suggest that survival in ALS with DPN is similar to that in classic ALS, and electrophysiologic nerve conduction abnormalities indicating DPN may not be the key determinant of survival in ALS.

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Inflammatory changes in infantile-onset *LMNA*-associated myopathy

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Abstract

Mutations in *LMNA* cause wide variety of disorders including Emery–Dreifuss muscular dystrophy, limb girdle muscular dystrophy, and congenital muscular dystrophy. We recently found a *LMNA* mutation in a patient who was previously diagnosed as infantile onset inflammatory myopathy. In this study, we screened for *LMNA* mutations in 20 patients suspected to have inflammatory myopathy with onset at 2 years or younger. The diagnosis of inflammatory myopathy was based on muscle pathology with presence of perivascular cuffing and/or endomysial/perimysial lymphocyte infiltration. We identified heterozygous *LMNA* mutations in 11 patients (55%), who eventually developed joint contractures and/or cardiac involvement after the infantile period. Our findings suggest that *LMNA* mutation should be considered in myopathy patients with inflammatory changes during infancy, and that this may help avoid life-threatening events associated with laminopathy.

Keywords: Inflammatory myopathy; Laminopathy; Emery–Dreifuss muscular dystrophy; Limb girdle muscular dystrophy; Congenital muscular dystrophy; *LMNA*; Infantile; Pathology; Steroid therapy; Muscle image

1. Introduction

Laminopathy is a group of disorders caused by mutations in the *LMNA* gene encoding A-type lamins that includes autosomal forms of Emery–Dreifuss muscular dystrophy (AD- and AR-EDMD) and limb girdle muscular dystrophy type 1B (LGMD1B). EDMD is characterized by the triad of: (1) early contractures of the elbows, Achilles tendons, and posterior cervical muscles; (2) slowly progressive muscle weakness and atrophy that begins in a humeroperoneal distribution; and (3) cardiomyopathy with conduction defects which culminates in complete heart block and atrial paralysis [1]. LGMD1B patients show progressive proximal dominant muscle involvement and

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cardiomyopathy with conduction defects, but joint contracture is not prominent. The onset of these diseases is usually 2 years or later. Recently, *LMNA*-related congenital muscular dystrophy (L-CMD) was reported as a novel and severe form of laminopathy [2]. L-CMD has variable severity and can be divided in two main groups: a severe group with absent motor development and patients with dropped-head syndrome.

We recently came across an infantile-onset laminopathy patient with marked mononuclear cell infiltrations in his muscle mimicking inflammatory myopathy (Patient 1 in Table 1, Fig. 1A). This patient showed hypotonia and delayed motor milestones with elevation of serum CK levels from 3 months of age. Although, he became ambulant at 15 months of age, he presented proximal dominant muscle weakness and atrophy with no dropped-head at 2 years of age. Corticosteroid therapy was started based on the muscle pathological findings that had beneficial effects on his motor development. *LMNA* gene analysis was done at 6 years of age when his ankle and elbow joint contractures appeared and a heterozygous p.Glu358Lys mutation was identified.

From this result, we screened *LMNA* mutation in the 20 patients with the onset at 2 years or younger who were pathologically suspected as inflammatory myopathy.

2. Patients and methods

2.1. Patients

All clinical materials used in this study were obtained for diagnostic purposes and written informed consent was obtained from guardians of all patients. This work was approved by the Ethical Committee of National Center of Neurology and Psychiatry (NCNP). We retrospectively recruited patients with onset at 2 years or younger who were pathologically suspected to have inflammatory myopathy from a total of 10,874 muscle biopsies stored in the

Table 1
Clinical, radiological, and genetic findings of patients with *LMNA* mutations and inflammatory changes.

Patient #/gender/ *LMNA* mutations	Age at onset /age at biopsy/ age at last consultation	Initial signs/ CK at biopsy	Muscle pathology	Steroid treatment: responsiveness/ age at start of administration/ duration of administration	Age at acquired ambulation/ maximum motor ability	Cardiac involvement	Joint contracture	Respiratory dysfunction	CT/MRI (age)/imaging at thigh	CT/MRI (age)/imaging at calf
1/M/E358K*	3 m/2 y/11 y	Motor delay/900	IC: marked, diffuse; NR: moderate; Fib: mild	Effective/2 y/9 y	15 m/Ambulant	No	6 y: Ankles, elbows, 8 y: rigid spine	No	MRI (8 y)/ selective involvement of VL, VI, VM	MRI (8 y)/ selective involvement of SO, mGC
2/M/R249W*	10 m/10 m/12 y (Died by respiratory failure)	Motor delay/1000	IC: marked, pathy; NR: mild; Fib: mild	Effective/10 m/11 y	Unknown/ambulant	9 y: Heart failure	4 y: Ankles, knees	9 y: Nocturnal NPPV	ND	ND
3/M/N39D	11 m/1 y/16 y	Motor delay/1100	IC: marked, pathy; NR: marked; Fib: mild	Effective/1 y/15 y	18 m/Ambulant	13 y: 200B0 A–V block, 15 y 3° A–V block, pacemaker implantation	1 y: Ankles, knees, hips, Rigid spine from childhood	No	CT (13 y)/DI with relative sparing of RF, GR, SA	CT (13 y)/DI
4/F/R249Q*	2 y/2 y/15 y	High CK/2000	IC: moderate, focal; NR: moderate; Fib: moderate	Effective/3 y/6 m	14 m/Ambulant	12 y: 1° A–V block	3 y: Ankles, 8 y: elbows	No	CT (6 y)/DI with relative sparing of RF, GR	CT (6 y)/ selective involvement of SO, mGC
5/M/R28Q	5 m/1 y/11 y	Motor delay/800	IC: marked, pathy; NR: moderate; Fib: moderate	Ineffective/1 y/2 y	18 m/9 y: Inability to walk	Atrial fibrillation, A–V block, PAC, PVC	No	No	CT (11 y)/DI with relative sparing of RF, GR, SA	ND
6/M/R41S	9 m/1 y/13 y	Motor delay/900	IC: moderate, diffuse, NR: moderate Fib: moderate	Ineffective/1 y/8 y	16 m/9 y: Inability to walk	11 y: PSVT attack	6 y: Ankles, elbows	11 y: Nocturnal NPPV	MRI (10 y)/ DI/DI	MRI (10 y)/ DI/DI
7/F/K32del*	1 y/2 y/6 y	Unsteady gait/800	IC: mild, focal; NR: mild; Fib: mild	Ineffective/2 y/8 m	15 m/5 y: Inability to walk	No	2 y: Ankles	No	CT (4 y)/DI with relative sparing of RF, GR/Selective involvement of SO, mGC	CT (4 y)/DI with relative sparing of RF, GR/Selective involvement of SO, mGC
8/M/R249W*	11 m/1 y/24 y (Died by arrhythmia)	Motor delay/600	IC: marked, pathy; NR: mild; Fib: moderate	Ineffective/1 y/unknown	2 y/12 y: Inability to walk	17 y: 2° A–V block, 23 y complete A–V block	17 y: Ankles, knees	No	ND	ND
9/F/L292P	1 y/8 y/10 y	Motor delay/300	IC: mild, focal; NR: moderate; Fib: marked	Unadministered	16 m/4 y: Inability to walk	6 y: LV dysfunction, 8 y: PAC, PVC	No	No	MRI (8 y)/DI with relative sparing of RF, GR, SA	MRI (8 y)/DI
10/F/R377C*	2 y/4 y/7 y (Died by heart failure)	Unsteady gait/1000	IC: moderate, focal; NR: moderate; Fib: moderate	Unadministered	10 m/ambulant	7 y: DCM (EF:32%)	5 y: Ankles	No	ND	ND
11/F/N456H	2 y/5 y/10 y	Unsteady gait/3000	IC: moderate, focal; NR: moderate; Fib: marked	Unadministered	12 m/ambulant	No	6 y: Ankle, knee, neck, 8 y: rigid spine	No	MRI (10 y)/ DI with relative sparing of RF, GR, SA	MRI (10 y)/ DI

A–V block = atrioventricular conduction block, CK = creatine kinase, CT = computed tomography, DI = diffuse involvement, EF = ejection fraction, Fib = endomysial fibrosis, GR = gracilis, IC = inflammatory cellular infiltration, LV = left ventricle, mGC = medial head of gastrocnemius, MRI = magnetic resonance imaging, NPPV = noninvasive positive-pressure ventilation, NR = necrotic and regenerating process, PAC = premature atrial contraction, PSVT = paroxysmal supraventricular tachycardia, PVC = premature ventricular contraction, RF = rectus femoris, SA = Sartorius, SO = soleus, VI = vastus intermedius, VL = vastus lateralis, VM = vastus medialis.

