

## Research Report

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# Diagnostic Challenges of Neuromuscular Disorders after Whole Exome Sequencing

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### Abstract.

**Background:** Whole-exome sequencing (WES) facilitates the diagnosis of hereditary neuromuscular disorders. To achieve an accurate diagnosis, physicians should interpret the genetic report carefully along with clinical information and examinations. We described our experience with (1) clinical validation in patients with variants found using WES and (2) a diagnostic approach for those with negative findings from WES.

**Methods:** WES was performed on patients with the clinical impression of hereditary neuromuscular disorders. Information on clinical manifestations, neurological examination, electrodiagnostic studies, histopathology of muscle and nerve, and laboratory tests were collected.

**Results:** Forty-one patients (Male/Female: 18/23, age of onset:  $34.5 \pm 15.9$ ) accepted WES and were categorized into four scenarios: (1) patients with a positive WES result, (2) patients with an inconclusive WES result but supporting clinical data, (3) negative findings from WES, but a final diagnosis after further work-up, and (4) undetermined etiology from WES and in further work-ups. The yield rate of the initial WES was 63.4% (26/41). Among these, seventeen patients had positive WES result, while the other nine patients had inconclusive WES result but supporting clinical data. Notably, in the fifteen patients with negative findings from WES, four patients (26.7%) achieved a diagnosis after further workup: tumor-induced osteomalacia, metabolic myopathy with pathogenic variants in mitochondrial DNA, microsatellite expansion disease, and vasculitis-related neuropathy. The etiologies remained undetermined in eleven patients (myopathy: 7, neuropathy: 4) after WES and further workup.

**Conclusions:** It is essential to design genotype-guided molecular studies to correlate the identified variants with their clinical features. For patients who had negative findings from WES, acquired diseases, mitochondrial DNA disorders and microsatellite expansion diseases should be considered.

**Keywords:** Whole-exome sequencing, neuromuscular disorders, myopathy, neuropathy, motor neuron disease

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## INTRODUCTION

Neuromuscular disorders (NMDs) are a clinically and genetically heterogeneous group of disorders affecting peripheral nerves or muscles. Over the past decades, next-generation sequencing (NGS) has revolutionized the diagnostic paradigm in genetic neuromuscular disorders, and whole-exome sequencing (WES) is one of these powerful diagnostic tools. WES investigates protein-coding genes, approximately 3% of the whole genome, and the protein-coding genes accounts for up to 60% of disease-causing genomic variation [1]. The comprehensive interpretation of clinical manifestations, pathology, electrophysiological studies, and WES reports leads to accurate diagnosis of NMDs.

Previous studies have shown that the early utilization of WES facilitates more rapid and accurate NMD diagnosis, especially in monogenetic diseases [2–4]. The overall diagnosis yield rate ranges from 40–60% in myopathic patients and 30–40% in neuropathy patients, with a higher yield rate in pediatric patients and a lower yield rate in single proband studies [5–10]. The negative-exome results may be caused by somatic mosaicism, copy number variants, mitochondrial mutations, structural variants not properly captured through exome sequencing, and secondary causes not identified [11].

For exome-positive cases with known pathogenic variants that had been well investigated, the final diagnosis can be reached using phenotype-genotype correlations. However, careful interpretation along with further evaluation are essential for exome-positive cases without previously reported pathogenic variants and exome-negative cases [12]. Herein, we described our experience in integrating WES into a diagnostic approach in patients with NMDs. We focus on (1) advanced molecular tests in patients with variants found using WES and (2) a diagnostic approach for those with negative findings from WES.

## METHODS

### *Patient recruitment*

This retrospective cohort study (202109079RIBN) was approved by the Ethics Committee of the National Taiwan University Hospital (NTUH), Taipei, Taiwan. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964

Helsinki Declaration and its later amendments or comparable ethical standards. The inclusion criteria were: (1) an impression of hereditary NMDs, (2) no other etiology identified after selective genetic testing before the WES study, and (3) clinical data were available. We arranged WES to all the probands, and for the proband's family, Sanger sequencing on the specific variants revealed by the proband-only WES was arranged if they agreed.

### *Clinical evaluations, laboratory study, and electrophysiological examinations*

The clinical data, including age of disease onset, sex, and pedigree, were collected in all patients. In patients with myopathy, we additionally collected the serum creatinine kinase (CK) levels and the informations of muscle histopathology. According to previous studies, distribution of weakness and associated symptoms can be categorized into different patterns for clinical diagnosis. The presentation of our patients was categorized as follows: (1) limb-girdle weakness, (2) scapuloperoneal weakness, (3) distal arm and proximal leg weakness, (4) distal weakness, (5) ptosis with or without ophthalmoplegia, (6) episodic weakness/myalgia/rhabdomyolysis, and (7) asymptomatic hyperCKemia. The muscle histopathology was evaluated by the neurologist and pathologist together. Routine histochemical staining in our hospital encompassed hematoxylin and eosin staining, Gomori-Trichrome staining, succinate dehydrogenase staining, nicotinamide adenine dinucleotide staining, and staining for myosin adenosine triphosphatase at pH 4.3 and 10.42. Based on clinical information and routine histochemistry results, specific immunohistochemistry would be arranged. Electron microscopy studies of muscle biopsy were also performed on some samples.

In patients with polyneuropathy, we collected clinical data and performed nerve conduction study (NCS). We used the electrophysiological criteria for chronic inflammatory demyelinating polyradiculoneuropathy to define demyelination and axonal loss.

### *Whole-exome sequencing*

WES was performed using an exome capture kit probe (Agilent V6 (before Jan 2021) and Roche Hyperexome (after Feb 2021)). We then did sequencing with a NovaSeq6000 sequencer (Illumina). The protein-coding genes and flanking intronic sequences

(50 bp) were studied with a 300-bp paired-end run and an average of > 150-fold coverage.

The human reference genome is GRCH38 and we used the Burrows–Wheeler Aligner (BWA) for sequence alignment and used the Genome Analysis Tool Kit (GATK V3.5, Broad Institute) [13] for variant calling. Variants were first annotated using ANNOVAR (<http://wannovar.wglab.org/>) [14]. Then, the annotated profiles were tagged with information of the inheritance pattern from OMIM (<https://omim.org/>), variant pathogenicity from ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>), and the allele frequency from the Taiwan Biobank (<https://taiwanview.twbiobank.org.tw/>). The variant filtering criteria incorporated maximal minor allele frequency (included if < 0.01) and in-silico analysis of variant severity (nonsynonymous variant, nonsense, deletion, splicing, insertion, or splicing). We manually classified the pathogenicity of variants according to American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology guidelines [15–19]. We further defined the results of WES as “positive”, “inconclusive” and “negative” based on ACMG criteria. The positive results were defined as the variants that had been asserted as pathogenic or likely pathogenic in ACMG criteria and definite genetic diagnosis

according to the inheritance pattern of gene. That is, for those compound heterozygous variants, only both alleles showed pathogenic or likely pathogenic in ACMG criteria were defined as “positive”. The inconclusive results were those being classified as variants of uncertain significance in ACMG criteria. The results of the WES were defined as negative if (1) no possible culprit variant identified in the WES or (2) the identified variants were excluded by the segregation analysis. For copy number analysis (CNV), with SAM/BAM file as input, the GATK germline CNV (gCNV) caller can generate a VCF file containing the CNVs found during the variant calling process [20].

