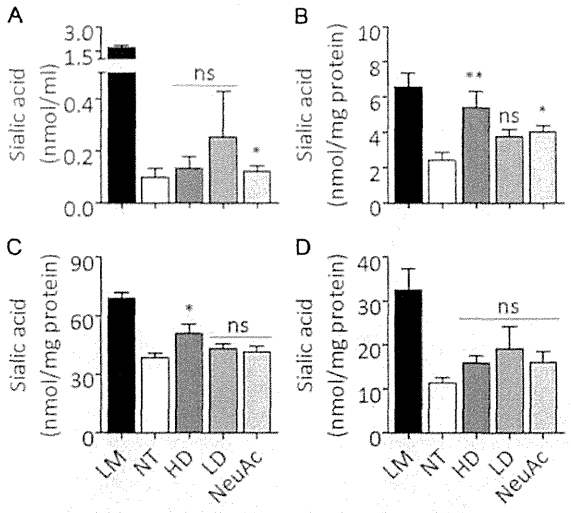

Figure 5 Total sialic acid levels in blood and tissues. (**A**) Total sialic acid concentration in blood. (**B–D**) Sialic acid levels in membranous fraction prepared from skeletal muscle (**B**), kidney (**C**), and liver (**D**). Control littermates (LM, black bars, $n = 14$); non-treated GNE myopathy mice (NT, white bars, $n = 15$); GNE myopathy mice with high dose 6′-sialyllactose (HD, dark grey bars, $n = 5$); GNE myopathy mice with low dose 6′-sialyllactose (LD, grey bars, $n = 7$); GNE myopathy mice with NeuAc (light grey bars, $n = 9$). $*P < 0.05$, $**P < 0.01$, ns = not significant.

On muscle pathology, the number of rimmed vacuole-containing fibres was decreased in all treated groups, especially in the high dose group (Fig. 3G). The high dose group also showed a smaller number of atrophic fibres than those in other treated groups (Fig. 3G). Figure 3I shows the representative data of histogram of myofibre diameters in gastrocnemius muscle of one mouse from each group. The average and distribution of myofibre size in gastrocnemius muscle in the high dose group were completely restored similarly to control littermates, but not in the low dose group (Fig. 3H and Supplementary Fig. 6). The NeuAc group showed no increase in fibre size (Fig. 3H) and the histogram was similar to that of the non-treated group (Supplementary Fig. 6).

By all treatment modalities, almost all of the GNE myopathy mice had few or only a small number of rimmed vacuoles, except for one mouse in the NeuAc group (Fig. 4B). Furthermore, amyloid-β_{1-40} and amyloid-β_{1-42} amounts, in addition to the number of amyloid-β inclusions, were also almost totally diminished by all three treatments (Fig. 4C–E). Overall, 6′-sialyllactose dose-dependently restored muscle degeneration in symptomatic GNE myopathy mice, while free NeuAc ameliorated muscle degeneration to some degree.

The sialic acid levels in blood were elevated by all treatments, high and low doses of 6′-sialyllactose, and NeuAc (Fig. 5A). Of note, sialic acid levels in blood did not reach the level of littermates either in the high dose or NeuAc group, but we found one mouse in the low dose group that had a similar level of blood sialic acid to littermates for unknown reason. Similarly, all three treatments increased sialic acid amounts in various tissues (Fig. 5B, C and D). In the high dose group, sialic acid levels in skeletal muscle and kidney were significantly and most efficiently increased among all three treatment groups (Fig. 5B and C). An increased level of sialic acid in skeletal muscle was also found in the NeuAc group (Fig. 5B).

In this study, we did not detect any improvement of the creatine kinase level in all treated groups: high dose group, 79.0 ± 55.8 ($n = 5$); low dose group, 80.9 ± 53.5 ($n = 7$); NeuAc group, 85.1 ± 48.8 IU/l ($n = 9$). We also confirmed no hepatic and renal toxicity by measurements of plasma aspartate aminotransferase, alkaline phosphatase, and blood urea nitrogen (Supplementary Table 1).

Discussion

We have reported the prophylactic effect of NeuAc, ManNAc, and 6′-sialyllactose against the development of GNE myopathy, but there were some limitations in the study (Malicdan *et al.*, 2009). First, the study design was prophylactic, limiting interpretation for clinical application. Second, we did not detect apparent dose-effect as the expected GNE myopathy phenotype was completely precluded, even at low doses. Third, to completely evaluate the choice of the compounds, there was a need to use severely affected animals. We address these issues in the present study. We explored a therapeutic strategy in symptomatic GNE myopathy mice, keeping in consideration the pharmacological properties of sialic acid in aiming to efficiently raise sialic acid levels in the blood and various tissues. We should take into account two major properties of potential therapeutic compounds, including effective incorporation into cells and pharmacokinetic properties. In this study, we tested 6′-sialyllactose, a compound that is slowly metabolized, allowing longer retention time in the circulation, and thereby effectively increasing sialic acid in the muscle.

In cultured human GNE muscle cells, a dose-dependent increase in sialic acid levels was seen by the addition of 6′-sialyllactose, similar to previous reports with NeuAc and ManNAc (Malicdan *et al.*, 2012). These results indicate that the incorporation rate or dosage of 6′-sialyllactose were not different from those of free NeuAc. It has been suggested that there is a difference in metabolism between 6′-sialyllactose and NeuAc or ManNAc. In the present pharmacokinetic results of oral 6′-sialyllactose, 6′-sialyllactose in blood was returned to the baseline at 240 min after its administration as compared to 120 min when NeuAc was administered (Fig. 1B) (Malicdan *et al.*, 2009). Similarly, 48–91% of 6′-sialyllactose was excreted into urine after 60 to 480 min (Fig. 1C), whereas 75% of NeuAc was excreted within 60 min (Malicdan *et al.*, 2009), showing that 6′-sialyllactose is less metabolized as compared to NeuAc. Previous reports also support these results that it took 24 h to excrete almost all amounts of sialyllactose and 6 h for 60–90% of NeuAc into urine when they were orally administered (Nöule and Schauer, 1981). Furthermore, the peak of free NeuAc in blood at 60 min after administration of 6′-sialyllactose (Fig. 1D) suggests that while circulating through the body, 6′-sialyllactose might be slowly metabolized into NeuAc and providing NeuAc source in the circulation within 50 min of delay from the time 6′-sialyllactose is given. In other

words, giving 6'-sialyllactose can extend the metabolizing time of sialic acid (compounds). These results support our pharmacological hypothesis that slowly metabolized compounds can circulate and stay in the body longer, allowing the tissues more time to incorporate the circulating sialic acid.

In this study, using a less intensive method to assess the motor performance in symptomatic mice, we showed that motor performance continues to deteriorate after 50 weeks of age, presumably because of severe muscle degeneration. By applying this method to all treatment groups, we found that a high dose of 6'-sialyllactose remarkably prevented further deterioration of motor performance. Our findings support the use of this less invasive and reliable tool in the evaluation of other models of myopathy.

As previously reported, GNE myopathy mice manifest muscle weakness and atrophy from 21 weeks of age, and show decreased specific twitch force after intracellular inclusions appear and parallel reduction in specific twitch and tetanic forces after rimmed vacuoles appear (Malicdan *et al.*, 2007, 2008). Indeed, 80-week-old GNE myopathy mice showed physiological findings congruent with progressive muscle degeneration. In terms of muscle contractile parameters, gastrocnemius muscles in the high dose group showed almost complete normalization in size and in both tetanic and twitch forces, suggesting that the properties of contractile machinery as well as contraction regulating systems have been repaired. Thus, these results imply that atrophy and weakness in older GNE myopathy mice are reversible by maintaining sufficient sialic acid levels in the muscle. We propose that muscle atrophy and weakness in patients with GNE myopathy can be rescued by sialic acid supplementation.

The low dose treatment did not restore muscle atrophy but ameliorated the contractile properties of gastrocnemius and tibialis anterior muscles and in the NeuAc group, sialic acid levels in skeletal muscle were increased to the level in the low dose group, but only minimal beneficial effect were seen on contractile properties and there was no effect on muscle size (Fig. 3E, F and H, and Supplementary Fig. 4). These findings suggest that there is a positive correlation between recovery of hyposialylation and improvement of muscle atrophy and weakness, and that the restoration of muscle size in GNE myopathy requires a high and steady amount of sialic acid incorporated into muscle cells.

The presence of intracellular protein deposits and rimmed vacuoles in the central portion of myofibres presumably interferes with the muscle force generation in rimmed vacuolar and autophagic vacuolar myopathies (Malicdan and Nishino, 2012; Raben *et al.*, 2012). Rimmed vacuoles are likely a secondary event to protein misfolding or aggregation in GNE myopathy (Malicdan *et al.*, 2008). However, intracellular inclusions and rimmed vacuoles almost disappeared in the affected mice after all three treatments (Fig. 4B and C), suggesting again that hyposialylation in muscles is associated with formation of such intracellular inclusions.