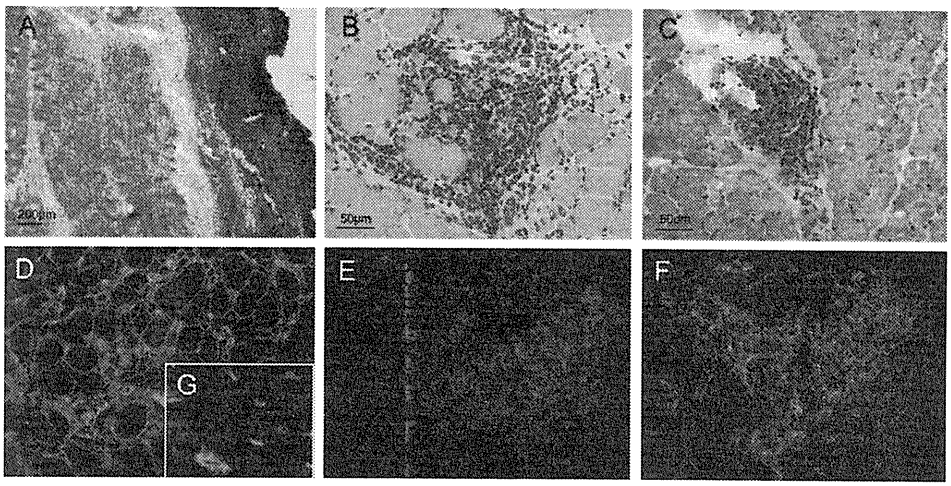

Fig. 1. Inflammatory cellular infiltration observed in the patients with *LMNA* mutations on hematoxilin and eosin staining (A: Patient 1, B: Patient 3, C: Patient 9). Serial frozen sections of muscle from Patient 5 were immunostained with HLA-ABC (D), double immunostained with CD4 (green) and dystrophin (red) (E), and CD20 (green) and dysrophin (red) (F). HLA-ABC stain in control muscle is shown in (G).

National Center of Neurology and Psychiatry. The diagnosis of inflammatory myopathy was based upon the mononuclear cell infiltrations at perimysial, endomysial, and perivascular sites [3]. Patients suspected to have dermatomyositis with skin rash and/or perifascicular atrophy on muscle pathology were excluded in this study. Then we gathered a total of 20 patients including one patient (Patient 2) who had previously been reported as infantile polymyositis [4].

2.2. Histopathological studies

All biopsied samples were taken from biceps brachii. Muscle specimens were frozen in isopentane chilled in liquid nitrogen. Serial frozen sections were stained with hematoxylin and eosin, modified Gomori trichrome, and a battery of histochemical methods. Immunohistochemical analysis was performed as described previously [5]. Antibodies used in this study are: dystrophin (DMDP-II [6], DYS1, DYS2, and DYS3 from Novocastra, Newcastle upon Tyne, UK); sarcoglycans (SGCA, SGCB, SGCG, and SGCD: Novocastra); laminin-α2 chain (ALEXIS, Farmingdale, NY); α-dystroglycan (Upstate Biotech, Lake Placid, NY); caveolin-3 (BD Transduction Laboratories, Franklin Lakes, NJ); dysferlin (Novocastra); emerin (Novocastra); collagen VI (Novocastra); CD4 and CD8 (Nichirei, Tokyo, Japan); CD20, and HLA-ABC (DAKO, Glostrup, Denmark).

2.3. Mutational analysis of LMNA

Genomic DNA was extracted from either frozen muscles or peripheral lymphocytes using standard protocols [7]. All exons and their flanking intronic regions of *LMNA* were amplified by PCR and directly sequenced using automated 3130 sequencer (PE Applied Biosystem, Foster City, CA). Primer sequences are available upon request.

2.4. Clinical information

Clinical characteristics collected from attending physicians were demographic data, age of onset, initial signs, motor functions, presence of cardiac involvement, presence of joint contractures, respiratory function, effectiveness of steroid, and pertinent laboratory examinations including serum creatine kinase (CK), electrocardiogram, Holter electrocardiogram, and echocardiogram.

2.5. Muscle imaging

Muscle computed tomography (CT) or magnetic resonance imaging (MRI) was done with some modifications depending on the facilities in each hospital. Scans were performed at thigh (the largest diameter of thigh) and calf (the largest diameter of lower leg) levels. Involvement of each muscle was evaluated at both scan levels.

3. Results

Ten types of heterozygous single nucleotide substitutions in *LMNA* were identified in 11 of 20 patients. Four (p.Arg249Gln, p.Leu292Pro, p.Asn456His and p.Arg377 Cys) mutations were previously reported in patients with AD-EDMD or LGMD1B, one (p.Arg249Trp) was found only in L-CMD patients, and two (p.Lys32del and p.Glu358Lys) were identified in AD-EDMD, LGMD1B, or L-CMD patients [2,8–10]. Another three (p.Arg28Gln, p.Asn39Asp, p.Arg41Ser,) were novel mutations and not detected in 300 control chromosomes. All 11 patients had neither consanguinity nor family history of myopathy or

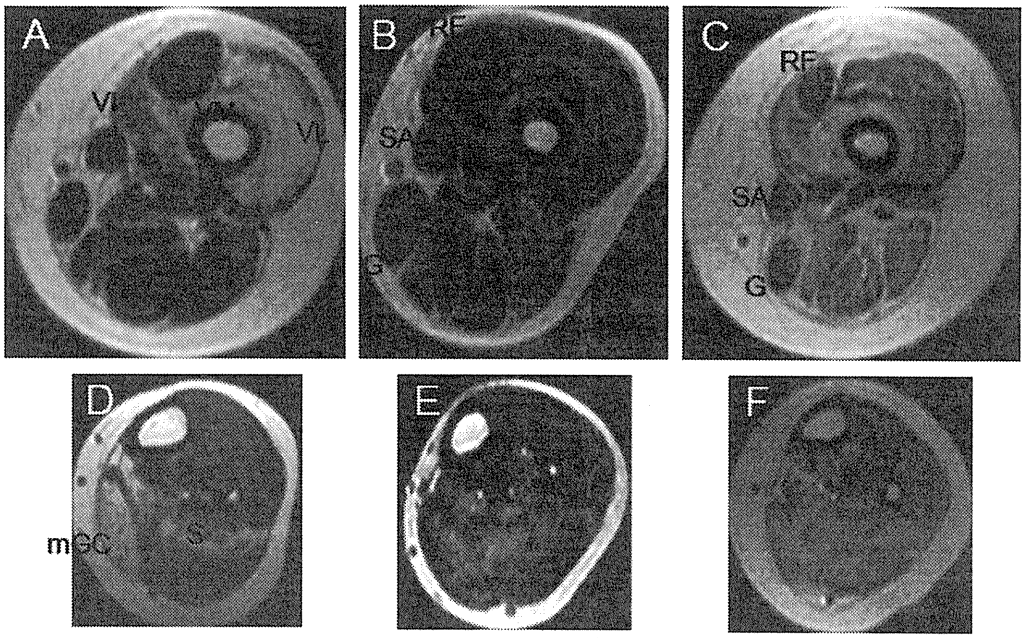

Fig. 2. Selective muscle involvement of thigh and calf muscles. Transverse sections of T1 weighted magnetic resonance imaging of thigh (A–C) and calf (D–F) in patients with *LMNA* mutations. Selective involvements of vastus lateralis (VL), vastus intermedius (VI), vastus medialis (VM), soleus (S), and medial head of gastrocnemius (mGC, A, D: Patient 1), relatively mild and diffuse involvements with relative sparing of rectus femoris (RF), gracilis (G), sartorius (SA, B, E: patient 11), and diffuse and severe involvement with relative sparing of rectus femoris, gracilis, sartorius (C, F: patient 9) are observed.

cardiomyopathy. DNA samples from the parents of 11 patients were not available.

Table 1 shows clinical summary of the 11 patients with *LMNA* mutations. Initial clinical signs were motor developmental delay or progressive muscle weakness. Head drop was not observed in any patient. Serum CK levels were mildly to moderately elevated in all patients. Joint contractures, spinal rigidity, and cardiac involvement were not observed at the time of the biopsy but became prominent in some patients in later age. Importantly, Patient 6 had an episodic paroxysmal supraventricular tachycardia during general anesthesia at age 11 years, and Patient 3 received pacemaker implantation due to complete atrioventricular conduction block at age 15 years. Patient 8 succumbed to sudden death due to arrhythmia at age 24 years and Patient 10 died by cardiac failure at age 7 years. Two patients developed chronic respiratory failure requiring non-invasive positive-pressure ventilation. Patient 2 died by respiratory failure at age 12 years. Steroid was used in eight patients but beneficial effects such as improvement of muscle power and reduction of serum CK levels were seen only in four.

On muscle biopsy, the most striking inflammatory change was observed in Patient 1 showing numerous inflammatory cells predominantly located in the perimysial connective tissue (Fig. 1A). This finding was diffusely seen in the whole muscle specimen. The other 10 patients also showed variable degrees of mononuclear cellular infiltration with active necrosis and regenerating process (Fig. 1B, C, Table 1). Fiber size variation and endomysial fibrosis were also seen. Fiber type grouping, groups of atrophic fibers, and abnormal oxidative stains were not observed. Immunohistochemically, sarcolemmal HLA staining was increased in many fibers in all patients examined (Fig. 1D). Infiltrated mononuclear cells were positive for lymphocyte markers of CD4 (Fig. 1E), CD8 (data not shown), or CD20 (Fig. 1F). No abnormal immunostaining was seen for the antibodies associated with muscular dystrophy (data not shown).

Muscle imaging was performed in relatively later stages of the disease in eight out of 11 patients with *LMNA* mutations (Fig. 2). At the level of thigh, Patient 1 showed selective involvement of vastus lateralis, vastus intermedius and vastus medialis. Patient 6 showed diffuse involvement of all thigh muscles. The remaining six patients showed diffuse involvement of thigh muscles with relative sparing of sartorius, gracilis and rectus femoris. At lower leg levels, three patients (Patients 1, 4, and 7) showed selective involvement of soleus and medial head of gastrocnemius. The remaining four patients showed diffuse involvement of calf muscles.

