의학박사 학위논문

Targeted massive parallel sequencing and comprehensive phenome-genome assessment in neuromuscular patients without genetic etiology by conventional diagnostic methods

기존 진단 방법으로 원인유전자를 발견하지 못한
신경근육 질환 환자에서 표적 병렬 시퀀싱 및
포괄적 표현형-유전형 분석 연구

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Targeted massive parallel sequencing and comprehensive phenome-genome assessment in neuromuscular patients without genetic etiology by conventional diagnostic methods

By

Anna Cho, M.D.

Directed by Jong Hee Chae, M.D., Ph.D.

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Approved by Thesis Committee:

Professor _____ (인) Chairman

Professor _____ (인) Vice chairman

Professor _____ (인)

Professor _____ (인)

Professor _____ (인)

Abstract

Targeted massive parallel sequencing and comprehensive phenome-genome assessment in neuromuscular patients without genetic etiology by conventional diagnostic methods

Anna Cho
Translational Medicine
The Graduate School
Seoul National University

Neuromuscular disorders are clinically, pathologically, and genetically heterogeneous groups of disorders. An accurate genetic diagnosis has often been challenging due to the heterogeneity and complexity of these groups of disorders. The advent of next generation sequencing (NGS) technologies have started a new era of molecular genetic diagnosis in neuromuscular disorders. It is an effective diagnostic tool for the parallel investigation of a large number of genes and has been increasingly used in the recent clinical and research fields. In this study, NGS-

based targeted sequencing panels were applied to the diagnostic workflow in the genetic characterization of 327 patients who were suspected of having a hereditary neuromuscular disorders but without a definite molecular diagnosis using traditional genetic analysis. Four sets of targeted sequencing panels prepared at different times and were sequentially applied. Fifty eight patients were sequenced for 579 genes, 29 patients for 383 genes, and 150 patients for 436 genes. The last 90 patients were analyzed with tests selected among 6 custom panels for congenital myopathies, congenital muscular dystrophies, limb girdle muscular dystrophies, metabolic myopathies, myofibrillar myopathies, and Charcot-Marie-Tooth diseases. Each panel contains 29 to 79 genes according to the clinical subgroups. Pathogenic mutations were confirmed in 161 cases (49.2%) out of 327 patient. The diagnostic yield by phenotype groups were 62.7% in congenital muscular dystrophies (n = 47), 45.3% in limb girdle muscular dystrophies (n = 43), 54.3% in congenital myopathies (n = 50), 80% in congenital myasthenic syndromes (n = 4), 14.3% in metabolic myopathies (n = 3), 35.3% in motor neuron disorders (n = 6), 50% in hereditary motor and sensory neuropathies (n = 3), and 31.3% in other neuromuscular disorders (n = 5) respectively. The causative mutations were found in 50 different genes and the six most frequently found genes were *COL6A1* (n = 21), *RYR1* (n = 18), *DMD* (n = 10), *COL6A2* (n = 9), *ACTA1* (n = 7), and *LMNA* (n = 7) in order. This study illustrates the clinical utility of targeted NGS as a powerful diagnostic tool in hereditary neuromuscular disorders. Integrated phenome-genome

analysis is very important for improving the diagnostic efficiency of this technique. It is necessary to establish a comprehensive diagnostic platform for the molecular diagnosis of neuromuscular disorders that integrates single gene testing and NGS based on the phenotype analysis.

Keywords: targeted parallel sequencing, next generation sequencing, molecular diagnosis, neuromuscular disorders, muscular dystrophy, congenital myopathy

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Contents

List of Tables

List of Figures

List of Abbreviations

ACMG: American College of Medical Genetics and Genomics

AD: autosomal dominant

AR: autosomal recessive

BMD: Becker muscular dystrophy

CCD: central core disease

CFTD: congenital myopathy with fiber type disproportion

CMA: chromosome microarray

CMD: congenital muscular dystrophy

CMP: congenital myopathy

CMS: congenital myasthenic syndromes

CMT: Charcot-Marie-Tooth disease

CNM: centronuclear myopathy

CNV: copy number variation

CK: creatine kinase

DCMP: dilated cardiomyopathy

DM: dermatomyositis

DMD: Duchenne muscular dystrophy

EMG: electromyography

FSHD: facioscapulohumeral muscular dystrophy

HGMD: Human Gene Mutation Database

HMSN: hereditary motor and sensory neuropathies

IHC: immunohistochemistry

IMNM: immune mediated necrotizing myopathy

LGMD: limb girdle muscular dystrophy

MD: muscular dystrophy

MDCMD: merosin deficient congenital muscular dystrophy

MFM: myofibrillar myopathy

MLPA: multiplex ligation dependent probe amplification

MM: metabolic myopathy

MMD: multi-minicore disease

MND: motor neuron diseases

MRI: magnetic resonance imaging

MTM: myotubular myopathy

MyoD: myotonic dystrophy

NCV: nerve conduction velocity

NGS: next generation sequencing

NM: nemaline myopathy

NMD: neuromuscular disorders

ONM: other neuromuscular disorders

P: pathogenic

LP: likely pathogenic

SMA: spinal muscular atrophy

SNUH: Seoul national university hospital

UCMD: Ullrich congenital muscular dystrophy

VUS: variants of unknown significance

WB: Western blot

WES: whole exome sequencing

XD: X linked dominant

XR: X linked recessive

Introduction

Neuromuscular disorders (NMD) are clinically, pathologically, and genetically heterogeneous groups of disorders. The clinical presentation shares similar features such as hypotonia, weakness, and motor developmental delay, which make it difficult to reach a specific diagnosis even for the experienced clinicians (1-4). Traditionally, most of neuromuscular disorders have been categorized and diagnosed according to their clinical phenotypes and histological presentations (5, 6) and genetic diagnosis has not been always possible because of the diversity of disease causing genes.

There are at least three major reasons that many cases with probable hereditary neuromuscular disorders have been genetically undiagnosed. Firstly, it is often difficult to determine the target gene to be analyzed due to genetic complexity and heterogeneity. For example, nemaline myopathy, one of the most common of the congenital myopathies, is caused by at least 12 different genes including *ACTA1*, *NEB*, *TPM2*, *TPM3*, *MYPN*, *TNNT1*, *TNNT3*, *KLHL40*, *KLHL41*, *KBTBD13*, *CFL2*, and *LMOD3* (7). While *ACTA1*, a representative causative gene of nemaline myopathy, shares various phenotypes including intra-nuclear rod myopathy, congenital myopathy with fiber type disproportion, and zebra body myopathy. Secondly, muscle genes are often big in size such as *TTN* with 362 exons, making it difficult to routinely sequence such genes even though there are known causative

genes (8). Thus far, we have often performed routine gene sequencing of small genes such as *ACTA1* and *MTM1* and large genes such as *RYR1* and *COL6A1* were occasionally subjected to sequencing analysis only at hot spots. Thirdly, most cases are sporadic without parental consanguinity, which makes us difficult to do linkage study to identify new causative genes.

The advent of next generation sequencing (NGS) technologies have started a new era of molecular genetic diagnosis in neuromuscular disorders (9-12). It is an effective diagnostic tool for the parallel investigation of a large number of genes and has been increasingly used in the recent clinical and research fields (13-16). Genome-wide approaches such as whole exome sequencing (WES) or whole-genome sequencing (WGS) can be the ultimate goal in genetic testing, in that it allows the screening of numerous known gene mutations and can potentially identify new disease causative genes (12). But selected gene panel tests do have multiple clinical advantages over genome-wide sequencing methods in terms of time and cost effectiveness (17, 18). The important point is to establish a comprehensive diagnostic system in patients with hereditary neuromuscular disease that is clinically very complex and contains a wide range of genes.

In this study, we applied an NGS-based platform to the diagnostic workflow in the genetic characterization of neuromuscular patients who were suspected of having

a Mendelian disorder but without a definite molecular diagnosis using the traditional genetic analysis.

Methods

Design and subjects

The study enrolled a total of 327 patients with suspected hereditary neuromuscular disorders based on their clinical and/or muscle histopathological analysis but without a definite molecular diagnosis. All cases were ascertained from the Seoul National University Hospital (SNUH) – Neuromuscular Disorders (NMD) database, which includes a cohort of more than 2000 cases from 2000 to 2020. All clinical information and samples used form diagnostic purpose in this study were collected after obtaining written informed consent from.

Before the NGS testing, several genetic testing including *SMN1/2* Multiplex ligation dependent probe amplification (MLPA), *DMD* MLPA, *DMPK* PCR and Southern blot analysis, and Sanger sequencing targeting entire genes or hot spots of severe genes (*ACTA1*, *CPT2*, *FKTN*, and *MTM1*) were performed based on the clinical and pathologic findings in selected patients, but were negative. Patients whose molecular diagnosis was confirmed by these conventional genetic analysis methods were excluded from this study.

Four sets of targeted sequencing panels prepared at different times were sequentially applied for the analysis of 327 patients. In 2013, the first 58 patients were analyzed with NGS in a collaborative study with Seattle Children's Research

Institute (10). Then we developed the first in-house panel for the neuromuscular disorders and analyzed 29 patients in 2014. The gene panel library was updated once in 2015 and an additional 150 patients were analyzed. As Korea's National Health Insurance Service coverage became available for the panel NGS analysis of hereditary diseases in 2017 (Ministry of Health and Welfare Notice No.2017-15), the validated NGS custom panels by the Department of Laboratory Medicine at Seoul National University Hospital has begun. We set up 6 kinds of different targeted panels for the hereditary neuromuscular disorders according to the clinical subgroups: 1) congenital muscular dystrophy (CMD), 2) limb girdle muscular dystrophy (LGMD), 3) congenital myopathy (CMP), 4) metabolic myopathy (MM), 5) Charcot-Marie-Tooth disease (CMT), and 6) myofibrillar myopathy (MFM). Total 90 patients underwent the SNUH-NGS panel analysis corresponding to the clinical diagnosis. The diagnostic workflow and study design is summarized as a flow chart in Figure 1.

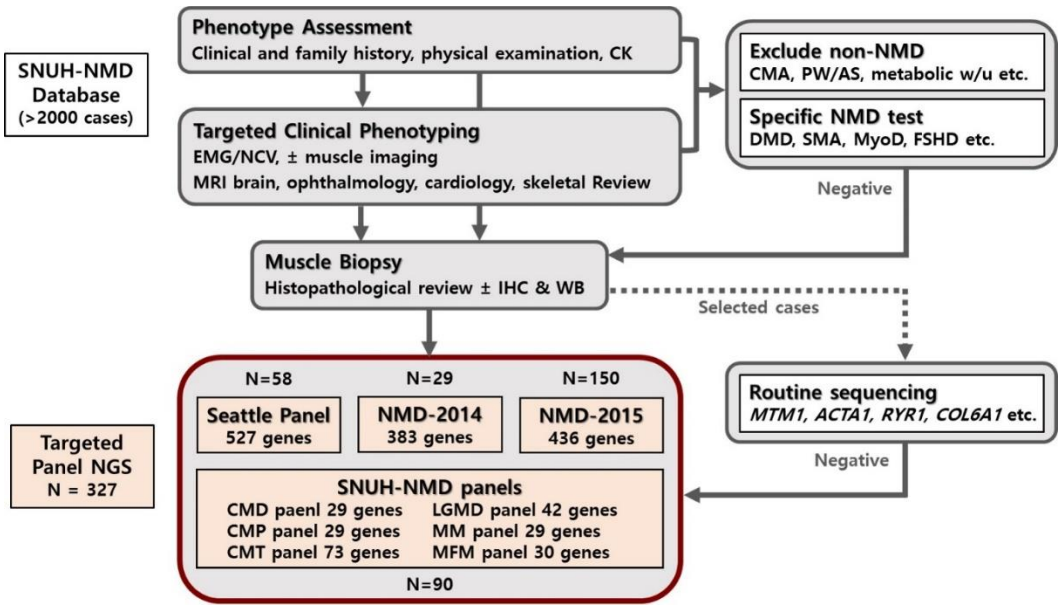

Figure 1. Flowchart of the diagnostic workflow with NGS application

SNUH, Seoul National University Hospital; NMD, neuromuscular disorders; CK, creatine kinase; EMG/NCS, electromyography & nerve conduction velocity; MRI, Magnetic Resonance Imaging; IHC & WB, immunohistochemistry & Western blot; NGS, next generation sequencing; N, numbers; CMD, congenital muscular dystrophy; LGMD, limb girdle muscular dystrophy; CMP, congenital myopathy; MM, metabolic myopathy; CMT, Charcot-Marie-Tooth disease; MFM, myofibrillar myopathy; CMA, chromosome microarray; PW/AS, Prader-Willi/Angelman Syndrome methylation test; DMD, Duchenne muscular dystrophy; SMA, spinal muscular atrophy; MyoD, myotonic dystrophy; FSHD, facioscapulohumeral muscular dystrophy

Clinical and histological data analysis

Clinical information was reviewed including demographic information, the age of onset, clinical presentation, and serum creatine kinase (CK) levels.

1) Clinical phenotype classification

All patients were classified into 8 diagnostic subgroups based on their clinical and muscle histopathological analyses: congenital muscular dystrophies (CMD), limb girdle muscular dystrophies (LGMD), congenital myopathies (CMP), congenital myasthenic syndromes (CMS), metabolic myopathies (MM), motor neuron diseases (MND), hereditary motor and sensory neuropathies (HMSN), and other neuromuscular disorders (ONM). CMD are categorized based on high CK and/or presence of necrotic and regenerating muscle fibers on muscle pathology with the clinical onset in infancy or before independent gait. LGMD are categorized based on high CK and/or presence of necrotic and regenerating muscle fibers on muscle pathology with the onset of weakness after independent gait. CMP are categorized based on pathological diagnosis and/or characteristic clinical features including hypotonia and facial weakness at birth. CMS are categorized based on clinical diagnosis with characteristics of weakness with diurnal variation, ptosis, and/or positive Jolly test. MM are categorized based on clinical diagnosis with characteristics of exercise intolerance, rhabdomyolysis, and/or muscle weakness

with high CK. MND and HMSN are categorized based on electrophysiological findings and/or presence of fibers type grouping (denervation pattern) on muscle pathology. Patients who could not be classified into a specific group were classified as ONM.

2) Muscle pathology analysis

Histopathological phenotype was reviewed in 261 cases that underwent muscle biopsies at Seoul National University Children's Hospital. Serial frozen sections from each muscle sample were stained using a set of histochemical methods including hematoxylin and eosin (H&E), modified Gomori trichrome, NADH-Tetrazolium Reductase (NADH-TR), succinyl dehydrogenase, ATPase, and immunohistochemistry for dystrophin, merosin and dysferlin. Additional immunohistochemical stainings with mouse monoclonal anti-human collagen VI primary antibodies (Chemicon, MAB1944) or monoclonal antibodies against alpha-dytroglycan with glycoepitope-dependent antibody (VIA4-1) (Merck Millipore, Catalogue No. 05-298) were performed on clinically suspected. Reports or slides of muscle histopathology performed at an external institution in 10 cases were reviewed.

Gene selection, sequencing, and variant annotation

1) Seattle Panel

The first NGS series were performed at Seattle Children's Research Institute, Seattle, Washington, USA (10). A DNA library was prepared for each sample by capturing the exons of the genes of interest using custom made DNA probes (Haloplex Agilent, Santa Clara, CA). The selected 579 genes (Table 1) comprised 10,706 exons and a total of 3.88 Mb. Fifty eight samples were sequenced at 5-8 samples per lane on a GAIIx instrument (Illumina, San Diego, CA) using 2x100 paired-ends reads. Reads were aligned using Burrows-Wheeler Aligner (BWA, version 0.7.3) and data were analyzed with the Genome Analysis Toolkit (GATK, version 2.4.9) (Broad Institute, Cambridge, MA) as previously described (19).

Variants were annotated using SeattleSeq (20) and wAnnovar (21). Variants found within the targeted regions were further evaluated for their possible clinical significance by cross-referencing to the Single Nucleotide Polymorphism Database (dbSNP), the Exome variant server, and the 1000 Genomes browser. An internal database of polymorphisms was also used for quality assurance during this analysis. PolyPhen-2 and SIFT (sorting intolerant from tolerant) scores were used only for reference but were not used for filtering the variants. For variants in genes with autosomal dominant and X-linked disease inheritance, we used the minor allele frequency (MAF) cut-off of 0.2%, and for variants in genes with autosomal

recessive disease inheritance, we used the MAF cut-off of 0.5% (19). Variants that exceeded these frequencies were not considered as potential mutations, even if they were previously reported as such in the Human Gene Mutation Database (HGMD). Finally, variants were searched in the HGMD by Genometrax (22, 23).