#### *Clinical validation of WES results: Clinical-Molecular diagnosis*

In patients with myopathy, the pathogenicity was defined as “definite” in two situations: (1) patients presented with the representative clinical phenotype with positive WES results, or (2) those who had a typical phenotype but with inconclusive WES results, the diagnosis validated by typical pathognomonic findings in clinical manifestations, pathology, or other examinations. Notably, there should be negative results for the other known genes with the

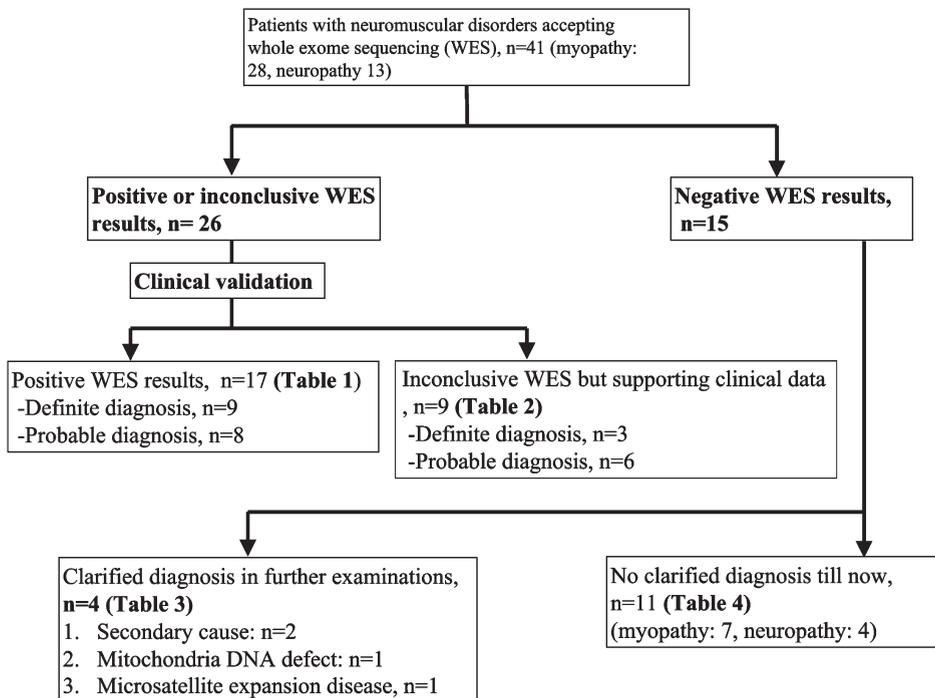


Fig. 1. Algorithm of the study.

similar phenotype in both situations [21]. Instead, pathogenicity was defined as “probable” if the criteria were not met. Among the patients with neuropathy or motor neuron disease, the clinical-molecular diagnosis was regarded “definite” only if the clinical manifestations, culprit variants, and electrophysiological study were consistent with the literature. Instead, pathogenicity was defined as “probable” if the above criteria were not met. For those with two allelic variants, the definite diagnosis required that compound heterozygous variants were confirmed. The final clinical-molecular diagnosis was made based on the genotype-phenotype correlation and was referred to Online Mendelian Inheritance in Man (OMIM) database.

## RESULTS

In total, we recruited 41 patients with neuromuscular disorders who accepted the WES study. Most of the patients ( $n=28$ ) had a clinical diagnosis of myopathy, while the other 10 patients were clinically diagnosed with neuropathy, and 3 patients were diagnosed with motor neuronopathy. The clinical phenotypes, laboratory findings including electrophysiological and pathology, and genetic findings are summarized in Tables 1, 2, 3, & 4, Fig. 1, and supplementary. Overall, twenty-six patients (63.4%) had a likely diagnosis from the WES, meeting the clinical-molecular diagnosis [21], most of whom were diagnosed with myopathy, except four patients who were diagnosed with neuropathy or neuronopathy. Eight cases had biallelic variants, and compound heterozygosity was confirmed in four cases. Five patients could not meet the “definite” clinical-molecular diagnosis due to lack of segregation analysis. There were four scenarios among these 41 patients:

### *Scenario 1: Patients with a positive WES result*

Among these 17 patients with positive WES result (Table 1), most presented with a typical clinical phenotype. We made the diagnosis with genotype-phenotype correlation. In the group of definite diagnosis, two cases with atypical presentations (Cases 6 and 7). Case 6 was a male with exercise intolerance and high hyperCKemia. The work-up of metabolic myopathy and mitochondrial myopathy was normal. However, the patient had partial loss of dystrophin staining in muscle biopsy, supporting the diagnosis of dystrophinopathy. Case 7 had hand weakness as an initial symptom but later developed

lower limb distal weakness, which is rarely reported in GNE myopathy.

There were 8 patients classified as members of the probable diagnosis group (myopathy: 5, neuropathy: 3). Three patients (*CAPN3*, *TTN*, and *SCN4A*) with atypical or uncommon clinical phenotypes could not meet the definite diagnosis criteria, and segregation analysis and molecular studies were needed. Four patients (*FHL1*, *FBXO38*, *POLG*, and *OPA1*) had variants that had been asserted as pathogenic or likely pathogenic in ClinVar database, and one patient (*DYSF*) need a segregation analysis to ascertain compound heterozygote.

### *Scenario 2: Patients with an inconclusive WES result but supporting clinical data*

There were 9 patients showing inconclusive WES result but having supporting clinical data. Of note, 3 patients with myopathy who carried the new variants fulfilled the proposed definite diagnosis criteria. Their diagnosis was validated by (1) typical pathognomonic findings yielded from the clinical manifestations or muscle biopsy (e.g., distal myopathy and atrophic myocytes with desmin aggregates in *DES*-related myofibrillar myopathy 1; limb-girdle weakness with partial loss of dystrophin staining in muscle biopsy), or (2) further molecular examinations (e.g., mitochondria DNA sequencing and muscle electron transfer chain activity assay in *TK2*-related myopathy [22]).

