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RESEARCH ARTICLE

Novel compound heterozygous missense mutations in *GDAP1* cause Charcot–Marie–Tooth type 4A

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Abstract. Homozygous or compound heterozygous mutations in the *GDAP1* gene cause Charcot–Marie–Tooth (CMT4A) that are consistent with an autosomal recessive mode of inheritance. The case reported in this study is clinically and genetically diagnosed with recessive CMT4A that is caused by a compound novel heterozygous *GDAP1* mutation. The genomic DNA of the proband with the clinical diagnosis of CMT was screened for *GDAP1* mutations using a targeted next-generation sequencing (NGS) gene-panel that comprised of 27 CMT genes. Two novel compound heterozygous amino acid changing variants were identified in the *GDAP1* gene, c.246C>G p.His82Gln in exon 2 and c.614T>G p.Leu205Trp in exon 5. The two amino acid changing variants were not previously reported in the 1000 Genome, Mutation Taster and gnomAD. Our findings expand the phenotypic characterization of the two novel heterozygous mutations associated with CMT4A (AR-CMT1A) and add to the repertoire of *GDAP1* mutations related to autosomal recessive CMT in Chinese populations.

Keywords. Charcot–Marie–Tooth type 4A; autosomal recessive; *GDAP1* gene; compound mutation.

Introduction

Charcot–Marie–Tooth disease (CMT) or hereditary motor and sensory neuropathy (HMSN) is a hereditary demyelinating neuropathy characterized by progressive distal muscle weakness, atrophy, pes cavus deformity, sensory loss, decreased tendon reflexes, and reduced nerve conduction velocity (less than 38 m/s) (Garcia-Sobrino *et al.* 2017). CMT is a highly heterogeneous genetic disorder which has an autosomal dominant, autosomal recessive or X-linked inheritance pattern (Ho *et al.* 2017). On the basis of electrophysiologic properties and histopathology, the CMT disease is classified in two main groups: primary peripheral demyelinating neuropathy, CMT1; and primary peripheral axonal neuropathy, CMT2. However, genetically CMT has been classified into six main types: CMT1, CMT2, CMT3, CMT4, CMTX and DI-CMT. CMT4A also named AR-CMT1A (Vallat *et al.* 2018). Prevalence of CMT or HMSN varies among different populations. In Western Europe, USA and Japan, dominantly inherited CMT is the most common form, while in other countries, such as those of the Mediterranean Basin, the autosomal recessive form (AR-CMT) is more common. Autosomal recessive CMT cases are generally characterized by earlier onset, rapid clinical progression and more marked distal limb deformities such as pes equinovarus, claw-like hands, and often major spinal deformities (Tazir *et al.* 2013).

Autosomal recessive forms of demyelinating CMT disease is also named as CMT disease type 4 (CMT4). CMT4A (or AR-CMT1) has been shown to be caused by mutations in the gene encoding ganglioside induced differentiation

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associated protein-1 (GDAP1; OMIM: 606598) which is located on chromosome 8q21 (Ben *et al.* 1993). CMT4 is a peripheral neuropathy disease and characterized by gait difficulties, foot deformities and claw-like hands (Parman *et al.* 2004). CMT4 patients have distal motor and sensory impairment, which means motor nerve conduction velocities are decreased, and sural nerve biopsies show loss of myelinated fibers, but the age at onset and severity is variable (Patzko and Shy 2012). In this study, we describe the clinical features and the molecular characterization of compound heterozygous novel point mutations in the *GDAP1* gene in a Chinese patient with autosomal recessive CMT4A.

Materials and methods

Patients

The proband was referred to the Children's Hospital of Shanxi Province, Genetic Central for muscle weakness in the lower limbs. The diagnosis of CMT4A was verified by clinical examination, nerve conduction studies and pedigree analysis.