Table 1. List of 579 Genes for Targeted NGS Sequencing [Seattle Panel]

Congenital myopathy (30 genes)
ACTA1, ACTG2, ATP2A1, BIN1, CCDC78, CFL2, CLCN1, CNTN1, DNM2, FAM123B, FLNC, GNE, HOXD10, HRAS, KBTBD13, LDB3, MEGF10, MTM1, MTMR14, MYF6, MYH2, MYH7, NEB, TIA1, TNNT1, TPM2, TPM3, TTN, VCP, VMA21
Muscular dystrophy (48 genes)
ANO5, BAG3, CAPN3, CAV3, CHKB, CNBP, COL6A1, COL6A2, COL6A3, CRYAB, DAG1, DES, DMD, DMPK, DNAJB6, DPM2, DPM3, DYSF, EMD, FHL1, FKBP14, FKRP, FKTN, GTDC2, ISPD, ITGA7, ITGA9, KLHL9, LAMA2, LARGE, LMNA, MTAP, MYOT, PABPN1, PLEC, PLOD3, POMGNT1, POMT1, POMT2, SECISBP2, SGCA, SGCB, SGCD, SGCG, SPP1, SYNE2, TCAP, TRIM32
Congenital myopathy or Muscular dystrophy (2 genes)
RYR1, SEPN1
Metabolic myopathy (202 genes)
AARS2, ACACA, ACAD9, ACADM, ACADVL, ACSF3, ACY1, ADCK3, AGK, AGL, AIFM1, ALDH1B1, ALDOA, ALG1, ALG11, ALG12, ALG13, ALG2, ALG3, ALG6, ALG8, ALG9, ALPL, AMACR, AMPD1, ATP5A1, ATP5E, ATP5G3, ATPAF2, B4GALT1, BCS1L, BRP44L, C10orf2, C12orf65, CACNA1S, COA5, COG1, COG4, COG5, COG6, COG7, COG8, COQ2, COQ4, COQ6, COQ9, COX10, COX14, COX15, COX20, COX4I2, COX6B1, COX7B, CPT1A, CPT1B, CPT2, CYC1, CYCS, D2HGDH, DARS2, DGUOK, DMGDH, DNA2, DOLK, DPM1, EARS2, ENO3, ETFA, ETFB, ETFDH, FARS2, FASTKD2, FOXRED1, GAA, GALC, GBE1, GFER, GFM1, GPR172B, GYG1, GYS1, HADH, HADHA, HADHB, HARS2, IARS, ISCU, LAMP2, LARS2, LDHA, LDHB, LIAS, LPIN1, LRPPRC, LYRM4, LYRM7, MARS, MATR3, MGAT2, MGME1, MPV17, MRPL12, MRPL3, MRPL44, MRPS16, MRPS22, MTFMT, MTHFD1, MTHFR, MTO1, NDUFA1, NDUFA10, NDUFA11, NDUFA12, NDUFA2, NDUFA4, NDUFA9, NDUFAF1, NDUFAF2, NDUFAF3, NDUFAF4, NDUFAF5,

NDUFAF6, NDUFB3, NDUFB9, NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS6, NDUFS7, NDUFS8, NDUFV1, NDUFV2, NEU1, NFU1, NUBPL, OAT, OPA1, OXCT1, PDSS1, PDSS2, PEX12, PEX14, PEX26, PEX3, PEX5, PEX6, PEX7, PFKM, PGAM2, PGK1, PGM1, PHKA1, PHKA2, PHKB, PHKG2, PHYH, PMM2, PNPLA2, PNPT1, POLG, POLG2, PSAP, PTEN, PTRF, PUS1, PYGL, PYGM, RMND1, RRM2B, RSPH9, SCO1, SCO2, SDHA, SDHAF1, SDHAF2, SDHB, SDHC, SDHD, SLC22A5, SLC25A20, SLC25A3, SLC25A4, SUCLA2, SUCLG1, SURF1, TACO1, TARS2, TAZ, TIMM44, TK2, TMEM165, TMEM70, TSFM, TTC19, TYMP, UQCRB, UQCRC2, UQCRQ, VARS2, YARS2

Motor neuron or peripheral nerve disease (75 genes)

AARS, AFG3L2, APTX, ARHGEF10, ATL1, ATXN2, BSCL2, CCT5, COLQ, DCTN1, DHTKD1, DNAJB2, DNMT1, DYNC1H1, EGR2, ELAVL4, ERLIN2, FAM134B, FGD4, FOXE1, GAN, GARS, GDAP1, GJB1, HARS, HINT1, HOXB1, HSPB1, HSPB3, HSPB8, HSPG2, IGHMBP2, IKBKAP, INF2, KARS, KIF1B, KIF5A, LITAF, MED25, MFN2, MPZ, MTMR2, MYH14, NAGA, NDRG1, NEFL, NGF, NIPA1, PDK3 , PLEKHG5, PLP1, PMP22, PNPLA6, PRX, RAB7A, REEP1, SACS, SBF2, SH3TC2, SLC12A6, SLC5A7, SMN1, SMN2, SNX25, SPTLC1, SPTLC2, SYNE1, TDP1, TFG, TGM6, TRPV4, UBA1, VRK1, WNK1, YARS

Myasthenic syndrome (12 genes)

AGRN, CHAT, CHRNA1, CHRNB1, CHRND, CHRNE, DOK7, DPAGT1, GFPT1, LAMB2, MUSK, SCN4A

Cardiomyopathy (3 genes)

MYBPC3, MYLK2, RYR2

Other candidate genes (207 genes)

ACAD10, ACAD11, AIFM2, ATP5B, ATP5C1, ATP5D, ATP5F1, ATP5G1, ATP5G2, ATP5H, ATP5I, ATP5J, ATP5J2, ATP5L, ATP5O, ATP5S, ATPAF1, ATPIF1, C14orf2, CARS, CARS2, CD36, CHCHD1, CHCHD7, CMC1, CMC2, COA1, COA3, COA6,

COQ10A, COQ10B, COQ3, COQ5, COQ7, COX11, COX16, COX17, COX18, COX19, COX4I1, COX5A, COX5B, COX6A1, COX6A2, COX6B2, COX6C, COX7A1, COX7A2, COX7A2L, COX7B2, COX7C, COX8A, COX8C, ECi1, ECSIT, EPRS, FARSA, FARSB, FRG1, FUNDC2, HINT3, IARS2, ICT1, LACTB, LYRM1, MCAT, MDH2, METTL17, MNF1, MRP63, MRPL1, MRPL10, MRPL11, MRPL13, MRPL14, MRPL15, MRPL16, MRPL17, MRPL18, MRPL19, MRPL2, MRPL20, MRPL21, MRPL22, MRPL23, MRPL24, MRPL27, MRPL28, MRPL30, MRPL32, MRPL33, MRPL34, MRPL35, MRPL36, MRPL37, MRPL38, MRPL39, MRPL4, MRPL40, MRPL41, MRPL42, MRPL43, MRPL45, MRPL46, MRPL47, MRPL48, MRPL49, MRPL50, MRPL51, MRPL52, MRPL53, MRPL54, MRPL55, MRPL9, MRPS10, MRPS11, MRPS12, MRPS14, MRPS15, MRPS17, MRPS18A, MRPS18B, MRPS18C, MRPS2, MRPS21, MRPS23, MRPS24, MRPS25, MRPS26, MRPS27, MRPS28, MRPS30, MRPS31, MRPS33, MRPS34, MRPS35, MRPS36, MRPS5, MRPS6, MRPS7, MRPS9, MRS2, MTCH1, MTERF, MTG1, MTHFD1L, MTHFS, MTIF3, MTRF1L, NARS, NARS2, NDUFA3, NDUFA4L2, NDUFA5, NDUFA6, NDUFA7, NDUFA8, NDUFAB1, NDUFB1, NDUFB10, NDUFB11, NDUFB2, NDUFB4, NDUFB5, NDUFB6, NDUFB7, NDUFB8, NDUFC1, NDUFC2, NDUFS5, NDUFV3, NIPSNAP3A, NRF1, OXA1L, OXSM, PARS2, PGAM1, POLRMT, PRELID1, PRELID2, PTCD1, PTCD2, PTCD3, PTRH2, QARS, RARS, SLIRP, SMCR7, SMCR7L, SUCLG2, TARS, TARSL2, TIMM21, TIMM23, TOMM70A, TOP1MT, TYMS, UQCR10, UQCR11, UQCRC1, UQCRFS1, UQCRH, USMG5, VARS, WARS, WARS2, YME1L1

This table was reproduced from [Supplement Table 1] in "Utility of next generation sequencing in genetic diagnosis of early onset neuromuscular disorders" J Med Genet 52(3):208-16 (10).

2) NMD-2014 and NMD-2015 Panels

The custom-designed SureSelect Target Enrichment System Kit (Agilent Technologies, USA) was used to assess hereditary neuromuscular genes at SNUH. The targeted genes were selected using the 2014 and 2015 version of the gene table of monogenic neuromuscular disorders (24, 25) and some of the genes were added based on a literature search. The capture kits were updated once to include newly identified genes. Twenty nine patients were sequenced with the first kit (NMD-2014 including 383 genes, Table 2) and 150 with the second kit (NMD-2015 including 436 genes, Table 3). Library preparation was completed according to the manufacturer's instructions (Agilent Technologies). The library was paired-end sequenced on an Illumina HiSeq 2500 sequencing system.

Paired-end sequencing reads with a read length of 101 bp were aligned to Genome Reference Consortium Human Genome build 37 (patch release 13) using BWA-0.7 (26). The Picard software (v.2.1.1), SAMtools (v.1.3.1) (27), and Genome Analysis Toolkit (v.3.8) (28) best-practice pipelines were used in data analyses. Variant calling was performed using HaplotypeCaller and variant annotation using ANNOVAR (29). mTect2 (30) was used for low-frequency variant detection to search for variants with a variant allele frequency from 0.05 to 0.25. Only low-frequency variants with a variant allele count $\geq$ 30 were selected.

Table 2. List of 383 Genes for Targeted NGS Sequencing [NMD-2014]

The 2014 version of the gene table of monogenic neuromuscular disorders (361 genes)

AARS, ABCC9, ABHD5, ACADVL, ACTA1, ACTC1, ACTN2, ACVR1, AFG3L2, AGL, AGRN, AIFM1, AKAP9, ALDH3A2, ALG13, ALG14, ALG2, ALS2, ANG, ANK2, ANKRD1, ANO5, AP4E1, AP4M1, AP5Z1, APTX, AR, ARHGEF10, ASAH1, ATL1, ATM, ATP2A1, ATP7A, ATXN1, ATXN10, ATXN2, ATXN3, ATXN7, ATXN8OS, B3GALNT2, B3GNT1, BAG3, BEAN, BICD2, BIN1, BSCL2, C9orf72, CABC1, CACNA1A, CACNA1C, CACNA1S, CACNB2, CACNB4, CAPN3, CASQ2, CAV3, CFL2, CHAT, CHKB, CHMP2B, CHRNA1, CHRNB1, CHRND, CHRNE, CHRNG, CLCN1, CNTN1, COL6A1, COL6A2, COL6A3, COLQ, COX15, CPT2, CRYAB, CSRP3, CTDP1, CYP7B1, DAG1, DCTN1, DES, DMD, DMPK, DNAJB2, DNAJB6, DNM2, DNMT1, DOK7, DOLK, DPAGT1, DPM1, DPM2, DPM3, DSC2, DSG2, DSP, DTNA, DUX4, DYNC1H1, DYSF, EGR2, EMD, ENO3, ERBB3, ETFA, ETFB, ETFDH, EXOSC3, EYA4, FA2H, FBLN5, FGD4, FGF14, FHL1, FIG4, FKRP, FKTN, FLNA, FLNC, FUS, FXN, GAA, GAN, GARS, GATAD1, GBE1, GDAP1, GDF8, GFPT1, GJA5, GJB1, GLE1, GMPPB, GNB4, GNE, GPD1L, GTDC2, GYG1, GYS1, HCN4, HEXB, HINT1, HK1, HOXD10, HSPB1, HSPB3, HSPB8, HSPD1, HSPG2, ICSU, IFRD1, IGHMBP2, IKBKAP, ILK, INF2, ISPD, ITGA7, ITPR1, JPH2, JUP, KARS, KBTBD13, KCNA1, KCNA5, KCNC3, KCNE1, KCNE2, KCNE3, KCNH2, KCNJ18, KCNJ2, KCNJ5, KCNQ1, KIAA0196, KIF1A, KIF1B, KIF21A, KIF5A, KLHL40, KLHL9, L1CAM, LAMA2, LAMA4, LAMB2, LAMP2, LARGE, LDB3, LDHA, LITAF, LMNA, LPIN1, LRSAM1, MARS, MATR3, MED25, MEGF10, MFN2, MPZ, MRE11A, MRPL3, MTM1, MTMR2, MTPAP, MURC, MUSK, MYBPC3, MYH2, MYH3, MYH6, MYH7, MYH8, MYL2, MYL3, MYLK2, MYOT, MYOZ2, MYPN, NDRG1, NDUFAF1, NEB, NEFH, NEFL, NEXN, NGFB, NIPA1, NOP56, NPPA, OPA1, OPTN, PABPN1, PDK3, PEO1, PEX7, PFKM, PFN1, PGAM2, PGK1, PGM1, PHKA1, PHOX2A, PHYH, PIP5K1C, PKP2, PLEC1, PLEKHG5, PLN, PLP1, PMP22, PNPLA2 ,PNPLA6, POLG, POLG2, POMGNT1, POMK, POMT1, POMT2, PPP2R2B, PRKAG2, PRKCG, PRPH,

PRPS1, PRX, PSEN2, PTPLA, PTRF, PYGM, RAB7, RAPSN, RBCK1, RBM20, REEP1, RRM2B, RTN2, RYR1, RYR2, SACS, SBF1, SBF2, SCN1B, SCN3B, SCN4A, SCN4B, SCN5A, SDHA, SEPN1, SEPTIN9, SETX, SGCA, SGCB, SGCD, SGCE, SGCG, SH3TC2, SIGMAR1, SIL1, SLC12A6, SLC1A3, SLC22A5, SLC25A20, SLC25A4, SLC33A1, SLC52A2, SLC52A3, SLC5A7, SMCHD1, SMN1, SNTA1, SOD1, SPAST, SPG11, SPG20, SPG21, SPG3A, SPG7, SPTBN2, SPTLC1, SPTLC2, SUCLA2, SYNE1, SYNE2, TARDBP, TAZ, TBP, TCAP, TDP1, TFG, TGFB3, TIA1, TK2, TMEM43, TMEM5, TMPO, TNNC1, TNNI2, TNNI3, TNNT1, TNNT2, TNNT3, TNPO3, TOR1A, TPM1, TPM2, TPM3, TRAPPC11, TRIM32, TRPV4, TTBK2, TTN, TTPA, TTR, TUBB3, UBA1, UBQLN2, VAPB, VCL, VCP, VRK1, WNK1, YARS, ZFYVE26, ZFYVE27, ZNF9
Genes added after the literature review (22 genes) *ANKB, CACNA2D1, CACNB2b, CTF1, DNAJC19, FHL2, FOXD4, GATA4, GATA5, GATA6, GLA, GREM2, KCND3, KCNE5, KCNH2, KCNJ8, MOG1, NKX2.5, NUP155, PITX2c, PSEN1, SCN2B*

Table 3. List of 436 Genes for Targeted NGS Sequencing [NMD-2015]

The 2015 version of the gene table of monogenic neuromuscular disorders (406 genes)