4. Discussion

In our series, surprisingly, more than half of the infantile patients showing inflammatory changes are due to *LMNA* mutations. Prominent mononuclear cell infiltrations can sometimes be evident in biopsies from muscular dystrophy patients including CMD, LGMD, and facioscapulohumeral muscular dystrophy, leading to misdiagnosis of inflammatory myopathy [11–16]. Apparently, however, frequency of inflammatory changes is much higher in infantile striated muscle laminopathy patients, suggesting a possibil-

ity that *LMNA* mutations may cause active inflammation in skeletal muscle during infancy by a certain mechanism. In support of this notion, three of 15 L-CMD patients report by Quijano-Roy et al. had inflammatory cell infiltration [2]. In Patients 4, 7, 9, 10 and 11, muscle biopsies were done at the age of 2 years or later and inflammatory changes were relatively milder compared to the other earlier biopsies. These findings suggest that severities of inflammation may be related to the age of biopsies.

Inflammatory myopathy manifesting with muscle weakness starting during infancy is a poorly defined muscle disorder and limited number of patients were described in the literature [4,17–20]. Thompson emphasized that responsiveness to corticosteroid is one of the crucial findings that define the infantile myositis [17]. However, this is unlikely to be always the case as some of our laminopathy patients, who were initially diagnosed as infantile-onset inflammatory myopathy also showed some clinical improvement by corticosteroid therapy. Good response to steroids is not only a feature of myositis but can also be seen in other muscular dystrophies including Duchenne muscular dystrophy. Therefore, the possibility of laminopathy should not be excluded solely based upon steroid responsiveness. Interestingly, all steroid-responsive patients were ambulant whereas non-responsive patients could not walk, which might imply some genotype–phenotype correlation. Nonetheless, the correlation between genotype and steroid responsiveness cannot be discussed at this moment as all patients for whom steroid was used had distinct mutations. In any case, corticosteroid therapy could be considered for infantile striated muscle laminopathy patients as some patients respond, although its long-term efficacy is still unknown.

The p.Arg249Trp mutation found in this study was previously reported in L-CMD patients [2], but not in AD-EDMD or LGMD1B. In contrast, p.Glu358Lys mutation has also been reported with extremely variability of phenotypes, including AD-EDMD, LGMD1B, or L-CMD [10]. Thus, the same mutation can result in different phenotypes and severities. These findings raise a possibility that other unknown factor(s) may play a role in the development of laminopathy phenotype.

Muscle imaging demonstrated selective muscle involvement in all eight patients examined. Vastus lateralis and intermedius were markedly affected, while involvement of adductor magnus was minimal. In addition, medial head of the gastrocnemius was remarkably involved while lateral head was relatively spared in most patients. This selective muscle involvement is basically identical to that observed in AD-EDMD/LGMD1B patients [21] and may be helpful for the diagnosis of laminopathy in children.

Cardiomyopathy with conduction defects is a common serious clinical problem in patients with EDMD and LGMD1B [1]. In the present study, 8 of 11 patients developed cardiac complications such as arrhythmia and heart failure in their childhood and two died due to arrhythmia and heart failure, respectively. These findings clearly demonstrate that accurate diagnosis followed by periodic examination of cardiac function including electrocardiogram, holter electrocardiogram and echocardiogram, and appropriate implantation of defibrillators is necessary to avoid unexpected sudden death [22,23].

Our results expand clinical and pathological variation of striated muscle laminopathy and the inflammatory histology is an important diagnostic clue to the *LMNA* related myopathy patients. Further analysis is needed to elucidate the role of mutant A-type lamins in inducing inflammatory process during infancy.

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Heterozygous UDP-GlcNAc 2-epimerase and *N*-acetylmannosamine kinase domain mutations in the *GNE* gene result in a less severe GNE myopathy phenotype compared to homozygous *N*-acetylmannosamine kinase domain mutations

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ABSTRACT

Background: Glucosamine (UDP-N-acetyl)-2-epimerase/*N*-acetylmannosamine kinase (GNE) myopathy, also called distal myopathy with rimmed vacuoles (DMRV) or hereditary inclusion body myopathy (HIBM), is a rare, progressive autosomal recessive disorder caused by mutations in the *GNE* gene. Here, we examined the relationship between genotype and clinical phenotype in participants with GNE myopathy.
Methods: Participants with GNE myopathy were asked to complete a questionnaire regarding medical history and current symptoms.
Results: A total of 71 participants with genetically confirmed GNE myopathy (27 males and 44 females; mean age, 43.1 ± 13.0 (mean ± SD) years) completed the questionnaire. Initial symptoms (*e.g.*, foot drop and lower limb weakness) appeared at a mean age of 24.8 ± 8.3 years. Among the 71 participants, 11 (15.5%) had the ability to walk, with a median time to loss of ambulation of 17.0 ± 2.1 years after disease onset. Participants with a homozygous mutation (p.V572L) in the *N*-acetylmannosamine kinase domain (KD/KD participants) had an earlier disease onset compared to compound heterozygous participants with mutations in the uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) 2-epimerase and *N*-acetylmannosamine kinase domains (ED/KD participants; 26.3 ± 7.3 vs. 21.2 ± 11.1 years, respectively). KD/KD participants were more frequently non-ambulatory compared to ED/KD participants at the time of survey (80% vs. 50%). Data were verified using medical records available from 17 outpatient participants.
Conclusions: Homozygous KD/KD participants exhibited a more severe phenotype compared to heterozygous ED/KD participants.

1. Introduction

Glucosamine (UDP-N-acetyl)-2-epimerase/*N*-acetylmannosamine kinase (GNE) myopathy, also known as distal myopathy with rimmed vacuoles (DMRV), Nonaka myopathy (MIM: 605820) or hereditary inclusion body myopathy (HIBM; MIM: 600737), is an early adult-onset, progressive myopathy that affects the tibialis anterior muscle, but spares quadriceps femoris muscles [1,2]. The disease is caused by a mutation in the *GNE* gene, which encodes a bifunctional enzyme [uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) 2-epimerase (GNE) and *N*-acetylmannosamine kinase (MNK)] known to catalyze two rate-limiting reactions involved in cytosolic sialic acid synthesis [3–7]. Mutations in the *GNE* gene result in decreased enzymatic activity *in vitro* by 30–90% [7–10]. Therefore, hyposialylation is thought to

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contribute to the pathogenesis of GNE myopathy. This is supported by the myopathic phenotype associated with a mouse model expressing the human D176V mutant GNE protein (GNE−/−hGNED176V-Tg) [11]. Muscle atrophy and weakness are prevented by oral treatment with sialic acid metabolites in this mouse model [12].

A phase I clinical trial using oral sialic acid therapy has recently been performed in Japan for the treatment of GNE myopathy (ClinicalTrials.gov; NCT01236898). A similar phase I study is currently underway in the United States (ClinicalTrials.gov; NCT01359319). Natural history and genotype–phenotype correlations need to be established for a successful phase II clinical trial for the treatment of GNE myopathy. However, only a small number of studies have been conducted that review the natural course of this disease. In addition, the presence of genotype–phenotype correlations is controversial in GNE myopathy, with most reports denying significant correlations [7]. In fact, substantial heterogeneity is observed among participants who have the same mutations. For example, few subjects with p.D176V and p.M712T mutations exhibited a normal or very mild phenotype, with disease onset after the age of 60 [3,13]. Furthermore, only a limited number of studies that analyze compound heterozygous patients are available. Nonetheless, such studies report a variable degree of severity [14–17].

To clarify the potential relationship between genotype and clinical phenotype (*i.e.*, age at onset, disease course, and current symptoms) of GNE myopathy, we performed a questionnaire-based survey of participants with confirmed GNE myopathy.

2. Participants and methods

2.1. Study population

We obtained approval for this study from the Medical Ethics Committee of the National Center of Neurology and Psychiatry (NCNP). Seventy-eight participants with known GNE myopathy were seen at 8 hospitals specializing in muscle disorders in Japan and 83 participants (not all genetically diagnosed) from the Participants Association for Distal Myopathies (PADM) were recruited. Participants provided written informed consent prior to completing the questionnaire.

A total of 75 participants completed and returned the questionnaire. Of the 75 participants analyzed, 4 were found to have only one heterozygous mutation. Because single heterozygous mutations have not been confirmed to cause GNE myopathy, these 4 participants were excluded from this study.

2.2. Study design

The present study is a retrospective and cross-sectional analysis, which includes 71 participants with genetically confirmed GNE myopathy. Clinical information was collected from participants using a questionnaire and genetic information was acquired from available medical records.

2.3. Questionnaire

Participants completed a self-reporting questionnaire regarding 1) developmental and past symptoms, 2) past and present ambulatory status, and 3) information about diagnosis and medical services (Supplementary material, original version in Japanese).

To determine developmental history, we collected the following information: 1) trouble before and/or during delivery, 2) body weight and height at birth, 3) age at first gait, 4) exercise performance during nursery, kindergarten, or school, and 5) age at onset and signs of first symptoms. Participants were also asked about the onset of 1) gait disturbance, 2) walking with assistance (*i.e.*, cane and/or orthotics and/or handrails), 3) wheelchair use, 4) loss of ambulation, and 5) current gait performance. With regard to medical history, participants were asked about 1) age at the time of first hospital visit, 2) whether or not they had symptoms at the time of visit, 3) age at the time of final diagnosis, 4) how many hospitals/clinics were visited before final diagnosis, and 5) whether a biopsy was performed.