There were another 6 patients classified in the probable diagnosis group (myopathy: 3, neuropathy: 3). Three patients (*SLCO2A1*, *POLG*, and *SCO2*) with representative clinical phenotypes could not meet the definite diagnosis criteria due to the lack of segregation analysis to ascertain compound heterozygote. Notably, in a patient with *SLCO2A1*-related hypertrophic osteoarthropathy we found a nonspecific myopathic change in the proximal muscles, which has not been previously reported. The patient with variant in *TTN* gene had early respiratory failure when she could still ambulate. However, we classified the case as a probable diagnosis because the variant was not located in the common region related to hereditary myopathy with early respiratory failure (HMERF). Although the patient had no necklace body in the muscle pathology, the abnormal desmin and dystrophin subsarcolemmal in light microscopy and Z-disk alterations in electron microscopy are compatible with the pathological diagnosis of myofibrillar myopathy, which has been reported in *TTN*-related

Table 1  
Patients with a positive whole-exome sequencing result

Case/Age of onset/sex	Type of weakness	CK (U/L)	Pathology	Genetic diagnosis				Final diagnosis	Key points for definite/probable diagnosis
				Gene	Variants	Zygoty	ACMG class.		
<b>Myopathy-definite diagnosis</b>									
1/23/F	Distal weakness	171	Myopathy change with rimmed vacuoles	GNE	c.[1618C>T];[829C>T] p.[His540Tyr];[Arg277Trp]	Het/Het	LP/P	GNE myopathy	Clinical manifestations and muscle biopsy
2/40/F	Episodic pain and weakness	63	Lipid myopathy	ETFDH	c.[250G>A];[250G>A] p.[Ala84Thr];[Ala84Thr]	Hom	P	Multiple acyl-CoA dehydrogenase deficiency	Clinical manifestations, laboratory findings (tandem mass) and muscle biopsy
3/11/M	Episodic pain and weakness	192	-	ETFDH	c.[250G>A]; [250G>A] p.[Ala84Thr]; [Ala84Thr]	Hom	P	Multiple acyl-CoA dehydrogenase deficiency	Clinical manifestations and laboratory findings (tandem mass)
4/25/F	Distal Arm/Proximal Leg weakness	253	Nonspecific myopathy change	TCAP	c.23_24insCGAGGTGT p.Glu12_ArgfsTer20	Hom	P	TCAP-related distal myopathy	Clinical manifestations and muscle biopsy
5/18/M	Limb-girdle weakness	655	Myopathy change with rimmed vacuoles	TCAP	c.26_33dup p.Glu12_Argfs*20	Hom	P	LGMD R7	Clinical manifestations and muscle biopsy
6/Child-hood/M	Episodic pain related to exercise	3282	Myogenic myopathy with partial loss of dystrophin staining	DMD	g.(?–31929601)_(32035615-?)del Exon 45–47 deletion	Het	P	Dystrophinopathy	Muscle biopsy.
7/14/F	Distal weakness	786	Myopathy change with rimmed vacuoles	GNE	c.[1399C>T];[815T>G] p.[Gln467X];[Ile272Ser]	Het/Het	P/LP	GNE myopathy	Clinical manifestations and muscle biopsy
8/Child-hood/M	Distal weakness	105	Chronic atrophic myocytes with predominant type I fibers	CLCN1	c.[178C>T];[1444G>A] p.[Gln60Ter];[Gly482Arg]	Het/Het	P/LP	Myotonia congenita	Pathognomonic clinical manifestations, electrophysiology studies and muscle biopsy

Myopathy-probable diagnosis									
9/15/M	Episodic pain and weakness	448	Predominant type II fibers	CAPN3	c.[1621C>T];[1621 = ] p.[Arg541Trp];[Arg541 = ]	Het	LP	LGMD D4	Atypical clinical manifestations, need segregation analysis
10/40/M	Limb-girdle weakness	728	-	TTN	c.[95136T>G];[95136 = ] p.[Cys31712Trp];[Cys31712 = ]	Het	LP	Muscular dystrophy	Uncommon phenotypes of TTN myopathy, need segregation analysis
11/40/F	Limb-girdle weakness	1586	Dystrophic myopathy	DYSF	c.1277G>T(;);1667T>C p.Gly426Val(;);Leu556Pro	Het/Het	P/LP	LGMD R2	Need segregation analysis to ascertain compound heterozygotes.
12/47/F	Ptosis with ophthalmoplegia	426	Myopathy with combined neurogenic and myopathic features. Rimmed vacuoles, but no ragged-red fiber	SCN4A	c.[2297T>C];[2297T = ] p.[Met766Thr];[Met766 = ]	Het	LP	SCN4A-related myopathy (Single fiber EMG: normal jitter)	Atypical clinical manifestations, need segregation analysis and molecular studies.
13/48/F	Scapulo-peroneal	303	Myogenic myopathy	FHL1	c.[394T>C];[394 = ] p.[Cys132Arg];[Cys132 = ]	Het	LP	X-linked dominant scapulo-peroneal myopathy	Unreported variant, need segregation analysis and molecular studies.
Neuropathy-definite diagnosis									
Case/Age of onset/sex	Clinical manifestations	Electrophysiology studies		Genetic diagnosis				Final diagnosis	Key points for definite/probable diagnosis
				Gene	Variants	Zygosity	ACMG class.		
14/14/F	Distal numbness and weakness	Sensorimotor polyneuropathy with multiple conduction block		PMP22	g.(?-15230907)_(15569020_?)de	Het	P	HNPP	Clinical manifestations and nerve conduction studies

(Continued)

Table 1  
(Continued)

Case/Age of onset/sex	Type of weakness	CK (U/L)	Pathology	Genetic diagnosis				Final diagnosis	Key points for definite/probable diagnosis
				Gene	Variants	Zygoty	ACMG class.		
<b>Neuropathy-probable diagnosis</b>									
15/50/M	Distal weakness	Motor neuropathy/motor neuronopathy	FBXO38	c.[1389G>A];[1389G = ] p.[Met463Ile];[Met463 = ]	Het	LP	Probable neuropathy, distal hereditary motor, type IID	Genetic susceptibility to neuropathy; typical electrophysiology studies, need segregation analysis and molecular studies.	
16/38/M	Distal numbness, parkinsonism	Sensory-predominant polyneuropathy or sensory neuronopathy	POLG	c.[3535T>C];[3535T = ] p.[Phe1179Leu];[Phe1179 = ]	Het	LP	POLG related parkinsonism with sensory neuronopathy	Gene related to parkinsonism and sensory neuronopathy, neuropathy; typical electrophysiology studies, need segregation analysis and molecular studies.	
17/46/F	Limb-girdle weakness	Motor-predominant polyneuropathy with minimal sensory findings	OPA1	c.2600–2601del p.Val867Glufs*	Het	P	Probable OPA1-related motor dominant neuropathy	Gene related to motor-predominant polyneuropathy; neuropathy; typical electrophysiology studies, need segregation analysis and molecular studies.	