AARS, AARS2, ABCC9, ABHD5, ACADVL, ACTA1, ACTC1, ACTN2, ACVR1, AFG3L2, AGL, AGRN, AIFM1, AKAP9, ALDH3A2, ALG13, ALG14, ALG2, ALS2, AMPD2, ANG, ANK2, ANKRD1, ANO5, AP4B1, AP4E1, AP4M1, AP4S1, AP5Z1, APTX, AR, ARHGEF10, ARL6IP1, ASAH1, ATL1, ATM, ATP2A1, ATP7A, ATXN , ATXN10, ATXN2, ATXN3 ,ATXN7, ATXN8OS, B3GALNT2, B3GNT1, B4GALNT1, BAG3, BEAN, BICD2, BIN1, BSCL2, C12orf65, C19orf12, C9orf72, CABC1, CACNA1A, CACNA1C, CACNA1S, CACNB2, CACNB4, CAPN3, CASQ1 CASQ2, CAV3, CCDC88,. CFL2, CHAT, CHCHD10, CHKB, CHMP2B, CHRNA1, CHRNB1, CHRND, CHRNE, CHRNG, CLCN1, CNTN1, COL6A1, COL6A2, COL6A3, COLQ, COX15, COX6A1, CPT2, CRYAB, CSRP3, CTDP1, CYP2U1, CYP7B1, DAG1, DCTN1, DDHD1, DDHD2, DES, DMD, DMPK, DNAJB2, DNAJB6, DNM2, DNMT1, DOK7, DOLK, DPAGT1, DPM1, DPM2, DPM3, DSC2, DSG2, DSP, DTNA, DUX4, DYNC1H1, DYSF, EEF2, EGR2, ELOVL4, ELOVL5, EMD, ENO3, ENTPD1, ERBB3, ERLIN2, ETFA, ETFB, ETFDH, EXOSC3, EXOSC8, EYA4, FA2H, FBLN5, FBXO38, FGD4, FGF14, FHL1, FIG4, FKRP, FKTN, FLNA, FLNC, FUS, FXN, GAA, GAN, GARS, GATAD1, GBA2, GBE1, GDAP1, GDF8, GFPT1, GJA5, GJB1, GJC2, GLE1, GMPPB, GNB4, GNE, GPD1L, GTDC2, GYG1, GYS1, HCN4, HEXB, HINT1, HK1, HNRPDL, HOXD10, HSPB1, HSPB3, HSPB8, HSPD1, HSPG2, ICSU, IFRD1, IGHMBP2, IKBKAP, ILK, INF2, ISPD, ITGA7, ITPR1, JPH2, JUP, KARS, KBTBD13, KCNA1, KCNA5, KCNC3, KCND3, KCNE1, KCNE2, KCNE3, KCNH2, KCNJ18, KCNJ2, KCNJ5, KCNQ1, KIAA0196, KIF1A, KIF1B, KIF21A, KIF5A, KLHL40, KLHL41, KLHL9,L1CAM, LAMA2, LAMA4, LAMB2, LAMP2, LARGE, LDB3, LDHA, LITAF, LMNA, LPIN1, LRSAM1, MARS, MATR3, MED25, MEGF10, MFN2, MPZ, MRE11A, MRPL3, MRPL44, MTM1, MTMR2, MTO1, MTPAP, MURC, MUSK, MYBPC3, MYH2, MYH3, MYH6, MYH7, MYH8, MYL2, MYL3, MYLK2, MYOT, MYOZ2, MYPN, NDRG1, NDUFAF1, NEB, NEFH, NEFL, NEXN, NGFB, NIPA1, NOP56, NPPA, NT5C2,

NUP155, OPA1, OPTN, ORAI1, PABPN1, PDK3, PDYN, PEO1, PEX7, PFKM, PFN1, PGAM2, PGK1, PGM1, PHKA1, PHOX2A, PHYH, PIP5K1C, PKP2, PLEC1, PLEKHG5, PLN, PLP1, PMP22, PNPLA2, PNPLA6, POLG, POLG2, POMGNT1, POMK, POMT1, POMT2, PPP2R2B, PRKAG2, PRKCG, PRPH, PRPS1, PRX, PSEN2, PTPLA, PTRF, PUS1, PYGM, RAB7, RAPSN, RBCK1, RBM20, REEP1, RNF216, RRM2B, RTN2, RYR1, RYR2, SACS, SBF1, SBF2, SCN1B, SCN2B, SCN3B, SCN4A, SCN4B, SCN5A, SDHA, SEPN1, SEPTIN9, SETX, SGCA, SGCB, SGCD, SGCE, SGCG, SH3TC2, SIGMAR1, SIL1, SLC12A6, SLC1A3, SLC22A5, SLC25A20, SLC25A4, SLC33A1, SLC52A2, SLC52A3, SLC5A7, SMCHD1, SMN1, SNTA1, SOD1, SPAST, SPEG, SPG11, SPG20, SPG21, SPG3A, SPG7, SPTBN2, SPTLC1, SPTLC2, STIM1, SUCLA2, SYNE1, SYNE2, SYT2, TARDBP, TAZ, TBP, TCAP, TDP1, TECPR2, TFG, TGFB3, TGM6, TIA1, TK2, TMEM43, TMEM5, TMPO, TNNC1, TNNI2, TNNI3, TNNT1, TNNT2, TNNT3, TNPO3, TOR1A, TOR1AIP1, TPM1, TPM2, TPM3, TRAPPC11, TRIM32, TRPV4, TSFM, TTBK2, TTN, TTPA, TTR, TUBB3, UBA1, UBQLN2, VAPB, VCL, VCP, VMA21, VRK1, WNK1, YARS, YARS2, ZFYVE26, ZFYVE27, ZNF9

Genes added after the literature review (30 genes)

ACADL, ACADM, ACADS, ALDOA, AMPD1, CACNA2D1, CTF1, DGUOK, DNAJC19, FDX1L, FHL2, FOXD4, GATA4, GATA5, GATA6, GLA, GREM2, HADHA, HADHB, ISCU, KCNE5, KCNH2, KCNJ8, NKX2.5, PHKB, PITX2c, PSEN1, RANGRF, SIL1, TSEN54

3) SNUH-NMD Panel

The library preparation was performed according to SureSelectXT Target Enrichment protocol (Agilent, Santa Clara, CA, USA). The target genes were selected for 6 custom SNUH-NMD panels (Table 4) including congenital muscular dystrophy panel (29 genes), limb girdle muscular dystrophy panel (42 genes), congenital myopathy panel (29 genes), metabolic myopathy (29 genes), Charcot-Marie-Tooth disease panel (73 genes), and myofibrillar myopathy panel (30 genes). Paired-end 150-bp sequencing was performed using the MiSeq platform (Illumina, San Diego, CA, USA). The raw data of targeted sequencing was obtained in the FASTQ format. The sequencing data were mapped to the human reference genome sequence (GRCh37/hg19), and per-base coverage was calculated using the NextGENe software v2.4.0.1. (SoftGenetics).

Table 4. List of Genes for Targeted NGS Sequencing [SNUH-NMD]

Congenital muscular dystrophy (29 genes) *ACTA1, ALG13, B3GALNT2, CHKB, COL6A1, COL6A2, COL6A3, CRPPA, DNM2, DPM1, DPM2, FHL1, FKRP, FKTN, GAA, GMPPB, ITGA7, LAMA2, LARGE1, LMNA, POMGNT1, POMGNT2, POMK, POMT1, POMT2, RXYLT1, SELENON, TCAP, TRAPPC11*
Limb girdle muscular dystrophy (42 genes) *ANO5, CAPN3, CAV3, CAVIN1, CRPPA, DAG1, DES, DMD, DNAJB6, DPM3, DYSF, EMD, FHL1, FKRP, FKTN, GAA, GMPPB, GNE, HNRNPDL, ITGA7, LIMS2, LMNA, MYOT, PLEC, POMGNT1, POMT1, POMT2, SGCA, SGCB, SGCD, SGCG, SMCHD1, SYNE1, SYNE2, TCAP, TMEM43, TNPO3, TOR1AIP1, TRAPPC11, TRIM32, TTN, VCP*
Congenital myopathy (29 genes) *ACTA1, AGRN, BIN1, CFL2, CHAT, CHRNA1, CHRNB1, CHRND, CHRNE, COLQ, DNM2, DOK7, GFPT1, IGHMBP2, KBTBD13, KLHL40, LAMB2, MTM1, MUSK, MYH7, NEB, RAPSN, RYR1, SEPN1, SLC5A7, TNNT1, TPM2, TPM3, TTN*
Metabolic myopathy (29 genes) *ACTB, ACTG1, AKT3, ARFGEF2, ARX, CDK5, COL4A1, DCX, DYNC1H1, EMX2, FLNA, KIF2A, KIF5C, MCPH1, MTOR, NDE1, PAFAH1B1, PIK3CA, RELN, TUBA1A, TUBA8, TUBB, TUBB2A, TUBB2B, TUBB3, TUBG1, VLDLR, WDR62*
Charcot-Marie-Tooth disease (73 genes) *AARS, ABHD12, AIFM1, ARHGEF10, ATP1A1, ATP7A, BAG3, BSCL2, CNTNAP1, COA7, DCTN1, DCTN2, DGAT2, DHTKD1, DNAJB2, DNM2, DNMT1, DRP2, DYNC1H1, EGR2, FGD4, FIG4, GARS, GDAP1, GJB1, GNB4, HARS1, HINT1, HSPB1, HSPB3, HSPB8, IGHMBP2, INF2, KIF1B, KIF5A, LITAF, LMNA, LRSAM1, MARS, MCM3AP, MED25, MFN2, MME, MORC2, MPV17, MPZ, MTMR2, NAGLU, NDRG1, NEFH, NEFL, PDK3, PLEKHG5, PMP2, PMP22, PRPS1, PRX, PTRH2, RAB7A, SBF1, SBF2, SCO2, SETX, SGPL1, SH3TC2, SIGMAR1, SPG11, SPTLC1, TRIM2, TRPV4, VCP, WARS, YARS*

Myofibrillar myopathy (30 genes)

ACTA1, BAG3, CFL2, CRYAB, DES, DNAJB6, FHL1, FLNC, GNE, KBTBD13, KLHL40, LAMP2, LDB3, MATR3, MEGF10, MYH2, MYOT, NEB, ORAI1, PABPN1, PLEC, SELENON, SIL1, STIM1, TCAP, TIA1, TRIM32, TTN, VCP, VMA21

Variant interpretation and validation

All selected sequence variants were further confirmed with Sanger sequencing, which was also conducted for available family members. Sequence variants were classified according to the international guidelines of the American College of Medical Genetics and Genomics (ACMG) (31); those classified as ‘pathogenic’ or ‘likely pathogenic’ were considered causative for the phenotype. Low-frequency variants were further validated with amplicon sequencing in which six nucleotide barcode sequences unique to each sample as well as adaptor sequences (AGAT) were added to forward PCR primer to identify individual samples. The same amounts of PCR products for each sample were then pooled using an Illumina dual-indexed PCR free library preparation kit and sequenced on the Illumina HiSeq 2500 sequencing system. During sequence analysis, each paired-end read was assigned to an individual by barcode sequences, and read numbers with or without the variant for each sample were counted.

Comprehensive phenome-genome analysis after the NGS application

All patients underwent a clinical follow-up period of at least 1 year after the initial panel analysis. The patients whose final genetic diagnosis was confirmed through NGS analysis were reclassified into the 8 diagnostic subgroups through integrated phenome-genome analysis. CMD and LGMD are reclassified as cases in which genetic mutations reasonable for MD were found in addition to the clinical findings. CMPs are reclassified as cases in which compatible genetic variants were confirmed based on clinical and pathological findings. In the CMP patients who underwent muscle tissue examination, it was confirmed whether the results of the genetic test and the pathological test are consistent. All patients in whom the pathogenic mutations of CMS were found are categorized into the CMS group. Patients with confirmed causal variants of MM that were compatible with their clinical findings are finally classified into the MM group. MND and HMSN patients were also reclassified based on the confirmed genotypes and comprehensive phenotypes including clinical, histopathological, and/or electrophysiological findings. Patients who could not be classified into these 7 groups were classified as ONM.

For patients with negative results, further medical record reviews were performed to check for additional diagnoses through other tests including single genetic tests,

WES, WGS, RNA sequencing, etc. After reassessing the clinical phenotype, the autoantibodies were analyzed through a commercial line immunoblot assay (EUROLine, EUROIMMUN) on selected patients.

Result

Clinical spectrum

A total of 327 patients (211 males and 116 females) were recruited (Figure 2). Koreans accounted for the majority with 322 unrelated patients and two Emirates, two Mongolians, and one Indian were included. The mean age was 17.6 (± 10.5) years and patients aged 5 to 20 years were included the most in this study (64.5%, n = 211). The youngest subject was 1.2 years old and the oldest subject was 68.1 years old. MD were the most common clinical diagnosis with 170 patients, among which 75 patients (22.9%) were classified as CMD with the clinical onset in infancy or before independent gait. And the remaining 95 patients (29.1%) were classified as LGMD with the onset of weakness after independent gait. Ninety two patients (28.1%) were classified as CMP and 5 patients (1.5%) were classified as CMS. Twenty one patients were MM (6.4%). Seventeen patients (5.2%) with MND and six patients (1.8%) with HMSN were included. Sixteen patients (4.9%) were grouped as ONM because they did not belong to any of the above 7 clinical subgroups. Serum CK was elevated in CMD, LGMD, and MM groups with the mean values of 1231 (± 1803) IU/L, 3599 (± 5077) IU/L, and 27226 (± 73228) IU/L respectively (Figure 3). On the other hand, the mean CK of patients with CMP, CMS, MND, HMSN, and ONM were all within the normal range, in order of 89.6 (± 59.1) IU/L, 88.5 (± 30.0) IU/L, 121.3 (± 58.7) IU/L, 124.0 (± 15.6) IU/L, and

129.5 (± 108.6) IU/L. This is the result of reflecting the clinical classification and characteristics of each subgroup.

