Journal of Human Genetics (2016), 1–12

www.nature.com/jhg

ORIGINAL ARTICLE

Target resequencing of neuromuscular disease-related genes using next-generation sequencing for patients with undiagnosed early-onset neuromuscular disorders

Yuri Kitamura[1,2], Eri Kondo[2,3], Mari Urano[2], Ryoko Aoki[2] and Kayoko Saito[1,2]

Neuromuscular disorders are clinically and genetically heterogeneous diseases with broadly overlapping clinical features. Progress in molecular genetics has led to the identification of numerous causative genes for neuromuscular disorders, but Sanger sequencing-based diagnosis remains labor-intensive and expensive because the genes are large, the genotypes and phenotypes of neuromuscular disorders overlap and multiple genes related to a single phenotype exist. Recently, the advent of next-generation sequencing (NGS) has enabled efficient, concurrent examination of several related genes. Thus, we used NGS for target resequencing of neuromuscular disease-related genes from 42 patients in whom undiagnosed early-onset neuromuscular disorders. Causative genes were identified in 19/42 (45.2%) patients (six, congenital muscular dystrophy; two, Becker muscular dystrophy (BMD); three, limb-girdle muscular dystrophy; one, concurrent BMD and Fukuyama congenital muscular dystrophy; three, nemaline myopathy; one, centronuclear myopathy; one, congenital fiber-type disproportion; one, myosin storage myopathy; and one, congenital myasthenic syndrome). We detected variants of uncertain significance in two patients. In 6/19 patients who received a definitive diagnosis, the diagnosis did not require muscle biopsy. Thus, for patients with suspected neuromuscular disorders not identified using conventional genetic testing alone, NGS-based target resequencing has the potential to serve as a powerful tool that allows definitive diagnosis.
Journal of Human Genetics advance online publication, 30 June 2016; doi:10.1038/jhg.2016.79

INTRODUCTION

Neuromuscular disorders such as congenital muscular dystrophy (CMD), Duchenne muscular dystrophy (DMD), limb-girdle muscular dystrophy (LGMD), congenital myopathy (CM) and congenital myasthenic syndrome (CMS) manifest during the neonatal and childhood periods together with the onset of hypotonia and muscle weakness. These disorders form a clinically and genetically heterogeneous group of diseases that present common features, mainly hypotonia, muscle weakness, motor retardation and abnormal posture. The broadly overlapping clinical characteristics can make diagnosis challenging.

Neuromuscular disorders have traditionally been diagnosed based on the onset period, clinical symptoms, clinical course and neurological findings. However, because of major advances in molecular genetics over the past 20 years, neuromuscular disorders can now be definitively diagnosed through molecular genetic testing. Starting with the discovery of the dystrophin gene in 1987,[1] the causative genes related to numerous neuromuscular disorders have been successively identified, and these genes currently number 519.[2] Today, neuromuscular disorders are diagnosed based on both characteristic pathological findings and causative genes.[3–6] For CMD and spinal muscular atrophy (SMA) in particular, diagnosis through molecular genetic testing without muscle biopsy is becoming the gold standard. Furthermore, although CM can be differentiated to a certain degree based on muscle computed tomography and magnetic resonance imaging (MRI) findings mainly pertaining to the distribution of affected muscles, the findings are not sufficiently specific for CM and similar disorders. Therefore, muscle biopsy and genetic testing are essential for definitive diagnosis. However, during genetic diagnoses of neuromuscular disorders, difficulties arise due to the particular features of these disorders, and a definitive diagnosis is thus not obtained in nearly 40% of affected patients.[7–9] The critical genetic features of the aforementioned neuromuscular disorders include several of the causative genes being very large, broadly overlapping genotypes and clinical phenotypes, and the existence of several genes related to a single phenotype.

With regard to causative genes, many of the proteins expressed in muscle are extremely large, and their genes thus contain numerous exons. For example, the *DMD* gene is 2200 kb in total length and contains 79 exons, the *TTN* gene encoding giant titin proteins contains 363 exons, and the *NEB* gene encoding nemaline proteins comprises 183 exons. One example of genotype/phenotype overlap is

[1]Affiliated Field of Medical Genetics, Division of Biomedical Engineering and Science, Graduate School of Tokyo Women's Medical University, Tokyo, Japan; [2]Institute of Medical Genetics, Tokyo Women's Medical University, Tokyo, Japan and [3]Department of Pediatrics, Imperial Gift Foundation AIIKU Maternal and Child Health Center, AIIKU Clinic, Tokyo, Japan
Correspondence: Professor K Saito, Institute of Medical Genetics, Tokyo Women's Medical University, 10-22 Kawada-cho, Shinjuku-ku, Tokyo 162-0054, Japan.
E-mail: saito.kayoko@twmu.ac.jp
Received 5 February 2016; revised 23 April 2016; accepted 16 May 2016

α-dystroglycanopathy: nearly 20 genes have been identified that encode proteins involved in α-dystroglycan-related pathways, and these disorders are considered to be manifested through a common mechanism. The clinical phenotypes cover a very wide range from Walker-Warburg syndrome, which is a severe form of CMD accompanied by severe brain-structure abnormalities and ophthalmic abnormalities, to mild LGMD in the form of late-onset muscle weakness.[10,11] Lastly, an example of several genes being related to a single phenotype is nemaline myopathy (NM), the most frequent form of CM and a disease group characterized by the presence of nemaline bodies.[12,13] To date, nine genes have been identified that encode thin-filament proteins and related proteins: *TPM3*, *NEB*, *ACTA1*, *TPM2*, *TNNT1*, *KBTBD13*, *CFL2*, *KLHL40* and *LMOD3*.

Because several of the aforementioned genes are large, substantial labor, time and costs are required for the individual analysis of causative genes through conventional Sanger sequencing for differential diagnosis of clinically and genetically heterogeneous neuromuscular disorders. However, the recent advent of next-generation sequencing (NGS), which enables investigators to perform rapid high-throughput analysis, has made it possible to easily reduce both costs and time. Furthermore, by combining NGS results and pathological findings from muscle biopsy, causative genes can now be identified rapidly, efficiently and reliably.[14,15] Herein, we performed NGS-based target resequencing on causative genes related to neuromuscular disorders in 42 patients in whom undiagnosed neuromuscular disorders were suspected based on the manifestations that had developed during the neonatal through childhood period.

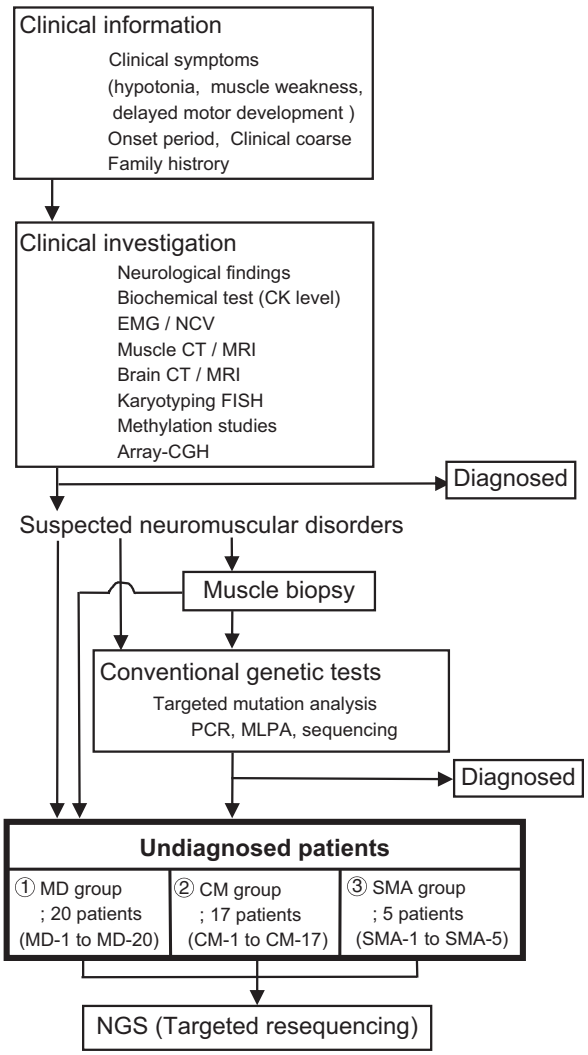

Figure 1 Diagnostic process of this study and three clinical categories of undiagnosed patients. CGH, comparative genomic hybridization; CK, creatine kinase; CM, congenital myopathy; CT, computed tomography; EMG, electromyogram; FISH, fluorescence *in situ* hybridization; MD, muscular dystrophy; MLPA, multiplex ligation-dependent probe amplification; NCV, nerve conduction velocity; SMA, spinal muscular atrophy.

