One PMP22/MPZ and Three MFN2/GDAP1 Concomitant Variants Occurred in a Cohort of 189 Chinese Charcot-Marie-Tooth Families

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Background and Aims: Charcot-Marie-Tooth (CMT) disease is a clinically and genetically heterogeneous group of inherited peripheral neuropathies. The wide phenotypic variability may not be completely explained by a single mutation.

Aims and Methods: To explore the existence of concomitant variants in CMT, we enrolled 189 patients and performed molecular diagnosis by application of next-generation sequencing combined with multiplex ligation-dependent probe amplification. We conducted a retrospective analysis of patients harboring coinherited variants in different genes.

Results: Four families were confirmed to possess variants in two genes, accounting for 2.1% (4/189) of the total in our cohort. One CMT1 patient with PMP22 duplication and MPZ variant (c.286A>C, p.K96Q) exhibited moderate neuropathy with infantile onset, while her father possessing MPZ variant was mildly affected with adolescence onset. A CMT2 patient with heterozygous variants in MFN2 (c.613_622delGTCACCAG, p.V205Sfs*26) and GDAP1 (c.713G>T, p.W238L) exhibited childhood onset mild phenotype, while his mother with MFN2 variant developed bilateral pes cavus only. A CMT2 patient with heterozygous variants in MFN2 (c.839G>A, p.R280H) and GDAP1 (c.3G>T, p.M1?) presented infantile onset and rapid progression, while her father with MFN2 variant presented with absence of deep tendon reflexes. One sporadic CMT2 patient with early onset was confirmed harboring de novo MFN2 variant (c.839G>A, p.R280H) and GDAP1 (c.3G>T, p.M17?) presented infantile onset and rapid progression, while her father with MFN2 variant presented with absence of deep tendon reflexes. One sporadic CMT2 patient with early onset was confirmed harboring de novo MFN2 variant (c.1835C>T, p.S612F) and heterozygous GDAP1 variant (c.767A>G, p.H256R).

Conclusion: Our results suggest that the possibility of concomitant variants was not uncommon and should be considered when significant intrafamilial clinical heterogeneity is observed.

Keywords: Charcot-Marie-Tooth diseases, concomitant variants, intrafamilial clinical heterogeneity, double trouble, genetic modifier
INTRODUCTION

Charcot-Marie-Tooth (CMT) disease is the most common inherited neuropathy with an estimated prevalence of 1 in 2,500 (1). It is a clinically and genetically heterogeneous group of disorders that is characterized by progressive weakness and atrophy of the extremities and loss of sensory function (2). Neurophysiological findings in median nerve differentiate CMT into two groups: demyelinating CMT (CMT1) with slow motor nerve conduction velocity (MNCV) (< 38 m/s) and axonal CMT (CMT2) with normal or a slight reduction of MNCV (≥ 38 m/s) (2). It is generally accepted that CMT is a monogenic condition. Up till now, more than 100 causative genes have been reported as causal for CMT (http://neuromuscular.wustl.edu/time/hmsn.html), among which PMP22 and MFN2 are the most common causative genes for CMT1 and CMT2, respectively (3). Of note, patients with PMP22 duplication or MFN2 mutation, such as p.I126S and p.R94W, have a heterogeneous clinical presentation in terms of age at onset, disease severity, and clinical progression (4, 5). Thus, a single mutation may not completely explain the intrafamilial heterogeneity of CMT. Next-generation sequencing (NGS) affords opportunities to detect the co-occurrence of variants in dual or multiple genes, providing insights into the wide phenotypic variability of the disease.

In this study, we screened 189 Chinese CMT families by using multiplex ligation probe amplification (MLPA) combined with NGS technologies. We are further reporting families with significant intrafamilial phenotypic heterogeneity in the presence of concomitant heterozygous MFN2 and GDAP1 variants and a PMP22 duplication combined with an MPZ variant.

MATERIALS AND METHODS

Patients and Clinical Analysis

We recruited 189 unrelated Chinese CMT families from the outpatient neurology clinic of the Third Xiangya Hospital from 2016 to 2020. All the patients were diagnosed by two experienced neurologists according to the CMT diagnostic criteria formulated by the European CMT Consortium (6). Electrophysiological examinations were performed on probands and available family members. Patients were classified into CMT1 (median MNCV < 38 m/s) and CMT2 (median MNCV ≥38 m/s) subtype accordingly (2). Disease severity was evaluated with the application of the CMT neuropathy score (CMTNS) (7). This study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University. Written informed consent was obtained from all the participants.

