Target-enrichment sequencing and copy number evaluation in inherited polyneuropathy

Wei Wang, MD
Chen Wang, PhD
D. Brian Dawson, PhD
Erik C. Thorland, PhD
Patrick A. Lundquist
Bruce W. Eckloff
Yanhong Wu, PhD
Saurabh Baheti, MS
Jared M. Evans, MS
Steven S. Scherer, MD,
PhD
Peter J. Dyck, MD
Christopher J. Klein, MD

Correspondence to
Dr. Klein:
klein.christopher@mayo.edu

ABSTRACT

Objective: To assess the efficiency of target-enrichment next-generation sequencing (NGS) with copy number assessment in inherited neuropathy diagnosis.

Methods: A 197 polyneuropathy gene panel was designed to assess for mutations in 93 patients with inherited or idiopathic neuropathy without known genetic cause. We applied our novel copy number variation algorithm on NGS data, and validated the identified copy number mutations using CytoScan (Affymetrix). Cost and efficacy of this targeted NGS approach was compared to earlier evaluations.

Results: Average coverage depth was $\sim 760\times$ (median = 600, 99.4% > 100 $\times$). Among 93 patients, 18 mutations were identified in 17 cases (18%), including 3 copy number mutations: 2 *PMP22* duplications and 1 *MPZ* duplication. The 2 patients with *PMP22* duplication presented with bulbar and respiratory involvement and had absent extremity nerve conductions, leading to axonal diagnosis. Average onset age of these 17 patients was 25 years (2–61 years), vs 45 years for those without genetic discovery. Among those with onset age less than 40 years, the diagnostic yield of targeted NGS approach is high (27%) and cost savings is significant ($\sim 20\%$). However, the cost savings for patients with late onset age and without family history is not demonstrated.

Conclusions: Incorporating copy number analysis in target-enrichment NGS approach improved the efficiency of mutation discovery for chronic, inherited, progressive length-dependent polyneuropathy diagnosis. The new technology is facilitating a simplified genetic diagnostic algorithm utilizing targeted NGS, clinical phenotypes, age at onset, and family history to improve diagnosis efficiency. Our findings prompt a need for updating the current practice parameters and payer guidelines. *Neurology*® 2016;86:1762–1771

GLOSSARY

CIAP = chronic idiopathic axonal polyneuropathy; **CMT** = Charcot-Marie-Tooth; **CNV** = copy number variation; **DGV** = Database of Genomic Variants; **HMSN** = hereditary motor and sensory polyneuropathy; **MAF** = minor allele frequency; **NGS** = next-generation sequencing; **VUS** = variants of unclear significance; **WES** = whole exome sequencing.

Polyneuropathies are common, with overall prevalence of 1.7%, and 6.6% among persons over 60 years old.¹ The high prevalence, multiple impairments, and complicated diagnostic procedures lead to high health care cost.^{1,2} Separating inherited from acquired causes is often difficult for insidious adult-onset cases, due to overlapping phenotypes.^{3,4} Furthermore, inherited and acquired polyneuropathies may coexist, resulting in worse severity.^{5–8} One large prospective study indicated that as many as 42% of patients with previously undiagnosed polyneuropathy coming to tertiary care referral had familial occurrence.⁹

Pragmatic diagnostic strategies have long been sought to improve testing effectiveness and reduce costs in neuropathy diagnosis.^{10–12} In Charcot-Marie-Tooth clinics, algorithms have been developed for selecting candidate genes.¹³ This approach is helpful for patients who have confirmed family history and have undergone nerve conduction studies, but a minority of US patients undergoes nerve conduction studies during the neuropathy diagnosis.^{1,2} Also, using

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From the Departments of Neurology, Peripheral Nerve Division (W.W., P.J.D., C.J.K.), Department of Health Science Research (C.W., S.B., J.M.E.), Laboratory Medicine and Pathology (D.B.D., E.C.T., P.A.L., Y.W., C.J.K.), Medical Genome Facility (B.W.E., Y.W.), and Medical Genetics (C.J.K., D.B.D.), Mayo Clinic, Rochester, MN; Department of Neurology (W.W.), China-Japan Friendship Hospital, Beijing, China; and Department of Neurology (S.S.S.), Perelman School of Medicine, University of Pennsylvania, Philadelphia.