Table 2  
Patients with an inconclusive whole-exome sequencing result but supporting clinical data

Case/Age of onset/sex	Type of weakness	CK (U/L)	Pathology	Genetic diagnosis				Final diagnosis	Key points for definite/probable diagnosis
				Gene	Variants	Zygoty	ACMG class.		
<b>Myopathy-definite diagnosis</b>									
18/43/F	Distal weakness	704	Myofibrillar myopathy	DES	c.1189_1206del p.397_402del	Het	VUS	Myofibrillar Myopathy 1	Clinical manifestations and muscle biopsy
19/44/F	Limb-girdle weakness	2701	Myogenic myopathy with partial loss of dystrophin staining	DMD	g.(?_32287478)_(32699344-?)dup Exon 8~43 duplication	Het	VUS	Dystrophinopathy	Muscle biopsy
20/37/F	Limb-girdle weakness	201	Myopathy change with some ragged red fibers	TK2	c.[367C>G];[157-2A>C] p.[R123G];[splice site]	Het/Het	VUS/LP	TK2-related myopathy	Muscle biopsy, mitochondria DNA sequencing and muscle electron transfer chain activity assay
<b>Myopathy-probable diagnosis</b>									
21/13/M	Proximal leg weakness	43	Mild atrophic myocytes	SLCO2A1	c.T1069C(;) G1106A p.Tyr357His(;)Gly369Asp	Het/Het	VUS/LP	Hypertrophic osteoarthropathy	Pathognomonic clinical manifestations, need segregation analysis to ascertain compound heterozygotes.
22/40/F	Limb-girdle weakness	564	Myogenic myopathy, subsarcolemmal accumulation, abnormal Z-lines and myofibrils	TTN	c.[1800 + 1G>A];[1800 + 1 = ]	Het	VUS	Hereditary myopathy with early respiratory failure (HMERF)	Unreported variant in the region not typically associated to HMERF.
23/20/F	Ptosis with ophthalmoplegia	195	-	POLG	c.2653A>C(;)924G>C p.Thr885Pro(;)Gln308His	Het/Het	VUS/LP	CPEO	Need segregation analysis to ascertain compound heterozygotes.
<b>Neuropathy-probable diagnosis</b>									
Case/Age of onset/sex	Type of weakness	Electrophysiology studies		Gene	Variants	Zygoty	ACMG class.	Final diagnosis	Key points for probable diagnosis
24/17/F	Isolated upper limbs weakness	Motor neuropathy/motor neuronopathy		NEFH	c.[G559T];[G559 = ] p.[Asp187Tyr];[Asp187 = ]	Het	VUS	Brachial amyotrophic diplegia	Genetic susceptibility to neuronopathy; typical electrophysiology studies; molecular research should be done
25/47/F	Distal numbness and weakness	Grossly normal		NEFH	c.2230_2247del p.744_749del	Het	VUS	Probable PLS	Genetic susceptibility to neuronopathy; molecular research should be done
26/30/F	Ptosis, hands numbness and gait disturbance	Motor-predominant polyneuropathy		SCO2	c.544C>T(;)358C>T p.Gln182Ter(;)Arg120Trp	Het/Het	P/VUS	CMT4	Gene related to CMT4; neuronopathy; typical electrophysiology studies; need segregation analysis and molecular studies.

Table 3  
Patients with negative WES result, but with final clinical diagnosis after further work-up

Age of onset	Clinical symptoms	CK (U/L)	Pathology	Clinical diagnosis	Genetic diagnosis				Final diagnosis
					Gene	Variants	Zygoty	ACMG class.	
<b>Myopathy-definite diagnosis</b>									
27/29/M	Limb-girdle weakness	51	- Muscle pathology: nonspecific findings - Tumor pathology: phosphaturic mesenchymal tumor	Chronic myopathy, hypophosphastemia	Not found	-	-	-	Tumor-induced osteomalacia
28/40/F	Episodic weakness related to exercise	248	Mitochondrial or lipid myopathy	Metabolic myopathy	MT-TL1	m.3243A>G	Heteroplasmy P level: 33.41%		Mitochondrial myopathy
<b>Neuropathy-definite diagnosis</b>									
Case/Age of onset/sex	Type of weakness	Electrophysiology studies		Clinical diagnosis	Genetic diagnosis				Final diagnosis
					Gene	Variants	Zygoty	ACMG class.	
29/61/M	Sensory dominant polyneuropathy with nystagmus	Sensory-predominant polyneuropathy with axonal degeneration features		Hereditary sensory autonomic neuropathy	ATXN3	CAG repeat numbers: 28/63* (Pathogenic $\geq 52$ )	Het	P	ATXN3-related sensory neuropathy and cerebellar ataxia
<b>Neuropathy-probable diagnosis</b>									
Case/Age of onset/sex	Type of weakness	Electrophysiology studies		Clinical diagnosis	Genetic diagnosis				Final diagnosis
					Gene	Variants	Zygoty	ACMG class.	
30/27/F	Episodic numbness and weakness	Sensorimotor polyneuropathy with axonal degeneration features and asymmetric distribution		Suspect mononeuropathy multiplex, or HNPP	Not found	-	-	-	Mononeuropathy multiplex, suspect vasculitis related

Table 4  
Patients with negative WES result and undetermined etiology in further work-ups

Age of onset	Type of weakness	CK (U/L)	Pathology	Clinical diagnosis	Genetic diagnosis				Further workup
					Gene	Variants	Zygosity	ACMG class.	
<b>Myopathy</b>									
31/10/M	limb-girdle weakness	352	-	Suspect mitochondrial disease	Not found	-	-	-	Blood mtDNA NGS: no culprit variants found; Consider muscle mtDNA sequencing, long-read sequencing, RNAseq or WGS
32/33/F	3 episodes of rhabdomyolysis	304	Variation of fiber size; most atrophic and angular fibers are type II, mild endomysial fibrosis	Suspect mitochondrial disease	Not found	-	-	-	Blood mtDNA NGS: no culprit variants found; Consider muscle mtDNA sequencing or WGS
33/55/F	Proximal Arm (Scapular winging)	43	Myogenic myopathy, rare ragged-red fibers	mitochondrial myopathy	ACAD9	c.[797G>A];[797G=] p.[Arg266Gln];[Arg266=]	Het	LP	Blood mtDNA NGS: no culprit variants found; Consider muscle electron transfer chain assay/muscle mtDNA sequencing
34/26/M	Limb-girdle weakness	145	-	Suspect hereditary hypophosphatemic rickets	Not found	-	-	-	Reanalysis WES 1-2 years later or check long-read sequencing, RNAseq, or WGS
35/19/M	Limb-girdle weakness	437	Congenital myopathy with myofibrillar abnormalities	Suspect muscular dystrophy, mitochondrial or other congenital myopathy	Not found	-	-	-	Reanalysis WES 1-2 years later or check long-read sequencing, RNAseq, or WGS
36/25/F	Limb-girdle weakness	52	Dystrophic myopathy	Suspect muscular dystrophy	Not found	-	-	-	Reanalysis WES 1-2 years later or check long-read sequencing, RNAseq, or WGS
37/52/M	No definite weakness, subjectively felt smaller size in right leg	fluctuate between 300 U/L to 1000 U/L	-	Asymptomatic hyperCKemia	ANO5	c.191dupA p.Asn64LysfsTer15	Het	P	Segregation analysis and muscle biopsy was suggested, but patient hesitated.
					FKRP	c.[826C>A]; [826=] p.[Leu276Ile]; [Leu276=]	Het	P	

