

Expanding genotype/phenotype of neuromuscular diseases by comprehensive target capture/NGS

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ABSTRACT

Objective: To establish and evaluate the effectiveness of a comprehensive next-generation sequencing (NGS) approach to simultaneously analyze all genes known to be responsible for the most clinically and genetically heterogeneous neuromuscular diseases (NMDs) involving spinal motoneurons, neuromuscular junctions, nerves, and muscles.

Methods: All coding exons and at least 20 bp of flanking intronic sequences of 236 genes causing NMDs were enriched by using SeqCap EZ solution-based capture and enrichment method followed by massively parallel sequencing on Illumina HiSeq2000.

Results: The target gene capture/deep sequencing provides an average coverage of $\sim 1,000\times$ per nucleotide. Thirty-five unrelated NMD families (38 patients) with clinical and/or muscle pathologic diagnoses but without identified causative genetic defects were analyzed. Deleterious mutations were found in 29 families (83%). Definitive causative mutations were identified in 21 families (60%) and likely diagnoses were established in 8 families (23%). Six families were left without diagnosis due to uncertainty in phenotype/genotype correlation and/or unidentified causative genes. Using this comprehensive panel, we not only identified mutations in expected genes but also expanded phenotype/genotype among different subcategories of NMDs.

Conclusions: Target gene capture/deep sequencing approach can greatly improve the genetic diagnosis of NMDs. This study demonstrated the power of NGS in confirming and expanding clinical phenotypes/genotypes of the extremely heterogeneous NMDs. Confirmed molecular diagnoses of NMDs can assist in genetic counseling and carrier detection as well as guide therapeutic options for treatable disorders. *Neurol Genet* 2015;1:e14; doi: 10.1212/NXG.0000000000000015

GLOSSARY

AD = autosomal dominant; **AR** = autosomal recessive; **BCM** = Baylor College of Medicine; **CACTD** = carnitine acylcarnitine translocase deficiency; **CCD** = central core disease; **CM** = congenital myopathy; **CMD** = congenital muscular dystrophy; **CMT** = Charcot-Marie-Tooth disease; **CMys** = congenital myasthenic syndrome; **CNM** = centronuclear myopathy; **EDMD** = Emery-Dreifuss muscular dystrophy; **HSAN** = hereditary sensory and autonomic neuropathy; **LGMD** = limb-girdle muscular dystrophy; **MH** = malignant hyperthermia; **MM** = metabolic myopathy; **MTS** = myotonic syndrome; **NGS** = next-generation sequencing; **NM** = nemaline myopathy; **NMD** = neuromuscular disease; **PFIS** = paralytic floppy infant syndrome; **SMARD1** = spinal muscular atrophy with respiratory distress type 1; **VUS** = variants of unknown significance.

Neuromuscular diseases (NMDs) are genetically and clinically heterogeneous. To date, more than 360 genes have been reported to cause NMDs.¹ As a group, the combined NMD prevalence is greater than 1 in 3,000.²

The majority of NMDs are inherited, degenerative, and rare.^{3,4} An early definitive molecular diagnosis is crucial for genetic counseling, family planning, prognosis, therapeutic strategies, and

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Supplemental data
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long-term care plans.^{3–7} The recent development of next-generation sequencing (NGS) has accelerated the discovery of novel NMD phenotypes and genotypes,^{8–11} including the identification of mutations in 5 large NMD genes (*TTN*, *NEB*, *SYNE1*, *RYR1*, and *DMD*^{1,4,8–12}) (table e-1 at Neurology.org/ng). With the ever-increasing number of causative genes and clinical heterogeneity, a comprehensive molecular approach with the feasibility to add newly discovered genes for analysis in a cost- and time-effective manner is needed.^{1,4,7,12–16}

A recent study using PCR enrichment and NGS approach to analyze 12 genes known to cause congenital muscular dystrophy (CMD) on 26 samples with known mutations¹⁷ reported that 49 exons (15%) had insufficient coverage ($<20\times$).¹⁷ Among 15 known variants, 6 (40%) were not detected. Similar studies on congenital myasthenic syndrome (CMyS),¹⁸ Charcot-Marie-Tooth disease (CMT),¹⁹ Duchenne/Becker muscular dystrophy,²⁰ and metabolic myopathy (MM)^{21,22} have demonstrated the clinical utility of NGS in specific disease categories. Nevertheless, these are small-scale studies focusing on subcategories of NMDs. Here, we describe a comprehensive target gene capture/NGS approach, analyzing 236 genes.

METHODS **Standard protocol approvals, registrations, and patient consents.** This study was conducted according to the Institutional Review Board–approved protocols of both Kaohsiung Medical University Hospital, Taiwan, and Baylor College of Medicine (BCM), Houston, TX. A signed informed consent was obtained for each participant.

Patients and DNA samples. Patients were clinically evaluated in Taiwan, and DNA samples were analyzed at BCM. DNA samples from 35 unrelated families (38 patients) with clinical diagnoses of NMD who underwent electrophysiologic examination and muscle imaging and/or muscle biopsies were analyzed. Patients with a proven common genetic diagnosis of spinal muscular atrophy, Duchenne muscular dystrophy, myotonic dystrophy types 1 and 2, CMT type 1A, or facioscapulohumeral muscular dystrophy were not included in the study. The initial diagnoses included congenital myopathy (CM) (23 patients), CMD (5), limb-girdle muscular dystrophy (LGMD)²³ (4), CMT (3), MM (2), and myotonic syndrome (MTS) and ion channel muscle disease (1). DNA was extracted from peripheral blood using a Puregene DNA extraction kit according to manufacturer's instructions (Gentra Systems Inc., Minneapolis, MN).

Design of capture probes and target gene enrichment. The capture probe library contained 236 genes, most of which were selected from the 2012 version of the gene table of monogenic

NMDs.²⁴ We categorized NMDs and their causative genes into 10 groups, including MM, CMD, CM, other myopathies, motor neuron disease, CMyS, arthrogryposis multiplex congenita, MTS and ion channel muscle diseases, CMT, and other muscular dystrophies as listed in table 1. Mitochondrial genes were not included.