From the biochemical point of view, we highlight our finding that the high dose group had higher sialic acid levels in skeletal muscle than the NeuAc group (Fig. 5B), leading to near-complete recovery of muscle function. Given that large amounts of sialic acid may be needed to rescue muscle atrophy, in addition to contractile property, there are several points that we must consider to achieve an efficient increase in sialic acid level in peripheral tissues, including the pharmacological advantages of the less metabolized compounds such as 6'-sialyllactose; high cellular uptake of compounds such as peracetylated ManNAc (Malicdan *et al.*, 2012); and the use of sustained-released preparations of free sialic acid. In addition, the finding that 6'-sialyllactose and peracetylated ManNAc remarkably increased sialic acid level in kidney (Malicdan *et al.*, 2012) also suggest that the other diseases associated with *GNE* gene mutation, such as severe renal disorders in *Gne* mutants (Galeano *et al.*, 2007; Ito *et al.*, 2012) and other transgenic models (Clement *et al.*, 2011), would be treatable strategies.

In this study, survival rate did not differ among all GNE myopathy groups during treatment (Fig. 4C). We also attempted to characterize respiratory and cardiac function in older mice by using whole body plethysmography and ECG, however, we did not detect physiological deterioration in the non-treated group compared to the littermates (data not shown). As previously demonstrated, GNE myopathy mice started to die from 3 weeks of age and showed a gradual increment of mortality from 25–55 weeks, but thereafter GNE myopathy mice showed a reduced mortality (Malicdan *et al.*, 2007). The surviving GNE myopathy mice older than 55 weeks of age might be resistant to the death related to vital organ functions, such as lung and heart, and that the survival rate may not be informative in evaluating efficacy of treatment in the experiments using symptomatic myopathic older mice; specific methods should be chosen on their phenotypes. In this study, spontaneous locomotion activity was applicable to older GNE myopathy mice when repeatedly performed, and indeed we demonstrated that the high dose group showed reversed motor function in the same GNE myopathy mice.

Our results emphasize that GNE myopathy is a treatable disease, requiring enhanced sialylation for recovery of skeletal muscle function. This study provided a proof of concept in the use of slowly metabolized sialic acid in the clinical trial of patients with GNE myopathy.

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Supplementary material

Supplementary material is available at *Brain* online.

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LETTER TO THE EDITOR

Absence of beta-amyloid deposition in the central nervous system of a transgenic mouse model of distal myopathy with rimmed vacuoles

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Keywords

Distal myopathy, rimmed vacuole, Gne knockout mouse, amyloid deposition

History

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Distal myopathy with rimmed vacuoles (DMRV) is an autosomal recessive disorder characterised by early adult-onset (15–40 years), slowly progressive myopathy with preferential weakness of the tibialis anterior and sparing of the quadriceps femoris muscles [1]. The muscle biopsy shows rimmed vacuoles containing deposits immunoreactive for β-amyloid (Aβ) and other proteins. DMRV is due to mutations in the *GNE* gene, which encodes a bifunctional enzyme (uridine diphosphate-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase) in the sialic acid synthetic pathway. A recently developed mouse model of DMRV that expresses the human *GNE* D176V mutation in a Gne knockout background reproduces the clinical, pathological and biochemical features of human DMRV [2]. As in the human disease the model exhibited muscle atrophy and weakness, and low levels of sialic acid in serum and solid organs. Moreover, Aβ was also found to be associated with the rimmed vacuoles and myofibrillar degeneration in the muscle fibres [2]. Because Alzheimer's disease (AD) is characterised by Aβ deposition in the brain, we examined the central nervous system of the DMRV mouse model for evidence of Aβ deposition and other pathological abnormalities to determine if there is any link between DMRV and AD.

Brain and skeletal muscle tissues from 30 DMRV mice older than 40 weeks were harvested, formalin-fixed, processed and paraffin embedded. Sixteen spinal cords were also available for examination. Tissues sections were stained by hematoxylin and eosin for light microscopy. Immunohistochemistry (IHC) to detect Aβ was performed using a mouse monoclonal primary antibody (clone 6E/10; Covance, Princeton, NJ) and the Envision method with minor modifications [3]. As a positive IHC control, we used tissues from an established mouse model of AD, the Tg2576 transgenic mouse (Taconic, Germantown, NY) that expresses the Swedish mutation of amyloid precursor protein. Brain tissue sections from a human case of AD were also included as a positive control. For negative controls, sections were incubated with normal goat serum to replace the primary antibody.

In the 30 aged DMRV mice examined there was no evidence of Aβ deposits in the brains or spinal cords (Figure 1B). However, Aβ deposits were detected in the skeletal muscles of all these animals. The deposits were usually linear, beaded and found in the central part of the fibres (Figure 1C, D). Occasionally, these deposits were associated with vacuoles (Figure 1C) corresponding to rimmed vacuoles as shown by the hematoxylin and eosin stains (data not shown). The three AD mouse brain positive controls showed plaque-like amyloid deposits in the cerebral cortex (Figure 1A). The human AD brain tissues showed Aβ deposition in the brain neuropil and around blood vessels (data not shown). There were no light microscopic abnormalities such as neurofibrillary tangles, amyloid angiopathy and other features of AD in the central nervous system of all test and control mice.

Although the aged DMRV mouse model demonstrated convincing Aβ deposition in skeletal muscles, there was no Aβ deposition in the CNS. This suggests that amyloidogenesis in AD and DMRV may be different. Amyloidogenesis in AD is mainly associated with post-translational proteolytic processing of amyloid precursor protein by α-, β- and γ-secretases. However, hyposialylation is an important factor in the pathogenesis of the disease and amyloidogenesis in DMRV. Hyposialylation of several important muscle glycoproteins

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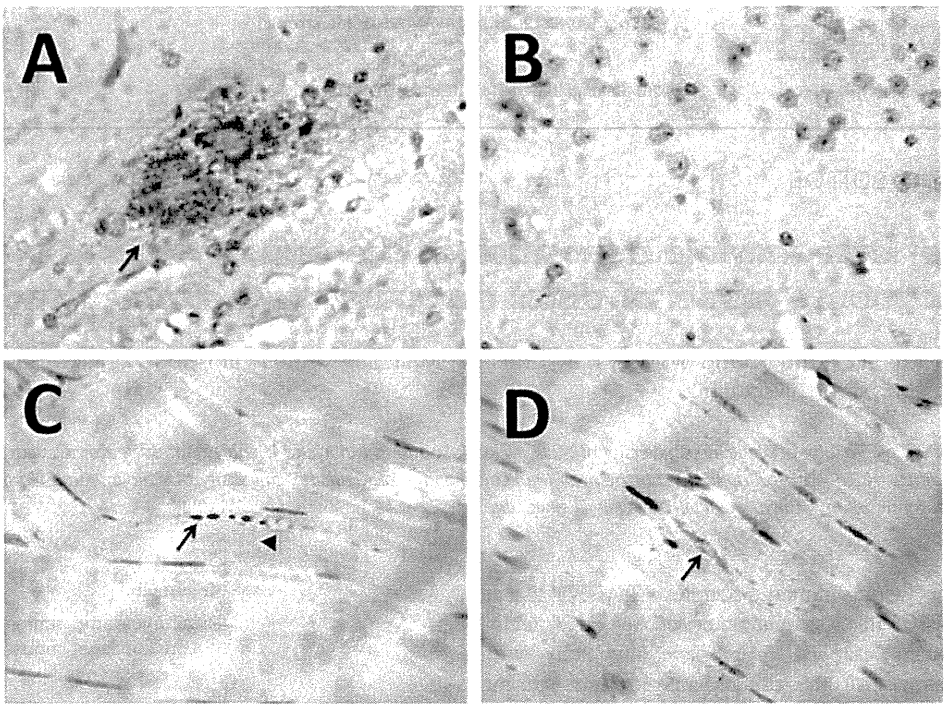

Figure 1. (A) Brain sections from Tg2576 transgenic mouse model of Alzheimer's disease shows plaque-like amyloid deposits (arrow). (B) β-amyloid deposition is not detected in brain sections of DMRV mouse. (C and D) Aβ deposits were detected in the skeletal muscle of DMRV mouse (arrow). (C) Occasionally these depositions were associated with vacuoles (arrow head).

such as neprilysin, a membrane proteinase involved with the normal breakdown of Aβ has been observed [4,5]. In DMRV, reduced muscle neprilysin activity may cause toxic Aβ accumulation in vulnerable fibres and a failure in repair or regeneration of muscle fibres possibly through modulation of the insulin-like growth factor I-dependent pathways [5]. In addition, it was demonstrated that reduced neprilysin activity in the DMRV mice was restored after treatment with sialic acid analogues [6]. Interestingly, the DMRV mouse brain showed normal sialylation levels [2,7], although the other organs showed hyposialylation. This is attributed to a strong sialic acid uptake mechanism in neuronal cells. It was suggested that sialin, a lysosomal sialic acid transporter protein, is involved in the uptake of exogenous sialic acid to maintain normal cellular sialylation in the brain [8]. This may be the reason there is no abnormal Aβ deposition in the DMRV mouse brain. This is also supported by the finding that sialic acid levels in the cerebrospinal fluid of the DMRV mice remain unaltered (Malicdan and Noguchi, unpublished data). In the DMRV mouse brain, normal levels of sialic acid could actually mitigate Aβ toxicity, if Aβ levels were to increase [6]. The level of sialic acid in brains of human DMRV is unknown and further investigations are needed. If there is no hyposialylation in the brains of DMRV patients, Aβ deposition may not occur. Nonetheless, based on current findings, we speculate that brain Aβ deposition is unlikely to be increased in human DMRV and hence, an increased incidence of AD in these patients is also probably unlikely.

