Original Article

A Novel Missense Mutation in Peripheral Myelin Protein-22 Causes Charcot-Marie-Tooth Disease

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Abstract

Background: Charcot-Marie-Tooth disease (CMT) is the most common inherited peripheral neuropathy. A great number of causative genes have been described in CMT, and among them, the heterozygous duplication of peripheral myelin protein-22 (*PMP22*) is the major cause. Although the missense mutation in *PMP22* is rarely reported, it has been demonstrated to be associated with CMT. This study described a novel missense mutation of *PMP22* in a Chinese family with CMT phenotype.

Methods: Targeted next-generation sequencing (NGS) was used to screen the causative genes in a family featured with an autosomal dominant demyelinating form of CMT. The potential variants identified by targeted NGS were verified by Sanger sequencing and classified according to the American College of Medical Genetics and Genomics standards and guidelines. Further cell transfection studies were performed to characterize the function of the novel variant.

Results: Using targeted NGS, a novel heterozygous missense variant in *PMP22* (c.320G>A, p.G107D) was identified. *In vitro* cell functional studies revealed that mutant PMP22 protein carrying p.G107D mutation lost the ability to reach the plasma membrane, was mainly retained in the endoplasmic reticulum, and induced cell apoptosis.

Conclusions: This study supported the notion that missense mutations in *PMP22* give rise to a CMT phenotype, possibly through a toxic gain-of-function mechanism.

Key words: Apoptosis; Charcot-Marie-Tooth Disease; Endoplasmic Reticulum; Missense Mutation; Peripheral Myelin Protein-22

Introduction

Charcot-Marie-Tooth disease (CMT), also termed hereditary motor and sensory neuropathy (HNSN), is the most common inherited neuromuscular disorders with an incidence of one in 2500 individuals.[1] It is clinically characterized by progressive distal muscle weakness, muscle atrophy, areflexia, sensory deficit, and skeletal deformities. In light of electrophysiological studies, CMT can be classified into a demyelinating form (CMT1) and axonal form (CMT2). To date, a large number of disease-causing genes have been identified to be associated with CMT. Among them, the heterozygous duplication of the peripheral myelin protein-22 (*PMP22*) gene is mainly responsible for CMT1 patients.[2]

PMP22 is located in chromosome 17p11.2, and the protein is a tetraspan integral membrane protein primarily expressed in the peripheral nervous system. In addition to the duplication mutation, a deletion mutation and point mutations in *PMP22* have been described in patients with inherited peripheral neuropathies as well.[3] The heterozygous deletion of *PMP22* is the major cause for the disease, hereditary neuropathy with liability to pressure palsies (HNPP), whereas point mutations in *PMP22* are connected with patients who display clinical features ranging from mild HNPP to severe CMT1. Point mutations in *PMP22* are rarely reported, although a variety of mutation types, including missense, nonsense, frameshift, and splicing mutations, have been mentioned.[4] It is believed that, through a toxic gain-of-function mechanism, most

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missense mutations in *PMP22* always result in the CMT1 phenotypes.[5-7]

In this study, using targeted next-generation sequencing (NGS) technology, we identified a novel *PMP22* missense mutation in an autosomal dominant demyelinating CMT family. The clinical features of these participants were well described, and the *in vitro* functional characteristics of the novel variant were further investigated.

Methods

Ethical approval

All participants signed written informed consent in accordance with the ethical protocol approved by the Ethics Board of Second Affiliated Hospital of Zhejiang University School Medicine.

Subjects

This study involved a family that presented with inherited peripheral neuropathy from the Second Affiliated Hospital of Zhejiang University School Medicine in 2016. Clinical features were carefully evaluated in all individuals. Electrophysiological examinations were performed in the proband. Five hundred healthy individuals without a history of peripheral neuropathy were selected as a control group.