MATERIALS AND METHODS

Patients

The study participants were 42 Japanese patients who had been referred to the Institute of Medical Genetics at Tokyo Women's Medical University between 2004 and 2014. The patients had shown symptoms of muscle weakness and hypotonia neonatally and/or during childhood, and neuromuscular disorders were thus suspected based on their clinical symptoms and muscle pathology findings. Pediatric neurology specialists made diagnoses based on the presence of muscle weakness and hypotonia, other physical findings, clinical course, muscle imaging features and muscle pathology findings, and divided the 42 patients into three groups accordingly: (1) MD group, 20 patients (MD-1 to MD-20); (2) CM group, 17 patients (CM-1 to CM-17); and (3) SMA group, five patients (SMA-1 to SMA-5) (Figure 1 and Table 1).

Muscle biopsy was performed in 16 of the 42 patients. For 34 patients, a definitive diagnosis was not yielded by conventional genetic tests (*DMD* gene: multiplex ligation-dependent probe amplification (MLPA); and/or *SMN1* gene exons 7, 8: MLPA; and/or *FKTN* gene: detection of ancestral founder mutation containing a 3-kb retrotransposal insertion in the 3′-non-coding region; and/or *DMPK* gene: CTG repeat detection). However, in patient MD-12, deletion of exons 45–53 in the *DMD* gene causing BMD was detected using MLPA, but this did not correspond to the patient's clinical findings, which were thus attributed to a potentially more pathogenic mutation.

Patients with suspected genetic chromosomal disorders such as trisomy 21 and Prader–Willi syndrome, central nervous system disorders or spinal cord injuries were excluded. Among neuromuscular disorders, myotonic dystrophy is caused by the expansion of a CTG trinucleotide repeat in the non-coding region of *DMPK* gene. Patients with these gene expansions were excluded because the expansions cannot be detected using NGS.

This study was conducted after explaining its purpose and contents to all patients and their parents, and obtaining written consent. Moreover, this study was approved after review by the ethics committee of Tokyo Women's Medical University (approval number: 2709).

Target resequencing

We used the SOLiD 4 system platform and the Ion PGM platform (Thermo Fisher Scientific, Waltham, MA, USA). We first used the SOLiD 4 system in 25 patients. Later, Ion PGM was introduced. Thirteen patients whose pathogenic mutations were not identified using the SOLiD 4 system and 18 patients who had been referred after May 2012 underwent testing using the Ion PGM platform.

Panel design

SOLiD. The 42-gene panels (Supplementary Table S1) were designed using the eArray tool (Agilent Technologies, Santa Clara, CA, USA) for the capture of non-contiguous target muscle-disease genomic regions that were approximately 3 Mb in total length.

Ion PGM. The 74-gene panels (Supplementary Table S1) were designed automatically using Ion Ampliseq Designer (Thermo Fisher Scientific); two pools of 3786 primers were generated for amplifying genomic regions. Coverage included the coding DNA sequence region and at least five intronic flanking nucleotides of each targeted gene to capture intron/exon splice junctions, as well as both 5′ promoter regions and 3′ untranslated regions. The amplicons covered 95.32% of the target sequence (515.15 kb).

Table 1 Summary of clinical information and investigations, and conventional genetic tests performed

										Conventional genetic tests			
Patient	*Sex*	*Age*[a]	*Clinical features*	*Family history*	*Maximum motor ability*	*Current motor ability*	*Age of death (cause)*	*CK level*	*Muscle biopsy findings*	*FCMD*[b]	*DMD*[c]	*SMA*[d]	*MD*[e]
MD group													
MD-1	M	11y 1m	Severe hypotonia and muscle weakness from prenatal period, cognitive disability, delayed motor development, bilateral cataracts, repeated afebrile seizures, pachygria and cerebellar cysts	—	Sitting with aid	Sitting with aid		X40	NE	△[f]			
MD-2	M	3y 0m	Severe hypotonia and muscle weakness from prenatal period, cognitive disability, delayed motor development, retinal detachment, pachygyria and cerebellar cysts	—	Poor head control	Poor head control		X60	NE	△			
MD-3	M	6y 6m	Severe hypotonia and muscle weakness from prenatal period, cognitive disability, delayed motor developmentpolymicrogyria and lissencephaly	—	Poor head control	Poor head control		X30	Dystrophic change	△			
MD-4	M	6y 0m	Muscle weakness from 5 months of age, cognitive disability, delayed motor development, pachygyria	—	Independent walking	Independent walking		X25	NE	×[g]	×		
MD-5	F	16y 2m	Muscle weakness from 1 year of age, cognitive disability, delayed motor development, pachygyria	—	Independent walking	Independent walking		X25	Dystrophic change, absent of α-dystroglycan staining		×		
MD-6	F	4y 6m	Severe hypotonia and muscle weakness from prenatal period, delayed motor development, joint contractures, scoliosis, diffuse white matter hypodensities on brain MRI	—	Sitting unaided	Sitting unaided		X8	Dystrophic change, absence of merosin staining				
MD-7	M	13y 3m	Lower limb muscle weakness from 3 years of age with slowly progressive, difficulties in climbing stairs, positive Gowers sign diffuse white matter hypodensities on brain MRI	—	Independent walking	Ambulant with aid		X35	Dystrophic change, decreased of merosin staining				
MD-8	F	19y 4m	Mild hypotonia and muscle weakness from prenatal period, proximal joint contractures, distal joint laxity, progressive respiratory impairment necessitating tracheostomy	—	Independent walking	Wheelchair		Normal	Variation in fiber size			×	
MD-9	M	9y 5m	Proximal muscle weakness from 3 years of age, frequent falls from 3 years of age, myocardial symptoms	—	Independent walking	Independent walking		X13	NE		×		
MD-10	M	6y 4m	Proximal muscle weakness from 6 years of age, slow running	—	Independent walking	Independent walking		X13	NE		×		
MD-11	F	32y 1m	Proximal muscle weakness slow progressive from 10 years of age, joint contractures,	—	Independent walking	Wheelchair		X4	Dystrophic change				

Table 1 (Continued)

Patient	*Sex*	*Age*[a]	*Clinical features*	*Family history*	*Maximum motor ability*	*Current motor ability*	*Age of death (cause)*	*CK level*	*Muscle biopsy findings*	*Conventional genetic tests*			
										FCMD[b]	*DMD*[c]	*SMA*[d]	*MD*[e]
			Inability to walk from 28 years of age										
MD-12	M	5y 5m	Severe hypotonia and muscle weakness from prenatal period, respiratory impairment treated with non-invasive ventilation, moderate cognitive disability, delayed motor development, pachygyria and hypoplastic operculum	—	Poor head control	Poor head control		X6	Dystrophic change	×	○[h]		
MD-13	M	10m	Mild hypotonia and muscle weakness from prenatal period, delayed motor development, pachygria	—	Poor head control	Poor head control		X30	NE	△			
MD-14	M	3y 9m	Severe hypotonia and muscle weakness from prenatal period, cognitive disability, delayed motor development, polymicrogyria	—	Sitting with aid	Sitting with aid		X35	NE	△			
MD-15	M	10m	Severe hypotonia and muscle weakness from prenatal period, joint contractures, cognitive disability, delayed motor development, polymicrogyria	—	Poor head control	Poor head control		X33	NE	×			
MD-16	F	2y 8m	Mild hypotonia and muscle weakness from prenatal period, delayed motor development	—	Sitting unaided	Sitting unaided		X15	Dystrophic change	×		×	
MD-17	F	7m	Severe hypotonia and muscle weakness from prenatal period, delayed motor development, acrocephaly	—	Poor head control	Poor head control		X25	NE	×			
MD-18	F	3y 3m	Mild hypotonia and muscle weakness from prenatal period, delayed motor developmentspasticity quadriplegia	—	Rolling over	Rolling over		Normal	NE				
MD-19	M	39y 5m	Muscle weakness from 3 months of age, cognitive disability, delayed motor development	—	Ambulant with aid	Wheelchair		X10	NE		×		
MD-20	F	3y 3m	Muscle weakness from 1 year of age, pseudohypertrophy of calf muscles	—	Independent walking	Independent walking		X60	NE		×		
CM group													
CM-1	M	17y 6m	Mild hypotonia and muscle weakness from prenatal period, myopathic face with high-arched palate, lower limb muscle pain	—	Independent walking	Independent walking		Normal	Nemaline bodies				
CM-2	F	11y 6m	Mild hypotonia from prenatal period, wadding gait, proximal and distal muscle weakness slowly progressive	—	Independent walking	Independent walking		Normal	NE			×	
CM-3	M	4y 5m	Severe hypotonia and muscle weakness from prenatal period, respiratory impairment,	—	Rolling over	Rolling over		Normal	Nemaline bodies				×