Declaration of interest

The authors report no conflicts of interest. This study was partially supported by Intramural Research Grant (25-5) for Neurological and Psychiatric Disorders of NCNP, by Comprehensive Research on Disability Health and Welfare from the Ministry of Health, Labour and Welfare in Japan; JSPS KAKENHI (23390236) and University of Malaya HIR grant (H-20001-00-E000031).

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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.

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).

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

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

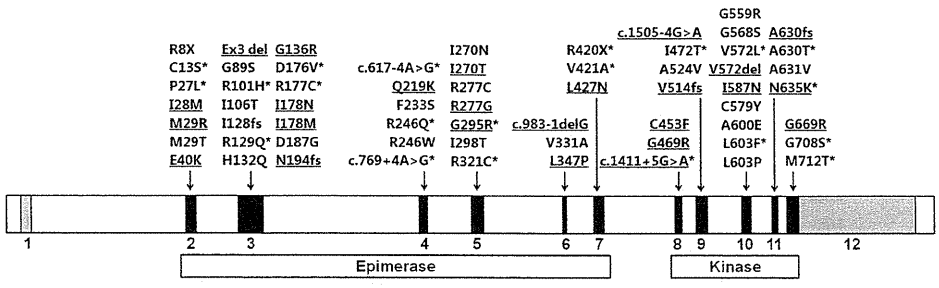

Figure 1 Mutation spectrum of *GNE* in the Japanese population. The mutations are located throughout the whole open reading frame. Twenty-five novel mutations are underlined, and 21 shared mutations are indicated with asterisks.

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.

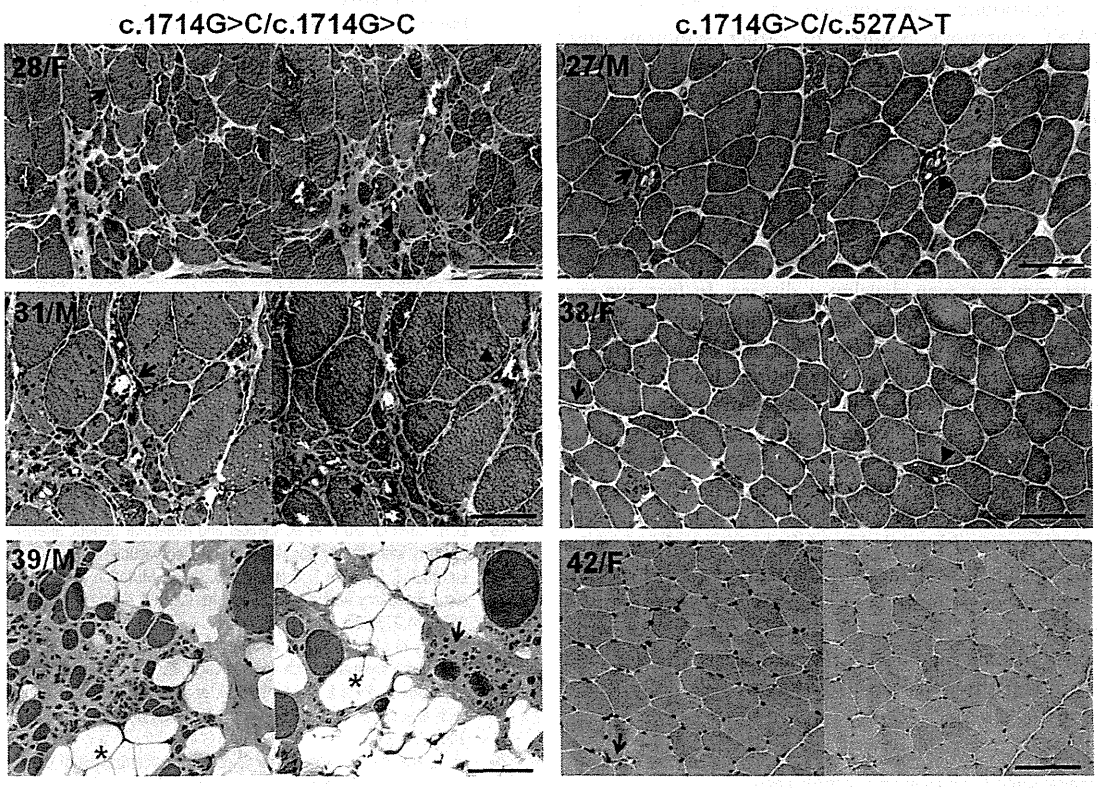

Figure 2 Comparison of muscle pathology between patients with homozygous c.1714G>C (p.Val572Leu) and with compound heterozygous c.1714G>C (p.Val572Leu)/c.527A>T (p.Asp176Val) mutations. Homozygous c.1714G>C (p.Val572Leu) mutations have led to much more advanced histopathological changes compared with 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.

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Congenital fiber type disproportion myopathy caused by *LMNA* mutations

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ABSTRACT

A boy, who had shown muscle weakness and hypotonia from early childhood and fiber type disproportion (FTD) with no dystrophic changes on muscle biopsy, was initially diagnosed as having congenital fiber type disproportion (CFTD). Subsequently, he developed cardiac conduction blocks. We reconsidered the diagnosis as possible LMNA-myopathy and found a heterozygous mutation in the *LMNA* gene. This encouraged us to search for *LMNA* mutations on 80 patients who met the diagnostic criteria of CFTD with unknown cause. Two patients including the above index case had heterozygous in-frame deletion mutations of c.367_369delAAG and c.99_101delGGA in *LMNA*, respectively. Four of 23 muscular dystrophy patients with *LMNA* mutation also showed fiber type disproportion (FTD). Importantly, all FTD associated with LMNA-myopathy were caused by hypertrophy of type 2 fibers as compared with age-matched controls, whereas CFTD with mutations in *ACTA1* or *TPM3* showed selective type 1 fiber atrophy but no type 2 fiber hypertrophy. Although FTD is not a constant pathological feature of LMNA-myopathy, we should consider the possibility of LMNA-myopathy whenever a diagnosis of CFTD is made and take steps to prevent cardiac insufficiency.

1. Introduction

Mutations in the gene encoding nuclear envelope proteins of A-type lamins (*LMNA*) cause several disorders referred to as laminopathies, which include skeletal and cardiac muscle disorders, lipodystrophy, peripheral neuropathy, and premature aging syndromes. Laminopathies predominantly affecting skeletal muscles (LMNA-myopathy) are clinically classified into three different phenotypes; Emery-Dreifuss muscular dystrophy (AD-, AR-EDMD), limb girdle muscular dystrophy type 1B (LGMD1B), and LMNA-related congenital muscular dystrophy (L-CMD). EDMD has distinctive clinical features including early joint contractures, humero-peroneal muscle weakness and dilated cardiomyopathy with conduction defects. LGMD1B is characterized by proximal muscle involvement and cardiomyopathy with conduction defects, but joint contracture is not prominent. L-CMD is an early onset form showing severe weakness of respiratory and neck muscles from infancy. Serum CK levels in LMNA-myopathy are normal to moderately elevated (2–20 times the upper limit of the normal range). Cardiac involvement, such as conduction blocks, dilated cardiomyopathy and sudden death, usually appears after the second decade of life. To minimize the risk of sudden cardiac death, early diagnosis and appropriate cardiac defibrillator implantation is recommended [1–3].

Pathologically, LMNA-myopathy is usually characterized by nonspecific dystrophic changes with variation in fiber size, mild necrotic and regenerating processes, and an increased number of muscle fibers with internalized nuclei. Both type 1 and type 2 fibers are affected. Nuclear abnormalities are common [4]. Interestingly, marked mononuclear cellular infiltrations mimicking inflammatory myopathy can be seen in some patients with the infantile onset form of LMNA-myopathy [5].

We recently experienced a patient with a *LMNA* mutation whose initial diagnosis was congenital fiber type disproportion (CFTD). This patient had shown muscle weakness, hypotonia, and unstable gait from early childhood with no dystrophic changes, but prominent fiber type disproportion (FTD) on his muscle biopsy performed at 4years of age. At his age of 16 years, he was pointed out to have atrial-ventricular conduction block and incomplete right bundle branch block. We thus reconsidered a possible diagnosis of LMNA-myopathy and identified a mutation in the *LMNA* gene.

CFTD is one of the congenital myopathies pathologically defined by smaller type 1 fibers, by at least 12%, than type 2 fibers without structural abnormalities such as nemaline bodies, cores, and central nuclei.

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Clinically, CFTD patients show generalized muscle hypotonia and weakness from infancy, multiple joint contractures, scoliosis, long thin face, and high arched palate. Approximately 30% of individuals with CFTD have mild-to-severe respiratory involvement. Cardiac involvement is seen in less than 10% of affected individuals [6,7]. Six causative genes for CFTD have been identified: *ACTA1* [8], *TPM3* [9], *RYR1* [10], *TPM2* [11], *MYH7* [12] and *SEPN1* [13] encoding α-skeletal actin, α-tropomyosin slow, ryanodine receptor type 1, β-tropomyosin, slow β-myosin heavy chain and selenoprotein N1, respectively.

In this study, we genetically screened CFTD patients for mutations in *LMNA*. We also re-evaluated clinical and pathological findings in patients previously diagnosed as having LMNA-myopathy to ascertain whether these patients have features similar to those of CFTD.

2. Materials and methods

All clinical materials used in this study were obtained for diagnostic purposes with written informed consent. This work was approved by the Ethics Committee of the National Center of Neurology and Psychiatry (NCNP).