(Continued)

Table 4  
(Continued)

Age of onset	Type of weakness	CK (U/L)	Pathology	Clinical diagnosis	Genetic diagnosis				Further workup
					Gene	Variants	Zygoty	ACMG class.	
<b>Neuropathy</b>									
Age of onset	Initial symptoms	Electrophysiology studies		Clinical diagnosis	Genetic diagnosis				Further workup
					Gene	Variants	Zygoty	ACMG class.	
38/42/F	Distal numbness and weakness	Sensorimotor polyneuropathy with demyelinating features		CMT1	POLG	c.[2890C>T],[2890C=] p.[Arg964Cys]; [Arg964=]	Het	P	Molecular study for the variants into CMT1 cell/animal model. Reanalysis WES 1-2 years later or check long-read sequencing, RNAseq, or WGS
39/52/M	Distal numbness	Sensorimotor polyneuropathy with demyelinating features. Autonomic dysfunction		CMT1	SPTLC1	c.[766C>T];[766C=] p.[Pro256Ser];[Pro256=]	Het	VUS	Reanalysis WES 1-2 years later or check long-read sequencing, RNAseq, or WGS
					*The segregation analysis: not cosegregation				
40/Child-hood/M	Distal numbness	Sensorimotor polyneuropathy with axonal degeneration features		CMT2	HARS	c.1255_1256delinsCT p.Lys419Leu	Het	VUS	Reanalysis WES 1-2 years later or check long-read sequencing, RNAseq, or WGS
					*The segregation analysis: not cosegregation				
41/79/M	Abnormal sensation below ankle	Motor-predominant polyneuropathy		Sensorimotor polyneuropathy. Left ventricular hypertrophy.	Not found				Reanalysis WES 1-2 years later or check long-read sequencing, RNAseq, or WGS

HMERF [23, 24]. Two patients had variants in the *NEFH* gene. We made the diagnosis of brachial amyotrophic diplegia, primary lateral sclerosis, neuronopathy, distal hereditary motor neuropathy, and type IID based on the clinical presentation, electrophysiological studies, and WES results.

*Scenario 3: Patients with negative WES result, but with final clinical diagnosis after further work-up*

Notably, in the 15 patients without a molecular diagnosis from WES, four patients (26.7 %) had a diagnosis after further work-up. The details of these four patients are described below.

**Case 27: Tumor-induced osteomalacia:** A 32-year-old man presented with a 3-year history of progressive proximal muscle weakness, atrophy, and generalized back soreness. Electromyography performed at other hospitals showed myopathic changes with spontaneous activities and a right proximal tibial tumor. WES was performed but only found some benign variants. The clinical evaluation revealed a reduction in body height from 185 cm to 183 cm, kyphosis, and a palpable tender lump at the right proximal lateral tibia. Laboratory tests showed markedly reduced phosphate (1.3 mg/dL; normal range 2.5–5 mg/dL) and normal creatine kinase levels. The workup for the hypophosphatemia revealed normal calcium and iPTH, decreased 25-OH vitamin D (16 ng/mL; normal range 25–80 ng/mL), and elevated alkaline phosphatase (439 U/L; normal range 34–104 U/L). The urine analysis showed a markedly elevated urinary phosphate level (5332 mg/24 hrs). Furthermore, the bone scan showed a bone tumor with markedly increased osteoblastic activity at the right proximal femur. Combined with the above information, tumor-induced osteomalacia was highly suspected. Elevated serum fibroblast growth factor 23 (FGF23) confirmed the diagnosis. The pathology of the bone tumor was consistent with a phosphaturic mesenchymal tumor. The patient received tumor excision, his FGF23 was undetectable five hours after the operation, and his serum phosphate returned to normal three days after the surgery (Fig. 2). On follow-up, his bone pain and whole back soreness resolved. He could walk with a cane for more than 100 meters three months later and could stand without assistance for more than 10 minutes at the 5-month follow-up. (Supplementary Video)

**Case 28: Mitochondrial myopathy:** A 41-year-old woman with no systemic disease visited our

hospital for a one-month history of progressive proximal muscle weakness along with generalized myalgia. In addition, she lost eight kg in two months. She first received steroid therapy and her muscle strength improved in the first three months but later worsened even under treatment with prednisolone and azathioprine. Moreover, she developed obvious exercise intolerance. Her lactate level was 6.77 mmol/L at baseline (normal range 0.5–2.2 mmol/L), and increased to 11.96 mmol/L after exercise. Large amounts of lactate and pyruvate were noted in the urine as well as the presence of TCA cycle intermediates. The histopathology of the left biceps brachii revealed excessive lipid accumulation. (Fig. 3) Given the impression of metabolic myopathy, WES was ordered but yielded only benign variants. Based on the clinical information, we further arranged mitochondrial DNA (mtDNA) next-generation sequencing and a pathogenic variant (m.3243A>G) was found, confirming the diagnosis of mitochondrial myopathy.

**Case 29: Microsatellite expansion disease:** A 65-year-old man with congenital deafness developed unsteady gait at the age of 61. Bilateral upper limb clumsiness, dysmetria, and dysphagia appeared successively from the age of 63. With disease progression, he became unable to walk without assistance and was brought to our hospital for help. He reported a family history of gait problems in his mother and siblings. The neurological examination showed saccadic pursuit, dysphagia, decreased muscle strength in all four limbs, areflexia, positive Romberg test, and ataxic gait. The brain and spine MRI findings were normal. NCS revealed sensorimotor polyneuropathy. With multisystem involvement, WES was arranged but showed negative results. Notably, a heterozygous CAG repeat expansion (repeat number: 28 and 63) was noted in the *ATX3* gene, and compatible with Machado-Joseph Disease.

