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RESEARCH PAPER

Necklace cytoplasmic bodies in hereditary myopathy with early respiratory failure

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ABSTRACT

Background In hereditary myopathy with early respiratory failure (HMERF), cytoplasmic bodies (CBs) are often localised in subsarcolemmal regions, with necklace-like alignment (necklace CBs), in muscle fbres although their sensitivity and specifcity are unknown.

Objective To elucidate the diagnostic value of the necklace CBs in the pathological diagnosis of HMERF among myofbrillar myopathies (MFMs).

Methods We sequenced the exon 343 of TTN gene (based on ENST00000589042), which encodes the fbronectin-3 (FN3) 119 domain of the A-band and is a mutational hot spot for HMERF, in genomic DNA from 187 patients from 175 unrelated families who were pathologically diagnosed as MFM. We assessed the sensitivity and specifcity of the necklace CBs for HMERF by re-evaluating the muscle pathology of our patients with MFM.

Results TTN mutations were identifed in 17 patients from 14 families, whose phenotypes were consistent with HMERF. Among them, 14 patients had necklace CBs. In contrast, none of other patients with MFM had necklace CBs except for one patient with reducing body myopathy. The sensitivity and specifcity were 82% and 99%, respectively. Positive predictive value was 93% in the MFM cohort.

Conclusions The necklace CB is a useful diagnostic marker for HMERF. When muscle pathology shows necklace CBs, sequencing the FN3 119 domain of A-band in TTN should be considered.

INTRODUCTION

Hereditary myopathy with early respiratory failure (HMERF; OMIM 603689) is an adult-onset progressive myopathy characterised by early presentation of respiratory insufficiency usually during ambulant stage.[1–3] Pathologically, HMERF shares features of myofibrillar myopathy (MFM) besides the key finding of cytoplasmic bodies (CBs).[2 3] Its causative gene TTN, which encodes a gigantic protein, titin,[2 3] is known to be causative also for tibial muscular dystrophy, limb girdle muscular dystrophy type 2J, early-onset myopathy with fatal cardiomyopathy and dilated or hypertrophic cardiomyopathy.[4–10] Interestingly, all patients with HMERF so far identified carry a mutation in exon 343 (based on ENST00000589042) encoding the fibronectin-3 (FN3) 119 domain in the A-band region of titin.[2 3 11–17]

CBs are abnormal protein aggregates visualised usually as red-colored objects on modified Gomori trichrome stain and can be observed in a wide range of myopathic conditions. Nevertheless, they are often conspicuous in MFM and considered as one of the representative pathological findings in MFM.[18] In muscle specimens of HMERF, CBs are often located in the subsarcolemmal region,[12 13 14 19] with a necklace-like alignment, which here we call 'necklace CBs'. However, the utility of necklace CBs in the pathological diagnosis of HMERF is unknown. We therefore tested the sensitivity and specificity of necklace CBs in the diagnosis of HMERF.

METHODS

Patients

National Center of Neurology and Psychiatry (NCNP) functions as a referral centre for muscle pathology and muscle biopsy samples are sent from all over Japan. From 1991 to 2013, 187 patients from unrelated 175 Japanese families have been pathologically diagnosed as MFM at NCNP. In this cohort, mutations were found in known MFM-related genes: DES: 8 families (4.6%), VCP: 8 families (4.6%), FLNC: 6 families (3.4%), DNAJB6: 6 families (3.4%), ZASP: 5 families (2.9%), FHL1: 5 families (2.9%), MYOT: 4 families (2.3%) and BAG3: 1 family (0.6%). No mutation was identified in CRYAB. Clinical information at the time of muscle biopsy was available in all patients.