2.1. Patients

We examined 80 unrelated muscle biopsies from the NCNP muscle repository. All specimens were from patients who had been diagnosed as having CFTD based on pathological findings as well as clinical features. All cases satisfied the pathological criteria for CFTD; mean type 1 fiber diameter is at least 12% smaller than the mean type 2 fiber diameter, with no structural abnormalities such as nemaline bodies, cores, and increased number of fibers with internal nuclei. In addition, we re-evaluated muscle pathology findings from 23 unrelated patients who had previously been diagnosed as having LMNA-myopathy. We chose genetically confirmed CFTD patients including 7 with *ACTA1* mutation and 2 with *TPM3* mutation for comparison of clinicopathological features. Clinically, all of the patients including in this study had muscle weakness and/or hypotonia from the preschool years (onset age; <6 years).

2.2. Mutation analysis

Genomic DNA was extracted from peripheral lymphocytes or frozen muscle specimens using standard techniques. For mutation screening of *LMNA*, *ACTA1* and *TPM3*, all exons and their flanking intronic regions were amplified by PCR and directly sequenced using an ABI PRISM 3100 automated sequencer (PE Applied Biosystems, Foster City, CA). Primer sequences are available on request.

2.3. Histochemical analysis of biopsied muscles

Biopsied skeletal muscles were frozen with isopentane cooled in liquid nitrogen. Serial frozen sections, 10 μm in thickness, were stained employing histochemical methods including hematoxylin and eosin (H&E), modified Gomori-trichrome (mGT), NADH-tetrazolium reductase (NADH-TR), and ATPases (pH 10.6, pH 4.6 and pH 4.3). For each muscle specimen, the mean fiber diameter was calculated by obtaining the shortest anteroposterior diameters of 100 type 1 and type 2 (A + B) fibers each using ATPase stains. Fiber size disproportion (FSD) was computed as; difference between type 2 fiber diameter (mean) and type 1 fiber diameter (mean) divided by type 2 fiber diameter (mean) × 100%. To obtain muscle fiber size information for age-matched controls, a total of 18 muscle specimens with minimal pathological changes from each age were examined.

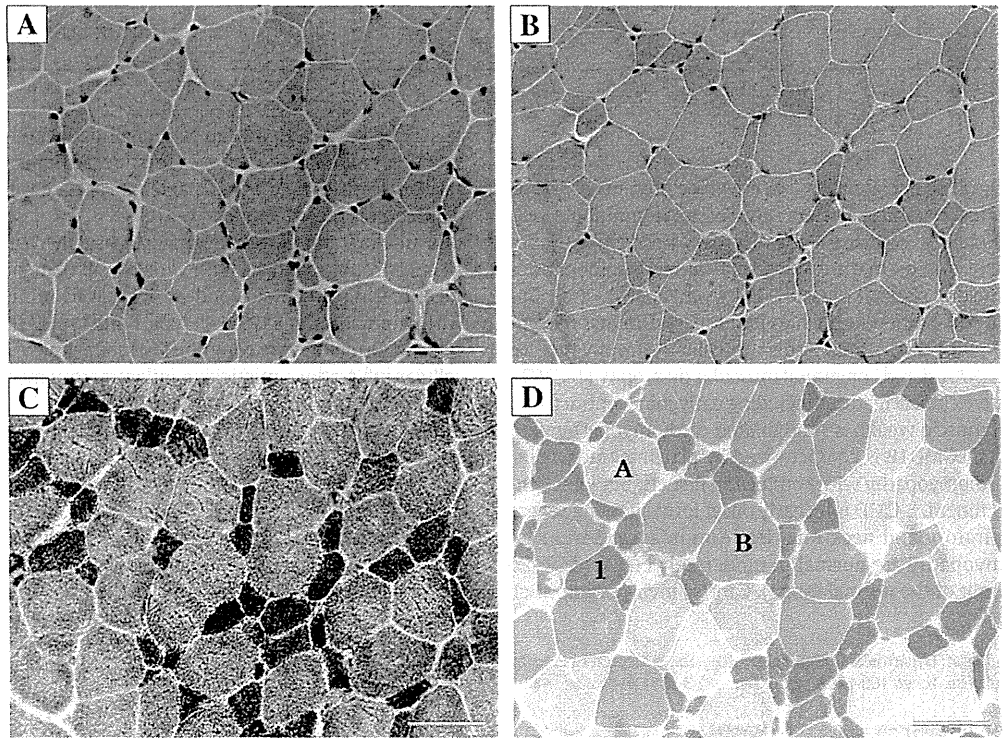

Fig. 1. Muscle biopsy from Patient 1 taken at age 4 years. (A) H&E stain shows marked variation in fiber size with neither fiber necrosis nor regeneration. (B) No nemaline bodies or cytoplasmic inclusions are revealed by mGT stain. On NADH-TR, intermyofibrillar networks are well organized. (D) On ATPase (pH 4.6), type 2A (A) and 2B (B) fibers are larger than type 1 (1) fibers. Bar = 50 μm.

Table 1
Histological features of LMNA-myopathy patients with FTD, CFTD patients with *ACTA1* and *TPM3* mutations.

Patient No.	Muscle Biopsied	Age at Biopsy	Type 1			Type 2A			Type 2B			Type 2C	%FSD	Mutation
			%	Mean Diameter (μM)	SD	%	Mean Diameter (μM)	SD	%	Mean Diameter (μM)	SD			
LMNA mutation														
1	Biceps	4y	52	16.5	5.0	30	39.1	5.3	18	37.1	7.5	0	57	c.367_369delAAG (p.K123del)
2	Quadriceps	2y	48	20.8	3.7	33	24.1	4.2	19	23.8	4.8	0	13	c.99_101delGGA (p.E33del)
3	NA	2y	38	28.6	7.7	50	36.3	4.4	7	31.3	10.5	5	15	c.1583C>A (p.T528K)
4	Biceps	4y	32	22.1	5.9	52	31.2	5.2	15	28.2	5.3	1	25	c.1357C>T (p.R453W)
5	Biceps	4y	56	21.6	5.6	32	40.0	5.8	10	34.0	8.4	2	42	c.1357C>T (p.R453W)
6	Biceps	5y	60	27.5	7.4	28	33.2	7.9	8	29.8	6.3	4	15	c.907T>C (p.S303P)
ACTA1 mutation														
1	Biceps	4y	73	14.5	3.7	26	17.8	3.7	1	–	–	0	18	c.16G>A (p.E6K)
2	Quadriceps	0y6m	60	11.9	3.1	10	18.0	2.8	20	18.8	2.8	10	35	c.143G>T (p.G48C)
3	Quadriceps	0y7m	60	6.8	1.6	29	11.5	2.1	3	–	–	8	44	c.143G>T (p.G48C)
4	NA	0y1m	52	5.6	1.5	28	14.4	2.0	12	10	2.8	8	57	c.668 T>C (p.L223P)
5	Biceps	10y	70	11.9	2.3	27	17.2	3.2	2	–	–	1	31	c.682G>C (p.E228Q)
6	Biceps	0y9m	62	10.5	2.8	23	17.2	2.8	10	18.8	2.8	5	42	c.981T>A (p.M326K)
7	Biceps	0y10m	72	12.0	1.8	22	19.5	3.5	3	–	–	3	36	c.1000C>T (p.P332S)
TPM3 mutation														
1	Biceps	0y5m	56	9.0	2.4	44	24.4	3.2	0	–	–	0	63	c.502C>T (p.R168C)
2	Biceps	0y6m	58	9.7	2.0	20	17.9	2.5	16	17.1	2.4	6	45	c.502C>T (p.R168C)

SD = standard deviation; NA = data not available; dash = not applicable.

2.4. Electron microscopic observation

Muscle specimens were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer. After shaking with a mixture of 4% osmium tetroxide, 1.5% lanthanum nitrate, and 0.2 M s-collidine for 2–3 h, samples were embedded in epoxy resin. Semi-thin sections (1 μm-thickness) were stained with toluidine blue. Ultrathin sections, 50 nm in thickness, were stained with uranyl acetate and lead citrate, and then examined