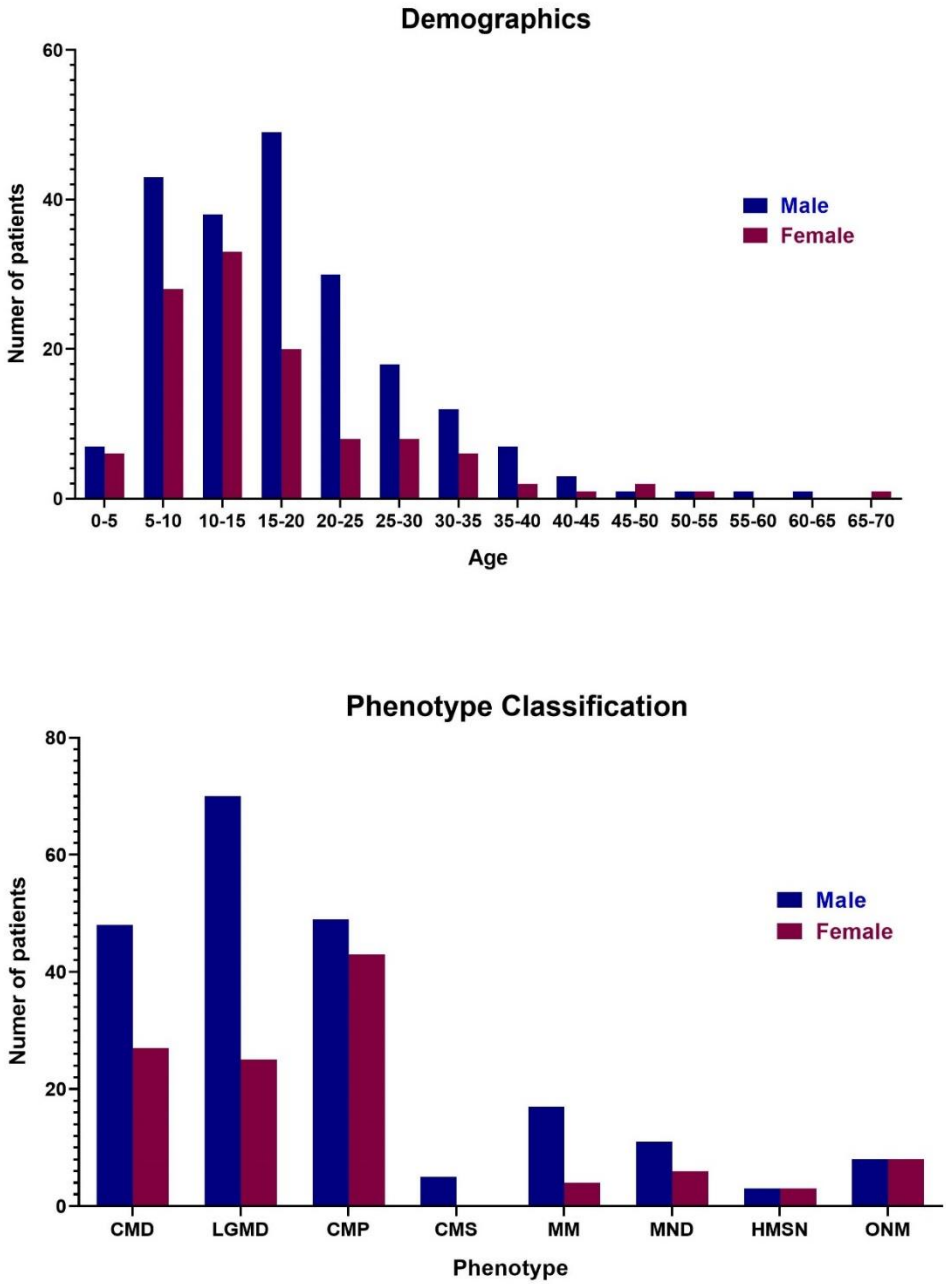

Figure 2. Demographics and phenotype classification

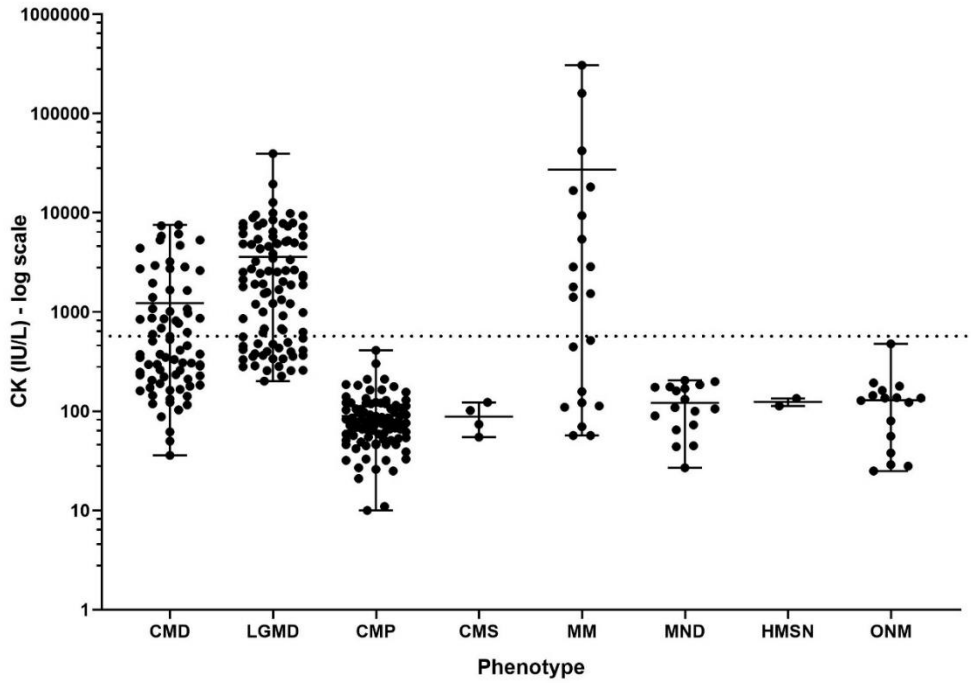

Figure 3. Serum creatine kinase (CK) by phenotype groups

Dotted horizontal line = upper limit of normal CK (270 IU/L)

CMD, congenital muscular dystrophies; LGMD, limb girdle muscular dystrophies; CMP, congenital myopathies; CMS, congenital myasthenic syndromes; MM, metabolic myopathies; MND, motor neuron diseases; HMSN, hereditary motor and sensory neuropathies; ONM, other neuromuscular disorders

Identification of pathogenic variants with phenotypic characteristics

Pathogenic mutations were confirmed in 161 cases (49.2%) out of 327 patient. The diagnostic yield by panel sets were 53.4% (n = 31/58) in Seattle Panel, 51.7% (n = 15/29) in NMD-2014 Panel, 46% (n = 69/150) in NMD-2015 Panel, and 51.1% (n = 46/90) in SNUH-NMD Panels.

The diagnostic yield by phenotype subgroups were 62.7% (n = 47/75) in CMD, 45.3% (n = 43/95) in LGMD, 54.3% (50/92) in CMP, 80% (n = 4/5) in CMS, 14.3% (n = 3/21) in MM, 35.3% (n = 6/17) in MND, 50% (n = 3/6) in HMSN, and 31.3% (5/16) in ONM respectively (Figure 4).

The causative mutations were found in 50 different genes (Figure 5) including *ACADVL, ACTA1, AGRN, BAG3, BICD2, CAPN3, CAV3, CCDC78, CHRNE, COL6A1, COL6A2, COL6A3, COLQ, CRYAB, DMD, DNM2, DOK7, DYNC1H1, DYSF, FHL1, FKRP, GAA, GARS1, GFPT1, GMPPB, GNB4, GNE, HADHA, HADHB, ISPD, KLHL40, LAMA2, LMNA, MARS1, MTM1, MYBPC3, MYH7, NEB, POMGNT1, POMT1, PYGM, RAPSN, RYR1, SELENON, SGCA, SLC5A7, TPM2, TPM3, TRPV4, TTN*. Only 24 genes were identified in two or more cases, of which 6 most frequently found genes were *COL6A1* (n = 21), *RYR1* (n = 18), *DMD* (n = 10), *COL6A2* (n = 9), *ACTA1* (n = 7), and *LMNA* (n = 7) in that order.

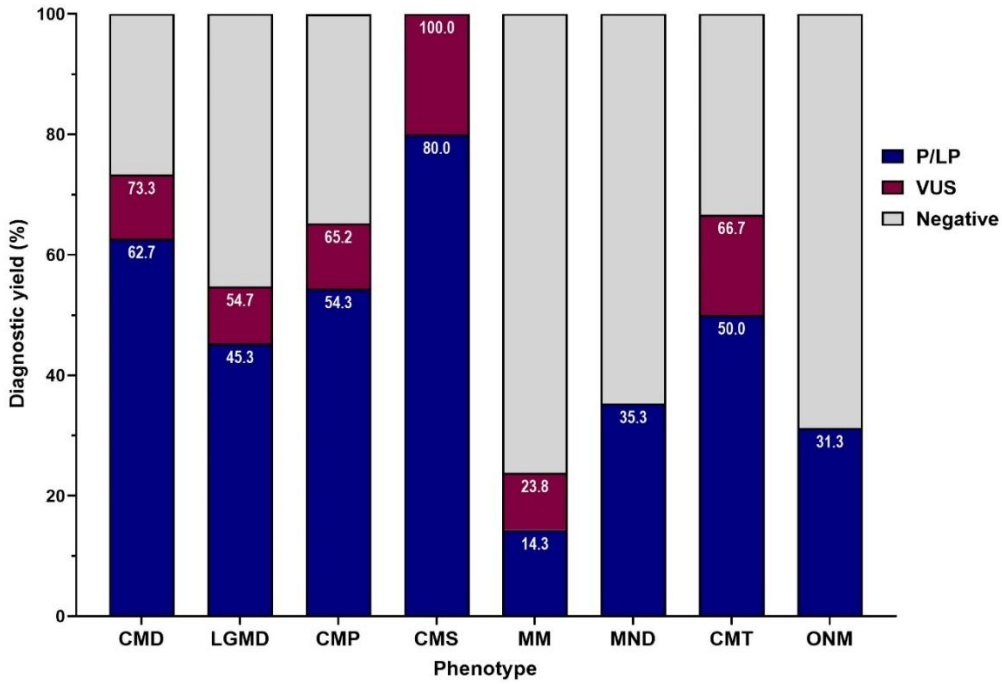

Figure 4. Fraction of diagnostic yields by phenotype groups

The total diagnostic yield was 49.2%. The diagnostic yield in CMD was 62.7%, LGMD was 45.3%, CMP was 54.3%, CMS was 80%, MM was 14.3%, MND was 35.3%, CMT was 50%, and OMN was 31.3%.

CMD, congenital muscular dystrophies; LGMD, limb girdle muscular dystrophies; CMP, congenital myopathies; CMS, congenital myasthenic syndromes; MM, metabolic myopathies; MND, motor neuron disease; CMT, Charcot-Marie-Tooth disease; ONM, other neuromuscular disorders; P/LP, pathogenic and likely pathogenic variants identified; VUS, variants of uncertain significance

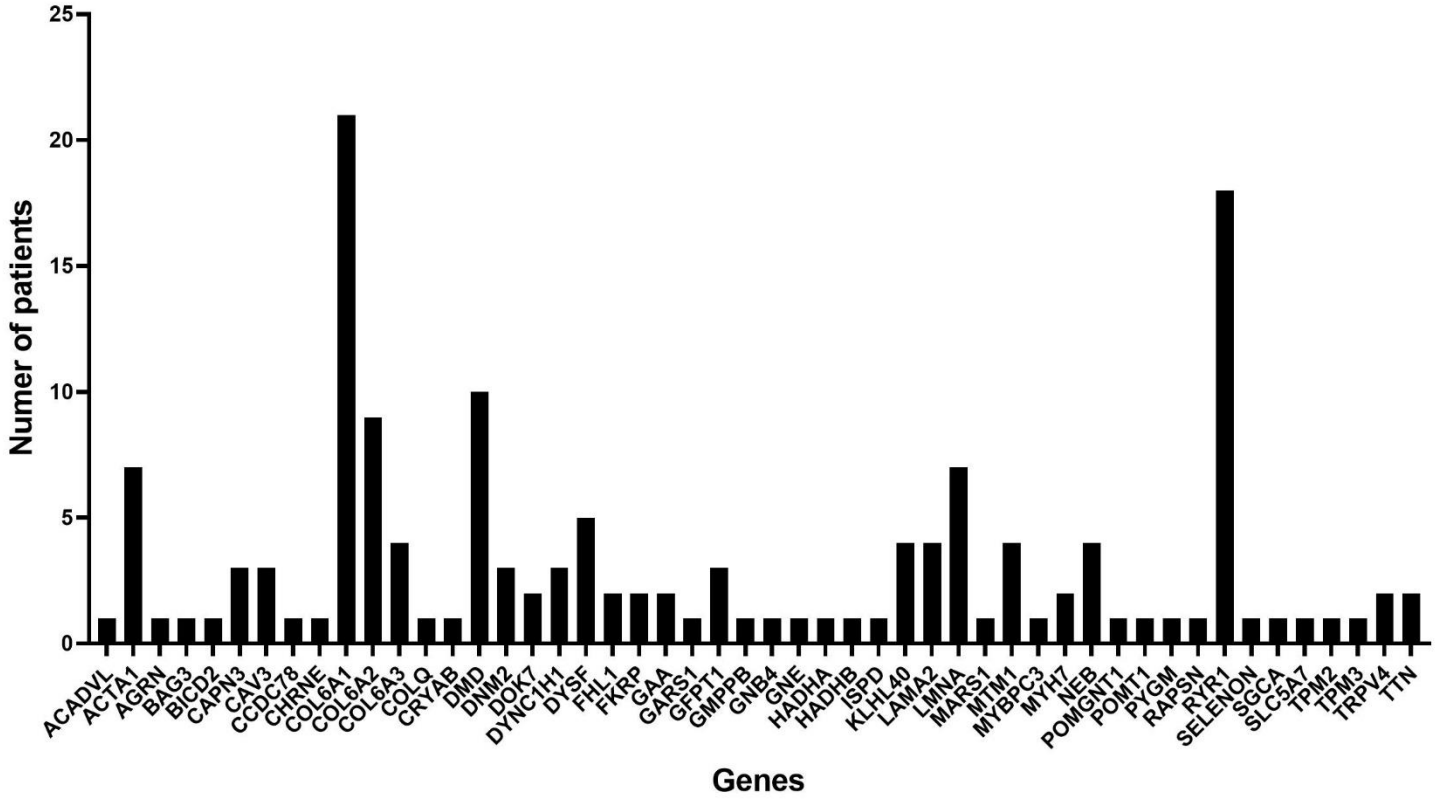

Figure 5. Diagnosis frequency in 50 genes with mutations

Genetically confirmed 161 patients were reclassified by integrating genotypes and phenotypes.

Muscular dystrophies

Eighty three patients were diagnosed with MD (Table 5). Pathogenic and likely pathogenic variants were identified in *ACTA1* (n = 1), *CAPN3* (n = 4), *CAV3* (n = 3), *COL6A1* (n = 21), *COL6A2* (n = 9), *COL6A3* (n = 5), *DMD* (n = 12), *DYSF* (n = 6), *FKRP* (n = 2), *GAA* (n = 2), *GMPPB* (n = 2), *ISPD* (n = 1), *LAMA2* (n = 4), *LMNA* (n = 7), *POMGNT1* (n = 1), *POMT1* (n = 1), *RYR1* (n = 1), and *SGCA* (n = 1). Clinical features of all the patients were consistent with the detected genotypes. Type 6 collagenopathies (*COL6A1, 2, 3*) and dystrophinopathies (*DMD*) are the most commonly diagnosed genes in MD (Figure 6).

Table 5. Pathogenic and likely pathogenic variants in 83 muscular dystrophy patients

ID	Sex	Age	Phenotype	Clinical presentation	Onset	CK	Gene	Mode	Mutation	Class
1	M	13.7	CMD	Floppy infant	Birth	868	ACTA1	AD	c.487C>G:p.His163Asp	LP
2	M	35.7	LGMD	Asymptomatic high CK		2653	CAPN3	AR	c.1318C>T:p.Arg440Trp c.2305C>T:p.Arg769Trp	LP LP
3	M	25.0	LGMD	Asymptomatic high CK		5926	CAPN3	AR	c.473delA:p.Asn158Thrfs*21 c.2120A>G:p.Asp707Gly	P LP
4	M	22.8	LGMD	Asymptomatic high CK		4891	CAPN3	AR	c.316dup:p.Cys106Leufs*9 c.2120A>G:p.Asp707Gly	P LP
5	M	14.5	LGMD	Weakness from teenage	12Y	4343	CAPN3	AR	c.946-2A>C c.2442G>A:p.Trp814*	LP LP
6	M	19.4	LGMD	Asymptomatic high CK		680	CAV3	AD	c.307_312del:p.Val103_Val104del	LP
7	M	21.9	LGMD	Exercise induced muscle cramping	11Y	1886	CAV3	AD	c.84C>A:p.Asp28Glu	P
8	M	18.0	LGMD	Asymptomatic high CK		564	CAV3	AD	c.307_3012delGTGGTG:p.Val103_Val104del	LP
9	M	22.0	Bethlem	Gait abnormality, weakness from toddler	1-2Y	50	COL6A1	AD	c.1002+1delG	P
10	M	15.3	Bethlem	Muscle weakness with joint contractures	1-2Y	190	COL6A1	AD	c.1056+1delG	LP
11	F	32.8	LGMD	Gait abnormality	13Y	259	COL6A1	AD	c.877G>A, p.Gly293Arg	P
12	F	26.0	LGMD	Gait abnormality	7Y	282	COL6A1	AD	c.850G>A:p.Gly284Arg	P
13	F	16.3	LGMD	Gait abnormality	1-2Y	366	COL6A1	AD	c.1003-2A>G	LP