Genetic analysis

Genomic DNA was isolated from peripheral lymphocytes or frozen muscle as previously described.[20] Exon 343 of TTN was directly sequenced using ABI PRISM 3130 automated sequencer (PE Applied Biosystems). Sequence variants were assessed using publically available databases including 1000 Genomes Project database (http://www.1000genomes.org/), NHLBI Exome Sequencing Project 5400 database (http://evs.gs.washington.edu/EVS/), dbSNP135 (http://www.ncbi.nlm.nih.gov/SNP/) and Human Genetic Variation Browser (http://www.genome.med.kyoto-u.ac.jp/SnpDB/); and

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Table 1 Clinical features of the patients with TTN variants

Patient Number	Age (years)/ sex	Relationship	Mutation (protein level)	Family history (years)	Initial manifestations (years)	Age at gait disturbance/ ambulant	Foot drop (years)	Respiratory disturbance (years)	Artificial ventilation (years)	Cardiac involvement	Dysphagia (years)	Other features
A-1	49/M	Father	p.C31712R		Tripping (46)	46/yes	Yes (NA)	Yes (48)	Yes (49)	RHF	No	No
A-2	26/M	Son			Tripping (20)	22/yes	Yes (22)	Yes*	No	No	No	No
B-1	45/M	Older brother	p.C31712R	Father: Foot drop; sudden death (42) Oldest brother: gait disturbance (32); sudden death (40)	Foot drop (31)	31/yes	Yes (31)	Yes (44)	Yes (45)	No	No	No
B-2	37/M	Younger brother			Foot drop (27)	27/yes	Yes (27)	Yes (34)	Yes (37)	No	No	No
C	34/F		p.C31712R		Fatiguability (26)	29/yes	Yes (31)	Yes (26)	Yes (31)	–	No	Difficulty in opening mouth
D	38/M		p.C31712R		Difficulty in lifting thigh (20)	20/no (32)	Yes (20 s)	Yes (29)	Yes (29)	STC	Yes (28) Artificial nutrition (35)	
E	40/M		p.C31712R	Mother: sudden death (38) Older sister: proximal muscle weakness; respiratory failure (35)	Fatiguability; respiratory failure (31)	31/yes	Yes (NA)	Yes (31)	Yes (37)	RHF, PH	–	
F	52/M		p.C31712R		Foot drop (47)	47/yes	Yes (47)	Yes (50)	Yes (52)	–	Yes (47)	
G-1	58/M	Father	p.C31712R	Grandfather of G-2: died of respiratory failure (45)	Tripping (57)	57/yes	Yes (58)	Yes*	–	–	–	
G-2	29/M	Son			Tripping (20)	20/yes	Yes (20 s)	Yes (29)	Yes (29)	RHF	–	
H	68/F		p.C31712R	Son: distal myopathy; sudden death	Difficulty in standing on right toe (56)	56/yes	No	Yes (68)	Yes (68)	RHF, Af	–	Head drop; forward bent posture
I	43/M		p.C31712R		Fatiguability; weight loss (39)	42/yes	No	Yes (39)	Yes (42)	–	Yes (42)	Myalgia muscle cramp
J	42/F		p.C31712Y		Difficulty lifting thighs (36)	38/Yes	No	Yes*	–	–	–	
K	38/M		p.G31791D		Gait disturbance (28)	28/Yes	Yes (30)	Yes*	Yes (38)	–	Yes (36)	Head drop
L	44/F		p.G31791R		Fatiguability; loss of appetite (40)	41/yes	No	Yes (40)	Yes (44)	STC	–	
M	40/M		p.G31791V	Mother and younger sister: lower leg muscle weakness	Gait disturbance (24)	24/yes	Yes (27)	Yes*	–	2° AVB (type 1)	–	
N	46/M		p.R31783_V31785del		Foot drop; difficulty in opening a bottle (41)	41/yes	Yes (41)	Yes*	–	–	–	Myalgia

*Asymptomatic but found by laboratory tests.
2° AVB (type 1), Mobitz type 1 second degree atrioventricular block; Af, atrial fibrillation; F, female; M, male; NA, not available; PH, pulmonary hypertension; RHF, right-sided heart failure; STC, sinus tachycardia.

softwares to predict functional effects of mutations such as PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and Mutation Taster (http://www.mutationtaster.org/). The description of mutations of TTN conforms to Ensemble sequence ENST00000589042.

In this study a diagnosis of HMERF was made based on the presence of a mutation in exon 343 of TTN, although the possibility that mutations in other parts of the gene cause HMERF cannot be totally excluded.