Genetic Analysis

Genomic DNA was isolated from peripheral blood obtained from all participants using standard Phenol-Chloroform procedures. We first utilized the application of MLPA (P033 kit, MRC Holland, the Netherlands) for the detection of PMP22 duplication in patients with CMT1. The target NGS was a well-suited and cost-effective strategy for efficient molecular diagnosis of CMT (8–11). Thus, inherited peripheral neuropathy multigene panel (Supplementary Table 1) sequencing was further applied in patients with CMT1 who failed to achieve molecular diagnosis and in patients with CMT2. For patients with concomitant variants, whole-exome sequencing (WES) was carried out to exclude other potential genetic variants. The sample was captured by SureSelect Human All Exon V5 Kit (Agilent). Genomic DNA sequencing was performed on the Illumina HiSeq 2500 platform (San Diego, CA, USA).

Data Analysis for the Determination of Pathogenic Mutations

All the variants were filtered against the following population database: Genome Aggregation Database (gnomAD), 1,000 Genome project (1,000 genomes), and dbSNP129. In silico analyses were performed by Mutation Taster, PolyPhen-2, SIFT, and CADD (Combined Annotation Dependent Depletion) to predict the biological relevance of the amino acid changes and phylogenetic conservation of the mutation sites. Co-segregation analysis was performed utilizing Sanger sequencing to verify the variants. All variants were interpreted according to the American College of Medical Genetics and Genomics (ACMG) standards and guidelines (12).

RESULTS

Genetic Findings and Analysis

Four families were confirmed to possess variants in two distinct CMT genes. One CMT1 family (F1) harbored a heterozygous PMP22 duplication and an MPZ variant (c.286A>C, p.K96Q) (Figure 1A). Three families with CMT2 carried simultaneous heterozygous variants in MFN2 and GDAP1, among which F2 possessed variants c.613_622delGTCACCACAG (p.V205Sfs*26) in MFN2 and c.713G>T (p.W238L) in GDAP1, F3 harbored variants c.839G>A (p.R280H) in MFN2 and c.3G>T (p.M1?) in GDAP1, and F4 with de novo c.1835 C>T (p.S612F) variant in MFN2 and c.767A>G (p.H256R) variant in GDAP1 (Figures 1B–D). All the variants were absent or very rare in gnomAD, 1000 Genomes and dbSNP129 (Table 1), were predicted to be damaging by utilizing the application of bioinformatic tools (Table 1), and were well conserved among different species (Figure 1E). We classified them according to the ACMG standards and guidelines and illustrated the results of in silico analysis and predicted the pathogenicity of these variants in Table 1. WES was further performed in two families with CMT2 (F2 and F3), and no other potential disease-causing variants were identified.

We further made frequency comparisons of GDAP1 variants between patients with CMT and controls (gnomAD) using Pearson chi-squared test. In our cohort, the c.713G>T variant was identified in 1/189 patients (T = 1/378), the c.3G>T variant was identified in 2/189 patients (T = 2/378), and the c.767A>G variant was identified in 4/189 patients (G = 4/378). These three GDAP1 variants are more frequent in patients with CMT than in controls (p < 0.0001).
Clinical Features of Four Families With Concomitant Variants

F1. The proband, a 6-year-old girl, harboring PMP22 duplication and MPZ variant c.286A>C (p.K96Q) presented with delayed motor milestones and walked independently until 3 years of age. Subsequently, she developed frequent falls and had difficulty walking upstairs or downstairs. The symptoms progressed into her upper limbs with difficulty in buttoning at age 6. Neurological examination revealed atrophy and weakness of the distal upper (scored 3/5) and lower extremities (scored 3/5) and decreased superficial sensations under the ankle. Tendon reflexes were absent in the upper and lower limbs. She presented with bilateral pes cavus deformity and walked with a steppage gait. Electrophysiological studies revealed that the amplitudes of compound muscle action potentials (CMAPs) and sensory nerve action potentials (SNAPs) were absent in both the upper and lower limbs. The CMTNS was 15. Her father, a 28-year-old male, with a single MPZ variant, noticed his high-arched feet at age 15. He was presented with the reduced athletic ability (running and jumping) compared with his peers and was unable to maintain balance during squatting at age 16. The weakness was gradually progressive without the involvement of the upper extremities. Neurological examination revealed the distal weakness of his lower limbs (scored 4/5), normal sensations, bilateral pes cavus, and steppage gait. Tendon reflexes were absent in the lower limbs and reduced in the bilateral upper limbs. Electrophysiological studies revealed that the CMAPs amplitudes were decreased in the upper limbs and absent in the lower limbs and the SNAPs amplitudes were absent in the upper and lower limbs. His CMTNS was 6.