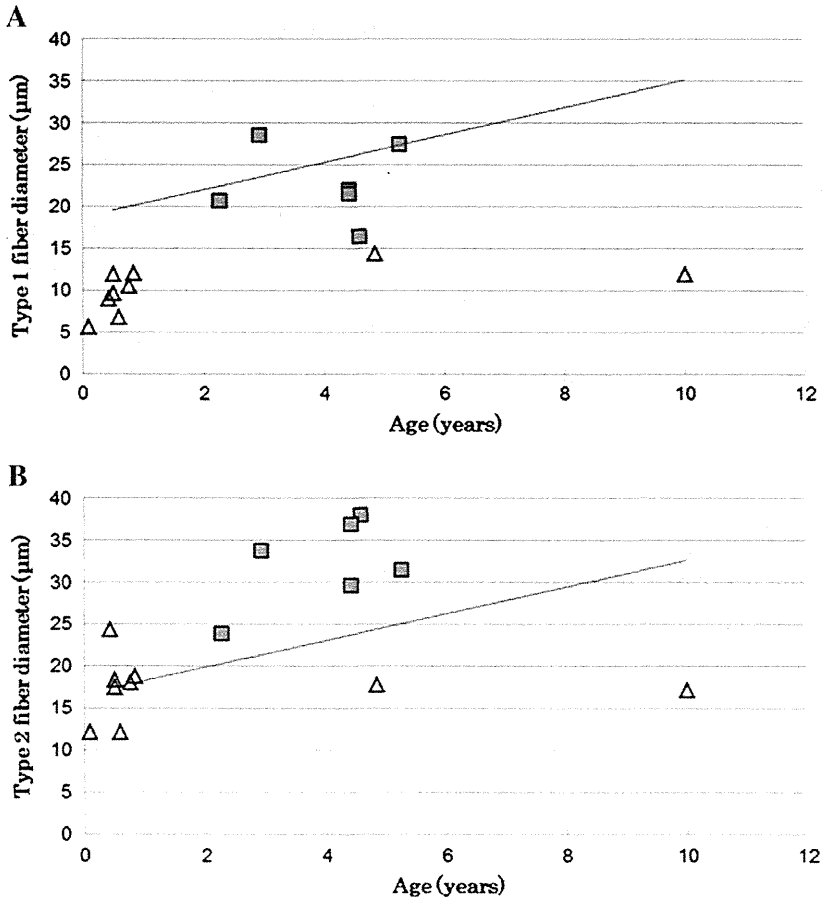

Fig. 2. Composition of mean muscle fiber diameter in each patient. (A) Mean diameters of type 1 fibers, (B) Mean diameters of type 2 fibers. Filled squares represent LMNA-myopathy with FTD, open triangles show CFTD with *ACTA1* or *TPM3* mutations, and the solid line indicates the mean fiber diameter of age-matched controls for children at various ages taken from biopsies classified as normal. CFTD with *ACTA1* and *TPM3* mutations show type 1 fiber atrophy whereas LMNA-myopathy with FTD shows type 2 fiber hypertrophy.

Table 2
Clinical and pathological summary of LMNA-myopathy patients with FTD.

Patient No	Sex	Age at Diagnosis (yr)	Age at biopsy (yr)	Pathological diagnose	Age at walk (mo)	Hypotonia	High arched palate	Respiratory involvement	Cardiac symptoms	Other presenting signs/Symptoms (age/yr)	CK (IU/L)	FSD (%)
1	M	16	4	CFTD	12	Yes	No	No	AV-b, ICRBBB (16 yr)	Joint contractures (4)	330	57
2	M	4	2	CFTD	14	Yes	No	No	No	Joint contractures (2) Dropped head (4) Rigid spine (4)	367	13
3	M	10	2	MD	15	Yes	No	No	No	Joint contractures (2) Rigid spine (8)	1098	15
4	F	4	4	MD	12	Yes	No	No	No	No	1408	25
5	F	13	4	MD	14	No	No	No	No	Lordosis (4) Joint contracture (6) Rigid spine (10)	1985	42
6	F	5	5	MD	18	No	No	No	No	No	303	15

MD; muscular dystrophy, AV-b; atrioventricular block, IRBBB; incomplete right bundle-branch block, PAF; paroxysmal atrial fibrillation.
Patients 1 and 2 were initially diagnosed as having CFTD. Patients 3 to 7 were genetically confirmed to have LMNA-myopathy with FTD. None of the patients had a high arched palate and/or respiratory involvement. Serum creatine kinase (CK) was mildly elevated in all patients.

under a tecnai spirit transmission electron microscope (FEI, Japan) at 120 kV.

2.5. Statistical analysis

All data are presented as means ± SD. Comparisons among groups were made using Student's *t* test and analysis of variance (ANOVA). A difference was considered to be statistically significant at a *p* value less than 0.05.

3. Results

3.1. Mutation analysis

Among the 80 unrelated patients who were diagnosed as having CFTD based on clinical and pathological findings, a heterozygous *LMNA* mutation was identified in two; a previously reported c.367_369delAAG (p.Lys123del) in Patient 1 and a novel c.99_101delGGA (p.Glu33del) in Patient 2 [14]. *ACTA1* mutations found in the 7 CFTD patients were c.16G>A (p.Glu6Lys), c.142G>T (p.Gly48Cys), c.668T>C (p.Leu223Pro), c.682G>C (p.Glu228Gln), c.980T>A (p.Met327Lys), and c.1000C>T (p.Pro334Ser). Two CFTD patients had the same heterozygous c.502C>T (p.Arg168Cys) mutation in *TPM3*. The novel mutations of *LMNA* c.99_101delGGA (p.Glu33del) and *ACTA1* c.980T>A (p.Met327Lys), were not found in either 100 Japanese control chromosomes or the dbSNP and 1000 Genomes databases.

3.2. Histological findings

Histologically, type 1 fiber predominance (more than 55% of type 1 fibers) and type 2B fiber deficiency (less than 5% of type 2B fibers) were observed in 61% and 28%, respectively, of our 80 CFTD cohort. These results are consistent with those of a previous report [7].

Two patients with *LMNA* mutations showed a marked difference in the sizes of type 1 and type 2 fibers, resulting in FSD of 57% and 13%, respectively (Fig. 1). Neither type 1 fiber predominance nor type 2B fiber deficiency was seen (Table 1).

Re-evaluation of genetically confirmed LMNA-myopathy revealed that 4 of 23 patients (17%) had fiber type disproportion (FTD). Their FSD was ranged from 15 to 42%. All 4 patients with FTD also showed some necrotic and/or regenerating fibers in their muscle biopsy and had a diagnosed of muscular dystrophy. These 4 patients with FTD had 3 different mutations. Two mutations of c.1583C>A (p.Thr528Lys) and c.1357C>T (p.Arg453Trp) have already been reported [15,16], whereas the c.907 T>C (p.Ser303Pro) mutation was not reported previously. These mutations were distributed in both central rod and tail domains, but not in the head domain (Table 1).

To clarify whether LMNA-myopathy patients with FTD have specific pathological findings different from those affecting CFTD muscles with known gene mutations, we carefully re-evaluated the muscle pathologies of the 6 LMNA-myopathy patients with FTD, 7 CFTD patients with *ACTA1* mutations, and 2 CFTD patients with *TPM3* mutations. FSD in LMNA-myopathy with FTD, and in CFTD with *ACTA1* and *TPM3* mutations were calculated to be 27.8 ± 17.9% (mean ± SD), 37.7 ± 12.1%, and 54.1 ± 13.1%, respectively. No significant differences were seen in FSD among the 3 groups. We also compared fiber sizes among LMNA-myopathy with FTD, CFTD with *ACTA1* or *TPM3* mutations and age-matched controls. Surprisingly, CFTD with *ACTA1* and *TPM3* mutations showed type 1 fiber atrophy, whereas LMNA-myopathy with FTD showed type 2 fiber hypertrophy with lack of type 1 fiber atrophy (Fig. 2).

In this study, type 1 fiber predominance was seen in 86% of CFTD patients with *ACTA1* mutations and in 100% of those with *TPM3* mutations, but in only 33% of LMNA-myopathy patients with FTD. The percentage of type 1 fibers in LMNA-myopathy was calculated to be 44.6 ± 12.8 (mean ± SD), which was significantly lower than that in CFTD with *ACTA1* mutations (64.1 ± 7.1%) and that with *TPM3* mutations (57.0 ± 1.4%) ($p < 0.05$). Type 2B fiber deficiency was not seen in LMNA-myopathy with FTD (Tables 1, 3), whereas 4 of 7 (57%) patients with *ACTA1* mutations and one (50%) with *TPM3* mutation showed type 2B fiber deficiency.

On electron microscopic (EM) observations, nuclear changes are important pathological findings in skeletal muscles of LMNA-myopathy [4]. We examined the nuclear changes in Patients 2, 4 and 5 on EM, and found a few myonuclei showing abnormal shapes and chromatin disorganization (Fig. 3). Smaller nuclei arranged in a row, giving the appearance of a 'nuclear chain', were also seen (data not shown). However, nuclear abnormalities in patients who had LMNA-myopathy with

Table 3
Comparison of clinical and pathological information between LMNA-myopathy with FTD and CFTD with *ACTA1* and *TPM3* mutations.

Gene mutation	*LMNA*	*ACTA1*	*TPM3*
Number of patients	6	7	2
Onset	Infantile	at birth	< 2 months
Hypotonia	67% (4/6)	100% (7/7)	100% (2/2)
High arched palate	0% (0/6)	57% (4/7)	50% (1/2)
Respiratory involvement	0% (0/6)	57% (4/7)	0% (0/2)
Joint contracture	67% (4/6)	14% (1/7)	0% (0/2)
CK level (IU/L)	963 ± 662	53 ± 15	42 ± 16
Type 1 fiber predominance	33% (2/6)	86% (6/7)	100% (2/2)
Type 2B fiber deficiency	0% (0/6)	57% (4/7)	50% (1/2)

Type 1 fiber predominance and absence of type 2B fibers were common in CFTD caused by *ACTA1* or *TMP3* mutations. Type 2B fiber deficiency was not seen in LMNA-myopathy with FTD. Serum creatine kinase (CK) levels were significantly higher in LMNA-myopathy than in CFTD with *ACTA1* and *TPM3* mutations ($p < 0.05$).

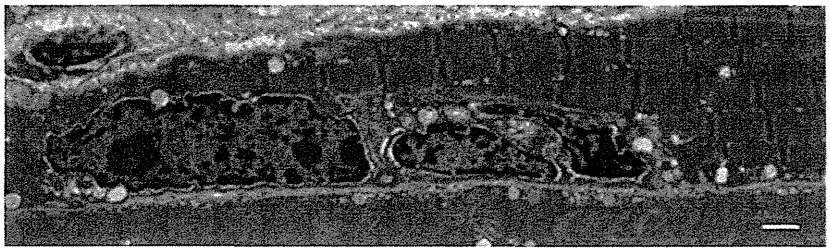

Fig. 3. Myonuclear shape changes in patient 2. Nuclear contours are irregular with a serpentine appearance. Bar = 1 μm.