14	M	19.7	UCMD	Motor developmental delay	<1Y	296	COL6A1	AD	c.G868A:p.G290R	P
15	M	22.1	UCMD	Weakness with joint laxity	<1Y	116	COL6A1	AD	c.958-2A>G	P
16	M	17.9	UCMD	Weakness with joint laxity	Birth	626	COL6A1	AD	c.850G>A:p.Gly284Arg	P
17	F	16.9	UCMD	Gait abnormality, weakness from toddler	1-2Y	350	COL6A1	AD	c.877G>A:p.Gly293Arg	P
18	M	20.8	UCMD	Floppy infant	<1Y	259	COL6A1	AD	c.958-2A>G	P
19	M	18.6	UCMD	Gait abnormality, weakness from toddler	1-2Y	161	COL6A1	AD	c.G814A:p.Gly272Ser	LP
20	F	15.5	UCMD	Hypotonia with joint laxity	Birth	119	COL6A1	AD	c.1461+3G>C	LP
21	M	17.7	UCMD	Floppy infant	Birth	144	COL6A1	AD	c.859-2A>G	P
22	M	20.4	UCMD	Floppy infant	Birth	232	COL6A1	AD	c.850G>A:p.Gly284Arg	P
23	F	9.3	UCMD	Floppy infant	<1Y	332	COL6A1	AD	c.850G>A:p.Gly284Arg	P
24	F	10.6	UCMD	Motor developmental delay	<1Y	133	COL6A1	AD	c.850G>A:p.Gly284Arg	P
25	F	8.9	UCMD	Motor developmental delay	<1Y	297	COL6A1	AD	c.850G>A:p.Gly284Arg	P
26	M	13.1	UCMD	Motor developmental delay	<1Y	262	COL6A1	AD	c.850G>A:p.Gly284Arg	P
27	F	8.4	UCMD	Motor developmental delay	<1Y	286	COL6A1	AD	c.877G>A, p.Gly293Arg	P
28	F	14.9	UCMD	Weakness with joint laxity	1-2Y	163	COL6A1	AD	c.868G>A:p.Gly290Arg	P
29	F	14.1	UCMD	Weakness with joint laxity	<1Y	223	COL6A1	AD	c.833G>T:p.Gly278Val	LP
30	M	33.1	LGMD	Gait abnormality	13Y	287	COL6A2	AD	c.349_350delinsAA:p.Ser117Asn	LP
31	M	37.6	LGMD	Gait abnormality	1-2Y	226	COL6A2	AD	c.883G>A:p.Gly295Arg	LP
32	M	14.0	LGMD	Gait abnormality	6Y	358	COL6A2	AD	c.736-2A>G	LP

33	M	7.7	UCMD	Floppy infant	<1Y	173	COL6A2	AD	c.1615C>T:p.Arg539Ter	P
34	M	17.7	UCMD	Floppy infant	<1Y	247	COL6A2	AD	c.875G>A:p.Gly292ASP	
35	M	5.1	UCMD	Gait abnormality	1-2Y	166	COL6A2	AD	c.801+1G>A	P
36	F	36.0	UCMD	Motor developmental delay	<1Y	62	COL6A2	AD	c.801+1G>A	P
37	F	36.4	LGMD	Gait abnormality	12Y	678	COL6A2	AD / AR	c.1189_1196del:p.G397fs c.2843C>G:p.Thr948Arg	LP VUS
38	M	18.8	UCMD	Floppy infant	<1Y	228	COL6A2	AR	c.2386A>T:p.Lys796X c.1816+1G>T	P LP
39	M	12.0	Bethlem	Gait abnormality, distal joint contractures.	1-2Y	142	COL6A3	AD	c.4367A>G:p.Tyr1456Cys	LP
40	F	14.1	UCMD	Gait abnormality, weakness from toddler	1-2Y	377	COL6A3	AD	c.6282+1G>C	P
41	M	13.7	UCMD	Motor developmental delay	<1Y	308	COL6A3	AD	c.6210+1G>A	P
42	M	9.1	UCMD	Motor developmental delay	<1Y	88	COL6A3	AD	c.4389+1G>A	P
43	M	38.9	Bethlem	Gait abnormality, high arched palate, scoliosis.	2-3Y	164	COL6A3	AR	c.1825C>T, p.Arg609* c.6690+1G>A	P LP
44	M	25.3	BMD	Exercise induced muscle cramping	5Y	4866	DMD	XR	c.48G>A:p.Trp16Ter	P
45	M	13.2	BMD	Asymptomatic high CK		4985	DMD	XR	c.31+36947G>A	P
46	M	11.6	BMD	Asymptomatic high CK		7892	DMD	XR	exons 30 - 42 deletion	P
47	M	8.2	BMD	Asymptomatic high CK		3470	DMD	XR	c.23A>G:p.Glu8Gly	LP
48	M	6.3	BMD	Asymptomatic high CK		12708	DMD	XR	c.9959C>T:p.Pro3320Leu	LP
49	M	10.8	DMD	Gait abnormality	3Y	8900	DMD	XR	c.984C>T:p.Gln984Ter	P

50	M	15.3	DMD	Gait abnormality	6Y	5769	DMD	XR	c.2739G>T:p.Lys913Asn	LP
51	M	9.9	DMD	Motor developmental delay	1-2Y	7800	DMD	XR	c.9640A>T:p.Lys3214*	LP
52	F	30.7	Carrier	Asymptomatic high CK		2525	DMD	XR	c.5921+2T>A	P
53	F	10.6	Carrier	Asymptomatic high CK		7310	DMD	XR	c.433C>T:p.Arg145Ter	P
54	F	12.0	Carrier	Asymptomatic high CK		3372	DMD	XR	c.4001dup:p.Val1335Serfs*4	LP
55	F	13.2	Carrier	Asymptomatic high CK		2536	DMD	XR	c.9450_9453del:p.Asn3152Valfs*2	LP
56	F	45.6	LGMD	Gait abnormality	22Y	8482	DYSF	AR	c.663+1G>C c.2997G>T:p.Trp999Cys	P LP
57	F	45.0	LGMD	Gait abnormality	27Y	3246	DYSF	AR	c.1284+2T>C c.2974T>C:p.Trp992Arg	P P
58	F	42.7	LGMD	Gait abnormality	18Y	2033	DYSF	AR	c.663+1G>C c.6057-2A>C	P P
59	M	20.3	LGMD	Asymptomatic high CK		7888	DYSF	AR	c.663+1G>C c.2997G>T:p.Trp999Cys	P LP
60	M	58.0	LGMD	Gait abnormality	20Y	NA	DYSF	AR	c.2548C>T:p.Gln850Ter c.3051G>T:p.Trp1017Cys	P LP
61	M	19.9	LGMD	Gait abnormality	15Y	7407	DYSF	AR	c.2494C>T:p.Gln832* c.2494C>T:p.Gln832*	P P
62	M	3.9	CMD	Floppy infant	Birth	7529	FKRP	AR	c.1170_1171delCG, p.Gly391Leufs*72 c.1136G>C, p.Arg379Pro	P LP
63	F	5.7	LGMD	Gait abnormality, weakness from toddler	1-2Y	9546	FKRP	AR	c.501_502delinsCC:p.Arg167_Cys168delinsSerArg c.1176C>G:p.Phe392Leu	LP LP

64	M	26.4	LGMD	Gait abnormality	8Y	661	GAA	AR	c.1316T>A:p.Met439Lys c.2238G>C:p.Trp746Cys	LP LP
65	M	19.3	LGMD	Asymptomatic high CK		1920	GAA	AR	c.2015G>A:p.Arg672Gln c.546G>T:p.Thr182=	P P
66	M	12.9	CMD	Gait abnormality, weakness from toddler	1-2Y	817	GMPPB	AR	c.391G>T:p.Gly131Cys c.391G>T:p.Gly131Cys	LP LP
67	M	4.5	CMD	Floppy infant	Birth	1014	GMPPB	AR	c.343T>C, p.Phe115Leu c.787G>A, p.Gly263Ser	LP LP
68	M	18.1	CMD	Floppy infant	<1Y	4710	ISPD	AR	c.894delT:p.Phe298fs*7 c.964_966del:p.Val322del	P LP
69	M	27.5	CMD	Motor developmental delay	<1Y	845	LAMA2	AR	c.595T>C:p.Cys199Arg c.7605delT:p.Pro2535fs	LP P
70	F	10.8	CMD	Floppy infant	Birth	5307	LAMA2	AR	c.4987C>T:p.Gln1663* c.5974G>T:p.Glu1992*	P P
71	M	9.2	CMD	Motor developmental delay	<1Y	2951	LAMA2	AR	c.648delC, p.Ser217Leufs c.7156-4A>G	P LP
72	F	14.9	CMD	Floppy infant	<1Y	2623	LAMA2	AR	c.5866-2A>G c.8952delG:p.Met2984*	P LP
73	M	18.1	CMD	Weakness with ankle contractures, neck muscle weakness	1-2Y	1071	LMNA	AD	c.1406T>C:p.Ile469Thr	P
74	F	17.3	CMD	Gait abnormality	1-2Y	1081	LMNA	AD	c.149G>C:p.Arg50Pro	P
75	F	13.2	CMD	Weakness with ankle contractures	<1Y	975	LMNA	AD	c.745C>T:p.Arg249Trp	P
76	F	10.7	CMD	Motor developmental delay	<1Y	1651	LMNA	AD	c.1156A>T:p.Arg386Trp	LP

77	M	6.7	CMD	Gait abnormality, weakness from toddler	1-2Y	867	LMNA	AD	c.1081G>A:p.Glu361Lys	P
78	M	8.4	CMD	Gait abnormality, neck muscle weakness.	1-2Y	769	LMNA	AD	c.122G>A:p.Arg41His	P
79	F	7.3	CMD	Floppy infant	Birth	1671	LMNA	AR	c.1129C>T:p.Arg377Cys c.1129C>T:p.Arg377Cys	LP LP
80	M	7.4	CMD	Floppy infant	<1Y	2743	POMGNT1	AR	c.1011_1012insT c.1768T>C:p.Trp590Arg	P LP
81	F	6.8	CMD	Floppy infant	Birth	2848	POMT1	AR	c.2167del:p.Asp723Thrfs*21 c.2167del:p.Asp723Thrfs*21	P P
82	F	23.9	CMD	Weakness	<1Y	855	RYR1	AR	c.1654C>T:p.Arg552Trp c.2287G>A:p.Val763Met	P LP
83	M	10.2	LGMD	Asymptomatic high CK		495	SGCA	AR	c.220C>T:p.Arg74Trp c.320C>T:p.Ala107Val	P LP

Abbreviations: M, male; F, female; CMD, congenital muscular dystrophy; LGMD, limb girdle muscular dystrophy; Bethlem, Bethlem myopathy; UCML, Ullrich congenital muscular dystrophy; BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; Carrier, female carriers of DMD; CK, Creatine kinase; NA, not available; Y, years; AD, autodomal dominant; AR, autosomal recessive; XR, X linked recessive; P, pathogenic; LP, likely pathogenic; VUS, variants of unknown significance

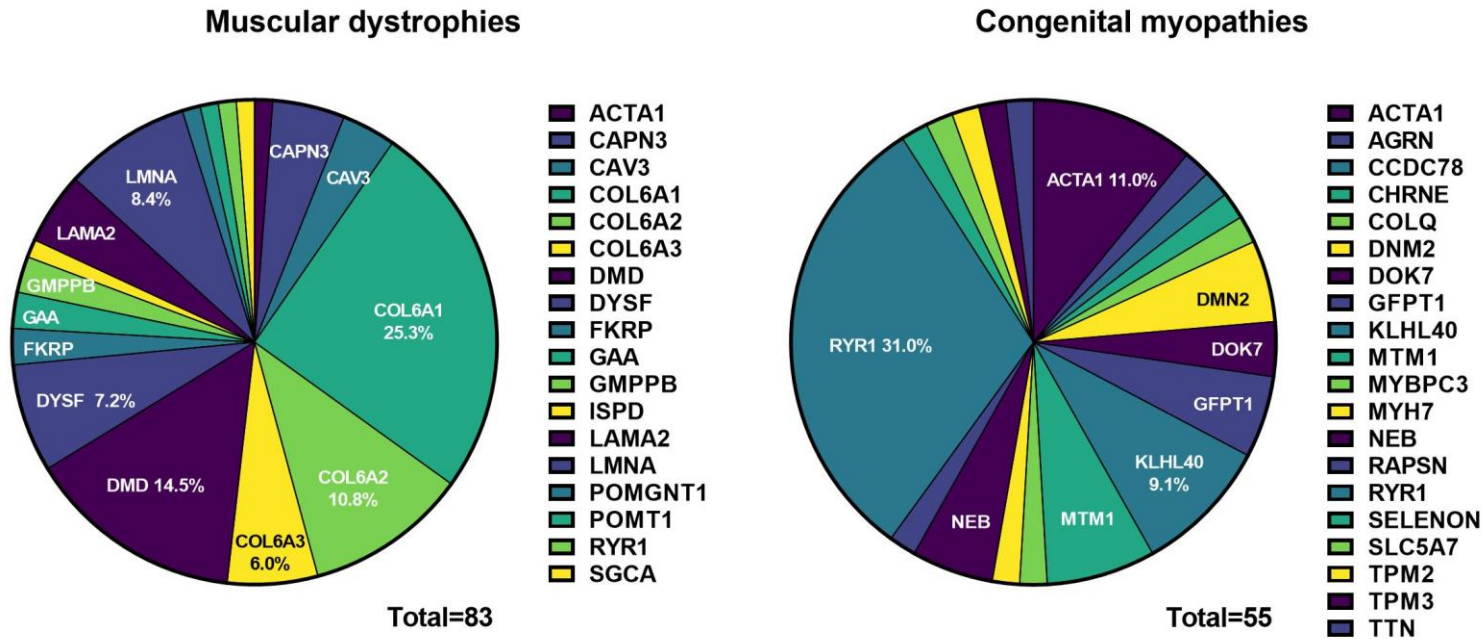

Figure 6. Distribution of genotypes in muscular dystrophies and congenital myopathies by NGS analysis

Molecular diagnosis was newly confirmed in 83 muscular dystrophy patients and 55 congenital myopathy patients who had not been diagnosed in the previous conventional genetic analysis.

Congenital myopathies and congenital myasthenic syndromes

Forty-five patients were diagnosed with congenital myopathies and 10 patients were diagnosed with congenital myasthenic syndromes (Table 6). Pathogenic and likely pathogenic variants were identified in *ACTA1* (n = 6), *AGRN* (n = 1), *CCDC78* (n = 1), *COLQ* (n = 1), *DNM2* (n = 3), *DOK7* (n = 2), *GFPT1* (n = 3), *KLHL40* (n = 5), *MTM1* (n = 4), *MYBPC3* (n = 1), *MYH7* (n = 1), *NEB* (n = 3), *RAPSN* (n = 1), *RYR1* (n = 17), *SELENON* (n = 1), *SLC5A7* (n = 1), *TPM2* (n = 1), *TPM3* (n = 1), and *TTN* (n = 1). Variants were prioritized in genes that were consistent with histological phenotypes in 52 cases and 32 patients showed characteristic diagnostic findings on muscle biopsy (Figure 7). Eight patients were nemaline myopathies, 8 patients were congenital fibers type disproportion, 8 patients were central core diseases, 3 patients were myotubular myopathies, 3 patients were centronuclear diseases, and 2 patients were multi-minicore diseases. Muscle biopsy was not performed in one patient (ID 116) suspected as having CMS and two patients (ID 108 and 115) with CMP. Core myopathies with *RYR1* mutations and nemaline myopathies with *ACTA1* were the most common diagnoses of congenital myopathy in this study (Figure 6).