Table 1 (Continued)

Patient	Sex	Age[a]	Clinical features	Family history	Maximum motor ability	Current motor ability	Age of death (cause)	CK level	Muscle biopsy findings	Conventional genetic tests			
										FCMD[b]	DMD[c]	SMA[d]	MD[e]
			delayed motor development, ophthalomoplegia, dysphagia, myopathic face with high-arched palate										
CM-4	F	1y 5m	Severe hypotonia and muscle weakness from prenatal period, respiratory impairment treated with non-invasive ventilation, myopathic face with high-arched palate, dilated cardiomyopathy	—	Poor head control	—	3y 5m (dilated cardiomyopathy)	Normal	Fiber type disproportion			×	×
CM-5	M	17y7m	Frequent falls from 6 years of age, joint contractures, waddling gait	—	Independent walking	Independent walking		X2	Type 2 fiber predominance			×	
CM-6	F	12y 1m	Frequent falls from 9 years of age,Achilles tendon contractures, difficulties in sporting activities in school	Grand-mother-mater-nal aunt	Independent walking	School sports difficulty		X4	Internal nuclei				
CM-7	M	6y 9m	Fetal akinesia, multiple joint contractures, pulmonary hypoplasia, delayed motor development, myopathic face with high-arched palate and elongated face, rocker-bottom feet	—	Sitting unaided	Sitting unaided		Normal	Variation in fiber size				×
CM-8	M	6y 2m	Mild hypotonia and muscle weakness from prenatal period, myopathic face with open mouth and elongated face, scoliosis	—	Independent walking	Independent walking		Normal	NE			×	
CM-9	M	2y 2m	Severe hypotonia and muscle weakness from prenatal period, respiratory impairment, delayed motor development, multiple joint contractures, ophthalomoplegia, myopathic face with high-arched palate and open mouth	—	Rolling over	Rolling over		Normal	NE			×	×
CM-10	M	8y 10m	Mild hypotonia and muscle weakness from prenatal period, myopathic face with high-arched palate	—	Independent walking	Independent walking		X2	NE			×	
CM-11	F	18y 3m	Mild hypotonia from prenatal period, distal and upper limb muscle weakness slowly progressive, delayed motor development	—	Independent walking	Independent walking		Normal	NE				×
CM-12	M	2y 0m	Mild hypotonia and muscle weakness from prenatal period, myopathic face with high-arched palate, scoliosis	—	Independent walking	Independent walking		Normal	NE			×	
CM-13	M	12y 4m	Mild hypotonia and muscle weakness from prenatal period, severe respiratory impairment treated with invasive ventilation, scoliosis	—	Sitting alone	Wheelchair		Normal	NE			×	

Table 1 (Continued)

Patient	*Sex*	*Age*[a]	*Clinical features*	*Family history*	*Maximum motor ability*	*Current motor ability*	*Age of death (cause)*	*CK level*	*Muscle biopsy findings*	*Conventional genetic tests*			
										FCMD[b]	*DMD*[c]	*SMA*[d]	*MD*[e]
CM-14	F	2y 0m	Severe hypotonia and muscle weakness from prenatal period, respiratory impairment with invasive ventilation, dysphagia, multiple joint contractures	—	Poor head control	—	2y 0m (respiratory failure)	Normal	NE			×	×
CM-15	M	7m	Severe hypotonia and muscle weakness from prenatal period, severe respiratory impairment with diaphragmatic paralysis necessitating tracheostomy	—	Poor head control	Poor head control		Normal	Variation in fiber size			×	×
CM-16	F	13y 5m	Mild hypotonia and muscle weakness from prenatal period, myopathic face with high-arched palate	—	Independent walking	Independent walking		X2	NE				
CM-17	F	1y 10m	Fetal akinesia, respiratory impairment, delayed motor development, multiple joint contractures, myopathic face with high-arched palateover-lapping fingers, rocker-bottom feet	—	Sitting alone	Sitting alone		Normal	NE				
SMA group													
SMA-1	M	10m	Severe hypotonia and muscle weakness from prenatal period, presence of finger fasciculations	—	Poor head control	Poor head control		Normal	NE			×	
SMA-2	M	1y 6m	Severe hypotonia and muscle weakness from prenatal period	—	Poor head control	—	1y 6m (respiratory failure)	Normal	NE			×	
SMA-3	M	7m	Severe hypotonia and muscle weakness from prenatal period, multiple joint contractures, tespiratory impairment, myopathic face with high-arched palate	—	Poor head control	Poor head control		Normal	NE			×	
SMA-4	F	7m	Severe hypotonia and muscle weakness from prenatal period, presence of finger fasciculation, respiratory insufficiency treated with invasive ventilation	—	Poor head control	Poor head control		Normal	NE			×	×
SMA-5	F	1y 6m	Severe hypotonia and muscle weakness from prenatal period, Presence of tongue fasciculation, respiratory impairment treated with invasive ventilation	—	Rolling over	Rolling over		Normal	Predominant large group atrophy			×	×

Abbreviations: MD, muscular dystrophy; CM, congenital myopathy; CK, creatine kinase (normal: <150); NE, not examined; SMA, spinal muscular atrophy.
[a]Age at last follow-up.
[b]Detection of Japanese founder mutation containing 3-kb retrotransposal insertion in the 3' non-coding region of *FKTN*.
[c]Multiplex ligation-dependent probe amplification (MLPA) of the *DMD*.
[d]MLPA of *SMN1* exons 7 and 8.
[e]Triplet repeat CTG expansion of *DMPK* of congenital myotonic dystrophy.
[f]Detected pathogenic mutation was heterozygous.
[g]Not detected.
[h]Deletion of exons 45–53 in the *DMD* was detected.

Table 2 Clinical findings and prediction scores of novel missense mutations identified by NGS

					Clinical findings								
			Nucleotide	*Amino acid*			*PolyPhen-2*[a]				*1000 Genomes*		
Patient	*Gene*	*Accession no.*	*change*	*change*	*Muscle biopsy*	*Brain imaging*	*(Hum Div)*	*SIFT*[b]	*GERP++*[c]	*PhyloP*[d]	*Project*	*dbSNP132*
MD-4	*POMT2*	NM_013382	c.1248C>G	p.His416Gln	NE	Mild pachygyria[e]	1	0	3.67	1.239	ND	ND
MD-5	*POMT2*	NM_013382	c.422C>T	p.Tyr141Ser	α-Dystroglycan immunostaining: absent[f]	Mild pachygyria[g]	1	0	5.97	2.283	ND	ND
	POMT2	NM_013382	c.559C>T	p.Leu187Phe			0.979	0.01	4.63	1.297	ND	ND
CM-3[h]	*RYR1*	NM_000540	c.4718C>T	p.Pro1573Leu	Nemaline bodies	NE	1	0	5.01	4.05	ND	ND
	RYR1	NM_001042723	c.7585G>A	p.Asp2529Asn			0.987	0	4.41	2.416	ND	ND
CM-8	*RAPSN*	NM_005055	c.1004T>G	p.Ile335Ser	HE staining: fiber size variation, electron microscopy of NMJ: simplification of the primary fold[i]	NE	0.99	0.01	5.88	2.252	ND	ND

Abbreviations: HE staining, hematoxylin–eosin staining; ND, no detected; NE, not examined; NMJ, neuromuscular junction.
[a]PolyPhen-2 scores to 1 are likely to be pathogenic.
[b]SIFT scores less than 0.05 are likely to be pathogenic.
[c]GERP++ scores above 5 are highly conserved.
[d]PhyloP scores above 2.5 are highly conserved.
[e]shown in Figure 2a.
[f]shown in Figure 2f.
[g]shown in Figure 2b.
[h]Patient CM-3 was previously reported as case report in Kondo *et al*,[43]
[i]shown in Figure 2m.