FTD were milder and less frequent than previously reported for AD-EDMD and LGMD1B muscles [4].

3.3. Clinical findings

Table 2 summarizes the characteristics of the 6 LMNA-myopathy patients with FTD. Patients 1 and 2 were initially diagnosed as having CFTD, and the 4 remaining patients (patients 3 to 6) showed FTD together with dystrophic changes on muscle pathology. All patients had normal antenatal courses and uneventful births. All patients had started walking without delay, but showed a waddling gait and muscle weakness and/or hypotonia from the preschool years. None had a high arched palate or respiratory dysfunction. Four of the 6 (67%) patients had contractures of the ankles and/or elbows which had not been present at birth but appeared with age. Serum creatine kinase (CK) was mildly elevated in all patients.

Sixteen of the 78 (21%) CFTD patients with unknown cause had high CK levels (>200 IU/l), and four of these 16 showed a high arched palate and respiratory involvement.

4. Discussion

FTD can be seen in a single muscle biopsy from patients with several diseases including congenital myotonic dystrophy and centronuclear myopathy [17–20]. Here we identified 2 LMNA-myopathy among patients diagnosed as CFTD. We also found FTD in 17% of muscular dystrophy patients with LMNA mutations. These results suggest that FTD may not be rare in LMNA-myopathy. None of these patients had either a high arched palate or respiratory insufficiency, and serum CK levels were mildly elevated. Pathologically, FTD in LMNA-myopathy is associated with type 2 fiber hypertrophy with lack of type 1 fiber atrophy, whereas type 1 fiber atrophy is seen in CFTD with *ACTA1* or *TPM3* mutations. Unlike CFTD due to *ACTA1* or *TPM3* mutations, type 1 fiber predominance and type 2B fiber deficiency are absent in LMNA-myopathy. These results suggest that *LMNA* analysis should be performed in CFTD patients who has the clinical features such as no high arched palate, no respiratory insufficiency and high CKemia, and has pathological features such as type 2 fiber hypertrophy and lack of type 1 fiber atrophy, type 1 fiber predominance, and type 2B fiber deficiency.

LMNA-myopathy is categorized as muscular dystrophy, and mild necrotic and regenerating processes are usually seen. However, no dystrophic features can be seen as reported herein. Higher CK levels raise the possibility of LMNA-myopathy being dystrophic in nature. On the other hand, in our series, 16 of the 78 (21%) CFTD patients with unknown cause had high CKemia. This result suggests a difficulty in making a differential diagnosis between congenital myopathy and muscular dystrophy in some cases.

Clinically, respiratory insufficiency is common, reportedly being seen in 30% of CFTD patients [7], and in 73% of L-CMD patients [4]. However, 2 CFTD patients with *LMNA* mutations in this study showed no respiratory involvement. Furthermore, in CFTD associated with *LMNA* mutations, FTD is the only pathological abnormality, while prominent dystrophic and/or inflammatory changes are seen in L-CMD. There results suggest that CFTD is the milder form of early onset LMNA-myopathy.

Acknowledgments

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GNE myopathy: A prospective natural history study of disease progression

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Abstract

Mutations in the glucosamine (UDP-*N*-acetyl)-2-epimerase/*N*-acetylmannosamine kinase gene cause GNE myopathy, a mildly progressive autosomal recessive myopathy. We performed a prospective natural history study in 24 patients with GNE myopathy to select evaluation tools for use in upcoming clinical trials. Patient clinical conditions were evaluated at study entry and one-year follow-up. Of the 24 patients, eight (33.3%) completed a standard 6-min walk test without assistance. No cardiac events were observed. Summed manual muscle testing of 17 muscles, grip power, and percent force vital capacity (%FVC) were significantly reduced ($p < 0.05$), and scores for 6-min walk test and gross motor function measure were decreased ($p < 0.1$) after one year. The decrement in %FVC was significant among non-ambulant patients, whereas the decrement in grip power tended to be greater among ambulant patients. The 6-min walk test, gross motor function measure, manual muscle testing, grip power, and %FVC reflect annual changes and are thus considered good evaluation tools for clinical trials.

Keywords: GNE myopathy; Distal myopathy with rimmed vacuoles (DMRV); Natural history; Respiratory function

1. Introduction

GNE myopathy, also known as distal myopathy with rimmed vacuoles (DMRV), is an early adult-onset myopathy with slow progression that preferentially affects the tibialis anterior muscle and commonly spares the quadriceps femoris muscles [1,2]. The disease cause is a mutation in the *GNE* gene encoding a bifunctional enzyme [uridinediphosphate-*N*-acetylglucosamine (UDP-GlcNAc) 2-epimerase and *N*-acetylmannosamine kinase] that catalyzes two rate-limiting reactions in cytosolic sialic acid synthesis [3–7]. Oral sialic acid metabolite treatment has been shown to prevent muscle atrophy and weakness in a mouse GNE myopathy model [8].

A recent phase I clinical trial with oral sialic acid was performed in Japan (ClinicalTrials.gov; identifier: NCT 01236898), and a phase II study is currently underway in the United States and Israel (ClinicalTrials.gov; identifier:

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0960-8966/

NCT01517880). A prospective natural history must be well understood prior to phase III clinical trials. We have identified genotype–phenotype correlations in our previous retrospective study [9], and found that a standard 6-min walk test (6MWT) might not be sufficient for evaluating most of patients, because the majority of Japanese patients were non-ambulant. On the other hand, respiratory function is impaired in patients with advanced GNE myopathy, and may serve as a useful evaluation tool especially among non-ambulant patients [10].

We performed a prospective study of confirmed GNE myopathy patients to assess the prospective natural history of GNE myopathy and obtain an appropriate evaluation tool. We aimed to identify evaluation items that can be used to detect disease progress within a year, with respect to observation duration of clinical trials.

2. Patients and methods

2.1. Study population and design

The present study included prospective data from genetically confirmed GNE myopathy patients who were evaluated twice (baseline and one-year follow-up) at a National Center of Neurology and Psychiatry (NCNP) hospital. All candidate patients were invited to participate in this study by mail and/or telephone. Patients who could not attend the follow-up visit were excluded from the study. The first patients were enrolled in April 2009, and last data were examined on November 25, 2013.

Approval for this study was obtained from the Medical Ethics Committee of the NCNP. Study objectives, design, risks, and benefits of participation were explained to all patients, and their written informed consent was obtained prior to enrollment.

2.2. Patients and Methods

A total of 27 Japanese patients (9 men, 18 women) participated in this study. Among them, 25 patients who completed 1-year follow up were included and two non-ambulant patients who could not visit annual evaluation were excluded. Of 25, one ambulant patient who got nephritic syndrome and resulted to 3 months bedrest and steroid therapy (maximal 1 mg/kg body weight) were excluded as it might have influenced the motor performance. A total of 24 Japanese patients (9 men, 15 women) participated in this study, of whom two women were siblings and the rest were unrelated.

Mean age at the time of data collection was 43.0 ± 12.9 years (mean ± SD). Mean age at disease onset was 25.9 ± 10.3 years (range, 15–58 years; median, 24 years). Of the 24 patients, 9 (36.0 %) were ambulant, 8 completed the 6MWT test without assistance, 1 required assistance (e.g., canes and ankle braces) and could not complete the 6MWT, and 15 (64.0 %) had lost ambulation. Among 9 ambulant patients, 4, 2, 1, and 1 patients used both cane and ankle brace, ankle brace only, cane only, and both walker and cane, respectively. Of 19 patients who used a wheelchair for transportation (4, part-time; 15, full time), 7 used wheelchair headrests, and 1 used a neck collar to prevent falling.

Medical complications and history were as follows: 3 patients had hypertension, 2 had obstructive sleep apnea syndrome, with 1 receiving treatment by continuous positive airway pressure, 2 had diabetes mellitus, hyperlipidemia, and past history of idiopathic thrombocytopenia, and 1 had atopic dermatitis, idiopathic thrombocytopenia, hypermenorrhea resulting anemia, and mastopathy. Occurrences of these diseases were similar to those of the general population of Japan.

All patients rested for more than two hours before each muscle strength test. Measurements using a hand held dynamometer of knee extension in sitting position (HHD, μ-Tas F-1®, Anima, Japan), grip power (Dynamometer®, TTM Japan), pinch power (PinchTrack™, JTECH, Japan), and occlusal force meter GM10® (NAGANO KEIKI, Japan) were repeated three times on both the right and left sides, and all six measurements were averaged for data analysis.

Muscle strength tests, including manual muscle testing (MMT) and gross motor function measure (GMFM, Japanese version; range, 0–100 [%]), were performed [11]. The following 17 muscle groups were examined: neck flexion, truncal flexion, shoulder abduction, shoulder adduction, shoulder flexion, shoulder extension, elbow flexion, elbow extension, wrist flexion, wrist extension, hip flexion, thigh adduction, thigh abduction, knee extension, knee flexion, ankle dorsiflexion, and planter flexion. Right and left MMTs were averaged, except for those corresponding to neck and truncal flexion. Summed MMT (range, 0–85) was obtained from the sum of the 17 muscle groups examined. The 6MWT was performed according to the American Thoracic Society guidelines [12] for patients who were able to walk without any assistance (canes or braces). Pinch and grip powers and MMT were measured by M.M.Y., and HHD measurement, GMFM, and 6MWT were measured by H.Y., assisted by other physiotherapists.