2.4. Medical record examination

To verify the accuracy of the information provided by each participant, available medical records from 17 participants (23.9%) seen at outpatient clinics at NCNP were examined (9 males and 8 females).

2.5. Data handling and analysis

All variables were summarized using descriptive statistics, which included mean, standard deviation (SD), median, range, frequency, and percentage. Each variable was compared against age, sex, genotype, and domain mutation (*i.e.*, within the UDP-GlcNAc 2-epimerase domain: ED or *N*-acetylmannosamine kinase domain: KD). Student's *t* test was used to compare the means for each participant group (ED/ED, ED/KD and KD/KD participants). Data from the two participant groups were calculated using chi-square contingency table analysis. The time from disease onset to walking with assistance, time from disease onset to wheelchair use, and time from disease onset to loss of ambulation were evaluated using the Kaplan–Meier method with log-rank analysis. Questionnaire reliability was tested using intraclass correlation coefficients (ICCs), and two-sided 95% confidence intervals (CIs) were calculated using a one-way random effects analysis of variance model for inter-rater reliability. All analyses were performed using SPSS for Macintosh (version 18, SPSS Inc., Chicago, IL).

3. Results

3.1. General characteristics

A total of 71 Japanese individuals (27 males and 44 females) participated in the study. The mean age at data collection was 43.1 ± 10.7 years. None of the participants showed developmental abnormalities during infancy or early childhood.

3.2. GNE mutations

Forty-one percent of study participants (n = 29/71) had homozygous mutations, while 59% (n = 42/71) had compound heterozygous mutations (Table 1). Among homozygous participants, 86.2% (n = 25/29) harbored the p.V572L mutation, while the remaining participants had other mutations. No homozygous participants for the p.D176V mutation were identified. Among compound heterozygous participants, 28.5% (n = 12/42) had p.D176V/p.V572L mutations, while the remaining participants had other mutations. With respect to allelic frequency, 50.0% (71/142) were p.V572L, 20.4% (29/142) p.D176V, 3.5% (5/142) p.C13S, 2.8% (4/142) p.M712T, and 2.1% (3/142) p.A630T. All other mutations accounted for 2%. A total of 18.3% (n = 13/71) of participants were homozygous with a mutation in the GNE domain (ED/ED), 39.4% (n = 28/71) of participants were compound heterozygous with a mutation in the GNE domain and one in the MNK domain (ED/KD), and 42.3% (n = 30/71) of participants had a mutation in the MNK domain in both alleles (KD/KD).

3.3. Past and present symptoms

Mean participant age at symptom onset was 25.2 ± 9.2 years (range, 12–58 years; median, 24.5 years). There was no significant difference between males and females for current age, age at disease

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Table 1
Genotypes of the GNE myopathy patient population.

		Questionnaire	Outpatients
ED/ED	Total	13	4
	Homozygote	1	0
	p.C13S homozygote	1	
	Compound heterozygote	12	4
	p.C13S/p.M29T	1	1
	p.C13S/p.A63I	1	1
	p.D176V/p.F233S	1	1
	p.D176V/p.R306Q	2	
	p.R129Q/p.D176V	1	
	p.R129Q/p.R277C	1	
	p.D27L/p.D176V	1	1
	p.B89S/p.D176V	1	
	p.D176V/p.R246W	1	
	p.D176V/p.R321C	1	
	p.D176V/p.V331A	1	
ED/KD	Total	28	8
	Compound heterozygote	28	8
	p.D176V/p.V572L	12	3
	p.C13S/p.V572L	1	1
	p.D176V/p.I472T	1	1
	p.D176V/p.L603F	1	1
	p.R177C/p.V572L	1	1
	383insT/p.V572L	1	1
	p.D176V/p.G708S	2	
	p.D187G/p.V572L	2	
	p.R8X/p.V572L	1	
	p.D176V/p.G568S	1	
	p.D176V/p.H626R	1	
	p.D176V/p.A630T	1	
	p.I276T/p.V572L	1	
	p.G295D/p.A631V	1	
	p.A600E/p.D176V	1	
KD/KD	Total	30	5
	Homozygote	28	5
	p.V572L homozygote	25	4
	p.M712T homozygote	2	
	p.A630T homozygote	1	
	Compound heterozygote	2	0
	p.V572L/p.R420X	1	1
	1756Gdel (stop)/p.V572L	1	

onset, age at walking with assistance, age at wheelchair use, and current ambulatory status. Initial symptoms included gait disturbance (66.2%, n = 47/71), other lower limb symptoms (26.8%, n = 19/71), easily fatigued (23.9%, n = 17/71), and weakness of hands and fingers (8.5%, n = 6/71). In addition, 21.1% (n = 15/71) had onset of symptoms before the age of 20. When specifically asked, 47.8% (n = 34/71) described themselves as slow runners during childhood, and 42.5% reported having had difficulty with physical exercise during school years.

3.4. Diagnosis

Mean participant age at diagnosis was 33.9 ± 12.6 years (median, 29.5 years; range 17 to 67 years). Mean participant age at first physician visit was 29.6 ± 10.4 years (median, 27 years; range, 12–62 years), and mean time between first visit and diagnosis was 4.4 ± 8.3 years.

3.5. Walking with assistance and wheelchair use

At the time of the survey, 52.0% (n = 37/71) were ambulant (41.3 ± 12.8 years); however, only 15.5% (n = 11/71, 40.0 ± 13.6 years) could walk without assistance, with the remaining 35.2% requiring assistance (n = 25/71, 41.8 ± 12.7 years). Only 7.0% of these participants (n = 5/71) could walk up stairs, while 49.3% (n = 35/71) were non-ambulant. Wheelchairs were used by 63.6% (23.9% partially bound and 43.7% totally bound) and an electric wheelchair was used by 41.9% (n = 31/71). Mean participant age of wheelchair users was 34.9 ± 11.7 years (range, 18–70 years). Wheelchairs were not used by 32.4% (n = 26/71) of participants. Current age of wheelchair-free participants was 39.4 ± 12.3 years (range, 21–61 years; median, 34 years) and that of wheelchair-bound participants was 42.8 ± 12.6 years (range, 21–71; median, 42 years).

Kaplan–Meier analysis revealed a median proportional age at walking with assistance of 30.0 ± 1.4 years. Median proportional age of wheelchair users was 36.0 ± 2.7 years, and that for loss of ambulation was 45.0 ± 4.2 years. The time from disease onset to walking with assistance was 7.0 ± 0.4 years, time from disease onset to wheelchair use was 11.5 ± 1.2 years, and time from disease onset to loss of ambulation was 17.0 ± 2.1 years.

3.6. Correlation between disease genotype and phenotype

To determine if a correlation between genotype and phenotype existed, we compared domain mutations (ED/KD, or both) available from medical reports to questionnaire answers (Table 2). Participants with KD/KD mutations (both homozygous and heterozygous) were younger and more severely affected compared to participants with ED/KD or ED/ED mutations. No significant difference in current age or age at disease onset between ED/ED and ED/KD participants was identified. Kaplan–Meier analyses revealed that the proportional time from disease onset to wheelchair use and from disease onset to loss of ambulation was significantly shorter in KD/KD compared to ED/KD participants. ED/ED participants exhibited a shorter time of disease onset to wheelchair use compared to ED/KD participants (Table 3, Fig. 1).

3.7. Comparison between p.V572L homozygous and p.D176V/p.V572L compound heterozygous participants

To compare clinical features in patients with the same mutations, we specifically analyzed data from those with p.V572L (n = 25/71, 35.2%) and p.D176V/p.V572L (n = 12/71, 16.9%) mutations, as these two were the most frequent mutations in our study population (Table 2). Age at disease onset of homozygous participants (p.V572L) was 21.3 ± 5.7 years (range, 12–32 years) and time from disease onset to wheelchair use was 11.3 ± 5.4 years (range, 3–21 years). Only 16.0% (n = 4/25) of these homozygous participants reported that they were not currently using a wheelchair. In contrast, the mean age at disease onset of heterozygous participants (p.D176V/p.V572L) was 35.5 ± 14.1 years (range, 13.5–57 years) and time from disease onset to wheelchair use was 17.9 ± 7.0 years (range, 11–28 years). A total of 66.7% of these compound heterozygous participants (n = 8/12) reported that they were not using a wheelchair.

3.8. Questionnaire response compared to medical records

Questionnaires from 17 participants (NCNP outpatient participants) were compared to available medical records (Table 2). Age at disease onset, age at onset of gait disturbance, age at walking with assistance, and age at loss of ambulation were assessed for inter-rater reliability. Age at disease onset, age at onset of gait disturbance, age at walking with assistance, and age at loss of ambulation were assessed for inter-rater reliability. ICC values were 0.979 (95% CI 0.941–0.992) for age at disease onset, 0.917 (95% CI 0.752–0.972) for age at onset of gait disturbances, 0.985 (95% CI 0.949–0.995) for age at walking with assistance, and 0.967 (95% CI 0.855–0.993) for age at loss of ambulation.

4. Discussion

The present study provides a detailed overview of disease severity and progression in 71 Japanese participants with genetically confirmed GNE myopathy. Questionnaire-based surveys have been used to study

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Table 2
Comparison of disease course among genotypes.

