事戦略相談で指導を受けた必要な生殖発生毒性試験を実施し、その成績より生殖発生毒性のリスクは低いと考えられた。

F：健康危険情報

なし

G：研究発表

（発表雑誌名、巻号、頁、発行年なども記入）

１：論文発表

なし

２：学会発表

なし

H：知的所有権の取得状況（予定を含む）

１：特許取得

なし

２：実用新案登録

なし

３：その他

実施した生殖発生毒性試験の最終報告書。

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

発表者名	論文タイトル名	発表誌	巻・号	ページ	出版年
Nalini A, Gayathri N, Nishino I, Hayashi YK	GNE myopathy in India.	Neurol India.	61(4)	371-374	2013
Nemazanyy I, Blaauw B, Paolini C, Caillaud C, Protasi F, Mueller A, Proikas-Cezanne T, Russell RC, Guan KL, Nishino I, Sandri M, Pende M, Panasyuk G	Defects of Vps 15 in skeletal muscles lead to autophagic vacuolar myopathy and lysosomal disease.	EMBO Mol Med.	5(6)	870-890	2013
Stenzel W, Nishino I, von Moers A, Kadry MA, Glaeser D, Heppner FL, Goebel HH	Juvenile autophagic vacuolar myopathy – a new entity or variant?	Neuropathol Appl Neurobiol.	39(4)	449-453	2013

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Huizing M, Carrillo-Carrasco N, Malicdan MC, Noguchi S, Gahl WA, Mitrani-Rosenbaum S, Argov Z, Nishino I	GNE myopathy: New name and new mutation nomenclature.	Neuromuscul Disord.	24(5):	387-389	2014

Ⅳ. 研究成果に関する刊行物

Original Article

GNE myopathy in India

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Abstract

Background: *GNE* myopathy is a clinicopathologically distinct distal myopathy with autosomal-recessive inheritance. The *GNE* gene mutations are known to cause this form of distal myopathy **Materials and Methods:** Over the last 6 years, a total of 54 patients from 48 families were diagnosed to have *GNE* myopathy based on the clinical and histopathological findings. We have reported on 23 cases earlier and from this cohort 12 patients from 11 families underwent genetic testing for *GNE* mutation. **Results:** Nine patients belonging to eight families were confirmed as *GNE* myopathy by genetic analysis. There were six women and three men. Mean age of onset was 26.7 ± 5.47 years (20-36 years) and mean age at clinical examination was 32.3 ± 4.2 years (28-39 years). Mean duration of the illness was 5.7 ± 4.7 years (1-14 years). All had characteristic clinical features of progressive weakness and wasting of the anterior part of leg muscles, adductors of thighs and hamstrings with relative sparing of the quadriceps muscles. Biopsy from the tibialis anterior muscles revealed the presence of rimmed vacuoles. Mutation analysis of the *GNE* gene revealed that c. 2086G > A (p.Val696Met) change was common in our series like Thailand and six of eight families carried this mutation, heterozygously. **Conclusion:** These results show the presence of a common mutation in *GNE* gene in Southeast Asia.

Key words: *c. 2086G > A (p.Val696Met), GNE myopathy, mutation analysis*

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Introduction

GNE myopathy also called as distal myopathy with rimmed vacuoles (DMRV), hereditary inclusion body myopathy (hIBM), quadriceps-sparing myopathy or Nonaka myopathy is a clinicopathologically distinct distal myopathy with autosomal-recessive inheritance. It is clinically characterized by preferential involvement of anterior tibial muscles and sparing of quadriceps and pathologically by the presence of rimmed vacuoles (RV) on muscle biopsy.[1-3] The age of onset ranges from 15 to 40 years.[4] Patients generally become wheelchair bound between 26 and 57 years of age, on an average 12 years after the onset of symptoms.[4] Serum creatine kinase (CK) level is normal or mildly elevated.

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The *GNE* gene mutations are known to cause this form of distal myopathy.[5,6] *GNE* encodes a bifunctional enzyme, uridine diphosphate-N-acetylglucosamine 2-epimerase/ N-acetylmannosamine kinase, which plays a critical role in the production of sialic acid.[7] *GNE* mutations are reported to be responsible for a lower level of sialic acid in skeletal muscles, which is postulated to be causative of the diseases.[8] Recently, limb girdle phenotype is reported to be frequent presentation in patients with myopathy associated with *GNE* mutations.[9] In the present report, we describe 9 patients with *GNE* myopathy who underwent *GNE* mutation studies.

Materials and Methods

Institutional ethical approval was obtained for the study. Muscle biopsies are routinely performed for diagnostic purpose. Written informed consent was obtained from all patients who approached for genetic analysis. Nine adult

patients who belonged to the original cohort of 23 cases were included in the present study.[10] All these patients were recruited from the Neuromuscular Disorders clinic at National Institute of Mental Health and Neurosciences, Bangalore, India. All patients attending the clinic undergo a thorough phenotypic characterization. An exhaustive proforma is completed for the topography of muscle involvement. *GNE* myopathy was diagnosed based on the following proposed findings (i) autosomal-recessive inheritance, but may be sporadic, (ii) onset of symptoms in early adulthood (iii) weakness beginning in the distal leg muscles, typically in the anterior tibialis muscles, with the quadriceps muscles remaining relatively unaffected, (iv) myogenic changes on electromyography, (v) normal or mildly elevated serum CK, (vi) muscle biopsies displaying RV without obvious dystrophic features.[1,11,12]

Genetic analysis

Genomic DNA was extracted from peripheral blood lymphocytes using the standard techniques. All exons and their flanking intronic regions of *GNE* were sequenced directly using an ABI PRISM 3130 automated sequencer (PE Applied Biosystems). The primer sequences used in this study are available on request. We used the GenBank NM_001190383 for the description of the mutations.

Results

Clinical features

We examined nine patients (six women and three men) who had weakness and atrophy of peroneal muscles with relatively preserved to normal power in quadriceps muscles. Onset of the disease was in the second or third decade in the majority and the mean age of onset was 26.7 ± 5.4 (20-36) years. Mean age at clinical examination was 32.3 ± 4.2 years (28-39). Mean duration of illness was 5.6 ± 4.7 (1-14) years. Details of the clinical manifestations, investigations and mutation findings are represented in Table 1. The initial symptom in the majority of the patients was altered gait. Muscle weakness was particularly prominent in tibialis anterior, hip adductors and hamstrings in the lower limbs with foot drop. Neck flexors were weak in majority. In upper limbs, distal and shoulder girdle muscles were more affected than arm muscles. The long finger flexors were particularly weak. Within months to a few years, the patients developed muscle weakness of the proximal lower and the distal upper limbs. All patients had minimal or no involvement in quadriceps muscles and was ambulant at the time of evaluation except for three severely disabled patients (case three, four, five and seven). Serum CK levels were normal to only slightly elevated.