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conventional Sanger sequencing to detect mutations has become impractical owing to the large list of causal genes and high cost. The high cost in polyneuropathy evaluation needs to be addressed as the average Medicare expenditure was \$14,362 in the year that the diagnosis of a polyneuropathy was made.²

Next-generation sequencing (NGS) provides a powerful tool for both genetic research and potentially clinical diagnosis.^{14,15} One NGS application, whole exome sequencing (WES), screens the transcribed regions of all known genes. We previously explored WES utility in patients with undiagnosed inherited polyneuropathy and found that 21% had pathogenic nucleotide mutations.¹⁶ The discoveries from WES have also broadened gene-specific phenotypes.^{17,18} Despite the benefits, the current use of WES as a routine clinical test in polyneuropathy is suboptimal because of its performance metrics (such as depth of coverage), cost, and the difficulty in analysis. Furthermore, complex strategies in reporting and consenting individuals undergoing WES or whole genome sequencing are needed to address ethical issues when unexpected mutations are found in genes unrelated to the disease.¹⁹

We investigate the effectiveness of a custom-designed targeted NGS approach to identify nucleotide mutations, small insertion/deletions, and large copy number changes such as *PMP22* gene duplications for inherited polyneuropathy. The current technology combined with past experience prompts us to consider a new algorithm incorporating targeted NGS approach to improve the efficacy of polyneuropathy diagnosis in clinical practice.

METHODS **Standard protocol approvals, registrations, and patient consents.** The study was approved by our institutional review board. Patients provided written consent. We identified cases without known genetic cause and suspected to have inherited polyneuropathy with or without family history.

Targeted enrichment design. We designed a SureSelect Target-Enrichment kit (Agilent Technologies, Santa Clara, CA) for diagnostic testing of all types of hereditary neuropathies. This panel covers 197 genes (table e-1 on the *Neurology*[®] Web site at Neurology.org) and is part of a larger neuromuscular gene capture design also including genes for dystrophies such as those caused by deletions of the *DMD* gene. A total of 5,940 targets were included. We performed paired-end 101bp sequencing on HiSeq2000 (Illumina, San Diego, CA). Because all the targeted regions only cover less than 2M base, we indexed 48 samples per lane and still achieved high-depth coverage.

Bioinformatics analysis. Bioinformatics analysis was performed using GenomeGPS, a comprehensive analysis pipeline developed at Mayo Clinic.¹⁶ We filtered the variants using Ingenuity Variant Analysis (Qiagen, Venlo, Netherlands). The variants were filtered and annotated using dbSNP (build144), OMIM, 1KGenome, ESP6500, Exac databases, and Ingenuity community collected exome database totaling ~100,000 samples of which ~86,000 are exomes and 14,000 are whole genome sequencing, as well as Human Genome Mutation Database. Nonsynonymous/splicing variants were evaluated by Polyphen2, SIFT, and Genomic Evolutionary Rate Profiling score. The identified mutations and novel variants (minor allele frequency [MAF] <0.0001, SIFT or Polyphen2 damaging) were Sanger sequence confirmed.

Copy number analysis. We also developed a new copy number variation (CNV) detection algorithm (PatternCNV) to assess CNV with improved sensitivity/specificity and increased resolution compared to other approaches.²⁰ To validate our copy number detection algorithm, we include 1 sample with *PMP22* duplication and 2 samples with *DMD* deletions. The identified copy number mutations and novel CNV variants were confirmed using CytoScan (Affymetrix, Santa Clara, CA) in our CLIA-certified laboratory.

Cost analysis. We evaluated the cost of earlier evaluations using 4 categories: (1) electrophysiologic studies; (2) laboratory tests such as complete blood cell count, urinalysis routine, serum vitamin B₁₂ assay, fasting glucose, hemoglobinA1C, protein electrophoresis, high sensitivity thyrotropin, sedimentation rate, lipid panel, and rheumatoid factor, autoimmune testing such as paraneoplastic antibody evaluation, and pathologic testing such as abdominal fat aspiration and sural nerve biopsy; (3) imaging testing, including x-ray bone survey, and brain or spinal MRI; and (4) selected candidate gene testing by Sanger sequencing or multiplex ligation amplification for copy number change. Only costs at our institution ordered to define the etiology of the polyneuropathy were calculated.

