

A Chinese pedigree with a novel mutation in *GJB1* gene and a rare variation in *DHTKD1* gene for diverse Charcot-Marie-Tooth diseases

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Abstract. Charcot-Marie-Tooth (CMT) disease is a group of motor and sensory neuropathies with a high degree of pathological and genetic heterogeneity. The present study described 2 patients with CMT in a Chinese Han pedigree. The proband exhibited the classic manifestation of CMT with slowly progressing muscular atrophy and weakness. Electrophysiological examination highlighted axonal and demyelinating features. His mother did not have any symptoms, but did exhibit abnormal electrophysiological results. Next-generation sequencing technology was employed to screen mutations in the genes associated with inherited motor nerve diseases. A novel mutation, c.528_530delAGT, in the gap junction protein beta 1 (*GJB1*) gene for CMTX, and a rare variation, c.2369C>T, in the dehydrogenase E1 and transketolase domain containing 1 (*DHTKD1*) gene for CMT disease type 2Q (CMT2Q), were identified in the proband and his mother. The results were verified by Sanger sequencing. Although the *in silico* analysis predicted no change in the 3-dimensional structure, the clinical and electrophysiological presentation in the pedigree and the high evolutionary conservation of the affected amino acid supported the hypothesis that the c.528_530delAGT mutation in the *GJB1* gene may be pathogenic in this pedigree. *In silico* analysis and high evolutionary conservation suggested the pathogenicity of the c.2369C>T mutation in the *DHTKD1* gene; however, the clinical and electrophysiological performances of the proband and his mother did not conform to those of CMT2Q caused by the *DHTKD1*

gene. The present study provided additional information concerning the range of mutations of the *GJB1* gene, which facilitated the understanding of the genotype-phenotype association of CMT.

Introduction

Charcot-Marie-Tooth disease (CMT) is one of the most common inherited neurological disorders presenting with symmetrical, slowly progressive distal motor neuropathy (1). It results in varying degrees of muscle weakness and atrophy in the feet and/or hands. Electrophysiological examination may be used for classifying CMT into CMT1 with demyelinating features or CMT2 with axonal features. Certain patients exhibit central nervous system symptoms and cerebral white matter lesions. The disease exhibits marked clinical and genetic heterogeneity. At present, >80 unique genes are known to be involved in CMT in autosomal-dominant, recessive-dominant or X-linked manners (2).

X-linked CMT (CMTX), primarily caused by mutations in the gap junction protein beta 1 (*GJB1*) gene, is the second most common genetic variant of CMT (3). The *GJB1* gene encodes the gap junction beta 1 protein connexin 32 (Cx32) which is expressed in myelinating Schwann cells and formed gap junctions. Gap junctions provide a direct diffusion pathway for ions and small molecules and are conducive to rapid and special communication between the periaxonal and peripheral cytoplasm (4). Mutations in the *GJB1* gene lead to the dysfunction of Cx32 and cause damage to peripheral myelin. In small families, the mode of inheritance of disease may be incorrectly characterized using the pedigree analysis. In addition, various severe clinical presentations of CMTX are not easily differentiated from other types of CMTs. Therefore, next-generation sequencing has the potential to quickly screen for mutations in the diseases with extreme genetic heterogeneity, resulting in a precise diagnosis.

The present study describes a two-generation Chinese family with CMT. A novel mutation in the *GJB1* gene and a rare variation in the dehydrogenase E1 and transketolase domain containing 1 (*DHTKD1*) gene were detected using high-throughput sequencing followed by Sanger sequencing.

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Materials and methods

Patients. A two-generation Han Chinese CMT family with 3 family members, living in Southeast China, was studied (Fig. 1A). The proband was a 27-year-old male, with a 48-year-old father and 53-year-old mother. Standard clinical and electrophysiological evaluations were performed in all the patients. Peripheral blood samples (10 ml) were collected for mutational analysis from the elbow vein of each family member upon admission to the Department of Neurology, Fujian Provincial Hospital (Fuzhou, China) in December 2017. In addition, 500 healthy individuals (>65 years, 221 males and 279 females) with no known history of CMT were selected as a control group. The samples provided from Fujian Key Laboratory of Molecular Neurology, Fujian Medical University (Fuzhou, China) were collected from April 2014 to December 2016. The study was approved by the Ethics Committee of Fujian Provincial Hospital, Fujian Medical University. Informed consent was obtained from all individuals included in the study.