Mutation analysis of *GNE*

We identified mutations in *GNE* in all 8 families examined. One consanguineous family (F1) had a homozygousc. 484C > T (p.Arg162Cys) mutation, which is previously reported.[13] Four had a compound heterozygous mutations including two novel missense mutations of c. 910G > A (p.Gly304Arg) and c. 1703G > T (p.Gly568Val) and two previously reported nonsense mutations of c. 1258C > T (p.Arg420X) andc. 1539G > A (p.Trp513X).[14] A c. 2086G > A (p.Val696Met) mutation was commonly seen in our series and identified in the six of eight families (75%), heterozygously. Four patients in three families (F6-8) had only one heterozygous reported mutation.

Discussion

Familial vacuolar myopathy with autosomal-recessive inheritance characterized clinically by progressive distal and proximal muscle wasting and weakness beginning in early adulthood and almost always sparing the quadriceps femoris even in advanced stages was reported to occur in Jews of Persian origin.[15,16] The familial myopathy in Persian Jews has been considered to be an autosomal-recessive form of hIBM, which is now called as *GNE* myopathy.[17-19] Our patients all had the classical clinical and histopathological features of *GNE* myopathy.

Over the last 6 years, we have diagnosed 54 patients phenotypically having the classical features of *GNE* myopathy and muscle biopsy confirming the diagnosis of rimmed vacuolar myopathy. Our earlier report on 23 cases was based on the clinical and histopathological features.[10] The initial and preferential peroneal muscle involvement appears in the second or third decade of life in most patients and the disease is progressive, usually leading to a non-ambulant state within 10 years after the onset.[1-3]

Nonaka *et al.*, for the first time described three patients from two families of Japanese origin with autosomal-recessive inheritance and presence of RV in muscle biopsies.[11] DMRV or now termed as *GNE* myopathy is a distinct clinical entity inherited through an autosomal-recessive trait with female preponderance.[3] The mean age of onset in our group was 26.7 years and the initial symptoms of muscle weakness of the legs appeared in the majority in the second or third decade. In a review of 37 Japanese patients, the mean age of onset was 26.1 and onset in the third decade was noted in 64% of the patients.[20] Clinically in seven of their nine patients in whom duration of illness was more than 10 years, five of them became non-ambulatory. Serum CK was mildly

Table 1: Clinical characteristics and investigation findings in the nine cases with GNE mutation analyses

Parameters	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7 (Sibling of case 6)	Case 8	Case 9
Gender	M	F	F	F	M	F	F	M	F
Age at onset (year)	26	26	34	20	25	20	26	27	36
Duration of illness (year)	8	2	1	10	14	9	3	2	2
Symptom at onset	Foot drop	Foot drop	Foot drop	Foot drop	Foot drop	Foot drop	Foot drop	Foot drop	Foot drop
Symm/Asymat onset	Asym	Asym	Symm	Asym	Symm	Asym	Symm	Symm	Asym
Consanguinity	Yes	No	No	No	No	Yes	Yes	Yes	No
Family history	No	No	No	No	No	Yes	Yes	No	Yes
Serum CK (IU)	238	520	496	116	496	132	445	230	189
MRC power (grade)									
Shoulder	5	5	5	1	4	1	4	3	5
Elbow	5	5	5	1	4	2	4	3	5
Long flexors of hand	4	3	4	2	2	1	2	2	2
Small muscle of hand	4	4	4	1	2	1	2	2	2
Iliopsoas	4	1	2	0	2	1	1	2	1
Glut Max	4	4	4	0	3	1	2	3	3
Adductors	3	1	1	0	2	1	0	3	1
Abductors	4	4	5	0	3	1	3	3	3
Quadriceps	4	5	5	2	5	1	5	3	5
Hamstrings	3	2	2	0	2	1	1	2	2
Ankle									
Dorsiflexors	1	1	3	1	0	1	0	2	2
Plantarflexors	4	3	5	1	2	1	3	4	5
At evaluation	Ambulant	Ambulant	Ambulant	WCB	Ambulant with support	WCB	WCB	Ambulant	Assistance
Muscle biopsy (RV's)	Present	Present	Present	Present	Present	Present	Present	Present	Present
Mutation									
Nucleotide substitution	c.484C> T (homo)	c.910G>A/c. 2086G>A	c.1258C>T/c. 2086G>A	c.1539G>A/c. 2086G>A	c.1703G>T/c. 2086G>A	c.2086G> A/?	c.2086G >A/?	c.2086G >A/?	c.80C> T/?
Exon domain	Epimerase/ epimease	Epimerase/ kinase	Kinase/ kinase	Kinase/ kinase	Kinase/ kinase	Kinase/?	Kinase/?	Kinase/?	Epimerase/?
Amino acid substitution	Arg162Cys (homo)	Gly304Arg/ Val696Met	Arg420X/ Val696Met	Trp513X/ Val696Met	Gly568Val/ Val696Met	Val696Met/?	Val696Met/?	Val696Met/?	Pro27 Leu/?

Y - Years, Symm - Symmetrical, Asym - Asymmetrical, CK - Creatine kinase, Glut - Gluteus, Max - Maximus, RV - Rimmed vacuoles, WCB - Wheel chair bound, MRC - Medical research council

elevated or within the normal limits among the 37 Japanese patients. CK levels in our cohort were normal or minimally elevated.