Re-evaluation of muscle pathology

Muscle pathology was re-evaluated focusing on necklace CBs on modified Gomori trichrome. In the present study, we tentatively defined the presence of necklace CBs as at least two muscle fibres containing CBs exclusively localised in the subsarcolemmal area, covering more than 50% of circumference of each muscle fibre in three non-serial sections (each section was at least 250 µm apart and included at least 300 muscle fibres). CBs were evaluated in all MFM cases, and subsequently the sensitivity and specificity of necklace CBs for the diagnosis of HMERF were calculated. The positive predictive value (PPV) and 95% CIs were calculated with GraphPad Prism V.5.0 (Graph Pad Software, California, USA).

Electron microscopic observation

Biopsied muscle specimens were fixed in 2.5% glutaraldehyde and post-fixed with 2% osmium tetroxide. Semithin sections stained with toluidine blue were examined by light microscopy. Ultrastructural analysis was carried out on longitudinal and transverse ultrathin sections of muscles after staining with uranyl acetate and lead citrate, using Tecnai spirit transmission electron microscope (FEI, Hillsboro, Oregon, USA).

Re-evaluation of clinical data

The clinical information in our cohort together with previous reports[2 3 11–14] showed that respiratory insufficiency before being wheelchair users and selective involvement of semitendinosus muscles on muscle imaging were frequently observed, raising a possibility that these findings might be clues for the diagnosis of HMERF. We therefore reviewed clinical data of our patients with MFM in order to calculate sensitivities, specificities and PPVs of them at the time of muscle biopsy.

Ethics

All of the clinical information and materials used in this study were obtained for diagnostic purpose and permitted for scientific use with written informed consent. All experiments in this study were approved by the Ethical Committee of National Center of Neurology and Psychiatry.

RESULTS

Mutation analysis

Six different heterozygous mutations in exon 343 of TTN were identified in 17 patients from 14 families (table 1). Among them, two mutations, g.284701T>C (c.95134T>C; p.C31712R) and g.284939G>A (c.95372G>A; p.G31791D) were previously reported.[2 3 11 12 14] The former mutation was reported to be the most common in other populations.[11 12] This was also the case in our cohort and the mutation was shared by nine families, while all others were found in single families. The latter mutation was previously reported in a European-American family.[14] Among four novel mutations that we identified, three were missense: g.284702G>A (c.95135G>A; p.C31712Y), g.284938G>C (c.95371G>C; p.G31791R) and g.284939G>T (c.95372G>T; p.G31791V); and one was non-frameshift deletion: g.284913_284921delGAGGGCAGT (c.95346_95354del; p.R31783_V31785del). None of the variants was listed in the

Table 2 Laboratory findings at the time of muscle biopsy

Patient	CK IU/L (normal value)	Respiratory function (%VC, sitting position)	Selective involvement of muscles on imaging test		Muscle pathology		
			Semitendinosus muscle	Anterior compartment of lower legs	CB	Necklaces of CBs	RV
A-1 (49 years)	Normal (value: NA)	Abnormal (value: NA.)	NA	NA	+	+	+
A-2 (26 years)	65 (20–190)	77% (68%, lying)	+	+	+	+	+
B-1 (39 years)	425 (51–197)	84% → 67% (45 years)	+	+	+	+	−
B-2 (32 years)	659 (−200)	82% → 63% (37 years)	+	+	+	+	+
C (31 years)	488 (45–170)	32%	NA (+, 34 years)	NA (+, 34 years)	+	+	−
D (31 years)	375 (51–197)	VC: 0.97 L	+	−*	+	+	−
E (40 years)	234 (62–287)	ABG: $PaCO_2$ 86 mm Hg, PaO_2 56 mm Hg (RA)	+	+	+	+	−
F (50 years)	61 (NA)	41%	+	+	+	−	−
G-1 (58 years)	146 (50–170)	59%	+	+	+	−	−
G-2 (29 years)	142 (50–170)	31%	+	+	+	+	+
H (68 years)	140 (50–170)	ABG: PaCO2 60 mm Hg	+	+	+	+	−
I (43 years)	179 (62–287)	40%	+	+	+	−	−
J (42 years)	364 (45–163)	64%	+	+	+	+	+
K (34 years)	645 (51–197)	67% (55%, lying)	+	+	+	+	+
L (44 years)	139 (43–165)	36%	+	+	+	+	−
M (40 years)	190 (62–287)	61%	−*	+	+	+	+
N (46 years)	799 (62–287)	67%	+	+	+	+	+

In the column of Patient, the same alphabet indicates that they belong to the same family.
*Diffuse muscle involvement.
ABG, arterial blood gas; CB, cytoplasmic body; NA, not available; RV, rimmed vacuole; VC, vital capacity.