Peripheral myelin protein-22 duplication/deletion analysis, targeted NGS, and Sanger sequencing

Genomic DNA was extracted from peripheral blood cells using the Blood Genomic Extraction Kit (Qiagen, Hilden, Germany). The multiplex ligation-dependent probe amplification (MLPA) kit (MRC Holland, Amsterdam, the Netherlands) was used to test the *PMP22* duplication/deletion according to the manufacturer's protocol. With a negative result of the MLPA, targeted NGS was further carried out in the proband. A gene panel including 44 genes known to be associated with CMT has been designed.[8] The detailed information of targeted NGS can be found in our previously reported studies.[8,9] In brief, samples were sequenced on an Illumina HiSeq2000 platform (Genergy Biotechnology Co. Ltd., Shanghai, China). The variants were detected using the Genome Analysis Toolkit version 3.7 (Broad Institute, Cambridge, UK). All of the variants were then annotated using ANNOVAR software (version 2016Feb01, GitHub, San Francisco, USA). Variants were further filtered according to the following steps: exclusion of variants with frequency >0.05 in the 1000 Genomes (http://www.internationalgenome.org/) or ExAc (http://exac.broadinstitute.org/), exclusion of variants that were not in the exonic or splicing site, and exclusion of synonymous variants. SIFT (http://sift.jcvi.org/) and PhlyPhen-2 (http://genetics.bwh.harvard.edu/pph 2/) were used to predict the protein's function change due to the amino acid substitution. Sanger sequencing was performed to verify the identified variants using standard protocols. The PCR products were directly sequenced on an ABI 3730 DNA Sequencer (Thermo Fisher Scientific, California, USA).

Plasmid constructs

The cDNA encoding the wild-type (Wt) human PMP22 protein was amplified and cloned into the expression plasmid (pEGFP-N1). The mutations, c.47T>C (p.L16P), c.320G>A (p.G107D), c.320G>T (p.G107V), and c.449G>A (p.G150D), were introduced into the Wt expression plasmids, separately, using PCR mutagenesis. All constructs were confirmed by Sanger sequencing.

Cell culture and transient transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (HyClone, Logan Utah, USA) supplemented with 10% fetal bovine serum (HyClone, Logan Utah, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (HyClone, Logan Utah, USA) in a humidified 5% CO_2 incubator. Cells were transiently transfected with various expression vectors using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, California, USA).

Confocal analysis

Forty-eight hours after transfection with the plasmids, cells were treated with ER-Tracker Red (Thermo Fisher Scientific, Eugene, USA) and NucBlue Live Reagent (Hoechst 33342; Thermo Fisher Scientific, Eugene, USA). Microscopic images were obtained using a confocal microscope (Leica TCS SP8; Leica Microsystems, Mannheim, Germany).

Flow cytometric analysis

HeLa cells were transiently transfected with a GFP empty vector, or a Wt, L16P, G107D, G107V, or G150D vector. Forty-eight hours after transfection, cells were harvested and stained with Annexin V (BD, San Diego, USA) and 7-ADD (BD, San Diego, USA) according to the manufacturer's protocol. All of the flow cytometric analyses were performed on an LSR II Fortessa cytometer (BD, New Jersey, USA), and the data were analyzed using FlowJo software (FLOWJO, LCC, Ashland, USA).

Statistical analysis

Values were presented as the means ± standard error (SE). Data were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism version 5 (GraphPad Inc., California, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Clinical manifestations

The clinical features of affected individuals in the pedigree are summarized in Table 1. Among these patients, the age ranged from 31 to 65 years. The age at onset was around the second decade of their lives. The initial symptom was weakness of the distal part of the legs after standing or walking for a long time. All of these patients displayed a slowly progressive disease course. With the progression of the disease, signs of muscle atrophy in the distal lower limbs and then the upper limbs were observed. In this family, the clinical severity was diverse. The eldest one

(patient II-2) exhibited the most severe muscle disability. He was wheelchair dependent and had claw-like fingers. The youngest one (patient III-3) was walking abnormally but autonomously and had no functional disability of the hands. Neurological examinations revealed that symmetric muscle wasting predominated in the distal parts of the legs in all patients. The aged affected individuals had hand muscle weakness and presented with bilateral sensory deficits. All affected individuals had scoliosis and pes cavus. Areflexia was noticed in all patients. Member III-2 was a 34-year-old female [Figure 1a]. She displayed no signs of motor and sensory impairment. Nerve conduction studies of the proband (patient II-3) revealed that the motor and sensory nerve conduction velocity was markedly reduced in all limbs. In addition, needle electromyography examination showed a neurogenic pattern in the examined limb muscles, including the tibialis anterior muscle and first dorsal interosseous muscle.