A custom NimbleGen in-solution DNA capture library was designed to capture all 4,815 coding exons and at least 20 bp flanking intron regions of the 236 NMD-related genes. The NM accession numbers of the genes are listed in table e-1. The coding regions were enriched according to manufacturer's instructions (Roche NimbleGen Inc., Madison, WI) and sequencing was performed on HiSeq2000, as previously described.^{21,25}

Sequence alignment and analytical pipeline for variant calling. Conversion of raw sequencing data, demultiplexing, sequence alignment, data filtering, and analyses using CASAVA v1.7, NextGENe software were performed as previously described.^{21,25} Multiple in silico analytical tools, such as SpliceSiteFinder-like, MaxEntScan, NNSPLICE, and GeneSplicer, were used to predict the effects of splice site variants (Alamut, <http://www.interactive-biosoftware.com>). SIFT²⁶ and PolyPhen-2²⁷ were used to predict the pathogenicity of novel missense variants. The pathogenicity of the variants was categorized according to published databases, such as Human Gene Mutation Database (<http://www.biobase-international.com/product/hgmd>), PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>), and American College of Medical Genetics guidelines.²⁸ The analytical flowchart is depicted in figure e-1. All mutations and novel variants identified by NGS were confirmed independently by Sanger sequencing.^{21,25} Family members, if available, were also tested to evaluate the mode of inheritance, disease segregation, and clinical correlation.

Detection of deletions using sequence read coverage data from NGS. We used a newly developed analytical method to detect exonic deletions using the same set of NGS data by comparing the normalized coverage depth of each individual exon of the test sample with the mean coverage depth of the same exon from a group of 20 reference samples.²⁹

RESULTS **Characteristics of target gene capture and sequence depth.** More than 99.4% (4,787/4,815) of the target sequences were enriched in an unbiased fashion, with a minimal coverage of $20\times$ and a mean coverage depth of $1,136\times$ per base (figure 1). An average of 28 exons per sample was consistently insufficiently sequenced ($<20\times$) due to the high GC content, sequence homologies in the genome, short tandem repeats, or secondary structural difficulties (table e-2).

Clinical history. A total of 35 unrelated affected families (38 patients) with clinical diagnosis of NMD were studied. Among them, 3 families had 2 affected family members (patients 4/6, 19/20, and 25/26). The male to female patient ratio was about 1 to 1. The majority of patients (27/38, 71%) presented with paralytic floppy infant syndrome (PFIS). The clinical and pathologic features are summarized in table 2.

Identification of mutations. Figure e-1 illustrates the analytical algorithm for the identification of disease-causing variants. The approximate number of variants in each step of analysis is included. DNA

Table 1 Ten categories of neuromuscular diseases

Name of panel	No.	Genes
Metabolic myopathies	39 genes	ABHD5, ACADM, ACADS, ACADVL, AGL, ALDOA, CPT1A, CPT1B, CPT1C, CPT2, ENO3, ETFA, ETFB, ETFDH, G6PC, GAA, GBE1, GYG1, GYS1, HADH, HADHA, HADHB, LDHA, LPIN1, PFKM, PGAM2, PGK1, PGM1, PHKA1, PHKA2, PHKB, PHKG2, PNPLA2, PRKAG2, PYGM, PYGL, SLC22A5, SLC25A20, SLC37A4
Congenital muscular dystrophy	18 genes	CHKB, COL6A1, COL6A2, COL6A3, DNM2, FHL1, FKRP, FKTN, GTDC2, ISPD, ITGA7, LAMA2, LARGE, POMT1, POMT2, POMGNT1, SEPN1, TCAP
Congenital myopathy	21 genes (2)	ACTA1, BIN1, CCDC78, CFL2, CNTN1, DNM2 , KBTBD13, MTM1, MTMR14, MYBPC3, MYF6, MYH2, MYH7, NEB, RYR1, SEPN1 , TNNT1, TPM2, TRIM3, TTN, TPM3
Other myopathies	16 genes (3)	ACVR1, BAG3, CAV3, CRYAB, DES, FHL1 , FLNC, GDF8, ISCU, LAMP2, LDB3, PABPN1, PLEC1, SEPN1 , TTID, TTN
Motor neuron disease	37 genes	ALS2, ANG, AR, ATP7A, ATXN2, BSCL2, CHMP2B, DCTN1, DYNC1H1, ERBB3, FIG4, FUS, GARS, GLE1, HSPB1, HSPB3, HSPB8, IGHMBP2, MYBPC1, NEFH, OPTN, PFN1, PIP5K1C, PLEKHG5, PRPH, REEP1, SETX, SIGMAR1, SMN1, SOD1, TARDBP, TRPV4, UBA1, UBQLN2, VAPB, VCP, VRK1
Congenital myasthenia syndrome	14 genes (1)	AGRN, CHAT, CHRNA1, CHRNB1, CHRND, CHRNE, COLQ, DOK7, GFPT1, LAMB2, MUSK, PLEC1 , RAPSN, SCN4A
Arthrogryposis multiplex congenita	21 genes (6)	CHRNA1 , CHRND , CHRNA1 , CHRNA1 , CHRNA1 , CHRNA1 , DHCR24, DOK7 , ERCC2, ERCC6, FBN2, HRAS, LMNA, MYBPC1 , MYH3, MYH8, RAPSN, RIPK4, TPM2 , TNNT2, TNNT3, VIPAR, VPS33B, ZMPSTE24
Myotonic syndromes and ion channel muscle diseases	20 genes (2)	ATP2A1, CACNA1A, CACNA1S, CAV3 , CLCN1, DMPK, HSPG2, KCNA1, KCNE1, KCNE2, KCNH2, KCNJ5, KCNJ11, KCNJ18, KCNQ1, LIFR, SCN4A , SCN4B, SCN5A, ZNF9/CNBP
Charcot-Marie-Tooth diseases and peroxisomal disorders	58 genes (8)	AARS, ARHGEF10, ATL1, CTDP1, DNM2 , DNMT1, DYNC1H1 , EGR2, FGD4, FIG4 , GAN, GARS , GDAP1, GJB1, HOXD10, HSPB1 , HSPB8 , IKBKAP, KIF1A, KIF1B, LITAF, LMNA, LRSAM1, MED25, MFN2, MPZ, MTMR2, NDRG1, NEFL, NGFB, PEX1, PEX2, PEX3, PEX5, PEX6, PEX7, PEX10, PEX11B, PEX12, PEX13, PEX14, PEX16, PEX19, PEX26, PMP22, PRPS1, PRX, RAB7, SBF2, SEPT9, SH3TC2, SLC12A6, SPTLC1, SPTLC2, TFG, TRPV4 , WNK1, YARS
Other muscular dystrophy	28 genes (14)	ANO5, CAPN3, CAV3 , DAG1, DES , DMD, DNAJB6, DYSF, EMD, FHL1 , FKRP , FKTN , LMNA, MYH7, PLEC1 , POMGNT1 , POMT1 , POMT2 , SGCA, SGCB, SGCD, SGCG, SYNE1, SYNE2, TCAP, TRIM32, TTID, TTN
Total	236 genes	272 genes (36)