RESULTS **Included cases.** We identified 93 randomly selected genetically unresolved cases from our Neuropathy DNA Sample bank, including the following: hereditary motor and sensory polyneuropathy (HMSN) type 2 (HMSN2; n = 80); HMSN1 (n = 1); HMSN unclassified (n = 3); chronic idiopathic axonal polyneuropathy (n = 6), defined as polyneuropathy with distal leg sensory predominant symptoms, without family history^{21,22}; and hereditary motor neuropathy (n = 3). The mean age at the initial evaluation was 54 years (range 2–86), and the mean age at polyneuropathy onset was 41 years (range 2–85). Varied extent of family history of polyneuropathy was reported in 51 of 93 patients. Among these 51 patients, 37 reported similar weakness or sensory symptoms in one or more of their family members, and 14 had high arches with/without hammertoes or skinny legs.

Coverage of targeted polyneuropathy genes. We obtained high-quality calls. Eighty-five percent reads have Phred-Score >30 and average coverage depth of 760× for 5,940 regions (median = 600×). Almost all targeted regions (5,905, 99.4%) had >100× average coverage

depth (figure e-1). Among 35 regions $<100\times$ depth, 20 had $<25\times$. Of note, 18 of these 20 regions are in introns or untranslated region; the reason some targeted regions fall into introns is that our design requested 25bp surrounding sequence of exons. All under-covered regions overlap with repetitive elements that are difficult for all sequencing platforms.

Copy number change identification. Our analysis successfully detected the copy number mutations in 3 controls: one covering the entire *PMP22* gene region (~ 1.4 MB), one large *DMD* deletion (exon 2–exon 45, ~ 633 kb), and a small *DMD* deletion (exon 46–exon 47, ~ 3 kb), matching the findings from clinical testing (figure e-2).

Among 93 cases, we detected 3 CNV mutations: *PMP22* duplications (P2 and P78) (see chromosome- and gene-level graphs in figure 1A) and *MPZ* gene duplication (P46) within a large Chr1q duplication (figure 1B). *MPZ* duplication has been recently reported to cause hereditary neuropathy.²³ The unexpected *PMP22* duplications occurred in 2 HMSN2-labeled patients with onset in toddler years after initially making normal motor milestones; however, their electrophysiologic evaluations were done ~ 30 years after onset, when they had severe phenotypes with flail hands, wheelchair-assisted and needing nocturnal respiratory support. Nerve conductions were absent in the feet and hands but proximal stimulation and blink responses were not performed.

Pathogenic mutation identifications. We identified 15 nucleotide pathogenic mutations in 14 cases (1 case had mutations in both *MPZ* and *MFN2*). Combined with the aforementioned 3 cases with copy number mutations of *PMP22*, and *MPZ*, a total of 17 cases (18%) obtained genetic discovery. Eleven of them had previously negative candidate gene testing. Among these 17 cases, the mean onset age is 25 years (range 2–61), and the mean age at initial evaluation is 42 years (range 2–69). Among them, 12 patients (71%) had familial occurrence or tendency, of which 9 reported similar symptom in family members, and 3 reported family members with high arches with/without hammertoes or skinny legs. The identified mutations are summarized in table 1.