Genetic analysis

As the proband showed an autosomal dominant demyelinating form of CMT, the copy number variant of *PMP22* was first analyzed. The MLPA test confirmed that the proband was negative for *PMP22* duplication/deletion. Then, targeted NGS was carried out to screen the causative genes of CMT for the proband. In total, 69 variants, including 63 single-nucleotide variants and 6 small insertions and deletions, were identified. After filtering, three candidate heterozygous missense variants were found [Table 2]. These variants were verified by Sanger sequencing. Further co-segregation analysis in this family demonstrated that only one novel variant, c.320G>A (p.G107D) within *PMP22*, was present in all affected individuals and absent in the unaffected members [Figure 1a and 1b]. This variant was not present in 500 healthy controls and was absent in the 1000 Genomes and ExAc database. The variant was predicted to be

Table 1: Clinical features of the affected individuals with Charcot-Marie-Tooth disease

Characteristics	Patient II-2	Patient II-3	Patient II-5	Patient III-3
Age (years)	65	61	58	31
Sex	Male	Male	Male	Female
Age at onset (years)	15	20	18	21
FDS	7	3	2	1
Muscle weakness	UL + LL	UL + LL	UL + LL	LL
Muscle atrophy	UL + LL	UL + LL	UL + LL	LL
Areflexia	Yes	Yes	Yes	Yes
Sensory loss	Pain, vibration	Pain, vibration	Pain, vibration	NE
Skeletal deformities	Pes cavus, scoliosis	Pes cavus, scoliosis	Pes cavus, scoliosis	Pes cavus, scoliosis
R/L median nerve MNCV (m/s)	NE	12.9/12.8	NE	NE
R/L sural nerve SNCV (m/s)	NE	NP/NP	NE	NE

R/L: Right/left; UL: Upper limb; LL: Lower limb; NP: Not potential; NE: Not examined; MNCV: Motor nerve conduction velocity (normal values: Median MNCV ≥49 m/s); SNCV: Sensory nerve conduction velocity (normal values: Sural SNCV ≥40 m/s); FDS: Functional disability scale (0: Normal; 1: Normal but with cramps and fatigability; 2: Unable to run; 3: Walking with difficulty but without assistance; 4: Walking with a cane; 5: Walking with crutches; 6: Walking with walking frame; 7: Wheelchair bound; 8: Bedridden).

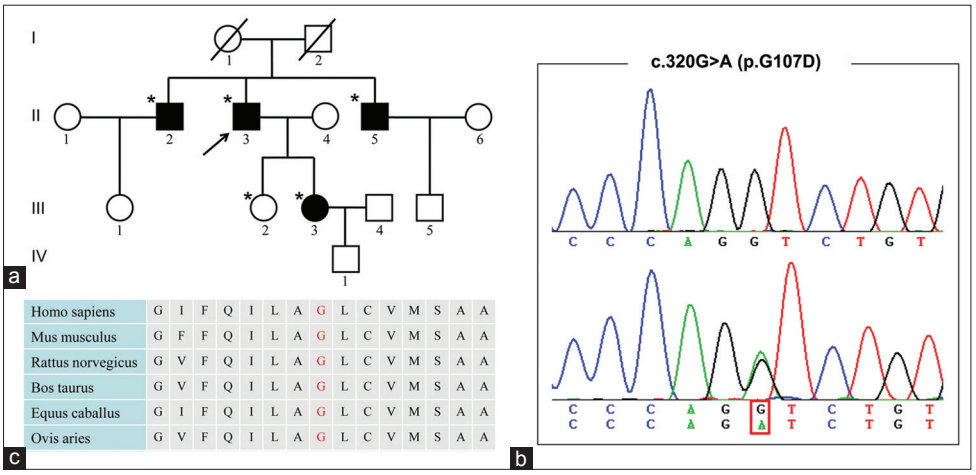

Figure 1: Pedigree, sequencing chromatograms, and conservation analysis of the novel variant in the *PMP22* gene. (a) Pedigree of the study family with Charcot-Marie-Tooth disease. Filled symbols indicate affected individuals, empty symbols indicate unaffected individuals, the arrow indicates the proband, and asterisks indicate individuals for whom DNA was available for sequencing. (b) The sequence variation (c.320G>A, p.G107D) in *PMP22* gene identified in this study. (c) The evolutionarily conserved protein alignments of multiple species were depicted to assess the conservation of the affected amino acid resident. PMP22: Peripheral myelin protein-22.