A total of 236 genes responsible for the neuromuscular disorders are listed as 10 categories of diseases. Repeated genes are shown in boldface type, and the number of repeated genes is in parentheses.

sequence variants were considered deleterious if they led to a translational frameshift, exon skipping, or stop codon or if they were previously reported in affected patients. Table 2 summarizes initial clinical diagnoses, muscle pathology findings, mutations

identified, and final diagnoses. Among 35 families, 21 families (60%) had definitive diagnoses with deleterious mutations in the expected causative genes that were confirmed in family members. Likely diagnoses were identified in an additional 8 families (23%) that had novel genotype/phenotype findings requiring functional, clinical, and/or genetic confirmation. Six families were without a specific diagnosis due to inconsistent genotype/phenotype correlation based on current knowledge. Similar to the proportion of clinical diagnoses, mutations in genes causing CM were most common. However, this observation is biased due to the prevalence of pediatric patients in our cohort. About half of the disorders are inherited in an autosomal recessive (AR) mode, with 2 X-linked recessive cases. Autosomal dominant (AD) disorders account for about 20%.

Molecular diagnoses of NMDs. Confirmation of NMD diagnosis: Identification of deleterious mutations in genes responsible for the observed clinical phenotype and muscle pathology. The diagnoses of 13 families have been confirmed by this capture/sequencing approach (R1 in table 2). Patient 2 had presented with MM due to PFIS and hyperammonemia since birth. His muscle pathology was consistent with lipid storage myopathy (figure 2A). He was homozygous for the c.199-10T>G Asian common splice mutation in

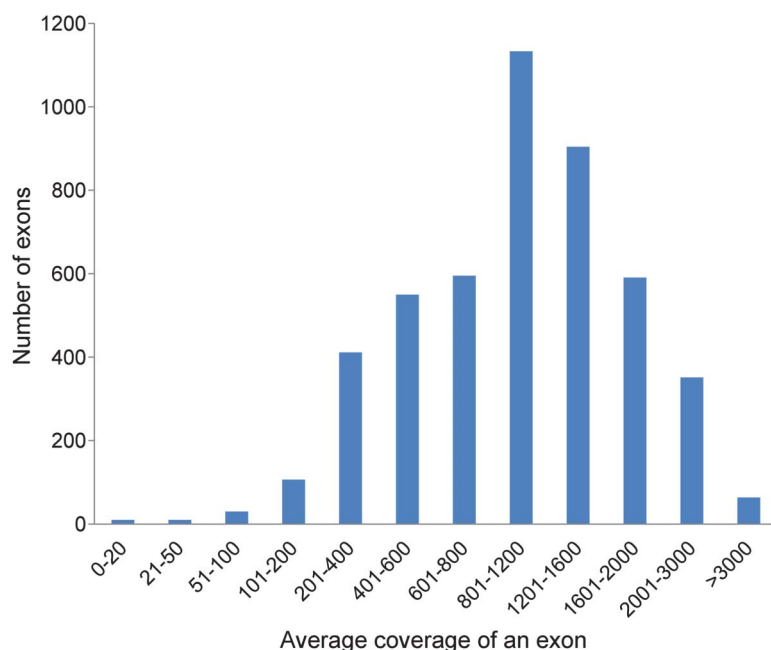
Figure 1 Coverage depth of 4,815 coding exons of the neuromuscular disease panel

Table 2 Summary of clinical and molecular diagnosis of patients with neuromuscular diseases

Patient	Age at onset	Current age, y	Sex	Clinical Dx	Pathologic Dx	Gene symbol	Inheritance	Mutation	Gene name	Genetic Dx	Final Dx	Category
Dx AR (14)												
2	NB	1.5 (exp)	M	MM	LSM	<i>SLC25A20</i>	Mat	c.199-10T>G ^a	Carnitine acylcarnitine translocase	CACTD	CACTD	R1
						<i>SLC25A20</i>	Pat	c.199-10T>G ^a				
3	NB	18.5	M	CM	CCD	<i>RYR1</i>	NA	c.11164T>C(p.Y3722H) ^b	Ryanodine receptor 1	CCD, <i>RYR1</i> -related	CCD, <i>RYR1</i> -related	R1
						<i>RYR1</i>	NA	c.14584A>G(p.K4862E) ^b				
11	3-4 y	10.9	F	CM	MMCD	<i>SEPN1</i>	Mat	c.1096G>T(p.E366*) ^{a,b}	Selenoprotein N1	MMCD, <i>SEPN1</i> -related	MMCD, <i>SEPN1</i> -related	R1
						<i>SEPN1</i>	Pat	c.1209dup(p.K404Qfs*32) ^{a,b}				
12	NB	16.2	F	CMD	DG-CMD	<i>POMT1</i>	Mat	c.793C>T(p.R265*) ^a	Protein o-mannosyltransferase 1	MDDGB1	CMD, <i>POMT1</i> -related	R2
						<i>POMT1</i>	Pat	c.1859G>C(p.R620P) ^b				
16	NB	24.7	M	CMD	MN-CMD	<i>LAMA2</i>	Mat	c.2945dup(p.S982Rfs*16) ^{a,b}	Laminin, α -2	MDC1A	MDC1A	R1
						<i>LAMA2</i>	Pat	c.8654T>C(p.L2885P) ^b				
17	10 y	17.8	M	MC	NSF	<i>CLCN1</i>	Mat	c.301G>T(p.D101Y) ^b	Chloride channel 1, muscle	MCAR	MCAR	R2
						<i>CLCN1</i>	Pat	c.1205C>T(p.A402V) ^a				
18	NB	9.1 (exp)	F	CMT	NC	<i>IGHMBP2</i>	Pat	c.711+1G>C ^a	Immunoglobulin mu-binding protein 2	SMARD1	SMARD1	R3
						<i>IGHMBP2</i>	Mat	c.2356del(p.A786Pfs*45) ^a				
21	Infancy	17.8	F	CM	MMCD	<i>SEPN1</i>	Mat	c.802C>T(p.R268C) ^a	Selenoprotein N1	MMCD, <i>SEPN1</i> -related	MMCD, <i>SEPN1</i> -related	R1
						<i>SEPN1</i>	Pat	c.1574T>G(p.M525R) ^b				
25	NB	22.5	M	CM	CNM	<i>RYR1</i>	NIP	c.9658A>G(p.T3220A) ^b	Ryanodine receptor 1	CNM, <i>RYR1</i> -related	CNM, <i>RYR1</i> -related	R1
						<i>RYR1</i>	Pat	exon39 het del ^{a,b}				
26	NB	19.0	M	CM	CNM	<i>RYR1</i>	NIP	c.9658A>G(p.T3220A) ^b	Ryanodine receptor 1	CNM, <i>RYR1</i> -related	CNM, <i>RYR1</i> -related	
						<i>RYR1</i>	Pat	exon39 het del ^{a,b}				
32	20 y	31.8	M	CM	MCNI	<i>TCAP</i>	Pat	c.26_33dup(p.E12Rfs*20) ^a	Titin-cap	LGMD2G	LGMD2G	R3
						<i>TCAP</i>	Mat	c.26_33dup(p.E12Rfs*20) ^a				
33	NB	2.7	F	CM	ESMC	<i>RYR1</i>	Pat	c.7795C>T(p.Q2599*) ^{a,b}	Ryanodine receptor 1	CM, <i>RYR1</i> -related	CM, <i>RYR1</i> -related	R2
						<i>RYR1</i>	Mat	c.11737A>G(p.N3913D) ^b				
51	18 y	35.5	F	LGMD	LGMD2B	<i>DYSF</i>	NA, cons	exon 5 del	Dysferlin	LGMD2B	LGMD2B	R1
						<i>DYSF</i>		exon 5 del				
						<i>SGCA</i>	NA	c.662G>A(p.R221H) ^a	Sarcoglycan, α	LGMD2D	LGMD2D	
						<i>SGCA</i>	NA	c.320C>T(p.A107V)				