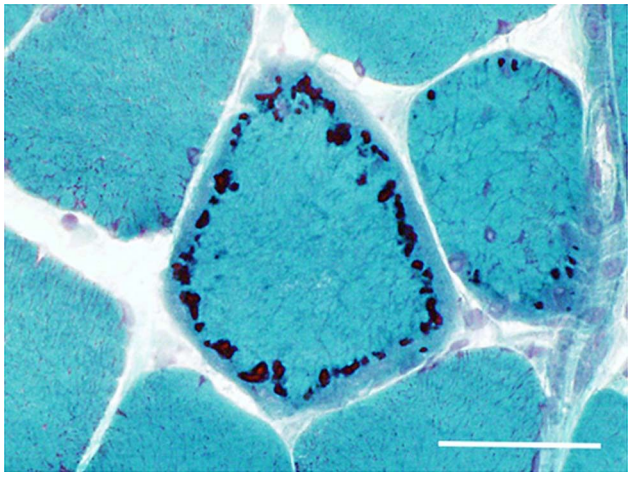

Figure 1 Necklace cytoplasmic bodies. Cytoplasmic bodies are located in line in subsarcolemmal region, often covering the total circumference of a muscle ibre. Modiied Gomori trichrome stain. Bar: 50 µm.

genomic variation databases. The mutated amino acids were highly conserved among species (UCSC Genome Browser). PolyPhen-2 predicted p.C31712Y, p.G31791R and p.G31791V mutations as probably damaging with scores 0.996, 1.000 and 1.000, respectively. Likewise, Mutation Taster predicted the p.C31712Y, p.G31791R, p.G31791V and p.V31782_A31784del mutations to be disease-causing with a probability of 1.000, 1.000, 1.000 and 0.998, respectively. No segregation analysis was possible in any family.

Clinical information of 17 participants with TTN mutations are summarised in tables 1 and 2 and online supplementary table. All patients show clinical signs consistent with HMERF previously reported.[1–3 11–14] The median age of onset was 31 years (range 20–57 years). Four patients developed dysphagia, and one of them required tube feeding.

Table 3 Cross tabulation of necklace cytoplasmic bodies and hereditary myopathy with early respiratory failure

	Patients with myofibrillar myopathies		
Necklace CBs	HMERF	Non-HMERF	Row total
+	14	1	15
% within column	82.4%	0.6%	
% within row	93.3%	6.7%	
–	3	169	172
% within column	17.6%	99.4%	
% within row	1.7%	98.3%	
Column total	17	170	187

CB, cytoplasmic body; HMERF, hereditary myopathy with early respiratory failure.

Sensitivity and speciicity of the necklace of CBs

Among 17 genetically-confirmed patients with HMERF, necklace CBs were found in 14 patients, comprising 0.1–0.8% of the muscle fibres (figure 1). In contrast, none of the 170 patients who had MFM other than HMERF had necklace CBs except for only one patient who had reducing body myopathy, which had been confirmed by the presence of reducing bodies in muscle fibres on menadione-linked α-glycerophosphate dehydrogenase (MAG) stain without substrate and a mutation in the second LIM domain of FHL1 (g.60438G>A; c.377G>A; p.C126Y, based on ENST00000543669; online supplementary figure S1). Based on these results, the sensitivity and specificity of the necklace CBs in HMERF were calculated as 82% (14/ 17, 95% CI 57% to 96%) and 99% (169/170, 95% CI 97% to 100%), respectively (table 3). Since the prevalence of HMERF in the MFM cohort was 9.1% (17/ 187), the PPV was calculated as 93% (95% CI 68% to 100%) based on Bayes' theorem.