Mutational analysis

DNA extraction. DNA extraction was performed using a Qiagen Blood and Tissue kit according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). DNA concentrations were determined using a NanoDrop 2000 UV/Vis Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA).

High-throughput sequencing, Sanger sequencing and variant analysis. High-throughput sequencing was applied for the mutation screening of the pedigree. Briefly, the coding exons and flanking regions of the 110 genes (Table I) associated with inherited motor nerve diseases in the panel were selected and captured with a GenCap custom enrichment kit (MyGenostics, Inc., Beijing, China). Then, the enriched libraries were sequenced by using an Illumina NextSeq500 Sequencer with a sequencing depth of 100x (Illumina, Inc., San Diego, CA, USA). The raw data were compared to the human reference genome (hg19) using the Burrows Wheeler Aligner Multi-Vision software package (version 0.7.10) (5). Duplicates were removed using Picard software version 1.119 (<http://broadinstitute.github.io/picard>). Then, the single nucleotide polymorphisms and insertions/deletions were identified by the SOAPsnv version 1.03 (6) and Genome Analysis Toolkit programs (version 3.3.0) (7). Synonymous variants and the variants with allele frequencies 1>% in the 1000Genome (<http://www.internationalgenome.org/>), ESP6500 (<https://esp.gs.washington.edu>) and inhouse databases were excluded. The Human Gene Mutation Database was used to detect the known disease-causing mutations. To validate the pathogenicity of the variant sites, Sanger sequencing was used to amplify the regions of interest by polymerase chain reaction performed using a GoldStar MasterMix PCR kit (Beijing CoWin Biotech Co., Ltd., Beijing, China) under the following conditions: 95°C for 5 min; 35 cycles of 95°C for 30 sec, 58°C for 45 sec and 72°C for 1 min; and 72°C for 5 min. The primers are listed in Table II. To analyze the evolutionary conservation of the two variation positions, sequence alignments with the human genes were performed for 10 other species (*Pan troglodytes*, *Macaca mulatta*, *Mus musculus*, *Felis catus*,

Gallus gallus, *Xenopus tropicalis*, *Takifugu rubripes*, *Danio rerio*, *Drosophila melanogaster* and *Caenorhabditis elegans*) using MutationTaster (<http://www.mutationtaster.org/>) (8). Matches were classed as 'all identical', 'conserved' or 'not conserved', depending on whether the aligned amino acid was the same, similar or different. A total of 500 unrelated Chinese healthy controls were screened for the *de novo* mutation position of the *GJB1* gene by Sanger sequencing. The effects of the mutations on protein function were assessed using the Swiss-Model tool (<http://swissmodel.expasy.org/>) (9-13). Mutation Taster (8) was used to assess the biological relevance of the novel amino acid changes.

Results

Clinical manifestation, physical examination and electromyography results. The proband (patient III1; Fig. 1A) was admitted for slowly progressing limb atrophy (Fig. 1B). He had developed distal muscle weakness and limb atrophy at 17 years of age. He had mild gait abnormalities, but was able to walk without assistance. Neurological examination revealed no deep tendon reflexes in the limbs. Electrophysiological examination identified axonal and demyelinating features. His median and ulnar motor nerve conduction velocities (MCVs) were 28.5 and 25.5 m/sec, respectively. In addition, his bilateral tibial MCVs were not detected. His median, ulnar and peroneal sensory nerve conduction velocities (SCVs) were 31.1, 32.7 and 35.5 m/sec, respectively. The compound motor action potentials (CAMPs) were markedly decreased in all nerves, particularly in sensory nerves and in the lower legs. His CMT disease neuropathy score was 8 points, which indicated mild severity of the disease (14). The proband's mother (Patient I2), a 48-year-old woman, was also examined by a neurology chief physician in Fujian Provincial Hospital in December 2017, and presented with no clinical symptoms. Her median, ulnar and peroneal MCVs were 43.7, 48.3 and 40 m/sec, respectively. Her median and ulnar SCVs were 43.1 and 41.1 m/sec, respectively. However, her bilateral tibial SCVs were not detected. The CAMPs were normal in her upper limbs, but were decreased in the lower limbs. She scored 0 points on the CMT disease neuropathy evaluation.