Genetic analysis

A targeted next-generation sequencing (NGS) gene-panel was used to screen 27 CMT genes in the proband (table 1). Briefly, genomic DNA was extracted from peripheral blood leukocytes of two available family members (the affected son proband (II-1) and the mother (I-2)) according to the manufacturer's standard procedure using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). Further, the genomic DNA was fragmented by Covaris LE220 (Covaris, Massachusetts, USA) to generate paired-end library (200–250 bp). All amplified libraries were subsequently sent to the Beijing Genomics Institute (BGI) for circularization and sequencing on the HiSeq2000 (Illumina, San Diego, USA). Further, the results were validated by Sanger sequencing using the ABI 3100 sequencer (Thermo Fisher, Waltham, USA). Variant filtering was performed via multiple databases, including NCBI dbSNP, HapMap, 1000 Human Genome dataset, Human Gene Mutation Database (HGMD). To assess the effect of the identified heterozygous variants, we used dbNSFP (Liu *et al.* 2011), which contains 20 *in silico* prediction algorithms (e.g., sorting intolerant from tolerant (SIFT), Polyphen2, LRT, MutationTaster, and PhyloP), as pathogenicity supporting evidence according to the American College of Medical Genetics and Genomics guideline (Richards *et al.* 2015). To better evaluate the pathogenicity of the variants, we used the combined annotation dependent depletion (CADD) method (Rentzsch *et al.* 2019; Kircher *et al.* 2014) and BLOSUM62 matrices (Henikoff and Henikoff 1992) for extra evolutionary conservation analysis. This study was approved by the Children's Hospital of Shanxi ethics committees (no. 201321), and informed consent was obtained from the two participants (proband's guardians and mother).

Results

Clinical findings

The 16-year-old boy had shown progressive gait disturbance, muscle wasting in the lower limbs and claw-like hands. He was unable to run, felt difficult to climbing stairs, and was unable to grasp objects from the age of 6. Physical examination showed marked wasting and weakness of leg, foot and hand muscles; high-arched feet; weakness in ankle dorsiflexion and steppage gait, however, he did not show any spinal deformities (figure 1). The deep tendon reflexes were decreased in both lower and upper extremities, while tactile and pain sensation were intact. Electrophysiological examination revealed slowing of motor nerve conduction velocity (MNCV) of 33.6 m/s (normal >38 m/s) in the left median nerve, right tibial nerve and right sural nerve were both

Table 1. General classification of CMT disease (Reilly *et al.* 2017) and the targeted 27 gene-panel used in this study.

CMT types (new names)	CMT subtypes	Implicated genes
Demyelinating CMT		
CMT1 (AD- CMT1)	CMT1 (A, B, C, etc.)	*MPZ*, *NEFL*, *EGR2*, *LITAF*, *PMP22*
CMT4 (AR- CMT1)	CMT4A	*GDAP1*
	CMT4 (B, C, etc.)	*SH3TC2*, *FIG4*, *NDRG1*, *SBF2*, *FGD4*, *PRX*, *MTMR2*
Axonal CMT		
CMT2 (AD- CMT2)	CMT2 (A, B, C, etc.)	*KIF1B*, *MFN2*, *RAB7A*, *GARS*, *HSPB1*, *HSPB8*, *AARS*, *DNM2*, *TRPV4*
CMT2 (AR- CMT2)		*LMNA*, *NEFL*, *GDAP1*, *MED25*
Intermediate CMT		
DI- CMT		*YARS*, *MPZ*, *DNM2*
CMT-X (XL-CMT)	CMTX (1, 2, etc.)	*GJB1*, *PRPS1*