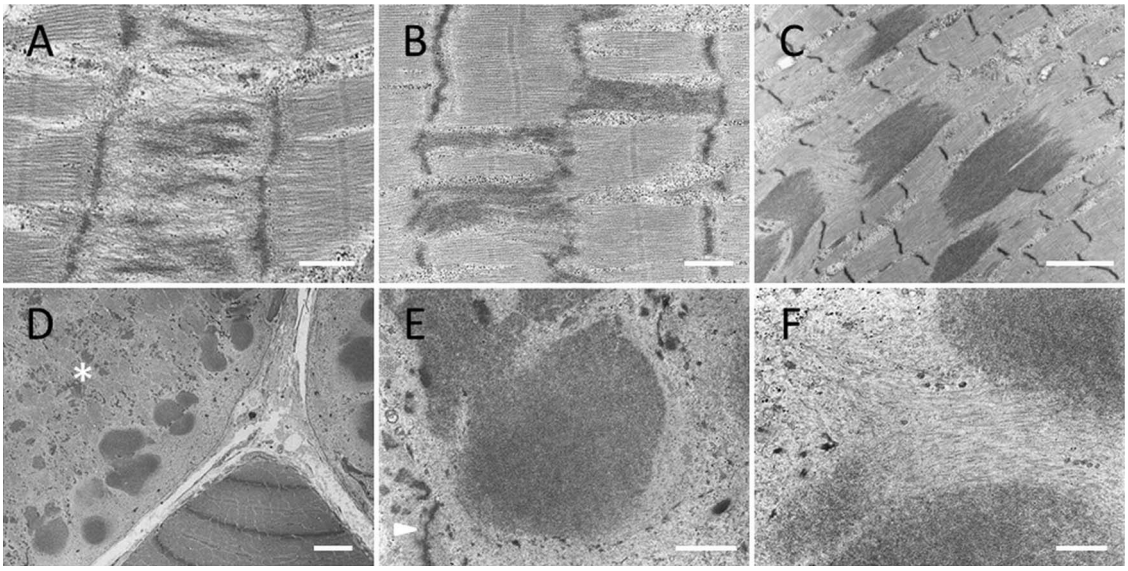

Figure 2 Electron microscope images. (A and B) Sarcomeric disarrangement limited to one sarcomere is observed. (C) Multiple electron-dense inclusions are present in association with Z lines. (D) A ibre containing necklace cytoplasmic bodies (CBs) shows marked myoibrillar disorganisation (asterisk), especially around the CBs. (E) CBs showing a necklace alignment with lower electron density as compared to that of Z line. ⅄: a remnant of Z line. (F) Thin ilamentous structure around the CBs. Lattice-like structure is not seen in the CBs. Bar: 0.5 µm (A, B and F), 1 µm (E), 2 µm (C), and 5 µm (D).

Muscle specimens of the three patients with HMERF had CBs, which are usually located in the subsarcolemmal regions, but did not show the definite necklace-like alignment pattern (online supplementary figure S2). Those three patients shared the same mutation, g.284701T>C (p.C31712R), albeit other nine patients harbouring the same mutation had definite necklace CBs.

Fibres with rimmed vacuoles were occasionally seen, but the sensitivity, specificity and PPV for HMERF were lower than those of the necklace CBs: 47% (8/ 17, 95% CI 23% to 72%), 61% (104/ 170, 95% CI 53% to 69%) and 11% (95% CI 20% to 48%), respectively.

Ultrastructural features

EM samples were available from four patients with HMERF. Sarcomeric disarrangement limited to one sarcomere was observed in all patients (figure 2A, B). In some areas, multiple electron-dense inclusions associated with Z line were surrounded by disorganised myofibrils (figure 2C). Fibres with necklace CBs were included only in one sample. These fibres showed marked myofibrillar disorganisation (figure 2D, asterisk), especially in the vicinity of the CBs (figure 2D, E). The CBs had a lower electron density as compared with that of the Z line and contained dense and mildly filamentous components, without lattice-like structure (figure 2E, F). Small number of CBs were partly surrounded by thin filaments (figure 2F).

Sensitivity and speciicity of respiratory dysfunction and muscle imaging data

Using the data at the time of muscle biopsy about the respiratory function of 102 participants in the MFM cohort, the sensitivity, specificity and PPV of the respiratory insufficiency before being wheelchair users (below 80% of vital capacity or over 45 mm Hg of $PaCO_2$) were calculated as 88% (14/16, 95% CI 62% to 99%), 94% (81/86, 95% CI 87% to 98%) and 74% (95% CI 49% to 91%), respectively (table 2 and 4). In five non-HMERF participants presenting respiratory insufficiency during the ambulant stage, one had a mutation in VCP, but no causative genes were identified in four participants as far as we have screened.