Although almost all 17 patients were previously diagnosed with or suspected to have HMSN2, our results indicate that 5 actually have HMSN1, 1 has HSAN1, 1 has giant axonal neuropathy, and 1 has Fabry disease. Three patients with mutations in *EGR2* and *GJB1* had been thought to have HMSN2 because their EMG revealed either no response or very low amplitude at upper limb ulnar motor nerve conduction (<0.1 mV), and they all had long disease duration (>30 years). The 1 patient with a *GJB1* mutation had a severe axonal polyneuropathy on EMG results

with intermediate slowing. The HSAN1 patient with *SPTLC1* mutation had prominent length-dependent motor nerve involvement, but no obvious foot ulcer or family history. The giant axonal polyneuropathy patient with a homozygous *GAN* mutation initially presented with early-onset axonal sensorimotor polyneuropathy and unclear family history since he was adopted. Neuropathy has been reported in patients with *GAN* mutation.²⁴ The 2 cases with *MPZ* nucleotide mutations were determined to have Charcot-Marie-Tooth (CMT) 2I based on further clinical review; 1 also had a *MFN2* mutation. The patient with a *BAG3* mutation was diagnosed with HMSN2 with adolescence-onset polyneuropathy, signs of distal atrophy in the legs, high arches, and curled toes. Dominant *BAG3* mutations typically cause myofibrillar myopathy or cardiomyopathy, but recently a *BAG3* mutation was found to cause an axonal sensorimotor polyneuropathy with giant axons.²⁵ The male patient with a hemizygous mutation *GLA*p.M1L (damaging by SIFT and Polyphen2) is recognized in retrospect to have Fabry disease with significant small-fiber involvements but without angiokeratomas. Five mutations at this position, *GLA*p.M1R, M1I, M1K, M1T, and M1V, were previously reported in patients with Fabry disease.^{26–28}

Rare variants and CNVs. In addition to 18 pathogenic mutations, we also identified 4 rare variants and 2 novel copy number changes in 6 cases, occurring in *AARS*, *LRSAM1*, *KIAA0196*, *DCTN1*, *SPAST*, and *TRIM2*. These nucleotide variants are either absent or have $MAF < 0.0001$ in variant databases (1KGenome, dbSNP, Exac, and Ingenuity Community Database). They occurred at highly conserved positions and are predicted damaging (table 2). In addition, 1 case (P43) was found with *SCN11A*p.L1158P (MAF in Exac = 0.06), a previously reported mutation for small fiber neuropathy,²⁹ although the patient did not have any phenotype of small fiber neuropathy. We did not include rare heterozygous variants found in the genes only linked to recessive disorders or CNVs that have been reported in Database of Genomic Variants (DGV). For the 6 patients with rare, damaging variants, the average onset age is 35 years (range 3–63), the average age at initial evaluation is 45 years (range 17–67), and 2 had family history. For the remaining 70 cases (75%) without causal mutation or rare damaging variant, the average onset age is 45 years (range 4–85) and the average age at initial evaluation is 58 years (range 11–86), indicating that genetic diagnosis remains to be found in the majority of patients with inherited axonal polyneuropathy (figure 2, A and B).³⁰ The novel CNV of *SPAST* and *TRIM2* (figure e-3) are not in DGV, and their disease association needs further investigation.

Figure 1 Pattern copy number variation (CNV) algorithm identifies copy number changes using next-generation sequencing (NGS) data

(A) The identification of *PMP22* gene duplication using PatternCNV and its confirmation by CytoScan (Affymetrix). (a) Schematic view of chromosome 17, where red boxes depict the position of duplicated regions. (b) Chromosome view of PatternCNV analysis shows increased CNV Log2 ratio for *PMP22*. (c) Genome view of PatternCNV analysis shows increased CNV Log2 ratio for *PMP22*. (d) CytoScan (Affymetrix) results reveal genomic duplication and confirm the duplication region covering *PMP22* gene. (B) The identification of duplication of chromosome 1q region (where *MPZ* gene resides) using PatternCNV and its confirmation by CytoScan (Affymetrix). (a) Schematic view of chromosome 1, where red boxes depict the position of duplicated regions. (b) Chromosome view of PatternCNV analysis shows increased CNV Log2 ratio for duplicated region. (c) Genome view of PatternCNV analysis shows increased CNV Log2 ratio for chromosome 1q region inclusive of *MPZ*. (d) CytoScan (Affymetrix) results reveal genomic duplication and confirm the duplication region covering *MPZ* gene.