Continued

Table 2 Continued

Patient	Age at onset	Current age, y	Sex	Clinical Dx	Pathologic Dx	Gene symbol	Inheritance	Mutation	Gene name	Genetic Dx	Final Dx	Category
52	13 y	37.4	F	LGMD	LGMD	CAPN3	Sister	c.1621C>T(p.R541W) ^a	Calpain 3	LGMD2A	LGMD2A	R1
						CAPN3	Mat/sister	c.2305C>T(p.R769W) ^a				
M516	NB	10.0	F	CM	NM	RYR1	Pat	c.1675dup(p.I559Nfs*11) ^{a,b}	Ryanodine receptor 1	NM, RYR1-related	NM, RYR1-related	R2
						RYR1	Mat	c.3800C>G(p.P1267R) ^a				
Dx X-R (1)												
27	NB	25.5	M	CM	MTM	MTM1	De novo	c.679G>A(p.V227M) ^a	Myotubularin	MTM, X-linked	MTM, X-linked	R1
Dx AD (6)												
1	NB	6	M	CM	NSF	SPTLC2 (AD)	Pat	c.1292G>A(p.G431D) ^b	SPT, long-chain base subunit 2	HSAN1C	HSAN1C with MHS1	R3
						RYR1 (AD)	Mat	c.7692G>C(p.K2564N) ^b	Ryanodine receptor 1	MHS1		
9	NB	0.5 (exp)	F	CM	NM	ACTA1	NA	c.413T>C(p.I138T) ^a	Muscle actin α 1	NEM3	NEM3	R1
19	7 y	34.9	F	CMT	NC	MFN2	S/B	c.281G>A(p.R94Q) ^a	Mitofusin 2	CMT2A2	CMT2A2	R1
20	5 y	9.1	M	CMT	NC	MFN2	Mat	c.281G>A(p.R94Q) ^a	Mitofusin 2	CMT2A2	CMT2A2	
29	15 y	22.1	M	MM	VM	CACNA1S	Mat	c.4639C>T(p.R1547W) ^b	Calcium channel, voltage-dependent, L type, α 1S	HOKPP1, with MHS5	HOKPP1, with MHS5	R3
46	NB	2.3	F	CM	CCD	RYR1	De novo	c.14581C>T(p.R4861C) ^a	Ryanodine receptor 1	CCD, RYR1-related	CCD, RYR1-related	R1
M495	3 mo	5.0	M	CM	NM	ACTA1	De novo	c.802T>C(p.F268L) ^a	Muscle actin α 1	NEM3	NEM3	R1
Likely Dx (8)												
7	NB	11.6	M	CM	MCM	SYNE1 (AD/AR)	Mat	c.16388A>C(p.E5463A) ^b	Spectrin repeat-containing nuclear envelope protein 1	EDMD4	Susp EDMD4	R4
						SYNE1 (AD/AR)	Pat	c.1859C>T(p.S620F)				
						CLCN1 (AD/AR)	Mat	c.1723C>T(p.P575S) ^a	Chloride channel 1, muscle			
						TTN (AD/AR)	Mat	c.57388C>T(p.R19130C)	Titin			
8	Infancy	12.8	F	CM	MCM	RYR1 (AD)	Pat	c.2203C>T(p.H735Y) ^b	Ryanodine receptor 1	MHS1	Susp CM, RYR1-related	R4
						TTN (AD/AR)	Pat	c.84995A>G(p.N28332S)	Titin			
						TTN (AD/AR)	Mat	c.7156 G>A(p.G2386S) ^b	Titin			
24	NB	21.8	F	CM	MTM	DMPK (AD)	Mat	c.625 G>T(p.D209Y) ^b	Dystrophin myotonic protein kinase	DM1	Susp DM1	R4
						TTN (AD/AR)	Mat	c.47680A>C(p.K15894Q) ^b	Titin			
30	NB	6.3	F	CM	CNM	SYNE1 (AD/AR)	NIM	c.1399A>T(p.K467*) ^{a,b}	Spectrin repeat-containing nuclear envelope protein 1	EDMD4	Susp EDMD4	R4
						TTN (AD/AR)	Mat	c.55265A>T(p.D18422V) ^b				