Table 6. Pathogenic and likely pathogenic variants in 55 congenital myopathy and congenital myasthenic syndrome patients

ID	Sex	Age	Phenotype	Clinical presentation	Onset	CK	GENE	Mode	Mutation	Class
84	M	22.6	CMP	Floppy infant	Birth	27	ACTA1	AD	c.493G>T:p.Val165Leu	P
85	F	8.1	NM	Motor developmental delay	<1Y	11	ACTA1	AD	c.599A>G:p.Tyr200Cys	P
86	F	27.4	NM	Floppy infant	Birth	10	ACTA1	AD	c.347C>T:p.Ala116Val	P
87	F	11.1	NM	Floppy infant	Birth	130	ACTA1	AD	c.215C>G:p.Pro72Arg	P
88	M	8.6	CMP	Floppy infant	Birth	122	ACTA1	AD	c.443G>A:p.Gly148Asp	LP
89	F	5.5	NM	Floppy infant	Birth	33	ACTA1	AD	c.220G>C:p.Glu74Gln	LP
90	M	11.9	CMS	Floppy infant	Birth	26	AGRN	AR	c.5012G>A:p.Arg1671Gln c.5012G>A:p.Arg1671Gln	LP LP
91	F	25.5	CMP	Weakness from toddler, respiratory distress, scoliosis	1-2Y	119	CCDC78	AD	c.1133+1G>C	LP
92	M	20.8	CMS	Weakness from infancy	<1Y	123	CHRNE	AD	c.850A>C:p.Thr284Pro	P
93	M	20.6	CMS	Motor developmental delay	<1Y	55	COLQ	AR	c.1354C>T:p.Arg452Cys c.107-2A>G	LP P
94	F	21.2	CNM	Gait abnormality, hypotonia	1-2Y	89	DNM2	AD	c.1553G>A:p.Arg518His	P
95	F	32.7	CNM	Gait abnormality	9Y	46	DNM2	AD	c.1106G>A:p.Arg369Gln	P
96	M	7.8	CNM	Floppy infant	Birth	86	DNM2	AD	c.1844C>T:p.Ser615Leu	P

97	M	7.5	CMS	Floppy infant	Birth	106	DOK7	AR	c.476C>A:p.Pro159His c.1502C>T:p.Ala501Val	LP LP
98	F	18.0	CMS	Floppy infant	Birth	46	DOK7	AR	c.1185C>G:p.Tyr395Ter c.1185C>G:p.Tyr395Ter	P P
99	M	24.8	CMS	Motor developmental delay	<1Y	376	GFPT1	AR	c.128A>T:p.Asp43Val c.706A>T:p.Lys236Ter	LP P
100	M	20.7	CMS	Gait abnormality, history of neonatal respiratory distress	7-8Y	257	GFPT1	AR	c.706A>T:p.Lys236Ter c.1549A>C:p.Ile517Leu	LP LP
101	M	22.9	CMS	Gait abnormality	7-8Y	162	GFPT1	AR	c.766G>C:p.Glu256Gln c.520G>A:p.Val174Met	LP LP
102	F	17.2	NM	Floppy infant	Birth	42	KLHL40	AR	c.1582G>A:p.Glu528Lys c.1582G>A:p.Glu528Lys	P P
103	F	14.0	CFTD	Floppy infant	Birth	72	KLHL40	AR	c.1582G>A:p.Glu528Lys c.1582G>A:p.Glu528Lys	P P
104	F	14.1	CMP	Floppy infant	Birth	53	KLHL40	AR	c.1582G>A:p.Glu528Lys c.1582G>A:p.Glu528Lys	P P
105	F	9.3	NM	Floppy infant	Birth	32	KLHL40	AR	c.1582G>A:p.Glu528Lys c.1582G>A:p.Glu528Lys	P P
106	F	9.3	NM	Floppy infant	Birth	92	KLHL40	AR	c.1582G>A:p.Glu528Lys c.1582G>A:p.Glu528Lys	P P
107	M	5.7	MTM	Floppy infant	Birth	55	MTM1	XR	c.1786_1795del:p.Met596Cysfs*22	P
108	M	26.1	CMP	Floppy infant	Birth	113	MTM1	XR	c.1237A>C:p.Ser413Arg	LP
109	M	7.1	MTM	Floppy infant	Birth	75	MTM1	XR	c.679G>A:p.Val227Met	P

110	M	3.9	MTM	Floppy infant	Birth	52	MTM1	XR	c.1261-10A>G	P
111	F	25.0	CMP	Weakness from infancy, DCMP	<1Y	80	MYBPC3	AD	c.713G>A:p.Arg238His	LP
112	F	15.3	CFTD	Weakness from infancy, DCMP	<1Y	71	MYH7	AD	c.5655+1G>A	LP
113	F	17.6	CFTD	Motor developmental delay	Birth	21	NEB	AR	c.5452-2A>G c.8425C>T, p.Arg2809*	P P
114	M	9.4	NM	Motor developmental delay	<1Y	156	NEB	AR	c.2590G>A:p.Asp864Asn c.8425C>T, p.Arg2809*	LP LP
115	M	6.3	CMP	Floppy infant	Birth	302	NEB	AR	c.9241A>T, p.Lys3081Ter c.10922C>T, p.Pro3641Leu	P LP
116	M	15.6	CMS	Motor developmental delay, ptosis	Birth	102	RAPSN	AR	c.G133A:p.Val45Met c.690G>A:p.Glu230=	P LP
117	M	6.4	CCD	Motor developmental delay	1-2Y	76	RYR1	AD	c.14815G>T, p.Asp4939Tyr	LP
118	M	22.8	CCD	Motor developmental delay, congenital hip dislocation	<1Y	66	RYR1	AD	c.14636G>G:p.Tyr4879Cys	LP
119	F	8.2	CCD	Gait abnormality	<1Y	45	RYR1	AD	c.14422T>G:p.Phe4808Val	LP
120	M	20.9	CFTD	Motor developmental delay, myopathic face	1-2Y	123	RYR1	AD	c.14762T>C:p.Phe4921Ser	LP
121	F	19.5	CCD	Motor developmental delay, myopathic face	1-2Y	98	RYR1	AD	c.14693T>C:p.Ile4898Thr	P
122	F	18.7	CCD	Gait abnormality	1-2Y	46	RYR1	AD	c.13897G>A:p.Gly4633Ser	P
123	F	17.6	CCD	Motor developmental delay	<1Y	92	RYR1	AD	c.14567G>A:p.Arg4856His	P

124	F	10.6	CCD	Gait abnormality	<1Y	115	RYR1	AD	c.13898G>A:p.Gly4633Asp	P
125	M	8.4	CMP	Floppy infant	Birth	71	RYR1	AR	c.14652C>G:p.Tyr4884Ter c.14710G>A:p.Glu4904Lys	P LP
126	M	34.3	CMP	Motor developmental delay	<1Y	80	RYR1	AR	c.A13705G:p.Ile4569Val c.A13705G:p.Ile4569Val	LP LP
127	M	18.6	CCD	Gait abnormality	1-2Y	72	RYR1	AR	c.3619G>A:p.Val1207Met c.12654C>A:p.Phe4218Leu	LP LP
128	F	10.0	CFTD	Floppy infant	<1Y	57	RYR1	AR	c.10316G>A:p.Gly3439Asp c.14595_14597delCAA:p.Asn4865del	LP LP
129	F	14.6	CMP	Floppy infant	<1Y	39	RYR1	AR	c.14427C>A:p.Phe4809Leu c.14798C>A:p.Ile4933Thr	LP LP
130	F	11.4	MMD	Motor developmental delay	<1Y	66	RYR1	AR	c.7835+1G>A c.9623C>T:p.Pro3208Leu	LP LP
131	F	8.7	MMD	Motor developmental delay	<1Y	62	RYR1	AR	c.14422_14423delinsAA: p.Phe4808Asn c.7615G>A:p.Ala2539Thr	LP LP
132	M	19.0	CMP	Motor developmental delay	<1Y	210	RYR1	AR	c.13545dupC:p.Glu4516Argfs*67 c.2287G>A:p.Val763Met	P LP
133	F	13.0	CFTD	Motor developmental delay	<1Y	103	RYR1	AD	c.14582G>A:p.Arg4861His	LP
134	F	7.7	CMP	Gait abnormality, myopathic face, high arched palate	<1Y	105	SELENON	AR	c.1574T>G, p.Met525Arg c.1574T>G, p.Met525Arg	LP LP
135	M	12.1	CMS	Floppy infant, ptosis	Birth	NA	SLC5A7	AR	c.571G>A:p.Ala191Thr c.571G>A:p.Ala191Thr	LP LP

136	M	12.4	CFTD	Floppy infant	Birth	51	TPM2	AD	c.121G>A:p.Glu41Lys	LP
137	M	33.0	CMP	Floppy infant	Birth	65	TPM3	AD	c.193C>G:p.Arg65Gly	LP
138	F	9.4	CFTD	Floppy infant	Birth	87	TTN	AR	c.98575C>T:p.Gln32859Ter c.36578-2A>C	P P

Abbreviations: M, male; F, female; CMP, congenital myopathy; NM, nemaline myopathy; CMS, congenital myasthenic syndrome; CNM, centronuculear myopathy; CFTD, congenital myopathy with fiber type disproportion; MTM, myotubular myopathy; CCD, central core disease; MMD, multi-minicore disease, DCMP, dilated cardiomyopathy; Y, years; CK, Creatine kinase; NA, not available; AD, autodomal dominant; AR, autosomal recessive; XR, X linked recessive; P, pathogenic; LP, likely pathogenic

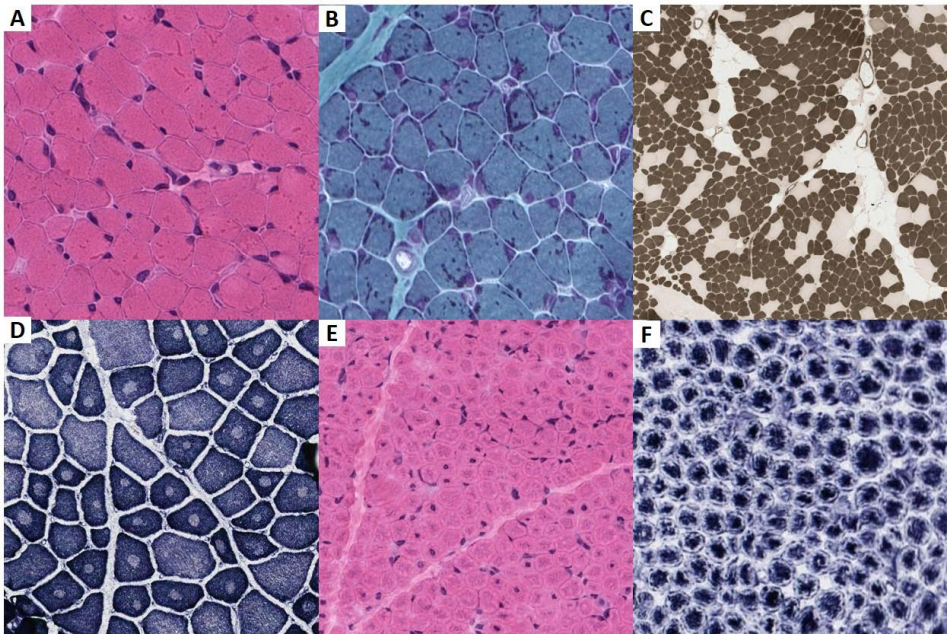

Figure 7. Muscle histopathology in congenital myopathies

Pathologic features that define the major subtypes of congenital myopathies. (A, B) Nemaline myopathy: Patient 85 with a dominant mutation in the *ACTA1* (A: H&E, B: modified Gomori trichrome). (C) Congenital myopathy with fiber type disproportion: Patient 128 with recessive mutations in the *RYR1* (ATPase preincubated at pH 4.3). (D) Central core disease: Patient 122 with a dominant mutation in the *RYR1* (NADH-TR). (E, F) Myotubular myopathy: Patient 110 with an X-linked hemizygous mutation in *MTM1* (E: H&E, F: NADH-TR).

Neuropathies

Five patients each were diagnosed with MND and HMSN, also known as Charcot-Ma Charcot-Marie-Tooth (CMT) disease (Table 7). Pathogenic and likely pathogenic variants were identified in *BICD2* (n = 1), *DYNC1H1* (n = 3), *GARS1* (n = 1), *GNB4* (n = 1), *MARS1* (n = 1), *PMP22* (n = 1), and *TRPV4* (n = 2). All mutations found in this group showed an autosomal dominant inheritance pattern. In patient 151, two likely pathogenic mutations were found in the *BICD2* gene, but the segregation study confirmed that the two mutations were present in the same allele.

Table 7. Pathogenic and likely pathogenic variants in 10 motor neuron disease and hereditary motor and sensory neuropathy patients

ID	Sex	Age	Phenotype	Clinical presentation	Onset	CK	Gene	Mode	Mutation	Class
151	F	27.4	MND	Motor developmental delay	<1Y	100	BICD2	AD	c.1955C>T:p.Ser652Leu / c.1930G>T:p.Ala644Ser (Cis)	LP/LP
152	M	27.4	MND	Motor developmental delay	1-2Y	119	DYNC1H1	AD	c.751C>T:p.Arg251Cys	LP
153	M	10.6	MND	Motor developmental delay	<1Y	132	DYNC1H1	AD	c.3179T>C:p.Leu1060Ser	LP
154	M	19.7	MND	Gait abnormality	1-2Y	161	DYNC1H1	AD	c.A917G:p.His306Arg	LP
155	M	10.3	MND	Floppy infant	<1Y	65	GARS1	AD	c.998A>G:p.Glu333Gly	P
156	M	19.6	HMSN	Gait abnormality, demyelinating pattern abnormality on NCS	1-2Y	NA	GNB4	AD	c.265A>G:p.Lys89Glu	P
157	M	25.7	HMSN	Gait abnormality, scoliosis	3Y	175	MARS1	AD	c.2398C>A:p.Pro800Thr	LP
158	F	3.6	HMSN	Gait abnormality, no evoked action potentials on NCS	1-2Y	135	PMP22	AD	c.233_234insA: p.Ser79Valfs*144	LP
159	F	10.3	HMSN	Hypotonia with foot deformity	<1Y	NA	TRPV4	AD	c.1866C>A:p.Ser622Arg	LP
160	M	12.3	HMSN	Hypotonia, arthrogryposis multiplex	<1Y	90	TRPV4	AD	c.694C>T:p.Arg232Cys	P

Abbreviations: M, male; F, female; MND, motor neuron disease; HMSN, hereditary motor and sensory neuropathy; NCS, nerve conduction study; Y, years; CK, Creatine kinase; NA, not available; AD, autodomal dominant; P, pathogenic; LP, likely pathogenic

Metabolic and other myopathies

Four patients were diagnosed with metabolic myopathies. Four and five patients were diagnosed with distal myopathies and myofibrillar myopathies (MFM), respectively (Table 8). Pathogenic and/or likely pathogenic variants in 4 patients with metabolic myopathies were identified in ACADVL (n = 1), HADHA (n = 1), HADHB (n = 1), and PYGM (n = 1). Mutations in DMN2 (n = 1), GNE (n = 1), MYH7 (n = 1), and NEB (n = 1) were confirmed in 4 patients with distal myopathies. Pathogenic or likely pathogenic variants were identified in BAG3 (n = 1), CRYAB (n = 1), FHL1 (n = 2), and TTN (n = 1) with the diagnosis of MFM. Among them, 4 cases (ID 140, 141, 143, and 151) underwent muscle biopsy and showed myofibrillar disorganization in histopathological analysis (Figure 8).

Table 8. Pathogenic and likely pathogenic variants in 13 metabolic and other myopathies patients.