F2. The proband, a 22-year-old male, harboring heterozygous variants in MFN2 c.1835 C>T (p.S612F) variant and heterozygous GDAP1 c.767A>G (p.H256R) variant. Clinical status: open symbols, unaffected family members; filled symbol, patients; black dot in open symbols, subclinical subjects with electromyographic or clinical examination perturbation; the black arrow, proband; wt, wild-type alleles; m, mutated alleles; the red arrow, mutated sites. (E) Conservation of amino acids at mutation sites in the different species.
TABLE 1 | Bioinformatics analysis and ACMG classification of the variants detected in 4 families.

| Population databases | In silico analysis | Amino acid changes | Mutation taster | Polyphen2 | SIFT | CADD | ACMG classification |
|----------------------|-------------------|--------------------|----------------|----------|------|------|---------------------|
| gnomAD               | 1000G             | NF                 | NF             | NA       | D    | D    | Pathogenic          |
|                      |                   | NF                 | NF             | NA       | D    | D    | Pathogenic          |
|                      |                   | NF                 | NF             | NA       | D    | D    | VUS                 |
|                      |                   | NF                 | NF             | NA       | D    | D    | VUS                 |

gnomAD, Genome Aggregation Database; 1000G, 1000 Genome project; SIFT, Sorts Intolerant From Tolerant; CADD, Combined Annotation Dependent Depletion; NF, Not found; D, Disease-causing/Damaging; B, Benign; T, Tolerable; ACMG, The American College of Medical Genetics and Genomics; VUS, Variants of uncertain significance.

D. Detailed clinical features of the 4 families are summarized in Table 2. Detailed nerve conduction studies are summarized in Supplementary Table 2.

DISCUSSION

In this study, the patient with CMT1 harboring a de novo PMP22 duplication and an inherited MPZ variant presented with an earlier disease onset and a severer phenotype than her father harboring only the MPZ variant (Figure 1A). Each variant was sufficient to cause disease, suggesting a cumulative “double trouble” effect of these two concomitant variants (Figure 2A). MPZ is the major structural protein of peripheral
## Clinical features of four families with concomitant variants in this study.

| Family | Individual | Variants | Age at onset/Initial Muscle atrophy | Distal muscle Reflexes Pes cavus findings | Sensory symptoms | CMTNS |
|--------|------------|----------|------------------------------------|----------------------------------------|-----------------|-------|
| F2     | II-1 (proband) | p.K96Q   | 15/28                              | Pes cavus                              | 5/4             | +/–   |
|        | F2         | p.V160fs*26 | 2/6                                | Inability to stand on his heels        | 5/5             | –/–   |
|        | II-1 (proband) | p.W238L  | 3/0                                | Delayed motor milestones               | 3/0             | +/–   |
| F4     | II-1        | p.H256R  | 4/2                                | Foot drop                              | –/–             | +/–   |

**Notes:**
- Muscle atrophy: –: no atrophy; ++: severe atrophy (involved in proximal muscle).
- Distal muscle weakness: ±: mild weakness; ++: severe weakness (involved in distal muscle).
- Sensory symptoms: –: normal; +: reduced; ++: hyperreflexia.
- **UL/LL:** upper limbs/lower limbs.
- **CMTNS:** Charcot-Marie-Tooth disease neuropathy score.

Mutation in *MPZ* was associated with unfolded protein response activation and protein aggregates, leading to the apoptosis and demyelination of the Schwann cell and altered axonal interaction (13, 14). *PMP22* is also highly expressed in myelinating Schwann cell (2–5%) and directly contribute to myelin organization (15). *PMP22* duplication disrupts the development and maintenance of normal myelin in the Schwann cell (16). Thus, simultaneous variants in these two myelin-related genes might exert double deleterious effects in Schwann cells, which finally leads to more severe defects in the myelination of axons. Of interest, the co-occurrence of *PMP22* duplication and variant in another CMT gene (*GJB1, LITAF*) had also been reported (17, 18). Considering *PMP22* duplication as the most common cause of CMT1 patients (approximately 70%) (3, 19, 20), one of the changes observed in cases with CMT1 with variants in two genes is usually the *PMP22* duplication.