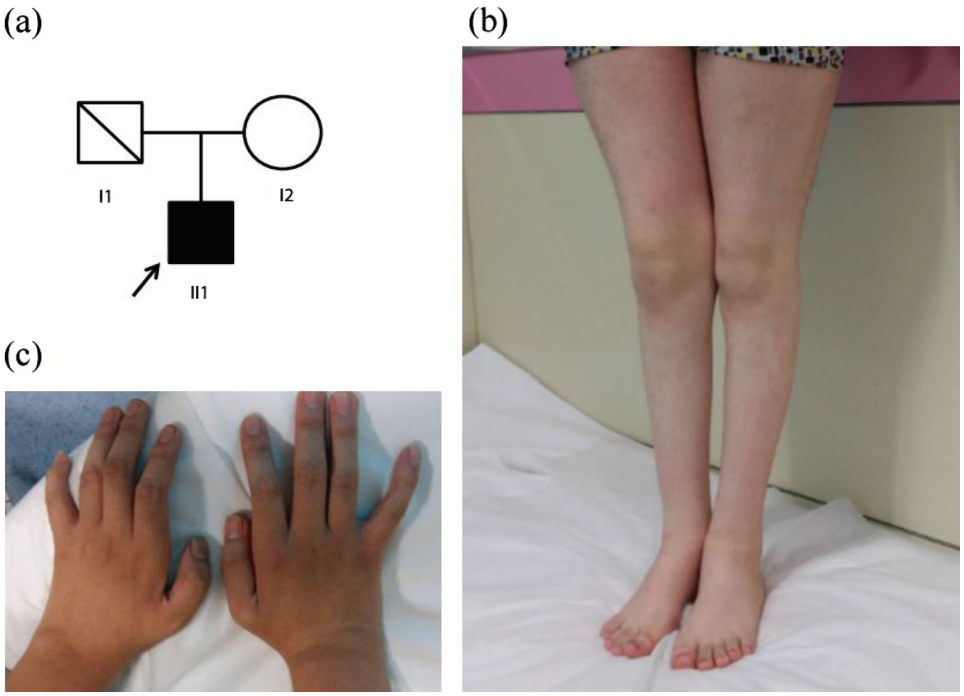

Figure 1. (a) Family tree of the affected individual with CMT4A (AR-CMT1). Squares represent men; circles, women; black filled figures, affected CMT4A; unfilled figures, unaffected; diagonal line, deceased individual, arrow represents proband. (b) The phenotypic characteristics of the affected proband with CMT4A (AR-CMT1), and the champagne bottle appearance of bilateral legs and foot drop. (c) The weakness of hand muscles and clawing of fingers.

Table 2. Nerve conduction score of the upper limb and lower limb.

Nerve	Lat (ms)	AMP (mv/ uv)	CV (m/s)
Right ulnar nerve (motor)	7.64	7.0	45.4
Left ulnar nerve (motor)	4.62	8.5	83.3
Left ulnar nerve (sensory)	2.25	–3.4	46.7
Right median nerve (motor)	8.52	5.8	48.4
Left median nerve (motor)	11.7	0.61	33.6
Left median nerve (sensory)	2.76	1.35	41.7
Right tibial nerve (motor)	–	–	np
Right sural nerve (motor)	–	–	np
Right sural nerve (sensory)	6.02	–0.35	35

CV, conduction velocity (m/s); AMP, amplitude (mv/uv); Lat, latency (ms); np, not performed; normal range: CV > 41m/s.

nonconductory (table 2). The patient had no family history of neuromuscular disease and his parents were normal.

Genetic characterization

Genetically CMT has been classified into six main types (table 1). The proband's genomic DNA was screened using the NGS targeted gene-panel covering 27 CMT causative genes with a coverage depth of >20×. Reads were mapped against the human genome 19 (hg19) to detect single-nucleotide polymorphisms (SNPs), single-nucleotide variants (SNVs), short deletions and insertions. Compound heterozygous mutations involving two novel missense mutations in the GDAP1 gene were defected in exon 2 and exon 5: Chr8:75263637 NM_018972:c.246C>G p.His82Gln and Chr8:75275208 NM_018972:c.614T>G p.Leu205Trp, respectively (figure 2), both are novel. Sanger sequencing confirmed the presence of both variants in the proband, the mother was found to carry the p.Leu205Trp missense variant (figure 2). The asymptomatic proband's father died without any genetic examination.

Both *GDAP1* variants identified in this study were absent in public databases of human genetic variation (1000 Genomes) (http://www.1000genomes.org), Mutation Taster (http://www.mutationtaster.org/) and gnomAD (http://gnomad.broadinstitute.org/). The nucleotide substitutions c.246C>G p.His82Gln and c.614T>G p.Leu205Trp alter evolutionarily conserved amino acids with BLOSUM62 scores 0 and –2, respectively, and both variants predicted to be damaging or disease causing by SIFT, PolyPhen-2 and Mutation Taster tools. CADD shows that both the variants are pathogenic (with C-scores 24.6 and 28.0, respectively, higher than threshold 10–20). In addition, we found the variant, rs878855054, resulting in p.Leu205Ser is on the same site of c.614T>G and it was annotated as 'uncertain significant', however, this annotation was evaluated by negative result using SIFT, which is opposite to ours. Based on molecular findings, both the identified variants were