Table 1 Summary of 17 patients with pathologic mutation

Patient	Mutation found by NGS	Onset age, y	Family history	Clinical feature/EMG results	Candidate gene testing	Genetic diagnosis	Initial diagnosis/suspicion
Copy number mutations							
P78	PMP22 duplication	4	Yes	Severe axonal polyneuropathy with bulbar and respiratory involvement, nonrecordable extremity nerve conduction responses, proximal nerve segments not examined	MFN2	HMSN1A ³³	HMSN2
P2	PMP22 duplication	3	Yes	Severe axonal polyneuropathy with bulbar and respiratory involvement, nonrecordable extremity nerve conduction responses, proximal nerve segments not examined	MFN2	HMSN1A ³³	HMSN2
P46	MPZ duplication	51	No	Length-dependent, predominantly axonal, sensorimotor polyneuropathy	None	HMSN2/MPZ-dup ^{23,32}	HMSN2
Nucleotide mutations							
P41	MPZ c.371C>T p.T124M	34	Yes	Stiffness and numbness in feet and toes; chronic, length-dependent, mixed axonal and demyelinating sensorimotor neuropathy	GJB1, PMP22 seq/dup/del	HMSN2I ^{38,39}	HMSN2, sleep apnea
P13	MFN2c.2146G>A p.A716T and MPZc.293G>T p.R98L	10	Yes	Progressive peroneal muscular atrophy of bilateral lower extremities	PMP22seq/dup/del, GJB1, SH3TC2	HMSN2A2 and HMSN2I ^{e1} (MPZp. R98S, R98C, R98P, and R98H reported ^{e2})	HMSN2, bilateral trigeminal neuralgia
P89	EGR2 c.1064A>G p.D355G	22	Yes	Severe length-dependent peripheral neuropathy with a limited autonomic neuropathy; all responses were absent in the upper and lower limbs on nerve conduction studies; the blink response was prolonged	PMP22 seq/dup/del, GJB1, MPZ, MFN2	HMSN1D (EGR2p. D355V reported ^{e3} in HMSN1)	HMSN2
P61	EGR2 c.1141C>T p.R381C	20	Yes	Progressive symmetric lower weakness and short of breath; mixed axonal and demyelinating sensorimotor neuropathy, with involvement of phrenic nerve; shortness of breath, hoarse voice, choking episodes	None	HMSN1D ^{e4}	HMSN2
P66	GJB1 c.83T>A p.I28N	10	No	Difficulty running, with foot drop; chronic, axonal, length-dependent sensorimotor neuropathy	None	HMSNIX1 ^{e5}	HMSN2
P63	MFN2 c.250A>G p.K84E	2	No	Progressive extremity weakness; chronic, axonal, sensorimotor neuropathy	None	HMSN2A2 ^{e6}	HMSN2
P87	MFN2c.1403G>A p.R468H	61	Yes	Numbness and weakness of bilateral lower extremities; axonal sensorimotor polyradiculoneuropathy	None	HMSN2A2 ^{e7}	HMSN2, Sjögren syndrome; diabetes mellitus
P1	MFN2c.2219G>C p.W740S	30	Yes	Progressive weakness in legs; axonal, motor greater than sensory neuropathy; median mononeuropathy, tremor, sleep apnea	TRPV4	HMSN2A2 ^{e8}	HMSN2, carpal tunnel syndrome; type 2 diabetes mellitus
P12	TRPV4 c.806G>A p.R269H	33	Yes	Chronic axonal sensorimotor neuropathy, with involvement of the right phrenic nerve	MFN2	HMSN2C ^{e9}	HMSN2, dyspnea on lying flat
P70	BAG3 c.625C>T p.P209S	18	Yes	Motor vehicle accident and chronic pain syndrome; severe length-dependent, axonal, sensory motor neuropathy	GJB1, MPZ, PMP22 seq/dup/del, EGR2, NEFL, GDAP1, LITAF, MFN2, SH3TC2, PRX	Axonal neuropathy (p.P209L reported in axonal neuropathy ²⁵)	HMSN2, chronic pain syndrome post motor vehicle accident
P39	HSPB1 c.116C>T p.P39L	51	Yes	Progressive distal sensory symptoms and weakness in the lower limbs; length-dependent, axonal, sensorimotor neuropathy	MFN2	HMSN2F ^{e10}	HMSN2, hypertension
P69	SPTLC1c.431T>A p.V144D	48	Yes	Pain and numbness in both feet, axonal, length-dependent, sensorimotor neuropathy with intermediate demyelinating features	PMP22 seq/dup/del, MPZ, MFN2, GJB1	HSAN1A ^{e11}	Intermediate HMSN, bilateral ulnar neuropathies
P16	GAN homozygous c.146C>A p.A49E	6	No	Axonal sensorimotor neuropathy, hypotonia, and progressive gait abnormalities	SMN	Giant axonal neuropathy ^{e12}	HMSN2
P07	GLA c.1A>C p.M1L	10	No	Length-dependent, predominantly axonal sensorimotor peripheral neuropathy	None	GLAp.M1R, M1I, M1K, M1T, M1V reported in Fabry disease ²⁶⁻²⁸	HMSN2