In the present study, genetic analysis identified a homozygous c. 484C > T (p.Arg162Cys) mutation in one family. This mutation was previously reported in an Italian family.[13] Four families had a compound heterozygous mutations including 2 novel missense mutations c. 910G > A (p.Gly304Arg) and c. 1703G > T (p.Gly568Val). Both these amino acids are well-preserved among species and a missense mutation p.Gly568Ser was also recently identified in a Japanese *GNE* myopathy patient.[21] The c. 2086G > A (p.Val696Met) mutation was common in this series and identified in the six of eight families (75%), heterozygously. This mutation was reported previously among patients from Thailand.[7,21]

In *GNE* myopathy, presence of the common mutation is known to occur in different ethnic backgrounds, i.e. p. Val572 Leu mutation in Japanese and Korean patients and p.Met712Thr in the Jewish population. Findings in the present study and earlier studies, p.Val696Met substitution appears to be the most common mutation in both India and Thailand.[22] In this study, four patients from three families had only one heterozygous mutation in *GNE* gene. The second mutation may not be detectable using the present method, which detects only large deletions in the *GNE* gene. In a previous study from Japan, patients with a nonsense mutation in one allele showed relatively severe clinical features.[21] However, in this series, one patient (case 3) of 34 years of age harboring p.Arg420X/Val696Met mutation was ambulant, but had illness onset at 33 years of age while case 4 (30 years of age) with p.Try513X/Val696Met was wheel chair-bound after 10 years of illness duration.

From the predicted structure models reported by Kurochkina, *et al.*, p.Arg420X can preserve its epimerase activity and the enzyme hexameric state, whereas p.Try513X causes its conformational change on glucose binding if these proteins are produced.[23] This study confirms that *GNE* myopathy may be indeed one among the common inherited myopathies among Indians. Further analysis in a larger cohort is required to clarify the genotype-phenotype correlations of *GNE* myopathy among Indian population.

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RESEARCH PAPER

Mutation profile of the *GNE* gene in Japanese patients with distal myopathy with rimmed vacuoles (GNE myopathy)

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ABSTRACT

Background GNE myopathy (also called distal myopathy with rimmed vacuoles or hereditary inclusion body myopathy) is an autosomal recessive myopathy characterised by skeletal muscle atrophy and weakness that preferentially involve the distal muscles. It is caused by mutations in the gene encoding a key enzyme in sialic acid biosynthesis, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE).

Methods We analysed the *GNE* gene in 212 Japanese GNE myopathy patients. A retrospective medical record review was carried out to explore genotype–phenotype correlation.

Results Sixty-three different mutations including 25 novel mutations were identified: 50 missense mutations, 2 nonsense mutations, 1 insertion, 4 deletions, 5 intronic mutations and 1 single exon deletion. The most frequent mutation in the Japanese population is c.1714G>C (p.Val572Leu), which accounts for 48.3% of total alleles. Homozygosity for this mutation results in more severe phenotypes with earlier onset and faster progression of the disease. In contrast, the second most common mutation, c.527A>T (p.Asp176Val), seems to be a mild mutation as the onset of the disease is much later in the compound heterozygotes with this mutation and c.1714G>C than the patients homozygous for c.1714G>C. Although the allele frequency is 22.4%, there are only three homozygotes for c.527A>T, raising a possibility that a significant number of c.527A>T homozygotes may not develop an apparent disease.

Conclusions Here, we report the mutation profile of the *GNE* gene in 212 Japanese GNE myopathy patients, which is the largest single-ethnic cohort for this ultra-orphan disease. We confirmed the clinical difference between mutation groups. However, we should note that the statistical summary cannot predict clinical course of every patient.

INTRODUCTION

GNE myopathy, which is also known as distal myopathy with rimmed vacuoles,[1] quadriceps sparing myopathy[2] or hereditary inclusion body myopathy (hIBM),[3] is an autosomal recessive myopathy characterised by skeletal muscle atrophy and weakness that preferentially involve the distal muscles such as the tibialis anterior. It is a progressive disease, whereby the symptoms of muscle weakness start to affect the patient from the second or third decade of life, and most of the patients become wheelchair-bound between twenties and sixties.[4] The characteristic histopathological features in muscle biopsy include muscle fibre atrophy with the presence of rimmed vacuoles and intracellular congophilic deposits.[4 5] GNE myopathy is caused by mutations in the gene encoding a key enzyme in sialic acid biosynthesis, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE).[6–8] Genetically confirmed GNE myopathy was initially recognised in Iranian Jews and Japanese,[7 9] but later appeared to be widely distributed throughout the world. More than 100 mutations in the *GNE* gene have been described up to date.

During the last decade, there has been extensive experimental work to elucidate the pathogenesis and to develop therapeutic strategies of GNE myopathy.[6 10–12] Better knowledge on the basis of those research achievements have currently enabled us to enter the era of clinical trial for human patients. At this moment, the identification of new GNE myopathy patients with precise genetic diagnosis and the expansion of global spectrum of *GNE* mutations are timely and important. Here, we report the molecular profile of Japanese GNE myopathy patients with a brief discussion of genotype–phenotype correlations.

METHODS

Patients

Two hundred and twelve patients from 201 unrelated Japanese families were included in this study. There were 117 female and 95 male patients. All cases were genetically confirmed as GNE myopathy. A retrospective medical record review was carried out to explore genotype–phenotype correlation. Informed consent was obtained for the collection of clinical data and extraction of DNA to perform mutation analysis.

Genetic analysis

DNA was extracted from peripheral blood leukocytes or skeletal muscle tissue. We used the previously described sequencing method to describe mutations at cDNA level.[7] All exons and splice regions of the *GNE* gene were sequenced. NM_005476.5 was used as a reference sequence. We screened 100 alleles from normal Japanese individuals to determine the significance of novel variations.

Pathological analysis

To evaluate histopathological phenotype according to genotype, we analysed muscle biopsies from two

most common genotype groups in Japanese population. Each of the three age-matched and biopsy site-matched samples from c.1714G>C homozygous group and c.1714G>C/c.527A>T compound heterozygous group was compared. Muscle samples were taken from biceps brachii and frozen with isopentane cooled in liquid nitrogen. Serial frozen sections of 10 μm were stained using a set of histochemical methods including haematoxylin-eosin and modified Gomori trichrome.

Statistical analysis

Statistics were calculated using GraphPad Prism 5 software (GraphPad Software, La Jolla, California, USA). Between-group comparison for clinical data was performed using one-way analysis of variance with Dunnett's post-test. All values are expressed as means±SD. We performed two-sided tests with a $p<0.05$ level of significance.