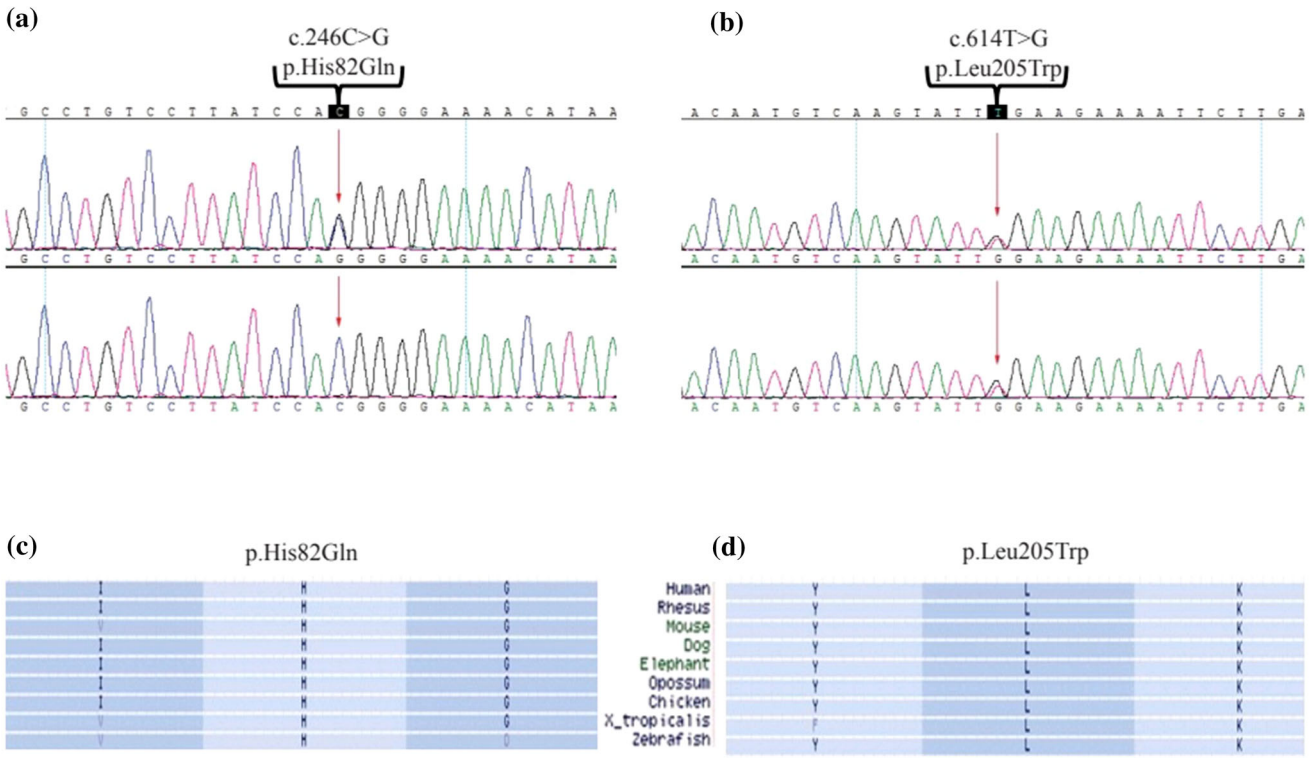

Figure 2. Sequencing of two novel mutations identified in ganglioside-induced differentiation-associated protein 1, *GDAP1*. Data were obtained by Chromas (from Sanger sequencing). (a) The heterozygous exonic point variant; (c.246C>G) (p.His82Gln) in exon 2 of *GDAP1*, (b) c.614T>G (p.L205W) of *GDAP1*. All were sequenced in the two family members, proband (top) and mother (bottom). (c) Evolutionary conservation of amino acid affected by the mutation (c.246C>G) (p.H82Q) of *GDAP1*. (d) Evolutionary conservation of amino acid affected by the mutation c.614T>G (p.L205W) of *GDAP1*.

annotated 'likely pathogenic' according to the American College of Medical Genetics (ACMG) clinical practice guidelines.