Abbreviations: HMSN = hereditary motor and sensory polyneuropathy; NGS = next-generation sequencing.

Table 2 Summary of 6 patients with variants of unknown significance

Patient	Rare variants	Onset age, y	Family history	MAF in ExAC or DGV	In silico analysis (SIFT, Polyphen2)	Disease linked to gene	Previous candidate gene testing	Initial diagnosis/suspicion
Copy number variation								
P29	SPAST duplication	10	No	Not in DGV	NA	Spastic paraplegia 4, autosomal dominant	None	HMSN2
P8	TRIM2 duplication	3	No	Not in DGV	NA	Axonal neuropathy, early-onset	None	HMN
Rare nucleotide mutations								
P30	AARSp.I470M	40	Yes	0	Damaging, benign	HMSN2	None	HMSN2
P18	LRSAM1 p.C694R	40	Adopted	<0.0001	Damaging, probably damaging	HMSN2	None	HMSN2
P47	KIAA0196 p.D81H	56	No	0	Damaging, probably damaging	Spastic paraplegia	None	HMSN2
P90	DCTN1p.509V	63	Yes	0	Damaging, benign	Perry syndrome, ALS, autosomal dominant motor polyneuropathy	None	HMSN2
P43	SCN11A	56	Yes	0.06*	Damaging, probably damaging	Previously reported causal for painful polyneuropathy*	PMP22, Cx32, MPZ, EGR2, NEFL	HMSN2

Abbreviations: ALS = amyotrophic lateral sclerosis; DGV = Database of Genomic Variants; ExAC = Exome Aggregation Consortium; HMN = hereditary motor neuropathy; HMSN = hereditary motor and sensory polyneuropathy; MAF = minor allele frequency; NA = not available.

Cost evaluation. Clinical testing of these 93 patients included 90 (97%) EMG, 76 (82%) fasting glucose/HbA1C, 67 (72%) B₁₂, 72 (77%) protein electrophoresis, 31 (33%) CSF, 19 (20%) nerve biopsy, and 46 had average of 4 (1–14) candidate genes tested. We categorized the above testing into 4 categories (see Methods), and randomly selected 10 patients who had candidate gene testing, 5 with (group 1) and 5 without (group 2) family history. The average cost of their clinical evaluation is summarized (table e-2). The average cost of a comprehensive evaluation with unclear etiology is estimated at ~\$13,000, similar to prior report.² The average cost of earlier candidate gene testing is ~\$2,800 (~\$250–\$8,500), and the most commonly screened genes were those recommended in earlier diagnostic algorithms *PMP22*, *MPZ*, *GJB1*, and *MFN2*.¹³ Because the current reagent cost of our targeted NGS approach is ~\$100, if the test is utilized routinely in the clinical setting, the cost of this comprehensive screening will be only a small fraction of candidate gene testing and the diagnosis rate can be improved. Using the data from the cost evaluation and diagnostic yield, we calculated the potential cost saving of utilizing NGS testing (table e-3). Specifically, if we estimate that targeted NGS testing costs \$1,000–\$1,500 per sample, with observed positive test rate of 20%, a cost savings of 8%–12% is achieved. If we focus on those with early-onset age and family history, the cost savings rise to 17%–21%.