As for the muscle imaging at the time of muscle biopsy, the sensitivity of selective muscle involvement of semitendinosus muscles as shown in figure 3 was 93% (14/ 15; table 4). The specificity could not be calculated due to the limited number of images available in non-HMERF participants.

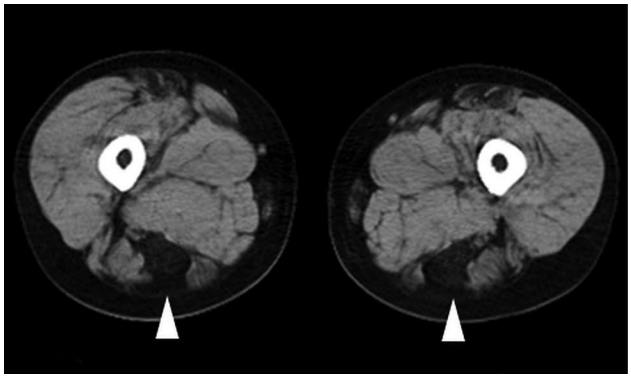

Figure 3 Muscle image. Representative image showing preferential involvement of semitendinosus muscles. CT images of skeletal muscles in proximal legs of the patient K. ⚠: semitendinosus muscles.

DISCUSSION

We have demonstrated that necklace CBs had high specificity for a diagnosis of HMERF (99%), with sensitivity 82% and PPV 93% in our MFM cohort. In this study, all 15 patients with necklace CBs had HMERF except for only one who had reducing body myopathy due to a mutation in FHL1, without mutation in exon 343 of TTN. Although we judged this case as having necklace CBs retrospectively, CBs in this case are smaller in size and scattered in the subsarcolemmal region in muscle fibres, giving an appearance different from typical necklace CBs in HMERF (online supplementary figure S1), suggesting that the specificity of necklace CBs could be substantially 100%. In addition, muscle pathology of this patient showed typical reducing bodies in scattered fibres on MAG stain without substrate, and thus differential diagnosis between reducing body myopathy and HMERF was not a problem in practise.

Interestingly, even in three patients with HMERF without typical necklace CBs, CBs are aligned in the subsarcolemmal regions in muscle fibres, albeit they do not encompass more than half of the myofibre circumference (online supplementary figure S2), suggesting that this unique subsarcolemmal alignment pattern is likely to be related to the dysfunction of titin due to exon 343 mutations although the detailed mechanism is still unknown.

On electron microscopy (EM), CBs in the patients differ slightly from typical CBs seen in other various diseases. While typical CBs consists of a core with electron density similar to Z line,[21] the CBs in HMERF show lower density. Furthermore, thin filaments, which compose a halo of typical CBs, are rarely seen around the CBs. Interestingly, the sarcomeric disarrangement appears to progress in order of figure 2A, C, suggesting a possibility that this sarcomeric disarrangement may ultimately produce the CBs.

Several groups reported that CBs in HMREF are reactive to antibodies to myofibrillar proteins including myotilin, αB-crystallin, actin (phalloidin), filamin C, dystrophin, and γ-sarcoglycan, but not for titin on immunohistochemical analysis.[11 12 14 19] Although similar findings were obtained in our observation (data not shown), the pathophysiological significance of CBs in HMERF has still not been elucidated.

We found four novel variants in exon 343 of TTN including three heterozygous missense mutations [g.284702G>A (p.C31712Y), g.284938G>C (p.G31791R) and g.284939G>T (p.G31791V)], and a heterozygous deletion variant [g.284913_284921del (p.R31783_V31785del)]. These variants were not described in any of the publically available databases. Mutated amino acids are highly conserved among species. Also

Table 4 Sensitivity, specificity, and positive predictive value of laboratory findings for HMERF

	Respiratory disturbance in the ambulant stage	Selective affected semitendinosus muscles on muscle imaging	Necklace CBs on muscle pathology
Sensitivity	88% (14/16)	93% (14/15)	82% (14/17)
Specificity	94% (81/86)	NA	99% (169/170)
PPV	74%	NA	93%

At the time of muscle biopsy.
Respiratory disturbance: <80% of %VC or >45 mm Hg of $PaCO_2$. PPVs were calculated by the HMERF prevalence of 9.1% in the MFM cohort.
CB, cytoplasmic body; HMERF, hereditary myopathy with early respiratory failure; MFM, myofibrillar myopathy; NA, not available, PPV, positive predictive value; VC, vital capacity.