Table 1 Allele frequency for GNE mutations in 212 Japanese GNE myopathy patients

	Allele frequency
Mutation type	
Missense	402 (94.8%)
Nonsense	3 (0.7%)
Insertion	1 (0.2%)
Small deletion	4 (0.9%)
Single exon deletion	2 (0.5%)
Intron	12 (2.8%)
Three most common mutations	
c.1765G>C (p.Val572Leu)	205 (48.3%)
c.578A>T (p.Asp176Val)	95 (22.4%)
c.38G>C (p.Cys13Ser)	15 (3.5%)
Total alleles	424

RESULTS

Mutation profile

We identified homozygous or compound heterozygous *GNE* mutations in all 212 patients (see online supplement 1). In total, 63 different mutations were found including 50 missense mutations, 2 nonsense mutations, 1 insertion, 4 deletions, 5 intronic mutations and 1 single exon deletion (figure 1). Twenty-five novel mutations were identified including 17 missense mutations, 4 small deletions, 3 intronic mutations and 1 single exon deletion (figure 1, see online supplement).

Twenty-one mutations were found to be shared between two or more unrelated families. The three mutations occurring most frequently in the Japanese population were c.1714G>C (p.Val572Leu), c.527A>T (p.Asp176Val) and c.38G>C (p.Cys13Ser); these comprised 48.3%, 22.4% and 3.5%, respectively, of the total number of alleles examined (table 1).

Genotype–phenotype correlations

The mean age of genetic analysis was 41.6±14.1 years (n=212), and the mean age of symptom onset based on the data available was 28.4±10.2 years (n=195). The earliest onset age was 10 and the latest was 61 years old in our cohort. Thirty-six among 154 patients (23.4%) were full-time wheelchair users at the point of genetic diagnosis with the average age at loss of ambulation being 36.8±11.3 years (n=36). The youngest wheelchair-bound age was 19, and the oldest ambulant age was 78. To investigate genotype–phenotype correlations in the major *GNE* mutations of Japanese population, we compared the age at symptom onset and loss of ambulation between the patients groups carrying either of the two most frequent mutations, c.1714G>C and c.527A>T (table 2). As with a previous report,[13] homozygous c.1714G>C mutations resulted in earlier symptom onset (23.9±7.1 years, $p<0.01$) and the majority of full-time wheelchair users were in this group. On the other hand, c.1714G>C/c.527A>T compound heterozygous patients first developed symptoms at a later age (37.6±12.6 years, $p<0.01$), and there were no wheelchair-bound patients at the time of genetic analysis in this group. Only three homozygous c.527A>T mutation patients were identified, and their average onset age (32.3±5.7 years) was also higher among total patients (28.4±10.2 years). All three patients were ambulant until the last follow-up visits (29, 40 and 44 years).

Among 212 cases, 80 patients underwent muscle biopsies. Overall pathological findings in our series were compatible with GNE myopathy. The characteristic rimmed vacuoles were observed in the majority (76/80, 95.0%) of the cases. Through the analysis of muscle biopsies from age-matched and biopsy site-matched samples, we found that the histopathological phenotypes were in line with these genotype–phenotype correlations (figure 2). Homozygous c.1714G>C mutations have led to much more advanced pathological changes with severe myofibre atrophy and increased numbers of rimmed vacuoles. Marked adipose tissue replacement was appreciated in a case with reflecting very advanced stage of muscle degeneration.

DISCUSSION

As shown in figure 1, mutations were located throughout the whole open reading frame of the *GNE* gene. The majority (94.8%, 402/424 alleles) of the mutations in our series were missense mutations (table 1), and there were no homozygous null mutations. These results are in accordance with previous reports[7 9] signifying that total loss of GNE function might be

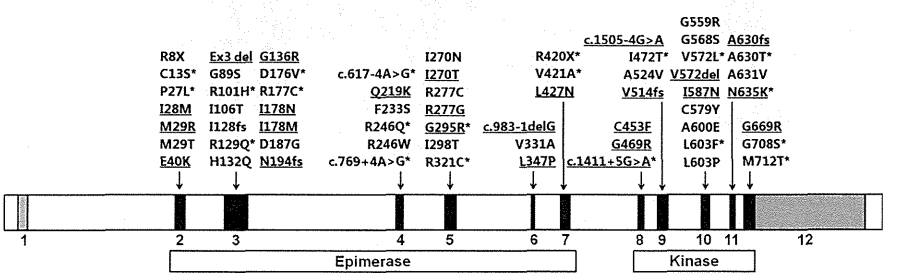

Figure 1 Mutation spectrum of *GNE* in the Japanese population. The mutations are located throughout the whole open reading frame. Twenty-five novel mutations are underlined, and 21 shared mutations are indicated with asterisks.

Table 2 Comparison of clinical course between two most frequent GNE mutations in Japanese population

Mutations	Age at exam (years)		Age at onset (years)		Age at WB (years)		Ambulant
c.1714G>C/c.1714G>C	38.6±13.4	(n=71)	23.9±7.1	(n=65)**	35.4±10.6	(n=28)	n=22
c.1714G>C/other	32.3±13.2	(n=25)	21.9±6.8	(n=22)*	37.0±8.6	(n=4)	n=16
c.1714G>C/c.527A>T	48.9±14.1	(n=38)	37.6±12.6	(n=35)**		(n=0)	n=29
c.527A>T/c.527A>T	37.7±7.7	(n=3)	32.3±5.7	(n=3)		(n=0)	n=3
c.527A>T/other	41.3±11.1	(n=51)	30.6±8.0	(n=46)		(n=2)	n=33
other/other	49.8±14.7	(n=24)	28.8±9.5	(n=24)		(n=2)	n=16
Total	41.6±14.1	(n=212)	28.4±10.2	(n=195)	36.8±11.3	(n=36)	n=118

Dunnett's multiple comparison test (control: total patients) *p<0.05, **p<0.01.
Other: a mutation other than c.1714G>C and c.527A>T; WB, wheelchair-bound.

lethal in human beings. The embryonic lethality of null mutation in *GNE* had also been proved in the mouse model.[14] Only three of total 212 patients carried a nonsense mutation; clinical data were available for two of them. Interestingly, one patient with compound heterozygous c.22C>T (p.Arg8X)/c.1714G>C (p.Val572Leu) mutations developed his first symptoms at the age of 15, while the other patient with c.1258C>T (p.Arg420X)/c.527A>T (p.Asp176Val) mutations developed her symptoms much later, at the age of 45. The similar difference was also observed in the phenotypes of patients with frame-shift mutations. A patient carrying c.383insT (p.I128fs) and c.1714G>C (p.Val572Leu) mutations developed his first symptom at the age of 13, whereas another two patients with c.1541-4del4 (p.Val514fs)/c.527A>T (p.Asp176Val) and c.581delA (p.N194fs)/c.527A>T (p.Asp176Val) mutations had later symptom onset, at the age of 30 and 32 years, respectively. This clinical variation can be explained as it reflects alternative missense mutations, because the two patients with very early onset shared the same missense mutation c.1714G>C, while the patients with the milder phenotype shared c.527A>T.