		Total	ED/ED	ED/LD	KD/KD
Questionnaire	*n*	71	13	28	30
	Age (years old)	43.1 ± 10.7	44.2 ± 11.2	45.3 ± 13.4	40.6 ± 13.0
	Age at onset (years old)	25.5 ± 9.2	26.3 ± 7.3^{+}	29.8 ± 11.0*	21.2 ± 5.5$^{*,+}$
	Age at walking with assistance	31.8 ± 10.0	34.0 ± 11.1	35.6 ± 10.9*	27.8 ± 6.8*
	Duration from onset to walking with assistance	8.4 ± 6.5	7.5 ± 7.3	9.2 ± 6.5	8.0 ± 6.6
	Wheelchair user (%)	48 (67.8)	10(76.9)	14 (50.0)*	24 (80.0)*
	Wheelchair use since (age)	37.6 ± 8.6	36.4 ± 12.0	43.0 ± 8.7*	31.2 ± 9.3*
	Number of patients with lost ambulation	35 (49.8)	6(46.2)	8 (28.6)*	21 (70.0)*
	Age at lost ambulation	33.6 ± 9.2	31.2 ± 6.0	39.7 ± 9.5	32.1 ± 9.3
	Duration from onset to loss of ambulation	12.2 ± 5.2	9.8 ± 3.5	13.8 ± 6.4	12.4 ± 5.1
NCNP outpatients	*n*	17	4	8	5
	Age (years old)	43.9 ± 14.1	53.5 ± 8.9^{+}	44.3 ± 16.3	35.6 ± 9.2^{+}
	Age at onset (years old)	25.8 ± 9.2	33.4 ± 9.2^{+}	29.6 ± 13.5	19.6 ± 4.2^{+}
	Duration from onset to walking with assistance	7.5 ± 4.2	8.9 ± 5.1	8.1 ± 4.7	5.2 ± 1.5
	Wheelchair user (%)	12 (70.6)	3 (75.0)	4 (50.0)	4 (100)
	Wheelchair use since (age)	33.3 ± 12.6	47.5 ± 17.7	35.2 ± 12.4	25.8 ± 6.3
	Number of patients with lost ambulation	9 (52.9)	3 (75.0)	3 (28.6)*	5 (100)*
	Age at lost ambulation	33.8 ± 9.3	40.0 ± 0.0	39.0 ± 16.5	31.0 ± 8.2
	Duration from onset to loss of ambulation	10.7 ± 4.2	11.2 ± 5.6	11.1 ± 7.8	6.2 ± 2.6

In the questionnaire group, age at onset and age at walking with assistance were significantly younger in KD/KD patients than in ED/KD patients. The number of wheelchair users and patients with loss of ambulation was significantly higher in the KD/KD group than in the ED/KD group. In contrast, with the exception of age at onset, there were no significant differences between ED/ED and ED/KD or KD/KD patients in these clinical parameters. The ED/ED patients were older than the others, and KD/KD patients tended to show the fastest progression.

* $p<0.05$ between ED/KD and KD/KD.

$^{+}$ $p<0.05$ between ED/ED and KD/KD.

the natural disease course of other rare neuromuscular disorders, such as Pompe disease [18] and spinal muscular atrophy type-1 [19]. It is difficult to establish the natural history of such rare disorders using medical records only because patients are typically seen in many different hospitals. In the present study, we used a self-reporting questionnaire and support its use for complementing medical records because it provides a more complete disease overview and establishes specific clinical trends or correlations. Indeed, our questionnaire demonstrates excellent inter-rater reliability against medical records and yields several findings regarding differences in disease progression among genetically distinct, GNE myopathy participants.

Only 15.5% of participants could walk and 7.0% could walk up stairs without assistance, which reflects the fact that GNE myopathy patients often require canes and/or leg braces at an early disease stage. This indicates that traditional six-minute walk or four-step walking tests often used to evaluate muscular dystrophies or myopathies can only be applied in a very limited number of cases, such as natural disease course studies or clinical trials. Therefore, alternate evaluation tools are required, which should include functional measurements that can be completed without canes or braces. For example, the Gross Motor Function Measure is a useful tool for evaluating mildly and severely affected patients [20].

The male to female ratio in our study population (27 males and 44 females) was skewed from the expected ratio for autosomal recessive inheritance. However, the male to female ratio of the 17 NCNP outpatient participants was 9:8. One possible explanation for the observed sex ratio in our study population is that female participants tend to be more enthusiastic toward questionnaire-based and/or PADM activities. There was no significant difference in age at survey and age at disease onset between male and female participants. However, in a mouse model of GNE myopathy, weight loss and muscle atrophy were more pronounced and occurred earlier in females compared to males [11].

We showed that KD/KD mutations are associated with a more severe phenotype compared to ED/KD mutations. Indeed, KD/KD participants had an earlier disease onset, a more rapid and progressive disease course, and a shorter time from disease onset to loss of ambulation. This was also observed in the 17 NCNP outpatient participants analyzed in our study. In contrast, ED/ED participants did not show significant differences across disease course parameters analyzed except for an earlier and later age at disease onset compared to ED/KD and KD/KD participants, respectively. Thus, ED/ED participants appear to have a disease severity intermediate between ED/KD and KD/KD participants. One possible explanation is that the major mutation, p.V572L, may be associated with a more severe phenotype. In general, the reasons for this earlier onset and disease progression remain unknown. Jewish GNE myopathy patients with homozygous p.M712T mutations have a milder phenotype compared to Japanese patients, as most of their quadriceps are spared and they usually become wheelchair-bound 15 years or more after disease onset [13,21]. Our study population included two women with homozygous p.M712T mutations: a 38 year-old ambulant and a 35 year-old non-ambulant participant. Although the two participants had a slightly later disease onset (ages 23 and 27 years, respectively) compared to KD/KD participants, the difference was not significant.

An asymptomatic patient with a p.D176V homozygous mutation was previously reported [3]. The study suggested that p.D176V homozygous patients may show a mild or late disease onset phenotype. The results presented here may support this observation as no p.D176V homozygous participants were present in our study

Table 3
Inter-rater reliability of the questionnaire.

	Onset	Age of gait disturbance	Age of gait with help	Age at loss of ambulant
Number of patients	17	17	13	9
ICC (95% CI)	0.979 (0.941–0.992)	0.917 (0.752–0.972)	0.985 (0.949–0.995)	0.967 (0.855–0.993)
p	0.000	0.000	0.000	0.000

Age at onset, age at onset of gait disturbances, age at walking with assistance, and age at loss of ambulation were assessed in a subgroup of 17 outpatients to evaluate the inter-rater reliability of the questionnaire.

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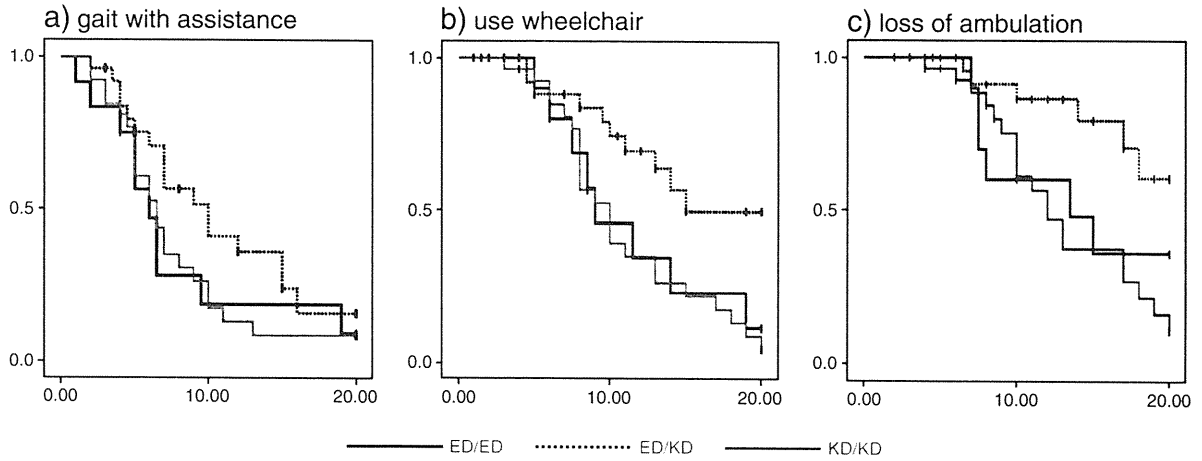

Fig. 1. Kaplan–Meier analysis of time from disease onset to (a) walking with assistance, (b) wheelchair use, and (c) loss of ambulation. Significant differences between ED/KD and KD/KD genotypes were identified. Age at disease onset was significantly different between ED/ED participants and ED/KD and KD/KD participants.

population, although p.D176V was the second most common mutation carried by 29 of our participants. In addition, a high variability was observed regarding age at disease onset and disease progression, underscoring the role of a yet-to-be identified factor(s) in determining disease phenotype.

The recruitment of participants from PADM and highly specialized neurology hospitals is a potential source of selection bias and thus a limitation of this study. These participants are likely to be more motivated because they are more severely affected compared to the general patient population. Furthermore, patients with lower disease severity may not yet be diagnosed with GNE myopathy. Therefore, our study may not accurately reflect the general patient population. Nevertheless, we believe our findings provide important information as our study population covers a broad range in age (22 to 81 years) and symptoms (minimal to wheelchair-bound). Finally, recall bias may also affect results presented in this retrospective study. Therefore, future studies should be performed with an emphasized prospective design.