Patient condition was assessed by physical examination, electrocardiography (ECG), echocardiography (UCG; EF, ejection fraction; FS, fraction shortening), Holter ECG, percent force vital capacity (%FVC), lean body mass (whole body, arms, and legs by standard procedure) by dual-energy X-ray absorptiometry (DEXA; Discovery bone densitometer, Hologic, Bedford, MA), and skeletal muscle mass index (SMI) [13]. Blood tests included creatine kinase (CK) measurement. For activities of daily living (ADL) and quality of life (QOL), the Barthel index (BI, range, 0–100), modified Rankin scale (mRS, Japanese version; range, 1–5), and a 36-item short form survey (SF-36; Japanese version) were used [14,15].

Patients were asked simple question at 1-year follow up visit whether they felt any changes about their symptoms.

2.3. Data analysis

Data were summarized using descriptive statistics, including mean, standard deviation (SD), median, range, frequency, and percentage. Each variable for ambulant (including patients requiring assistance) and non-ambulant patients was compared using *t*-test. In correlation analysis, Spearman correlations were used to determine the association between each of the variables. The paired *t*-test was used to compare differences between baseline and one-year follow-up data. For this comparison, items with no significant abnormalities in all patients at baseline were excluded from annual examinations. Data under measurement (=0) were also excluded from the follow-up analysis. All analyses were performed using SPSS for Macintosh (Version 18; SPSS Inc., Chicago, IL).

3. Results

3.1. GNE mutations

A total of 37.5% (9/24) of the patients harbored a p.V572L homozygous mutation, and 25.0% (6/24) harbored a compound heterozygous mutation. Of these, 12.5% (3/24) exhibited the p.D176V /p.V572L genotype; the rest had a different mutation (Supplementary Table 1).

3.2. Baseline tests

Baseline data are shown in Table 1A. MMT revealed significant weakness both in hip adduction and ankle dorsoflexion, whereas knee extension was markedly preserved (Fig. 1). MMT of the baseline visit showed that knee extension was relatively spared, especially among ambulant patients (Supplementary Fig. 1). With respect to HHD, grip and pinch power, the number of patients who were too weak to complete measurement was 8, 8, and 6, respectively. Non-ambulant patients showed a significantly low %FVC (74.7 ± 19.3 vs. 110.5 ± 12.1, $p < 0.01$, Table 1B). Non-invasive positive pressure ventilation (NPPV) toward respiratory failure of GNE myopathy was used in two patients at night due to respiratory dysfunction and hypoxemia during hospitalization for baseline evaluation.

3.3. Cardiac functions

All patients underwent ECG, but 2 and 4 of 24 patients did not undergo UCG and Holter ECG, respectively, due to their schedules. Twenty-one patients had normal sinus rhythms on the ECG. Two right bundle branch blocks (one complete and one incomplete), a 1st degree atrioventricular block with sinus bradycardia due to beta-blocker use, and a non-specific ST-T change (but normal UCG) were observed. Wall motions on UCG were normal in all patients except in one who had a history of myocardial infarction. In addition, EF and FS were normal in all patients. Holter ECG showed normal ranges in 15 of 20 patients, whereas non-specific ST-T changes in 2, sinus tachycardia in 2, and bradycaldia in 1 were observed. Patients with ST-T changes had diabetes mellitus and/or hypertension.

3.4. Annual changes

During the study period, no patients suffered from a systemic disease or from trauma; moreover, none

Table 1
Patient characteristics and annual changes.

		n	Baseline	1 Year	*p*
Muscle testing	Summed MMT	24	36.0 ± 21.0	33.2 ± 21.0	<0.001
	6MWT (m)	8	321 ± 141.3	273.0 ± 130.6	0.061
	GMFM (%)	24	41.1 ± 39.0	39.6 ± 39.3	0.089
	HHD (N)	16	165.5 ± 98.1	165.5 ± 150.0	0.999
	Grip power (kg)	16	6.8 ± 6.3	5.3 ± 5.6	0.034
	Pinch power (N)	20	22.2 ± 18.6	20.6 ± 21.0	0.261
Pulmonary function	FVC (%)	24	88.1 ± 24.3	84.8 ± 25.7	0.03
DEXA	Whole-body lean body mass (kg)	24	31.0 ± 7.0	30.6 ± 7.4	0.226
	Arm lean body mass (kg)	24	2.6 ± 1.0	2.6 ± 1.0	0.345
	Leg lean body mass (kg)	24	8.5 ± 2.5	8.4 ± 2.6	0.97
	SMI	24	4.1 ± 1.1	4.1 ± 1.1	0.148
Laboratory data	CK (IU/L)	24	222.8 ± 220.5	191.3 ± 199.1	0.087
ADL	Barthel index	24	49.0 ± 39.6	48.1 ± 39.3	0.213
	mRS	24	3.6 ± 1.0	3.6 ± 1.0	-
SF-36	SF-36 PCS	24	10.9 ± 13.2	7.9 ± 10.7	0.148
	SF-36 MCS	24	56.7 ± 11.1	57.9 ± 9.3	0.53
	SF-36 RCS	24	46.3 ± 19.0	43.0 ± 21.6	0.241

The results of baseline and one-year follow-up evaluations for all patients are shown. A total of 33.3% (8/24) of the patients completed the 6-min walk test without assistance. Paired *t*-tests revealed significant reductions in summed MMT of 19 muscles, grip power, and %FVC after one year ($p < 0.05$), and reductions in 6-min walk test scores and gross motor function measure ($p < 0.1$).

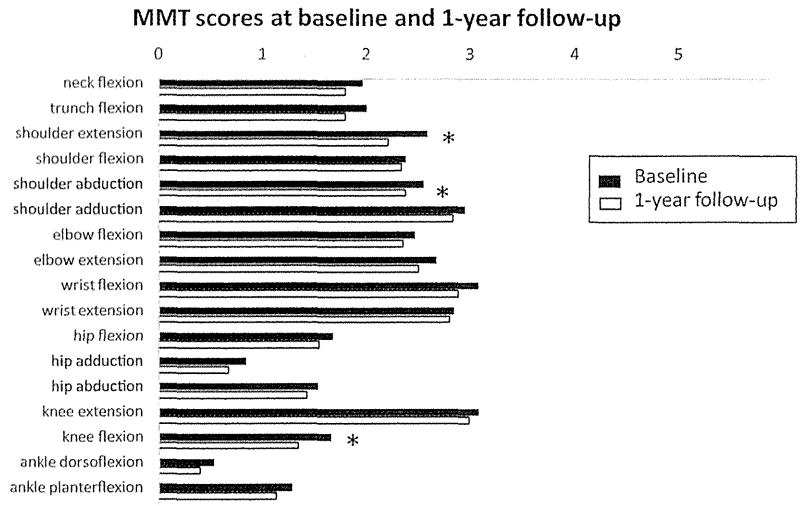

Fig. 1. MMT scores at baseline (black column) and 1-year follow-up (open column). Hip adduction and ankle dorsoflexion were markedly impaired, whereas knee extension was preserved among all the muscles examined. Shoulder extension ($p = 0.017$) and abduction ($p = 0.029$), and knee flexion ($p = 0.010$) showed significant annual decrements ($^{*}p < 0.05$).

required a major surgical intervention that could have influenced the natural course of the disease.

Of the 24 patients, the number of patients who were aware of worsening was 19 (79%). Among them, patients who were aware of worsening hand weakness, neck instability and weakness, gait disturbance, leg weakness, and/or arm weakness were 9, 7, 7, 6, and 2, respectively. Of the 8 ambulant patients not requiring assistance, 7 felt that their gait had become slower compared to the year before. In fact, one patient started using a wheelchair part-time during the one-year follow-up period. All patients who complained of neck instability and weakness were non-ambulant.

A significant reduction in summed MMT ($p < 0.01$), grip power ($p = 0.034$), and %FVC ($p = 0.030$), and a reduction in 6MWT ($p = 0.061$) scores, GMFM ($p = 0.089$), and CK ($p = 0.087$) were observed (Table 1). Among all the muscles examined, shoulder extension ($p = 0.017$) and abduction ($p = 0.029$), and knee flexion ($p = 0.010$) showed significant annual decrements (Fig. 1). Only one patient who succeeded in weight control and increased walking opportunity showed an improvement in 6MWT scores, while the results of other ambulant patients deteriorated in one year (Fig. 2A). Grip power decreased in ambulant patients (9.5 ± 6.9 to 7.1 ± 6.6, $p = 0.051$), but not in non-ambulant patients (3.3 ± 3.3 to 3.0 ± 3.0) (Table 2, Figs. 2D and E). On the other hands, changes in %FVC ($p = 0.034$) were greater in non-ambulant patients than in ambulant patients (Table 2, Figs. 2F and 2G). There were no significant changes in lean body mass, SMI, SF-36, BI, or mRS.