Discussion

CMT disease (Rentzsch *et al.* 2019) is the most common inherited peripheral neuropathy estimated to affect 1 in 1214 of the general population (Kircher *et al.* 2014). Over 60 nuclear genes have been implicated in the pathogenesis of peroneal progressive muscular atrophy disorders, and the genetic cause of about 50% of clinically diagnosed CMT remains unidentified (Henikoff and Henikoff 1992). Identification of the exact or most probable underlying genetic mutation is therefore of importance in the clinically and genetically heterogeneous group of monogenic disorders affecting the peripheral nerves (Reilly *et al.* 2017). Demyelinating CMT neuropathies are characterized by severely reduced nerve conduction velocities (less than 38 m/s). The proband in this study has a reduced MNCV in his upper and lower limbs. As a result, the proband was tentatively classified as demyelinating CMT disease (Braathen 2012; Tazir *et al.* 2013). As causative genes associated with CMT are constantly being identified, demyelinating CMT disease has been categorized into several subtypes, making it challenging to determine the correct subtype for a patient with CMT (Rossor *et al.* 2013). The phenotypic characteristics of the affected proband were: champagne bottle appearance of bilateral legs, foot drop, weakness of hand muscles, and clawing of fingers. These clinical manifestations were highly consistent with the phenotype induced by the mutations in the *GDAP1* gene (Tazir *et al.* 2014; Pla-Martin *et al.* 2015).

Mutations in *GDAP1* are responsible for both demyelinating and severe axonal autosomal recessive forms (CMT4A and AR-CMT2K, respectively), whereas the dominant *GDAP1* form (CMT2K) is much less frequent and milder (Braathen 2012; Tazir *et al.* 2013). Compound heterozygous mutations in *GDAP1* gene have been previously reported to cause CMT4A (https://uantwerpen.vib.be/), including single nucleotide substitutions (86%), insertions (13%), deletions (1%). The *GDAP1* gene, a member of the ganglioside-induced differentiation-associated protein family, is located on chromosome 8q13-q21 and is associated with mitochondrial dynamics fission, including fusion and transport of mitochondria in peripheral nerves (Ben *et al.* 1993; Kostera-Pruszczyk *et al.* 2014).

In this study, targeted NGS using a CMT gene panel comprised of 27 known CMT disease-causing genes (table 1) was used to screen for mutations in our proband. Compound heterozygous mutations in the *GDAP1* gene

c.246C>G p.His82Gln and c.614T>G p.Leu205Trp were detected by the NGS CMT gene panel, confirming the efficacy of gene-panel and NGS approaches to identify causative mutations for genetically heterogeneous disorders (Birouk *et al.* 2003).

The pathogenic effect of the His82Gln and Leu205Trp substitution in the *GDAP1* gene is supported by the following observations: (i) the tolerance of the two variant sites to base substitutions is very low. On the one hand, the two loci are highly conservative, with BLOSUM62 scores of 0 and –2, on the other hand, the variants are predicted to be harmful, and CADD, a tool of annotation method using large sample of human genetic variants databases, including GWAS data, suggests that the two variants will change protein function. (ii) Recessive inheritance. The father, though was not detected, which means we do not know p.His82Gln is *de novo* or paternal, could not overturn the pathogenicity of the variant. (iii) Most importantly, the phenotype of CMT4A found in the patient, which could be explained by *GDAP1* defects. GDAP1 expression in both neurons and Schwann cells in whole brain and spinal cord (Cuesta *et al.* 2002), and Gdap1-specific knockdown in rat resulted in a tubular mitochondrial morphology and it suggests that GDAP1 defects lead to axonal neuropathy and loss (Barneo-Muñoz *et al.* 2015). Unfortunately, pharmacological treatment options for CMT are currently unproven but there are ongoing clinical trials. Conservative therapies include supportive approaches and rehabilitation. Early stretching in the disease course can also delay the onset of early complications such as ankle contractures and joint deformities (Saporta 2014). Foot orthotics have also been strongly shown to improve outcomes in CMT. Orthopaedic surgery is more common in children and adolescents, with a number of surgical approaches predominately aimed at treating cavovarus deformities. Research is now focussed on developing further treatments for this evolving area and continuing clinical trials will hopefully yield meaningful data to guide future clinical practice, also to facilitate a more phenotypic-specific, and pharmaceutical approach.

In summary, novel compound heterozygous mutations c.246C>G p.His82Gln and c.614T>G p.Leu205Trp have been implicated as contributes to the CMT4A phenotype in this family, expanding the mutational spectrum of CMT4A in the Chinese population. Identification of the exact or most probable underlying genetic mutation is important, not only in clinical management but also for genetic and reproductive counselling. The NGS CMT gene panel was helpful in discovering new mutations involved in CMT and also in a better understanding of the pathogenesis of these disorders. Clinical research involving phenotype/genotype studies combined with NGS CMT gene panel studies should help provide targets for future medication and therapeutic trial development.

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