Among five intronic mutations identified in our series, c.617−4A>G and c.769 + 4A>G were previously reported as pathological mutations.[7 15] Three novel variants were located at splice junction of exon 6 (c.983−1delG), exon 8 (c.1411 +5G>A) and exon 9 (c.1505−4G>A), raising the high possibility of relevant exons skipping. These variants were not detected in 200 alleles from normal Japanese individuals and also in the single nucleotide polymorphism (SNP) database.

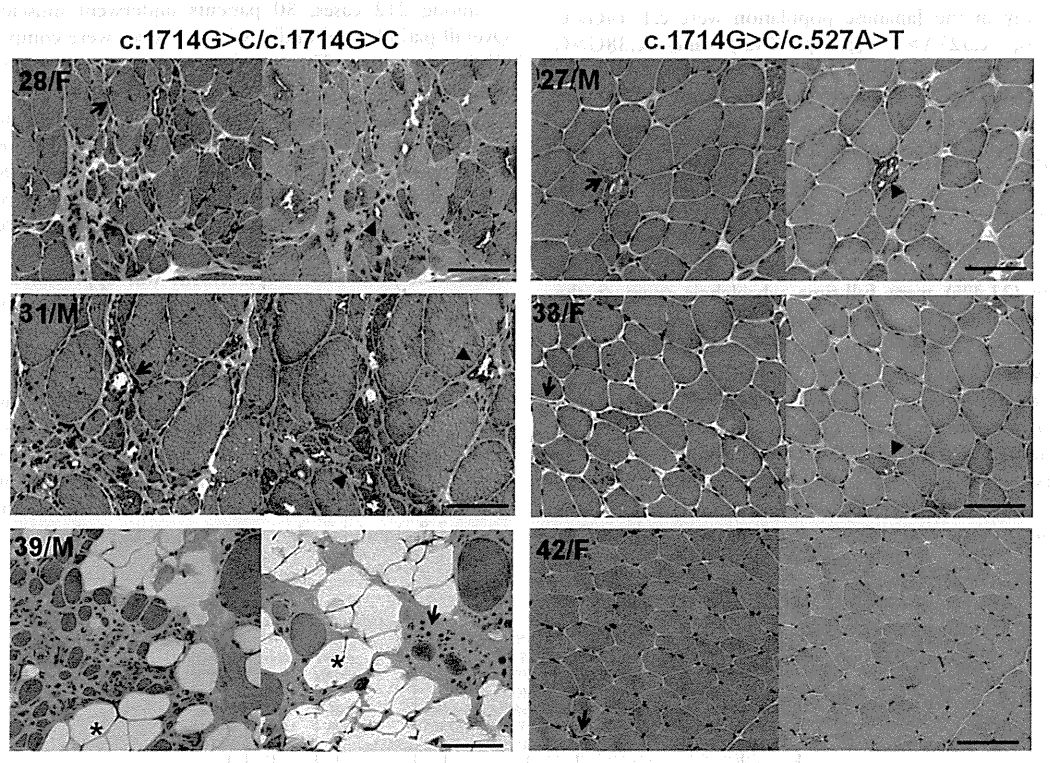

Figure 2 Comparison of muscle pathology between patients with homozygous c.1714G>C (p.Val572Leu) and with compound heterozygous c.1714G>C (p.Val572Leu)/c.527A>T (p.Asp176Val) mutations. Homozygous c.1714G>C (p.Val572Leu) mutations have led to much more advanced histopathological changes compared with compound heterozygous c.1714G>C (p.Val572Leu)/c.527A>T (p.Asp176Val) mutations. Haematoxylin-eosin (left) and modified Gomori trichrome (right) stains of muscle sections from age (c.1714G>C/c.1714G>C: 28, 31 and 39 years, c.1714G>C/c.527A>T: 27, 33 and 42 years) and biopsy site (biceps brachii muscles) matched samples. Bar=100μm; triangles: rimmed vacuoles; arrows: atrophic fibres; asterisks: adipose tissue.

As there are ethnic differences in *GNE* mutation frequencies,[9 16–19] establishing the mutation spectrum and defining predominant mutations in a certain population may be helpful for the diagnosis. Three most common mutations in the Japanese population and their allele frequencies (table 1) were in agreement with previous data.[7 13] The allele frequencies of top two mutations (c.1714G>C and c.527A>T) comprise more than two-third of the total number of alleles suggesting that founder effects are involved in the relatively higher incidence of GNE myopathy in Japan.

Although most of patients showed characteristic pathological features, the existence of exceptional cases with atypical biopsy findings implies that GNE myopathy cannot be totally excluded from the absence of rimmed vacuoles in muscle biopsies. On the other hand, we found 94 patients who were pathologically or clinically suspected but not had mutations in *GNE*. Several cases of VCP myopathy mutations in (*VCP*), myofibrillar myopathy mutations in (*DES*) and reducing body myopathy (*FHL1*) were later identified in this group, suggesting these diseases should be included as differential diagnosis of GNE myopathy.[20]

In terms of genotype–phenotype correlations, we confirmed that homozygosity for c.1714G>C (p.Val572Leu) mutation resulted in more severe phenotypes in clinical and histopathological aspects. In contrast, the second most common mutation, c.527A>T (p.Asp176Val), seems to be a mild mutation as the onset of the disease is much later in the compound heterozygotes with this mutation and c.1714G>C. Several evidences further strengthened the link between the more severe phenotype and c.1714G>C, and between the milder phenotype and c.527A>T. Compound heterozygosity for c.1714G>C and non-c.527A>T mutations resulted in earlier symptom onset (22.9±6.8 years, $p<0.05$) compared with the average onset age of the total group, whereas c.527A>T, both presented as homozygous and as compound heterozygous mutations, lead to slower disease progression (table 2). In addition, only three patients carrying this second most common mutation c.527A>T in homozygous mode were identified, which is much fewer than the number expected from high allele frequency (22.4%), raising a possibility that considerable number of c.527A>T homozygotes may not even develop a disease. In fact, we ever identified an asymptomatic c.527A>T homozygote at age 60 years.[7] Now he is at age 71 years and still healthy. Overall, these results indicate that different mutations lead to different spectra of severity. However, this is a result of a statistical summary that cannot predict clinical course of each individual patient.