Variant analysis results. Next-generation sequencing technology identified two potentially pathogenic mutations in the proband. The c.528_530delAGT mutation in the *GJB1* gene on the X chromosome was identified in the proband (III1) and his mother (I2; Fig. 1C), but not in his father (II1). The proband and his father presented homozygous genotype sequencing results and his mother exhibited a heterozygous sequencing result, as males carry 1 X chromosome and females carry 2 X chromosomes. The c.528_530delAGT mutation, passed from the mother to her son, matches the X-linked recessive inheritance disease pattern. This variation was not identified in 500 control subjects and, to the best of our knowledge, it has not been described previously. This mutation leads to a valine deletion at amino acid position 177. The amino acid V177 in Cx32 protein is highly conserved among species; the deletion mutation was identified as 'not conserved' (Fig. 1D). MutationTaster programs predicted that the pathogenicity of the c.528_530delAGT mutation in the *GJB1* gene may be

Table I. Genes associated with inherited motor neuron diseases in the panel for high-throughput sequencing.

No.	Gene	No.	Gene	No.	Gene	No.	Gene	No.	Gene	No.	Gene
1	AAAS	20	DHTKD1	39	GJB3	58	LAS1L	77	PLA2G6	96	SLC25A46
2	AARS	21	DNAJB2	40	GNB4	59	LITAF	78	PLEKHG5	97	SLC5A7
3	ABHD12	22	DNM2	41	HARS	60	LMNA	79	PMM2	98	SPTLC1
4	AIFM1	23	DNMT1	42	HEXA	61	LRSAM1	80	PMP22	99	SPTLC2
5	ARHGEF10	24	DPYSL5	43	HINT1	62	MARS	81	PRNP	100	SURF1
6	ARSA	25	DST	44	HK1	63	MED25	82	PRPS1	101	TDP1
7	ASAH1	26	DYNC1H1	45	HNRNPA2B1	64	MFN2	83	PRX	102	TFG
8	ATL1	27	EGR2	46	HOXD10	65	MPZ	84	RAB7A	103	TRIM2
9	ATL3	28	EXOSC3	47	HSPB1	66	MTMR2	85	RBM28	104	TRPV4
10	ATP7A	29	FAM134B	48	HSPB3	67	MYH14	86	REEP1	105	TTR
11	BICD2	30	FBLN5	49	HSPB8	68	NAGLU	87	RNF170	106	UBA1
12	BSCL2	31	FBXO38	50	IFRD1	69	NDRG1	88	SBF1	107	VPS13A
13	CCT5	32	FGD4	51	IGHMBP2	70	NEFL	89	SBF2	108	VRK1
14	COX6A1	33	FIG4	52	IKBKAP	71	NGF	90	SCN1A	109	WNK1
15	CTDP1	34	FLVCR1	53	INF2	72	NTRK1	91	SCN9A	110	YARS
16	CUL4B	35	GALC	54	KARS	73	PKD3	92	SETX		
17	DCAF8	36	GARS	55	KIF1A	74	PEX1	93	SH3TC2		
18	DCTN1	37	GDAP1	56	KIF1B	75	PEX7	94	SIGMAR1		
19	DHH	38	GJB1	57	KLHL9	76	PHYH	95	SLC12A6		

Table II. Primers for Sanger sequencing.