ID	Sex	Age	Phenotype	Clinical presentation	Onset	CK	Gene	Mode	Mutation	Class
139	F	23.6	Meta	Recurrent rhabdomyolysis	13Y	9392	ACADVL	AR	c.1349G>A:p.Arg450His c.619T>C:p.Ser207Pro	P LP
140	F	17.0	MFM	Gait abnormality, severe DCMP	9Y	1872	BAG3	AD	c.626C>T:p.Pro209Leu	P
141	M	27.7	MFM	Gait abnormality	1-2Y	398	CRYAB	AD	c.470G>A:p.Arg157His	P
142	M	16.0	Distal	Gait abnormality with foot deformity	13Y	453	DNM2	AD	c.1105C>T:p.Arg369Trp	P
143	M	17.3	MFM	Gait abnormality with joint contractures	9Y	474	FHL1	XR	c.310T>C, p.Cys104Arg	LP
144	M	38.8	MFM	Gait abnormality	10Y	29	FHL1	XD	c.496T>C:p.Cys166Arg	LP
145	M	37.6	Distal	Gait abnormality with lower legs atrophy	18Y	860	GNE	AR	c.1384G>C:p.Val462Leu c.131G>C:p.Cys44Ser	P LP
146	M	20.8	Meta	Recurrent rhabdomyolysis	15M	160000	HADHA	AR	c.2123T>G:p.Leu708Arg c.500C>T:p.Thr167Ile	LP LP
147	M	8.4	Meta	Recurrent rhabdomyolysis	<1Y	158	HADHB	AR	c.340A>G:p.Asn114Asp c.64+5G>A	LP LP
148	M	38.4	Distal	Gait abnormality, shoulder girdle atrophy, respiratory distress	6Y	128	MYH7	AD	c.2608C>T:p.Arg870Cys	LP
149	F	34.1	Distal	Gait abnormality, high arched palate	25Y	28	NEB	AR	c.23245C>T:p.Arg7714Ter c.21522+3A>G	LP LP
150	M	24.6	Meta	Muscle cramping with high CK	13Y	2226	PYGM	AR	c.1684C>T:p.Arg562Ter c.21522+3A>G	P LP

151	F	4.0	MFM	Floppy infant	<1Y	122	TTN	AR	c.11764+1G>A c.21522+3A>G	LP LP

Abbreviations: M, male; F, female; Meta, metabolic myopathy; MFM, myofibrillar myopathy; Distal, distal myopathy; DCMP, dilated cardiomyopathy; Y, years; CK, Creatine kinase; AR, autosomal recessive; AD, autodomal dominant; XR, X linked recessive; XD, X linked dominant; P, pathogenic; LP, likely pathogenic

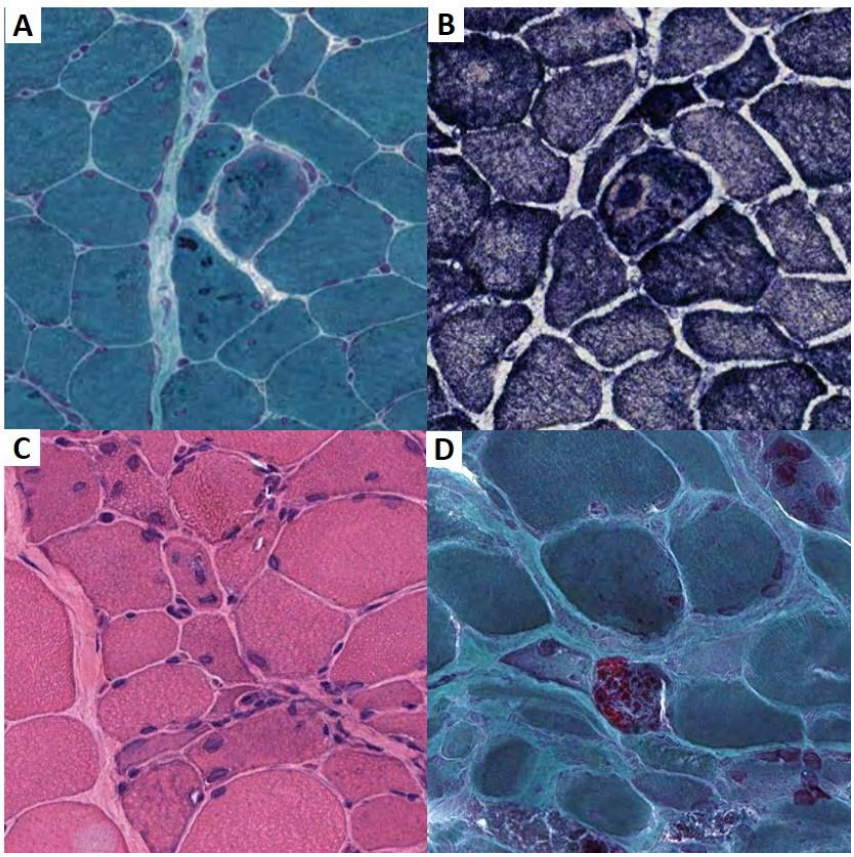

Figure 8. Muscle histopathology in myofibrillar myopathies

Myofibrillar myopathies (MFM) are histopathologically characterized by the presence of myofibrillar disorganization and accumulation of protein aggregates. (A, B) Patient 140 with a dominant mutation in the *BAG3* (A: modified Gomori trichrome, B: NADH-TR). (C, D) Patient 143 with an X-linked hemizygous mutation in the *FHL1* (C: H&E, D: modified Gomori trichrome).

Variants of uncertain significance

In 31 patients, variants consistent with the clinical phenotype were found but did not lead to a diagnosis due to lack of pathological evidence or only one allele pathogenic variant was found in AR genetic diseases (Table 9). These cases are highly likely to be further diagnosed in the future through parental testing, muscle biopsy, and additional analysis of the contralateral allele.

Table 9. Variants of uncertain significance in 31 patients

ID	Sex	Age	Phenotype	Clinical presentation	Onset	CK	Gene	Mode	Mutation	Class
162	M	22.7	Meta	Muscle cramping with high CK	13Y	1412	AGL	AR	c.1284-4A>G c.1936A>G:p.Ile646Val	VUS VUS
163	M	11.6	MFM	Floppy infant, DCMP, cataract	Birth	140	BAG3	AD	c.652C>T:p.Arg218Trp	VUS
164	M	5.6	CMS	Floppy infant, Jolly test positive	<1Y	74	CHRND	AD	c.255C>A, p.Asp85Glu	VUS
165	M	27.6	UCMD	Floppy infant	<1Y	36	COL6A2	AD AR	c.2927T>C:p.Leu976Ser c.1660_1668del: p.Lys554_Glu556del	VUS VUS
166	M	16.1	UCMD	Floppy infant	<1Y	306	COL6A3	AD	c.9329-4A>T	VUS
167	M	10.2	LGMD	Asymptomatic high CK		1583	DAG1	AR	c.331G>A:p.Asp111Asn c.785T>C:p.Lys262Phe	VUS VUS
168	F	22.2	Carrier	Gait abnormality	8Y	2621	DMD	XR	c.2780T>G:p.Ile927Ser	VUS
169	F	22.0	Carrier	Asymptomatic high CK		2126	DMD	XR	c.9164C>T:p.Thr3055Met	VUS
170	F	11.6	Carrier	Asymptomatic high CK		366	DMD	XR	c.2217_2225del:p.Ala740_Leu742del	VUS
171	M	6.2	BMD	Muscle cramping, high CK	5Y	>7800	DMD	XR	c.10922-5T>G	VUS
172	F	14.5	LGMD	Gait abnormality	1-2Y	2587	DYSF	AR	c.3175-2A>G not detected	P
173	M	52.2	LGMD	Gait abnormality, facial weakness, ptosis	42Y	988	EMD	XR	c.241G>A:p.ASP81Asn	VUS
174	M	4.4	LGMD	Asymptomatic high CK		357	EMD	XR	c.133A>G:p.Arg45Gly	VUS

175	M	5.8	CMD	Motor developmental delay, dysglycosylated aDG on IHC	<1Y	5315	FKTN	AR	c.49A>C, p.Ser17Arg c.165+6T>C	VUS VUS
176	F	4.3	CMD	Floppy infant	<1Y	4393	FKTN	AR	c.49A>C:p.Ser17Arg not detected	VUS
177	M	20.6	LGMD	Asymptomatic high CK		1216	FLNA	XR	c.3081C>G:p.Asp1027Glu	VUS
178	F	19.0	CMP	Floppy infant	<1Y	75	GFPT1	AR	c.1102C>T, p.Arg368Cys c.1189A>T, p.Met397Leu	VUS VUS
179	M	26.0	MDCMD	Floppy infant, merosin deficiency on IHC	Birth	529	LAMA2	AR	c.4987C>T, p.Gln1663Ter not detected	P
180	F	4.4	MDCMD	Floppy infant, merosin deficiency on IHC	Birth	1408	LAMA2	AR	c.910-1G>T c.909+7A>G	P VUS
181	M	32.3	CFTD	Gait abnormality, myopathic face, high arched palate	6Y	59	NEB	AR	c.1674+1G>T not detected	LP
182	F	11.2	NM	Floppy infant	Birth	59	NEB	AR	c.23014G>T:p.Glu7672* c.21522+3A>C	P VUS
183	M	8.6	NM	Floppy infant	<1Y	25	NEB	AR	c.15679A>T:p.Lys5227Ter not detected	P
184	F	6.2	NM	Floppy infant	Birth	83	NEB	AR	c.1359del, p.Asn453Lysfs*23 not detected	LP
185	M	26.4	Meta	Recurrent rhabdomyolysis	16Y	110	PYGM	AR	c.269G>A:p.Arg90Gln c.290G>A:p.Gly97Asp	VUS VUS
186	F	5.8	CMP	Floppy infant	Birth	86	RYR1	AR	c.3820C>G:p.Arg1274Gly c.14560G>A:p.Val4854Met	VUS VUS
187	M	16.3	CFTD	Floppy infant, myopathic face, high arched palate	Birth	70	RYR1	AD AR	c.3523G>A not detected	VUS
188	M	14.7	CFTD	Gait abnormality, scoliosis	1-2Y	80	RYR1	AD	c.2287G>A:p.Val763Met	VUS

								AR	not detected	
189	F	23.8	CMD	Floppy infant	Birth	348	SEPN1	AR	c.1574T>G, p.Met525Arg c.1574T>G, p.Met525Arg	VUS VUS
190	M	18.9	HMSN	Gait abnormality, HMSN type 2 on NCS	12Y	NA	TRPV4	AD	c.536G>A:p.Arg179His	VUS
191	M	9.9	CMD	Gait abnormality, neck muscle weakness	1-2Y	179	TTN	AR	c.91447G>A:p.Glu30483Lys not detected	VUS
192	M	19.7	CMP	Motor developmental delay	<1Y	121	TTN	AR	c.78559C>T:p.ARg26187Ter c.G12428C:p.R4143T	P VUS

Abbreviations: M, male; F, female; Meta, metabolic myopathy; MFM, myofibrillar myopathy; CMS, congenital myasthenic syndrome; UCMD, Ullrich congenital muscular dystrophy; LGMD, limb girdle muscular dystrophy; Carrier, female carriers of DMD, BMD, Becker muscular dystrophy; CMP, congenital myopathy; MDCMD, merosin deficient congenital muscular dystrophy; CFTD, congenital myopathy with fiber type disproportion; NM, nemaline myopathy; CMD, congenital muscular dystrophy; HMSN, hereditary motor and sensory neuropathy; DCMP, dilated cardiomyopathy; IHC, immunohistochemistry; NCS, nerve conduction study; Y, years; CK, Creatine kinase; AR, autosomal recessive; AD, autodomal dominant; XR, X linked recessive; VUS, variant of uncertain significance; P, pathogenic; LP, likely pathogenic

Further analysis in undiagnosed patients

Additional analyses were selectively performed on patients whose diagnosis was not confirmed. WES was performed in 94 cases, of which 40 were performed as single (patient alone), 53 as trio (patient and both parents), and one case as quad (patient, both parents and a symptomatic sibling). Transcriptome analysis was performed in 43 cases. Additional targeted gene analysis was also performed on selected patients according to clinical circumstances. Through these various analyzes, an additional diagnosis was confirmed in 28 patients (Table 10). A case (ID 176) in whom only one allele *FKTN* mutation was found in the previous panel analysis (Table 9) was confirmed the diagnosis by finding the retrotransposal mutation in a contralateral allele through the target analysis. In two patients with dystrophinopathy phenotypes (ID 201 and 202), RNA sequencing and subsequent WGS identified intronic variants producing aberrant splicing and premature termination (32). Homozygous large deletions of the *MICU1* (ID 205) and a small deletion of the *MYH7* (ID 206) were also found through transcriptome analysis. In 5 patients (ID 187, 192, 297, 202, and 203), WES revealed mutations in the genes included in the targeted panel tests. The reason that no mutations were found in the initial panel analysis was due to insufficient coverage depth of the mutation site or technical limitations of the variants calling pipeline. In 6 patients (ID 193-196, 199, and 209), mutations were found in genes not included in the large-scale target parallel sequencing of this study (*ADSSL1*, *BICD2*, *DHX16*, and *TRAPPC11*). In 4

patients (ID 198, 204, 207, and 208), mutations were found in genes not included in the initially selected one among 6 SNUH-NMD panels. Six patients (ID 210-215) were identified as having a genetic disease other than neuromuscular disorders through WES. Confirmed diagnoses, in order, include spondyloepimetaphyseal dysplasia (*AIFM1*), Ehlers-Danlos syndrome (*COL1A1*), autosomal dominant pontine microangiopathy and leukoencephalopathy (*COL4A1*), developmental and epileptic encephalopathy (*PCDH19*), Ghosal hematodiaphyseal syndrome (*MYMKAS1*), and Carey-Fineman syndrome (*TBXAS1*).

After reassessing the clinical phenotype, the autoantibodies were analyzed through a commercial line immunoblot assay (EUROLine, EUROIMMUN) on 3 patients. NXP2-positive dermatomyositis (ID 216) and SRP-positive immune-mediated necrotizing myopathy (ID 217) were diagnosed in one case each.

Table 10. List of diagnoses confirmed by further testing in 28 patients

ID	Sex	Age	Phenotype	Clinical presentation	Test	Gene	Mode	Mutation / Antibody	Class
176	F	4.3	CMD	Floppy infant	Targeted analysis	FKTN	AR	c.49A>C:p.Ser17Arg retrotransposal insertion	LP P
187	M	16.3	CMP	Floppy infant	WES	RYR1	AR	c.9413C>T:p.Pro3138Leu c.3523G>A:p.Glu1175Lys	LP LP
192	M	19.7	CMP	Motor developmental delay	RNAseq	TTN	AR	c.34212T>G:pTyr11404* c.105754C>T:p.Arg35252*	LP P
193	M	45.2	Distal	Gait abnormality	WES	ADSSL1	AR	c.362_363delAA:p.Lys121Argfs*33 c.910G>A:p.Asp304Asn	P LP
194	M	20.9	Distal	Gait abnormality	WES	ADSSL1	AR	c.910G>A:p.Asp304Asn c.1048delA:p.Ile350fs	LP P
195	F	17.0	Distal	Gait abnormality	WES	ADSSL1	AR	c.910G>A:p.Asp304Asn c.1048delA:p.Ile350fs	LP P
196	M	26.8	MND	Gait abnormality	WES	BICD2	AD	c.1645C>T:p.Pro549Ser	LP
197	M	16.6	Bethlem	Gait abnormality	WES	COL6A1	AD	c.957+5G>A	LP
198	M	11.0	Bethlem	Gait abnormality	WES	COL6A1	AD	c.1056+1G>A	P
199	M	17.9	Other MP	Gait abnormality, high CK	WES	DHX16	AD	c.1841C>T:p.Thr674Met	LP
200	M	14.6	DMD	Gait abnormality, high CK	RNAseq	DMD	XR	c.4846-157A>G	P
201	M	15.6	BMD	Asymptomatic high CK	RNAseq	DMD	XR	c.937-17665C>G	P
202	F	13.7	Carrier	Asymptomatic high CK	WES	DMD	XR	c.7645del:p.Ile2549fs	P
203	M	17.7	CMD	Motor developmental delay, high CK	WES	GMPPB	AR	c.391G>T:p.Gly131Cys c.787G>A:p.Gly263Ser	LP LP

204	M	7.8	HMSN	Motor developmental delay	WES	MFN2	AD	c.310C>T:p.Arg104Trp	LP
205	M	19.7	Other MP	Proximal limbs weakness High CK	RNAseq	MICU1	AR	Exons 5-8 deletion Exons 5-8 deletion	P P
206	M	19.2	CMP	Dilated cardiomyopathy, motor developmental delay	WES	MYH7	AD	c.5754_5768del: p.Asn1918_Ala1922del	P
207	F	5.7	CMP	Floppy infant	WES	NEB	AR	c.5364G>A:p.Trp1788* c.21623G>T:p.Ser7208Ile	P LP
208	M	19.8	CMP	Gait abnormality	WES	TNNT1	AR	c.611+1G>A c.724G>C:p.Ala242Pro	LP LP
209	M	14.8	LGMD	Gait abnormality, high CK	WES	TRAPPC11	AR	c.302A>G:p.Tyr101Cys c.302A>G:p.Tyr101Cys	LP LP
210	M	8.0	Non NMD	Motor developmental delay	WES	AIFM1	XR	c.710A>T:p.Asp237Val	LP
211	F	4.4	Non NMD	Motor developmental delay, joint laxity	WES	COL1A1	AD	c.2005G>T:p.Ala669Ser	P
212	M	15.7	Non NMD	Asymptomatic high CK, abnormal brain MRI	WES	COL4A1	AD	c.2850A>Tp.Lys950Asn	LP
213	F	16.9	Non NMD	Global developmental delay, joint laxity	WES	PCDH19	XL	c.67A>G:p.Ile23Val	LP
214	M	21.3	Non NMD	Motor developmental delay	WES	TBXAS1	AR	c.T397C:p.Trp133Arg c.692_693del:p.Leu231fs	LP LP
215	F	6.2	Non NMD	Floppy infant	WES	TMEM8C	AR	c.356C>T:p.Ser119Leu c.502C>T:p.Arg168Cys	LP LP
216	F	12.7	DM	Proximal limbs weakness High CK	Lineblot	NXP2 antibody strong positive			
217	M	8.8	IMNM	Gait abnormality, high CK	Lineblot	SRP antibody strong positive			