**Case 30: Mononeuropathy multiplex, vasculitis related:** The 37-year-old woman presented with several episodes of mononeuropathy in all four limbs with or without complete recovery from the age of 17. The NCS disclosed absent or decreased CMAP in the bilateral median, ulnar, tibial, and right peroneal nerves. Additionally, there was absent or reduced sensory nerve action potential (SNAP) in the bilateral median, ulnar, sural, superficial peroneal, and left superficial radial nerves. The laboratory tests and CSF studies were within normal limits except for elevated rheumatoid factor. The combined biopsy at the right superficial peroneal nerve and right peroneal

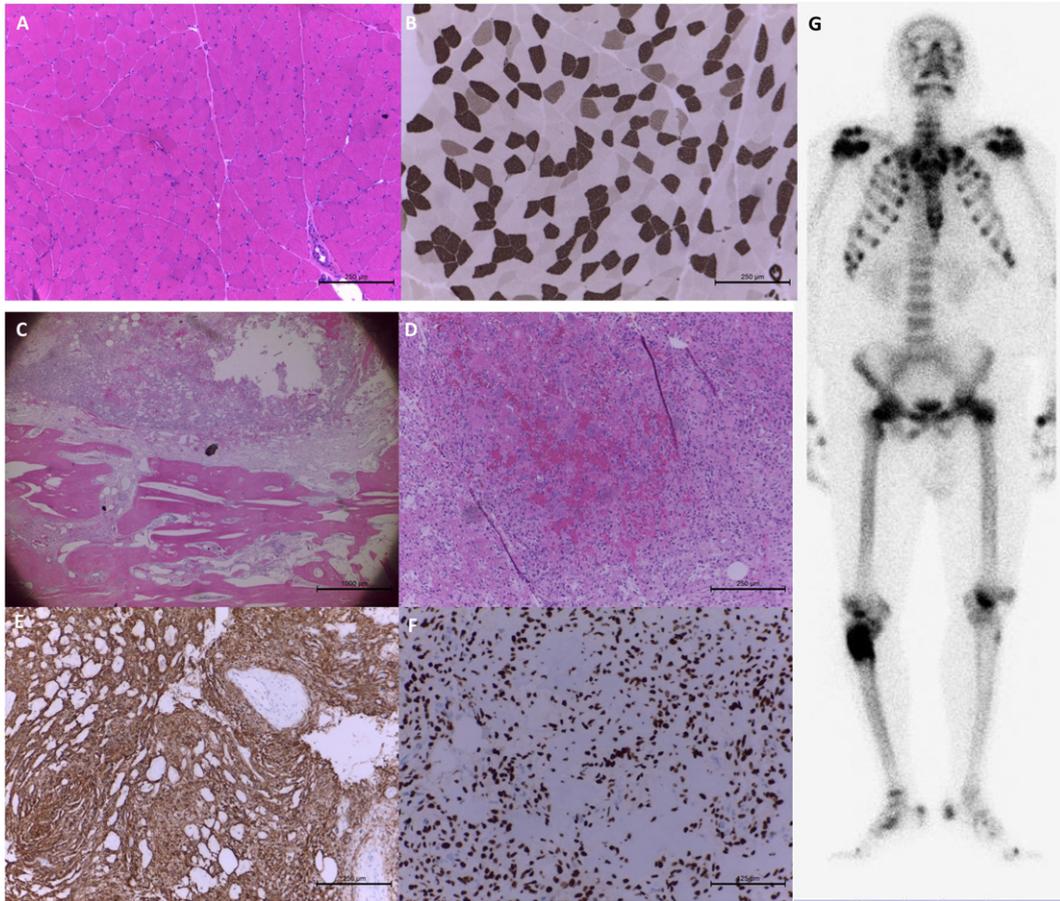


Fig. 2. Muscle and bone histopathology in patient 28 with tumor-induced osteomalacia. The staining of hematoxylin and eosin (H&E) (A) and myosin adenine triphosphatase at pH 4.3 (B) showed compacted muscle fibers with scattered atrophic fibers in the biopsy at left biceps brachii. The bone tumor pathology showed spindle tumor cells in myxohyalinized fibrotic stroma with eosinophilic matrix-like materials accompanying the bony components in H&E staining. (C&D) Focal bony destruction, woven bone formation and grungy calcification were also noted. The immunohistochemical staining of CD56 (E) and SATB2 (F) was strongly positive. Whole-body bone scintigraphy revealed a bone tumor with markedly increased osteoblastic activity at the right proximal femur. (G) Additionally, there were multiple posttraumatic changes in the thoracic cage, pubic bones, and bilateral femurs.

brevis muscle did not reveal evidence of vasculitis (Fig. 4). Under the impression of mononeuropathy multiplex with unknown cause, whole genome sequencing was arranged, yielding negative findings. Nevertheless, the patient-reported symptoms improved after receiving pentoxifylline and a short duration of oral steroids. Based on the clinical findings, the final diagnosis was vasculitis-related mononeuropathy multiplex.

#### *Scenario 4: Patients with negative WES result and undetermined etiology in further work-ups*

For the remaining 11 patients with a negative molecular diagnosis from WES, most ( $n=7$ ) had a

clinical diagnosis of myopathy, while others ( $n=4$ ) were clinically diagnosed with neuropathy. The detailed clinical features of patients without definite diagnosis are presented in Table 4. Among these, some had negative findings from NGS. Two had suspected variants at first but were later excluded by segregation analysis. WGS may be helpful for these patients. For patients with unreported pathological variants of *ANO5* and *FKRP* mutations, the definite diagnosis may be verified after segregation analysis and muscle biopsy with molecular studies. Currently, the patient is still hesitant about this plan. Some had suspected variants but without reported phenotypes, such as *POLG*-related Charcot-Marie-Tooth disease type 1 (CMT1) and *ACAD9*-related mitochondrial