Target gene mutation	Forward (5'-3')	Reverse (5'-3')
DHTKD1 c.2369C>T	TTGCGTCTATTAGGACATGGG	GGGAGATCTGCATCACCTG
GJB1 c.258_530delAGT	TGGTGGACCTATGTCATCAGC	CGCAGTATGTCTTTTCAGGGAG

DHTKD1, dehydrogenase E1 and transketolase domain containing 1; GJB1, gap junction protein beta 1.

disease causing. The mutation in the GJB1 gene was classified as 'Like Pathogenicity', according to the criteria of the American College of Medical Genetics and Genomics (ACMG) standards and guidelines (12).

The second mutation, a heterozygous mutation, was c.2369C>T in the *DHTKD1* gene from the proband (III) and his mother (I2; Fig. 1E). This mutation is listed in dbSNP (rs776497952) and was identified in the Exome Aggregation Consortium with a minor allele frequency of 0.004118% among 60,706 individuals. This mutation leads to an amino acid exchange of p.P790L. The amino acid P790 in the DHTKD1 protein is highly conserved, with the exception of *Drosophila melanogaster* and *Caenorhabditis elegans*; the p.P790L mutation was identified as 'not conserved' (Fig. 1F). The pathogenicity of the c.2369C>T variants in the *DHTKD1* gene was also assessed to be disease causing by Mutation Taster programs. The mutation in the *DHTKD1* gene was classified as 'Uncertain Significance', according to the criteria of the ACMG standards and guidelines (15).

The Swiss-Model tool revealed that, compared with the wild-type Cx32 (Fig. 2A), the deletion of amino acid Val did

not alter the 3-dimensional (3D) structure of Cx32, and a new hydrogen bond was formed between amino acid residues 178 and 166 (Fig. 2B). 3-D structures of Cx32 were also simulated for two other mutations, p.V177E (Fig. 2C) and p.V177A (Fig. 2D). The two mutations did not lead to any alterations in the 3-D structure, but the latter formed a new hydrogen bond between the amino acid residues 177 and 168. Potential mechanisms underlying the hypothesized pathogenicity of mutations affecting the amino acid residue 177 were presented in Fig. 2E.

Computer simulation of 3-D structure using the Swiss-Model tool revealed that the p.P790L mutation resulted in a structure alternation from an α -helix (Fig. 3A) to a β -fold (Fig. 3B), and the removal of the hydrogen bond between amino acid residues 790 and 789 (Fig. 3C).

Discussion

The *GJB1* gene is the primary cause of X chromosome linkage dominant inherited type 1 Charcot-Marie-Tooth disease (16). *GJB1* gene mutation usually results in classical