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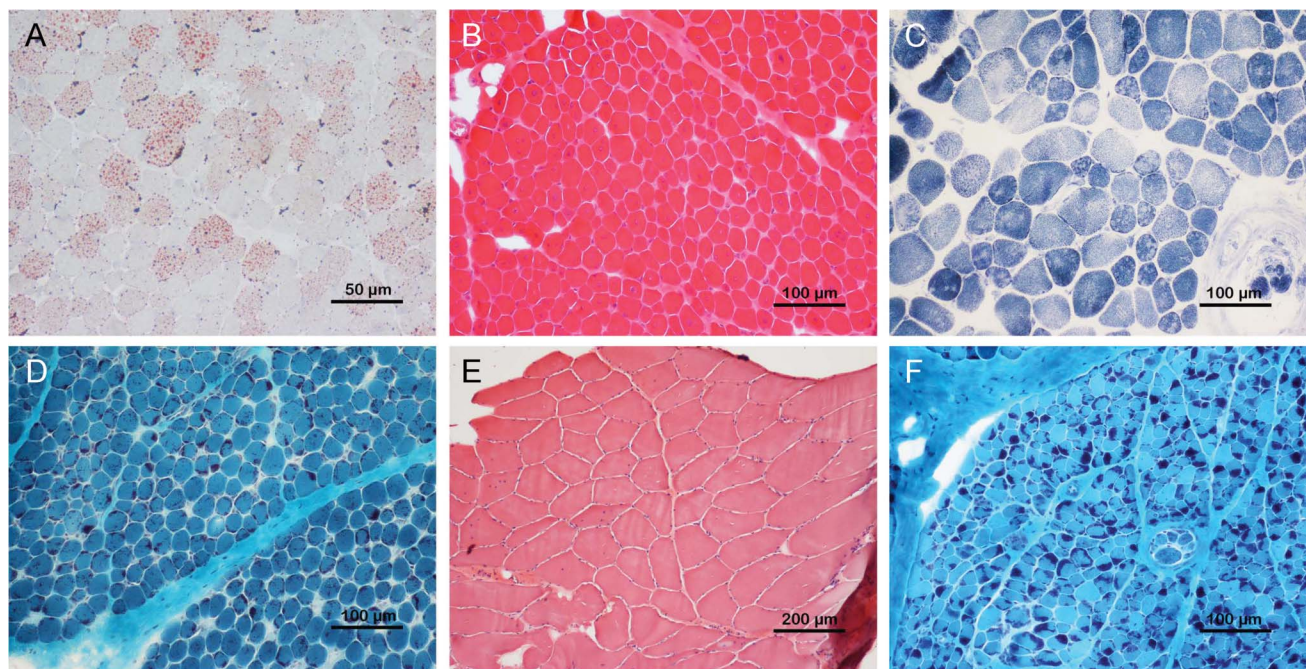
Table 2 Continued

Patient	Age at onset	Current age, y	Sex	Clinical Dx	Pathologic Dx	Gene symbol	Inheritance	Mutation	Gene name	Genetic Dx	Final Dx	Category
36	3 y	7.8	M	CM	2BA	TTN (AD/AR)	Pat	c.5740G>A(p.A1914T)	Titin	Titinopathy	Susp titinopathy	R4
						TTN (AD/AR)	Mat	c.71996A>G(p.N23999S)				
						NEB (AR)	Pat	c.12742-2A>G ^{a,b}	Nebulin	NEM2, AR		
48	NB	2.8	F	CM	NM	NEB (AR)	Pat	c.18176A>G(p.N6059S) ^b	Nebulin	NEM2, AR	Susp NEM2	R4
						NEB (AR)	Pat	c.7062_7063del(p.K2354Nfs*2) ^{a,b}				
						TTN (AD/AR)	Mat	c.99298A>C(p.S33100R) ^b	Titin			
						TTN (AD/AR)	Pat	c.13811G>A(p.G4604E)				
						MYH7 (AD)	Confirmed	c.1322C>T(p.T441M) ^b	Myosin, heavy chain 7, cardiac muscle, β			
						MYH2 (AD)	Confirmed	c.4258C>T(p.L1420F)	Myosin, heavy chain 2, muscle, adult			
54	53 y	55.9	M	LGMD	ESMC	EMD (X-R)	Mat	c.445G>C(p.D149H)	Emerin	EDMD1	Susp EDMD1	R4
						SYNE1 (AD/AR)	Pat	c.1507T>A(p.S503T) ^b	Spectrin repeat-containing nuclear envelope protein 1			
						TTN (AD/AR)	Pat	c.80173C>T(p.R26725C)	Titin			
						TTN (AD/AR)	Pat	c.26206G>A(p.A8763T)				
56	12 y	27.6	M	LGMD	MD	TTN (AD/AR)	Pat	c.92161+3A>T ^{a,b}	Titin	Titinopathy	Susp titinopathy	R4
						TTN (AD/AR)	Mat	c.6490G>A(p.A2164T)				
						TTN (AD/AR)	Mat	c.14987G>A(p.R4996Q) ^b				

Abbreviations: 2BA = type 2 B fiber atrophy; AD = autosomal dominant; AR = autosomal recessive; CACTD = carnitine acylcarnitine translocase deficiency; CCD = central core disease; CM = congenital myopathy; CMD = congenital muscular dystrophy; CMT = Charcot-Marie-Tooth disease; CMT2A2 = Charcot-Marie-Tooth disease, axonal, type 2A2; CNM = centronuclear myopathy; cons = consanguineous; DG = dystroglycanopathy; DM1 = myotonic dystrophy type 1; Dx = diagnosis; EDMD = Emery-Dreifuss muscular dystrophy; EDMD4 = Emery-Dreifuss muscular dystrophy 4, autosomal dominant; ESMC = end-stage myopathic change; exp = expired; HOKPP1 = hypokalemic periodic paralysis, type 1; HSN1C = hereditary sensory and autonomic neuropathy, type 1C; LGMD = limb-girdle muscular dystrophy; LGMD2B = dysferlinopathy; LGMD2G = limb-girdle muscular dystrophy type 2G; LGMD2J = limb-girdle muscular dystrophy type 2J; LSM = lipid storage myopathy; Mat = maternal; MC = myotonia congenita; MCAD = myotonia congenita, autosomal dominant; MCAR = myotonia congenita, autosomal recessive; MCM = minimal change myopathy; MCNI = myopathic change with nuclear internalization; MD = muscular dystrophy; MDC1A = muscular dystrophy, congenital merosin-deficient 1A; MDDGB1 = muscular dystrophy-dystroglycanopathy type B1; MHS1 = malignant hyperthermia susceptibility 1; MHS5 = malignant hyperthermia susceptibility 5; MM = metabolic myopathy; MMCD = multiminicore disease; MN = merosin negative; MTM = myotubular myopathy; NA = not available; NB = newborn; NC = neuropathic change; NEM2 = nemaline myopathy 2; NEM3 = nemaline myopathy 3; NIM = not in maternal; NIP = not in paternal; NM = nemaline myopathy; NSF = nonspecific finding; Pat = paternal; S/B = in son and brother; SMARD1 = spinal muscular atrophy with respiratory distress 1; Susp = suspected (phenotype/genotype now, may need further study); X-R = X-linked recessive; VM = vacuolar myopathy.