Here, we presented the molecular bases of 212 Japanese GNE myopathy patients with 25 novel *GNE* mutations. Based on the current status of knowledge, sialic acid supplementation may lead to considerable changes in the natural course of GNE myopathy within near future. The ongoing identification of *GNE* mutations and further studies regarding the clinicopathological features of each mutation will provide better understanding of GNE myopathy and lead to accelerated development of treatment for this disease.

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Contributors AC had full access to all of the data in the study and wrote the manuscript; YKH supervised all aspects of this study including study design, data interpretation and manuscript preparation; KM and YO participated in collecting and analysing all the clinical and genetic data; SN, I Nonaka and I Nishino were involved in data analysis and interpretation and also supervised manuscript preparation.

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Competing interests None.

Ethics approval This study was approved by the ethics committee of National Center of Neurology and Psychiatry.

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Mutation profile of the *GNE* gene in Japanese patients with distal myopathy with rimmed vacuoles (GNE myopathy)

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Scientific correspondence

Juvenile autophagic vacuolar myopathy – a new entity or variant?

Autophagic vacuolar myopathies (AVMs) comprise a heterogeneous cluster of diseases. These include lysosomal storage disease with or without abnormal acid maltase activity such as glycogen storage disease (GSD) type II (OMIM 232300) and lysosomal membrane protein LAMP-2-deficient Danon disease (OMIM 300257) [1] caused by mutations in the *LAMP-2* gene (OMIM 309060). X-linked myopathy with excessive autophagy (XMEA; OMIM 310440), infantile AVM (OMIM 609500) and adult onset AVM with multi-organ involvement are also included in the spectrum of AVMs [2–5]. These latter diseases demonstrate distinct pathomorphological features, including vacuoles with autophagy-associated proteins and immunoreactivity for a multitude of sarcolemmal proteins (e.g. dystrophin, β-spectrin, dysferlin, caveolin-3, sarcoglycans), prominent acetylcholine esterase (AChE) activity and complement deposition [3,6]. Particularly in XMEA, ultrastructural evidence of intravacuolar debris associated with multiplication of the basal lamina and fusion of the vacuoles with the sarcolemma have been described as specific hallmarks for the disease, suggesting an aberrant exocytotic process [2,6]. Furthermore, the *VMA21* gene on chromosome Xq28, which encodes a chaperone for assembly of lysosomal vacuolar ATPase, has been established as responsible for XMEA [7]. It is likely that AVMs share a common pathomechanism related to a dysfunctional autophagosomal machinery [1,6].

After a normal post-natal and infantile development, a 14-year-old boy from Yemen presented with mild proximal weakness affecting his lower extremities for a duration of 1 year. He showed Gowers sign upon neurological examination. Strength in his arms and distal leg muscles was unremarkable as was the rest of the entire neurological examination. In particular, no evidence of muscle or tendon contractures was found. Creatine kinase was elevated up to 15-fold, and a cardiac work-up disclosed a Wolf-Parkinson-White syndrome by 24-h electrocardiography, but no signs of cardiomyopathy by echocardiography, and no involvement of further internal organs was detectable. He had no intellectual deficits, and his family history was reported to be unremarkable, notably with an absence of a history of muscle diseases.

A biopsy specimen from the quadriceps muscle was subjected to routine enzyme histochemistry, immunohistochemistry and ultrastructural examination. In most fibres, numerous vacuoles inside the sarcoplasm exhibited sarcolemmal lining, demonstrated by the strong but variable expression of plasma membrane- or basal lamina-associated proteins, including β-spectrin, caveolin-3, laminin-α2 (300 kDa) (Figure 1B–D), dystrophin and dysferlin (not shown). Interestingly, invagination of the sarcolemma could be detected in Gomori trichrome, β-spectrin, caveolin 3, laminin-α2 and major histocompatibility complex (MHC) class I stains (Figure 1A–D, F). Vacuoles contained both AChE (Figure 2A) and non-specific esterase (Figure 2C) but lacked acid phosphatase activity (Figure 2B). Vacuoles were also lined by complement (C5b9), but none of the fibres demonstrated C5b9 staining of the sarcolemma (Figure 1E). Furthermore, MHC class I was upregulated on the sarcolemma and on the vacuolar lining (Figure 1F), while neither stained for MHC class II (not shown). Large autophagosomes were strongly immunopositive for LC3 (Figure 2D). Interestingly, LAMP-2 was strongly positive on numerous lysosomes (Figure 2E), which were more densely packed and larger when compared to normal controls (Figure 2E, inset). Ultrastructural studies identified cellular debris and myelin-like features in the centre of vacuoles (Figure 2F) as well as duplicated basal laminae (Figure 2G) and accumulation of glycogen, which was not membrane-bound (Figure 2H). Fusion of vacuoles with the sarcolemma of muscle fibres was not a detectable feature of this biopsy specimen.

Molecular sequencing was performed and ruled out GSD II (juvenile 'Pompe' disease), Danon disease and XMEA. Sequencing of the entire *GAA* gene on chromosome 17q25, *LAMP-2* gene on chromosome Xq24 as well as the *VMA21* gene on chromosome Xq28 failed to reveal deletions.

