for 10 min and concentrated with Amicon Ultra 30K (Millipore). The lysate and medium were subjected to SDS-PAGE and electroblotted onto a PVDF membrane. Membranes were incubated with a primary antibody, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. After washing, proteins were detected using the ECL chemiluminescence kit (GE Healthcare) and the LAS-4000 lumino-image analyzer (GE Healthcare). The primary antibodies were anti-c-Myc from Santa Cruz (A-14) and anti-GAPDH from Sigma (G9545). The secondary antibodies were anti-mouse-HRP from GE Healthcare (NA9310V) and anti-rabbit-HRP from GE Healthcare (NA9340V).

3. Results

3.1. Clinical features of the patient

The patient is currently an 11-year-old boy from non-consanguineous Japanese parents. He was born at 40 weeks of gestation with the birth weight of 3324 g, and had no asphyxia. His developmental milestones were normal with head control at 3 months and walking at 9 months. Although he began to walk normally, the parents noticed that his walking pattern was "rigid" with less flexion of knees and ankles. He was referred to the Kanagawa Children's Medical Center at age 2 years and 3 months. His mouth was protruded with a whistling-like face. He had mild blepharophimosis. His limb muscles were slightly contracted in a flexion posture and hypertonic with normal tendon reflexes. Laboratory data including creatine kinase were all normal. At age 2 years and 3 months, the patient was short (83.3 cm, −1.1 SD) and thin (10.3 kg, −1.4 SD). The whole bone X-ray images were compatible with mild chondrodysplasia but without overt bone deformities. Electromyography (EMG) showed the bursts of recurrently firing complex muscle action potentials with fixed frequency that lasted several minutes, which appeared and disappeared abruptly. Muscle biopsy of left biceps brachii showed fiber size variation, pyknotic nuclear clumps, necrotic fibers, and phagocytosis (Fig. 2A–D). ATPase reactions revealed type I fiber predominance with grouping and large cell sizes. At age 3 years, carbamazepine (60 mg/day for 2 months) and mexiletine (110 mg/day for 3 months) were prescribed but were not overtly effective. Phenytoin (60 mg/day) caused drug eruption and was discontinued within a month. Growth hormone (0.21–0.26 mg/kg/week) has been administered for treating short stature since age 6 years. Currently, at age 11 years and 10 months, he is still short (131.3 cm, −2.2 SD) and thin (26.6 kg, −1.5 SD). The myotonic features do not grossly affect his motor functions and he is able to move almost normally. His intelligence remains normal throughout his development.

3.2. Whole genome resequencing analysis and Sanger sequencing

We analyzed the patient's DNA using the whole genome resequencing method. The number of read tags after removing duplications was 606×10^6 covering 90.8 Gbp. Among these, 556×10^6 tags (91.9%) covering 83.5 Gbp were mapped to the human genome hg19/GRCh37 with the mean coverage of 29.2. Search for SNVs and indels detected 162 SNVs/indels in *HSPG2*. We first eliminated SNVs/indels registered in a non-clinical subset of dbSNP138, the 1000 genome project, the NHLBI ESP database, or the Japanese SNP database of HGVD at http://www.genome.med.kyoto-u.ac.jp/SnpDB/index.html, and obtained five SNVs/indels (Supplementary Table S1). Among them, two were in the coding region: p.Leu1088Pro and p.Gln3061Ter. We confirmed by Sanger sequencing that both p.Leu1088Pro and p.Gln3061Ter were heterozygous in the patient's DNA. We detected heterozygous p.Leu1088Pro in the mother and heterozygous p.Gln3061Ter in the father by Sanger sequencing. PolyPhen-2, SIFT, LRT, and Mutation Taster algorithms predicted that p.Leu1088Pro was "probably damaging", "damaging", "neutral", and "disease-causing", respectively. A search for CNVs with the CytoScan HD array detected no CNV in *HSPG2* in the patient.

3.3. p.Leu1088Pro in perlecan reduces cellular expression of domain III-2 and nullifies its secretion into the culture medium

In order to prove the pathogenicity of p.Leu1088Pro, we first confirmed that p.Leu1088Pro does not affect splicing of *HSPG2* exon 25 in the patient's mRNA (data not shown). We next transfected HEK293 cells with wild-type or mutant cDNA encoding perlecan domain III-2. The expression of domain III-2 in cells and medium were evaluated by Western blotting. Wild-type domain III-2 was detected in both the transfected cell lysate and the culture medium, whereas p.Leu1088Pro markedly reduced cytoplasmic expression and completely abolished secretion into the medium (Fig. 2E). To examine whether the lack of secretion is the primary event or is secondary to accelerated degradation of the p.Leu1088Pro-bearing domain III-2 in endoplasmic reticulum, we treated the transfected cells with the ubiquitin-proteasome inhibitor MG-132. MG-132 partly rescued the expression of p.Leu1088Pro-domain III-2, but not its secretion (Fig. 2E). Thus, p.Leu1088Pro is likely to primarily abolish the secretion of perlecan into the culture medium. The reduced cytoplasmic expression of p.Leu1088Pro-domain III-2 is likely to be accounted for by degradation of the intracellularly retained perlecan. Alternatively but less likely, exogenous proteases in the culture medium that specifically digest the p.Leu1088Pro-bearing domain III-2 might have degraded the secreted mutant domain III-2.