Library preparation and sequencing

SOLiD. We used standard protocols to extract genomic DNA from blood, and then used 3 μg of the DNA to construct a library composed of adaptor-ligated randomly fragmented DNA by employing the SOLiD Fragment Library Construction Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The adaptor-ligated DNA was captured through hybridization in solution with custom-designed cRNA oligonucleotide baits as per the manufacturer protocols. DNA sequencing was performed using Paired-End Sequencing (version 1.3) on the SOLiD 4 system.

Ion PGM. Genomic DNA was extracted from blood by using standard protocols, and 10 ng samples of the DNA were amplified and adaptor-ligated libraries were prepared by employing the Ion Ampliseq Library Kit with Ion Ampliseq Custom Primer Pool protocols according to the manufacturer's instructions (Thermo Fisher Scientific). DNA was sequenced using the Ion PGM 200 Sequencing Kit and an Ion Torrent PGM (Thermo Fisher Scientific).

Data analysis

Data from the SOLiD and the PGM runs were processed using SOLiD BioScope Software v1.3.1 and Ion Torrent Suite 3.2 software (Thermo Fisher Scientific), respectively. Sequences were aligned to the human genome reference (hg19). The obtained single-nucleotide variants and insertions/deletions were combined. All of the variants were filtered against dbSNP132 and annotated using ANNOVAR and a custom analysis pipeline employing dbNSFP,[16] which includes the relevant prediction scores such as SIFT[17] and PolyPhen-2,[18] conservation scores such as PhyloP[19] and GERP++,[20] and other related information, including allele frequencies observed in the 1000 Genomes Project data[21] and the NHLBI Exome Sequencing Project ESP6500 data.[22]

Sanger sequencing-based confirmation of variants detected using NGS

All potential pathological variants detected using NGS were confirmed by means of Sanger sequencing performed using a 3130xl Genetic Analyzer (Thermo Fisher Scientific), according to the manufacturer's protocol.

mRNA analysis

When aberrant splicing was suspected, total RNA was extracted from blood using a TRIzol RNA Isolation Reagents (Thermo Fisher Scientific) and reverse transcribed with an oligo$(dT)_{20}$ primer using SuperScript III reverse transcriptase (Thermo Fisher Scientific). mRNA was amplified by reverse transcriptase-PCR and the amplified fragment was directly sequenced by Sanger sequencing.

Validation of identified variants

We determined variants that had been reported previously in the literature or were classified as pathogenic mutations in databases, and we classified novel nonsense mutations, insertions and deletions, and splice-site mutations as pathogenic because they alter protein structures. We determined a variant to be a novel missense variant if it was not present in any of these databases: 1000 Genomes Project data, NHLBI Exome Sequencing Project ESP6500 data, the NCBI dbSNP database[23] and the Leiden Muscular Dystrophy database.[24] Furthermore, we classified these identified mutations as pathogenic missense mutations if the gene corresponded to the patient's clinical features, such as histological and ultrastructural findings from muscle biopsy or other specific and significant findings, and referred to the aforementioned prediction scores to evaluate allele frequency and determine whether the gene was pathogenic.

A disease diagnosis was considered 'definitive' if a pathogenic mutation was either detected in both alleles of a gene showing autosomal recessive inheritance, or detected in one allele of a gene showing autosomal dominant inheritance. If a patient carried two autosomal recessive mutations, we confirmed whether one gene carrying the mutation had come from each parent.

Finally, we considered a novel mutation to be a variant of uncertain significance if it was detected but could not be confirmed in either parent, or if we could not readily determine whether the mutation was pathogenic.

Table 3 Mutations detected by molecular genetic testing in the MD group

Patient	*Gene*	*Mutation*	*Detection NGS tool*	*Zygosity*	*Inheritance*	*Reference*	*Genetic diagnosis*
MD-1	*FKTN*	c.647+2084G>T	SOLiD[a]	het	AR	Leiden database[b]	FCMD
		founder mutation[c]	—[d]	het		Leiden database	
MD-2	*FKTN*	c.139C>T p.Arg47X	SOLiD	het	AR	Leiden database	FCMD
		founder mutation	—	het		Leiden database	
MD-3	*FKTN*	c.515A>G p.His172Arg	SOLiD	het	AR	Leiden database	FCMD
		founder mutation	—	het		Leiden database	
MD-4	*POMT2*	c.1139A>C p.Asp380Ala	SOLiD	het	AR	Leiden database	LGMD2N
		c.1248C>G p.His416Gln	SOLiD	het		Novel	
MD-5	*POMT2*	c.422A>C p.Tyr141Ser	SOLiD	het	AR	Novel	LGMD2N
		c.599C>T p.Leu187Phe	SOLiD	het		Novel	
MD-6	*LAMA2*	c.4048C>T p.Arg1350X	SOLiD	homo	AR	Leiden database	MDC1A
MD-7	*LAMA2*	c.4645C>T p.Arg1549X	SOLiD	het	AR	Leiden database	MDC1A
MD-8	*COL6A2*	c.812G>A p.Gly271Asp	ion PGM[e]	het	*De novo*	Leiden database	UCMD
MD-9	*DMD*	c.10033C>T p.Arg3345X	SOLiD	het	*De novo*	Leiden database	BMD somatic mosaicism
MD-10	*DMD*	c.1705-18T>G (splicing)	ion PGM	het	Unknown	Novel	BMD
MD-11	*CAPN3*	c.1790delA p.Lys597fs	ion PGM	het	AR	Novel	LGMD2A
		c.2264-1G>A (splicing)	ion PGM	het		Novel	
MD-12	*FKTN*	c.139C>T p.Arg47X	SOLiD	het	AR	Leiden database	FCMD+BMD
		c.165_166ins124	(mRNA)[f]	het		Novel	
	DMD	del exon45-53	—	het	X-linked	Leiden database	

Abbreviations: AR, autosomal recessive; BMD, becker muscular dystrophy; FCMD, fukuyama congenital muscular dystrophy; het, heterozygous; homo, homozygous; LGMD2N, limb-girdle muscular dystrophy type 2; MDC1A, merosin-deficient congenital muscular dystrophy 1A; UCMD, ullrich congenital muscular dystrophy.
[a]SOLiD 4 system.
[b]Leiden Muscular Dystrophy database: http://www.dmd.nl/.
[c]Japanese founder mutation is a 3-kb retrotransposal insertion in the 3' non-coding region of the *FKTN*.
[d]Target analysis.
[e]Ion PGMTM.
[f]mRNA analysis.

RESULTS

Sequencing summary

The SOLiD sequencing yielded an output of 0.79 Gb per sample, with a median sequencing depth of 84.17×. The Ion PGM sequencing yielded an output of 1.95 Mb per sample, with a median sequencing depth of 442.8×. Both approaches allow reliable detection of sequence variants with high accuracy.

Identified mutations and clinical and histological findings

Causative genes were identified in 19/42 (45.2%) patients, and 25 pathogenic mutations (12 novel and 13 previously reported mutations) were identified in 12 genes. Of these, six were novel missense mutations. The novel mutations were determined to be pathogenic based on the histological and ultrastructural findings from muscle biopsy or brain imaging features specific to each. All of these mutations were found to be associated with high levels of pathogenicity based on the prediction scores and minor allele frequencies (Table 2). Variant of uncertain significance were detected in 2 of the 42 patients (patients CM-8 and -9).

MD group. In six genes in the 20 patients with the MD phenotype (*FKTN*, *POMT2*, *LAMA2*, *COL6A2*, *DMD* and *CAPN3*), 14 pathogenic mutations were identified (six novel and eight previously reported mutations), and in 12 patients (60.0%) a definitive diagnosis was obtained (Table 3).

Definitive diagnoses were made in 12 cases, as follows: 5 cases with dystroglycanopathies (3, Fukuyama congenital muscular dystrophy (FCMD); 2, LGMD2N); cases with merosinopathies (one each, merosin-deficient congenital muscular dystrophy (MDC) 1A and partial MDC1A); 1 case with collagenopathy (Ullrich congenital muscular dystrophy); 2 cases with dystrophinopathy (BMD); 1 case of LGMD1A; and 1 case with concurrent BMD and FCMD. In 5 of the 12 cases that received a definitive diagnosis, muscle biopsy was not performed.