Taken together, the present case may represent the manifestation of a new disease or a variant among the genetically unidentified congenital infantile and adult AVM displaying some similarities with XMEA, but also distinctive clinicopathological features. We describe a juvenile male subject with normal intelligence suffering

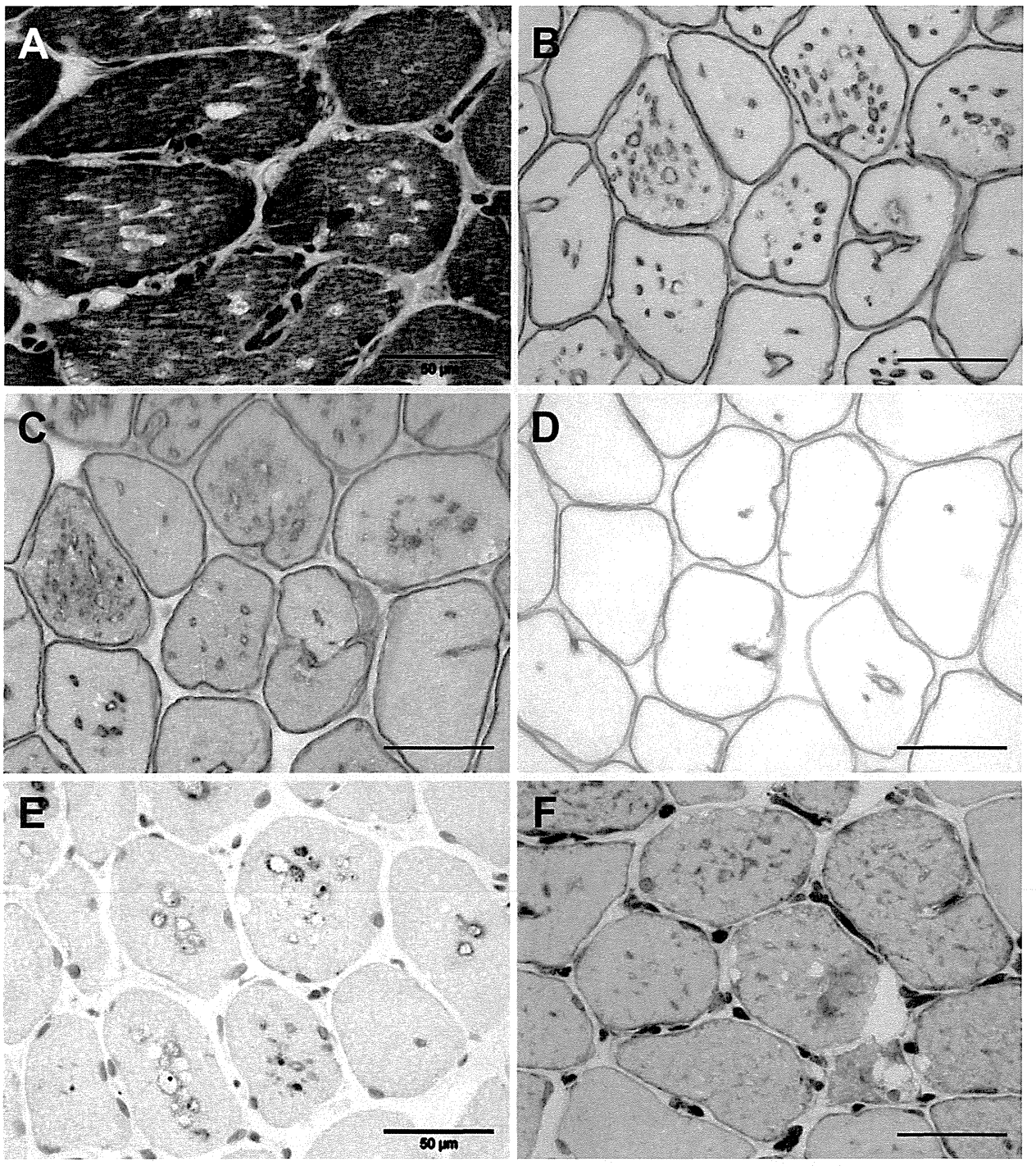

Figure 1. Myopathological characteristics of juvenile autophagic vacuolar myopathy with sarcolemmal features. Numerous vacuoles are illustrated by a Gomori trichrome stain in nearly every muscle fibre (A). Sarcolemmal features with lining of the vacuoles by sarcolemmal and basal lamina proteins at varying intensity are exemplified by β-spectrin (B), caveolin-3 (C) and laminin-α2 (300 kDa) stains (D). Complement deposition of the membrane attack complex (C5b9) was strongly positive in the vacuoles and in the vacuolar lining but negative on the sarcolemma of muscle fibres (E), while major histocompatibility complex class I is found on the vacuolar lining and on the sarcolemma (F).