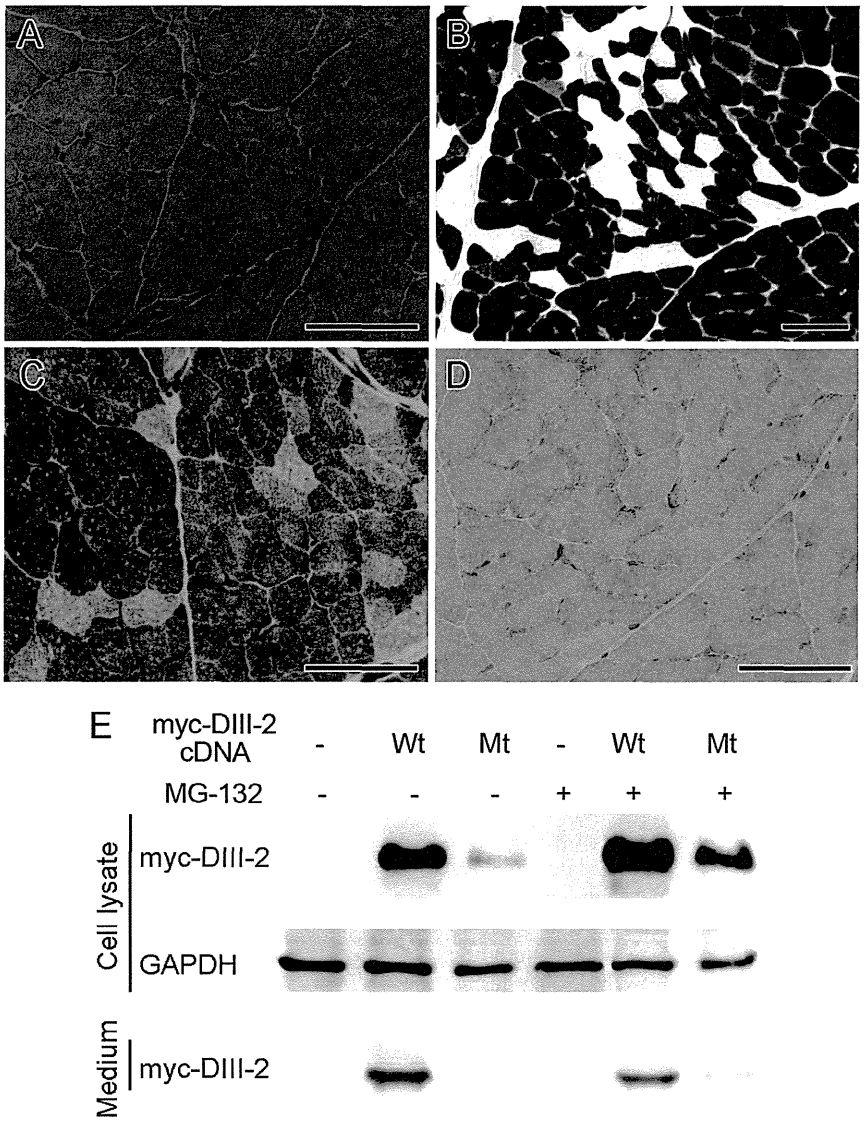

Fig. 2. Biopsied left biceps brachii (A–D) and Western blot analysis of wild-type and mutant perlecan domain III-2. (E). (A) Hematoxylin and eosin staining. (B) ATPase pH 4.3 staining. (C) NADH-TR staining. (D) Modified Gomori trichrome staining. Bars = 100 μm. (E) Western blotting of HEK293 cells transfected with wild-type (Wt) and p.Leu1088Pro-mutant (Mt) perlecan domain III-2 (DIII-2). Domain III-2 carries a myc tag. Proteins are detected with anti-myc and anti-GAPDH antibodies. p.Leu1088Pro mutation markedly decreases expression of domain III-2 in cell lysate and almost nullifies the expression in culture medium. A proteasome inhibitor, MG-132, suppresses degradation of the p.Leu1088Pro-mutant (Mt) domain III-2 in HEK293 cells. MG-132 fails to induce secretion of the mutant domain III-2 into culture medium.

4. Discussion

We have identified a heterozygous missense mutation p.Leu1088Pro in domain III-2 and a heterozygous nonsense mutation p.Gln3061Ter in domain IV in a Japanese patient with SJS and have shown that the p.Leu1088Pro mutation impairs secretion of perlecan domain III-2 in cultured cells. As p.Leu1088Pro and p.Gln3061Ter are inherited from the mother and the father, respectively, the patient is compound heterozygous for p.Leu1088Pro and p.Gln3061Ter. Recessive inheritance is consistent with loss-of-function nature of the two mutations and also of previously reported *HSPG2* mutations.

A total of 31 mutations in *HSPG2* in SJS have been reported to date: six missense (Table 1) [2,4,17], three nonsense [4,18], twelve splicing [2,4,18–20], and eleven indel mutations [4,19]. Although race and ethnicity of the patients with the 31 mutations have not been fully documented, only a single Japanese patient has been reported to our knowledge [3]. The current report underscores the notion that SJS is not clustered in any specific races/ethnicities and should be considered in differential diagnosis in patients representing chondrodysplasia and myotonia.

We have shown that p.Leu1088Pro-domain III-2 of perlecan cannot be secreted into the culture medium and is degraded