Table 2: The variants identified by targeted NGS in the proband

Gene	Nucleotide	Amino acid	Exon	Mutation type	SIFT	PolyPhen-2	1000G frequency	ExAc frequency	Family segregation
KIF1B (NM_015074)	c.3649C>T	p.P1217S	33	Missense	T	B	0.0002	0.000200	Not
PMP22 (NM_153321)	c.320G>A	p.G107D	5	Missense	D	D	0	0	Yes
DNMT1 (NM_001130823)	c.2741G>A	p.R914H	28	Missense	T	B	0	0.000016	Not

1000G: 1000 Genomes; ExAc: Exome Aggregation Consortium; T: Tolerated; B: Benign; D: Deleterious; NGS: Next-generation sequencing; *KIF1B*: Kinesin family member 1B; *PMP22*: Peripheral myelin protein-22; *DNMT1*: DNA methyltransferase 1.

damaging by the SIFT and PolyPhen-2 software programs. Moreover, the variant site was conserved in various animal species [Figure 1c]. One missense mutation affecting the same amino acid residue in *PMP22* (c.320G>T, p.G107V) had been previously reported, but the change was from glycine to aspartic acid instead of valine.[10]

In vitro functional characterization of the novel mutation within peripheral myelin protein-22

PMP22 has been demonstrated to be transported through the endoplasmic reticulum (ER) and Golgi apparatus to the plasma membrane.[11,12] To define whether the novel mutation we identified altered the intracellular transport of PMP22, five plasmids expressing Wt, L16P, G107D, G107V, and G150D PMP22-GFP fusion protein were generated and transiently transfected in HeLa cells. As the intracellular features of L16P and G150D PMP22 protein had been well described before,[12,13] they were used as positive controls in our study. A live cell confocal study showed that most of the Wt PMP22-GFP was located in the plasma membrane, and only a small proportion was co-localized with the ER marker in HeLa cells [Figure 2]. In contrast, all PMP22 mutants were mainly expressed in the ER and were not observable on the cell surface [Figure 2]. To further investigate the biologic effect of mutant PMP22 protein, flow cytometric analysis was performed in HeLa cells. As shown in Figure 3, we showed that, in cells expressing Wt or mutant PMP22, the percentage of live cells was decreased, while the ratio of apoptotic cells was increased. This phenotype was more obvious in cells transfected with mutant PMP22. All these data suggested that disease-causing missense mutations in *PMP22* could damage the intracellular trafficking of the protein and induce cell apoptosis.