^a Definitive mutation.

^b Novel variants.



(A) Markedly increased lipid droplets in both number and size were observed in patient 2 with homozygous *SLC25A20* mutations (oil red O). (B) Forty percent myofibers with centralized nuclei were found in patient 26 with compound heterozygous *RYR1* mutations (hematoxylin and eosin). (C) Multiminicore pattern was observed in patient 11 with compound heterozygous *SEPN1* mutations (nicotinamide adenine dinucleotide tetrazolium reductase). (D) Prominent nemaline rods were seen in patient 9 with heterozygous *ACTA1* mutations (modified Gomori trichrome). (E) Nonspecific myopathic change except for internalized nuclei and mild fiber size variation was shown in patient 32 with compound heterozygous *TCAP* mutations (hematoxylin and eosin). (F) Similar to patient 9, typical intracytoplasmic nemaline rods were present in patient 48 with compound heterozygous *NEB* mutations (modified Gomori trichrome).

SLC25A20,²² which is responsible for carnitine acylcarnitine translocase deficiency (CACTD). Each of his parents was a carrier. Patients 3 and 46 with central core disease (CCD) had AR *RYR1* mutations and an AD de novo *RYR1* mutation, respectively; patients 25 and 26 (siblings) with centronuclear myopathy (CNM) also had AR *RYR1* mutations (figure 2B). Patients 11 (figure 2C) and 21 with multiminicore disease had mutations in *SEPN1*; 2 had nemaline myopathy (NM) and heterozygous *ACTA1* mutations (patients 9 [figure 2D] and M495). Patient 16 with merosin-negative CMD had mutations in *LAMA2*; patients 19 and 20, a mother and son with CMT, had an AD mutation in *MFN2*; patient 51 with dysferlinopathy and LGMD2B, a consanguineous product, had a homozygous deletion of exon 5 of *DYSF*; patient 52 and her affected sister with LGMD had the same compound heterozygous mutations in *CAPN3*; and patient 27 with myotubular myopathy carried a de novo *MTM1* mutation (X-linked).

Ambiguous or nonspecific muscle pathology findings clarified by molecular diagnosis. Sometimes the pathology findings from a muscle biopsy can be ambiguous and/or not completely consistent with the known phenotype caused by a specific disease gene. Massively parallel sequencing of a group of genes can resolve the uncertainty. Four families belong to this

category (R2 in table 2). Patient 12 had reduced α -dystroglycan undiagnosed CMD with end-stage pathologic muscle changes. Compound heterozygous mutations in a protein glycosylation gene (*POMT1*) were identified and confirmed by parental studies. Patient 17 had nonspecific muscle findings with a clinical diagnosis of a myotonic disorder. He was found to have mutations in a chloride ion channel gene (*CLCN1*) and confirmed to have an AR myotonia congenita. Patient 33, a 2.7-year-old girl, presented with a myopathic face, high-arched palate, scoliosis, and swallowing and respiratory difficulties since birth. Her muscle pathology revealed nondiagnostic end-stage myopathic changes, although clinically she exhibited typical CM. NGS analysis revealed compound heterozygous mutations in *RYR1*, which confirmed the diagnosis of CM. Patient M516 had muscle pathology findings suggestive of an atypical NM and was found to have AR *RYR1* mutations: one frameshift mutation and another missense mutation predicted to be deleterious. Each parent is a carrier for one of the mutations. This patient adds to the short list of NM cases caused by *RYR1* mutations.³⁰

Intercategory expansion of phenotype and genotype. Four families had molecular diagnoses in genes belonging to a subcategory that was not suspected in the original clinical evaluation (R3 in table 2). Patient 18 was

originally diagnosed as having infantile-onset unclassified CMT. The identification of compound heterozygous mutations in *IGHMBP2* confirmed the diagnosis of spinal muscular atrophy with respiratory distress type 1 (SMARD1), mainly affecting motor neurons rather than peripheral nerves. Patient 32, a 32-year-old man, had gait disturbance and mild lower leg weakness with an elevated creatine kinase since he was first evaluated at age 20. Muscle biopsy revealed nuclear internalization without evidence of dystrophic changes in 10% scattered fibers, favoring a diagnosis of CNM (figure 2E). The identification of a homozygous frameshift c.26_33dup (p.Glu12Argfs*20) mutation in *TCAP* confirmed the diagnosis of LGMD type 2G. Patient 29, a 22-year-old man, was suspected of having an MM due to periodic muscle weakness, abnormal findings in his metabolic profile, and vacuolar myopathy in his muscle biopsy. An AD heterozygous novel mutation inherited from his mother was identified, c.4639C>T (p.Arg1547Trp) in *CACNA1S*, encoding a calcium channel protein, consistent with hypokalemic periodic paralysis. AD *CACNA1S* mutations have been found in Asian men with periodic muscle weakness and risk for malignant hyperthermia (MH), but they exhibit lower penetrance in women.³¹ Patient 1 showed a positive Gowers sign and waddling gait when first evaluated at 4 years of age; however, the EMG, nerve conduction velocity, and muscle pathology were unremarkable at that time. The finding of the AD *SPTLC2* mutation c.1292G>A (p.Gly431Asp), confirmed clinically and genetically in his biological father, established the unexpected diagnosis of a neuropathy (hereditary sensory and autonomic neuropathy [HSAN] type 1C).