In conclusion, our study shows that the KD/KD genotype (*i.e.*, p.V572L homozygous mutation) is associated with a more severe phenotype compared to compound heterozygous ED/KD mutations. Because only a small number of participants could walk, future studies should include ambulation-independent motor tests to yield a more comprehensive clinical overview in GNE myopathy patients with different genotypes.

Supplementary data to this article can be found online at doi:10.1016/j.jns.2012.03.016.

Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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A Congenital Muscular Dystrophy with Mitochondrial Structural Abnormalities Caused by Defective De Novo Phosphatidylcholine Biosynthesis

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Congenital muscular dystrophy is a heterogeneous group of inherited muscle diseases characterized clinically by muscle weakness and hypotonia in early infancy. A number of genes harboring causative mutations have been identified, but several cases of congenital muscular dystrophy remain molecularly unresolved. We examined 15 individuals with a congenital muscular dystrophy characterized by early-onset muscle wasting, mental retardation, and peculiar enlarged mitochondria that are prevalent toward the periphery of the fibers but are sparse in the center on muscle biopsy, and we have identified homozygous or compound heterozygous mutations in the gene encoding choline kinase beta (*CHKB*). This is the first enzymatic step in a biosynthetic pathway for phosphatidylcholine, the most abundant phospholipid in eukaryotes. In muscle of three affected individuals with nonsense mutations, choline kinase activities were undetectable, and phosphatidylcholine levels were decreased. We identified the human disease caused by disruption of a phospholipid de novo biosynthetic pathway, demonstrating the pivotal role of phosphatidylcholine in muscle and brain.

A spontaneous mutant mouse with a neonatal-onset autosomal-recessive rostral-to-caudal muscular dystrophy (*rmd* mouse) due to a loss-of-function mutation in choline kinase beta (*Chkb*) was identified in 2006.[1] Interestingly, *rmd* mice exhibit a unique mitochondrial morphology in muscle fibers, which show enlarged mitochondria at the periphery of the fiber but none at the center (Figure S1). These features are similar to those seen in a congenital muscular dystrophy (CMD) that we previously reported in four Japanese individuals.[2] We therefore screened 15 genetically undiagnosed cases of CMD with fairly homogenous clinical features (Table 1) for mutations in choline kinase beta (*CHKB*); we included the four cases from in our previous study in these 15 cases. Features included peculiar mitochondrial changes in muscle as well as motor delay followed by the appearance of severe mental retardation and microcephaly without structural brain abnormalities (Figure 1 and Table 1).

All clinical materials used in this study were obtained for diagnostic purposes with written informed consent. The study was approved by the Ethical Committee of the National Center of Neurology and Psychiatry. All mouse protocols were approved by the Ethical Review Committee on the Care and Use of Rodents in the National Institute of Neuroscience, National Center of Neurology and Psychiatry. For muscle pathology, samples of skeletal muscle were obtained from biceps brachii or quadriceps femoris in humans and from quadriceps femoris muscle in 8-week-old *rmd* mice. Muscles were frozen and sectioned at a thickness of 10 μm according to standard procedures, and a battery of routine histochemical stains, including hematoxylin and eosin (H&E), modified Gomori trichrome (mGT), NADH-tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH), cytochrome c oxidase (COX), and Oil Red O, were analyzed. For electron microscopic analysis, muscles were fixed as previously described,[3] and ultra-thin sections were observed at 120kV or 80kV. All affected individuals exhibited nonspecific dystrophic features (Figure 1A). However, in mGT, NADH-TR, SDH, and COX staining, prominent mitochondria at the periphery as well as central areas devoid of mitochondria were seen (Figures 1B and 1C). Oil Red O staining was unremarkable (data not shown). Electron microscopy confirmed enlarged mitochondria (Figure 1D).

We directly sequenced all exons and their flanking intronic regions in *CHKB* (MIM 612395, NM_005198.4, GenBank Gene ID 1120) in genomic DNA extracted from individuals' peripheral lymphocytes. All 15 individuals in three different populations (Japanese, Turkish, and British) had homozygous or compound heterozygous mutations in

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Table 1. Summary of Clinical and Laboratory Features

			Phenotypic Findings									Muscle Pathology					Mutations				
Individual	Sex	Origin	Age at Last Follow-Up	Floppy at Birth	Walk Alone	Serum Creatine Kinase (IU/liter)	Head Circumference (percentile)	Mental Retardation	Seizure	Cardiomyopathy	Skin Change	Age at Muscle Biopsy	Necrotic Fiber	Regenerative Fiber	Endomysial Fibrosis	Mitochondrial Enlargement	Status	cDNA	Consequence	Exon	Literature ref. on phenotype
1	F	Japanese	died at 13 yr	+	2 yr 6 mo	370	ND	+	-	+	-	7 yr3 mo	+	+	+	+	homo	c.810T>A	p.Tyr270X	7	2
2	M	Japanese	died at 23 yr	+	1 yr 9 mo	190–2676	25–50	+	+	+	-	1 yr 2 mo	+	+	+	+	homo	c.810T>A	p.Tyr270X	7	2
3	F	Japanese	28 yr	+	1 yr 6 mo	502	ND	+	+	+	-	8 yr	+	+	+	+	het	c.116C>A	p.Ser39X	1	2
																	het	c.458dup	p.Leu153PhefsX57	3	2
4	M	Japanese	22 yr	+	2 yr 6 mo	230	3–10	+	+	-	-	4 yr 11 mo	+	+	+	+	het	c.116C>A	p.Ser39X	1	
																	het	c.458dup	p.Leu153PhefsX57	3	
5	M	Turkish	7 yr	-	2 yr 6 mo	843	<3	+	-	-	+	6 yr	±	+	+	+	homo	c.611_612insC	p.Thr205AsnfsX5	5	
6[a]	M	Turkish	died at 2 yr 6 mo	+	no	258	<3	+	-	+	-	1 yr 3 mo	±	±	+	+	homo	c.922C>T	p.Gln308X	8	
7	F	Turkish	2 yr	-	no	368	3-10	+	-	-[b]	-	9 mo	-	±	+	+	homo	c.847G>A	p.Glu283Lys	8	
8	M	Turkish	13 yr	ND	2 yr	1122	ND	+	-	-	-	12 yr 10 mo	±	±	+	+	homo	c.1130 G>T	p.Arg377Leu	11	
9	F	Turkish	17 yr	+	3 yr	2669	<3	+	-	ND	-	17 yr	±	±	+	+	homo	c.554_562del	p.Pro185_Trp187del	4	
10	F	Turkish	16 yr	+	3 yr	1103	<3	+	-	-[c]	+	3 yr	-	±	+	+	homo	c.677+1G>A	ND	5	
11	F	Turkish	3 yr 3 mo	+	no	497	10-25	+	-	ND	-	3 yr	±	-	+	+	homo	c.677+1G>A	ND	5	
12	F	Turkish	5 yr	-	3 yr 6 mo	467	25–50	+	-	-[d]	+	4 yr 6 mo	±	+	+	+	homo	c.677+1G>A	ND	5	
13	M	Turkish	3 yr 6 mo	+	no	428	<3	+	-	+	+	3 yr	+	+	+	+	homo	c.1031+1G>A	aberrant splicing	9	
14	F	Turkish	6 yr 4 mo	-	1 yr 3 mo	1606	3–10	+	-	+	-	4 yr	+	+	+	+	homo	c.1031+1G>A	ND	9	
15	M	British	died at 8 yr	-	3 yr 4 mo	607–1715	<3	+	-	+	+	2 yr 2 mo	+	-	+	+	homo	c.852_859del	p.Trp284X	8	

Detailed clinical information for individual 1 to 4 was previously described (2). Eleven CHKB mutations were identified in 15 affected individuals. All exhibited generalized muscle hypotonia and weakness from early infancy. Ambulation was delayed, and gait in those who achieved walking was limited. In addition, all displayed marked mental retardation, and most never acquired meaningful language. Microcephaly with head circumferences at or below the 3rd to 10th percentile was observed in most cases. Cranial magnetic resonance imaging showed no developmental brain defects. Six individuals had dilated cardiomyopathy, and two had cardiac anomaly. Individuals 1, 2, 6, and 15 died from cardiomyopathy at ages 13 yr, 23 yr, 2 yr 6 mo, and 8 yr, respectively. No one had respiratory insufficiency. Ichthyosiform skin changes were frequent. All showed mildly to moderately elevated serum creatine kinase (CK) levels. Individuals 7 and 9 also had homozygous single-nucleotide substitutions, c.902C>T (p.Thr301Ile) and c.983A>G (p.Gln328Arg), respectively. CHK activities of recombinant CHK-β proteins with p.Thr301Ile and p.Gln328Arg were only mildly decreased (Figure S2), suggesting these are likely to be neutral polymorphisms or only mildly hypomorphic mutations. Individuals 10, 11, and 12, who have same c.677+1G>A mutation, and individuals 13 and 14, who have same c.1031+1G>A mutation, are not siblings. Abbreviations are as follows: ND, not determined; p, percentile; F, female; and M, male.

[a] An affected sibling had ichthyosis and died at age 6 years with cardiomyopathy.
[b] Patent ductus arteriosus.
[c] Atrial septal defect.
[d] Mitral valve prolapse.