DISCUSSION We designed a comprehensive inherited polyneuropathy evaluation that can handle an expanding genotype–phenotype complexity with increased efficiency at reduced cost. The comprehensive nature of this NGS approach reduces the need to order genetic testing based on the outcome of nerve conduction studies (axonal, demyelinating, intermediate). Our study indicates that clinical complexity or severity can incorrectly restrict candidate gene testing by clinical phenotypes or electrophysiologic categorization. Specifically, the genetic cause is often outside the original clinical diagnostic category, as shown in almost half of our patients with identified mutation. These genetic discoveries have significant implications to the affected patients and their families. For example, the patient with Fabry *GLA* mutation, previously diagnosed with HMSN2, is now known to have X-linked inheritance, and requires heart and kidney monitoring and consideration for enzyme replacement therapy. Similarly, the patient found to have a *SPTLC1* pathogenic mutation with intermediate nerve conduction slowing will need vigilant foot care to avoid mutilating ulcers, and can now consider participation in clinical trials using serine supplementation.³¹ One concern among physicians is that large targeted panel sequencing will result in many variants of unclear significance (VUS). We only found 7 VUS in 93 cases (table 2), and some of these VUS may lead to new genetic discoveries with further research investigations.

Importantly, by incorporating a newly developed CNV algorithm we are also able to screen for

Figure 2 Average onset age and next-generation sequencing (NGS) genetic test positive rate

(A) Mean onset age and initial evaluation for 17 cases with pathogenic mutations: 25 years (range 2–61); 6 cases with rare (minor allele frequency <0.0001) and damaging (by in silico analysis) variants: 35 years (range 3–63); and 70 cases without genetic discovery: 45 years (range 4–85). (B) Positive genetic test rate in different patient groups. VUS = variants of unclear significance.

PMP22 duplications, the most common CMT cause.¹³ This was especially helpful in 2 patients with *PMP22* duplication previously diagnosed with severe axonal disease. The copy number algorithm also identified a gene duplication of *MPZ*, which is recently recognized as a pathogenic mutation of *HMSN1*.^{23,32} This would have been missed by traditional Sanger sequencing. Our targeted NGS approach combined with an effective CNV analysis can accurately detect the copy number changes at a single exon level, and serve as a comprehensive screening test for all types of polyneuropathy, including CMT1A. Furthermore, our study emphasizes the importance of considering genes outside of those typically ordered by commercial testing, as 8 of 17 (47%) would have been missed

by testing only the most commonly mutated genes: *PMP22*, *MFN2*, *MPZ*, and *GJB1*.¹³

Based on the results of this study and the earlier published practice guidelines for polyneuropathy,¹² we propose a new diagnostic algorithm that utilizes the targeted NGS approach, onset age, family history, and bedside clinical findings to increase the efficacy of a polyneuropathy evaluation for inherited cause (figure 3). Since CMT1A is the most common type of hereditary neuropathy, if the patient underwent EMG study and showed uniform demyelinating with reduced conduction velocity (<35 m/s), *PMP22* duplication/deletion should still be considered as the first ordered test. Our results indicate that onset age appears to be a strong predicting factor for

Figure 3 The proposed diagnostic algorithm

The proposed algorithm for evaluating chronic slowly progressive length-dependent polyneuropathy utilizing targeted next-generation sequencing copy number evaluation, age at onset, and family history along with knowledge of inherited neuropathy phenotypes to determine testing. A negative or inconclusive evaluation prompts consideration of CIAP or research studies by whole exome sequencing/genome sequencing. *All adult patients undergo testing for HgBA1c or glucose tolerance, monoclonal proteins, and B₁₂ deficiency.

obtaining genetic diagnosis (figure 2A). The mean onset age of our patients with pathogenic mutations is much younger than that of patients with unclear etiology (25 years vs 45 years). Among slowly progressive and length-dependent polyneuropathy patients with early onset age, the NGS test positive rate is high at 27% (figure 2B). Even without family history, the diagnostic rate was at 20% among those with <40 years onset age. The diagnostic rate is also high for those with family history at 24%. Family history is useful but not necessary for genetic discovery. Among the 93 evaluated cases, 51 (55%) had family history, of which 24% (12/51) were identified with pathogenic mutations, but 12% (5/42) of cases without family history also had pathogenic

mutations. In addition, the family history described here is not always extensive or definitive; many patients only mentioned family members with high arches, hammertoes, or skinny legs. Although considered somewhat subjective, high arches have been reported to have ~40% possibility of having inherited polyneuropathy.³³