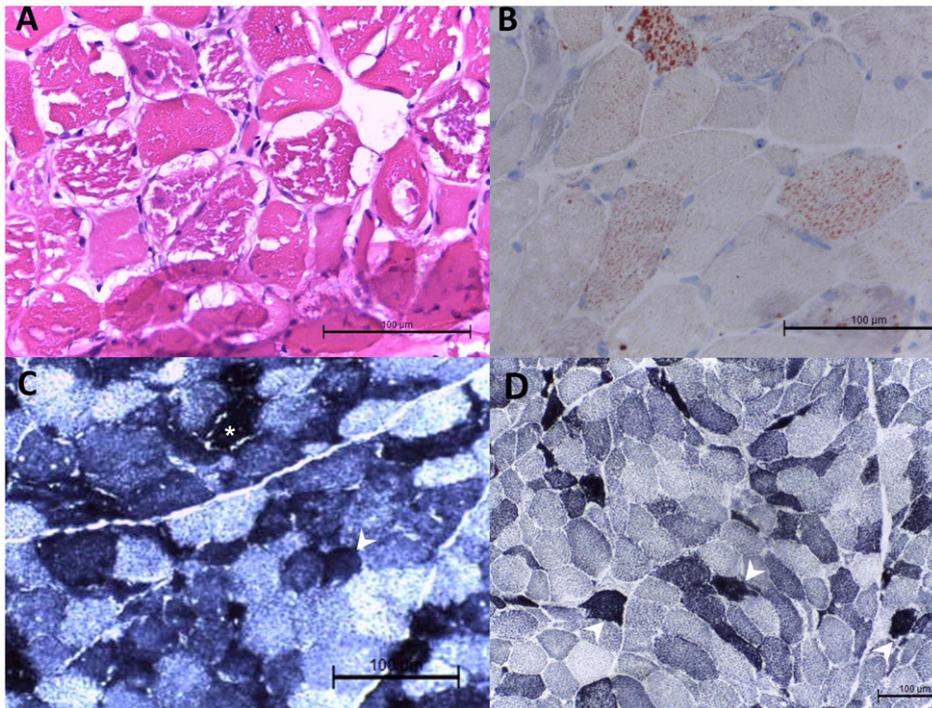


Fig. 3. Muscle histopathology in patient 29 with mitochondrial myopathy. Hematoxylin and eosin staining showed varied muscle fiber sizes, degenerative and atrophic muscle fiber sizes, and nuclear internalization in the biopsy at the left biceps brachii. There were muscle fibers with varied cytoplasmic vacuoles. The vacuole was strongly positive for Oil Red O staining (B). In the staining of nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) (C) and succinate dehydrogenase (D), dark staining was noted in some muscle fibers (arrowhead). Vacuoles were also seen in the NADH-TR staining (\*).

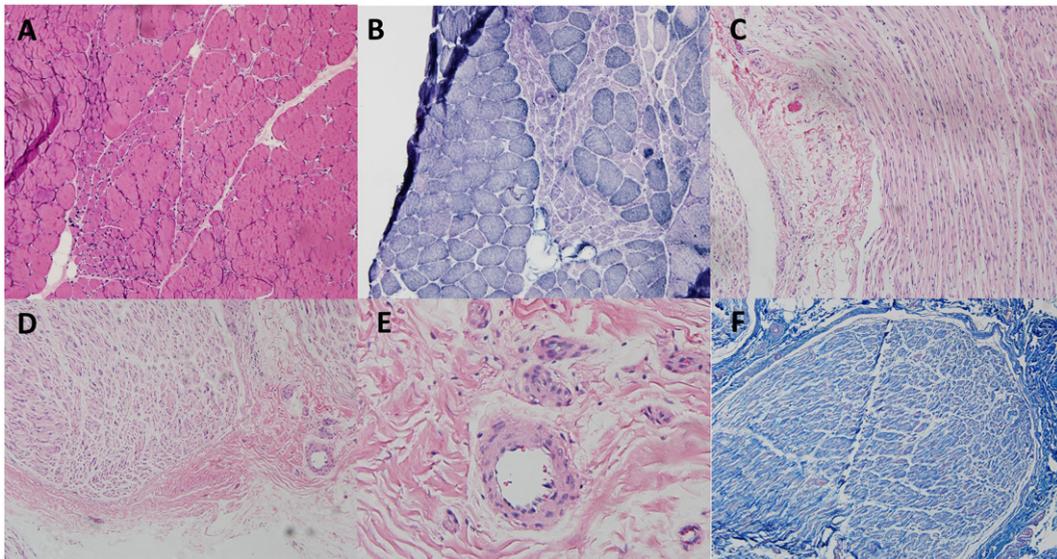


Fig. 4. Combined muscle and nerve biopsy in patient 31. The staining of hematoxylin and eosin (H&E) (A) and nicotinamide adenine dinucleotide-tetrazolium reductase (B) showed grouped atrophy in the right peroneal brevis muscle. The biopsy in the right superficial peroneal nerve did not show thickening of small vessels, lymphocyte infiltration, red blood cell extravasation in H&E (C-E) and Masson's trichrome staining (F).

myopathy. Segregation analysis and further molecular diagnosis are needed.

## DISCUSSION

WES is a powerful diagnostic approach for identifying pathogenic variants in patients with genetic diseases. Even when obtaining a positive finding from WES, the physician still needs to correlate the identified variants with the clinical phenotypes. Clinical challenges remain after obtaining a negative or undetermined WES. We focus our discussion on (1) designing molecular tests in patients with positive or inconclusive WES results and (2) a diagnostic approach for those with negative WES results.

In this study, our yield rate of WES is 63.4%, which is higher than the previous reports (yield rate of myopathic patients: 40–60%; yield rate of neuropathy patients: 30–40% [5–10]). Our WES pipeline is same as most of the study, and reports having different pipelines such as using higher minor allele frequency <0.02 or 0.05 as variant filtering criteria did not get higher yield rates. Several possibilities may explain this slightly higher yield rate. First, we considered the WES as the second-tier genetic test. After excluding common secondary cause, we would perform first-tier single gene test according to the deep phenotyping (e.g., D4Z4 repeats in the chromosome 4q in scapuloperoneal weakness and Ala117Ser in *TTR* gene in late-onset pan-modality neuropathy in Taiwanese). WES was served as the second-tier genetic test when the results of the first-tier test was negative. Second, we applied the segregation analysis with Sanger sequencing on the specific variants revealed by the proband-only WES, the cost of which is much lower than the cost of trio-WES. The procedure is mandatory for the cases with two variants but uncertain allele or the cases with only one previously unidentified variant. In our cohort, this procedure accompanied with muscle biopsy or further molecular examinations upgraded the patients with inconclusive WES result to definite clinical-molecular diagnosis in one case (Case 20), and exclude the variants as the pathogenic variants in two cases (Case 39 and 40). Of note, four patients could only meet the criteria of probable clinical-molecular diagnosis due to uncertainty of compound heterozygosity. Third, we arranged the specific genotype-guided molecular studies to investigate and confirm the proposed pathomechanism of

different genetic diagnosis. The process of extensive clinical, molecular, and pathological correlation may give us additional yield [12].

For those cases with positive WES result, the final diagnosis could be reached by phenotype-genotype correlations. Interestingly, *PMP22* deletion was found using WES in one patient who had negative results from extensive workups before receiving WES. In his previous genetic test, *PMP22* deletion screening was performed using PCR restriction fragment length polymorphism. However, since the patient had a DNA deletion that included the restriction site, the results of gel electrophoresis may miss the deletion [25]. In Case 10, he presented weakness and hyperCKemia, and had positive family history of early respiratory failure. The *TTN* gene variant c.95136T>G (p.Cys31712Trp) was identified from our patient, and this variant had been reported in one Korean family with clinical features of HMERF [26]. However, our patient has not developed with respiratory failure currently and refused the muscle biopsy. He was categorized into a probable diagnosis. In summary, a validation with clinical manifestations, pathology, or other examinations is still essential in patients with positive WES results.