Interesting cases with likely diagnosis and expansion of phenotype/genotype. Eight families had novel phenotype/genotype diagnoses that require further functional evidence of pathogenicity (R4 in table 2). Patient 56 had a clinical and pathologic diagnosis of LGMD. NGS revealed 3 *TTN* variants: the c.92161+3A>T from the father was predicted to abolish the normal splice site, while a novel c.14987G>A (p.Arg4996Gln) variant in *cis* with another variant, c.6490G>A (p.Ala2164Thr), were both inherited from the mother. These 2 missense variants were predicted to be deleterious by PolyPhen-2 and SIFT. Mutations in *TTN* can cause AR LGMD type 2J. The growing genetic complexity and emerging phenotypic variability for recessive and dominant *TTN* variants make the assignment of definitive pathogenicity to many *TTN* variants difficult and delay our understanding of the pathogenic mechanism of the largest protein known to date.³² In addition to compound heterozygous *TTN* variants of unknown significance (VUS), patients 36 and 48 each had a heterozygous truncating mutation (a splice

site novel mutation c.12742-2A>G and a frameshift mutation c.7062_7063del, respectively) in the *NEB* gene (table 2).³³ Each patient inherited the deleterious *NEB* allele from the father. Despite the absence of the second mutant allele, muscle biopsy of patient 36 revealed only a nonspecific type 2B fiber atrophy, while patient 48 did show an NM (figure 2F). Patient 54 was suspected of having Emery-Dreifuss muscular dystrophy (EDMD) due to the X-linked *EMD* gene, of which his mother was a carrier. His cardiac phenotype of complete right bundle branch block may be due to an emerin mutation, but his clinical and pathologic features are not typical of EDMD. The positive emerin staining in the muscle biopsy did not rule out a pathogenic role of this emerin variant.³⁴

Defective genes and diagnostic yields. In our patient cohort, CM was the most common diagnosis (23/38, 61%), followed by CMD and LGMD. Since parallel analysis of all 236 NMD-related genes in a clinically validated panel is a recent innovative approach to testing, the majority of mutations/variants are “novel.” Theoretically, all missense variants should be classified as VUS until a functional defect can be demonstrated. However, if a VUS is found in patients with a consistent clinical phenotype, family pedigree, and muscle biopsy findings and is predicted to be deleterious, then it is classified as likely pathogenic.

AR disorders account for about half of the cases, while AD cases account for about a quarter. Overall, our NMD gene panel analysis provides a diagnostic yield of 29/35 (83%), which is the highest reported in complex NMD cases in the NGS era.

DISCUSSION To date, except when using whole-exome sequencing and whole-genome sequencing for novel gene discovery, most NGS studies have focused on a specific NMD category, such as CMD,¹⁷ CMYs,¹⁸ CMT,¹⁹ dystrophinopathy,²⁰ glycogen storage disease,²¹ or CACTD.²² In this report, we analyzed 236 genes responsible for 10 NMD subcategories. Surprisingly, some patients were found to harbor mutations in genes responsible for disease categories that were not initially considered (*IGHMBP2*, *TCAP*, *SPTLC2*, *CACNA1S*). This observation confirms that clinical features, muscle imaging, and muscle pathology may be suggestive of a specific diagnosis but that the ultimate diagnosis relies on the identification of mutations in the causative gene(s). Furthermore, some patients are evaluated at a very early or late stage of their disease, when the details of their early clinical course may not be available and the muscle pathology may provide limited information. Under these circumstances, it is very difficult to focus on a specific disease category for the analysis of a single gene or a few genes. Thus, comprehensive NGS analysis of all NMD-related genes provides a cost-effective way

to identify causative mutations. Evidence for this is provided by patient 1. The mutation analysis not only established the unexpected diagnosis of HSAN type 1C but also underscored the importance of molecular diagnosis through NGS.³⁵ Similarly, patient 18 was originally diagnosed as having congenital neuropathy, CMT type. The identification of mutations in *IGHMBP2* changed the final diagnosis to SMARD1 and further expanded the intercategory phenotype/genotype.³⁶ The identification of *IGHMBP2* mutations facilitated the subsequent prenatal diagnosis that resulted in a normal fetus.

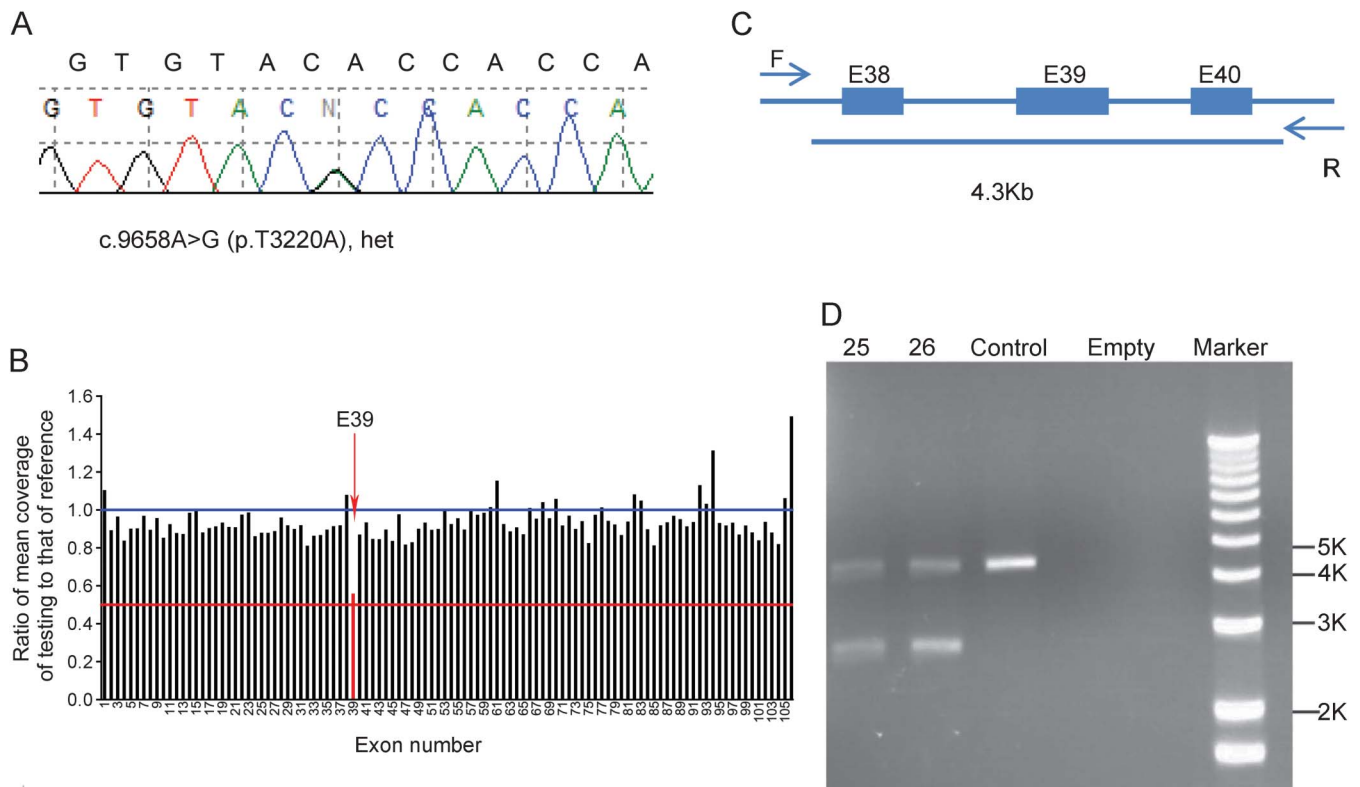
Our comprehensive NGS approach achieves a diagnostic yield of 83% for the highly genetically and clinically complex NMDs. Our capture/NGS approach has at least 2 unique advantages: (1) a comprehensive evaluation of 236 target genes to minimize variations in the coverage depth of individual exons, and (2) deep coverage depth at a mean of $>1,000\times$ per base, leaving only $\sim 0.6\%$ of coding exons containing insufficiently covered ($<20\times$) sequences requiring PCR/Sanger to complete. Thus, consistent coverage of individual exons allows the detection of deletions in $>99\%$ of targeted exons.²⁹ This is demonstrated by the identification of a single exon deletion in patient 51 (figure not shown) and by patients