Not surprisingly, those with early onset and family history had the highest diagnosis rate (33%). But only 1 of 22 patients with onset age of >40 years and no family history had genetic discovery. Thus, we suggest performing targeted NGS initially for those with young onset age or family history. Additionally, all patients should be tested for diabetes, monoclonal gammopathy, and B₁₂ deficiency, as they may further

worsen existing inherited polyneuropathy and the tests are inexpensive.^{34,35} However, unless there is specific clinical suspicion or indication, other laboratory tests can be delayed after the targeted NGS test. For patients with onset age >40 years and without family history, clinical phenotypes associated with inherited neuropathies, i.e., motor, large fiber sensory, large and small fiber sensory loss, can direct further appropriate evaluation.⁴ Specifically, motor predominant phenotypes are far more likely to have inherited cause; when large fiber sensory and pyramidal signs features are predominant, spinocerebellar syndromes should be considered. At present, repeat expansions cannot readily be assessed by targeted NGS, and MRI for cerebellar atrophy is often helpful in making the diagnosis.

Inherited disorders with Mendelian cause frequently have early onset relating to the strong deleterious influences of their pathogenic mutations.^{35,36} Cases with late onset have reduced likelihood of finding a genetic cause, highlighting the longstanding polygenic enigma of chronic idiopathic axonal polyneuropathy (CIAP).³⁷ In this study, no patients with CIAP phenotypes had pathologic mutations, consistent with evidence-based practice guidelines discouraging genetic testing in them.¹² However, accurately determining the age at onset can be difficult and careful clinical judgment is emphasized. In this study, we found the mean disease duration from the symptom onset to the first evaluation at our hospital is ~15 years. This further emphasizes the importance of affordable and comprehensive genetic testing available to general neurologists. When uncertainties remain, neuromuscular experts should be involved to assist in deep phenotyping, data interpretation, and potential kindred evaluations based on VUS discoveries.

The technology of NGS has evolved whereby we can appreciate meaningful clinical benefits. Our analysis suggests that the cost saving of utilizing targeted NGS testing with copy number evaluation will be significant. The actual savings could be greater than our calculation as we excluded patients with earlier positive gene testing of the most common phenotype-genotype associations.¹³ Targeted NGS approach combined with copy number analysis will expedite polyneuropathy diagnosis, reduce unnecessary treatments and testing, and allow clinicians to provide effective disease management, treatment, and research efforts. Our findings prompt a need for updating the current practice parameters and payer guidelines with respect to genetic testing for hereditary neuropathies.

AUTHOR CONTRIBUTIONS

Dr. Wang: study concept and design, acquisition of data, analysis and interpretation, drafting the manuscript, critical revision of the manuscript. Dr. Chen: acquisition of data, analysis and interpretation, critical

revision of the manuscript. Dr. Dawson: study concept and design, acquisition of data, analysis and interpretation, critical revision of the manuscript, laboratory funding support. Dr. Thorland: acquisition of data, analysis and interpretation. P.A. Lundquist: acquisition of data, analysis and interpretation. B.W. Eckloff: acquisition of data, analysis and interpretation. Dr. Wu: acquisition of data, analysis and interpretation, critical revision of the manuscript. S. Baheti: acquisition of data, analysis and interpretation. J.M. Evans: acquisition of data, analysis and interpretation. Dr. Scherer: study concept and design, critical revision of the manuscript. Dr. Dyck: study concept and design, acquisition of data, analysis and interpretation, critical revision of the manuscript, laboratory funding and support. Dr. Klein: study concept and design, acquisition of data, analysis and interpretation, drafting the manuscript, critical revision of the manuscript, study supervision, laboratory funding and support.

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DISCLOSURE

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