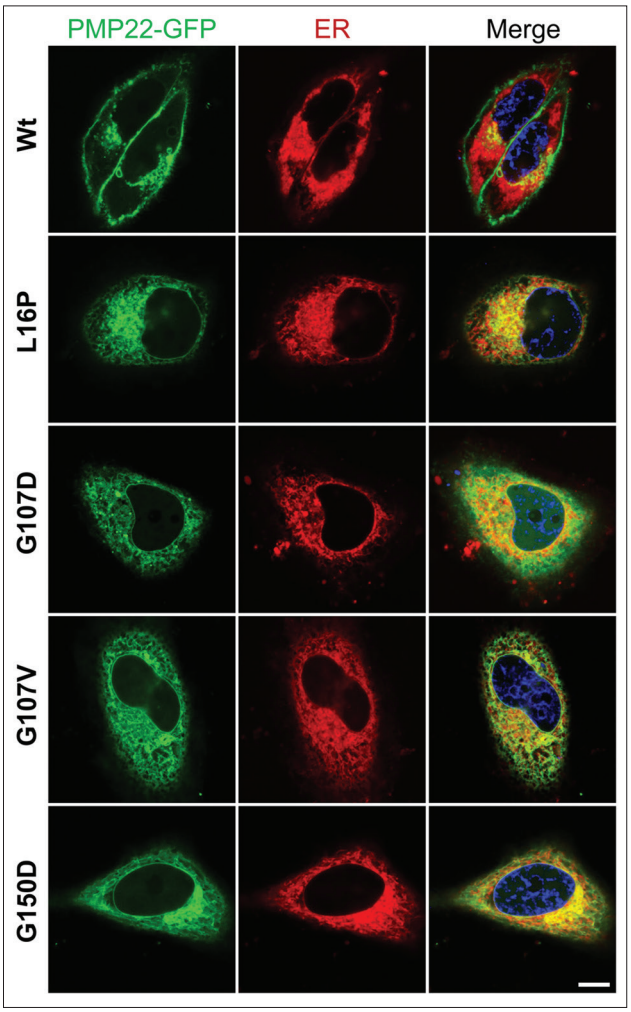

Figure 2: Subcellular localization pattern of PMP22 in HeLa cells. HeLa cells were transiently transfected with PMP22-GFP Wt or mutant expression plasmids (L16P, G107D, G107V, G150D). The intracellular ER was stained with ER-Tracker (red) and the cell nuclei were stained with Hoechst (blue). These microscopic images were obtained from a confocal microscope. Scare bar: 10 μm. Wt: Wild-type; PMP22: Peripheral myelin protein-22; ER: Endoplasmic reticulum.

Discussion

CMT is a heterogeneous inherited neuromuscular disorder. Due to the lack of a genotype-phenotype relationship, it is difficult for a laboratory to detect all of the possible genes using Sanger sequencing. Targeted NGS is well known to be an effective and rapid approach to simultaneously sequence thousands of genes of interest and has been successfully used to make a molecular diagnosis especially in clinical and genetic heterogeneous disorders.[14-16] In this study, we identified a novel heterozygous variant within *PMP22* (c.320G>A, p.G107D) using this technology. Further cell transfection studies revealed that mutant PMP22 carrying this variant could induce cell death. According to the American College of Medical Genetics and Genomics standards and guidelines,[17] this novel variant was classified as pathogenic with the following criteria: have a damaging effect supported by the *in vitro* functional study, located at the same amino acid where a different pathogenic missense change has been previously reported, absent from controls, predicted to be deleterious by various software programs, and shows co-segregation with the disease.

PMP22 is a 22-kDa glycoprotein that is primarily generated by myelinating Schwann cells and is a major component of