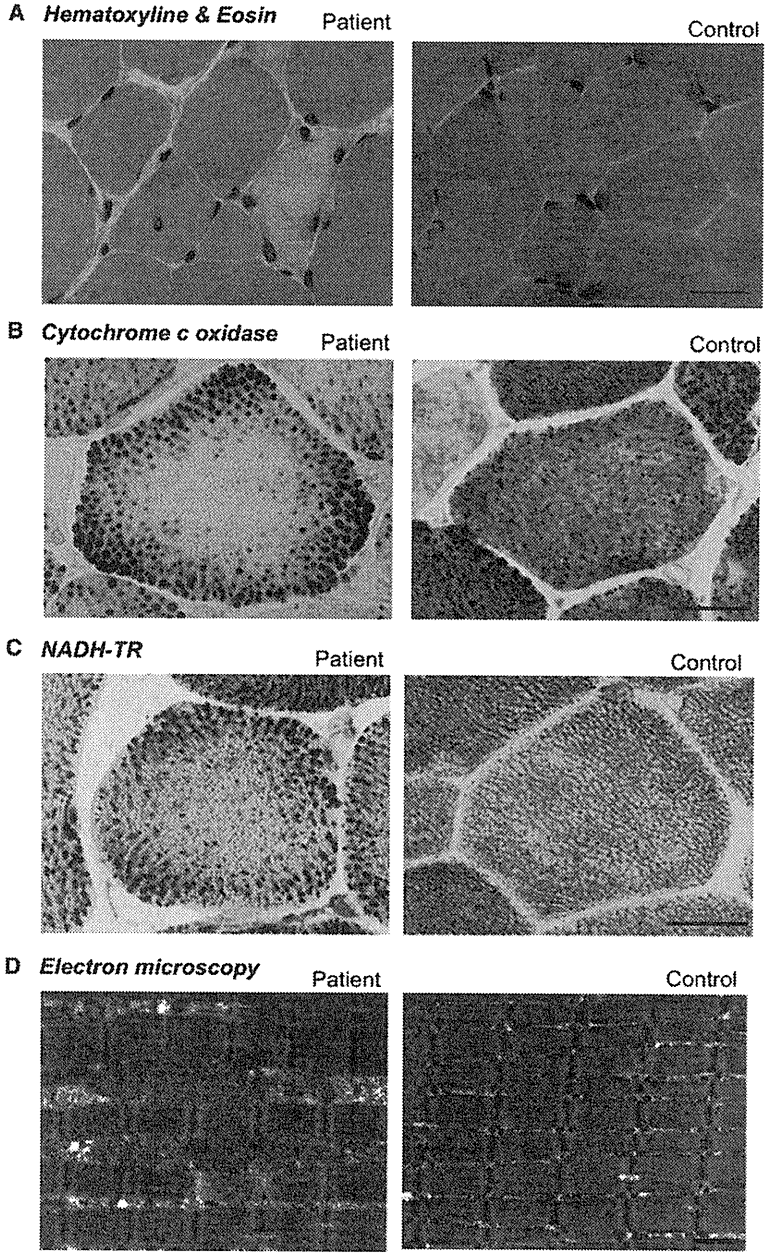

Figure 1. Muscle Pathology of the Affected Individuals

Cross-sections of muscle fiber from a human control and individual 4.

(A) On H&E staining, nonspecific dystrophic features with necrotic and regenerating fibers, internalized nuclei, and endomysial fibrosis are seen. The scale bar represents 25 μm.

(B) On cytochrome c oxidase staining, enlarged mitochondria at the periphery and central areas devoid of mitochondria were seen. The scale bar represents 20 μm.

(C) On NADH-TR staining, the intermyofibrillar network was preserved even in the central areas that are devoid of mitochondria, suggesting the presence of myofibrils and only absence of mitochondria. The scale bar represents 20 μm.

(D) Electron microscopy confirmed enlarged mitochondria. The scale bar represents 1 μm.

CHKB (Table 1). Among a total of 11 mutations identified, six were nonsense, two were missense, one was a 3 amino acid deletion, and two were splice-site mutations. The six nonsense mutations, c.116C>A (p.Ser39X), c.458dup (p.Leu153PhefsX57), c.611_612insC (p.Thr205AsnfsX5), c.810T>A (p.Tyr270X), c.852_859del (p.Trp284X), and c.922C>T (p.Gln308X), were predicted to truncate the protein and eliminate highly conserved domains of CHK.[4,5] Individuals 1 and 2 (unrelated, Japanese) had the same homozygous nonsense mutation of c.810T>A (p.Tyr270X). Individual 2's mother, who was healthy, had the heterozygous c.810T>A (p.Tyr270X) mutation. Unfortunately, a DNA sample from the father of individual 2 was not available. DNA samples from other family members of individual 1 and 2 were not available. Individuals 3 and 4 (siblings, Japanese) had the same compound heterozygous mutation c.116C>A (p.Ser39X) and c.458dup (p.Leu153PhefsX57). Both parents were healthy, and the father was heterozygous for mutation c.116C>A (p.Ser39X), whereas the mother was heterozygous for mutation c. 458dup (p.Leu153-PhefsX57), thus confirming a recessive inheritance pattern. These mutations cosegregated with the disease phenotype in all family members tested.

We therefore measured CHK activity in biopsied muscle. For all biochemical analyses, because of the limiting amounts of remaining tissue, biopsied muscle samples were available only from individuals 2, 3, and 4. Biopsied muscle samples from these three individuals were homogenized in 3 volumes of 20 mM Tris-HCl (pH 7.5), 154 mM KCl, and 1 mM phenylmethanesulfonyl fluoride with a sonicator (MISONIX), and supernatant fractions (105,000 × g, 60 min) were prepared and analyzed for CHK activity as previously described.[6] Similar to muscles of *rmd* mice,[1] muscles from individuals 2, 3, and 4, who carried homozygous or compound heterozygous nonsense mutations, did not have any detectable CHK activity (Figure 2A). Individuals 7, 8, and 9 had homozygous missense mutations c.847G>A (p.Glu283Lys) and c.1130 G>T (p.Arg377Leu) and a homozygous 3 amino acid deletion, c.554_562 del (p.Pro185_Trp187del), respectively.We screened 210 control chromosomes for the identified missense mutations and small in-frame deletion by direct sequencing or single-strand conformation polymorphism (SSCP) analysis. SSCP was performed with Gene Gel Excel (GE Healthcare) as previously described.[7] These missense mutations and this small in-frame deletion were not identified in control