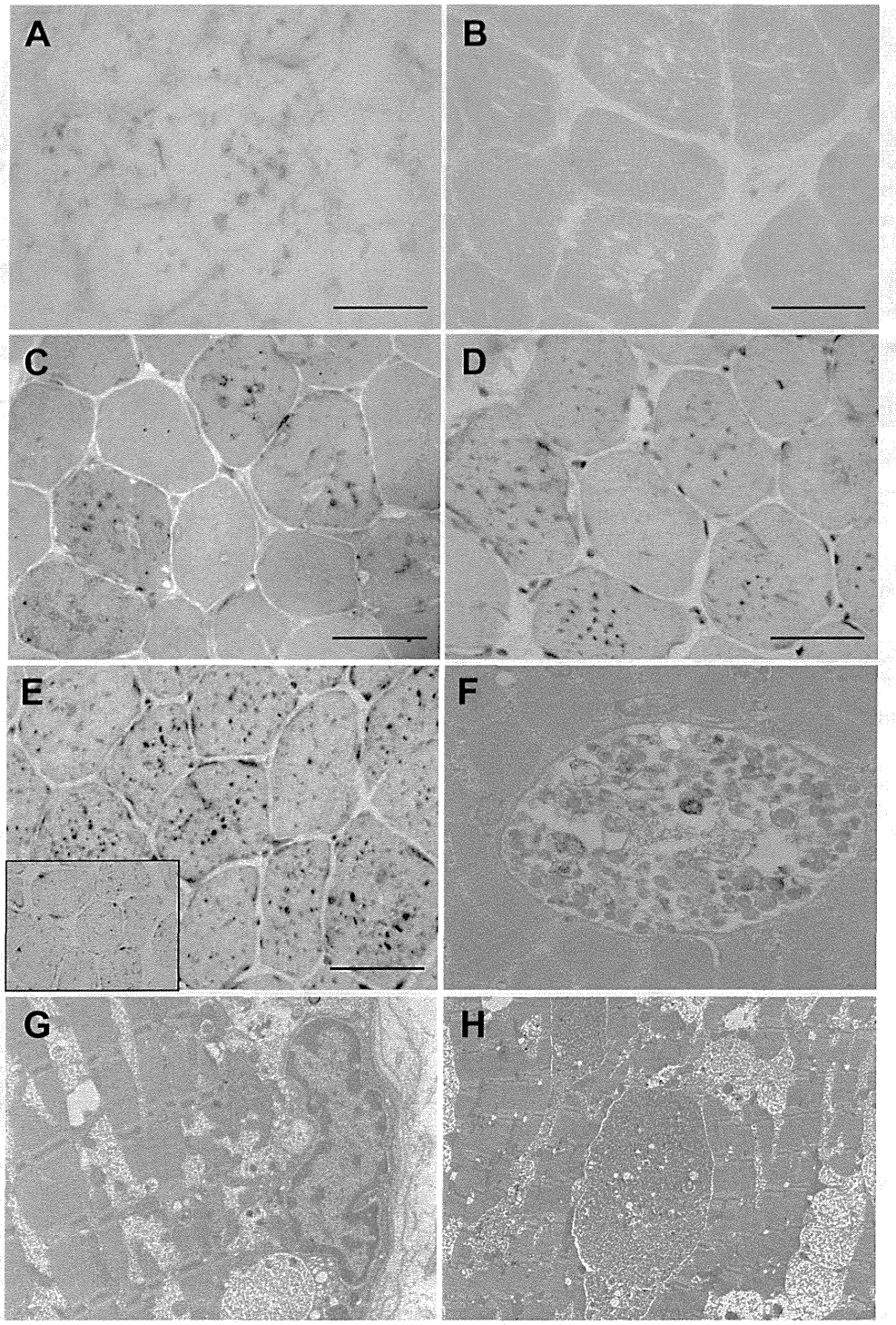

Figure 2. Light microscopic and ultrastructural characteristics of juvenile autophagic vacuolar myopathy with sarcolemmal features. The content of most vacuoles is positive for acetylcholine esterase (A), negative for acid phosphatase (B) but positive for non-specific esterase (C). Autophagic activity of large lysosomes is documented by LC3 immunostaining (D). Strong immunoreactivity of LAMP-2 protein is identified on these large lysosomes (E). The inset shows a healthy control muscle, with smaller lysosomes. Ultrastructural analysis reveals cellular debris, myelin-like formations and glycogen in vacuoles (F). Additionally duplicated basal laminae are illustrated (G), but exocytosed material between them is absent. Intermyofibrillar accumulation of glycogen which is not membrane-bound is illustrated (H).

from proximal myopathy and cardiac arrhythmia, but without manifesting signs of cardiomyopathy. Muscle biopsy showed morphological characteristics compatible with AVM, but genetic evidence for XMEA could not be found. Although unlikely, one caveat is that mutations in non-sequenced regions including the promoter and deep introns may have been missed. This is an issue in all sequencing endeavours across all hereditary diseases, and thus a general point of contention. Although not a probable diagnosis, atypical GSD II and Danon disease were also ruled out by genetic analyses as mentioned above. Of note, some features were particularly unique to the case and of diagnostic relevance; others were overlapping with details found in XMEA, GSD II and Danon disease and in the twin girls harbouring AVMs, described by Holton *et al.* [5]. Invaginations of the sarcolemma as illustrated here have also been described in Danon disease and in the report by Holton *et al.*, and support the concept of autophagosomal isolation of sarcolemmal membranes prior to fusion with lysosomes [1]. Importantly, acid phosphatase activity was consistently absent in the vacuoles in this juvenile patient's muscle tissue, which is known to be very strong in lysosomes and vacuoles of juvenile GSD II patients' muscle tissues. Further, acid phosphatase is present but less pronounced in Danon disease and in the twin girls' muscles reported by Holton *et al.*, while strong acid phosphatase positivity was reported in a Chinese patient's muscle who was one of seven affected boys suffering from congenital X-linked AVM [4]. Excessive LAMP-2 immunoreactivity on lysosomal structures has thus far not been described in infantile cases or in the adult case of AVM [4,8–11], but it was described to be increased in the report by Yan *et al.* in the patient of the congenital X-linked AVM family [4]. LAMP-2 immunoreactivity was not tested in the report on twin girls with AVM by Holton *et al.*, but mutations in the corresponding gene have formally been ruled out [5]. LAMP-2 staining in our case illustrates unusually large autophagosomes, which can be detected by the LC3 stain as well. Absence of complement deposition on the sarcolemma but strong presence on the vacuolar lining is a further relevant immunohistochemical feature of our patient's muscle tissue, presented here. The twin girls described by Holton *et al.* [5], and the patient from the family of congenital X-linked AVM [4], exhibited both sarcolemmal and vacuolar staining of complement. Finally, the ultrastructural morphology differs from the classical morphology of XMEA. We provide evidence of vacuoles containing cellular debris (Figure 2F) but absence of vacuoles having fused with the sarcolemma of muscle fibres as described in XMEA. Furthermore, multiplication of the basal lamina was clearly less prominent than in XMEA and in the report by Holton *et al.* [5]. Paucity of multilayered basal lamina may also be the underlying reason for an absence of complement deposition on the sarcolemma described here. Yet, another difference from XMEA is that exocytosed material between multilayered basal lamina could not be detected in our muscle biopsy specimen. The two male infants reported by Verloes *et al.* [9] and Morisawa *et al.* [8] had severe cardiomyopathy, which was lethal, as well as generalized muscle hypotonia. Interestingly, glycogen content was reported severely increased in skeletal muscle and especially in heart muscle in these aforementioned studies.

Taken together, a number of features in the present case are at variance with the morphology described in XMEA or the other AVMs with sarcolemmal features published to date. Further, the patient showed cardiac involvement, which also does not fit clinically with XMEA, where cardiac symptoms are generally absent. One example that contradicts this observation is a report of two male siblings with the severe congenital form of XMEA, one of whom suffered from cardiac right bundle branch block and left ventricular hypertrophy, while his brother did not show any cardiac involvement [4].

In conclusion, our 14-year-old patient is the first juvenile male subject reported with a proximal myopathy due to AVM with sarcolemmal features. Genetically, XMEA, which is due to mutations of the *VMA21* gene on chromosome Xq28, as the most probable cause was ruled out. Although very rare, it is important to consider AVM and thus perform muscle biopsy and appropriate analysis including electron microscopy to improve our understanding of the morphological spectrum of AVM. We believe that modern genetic analysis will lead to identification of further causative genes and functional concepts for these obviously heterogeneous entities grouped under the term AVM.

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Authors' contributions

Drs Werner Stenzel, Ichizo Nishino and Hans-Hilmar Goebel drafted and revised the manuscript, designed the