Specifically, in patient MD-1, a pathogenic mutation present within the *FKTN* intron was identified. In MD-9, mutant alleles were detected at a high rate (70.4%), and somatic mosaicism was identified. In MD-7, we found a heterozygous mutation in *LAMA2*, and the other mutation is missing, the patient's brain MRI showed diffuse white matter hypodensities in the bilateral periventricular areas (Figure 2d), so we made the diagnosis of MDC1A. In MD-12, hypotonia was severe, the patient's neck was unstable at age 5, and brain abnormalities were identified. In this case, an in-frame deletion of exons 45–53 in the *DMD* gene causing BMD was detected using MLPA, and then NGS analysis identified an *FKTN* point mutation. Furthermore, based on *FKTN* mRNA analysis, a 124-bp insertion mutation was identified near exon 4. Thus, we diagnosed concurrent BMD and FCMD.

CM group. In six genes in the 17 patients presenting the CM phenotype (*NEB*, *RYR1*, *ACTA1*, *MYH7*, *DNM2* and *RAPSN*), 11 pathogenic mutations were detected (six novel and five previously reported mutations), and in 7 patients (41.1%), a definitive diagnosis was obtained (Table 4). Two more patients harbored likely pathogenic mutations. Patient CM-8 presented clinical features of NM such as mild hypotonia and muscle weakness with a myopathic face and scoliosis. This patient was found to carry a *de novo* p.Ala155Val mutation in *TPM2*, which was not previously reported, and the level of pathogenicity was high according to the prediction scores (Polyphen-2: 1; SIFT: 0). Patient CM-9 presented severe hypotonia and was found to harbor the novel nonsense mutation p.Arg103X in *CCDC78*. We highly suspected that these two mutations may be

pathogenic; however, we had not taken a muscle biopsy from patient CM-8, so we could not confirm the pathological findings. Meanwhile, we could not confirm whether the parents of patient CM-9 harbored the same mutation because we could not follow-up on them any longer. Therefore, we could not readily determine whether the mutations were pathogenic.

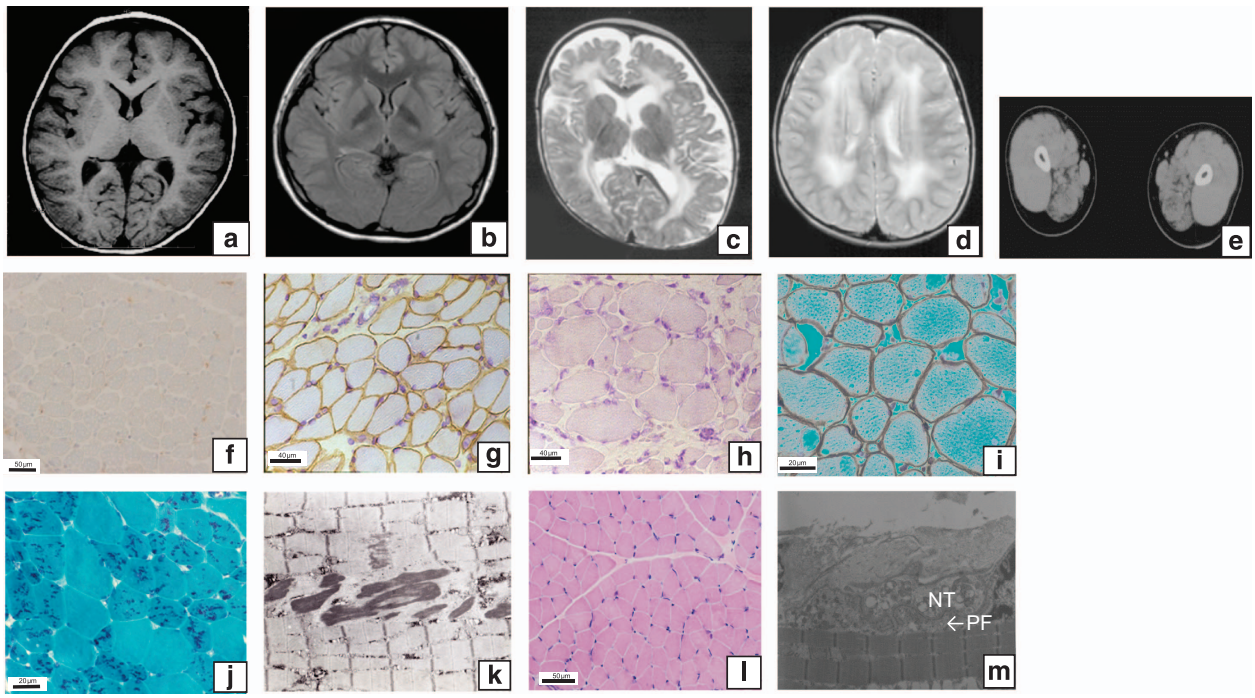

Figure 2 Findings of imaging and muscle biopsy. (**a**) T1-weighted brain MRI of patient MD-4 at age 8 months, showing mild pachygyria in the frontal region. (**b**) T2 FLAIR-weighted brain MRI of patient MD-5 at age 15 years, showing mild pachygyria in the frontal region. (**c**) T2-weighted brain MRI of patient MD-6 at age 12 months, showing diffuse white matter hypodensities in the bilateral periventricular areas. (**d**) T2-weighted brain MRI of patient MD-7 at age 5 years, showing diffuse white matter hypodensities in the bilateral periventricular areas. (**e**) CT imaging of patient MD-5 at age 15 years, showing dystrophic features predominantly in the thigh and bicep muscles. (**f**) Immunostained muscle biopsy specimen from patient MD-5 at age 3 years, showing absence of α-dystroglycan. (**g**) Control specimen for merosin immunostaining. (**h**) Muscle biopsy specimen obtained from patient MD-6 at age 8 months, showing dystrophic changes and absence of merosin immunostaining. (**i**) Merosin immunostaining of muscle biopsy specimen from patient MD-7 at age 6 years, showing diminished merosin immunostaining and non-uniform sarcolemma. (**j**) Gomori trichrome staining of the quadriceps femoris muscle of patient CM-1 at age 6 years, showing numerous cytoplasmic nemaline bodies, without dystrophic or inflammatory changes. (**k**) Electron microscopy image of a muscle specimen from patient CM-1 showing intracellular high electron-dense nemaline bodies. (**l**) HE staining of a muscle biopsy specimen from patient CM-5 at age 7 years, showing mild variation in fiber size and the predominance of type II fibers. (**m**) Electron microscopy image of a neuromuscular junction in patient CM-7 at age 8 months, showing simplified postsynaptic regions lacking junctional folds (NT, nerve terminal; PF, primary folds (arrow)).

Table 4 Mutations detected by molecular genetic testing in the CM group

Patient	*Gene*	*Mutation*	*Detection NGS tool*	*Zygosity*	*Inheritance*	*Reference*	*Genetic diagnosis*
Pathogenic mutations							
CM-1	*NEB*	c.1488_1489TCdel p.Asp497fs	ion PGM	het	AR	Novel	NEM
		c.24394-1G>A (splicing)	ion PGM	het		Novel	
CM-2	*NEB*	c.24580-1G>A (splicing)	ion PGM	het	AR	Leiden database	NEM
		c.8980delA p.Ile2994fs	ion PGM	het		Novel	
CM-3	*RYR1*	c.4718C>T p.Pro1573Leu	SOLiD	het	AR	Novel	NEM
		c.7585G>A p.Asp2529Asn	SOLiD	het		Novel	
CM-4	*ACTA1*	c.145A>G p.Met49Val	ion PGM	het	*De novo*	Leiden database	CFTD
CM-5	*MYH7*	c.5702A>T p.His1901Leu	ion PGM	het	*De novo*	Leiden database	MSM
CM-6	*DNM2*	c.1105C>T p.Arg369Trp	ion PGM	het	AD	Leiden database	CNM
CM-7	*RAPSN*	c.737C>T p.Ala246Val	ion PGM	het	AR	Leiden database	CMS
		c.1004T>G p.Ile335Ser	ion PGM	het		Novel	
Likely pathogenic mutations							
CM-8	*TPM2*	c.464C>T p.Ala155Val	ion PGM	het	*De novo*	Novel	—
CM-9	*CCDC78*	c. 307C>T p.Arg103X	ion PGM	het	Unknown	Novel	—

Abbreviations: AR, autosomal recessive; AD, autosomal dominant; CFTD, congenital fiber type disproportion; CMS, congenital myasthenic syndrome; CNM, centronuclear myopathy; MSM, myosin storage myopathy; NEM, nemaline myopathy.