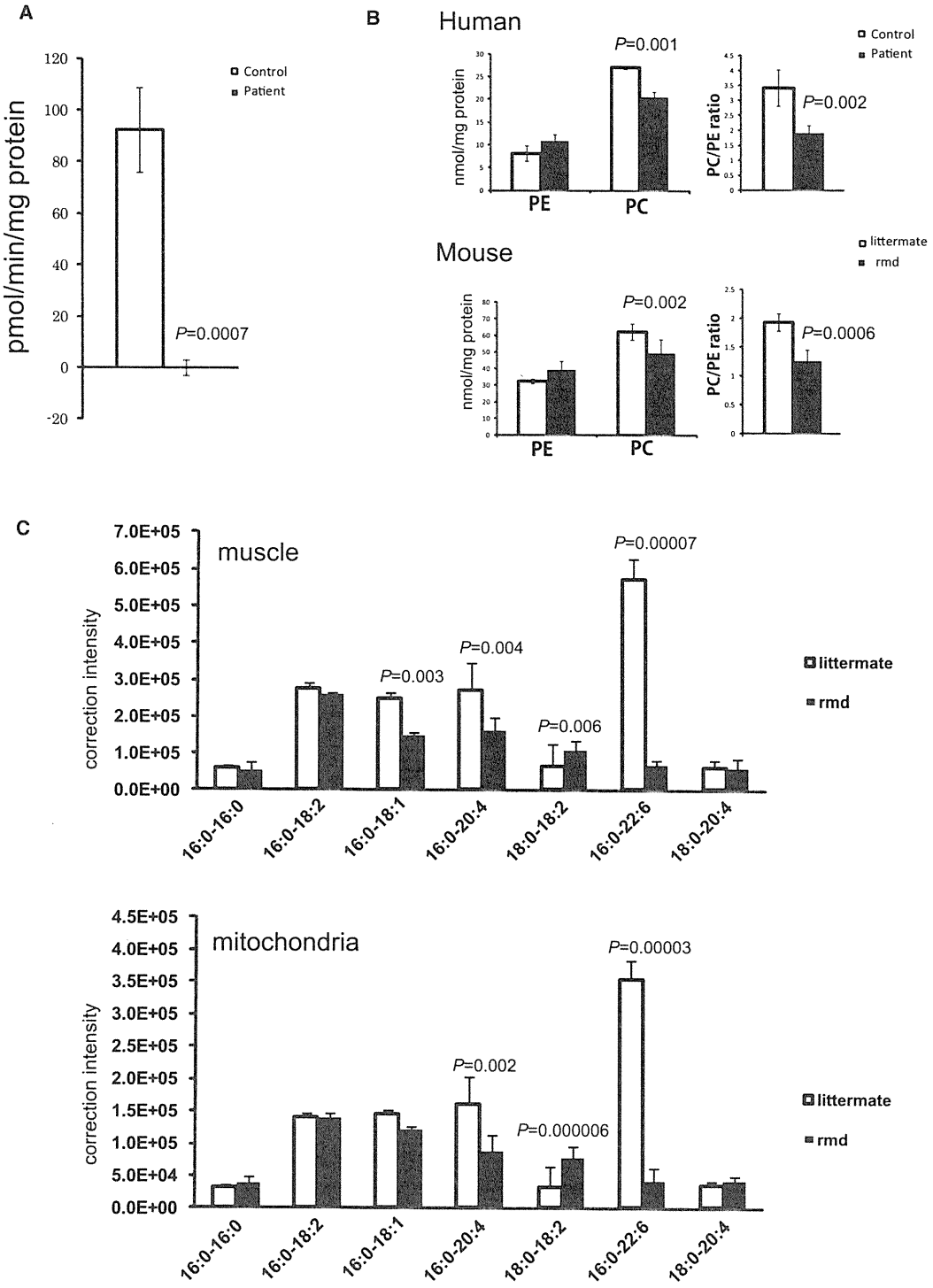

Figure 2. Choline Kinase Activity and Phospholipid Analyses
(A) In muscle tissue from individuals 2, 3, and 4, CHK activity cannot be detected (n = 3). Data represent the mean of three individuals. (B) PC and PE content in frozen biopsied muscle tissues from individuals 2, 3, and 4 and hindlimb muscles from 8-week-old *rmd* mice (n = 4) and control littermates (n = 5) were analyzed by thin-layer chromatography followed by phosphorus analysis. PC and the PC/PE ratio are significantly decreased in affected individuals and *rmd* mice (n = 3 for humans, n = 4 for *rmd* mice, n = 5 for littermates). (C) Fatty acid composition of PC molecular species in muscles and isolated mitochondria from hindlimb muscles of *rmd* mice are determined by electrospray ionization mass spectrometry (ESI-MS). We observed that 34:1-PC (16:0-18:1), 36:4-PC (16:0-20:4), and 38:6-PC (16:0-22:6) species are significantly decreased, whereas 36:2-PC (18:0-18:2) is increased in *rmd* muscle. Similarly, in isolated mitochondria from hindlimb muscle, 36:4-PC (16:0-20:4) and 38:6-PC (16:0-22:6) species are decreased, whereas 36:2-PC (18:0-18:2) is increased.

chromosomes. To elucidate the pathogenesis of these substitutions, we measured CHK activity in recombinant proteins with mutations. We cloned the open reading frame of *CHKB* into pGEM-T easy (Promega), then subcloned it into pET15b (Novagen) to make His-tagged CHK-β.[8] Each mutation was induced by site-directed mutagenesis.[7] Plasmids were transformed into *Escherichia coli* strain BL21 (DE3) and inoculated at 20°C to an OD_{600} of approximately 0.5, and the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside induced expression. The His-tagged CHK-β proteins were subjected to affinity purification on a nickel column (GE Healthcare) and eluted with 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 300 mM imidazol, and 1 mM phenylmethanesulfonyl fluoride, and 25 ng protein was analyzed for CHK activity. CHK activity of recombinant proteins with these mutations decreased to less than 30% of wild-type CHK activity, suggesting that these mutations are causative in these individuals (Figure S2). For individual 13, who had a mutation at the splice site of the exon-intron border after exon 9 (c.1031+1G>A), we also analyzed cDNA sequences. Exons 4 through 10 were amplified from the first-strand cDNAs, and direct sequencing followed. cDNA analysis of *CHKB* in skeletal muscle from individual 13 showed four splicing variants, all of which remove consensus domains for *CHKB* (Figure S3). This suggests the same loss-of-function mechanism in humans and *rmd* mice.

Because phosphorylation of choline by CHK is the first enzymatic step for phosphatidylcholine (PC) biosynthesis,[9] we anticipated that PC content should be altered in affected individuals' muscles. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and total phospholipid amounts were measured in biopsied muscles from individuals 2, 3, and 4 and in leg muscles from 8-week-old *rmd* mice by either one-dimensional or two-dimensional thin-layer chromatography (TLC) followed by phosphorus analysis.[10,11] As expected, PC levels decreased in affected individuals' skeletal muscle (Figure 2B), as they did in *rmd* mice (Figure 2B and Sher et al.[1]), suggesting that the CMDs due to *CHKB* mutations in humans and *rmd* mice are not only pathologically but also pathomechanistically similar.

PC is present in all tissues and accounts for around 50% of phospholipids in biological membranes in eukaryotes. Selective tissue involvement can be explained by the different tissue distribution of CHK isoforms. There are two CHK isoforms: CHK-α and CHK-β, encoded by distinct genes, *CHKA* (MIM 118491) and *CHKB*, respectively. They are known to form both homodimers and heterodimers, with differential tissue distribution.[12] In mice, disruption of *Chka* causes embryonic lethality,[13] suggesting the importance of CHK-α in embryonic development. In skeletal muscles from *rmd* mice, CHK activity is absent, and PC levels are decreased.[1] In other tissues, however, CHK activity is only mildly decreased, PC levels are not altered, and no obvious pathological change is seen.[1] CHK activity in skeletal muscle from individuals 2, 3, and 4 is barely detectable, and PC levels are significantly decreased, suggesting that CHK-β is the major isoform in human skeletal muscle. In support of this notion, CHK-α was not detected in human muscle (Figure S4). These results suggest that muscular dystrophy in affected individuals and *rmd* mice is caused by a defect in muscle PC biosynthesis. In addition, in *rmd* mice, hindlimb muscles are more significantly affected than forelimb muscles.[1] This is most likely explained by the fact that CHK activity is detected, though decreased, in forelimb muscles in *rmd* mice as a result of the continued post-natal expression of *Chka*.[14] This indicates that the severity of muscle involvement is determined by the degree of deficiency of CHK activity.

Generally, phospholipids have saturated or monounsaturated fatty acids at the *sn-1* position and polyunsaturated fatty acids at the *sn-2* position of glycerol backbone.[15] It has been shown that phospholipids have tissue-specific fatty acid composition.[15] For example, heart PC and muscle PC mainly contain docosahexaenoic acid (22:6) (Nakanishi et al.[15] and Figure 2C), but liver PC includes various fatty acids.[15] NanoESI-MS analyses of PC molecular species in muscle and isolated mitochondria were performed with a 4000Q TRAP (AB SCIEX, Foster City, CA, USA) and a chip-based ionization source, TriVersa NanoMate (Advion BioSystems, Ithaca, NY, USA).[16] Quadriceps femoris (hindlimb) and Triceps (forelimb) muscle from affected *rmd* mice and littermate controls were frozen with liquid nitrogen, and total lipid was extracted by the Bligh and Dyer method.[10] The ion spray voltage was set at −1.25kV, gas pressure at 0.3 pound per square inch (psi), and flow rates at 200 nl/min. The scan range was set at m/z 400~1200, declustering potential at −100V, collision energies at −35~−45V, and resolutions at Q1 and Q3 "unit." The mobile phase composition was chloroform: methanol (1/2) containing 5 mM ammonium formate and was normalized to the muscle weight. The total lipids were directly subjected by flow injection, and selectivity was analyzed by neutral loss scanning of the polar head

In muscle and isolated mitochondria, the 38:6-PC molecular species is profoundly decreased (n = 6 for muscle, n = 5 for isolated mitochondria).

Mitochondria from skeletal muscles of whole hindlimbs of *rmd* mice were isolated by the differential centrifugation method. Fresh muscle was minced and homogenized with a motor-driven Teflon pestle homogenizer with ice-cold mitochondrial isolation buffer (10 mM Tris-HCl [pH 7.2], 320 mM sucrose, 1mM EDTA, 1mM DTT, 1 mM PMSF, 1 mg/ml BSA, and protease inhibitor cocktail [Roche]) and centrifuged at 1,500 × *g* for 5 min. The supernatant fraction was centrifuged at 15,000 × *g* for 20 min, the pellet was resuspended in mitochondrial isolation buffer, and the centrifugation/resuspension was repeated twice more.

All data are presented as means ± standard deviation (SD). Means were compared by analysis with a two-tailed t test via R software version 2.11.0.

group for PC in negative-ion mode.[17] Interestingly, there was a 10-fold decrease (9.8%) in the 16:0-22:6-PC levels versus the control in *rmd* hindlimb muscle and also in muscle mitochondria (Figure 2C), indicating the importance of the PC de novo synthesis pathway for maintaining not only PC levels but also fatty acid composition of PC molecular species. Similarly, in forelimb muscle 16:0-22:6 PC levels were also decreased in comparison to the control, but to a milder extent (18.2%), suggesting an association between severity of muscle damage and fatty acid composition alteration of PC (data not shown). In *rmd* mice, it has been shown that muscle PC can be delivered from plasma lipoprotein,[18] suggesting that non-decreased PC molecular species might be derived from the plasma, whereas 16:0-22:6 PC might be synthesized only in muscle (and possibly in brain). However, confirmation of this requires further studies.

Individuals with *CHKB* mutations have severe mental retardation in addition to the muscular dystrophy. Interestingly, polymorphisms near the *CHKB* locus and decreased CHKB expression have been associated with narcolepsy with cataplexy, suggesting a link between CHK-β activity and the maintenance of normal brain function in humans.[19] Furthermore, brain damage in pneumococcal infection has been attributed to the inhibition of de novo PC synthesis, suggesting the importance of PC synthesis for the brain.[20] Our data provide evidence that altered phospholipid biosynthesis is a causative agent for a human congenital muscular dystrophy, and further studies will elucidate the detailed molecular mechanisms of the disease in both muscle and brain.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.cell.com/AJHG/.

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Web Resources

The URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org
R software version 2.11.0, http://www.r-project.org/

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