Abbreviations: M, male; F, female; CMD, congenital muscular dystrophy; Distal, distal myopathy; MND, motor neuron disease;

Bethlem, Bethlem myopathy, Other MP, other myopathy; DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; Carrier, female carrier of DMD, HMSN, hereditary motor and sensory neuropathy; CMP, congenital myopathy; LGMD, limb girdle muscular dystrophy; Non NMD, non-neuromuscular disorders; DM, dermatomyositis; IMNM, immune mediated necrotizing myopathy; CK, Creatine kinase; Test, test that confirm the diagnosis; WES, whole exome sequencing; RNAseq, RNA sequencing; Lineblot, line immunoblot assay; AR, autosomal recessive; AD, autodomal dominant; XR, X linked recessive; XL, X linked; LP, likely pathogenic; P, pathogenic

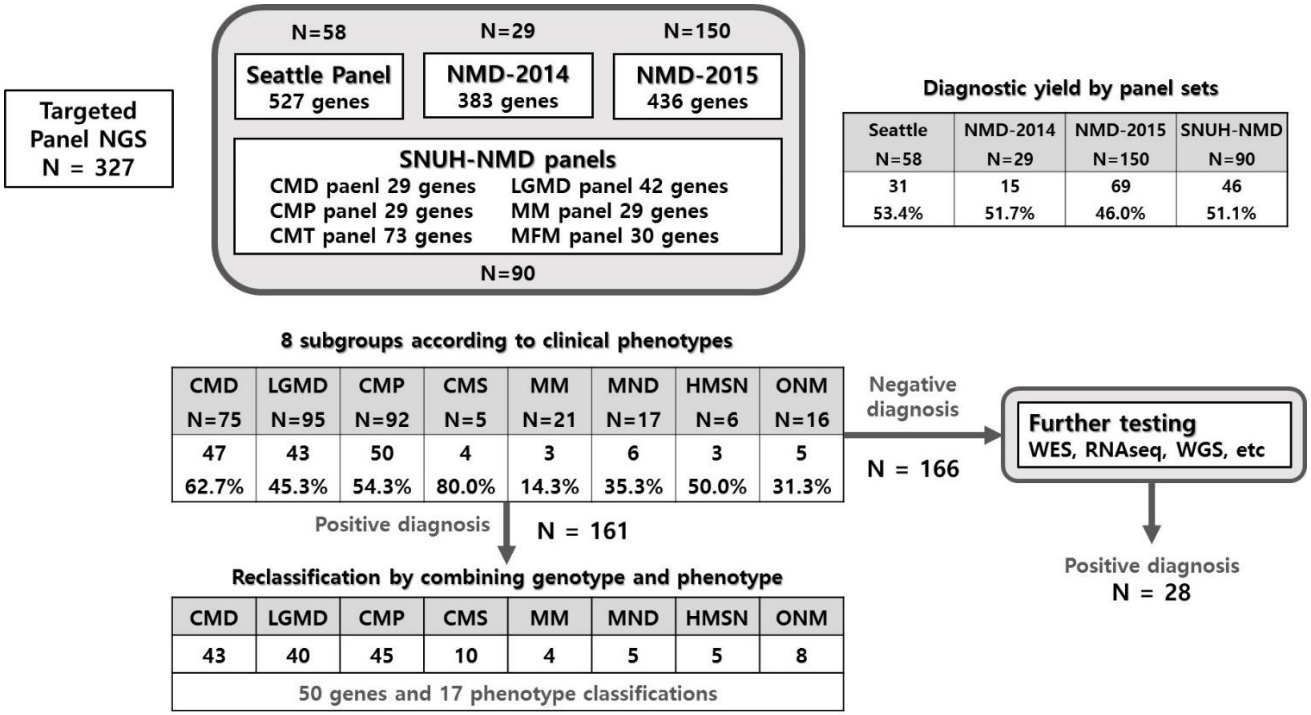

Figure 9. Summary of the targeted sequencing, phenotypic classification, and diagnostic yield

NGS, next generation sequencing; N, numbers; SNUH, Seoul National University Hospital; NMD, neuromuscular disorders; CMD, congenital muscular dystrophy; LGMD, limb girdle muscular dystrophy; CMP, congenital myopathy; MM, metabolic myopathy; CMT, Charcot-Marie-Tooth disease; MFM, myofibrillar myopathy; CMS, congenital myasthenic syndrome; MND, motor neuron disease; HMSN, hereditary motor and sensory neuropathy; ONM, other neuromuscular disorders; WES, whole exome sequencing; WGS, whole genome sequencing

Discussion

This study is a targeted massive parallel sequencing analysis conducted sequentially on the largest cohort of hereditary neuromuscular disorders in Korean. Genetic diagnosis was confirmed in total 161 patients who had not been diagnosed with the previous standard diagnostic methods.

MDs, the most common group in hereditary neuromuscular disorders, were confirmed in 83 patients, accounting for 51.5% of diagnosed cases in this study. Among them, type 6 collagenopathy was diagnosed with a very high frequency. When *COL6A1, COL6A2*, and *COLA3* are combined, a total of 35 cases were diagnosed, accounting for 42.4% of MD in this cohort. Since type 6 collagenopathy has a diverse clinical spectrum from floppy infant syndromes to adult-onset Bethlem myopathies and can show non-specific finding histologically, diagnosis can be often difficult (33, 34). Considering the clinical diagnosis is often challenging and the incidence is relatively high, the application of NGS panel at earlier diagnostic stage may be reasonable and effective in this disease.

As current clinical NGS assays are not yet reliable for copy number variant (CNV) detection, targeted panel tests need to be complemented with single gene analysis methods including CNV analysis for the time being. The most common type of MD worldwide is dystrophinopathy caused by *DMD* mutations. Most dystrophinopathy patients are not included in this study because 80% of them have exon deletion or

duplication mutations and are diagnosed with MLPA before NGS application (35). In addition, one of the most commonly diagnosed CMD subtype in Korea is Fukuyama CMD caused by *FKTN* mutation. Since most of Korean Fukuyama CMD patients have retrotransposal insertion and/or pseudoexon mutations as common genotypes (36), which are difficult to detect with NGS tests, they were also diagnosed in advance with a separate test and not included in this study. Therefore, it is inapproptiate to directly estimate the genetic epidemiology of muscular dystrophy in Korean population based on the frequency of genetic diagnosis in this study. Further epidemiologic study of the muscular dystrophy in Korean population with integrated NGS and single gene analysis is needed.

We were able to confirm the diagnosis of 45 congenital myopathies. CMPs are a clinically and genetically heterogeneous group of disorders defined in many cases by the presence of specific histopathological features (3). Our study also showed that the pathological phenotype and the genotype were consistent in most cases. *ACTA1, KLHL40*, and *NEB* genes were diagnosed in 8 patients with nemaline myopathies. All 5 patients with the *KLHL40* gene had homozygous c.1582G>A, p.Glu528Lys mutations, which can be considered a common genotype in Korean population. *RYR1* was diagnosed as the causative gene in all patients with central core diseases and multi-minicore diseases. It was the most common causative gene in CMPs and the second most common one in the entire cohort. In large-sized genes such as *RYR1* with 106 exons, variants are very frequently found in the NGS-based

analysis, but pathogenicity is often difficult to prove. Since the *RYR1* related diseases can be developed in both autosomal recessive and dominant modes and phenotypic penetrance may vary within families, it may be difficult to prove pathogenicity even with parental tests. In the pathological findings of *RYR1*-related myopathy, the core structures are highly diagnostic-specific (37), so in our study, the molecular diagnosis of many *RYR1* patients could be confirmed through pathological analysis. Thus, pathological findings can still play a very important role in the diagnosis of patients with hereditary neuromuscular disorders.

CMS was diagnosed in 10 patients, among which 4 were consistent with the initial clinical diagnosis but 6 were diagnosed with CMP, LGMD, or even unclassified ONM before molecular confirmation. Due to the wide heterogeneity of clinical and genetic features, the diagnosis of CMS remains challenging even for experienced clinicians (38-41). The number of causative genes continues to grow and the clinical differentiation of subtypes is usually very difficult (41-43). Since molecular diagnosis is crucial for the therapeutic decision making in CMS, it should be considered as a differential diagnosis in clinically distinct but genetically undiagnosed CMP or LGMD patients, especially when their pathological findings are nonspecific.

Although our cohort includes patients of all ages from 0 to 68 years, two-thirds of patients are younger than 20 years of age and the onset period of more than half of

the cases is infants or toddlers. Adult-type LGMDs, distal myopathies, MFM, and HMSN were therefore relatively rare in this study because their prevalence are high in adult patients. In the previous study that mainly targeted Korean adult patients, the *DYSF*, *GNE*, and *CAPN3* genes were reported at a higher frequency than in this study (44). Therefore, there are limitations in analyzing the characteristics and genotypes of adult-onset neuromuscular disorders in this study and further studies extending to older ages are needed.

Diagnosis rates differed according to the 8 clinical subgroups (Figure 4). Genetic confirmation rates were higher in CMD, CMP, CMS, and CMT with distinct clinical features and the ONM group with unclear clinical features showed a low diagnosis yield (31.3%). In particular, CMD showed a much higher diagnosis rate (62.7%) than LGMD (45.3%), reflecting the fact that the positive rate of genetic diagnosis was high in the early onset group. This was also the case in the CMP group, which had a very early age of onset in terms of clinical characteristics. Among the 8 clinical subgroups, the MM group had the lowest diagnosis rate (14.3%), which can be attributed to the inclusion of patients with transient clinical symptoms including the episodic rhabdomyolysis.

The overall diagnostic yield was 49.2%, which is quite high considering that the general diagnostic yields in previous studies are mostly between 20 to 40% and the maximum does not exceed 50% (14, 16, 45-47). It is comparable with or even

higher than those reported in several studies using WES (12, 15, 48-50). It can be thought that the reason why we were able to obtain a high diagnostic rate was because we initially constructed a large panel containing hundreds of genes. However, there was no difference in the diagnosis rate between the former panels containing a large number of genes and the latter panel consisting of a small number of genes divided by disease groups. Also, only 50 genes were confirmed for the final diagnosis among all genes. Therefore, in order to increase the diagnostic efficiency in the targeted NGS panels, it is more important to select appropriate genes rather than to increase the number of target genes. In the end, we were able to compose an efficient small size custom panels through the analysis experience of the previous three large scale panels and successfully maintain the high diagnosis rate.

Through more than 1 year of clinical follow-up and continuous diagnostic efforts after the initial panel analysis, we were able to obtain additional diagnostic results in 28 patients (Table 10). Twenty-one patients were additionally diagnosed by WES, and the genes diagnosed in 5 of them were included in the panel set of this study. It seems that the causative variants were not found in our panel NGS analysis due to the low depth coverage or the variant calling protocol failure. Since NGS technology deals with a wide range of data by conducting large-scale analysis, continuous efforts are needed to validate the internal diagnostic pipeline. Six patients had mutations in new genes not included in our panel set and 3 of them

were diagnosed with *ADSSL1* as the causative gene. *ADSSL1* was first reported in Korean patients with distal myopathies (51) after the time we last updated the panel gene sets. Since dozens of new genes in the monogenic neuromuscular disorders are discovered every year, continuous updating of panel gene sets and reanalysis of undiagnosed patients will be necessary for the time being.

Four patients were diagnosed with genes corresponding to different clinical subgroups after we limited the number of panel gene sets according to clinical diagnosis (SNUH-NMD panels). Since Korea's National Health Insurance Service coverage became available for the panel NGS analysis of hereditary diseases in 2017 (Ministry of Health and Welfare Notice No.2017-15), NGS panel testing is sorted into two types, Level I (The number of genes is 2 to 30, or the gene length is 150 kb or less) and Level II (If the gene length exceeds 150kb or more than 31 genes, it is recognized only for hereditary retinitis pigmentosa, hereditary hearing loss, and CMT) with different costs. Level I and Level II tests incur a 50% co-payment for the patient, with Level II tests having a higher price. Therefore, most of the neuromuscular gene panels except for CMT are level 1 and are limited to 30 genes to be analyzed. The number of causative genes for each phenotypic subgroup known to date exceeds 30 and the clinical diagnosis of neuromuscular patients often overlap with several phenotypic subgroups. Therefore, the current NGS panel classification system in the health insurance service must be improved.

Six patients were diagnosed with genetic disorders other than NMD, including skeletal dysplasia, connective tissue disease, and syndromic neurodevelopmental disorders. In infants and young children, it is often difficult to differentiate between neuromuscular disorders and other diagnoses when there are motor developmental delay and hypotonia accompanied by joint contracture or muscle atrophy (52). Re-evaluation of phenotypes that change over time will continue to be necessary and early application of WES can be considered for the patients whose clinical diagnosis has not been clearly confirmed.

Two patients with an initial diagnosis of MD were confirmed to have inflammatory myopathies through autoantibody-positive findings. MDs were initially suspected due to the younger age of onset and the chronic course of muscle weakness. However, the rapid and severe progression during the follow-up and negative NGS results prompted us to perform the antibody tests (53, 54). Serological findings can be very useful for confirming the diagnostically challenging cases, so as not to miss potentially treatable conditions.

In summary, this study illustrates the clinical utility of targeted NGS as a powerful diagnostic tool in hereditary neuromuscular disorders. Integrated phenotypic analysis is very important for improving the diagnostic efficiency of this technique. In addition, since neuromuscular disorders are genetically complex and include various types of mutations, a comprehensive genomic approach including single

gene analysis and CNV analysis is required.

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국문초록

유전성 신경근육질환은 골격근 또는 말초 운동신경계의 이상으로 인해 근위축 및 근력저하가 발생하는 다양한 질환을 통칭하는 복합적인 진단명으로 임상적, 병리학적, 분자유전학적 복합성으로 인해 정확한 유전 진단이 어려운 경우가 흔하다. 차세대 염기서열분석은 대량의 유전자에 대한 병렬 분석이 가능하다는 기술의 효율성으로 인해 임상 및 연구 분야에서 점점 더 많이 사용되고 있다. 본 연구에서는 기존의 표준 진단 방법으로 유전적 병인을 발견하지 못한 327명의 신경근육질환 환자에 대해 차세대 염기서열분석 기반 대규모 표적 병렬 시퀀싱 분석을 적용하였다. 순차적으로 대상 표적유전자를 업데이트한 패널이 분석에 이용되었고 58명의 환자는 579개의 유전자에 대해 29명의 환자는 383개의 유전자에 대해 150명의 환자는 436개의 유전자에 대해 시퀀싱을 진행하였다. 마지막 90명의 환자는 임상진단에 따라 선천성 근병증, 선천성 근디스트로피, 지대형 근디스트로피, 대사성 근병증, 근원섬유 근병증, 유전성 운동 및 감각 신경병증 진단을 위해 개발한 6개의 패널 중에서 선택하여 각각 29~79개의 표적유전자에 대해 분석하였다. 327명의 환자 중 161명 (49.2%)에서 유전적 원인을 확인하였다. 임상표현형에 따른 진단율은 각각 선천성 근디스트로피 62.7% (47명), 지대형 근디

스트로피 45.3% (43명), 선천성 근병증 54.3% (50명), 선천 근무력증후군 80% (4명), 대사성 근병증 14.3% (3명), 운동신경 질환 35.3% (6명), 유전성 운동 및 감각 신경병증 50% (3명), 기타 신경근육질환에서 31.3% (5명)이였다. 50개의 서로 다른 유전자에서 원인 돌연변이가 발견되었으며 가장 높은 빈도로 발견된 6개의 유전자는 순서대로 *COL6A1* (21명), *RYR1* (18명), *DMD* (10명), *COL6A2* (9명), *ACTA1* (7명), *LMNA* (7명)이었다. 본 연구는 유전성 신경근육질환의 진단에서 표적 병렬 시퀀싱의 임상적 유용성 제시하였다. 진단 효율성 향상을 위해서 적절한 표적유전자 선택과 통합적 임상 표현형 및 유전형 분석이 매우 중요하다.

주요어: 표적 병렬 시퀀싱, 차세대 염기서열분석, 분자진단, 유전성 신경근육질환, 근디스트로피, 선천성 근병증

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