The following diseases were definitively diagnosed: three cases of NM (due to mutations in *NEB*, *RYR1* and *ACTA1*) and one case each of centronuclear myopathy (CNM; due to a mutation in *DNM2*), congenital fiber-type disproportion (CFTD; *ACTA1*), myosin storage myopathy (MSM; *MYH7*) and CMS (*RAPSN*). Notably, in CM-2, muscle biopsy was not performed, but NM was identified because previously reported pathogenic mutations (*NEB* c.24580-1G>A and c.8980delA) were identified and the patient's clinical features clearly corresponded to the phenotypes of the reported cases.[25,26]

In the case of CM-5, who had childhood-onset slowly progressive muscle weakness, no specific results were obtained except for muscle biopsy findings of myogenic changes and type II fiber predominance (Figure 2l). However, a previously reported mutation (*MYH7* p.His1901Leu) was detected and the patient's clinical features were clearly consistent with those of definite cases;[27] thus, MSM was definitively diagnosed.

In CM-7, who presented a characteristic fatal akinesia deformation sequence, NGS analysis revealed compound heterozygous pathogenic mutations in *RAPSN*, and CMS was thereby definitively diagnosed. Subsequent electron microscopy confirmed simplification of the primary fold (Figure 2m). Thereafter, therapy with anticholinesterase agents was initiated and, currently, at 3 months after initiating therapy, ptosis of the eyelids has disappeared, and seated posture maintenance and other postural muscle improvements are ongoing.

SMA group. No causative genes were identified in any of the five patients with the SMA phenotype.

DISCUSSION

Neuromuscular disorders are genetically and clinically heterogeneous. When we performed NGS-based target resequencing for patients with undiagnosed neuromuscular disorders, we identified pathogenic mutations in 45.2% of the patients, and efficiently and quickly arrived at definitive diagnoses in these cases. Thus, NGS can be considered to demonstrate considerable utility in allowing an exhaustive analysis of neuromuscular disorders.

CMD disease types have been categorized according to evidence of unique protein deficiencies identified based on characteristic clinical symptoms and immunostaining results. However, in recent years, the identification of causative genes through a CMD diagnostic algorithm has emerged as the gold standard.[28] We obtained an MD diagnosis in 12 of the 20 patients (60.0%) with clinically suspected MD, and in 5 of these patients, muscle biopsy was not performed. Seven of the 17 patients (41.1%) in whom CM was suspected were diagnosed, and a definitive genetic diagnosis was obtained in 1 patient (CM-2) without performing a muscle biopsy. Furthermore, in CM-5, a lack of specific muscle biopsy findings precluded diagnosis, but a definitive genetic diagnosis was obtained based on the reported pathogenic mutations identified using NGS. Thus, if NGS is applied to the identification of pathogenic mutations, invasive muscle biopsies may be avoided in certain cases.

FCMD is the result of mutations in *FKTN*, and it is the most frequent form of CMD in Japan: 98% of FCMD patients in Japan carry homozygous or heterozygous founder mutations, and the cases that involve heterozygous founder mutations tend to be more severe than the homozygous cases.[29–31] We identified point mutations in the *FKTN* gene in four patients. In MD-1, we identified an intronic mutation that is specific to the Korean population.[32] In these four patients, severe hypotonia had been present since birth, and the clinical courses were more severe than in typical cases. Thus, if FCMD is suspected but is more severe than what is typically observed, then point mutations might be efficiently detected using NGS. Interestingly, in MD-12, we diagnosed concurrent FCMD and BMD due to mutations in *FKTN* and *DMD*, and, furthermore, the FCMD did not involve a founder mutation. Previously, Kondo *et al.*[33] reported a case of concurrent FCMD and Leigh syndrome. We must be aware of possible occurrence of cases in which pathogenic mutations in multiple genes have been identified together with overlapping neuromuscular disorders.

DMD, the causative gene for dystrophinopathy, is extremely large (genome size, 2200 kb), and approximately 30% of *DMD* contains microdeletions, insertions and point mutations.[34] When NGS was applied in this study, point mutations were efficiently identified in two patients (MD-9 and 10). Currently, considerable effort is being devoted to developing treatments such as exon-skipping therapy, read-through therapy and other forms of DMD molecular therapy.[35–37] Identification of genetic mutations in *DMD* would be extremely beneficial not only for definitive diagnosis, female-carrier diagnosis and prenatal diagnosis, but also when considering indications for the mutation-specific therapies that are currently under development.

Especially, phenotype–genotype correlations of merosinopathy (*LAMA2*) and Ullrich congenital muscular dystrophy (*COL6A1, COL6A2* and *COL6A3*) have been clear.[38–40]

LGMD is a disease group that shows a high degree of heterogeneity. To date, 31 genes (autosomal dominant: 8 genes; autosomal recessive: 23 genes) have been identified as causative genes of LGMD.[5] Furthermore, LGMD differs in its complications and degree of progression depending on the causative gene. Thus, we were able to provide detailed and accurate information about the possible complications and future disease progression for each diagnosed patient.

CM is a disease group that shows marked heterogeneity, and many of the causative genes contain numerous exons (e.g., *NEB*, 183 exons, and *RYR1*, 65 exons). Herein, we efficiently and quickly made definitive diagnoses by employing NGS. Specifically, in CM-1, NM was attributable to a mutation in *NEB* and nemaline bodies were detected in muscle biopsy specimens, but the patient's clinical phenotype was extremely mild, with no difficulties in daily life being reported. The patient was even able to ride a bicycle. Thus, even among previous descriptions of NM, this case can be considered to present a particularly mild clinical picture. In CM-3, a compound heterozygous *RYR1* mutation was identified as a cause of severe NM, and, furthermore, *RYR1* was identified as a new candidate gene for NM with external ophthalmoplegia and fiber-type disproportion. We reported this case previously.[41] *RYR1* mutations carry a risk of malignant hyperthermia, and we thus could provide genetic counseling to the patient and the parents carrying the heterogeneous mutant alleles to be mindful of malignant hyperthermia when undergoing general anesthesia.

CMS is phenotypically very similar to CM, and a definitive diagnosis is thus considered unobtainable in many of the patients with this disease. Herein, in CM-7, CMS was diagnosed when a fatal akinesia deformation sequence was suspected, which necessitated the initiation of treatment with anticholinesterase agents. Subsequently, improvements were observed, including the disappearance of ptosis and maintenance of a seated posture. Effective therapies for CMS exist in the form of treatment with anticholinesterase agents, acetylcholine receptor antagonists and 3,4-diaminopyridine.[6,42] Symptoms generally improve with treatment, and early diagnosis of the disease would thus be extremely valuable.

No causative genes were identified in any of the five patients in the SMA group. This is because the sequence of *SMN1*, which is a causative gene of SMA, is almost identical to that of its adjacent gene, *SMN2*, with the difference between the genes being only five nucleotides.[43] Thus, designing a primer pair exclusively for *SMN1* is extremely challenging; in our panel, the coverage rate of *SMN1* was 2.44%, and most of this gene could not be analyzed. Moreover, our panel included only three genes related to SMA. We speculate that additional genes related to SMA exist and remain to be identified. Several SMA patients harbor an exon 7 deletion or gene conversion in *SMN1*. These can be detected when MLPA is used for *SMN1* exon 7, but 2–5% of the cases are compound heterozygous for point mutations[44,45] and these are not detectable by MLPA performed for SMA. Recently, Kubo *et al.*[46] successfully isolated *SMN1* and *SMN2*. Thus, if no *SMN1* exon 7 deletion is detected but SMA is strongly suspected based on symptoms, use of the long-range PCR developed by Kubo *et al.* or exome sequencing is recommended.

Conventional Sanger sequencing would be highly time-consuming, expensive and labor-intensive, were it to be used for analyzing each conceivable causative gene one-by-one. However, NGS allows multiple genes to be analyzed concurrently, the amount of data obtained and the number of detected single-nucleotide variants are both large. However, patient information such as clinical and histopathological features remains extremely helpful. In addition to using bioinformatics to effectively identify causative genes, it is crucial to perform various types of filtering on large lists of candidate variants. In this study, we used multiple annotation databases to efficiently pinpoint the causative genes.