Table 5 Mutations in the FN3 119 domain of A-band in TTN in HMERF

DNA change	Amino acid change	Origin	Family	Reference
g.284693C>G	p.P31709R (p.P30068R)	French		3
g.284701T>C	p.C31712R (p.C30071R)	Swedish, British, Finnish, Italian, Spanish, Argentinian (European ancestry), East Indian, Japanese	A, B, C, D, E, F, G, H, I	2, 3, 11, 12, 14
g.284702G>A	p.C31712Y (p.C30071Y)	Japanese	J	*
g.284752T>C	p.W31729R (p.W30088R)	British		12
g.284754G>C	p.W31729C (p.W30088C)	German		12
g.284753G>T	p.W31729L (p.W30088L)	Japanese		13
g.284762C>T	p.P31732L (p.P30091 L)	Italian, French, British, Portuguese, Swedish		11, 12, 15, 16, 17
g.284913_284921del	p.R31783_V31785del (p.R30142_V30144del)	Japanese	N	*
g.284925C>G	p.N31786K (p.N30145 K)	British		11
g.284939G>A	p.G31791D (p.G30150D)	American (European ancestry), Japanese	K	14
g.284938G>C	p.G31791R (p.G30150R)	Japanese	L	*
g.284939G>T	p.G31791V (p.G30150V)	Japanese	M	*

*Possible novel mutation. Titin reference: ENST00000589042 and ENST00000591111, a former transcript, inside the brackets.
HMERF, hereditary myopathy with early respiratory failure.

the variants were predicted to be pathogenic by the plural prediction software programs. Furthermore, in single-base substitutions, other types of substitutions of the same amino acids (p. C31712R and p.G31791D) have already been reported in other families with HMERF (table 5).[2 3 11 12 14] Thus, although segregation analysis was not possible, the variants are highly likely to be pathogenic.

Previous reports suggested that HMERF might not be extremely rare in Caucasian populations.[11 12] In UK, in patients with HMERF with p.C31712R (p.C30071R, based on ENST00000591111), mutation in exon 343 of TTN was identified in 5.5% of the MFM cohort.[11] Patients have also been identified in Asian populations including Japanese and Indian, suggesting that patients with HMERF are likely to be distributed worldwide.[13 14 22] Here, we confirmed the presence of patients with HMERF in Japan. Furthermore, among all the 175 MFM families in our Japanese cohort, 14 families (8%) had HMERF with mutations in the exon 343 of TTN, which renders TTN the most frequent causative gene for MFM in our cohort although there still remains a possibility that there may be an undisclosed major causative gene as causative mutations have not been identified in more than 60% of the MFM families.

Clinical features of participants with HMERF described in this study coincide with those in previous reports for most parts: affected individuals usually present with predominant distal leg muscle weakness followed by chronic respiratory failure.[1–3 11–14] Interestingly, dysphagia was seen in 4 of the 17, which was rarely described in the literature.[13] Dysphagia seems to be mostly mild, but was severe in one patient, who required tube feeding.

Skeletal muscle imaging has been reported to show preferential involvement of semitendinosus, obturator, sartorius, gracilis, iliopsoas muscles and anterior compartment of lower legs, suggesting such imaging findings are useful for the diagnosis of HMERF.[3 11 12 19] Particularly, selective involvement of semitendinosus muscles is commonly observed. Our study showed a sensitivity of 93%, which is compatible with 95–100% reported by previous studies.[3 12] Unfortunately, skeletal muscle imaging was not available in many of our patients with MFM other than HMERF, and thus it was impossible to calculate the specificity and PPV of the selective involvement of semitendinosus muscles. However, it may not be so specific since such finding was observed also in other MFMs caused by mutations in DES, CRYAB and MYOT.[23 24]

In conclusion, the necklace CB is a useful pathological marker in the diagnosis of HMERF. When muscle pathology shows necklace CBs, sequencing the FN3 119 domain of the A-band in TTN should be considered.

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