25 and 26 (siblings), in whom both NGS and Sanger sequence analyses identified a heterozygous point mutation (p.Thr3220Ala) in *RYR1* (figure 3A), while copy number analysis using the same set of NGS data detected a heterozygous single exon deletion (figure 3B). The deletion was confirmed by using PCR across the deleted exon (figure 3C), showing the reduced size of PCR product (figure 3D).

Sequence variations in *RYR1* and *TTN* are frequent. This may be related to their relatively large size. Patients with mutations in these 2 genes may exhibit diverse clinical phenotypes and variable muscle pathology. For example, in our patient cohort, both AR and AD *RYR1* mutations were identified in muscle biopsies of patients with CCD (patient 3 AR and patient 46 AD), CNM (patient 26), CM with end-stage myopathic change (patient 33), atypical NM (patient M516), and nonspecific findings (patient 1). Patients with *RYR1* mutations may also be predisposed to MH. Thus, attention should be paid in anesthetic arrangements to avoid iatrogenic morbidity.^{37,38} Although NM is one of the most common types of CM,¹² we found only 2 patients with AD inheritance (9 and M495), both of whom had *ACTA1* mutations.

The *TTN* gene has not been extensively studied, likely in part due to its size. Sequencing of this

Figure 3 Confirmation of single exon deletion and point mutation in *RYR1* detected by next-generation sequencing



(A) Sanger sequence for heterozygous c.9658A>G (p.T3220A). (B) Exon 39 heterozygous deletion was detected by copy number variation analysis. (C) Designed primers for exon 39 deletion. Forward primer: F, reverse primer: R, the total length between the primers is 4.3 kb. (D) DNA gel for exon 39 deletion; patients 25 and 26 have extra small fragments on the gel (~ 2.7 kb).

gigantic gene (complementary DNA ~0.1 Mb) only became practical with NGS technology. Therefore, we have just begun to identify and understand its genetic diseases and mechanisms of inheritance. *TTN* mutations have been identified in patients with heterogeneous clinical phenotypes, including CM, LGMD, and others, with highly variable muscle biopsy findings.^{1,8,11,39,40} In this study, we have demonstrated the power of target gene capture followed by NGS. This comprehensive approach confirms clinical and pathologic diagnoses when the phenotype is consistent with the causative gene. It also clarifies a patient's underlying genetic cause when a muscle biopsy finding is ambiguous or does not comport with the clinical phenotype. Since the number of genes analyzed is large, it often identifies mutations in genes that may not have been considered in the clinical differential diagnosis, therefore expanding the phenotype/genotype relationship. Although ultimately this comprehensive noninvasive approach is much less expensive and more definitive for molecular diagnosis of the heterogeneous and complex NMDs, muscle pathology and thorough clinical evaluation are still important in the final clinical phenotype/genotype correlation, particularly at the early stage of expanding phenotype/genotype.

AUTHOR CONTRIBUTIONS

Dr. Tian: completed the statistical analysis; performed experiment; analyzed sequence results; and prepared figures and tables for publication. Dr. Liang: study concept and design; analysis and interpretation; and critical revision of the manuscript for important intellectual content. Dr. Feng: analyzed results; acquired data; and validated CNV detection using NGS data. Dr. Wang: helped with sequence data interpretation and critical review of the manuscript. Dr. Zhang: designed capture library and analytical pipeline; supervised NGS wet bench performance and sequence analysis; and determined the data filtering and variant annotation flow-chart. Mr. Chou: study concept and design. Dr. Huang: study concept and design and critical revision of the manuscript for important intellectual content. Dr. Lam: contributed 2 important cases. Dr. Hsu: study concept and design. Dr. Lin and Ms. Chen: critical revision of the manuscript for important intellectual content. Dr. Wong: interpreted results; designed and supervised the project; and drafted and critically revised the manuscript. Dr. Jong: acquisition of data; study concept and design; analysis and interpretation; critical revision of the manuscript for important intellectual content; and study supervision.

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DISCLOSURE

Dr. Tian is an employee of Baylor Miraca Genetics Laboratories. Dr. Liang reports no disclosures. Dr. Feng is an employee of Baylor Miraca Genetics Laboratories. Dr. Wang and Dr. Zhang are employees of Baylor Miraca Genetics Laboratories and are faculty members of BCM with joint appointment at BMGL. Mr. Chou reports no disclosures. Dr. Huang has served on the editorial boards of *BMC Bioinformatics*, *MicroRNA*, *Genomics*, *Proteomics & Bioinformatics*, and *Journal of Neuroscience and Neuroengineering* and has received research support from NSC. Dr. Lam, Dr. Hsu, Dr. Lin, and Ms. Chen report no disclosures. Dr. Wong is an employee of Baylor Miraca Genetics Laboratories, is a faculty member of BCM with joint appointment at BMGL, and serves on the editorial board of the journal *Mitochondrion*. Dr. Jong serves as a

consulting editor for *Pediatrics and Neonatology*, has served on the scientific advisory board for the Asian Oceanian Myology Center, and holds patents for a knockout-transgenic mouse model of spinal muscular atrophy, hydroxyurea treatment for spinal muscular atrophy, methods for diagnosing spinal muscular atrophy, and hnRNP A1 knockout animal model and use thereof. Go to Neurology.org/ng for full disclosure forms.

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