In NGS analysis, $2–4 \times 10^5$ single-nucleotide variants are reportedly detected per person in exomes, 4–9 in disease-related variant of uncertain significance, and 300–600 in variant of uncertain significance unrelated to disease.[47] The bioinformatics load is extremely high, and the possibility of detecting incidental findings exists.[48] The advantages of target resequencing are that, as compared with exome sequencing, it is inexpensive, the bioinformatics burden is low and there is no risk of incidental findings. Conversely, NGS cannot detect long deletions and insertions, and detects repeat sequences and copy number variations only weakly. As noted earlier in this section, 98% of Japanese patients with FCMD were found to carry the founder mutation, the size of which is about 3 kb. In the case of DMD, approximately two-thirds of the mutations are long deletions or insertions overlapping with multiple exons. Moreover, most SMA patients harbor an *SMN1* exon 7 deletion, but the sequences of *SMN1* and *SMN2* are nearly the same, which makes primer design challenging. NGS cannot be used for detecting these mutations, and MLPA is considerably more effective than NGS in such cases.

Another concern is that several more undiscovered causative genes are assumed to exist than have thus far been identified. In target resequencing, only known genes are covered, but untranslated region and regions other than the coding DNA sequence, which involve non-coding RNAs, are occasionally the disease sources.[49] Thus, the diagnostic capabilities of NGS are limited.

Herein, we combined conventional genetic testing and NGS and obtained a definitive diagnosis through rapid genetic testing for 19 of our 42 patients (45.2%) with undiagnosed neuromuscular disorders, in whom disease onsets had been during the neonatal and childhood periods. These results are almost identical to the 48.8% diagnosis rate obtained previously from target resequencing of 579 genes related to early-onset neuromuscular diseases.[50] Although fewer target genes were examined in our study than in the study by Chae *et al.*, we were able to obtain a similarly high diagnosis rate because we carefully examined each patient's clinical symptoms, the onset period and the clinical course, and investigated the neurological findings. Subsequently, we classified the cases into three categories based on their clinical diagnoses. Recently, projects have been initiated in several countries aimed at clarifying undiagnosed genetic diseases (nearly 50% of these patients have neurological disorders) through exome sequencing, but the diagnosis rate is only approximately 25%.[47,51,52] We suggest that as compared to simply analyzing all such cases by employing exome sequencing, a more useful approach would be to first carefully evaluate patient phenotypes, and then perform target resequencing when a neuromuscular disorder is suspected; subsequently, if pathogenic mutations are not detected through target resequencing, the next step could be exome sequencing.

Accurate molecular genetic diagnosis not only makes it possible to predict the progression of a disease, obtain information on possible future complications and implement appropriate health management, but also aids in providing appropriate genetic counseling, determining the odds of subsequent children being born with the same mutations and providing accurate prenatal diagnosis. Furthermore, because effective therapies exist for diseases such as CMS, efficient identification of causative genes, especially in the early stage, would be extremely useful. The cost of performing NGS is decreasing annually, raising the possibility of researchers being able to use NGS for clinical application in the near future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are grateful to the patients who agreed to participate in this study. We thank Drs Toru Furukawa, Ichizo Nishino and Rieko Takahashi for their valuable contributions to this study. This work was supported by an Intramural Research Grant (26-8) for Neurological and Psychiatric Disorders of NCNP, Practical Research Project for Rare/Intractable Diseases from the Japan Agency for Medical Research and Development (AMED), Grant Number 15ek0109086h0001, and the Problem-Solving Oriented Training Program for Advanced Medical Personnel: NGSD project.

1 Koenig, M., Hoffman, E. P., Bertelson, C. J., Monaco, A. P., Feener, C. & Kunkel, L. M. Complete cloning of the Duchenne muscular dystrophy (*DMD*) cDNA and preliminary genomic organization of the *DMD* gene in normal and affected individuals. *Cell* **50**, 509–517 (1987).
2 Laing, N. G. Genetics of neuromuscular disorders. *Crit. Rev. Clin. Lab. Sci.* **49**, 33–48 (2012).
3 D'Amico, A. & Bertini, E. Congenital myopathies. *Curr. Neurol. Neurosci. Rep.* **8**, 73–79 (2008).
4 Bonnemann, C. G., Wang, C. H., Quijano-Roy, S., Deconinck, N., Bertini, E., Ferreiro, A. *et al.* Members of International Standard of Care Committee for Congenital Muscular Dystrophies. Diagnostic approach to the congenital muscular dystrophies. *Neuromuscul. Disord.* **24**, 289–311 (2014).
5 Nigro, V. & Savarese, M. Genetic basis of limb-girdle muscular dystrophies: the 2014 update. *Acta Myol.* **33**, 1–12 (2014).
6 Cruz, P. M. R., Palace, J. & Beeson, D. Inherited disorders of the neuromuscular junction: an update. *J. Neurol.* **261**, 2234–2243 (2014).
7 Lo, H. P., Cooper, S. T., Evesson, F. J., Seto, J. T., Chiotis, M., Tay, V. *et al.* Limb-girdle muscular dystrophy: diagnostic evaluation, frequency and clues to pathogenesis. *Neuromuscul. Disord.* **18**, 34–44 (2008).
8 Peat, R. A., Smith, J. M., Compton, A. G., Baker, N. L., Pace, R. A., Burkin, D. J. *et al.* Diagnosis and etiology of congenital muscular dystrophy. *Neurology* **71**, 312–321 (2008).
9 Maggi, L., Scoto, M., Cirak, S., Robb, S. A., Klein, A., Lillis, S. *et al.* Congenital myopathies—clinical features and frequency of individual subtypes diagnosed over a 5-year period in the United Kingdom. *Neuromuscul. Disord.* **23**, 195–205 (2013).
10 Godfrey, C., Clement, E., Mein, R., Brockington, M., Smith, J., Talim, B. *et al.* Refining genotype phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan. *Brain* **130**, 2725–2735 (2007).