Two patients with concomitant variants in *MFN2* and *GDAP1* were severer affected than their parents harboring a single *MFN2* variant (Figures 1B,C). Heterozygous *MFN2* variants were the primary mutations that could establish the diagnosis. The variant c.3G>T in *GDAP1* changed the initiation codon, which has been reported in previous literature (21–23). The *GDAP1* c.713G>T missense change occurred at the same position (p.W238) as another pathogenic variant observed (21, 24). The bioinformatic analysis supported the deleterious effect of both variants. Of note, WES ruled out other candidate genetic variants that could be involved in phenotypic expression in these families. Taken together, the heterozygous state of *GADAP1* variants could increase the disease severity in association with the inherited *MFN2* variant, indicating their role of “genetic modifier” (Figure 2B). Another patient with CMT2 with de novo *MFN2* variant and inherited heterozygous *GDAP1* variant (Figure 1D) presented with early disease onset and moderate phenotype. The variant c.767A>G in *GADAP1* is widely recognized as causative of autosomal recessive (AR) CMT (25–27) and might also modulate phenotypic expression in this case.

*MFN2* and *GDAP1* serve as a protein partners in regulating mitochondrial dynamics (MFN2 for fusion, GDAP1 for fission) and are involved in the same mitochondrial function such as energy coupling (28, 29). Mutant GDAP1 exerts a loss of function mechanism and aggravate mitochondrial damage (dynamics dysfunction and energy deficit) caused by *MFN2* mutation, which could explain the exacerbation of the CMT2A phenotype. The deficits in mitochondrial function might be their underlying mechanism which still needs to be further studied. *MFN2* and *GDAP1* are among the more frequent causative genes of CMT2, and this might be the reason why concomitant variants in these two genes have been repeatedly encountered in patients with variants in two CMT2 genes. Of interest, the frameshift p.V205fs*26 variant in *MFN2* associated with subclinical neuropathy in our study suggested that *MFN2* is not sensitive to haploinsufficiency. Previous reports have described patients with heterozygous state of *MFN2* variants (p.E308X, p.V160fs*26 and del ex7–8) developing a subclinical phenotype or asymptomatic presentation (30, 31), indicating that null variants in *MFN2* might be associated with a minimal phenotype.
This study identified a 2.1% (4/189) prevalence of concomitant variants in our cohort. The concomitant variants in families with CMT have been documented in several studies. Recently, a Japanese cohort study identified 5 out of 1,005 families with CMT harboring co-inherited variants, accounting for 0.5% (5/1,005) of the total, in which the "double trouble" effect of concomitant variants were the underlying causes (32). Compared with the "genetic modifier" effect of GDAP1 variants observed in this study, the coexistence of homozygous AR-CMT2K (p.Q163X) or heterozygous autosomal dominant-CMT2K (p.H123R, p.E222K, and p.R120W) variant in GDAP1 and heterozygous variant in MFN2 had been reported associating with a more severe phenotype, suggesting the "double trouble" effect of concomitant MFN2 and GDAP1 variants (28, 30, 33, 34). Moreover, concomitant variants were also related to true digenic inheritance (DI) that has been defined as the coinheritance of two nonallelic mutations, both are indispensable to establish the diagnosis (35). The existence of true DI was reported in a family with CMT with heterozygous MFN2 p.L741V and GDAP1 p.Q163* variants (36). These results suggest that the occurrence of concomitant variants was not uncommon in CMT.

There was an important limitation to this study. Because we conducted a retrospective analysis of target NGS data, genetic variants in non-CMT-causing genes could not be detected. WES (or whole-genome sequencing [WGS]) would detect all possible genetic modifiers. The application of WES (WGS) in all the families with phenotypic variabilities could provide further insights into the clinical and genetic heterogeneity of CMT.

In summary, the coexistence of variants in PMP22/MPZ and MFN2/GDAP1 related to more severe phenotypes accounted for 2.1% of patients with CMT in our cohort. Cumulative deficits on myelination or mitochondrial function might be their underlying mechanism. The possibility of the coexistence of variants in distinct causative genes should be taken into consideration when significant clinical heterogeneity is observed.

DATA AVAILABILITY STATEMENT

The data presented in this study were deposited in Figshare open access repository doi: 10.6084/m9.figshare.17192927. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Third Xiangya Hospital of Central South University. Written informed consent to participate in this study was provided by the participants’ legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RZ designed and conceptualized study. YX, ZL, XL, LL, BW, WC, JG, LS, and RZ contributed patient material and clinical data. SH and HZ contributed acquisition of neurophysiological data. ZL and ZH interpreted the genetic data. YX provide the first draft of the manuscript. RZ and BT revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/neur.2021.736704/full#supplementary-material
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