4. Discussion

To our knowledge, this is the first study to assess the prospective natural history of GNE myopathy. Patients with GNE myopathy were disseminated across Japan and were not concentrated around the specialized muscle center hospital, because most patients did not require specialized cardiopulmonary treatment, such as those with Duchenne muscular dystrophy. Accordingly, we selected evaluation items that are commonly accepted among physiotherapists (MMT, 6MWT, and GMFM), and measurement instruments that are relatively inexpensive (e.g., grip, pinch power, and HHD); therefore, the method presented here can be readily implemented by clinical trials and hospitals. For us, it was important that GMFM were validated in the Japanese population [10].

We found that summed MMT, grip power and %FVC were significantly changed in one year. Although statistical significance was lower in the 6MWT, a larger cohort may clearly detect deterioration, given that our study included only a small number of ambulant patients. Severity of Japanese patients is one reason for small number of ambulant patients. It was difficult for us to correct more patients, as ambulant patients were relatively small numbers in Japan. Multicenter study should be required to resolve if the 6MWT are effective tools for annual evaluation.

The 6MWT and summed MMT are important end-point item candidates for clinical trials because they can be used to determine annual changes in disease progression. Our study showed respiratory function decrement especially among non-ambulant patients, suggesting that %FVC can be a useful outcome measure for non-ambulant patients. On the other hand, the decrement in grip power was greater in ambulant patients. These results indicate that evaluation tools should be selected according to the ambulation status of patients.

On the other hand, we could not find annual changes in HHD, lean body mass, BI, mRS, and SF-36. As muscle

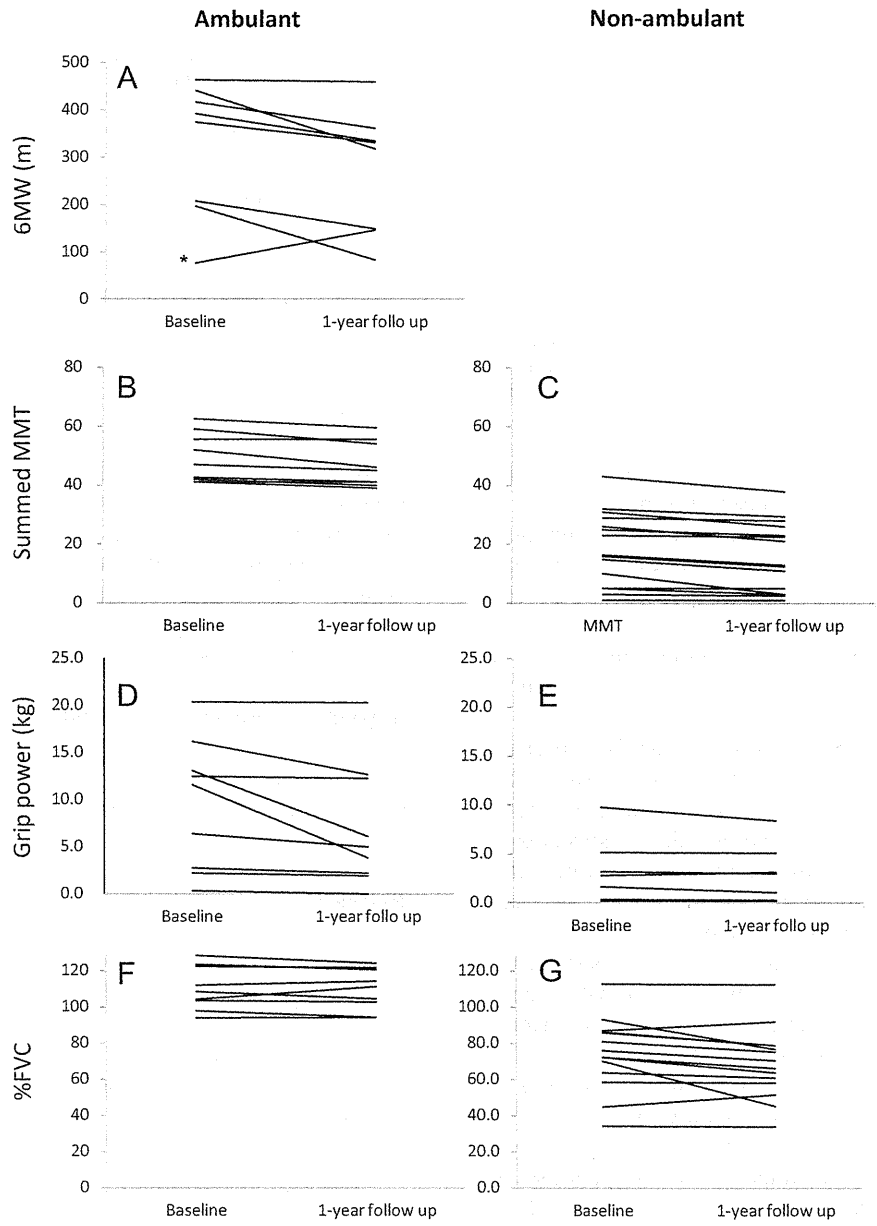

Fig. 2. Annual changes in motor functions. Right colomun: ambulant patients, left column: non-ambulant patients. A: 6MWT; B, C: summed MMT; D, E: grip power; F, G: %FVC. All patients but one (*) showed deterioration in 6MWT (A). Only one patient who showed an improved 6MWT had succeeded in weight control and had more opportunities to walk relative to baseline. Both ambulant (B) and non-ambulant (C) patient showed deterioration in summed MMT. The decrement in grip power was greater in ambulant patients (D, E), whereas the decrement in %FVC was greatern in non-ambulant patients.

strengths for knee extension were well preserved among patients with GNE myopathy, it may be difficult to detect disease progression during the one-year period. Indeed, MMTs of knee extension were preserved at follow-up evaluation. Weaker muscles, such as shoulder muscles or knee flexion muscles that showed deterioration over the one-year period, may be the possible candidates for evaluation. More detailed quantitative study must be carried out before the clinical trials. Although BI, mRS and SF-36 were unchanged, most of patients were aware of their symptoms, and indeed some parameters of muscle power were deteriorated. To detect disease progression, disease-specific QOL or ADL scales may be required.

Two patients started NPPV due to findings obtained during the study. They had been regularly followed by neurologists but had not been evaluated for respiratory function prior to the baseline visit. Both patients carried a V572L homozygous mutation with a more severely affected phenotype [9,16] and showed marked weakness, i.e., summed MMTs were under 5 and mRS was 5. It should be emphasized that patients with GNE myopathy are at risk of respiratory failure, and that physician

Table 2
Annual changes in ambulant and non-ambulant patients.

	Ambulant ($n = 9$)			Non-ambulant ($n = 15$)		
	pre	1 year	p	pre	1 year	p
Summed MMT	57.0 ± 9.5	55.0 ± 8.8	0.022	23.4 ± 14.8	20.1 ± 13.8	<0.001
GMFM (%)	87.6 ± 8.1	87.6 ± 7.9	0.933	13.2 ± 15.5	10.7 ± 11.9	0.078
HHD (N)	214.8 ± 83.2	221.6 ± 164.8	0.864	102.1 ± 80.9	93.2 ± 94.7	0.587
Grip power (kg)	9.5 ± 6.9	7.1 ± 6.6	0.051	3.3 ± 3.1	3.0 ± 3.0	0.179
Pinch power (N)	31.9 ± 18.5	30.4 ± 23.7	0.610	14.2 ± 15.2	12.6 ± 15.3	0.155
FVC (%)	110.5 ± 12.1	109.6 ± 11.5	0.624	74.5 ± 19.3	69.8 ± 19.2	0.034
CK (IU/L)	403.4 ± 273.8	343.9 ± 252.3	0.217	126.0 ± 117.0	108.3 ± 112.3	0.246

Annual changes according to ambulation status. Summed MMT showed a significant decrement in both ambulant and non-ambulant patients. On the other hands, %FVC tended to be preserved in ambulant patients, indicating that pulmonary functional impairment progressed only in non-ambulant patients. The decrement in grip power was also remarkable in non-ambulant patients.

should evaluate respiratory function if patients become non-ambulant and show advanced weakness.

Our study is the first to assess cardiac function in relation to GNE myopathy. However, we were unable to find any disease-related abnormalities in ECG, Holter ECG, and UCG even though cardiac involvement had been previously implicated in a mouse model [8]. Our data suggest that GNE myopathy does not involve cardiomyopathy.

There were limitations with our data analysis because of the small number of participants and short study period. Moreover, we are aware that recruitment of patients from NCNP, a national hospital highly specialized in muscle disease, is a potential source of selection bias, as they may be more severely affected than the general patient population. Japanese patients, especially those who carry a V572L homozygous mutation, show a more severely affected phenotype than previously reported [9,15]; in fact, no studies have ever reported on respiratory failure in GNE myopathy other than the one from Japan [10]. However, our study suggests that non-ambulant patients can be evaluated with %FVC, and that physician should pay attention to the yearly decrement in respiratory function. In rare diseases such as GNE myopathy, large-scale studies tend to be difficult. We have established a Japanese national GNE myopathy patient registry (Registration of Muscular Dystrophy; REMUDY, http://www.remudy.jp) to perform a broader investigation of associated conditions and for long-term observation of patients.

In conclusion, 6MWT, summed MMT, GMFM, grip power tests, and %FVC may be good clinical evaluation tools for trials and to correlate with disease progression, although %FVC and grip power should be used according to ambulation status. Our study revealed that both ambulant and non-ambulant GNE myopathy are basically progressive and do not involve cardiac abnormalities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nmd.2014.02.008.

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