11 Cirak, S., Foley, A. R., Herrmann, R., Willer, T, Yau, S., Stevens, E *et al. ISPD* gene mutations are a common cause of congenital and limb-girdle muscular dystrophies. *Brain* **136**, 269–281 (2013).
12 Shy, G. M., Engel, W. K., Somers, J. E. & Wanko, T. Nemaline myopathy. A new congenital myopathy. *Brain* **86**, 793–810 (1963).
13 Wallgren-Pettersson, C. & Laing, N. G. 138th ENMC Workshop: Nemaline Myopathy, 20–22 May 2005, Naarden, The Netherlands. *Neuromuscul. Disord.* **16**, 54–60 (2005).
14 Bohm, J., Vasli, N., Malfatti, E., Le Gras, S., Feger, C., Jost, B. *et al.* An integrated diagnosis strategy for congenital myopathies. *PLoS ONE* **8**, e67527 (2013).
15 Valencia, C. A., Ankala, A., Rhodenizer, D., Bhide, S., Littlejohn, M. R., Keong, L. M. *et al.* Comprehensive mutation analysis for congenital muscular dystrophy: a clinical PCR-based enrichment and next-generation sequencing panel. *PLoS ONE* **8**, e53083 (2013).
16 Liu, X., Jian, X. & Boerwinkle, E. dbNSFP: a lightweight database of human nonsynonymous SNPs and their functional predictions. *Hum. Mutat.* **32**, 894–899 (2011).
17 Kumar, P., Henikoff, S. & Ng, P. C. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat. Protoc.* **4**, 1073–1081 (2009).
18 Adzhubei, I. A., Schmidt, S., Peshkin, L., Ramensky, V. E., Gerasimova, A., Bork, P. *et al.* A method and server for predicting damaging missense mutations. *Nat. Methods.* **7**, 248–249 (2010).
19 Pollard, K. S., Hubisz, M. J., Rosenbloom, K. R. & Siepel, A. Detection of nonneutral substitution rates on mammalian phylogenies. *Genome Res.* **20**, 110–121 (2010).
20 Davydov, E. U., Goode, D. L., Sirota, M., Cooper, G. M, Sidow, A. & Batzoglou, S. Identifying a high fraction of the human genome to be under selective constraint using GERP++. *PLoS Comput. Biol.* **2**, e1001025 (2010).
21 The 1000 Genomes Project Consortium. A global reference for human genetic variation. *Nature* **526**, 68–74 (2015).
22 Exome Variant Server. NHLBI GO Exome Sequencing Project (ESP), Seattle, WA. https://esp.gs.washington.edu/drupal/. Accessed 5 June 2015.
23 Sherry, S. T., Ward, M. H., Kholodov, M., Baker, J., Phan, L., Smigielski, E. M. *et al.* dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res.* **29**, 308–311 (2001).
24 Aartsma-Rus, A., Van Deutekom, J. C., Fokkema, I. F., Van Ommen, G. J. & Den Dunnen, J. T. Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. *Muscle Nerve* **34**, 135–144 (2006).
25 Graziano, C., Bertini, E., Minetti, C. & Porfirio, B. α-Actin gene mutations and polymorphisms in Italian patients with nemaline myopathy. *Int. J. Mol. Med.* **13**, 805–809 (2004).
26 Malfatti, E., Lehtokari, V. L., Bohm, J., De Winter, J. M., Schaffer, U., Estournet, B. *et al.* Muscle histopathology in *nebulin*-related nemaline myopathy: ultrastrastructural findings correlated to disease severity and genotype. *Acta Neuropathol. Commun.* **2**, 44 (2014).
27 Bohlega, S., Abu-Amero, S. N., Wakil, S. M., Carroll, P., Al-Amr, R., Lach, B. *et al.* Mutation of the slow myosin heavy chain rod domain underlies hyaline body myopathy. *Neurology* **62**, 1518–1521 (2004).
28 Tawil, R., Kissel, J. T., Heatwole, C., Pandya, S., Gronseth, G. & Benatar, M. Evidence-based guideline summary: evaluation, diagnosis, and management of facioscapulohumeral muscular dystrophy: report of the Guideline Development, Dissemination, and Implementation Subcommittee of the American Academy of Neurology and the Practice Issues Review Panel of the American Association of Neuromuscular & Electrodiagnostic Medicine. *Neurology* **85**, 357–364 (2015).
29 Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura, K., Kondo-Iida, E., Nomura, Y. *et al.* An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. *Nature* **394**, 388–392 (1998).
30 Kondo-Iida, E., Kobayashi, K., Watanabe, M., Sasaki, J., Kumagai, T., Koide, H. *et al.* Novel mutations and genotype-phenotype relationships in 107 families with Fukuyama-type congenital muscular dystrophy (FCMD). *Hum. Mol. Genet.* **8**, 2303–2309 (1999).
31 Saito, K., Osawa, M., Wang, Z. P., Ikeya, K., Fukuyama, Y., Kondo-Iida, E. *et al.* Haplotype-phenotype correlation in Fukuyama congenital muscular dystrophy. *Am. J. Med. Genet.* **92**, 184–190 (2000).
32 Lim, B. C., Ki, C. S., Kim, J. W., Cho, A., Kim, M. J., Hwang, H. *et al.* Fukutin mutations in congenital muscular dystrophies with defective glycosylation of dystroglycan in Korea. *Neuromuscul. Disord.* **20**, 524–530 (2010).
33 Kondo, H., Tanda, K., Tabata, C., Hayashi, K., Kihara, M., Kizaki, Z. *et al.* Leigh syndrome with Fukuyama congenital muscular dystrophy: case report. *Brain Dev.* **36**, 730–733 (2013).
34 Takeshima, Y., Yagi, M., Okizuka, Y., Awano, H., Zhang, Z., Yamauchi, Y. *et al.* Mutation spectrum of the dystrophin gene in 442 Duchenne/Becker muscular dystrophy cases from one Japanese referral center. *J. Hum. Genet.* **55**, 379–388 (2010).
35 Welch, E. M., Barton, E. R., Zhuo, J., Tomizawa, Y., Friesen, W. J., Trifillis, P. *et al.* PTC124 targets genetic disorders caused by nonsense mutations. *Nature* **447**, 87–91 (2007).
36 Kinter, J. & Sinnreich, M. Molecular targets to treat muscular dystrophies. *Swiss Med. Wkly.* **144**, w13916 (2014).
37 Touznik, A., Lee, J. J. & Yokota, T. New developments in exon skipping and splice modulation therapies for neuromuscular diseases. *Expert Opin. Biol. Ther.* **14**, 809–819 (2014).
38 Geranmayeh, F., Clement, E., Feng, L. H., Sewry, C., Pagan, J., Mein, R. *et al.* Genotype-phenotype correlation in a large population of muscular dystrophy patients with *LAMA2* mutations. *Neuromuscul. Disord.* **20**, 241–250 (2010).
39 Brinas, L., Richard, P., Quijano-Roy, S., Gartioux, C., Ledeuil, C., Lacene, E. *et al.* Early onset collagen VI myopathies: genetic and clinical correlations. *Ann. Neurol.* **68**, 511–520 (2010).
40 Butterfield, R. J., Foley, A. R., Dastgir, J., Asman, S., Dunn, D. M., Zou, Y. *et al.* Position of glycine substitutions in the triple helix of *COL6A1*, *COL6A2*, and *COL6A3* is correlated with severity and mode of inheritance in collagen VI myopathies. *Hum. Mutat.* **34**, 1558–1567 (2013).
41 Kondo, E., Nishimura, T., Kosho, T., Inaba, Y., Mitsuhashi, S., Ishida, T. *et al.* Recessive *RYR1* mutations in a patient with severe congenital nemaline myopathy with ophthalomoplegia identified through massively parallel sequencing. *Am. J. Med. Genet. A* **158A**, 772–778 (2012).
42 Engel, A. G. The therapy of congenital myasthenic syndromes. *Neurotherapeutics* **4**, 252–257 (2007).
43 Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L. *et al.* Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* **80**, 155–165 (1995).
44 Parsons, D. W., McAndrew, P. E., Iannaccone, S. T., Mendell, J. R., Burghes, A. H. & Prior, T. W. Intragenic *telSMN* mutations: frequency, distribution, evidence of a founder effect, and modification of the spinal muscular atrophy phenotype by *cenSMN* copy number. *Am. J. Hum. Genet.* **63**, 1712–1723 (1998).
45 Wirth, B. An update of the mutation spectrum of the *survival motor neuron* gene (*SMN1*) in autosomal recessive spinal muscular atrophy (SMA). *Hum. Mutat.* **15**, 228–237 (2000).
46 Kubo, Y., Nishio, H. & Saito, K. A new method for *SMN1* and hybrid *SMN* gene analysis in spinal muscular atrophy using long-range PCR followed by sequencing. *J. Hum. Genet.* **60**, 233–239 (2015).
47 Yang, Y., Muzny, D. M., Reid, J. G., Bainbridge, M. N., Willis, A., Ward, P. A. *et al.* Clinical whole-exome sequencing for the diagnosis of Mendelian disorders. *N. Engl. J. Med.* **369**, 1502–1511 (2013).
48 Green, R. C., Berg, J. S., Grody, W. W., Kalia, S. S., Korf, B. R., Martin, C. L. *et al.* ACMG recommendations for reporting of incidental findings in clinical exome and genome sequencing. *Genet. Med.* **15**, 565–574 (2013).
49 Dusl, M., Senderek, J., Muller, J. S., Vogel, J. G., Pertl, A., Stucka, R. *et al.* A 3′-UTR mutation creates a microRNA target site in the *GFPT1* gene of patients with congenital myasthenic syndrome. *Hum. Mol. Genet.* **24**, 3418–3426 (2015).
50 Chae, J. H., Vasta, V., Cho, A., Lim, B. C., Zhang, Q., Eun, S. H. *et al.* Utility of next generation sequencing in genetic diagnosis of early onset neuromuscular disorders. *J. Med. Genet.* **52**, 208–216 (2015).
51 Gahl, W. A., Markello, T. C., Toro, C., Fajardo, K. F., Sincan, M., Gill, F. *et al.* The National Institutes of Health Undiagnosed Diseases Program: insights into rare diseases. *Genet. Med.* **14**, 51–59 (2012).
52 Zhu, X., Petrovski, S., Xie, P., Ruzzo, E. K., Lu, Y. F., McSweeney, K. M. *et al.* Whole-exome sequencing in undiagnosed genetic diseases: interpreting 119 trios. *Genet. Med.* **17**, 774–781 (2015).

Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)