Among the 9 patients with inconclusive WES result, three patients (*DES*, *DMD* and *TK2*) achieved a final diagnosis via further pathology or molecular studies. All had a typical clinical phenotype. In case 18, the deletion from 1189 to 1206 base pairs of the *DES* gene is a formerly unreported new variant. With atrophic myocytes with desmin aggregates in scattered fibers in the muscle pathology, the patient was diagnosed with myofibrillar myopathy-1 according to the concordance of genotype, phenotype, and pathology [27, 28]. In addition to the typical pathognomonic findings, in case 20 with *TK2* deficiency, we made a definite diagnosis using molecular examinations such as mitochondrial DNA sequencing and muscle electron transfer chain activity assays [29–31]. With a definite diagnosis, we could offer the patients more accurate genetic consultation, prognosis prediction, and the possibility of participation of clinical trial.

Not all patients or their families agreed to accept muscle biopsies or blood tests. Definite clinical-molecular diagnoses in our cohort were not achieved because of uncertain compound heterozygote (Cases 11, 21, 23, and 26), because of atypical manifestations according to literatures (Case 9, 10, and 12), because of unreported variants in the ClinVar database (Case 13 and 22) or because of current data limitations (Cases 15, 16, 17, 24 and 25). For

example, in Case 9, we only identified a variant in *CAPN3* gene, which had been reported in patients with both autosomal dominance inheritance and autosomal recessive inheritance [32, 33]. However, the details of the phenotype of the variant with autosomal dominance inheritance was not clear in the literature [32]. Whether the single variant could lead to his symptoms, milder than typical LGMD R2, requires segregation analysis and further molecular studies. Another example is two variants of the *NEFH* gene noted in two patients (Cases 24 and 25) with a clinical diagnosis of ALS or probable PLS. The *NEFH* gene encodes the heavy neurofilament protein playing a role in intracellular transport to axons and dendrites. In molecular and animal studies, mutation of the *NEFH* gene affects the efficiency of survival of motor and sensory axons, while in human studies, deletions of the *NEFH* tail have been found in approximately 1% of sporadic motor neuron diseases [34, 35]. The above findings support the hypothesis that the *NEFH* variant is associated with susceptibility to ALS. Apart from the above reasons, Case 16, with a diagnosis of *POLG*-related parkinsonism with sensory neuronopathy, was grouped into the probable diagnosis category. The function of the enzyme encoded by *POLG* is the repair and replication of mitochondrial DNA. Pathogenic variants in the *POLG* gene may cause variable clinical manifestations including neuropathy, parkinsonism, cerebellar ataxia, epilepsy, and chronic progressive external ophthalmoplegia [36]. Although the case matched none of the *POLG*-related syndromes, he indeed presented with two of the main features: sensory neuronopathy and parkinsonism [37]. Clinical or tissue evidence of mitochondrial dysfunction warrants further mitochondrial DNA testing. In summary, a genotype-guided molecular studies were warranted to ascertain the pathogenicity of the identified variants.

In cases with negative WES results, further pursuit of a diagnosis is challenging. For example, in Case 27 myopathy was suspected but WES did not yield a diagnosis. Clinical observation of decreased body height and kyphosis led to a finding of osteoporosis and renal wasting hypophosphatemia. Finally, we linked right tibial tumor, renal wasting-related hypophosphatemia, muscle weakness, and osteoporosis together and arrived at an impression of tumor-induced osteomalacia. Further evaluation of serum FGF23 and tumor biopsy confirmed our diagnosis. Careful evaluation of clinical findings and searching for acquired causes should be performed

before performing WES. If the WES result is undetermined, the acquired cause should be sought again.

Other than secondary causes, mitochondrial disease and microsatellite expansion disease should be kept in mind when WES is negative. Pathogenic variants in either the mitochondrial DNA or the nuclear DNA for mitochondria could lead to mitochondrial disease, but only nuclear DNA variants can be detected using WES. Therefore, it is crucial to consider mitochondrial disease in patients with maternally inherited patterns, exercise intolerance, ophthalmoplegia, or multisystemic disorders [38]. Of note, if a blood mtDNA yielded negative results, we would further arrange a muscle mtDNA test since heteroplasmy may be present. WES is incapable of detecting large-fragment copy number variations. Therefore, microsatellite expansion disease should still be put into the differential diagnosis when WES obtains negative findings. Additionally, a thorough evaluation of nerve system is essential to capture the deficits other than the peripheral nerve system. Of note, for those who had typical clinical presentation but an inconclusive pathology study, observation of treatment response could be helpful, for instance in our Case 30 with mononeuropathy multiplex.

The etiologies remained undetermined in eleven patients after WES and further workup. There are several possibilities. First, there should be secondary causes or culprit variants beyond our current knowledge that have not been evaluated. Second, under financial limitations, we checked mitochondrial genes only when clinically highly suspect. Although we did our best to evaluate all potential patients who had mitochondrial disease via detailed history taking, lactate exercise tests, muscle biopsies, and so on, a likelihood remains of missing patients with atypical phenotypes. Finally, the technological limitations of WES prevented us from detecting pathogenic variants in introns. Whole-genome sequencing or transcriptome studies in affected tissues may help to reach a final diagnosis for these patients.

Our study had limitations. First, this is a single-center study with a small cohort. Second, we did not set up a standard protocol for WES arrangement. To reduce selection bias, we recruited patients from only two attending physicians who reached a consensus about the inclusion criteria. Third, our study did not include pediatric patients. Expanding our findings to pediatric patients would require further study. Finally, the diversity of molecular studies for different genes was unavailable and resulted in many

patients being placed into the probable diagnosis category.

## CONCLUSION

WES facilitates the diagnosis of hereditary neuromuscular disorders. However, it is essential to pursue further molecular studies to correlate identified variants with clinical features. For patients who had negative findings from WES, secondary causes such as mitochondria and microsatellite expansion disease should be considered. Moreover, observation of treatment response, segregation analysis and further molecular evaluation should be beneficial to the final diagnosis.

## DECLARATIONS

### *Consent publication*

All authors have read and agreed the contents of this manuscript submitted to *Journal of Neuromuscular Diseases*.

## AVAILABILITY OF DATA AND MATERIAL

The datasets generated during analysis and/or during the current study are available from the corresponding author on reasonable request.

## COMPETING INTERESTS

None.

## FUNDING

None.

## AUTHOR'S CONTRIBUTIONS

All authors contributed to data acquisition and analysis. P.-S.C., N.-C.L., H.-W.H., and C.-C.Y. wrote the manuscripts with input from all authors.

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We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

## SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: <https://dx.doi.org/10.3233/JND-230013>.

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