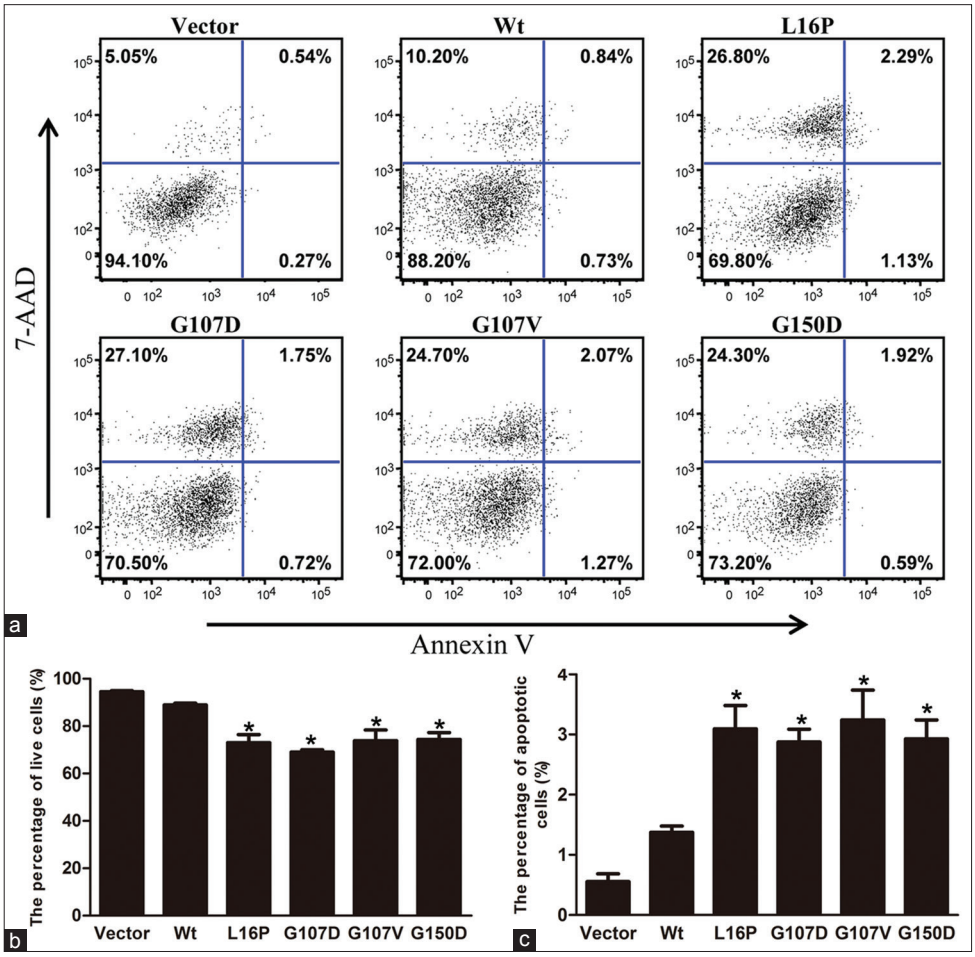

Figure 3: Flow cytometric study in transfected HeLa cells. (a) HeLa cells were transiently transfected with an empty vector, or a Wt, L16P, G107D, G107V, or G150D vector. Forty-eight hours later, flow cytometric analysis was performed in HeLa cells. (b) Note that the percentage of live cells was reduced after mutant PMP22 transfection. (c) The percentage of apoptotic cells was increased in cells expressing mutant PMP22 proteins. The results represent the means ± standard error of three independent experiments. *$P < 0.05$ versus the empty vector group. PMP22: Peripheral myelin protein-22.

peripheral myelin.[18] It has four putative transmembrane domains. Thanks to the *PMP22* transgenic animal models, we can infer that PMP22 plays an important role in the development and maintenance of peripheral nerves. A variety of missense mutations mainly localized in the four transmembrane domains of PMP22 has been demonstrated to be associated with CMT1.[3,4,19] Here, the novel variant we identified was situated in the third transmembrane domain. These disease-causing missense mutations may affect the protein's conformational stability and intracellular trafficking, resulting in a high level of mutant PMP22 in the ER.[12,20] Our live cell confocal study confirmed that mutant PMP22 carrying a missense mutation in the first (L16P), third (G107D and G107V), or fourth (G150D) transmembrane domain was mainly preserved in the ER. These mutants could not reach the plasma membrane.

The PMP22 protein has a rapid turnover rate. For newly synthesized PMP22, a large amount is rapidly degraded through the ER-associated degradation pathway due to misfolding, while only a small proportion is transported to the plasma membrane.[21] Mutant PMP22 retained in the ER had a deleterious effect on the protein degradation pathways, including the ubiquitin-proteasome pathway and autophagy-lysosome pathway, leading to the formation of intracellular aggregates.[7,21,22] The overload of mutant PMP22 may induce ER stress and trigger cell death, which can be observed in transgenic mice, such as Trembler and Trembler-J mice.[6,23] Furthermore, the heterozygous Trembler and Trembler-J transgenic mice had more severe phenotypes than the heterozygous *pmp22* knockout mice.[22,24] All of these studies confirmed that mutant PMP22 possess a toxic gain-of-function feature. In addition, mutant PMP22 could form heterodimers with the Wt PMP22 and then impair Wt protein transport through the ER to the cell surface, suggesting a dominant-negative mechanism.[22] This mechanism could explain the reason why patients with *PMP22* missense mutations always have a more severe phenotype than patients with a *PMP22* duplication mutation.[3,4]

Except for in a few cases, missense mutations in *PMP22* are believed to often lead to toxic gain-of-function and result in the CMT1 phenotype, while nonsense and frameshift

mutations cause haploinsufficiency of PMP22 and result in the HNPP phenotype.[25-27] In our study, patients with the G107D mutation in *PMP22* showed typical CMT1 features. Interestingly, in previously reported literature, patients carrying the G107V mutation in *PMP22* displayed a clinical phenotype ranging from symptomless to severely affected.[10] The type of the amino acid substitution may contribute to these distinct phenotypes.

In conclusion, utilizing targeted-NGS, we identified a novel mutation in *PMP22* in a CMT1 family. The missense mutation in *PMP22* led to the CMT1 phenotype, potentially through toxic gain-of-function mechanism.

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Conflicts of interest

There are no conflicts of interest.

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