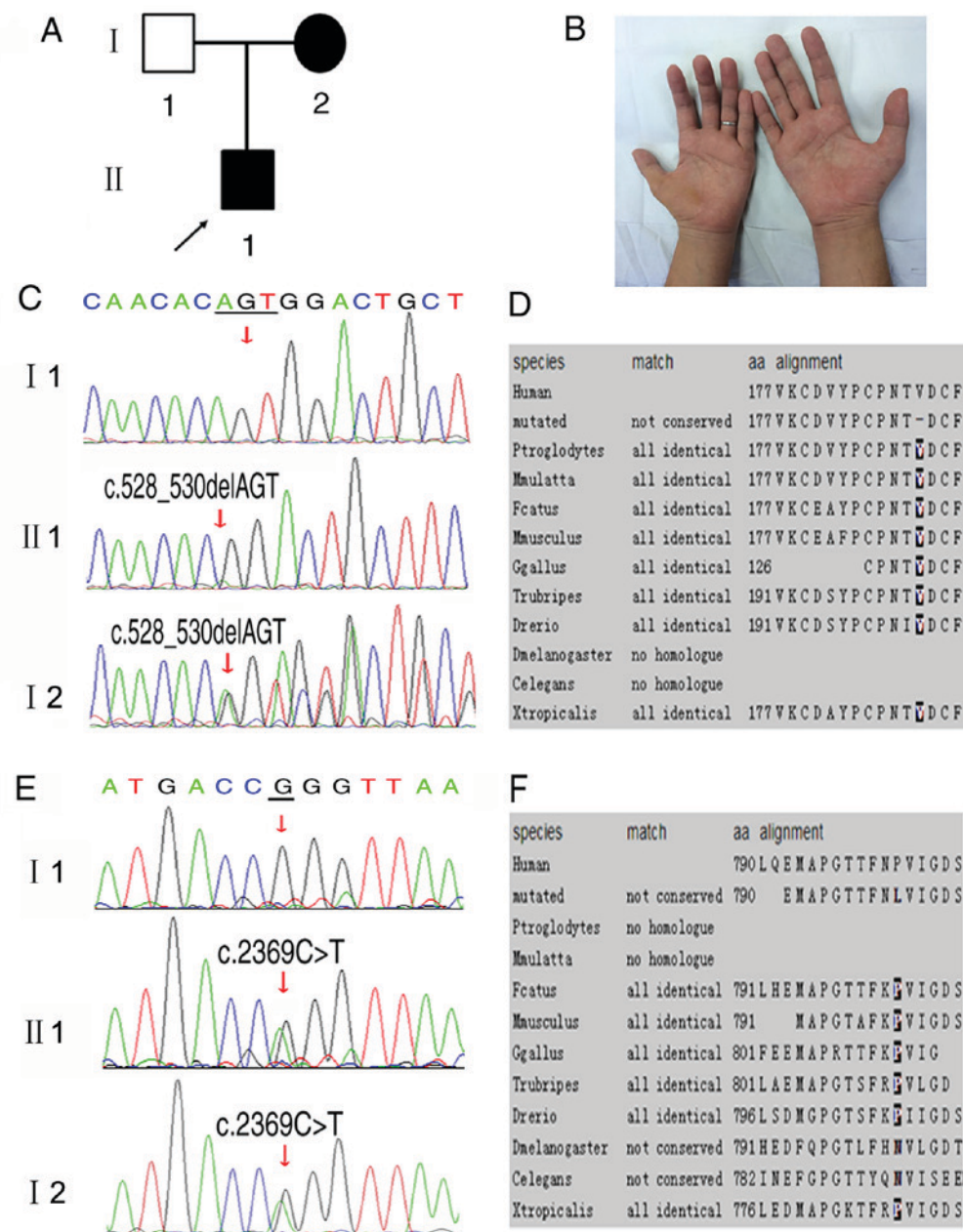


Figure 1. Mutation detection and evolutionary conservation analysis in a Chinese family affected by CMT. (A) CMT pedigree. (B) Proband (III) with distal upper-limb muscle atrophies. (C) Sequence chromatograms of the c.528_530delAGT mutation in the *GJB1* gene were identified in the proband (III) and his mother (I2), as indicated by the red arrows, and (D) sequence alignments of Cx32 across evolutionarily diverse species, as determined using MutationTaster. (E) Sequence chromatograms of the c.2369C>T mutation in the *DHTKD1* gene indicated by the red arrows were shown in the proband (III) and his mother (I2), and (F) sequence alignments of *DHTKD1* across evolutionarily diverse species, as determined using MutationTaster. CMT, Charcot-Marie-Tooth disease; *GJB1*, gap junction protein beta 1; Cx32, protein connexin 32; *DHTKD1*, dehydrogenase E1 and transketolase domain containing 1.

CMT, characterized by slowly progressive distal muscle weakness, atrophy, sensory abnormalities, pes cavus and hammer toes, usually starting during the teenage years (17). In pedigrees with CMTX, female patients always present with milder symptoms compared with males, due to the random X chromosome inactivation in each myelinating cell (18). In the present study, the proband presented with the classical clinical and electrophysiological symptoms of CMT caused by *GJB1* mutation (19). It was identified that the c.528_530delAGT mutation, leading to a valine deletion at amino acid position 177, was a novel pathogenicity mutation. Valine at amino acid position 177 is an important site for the

function of the *GJB1* gene, which is supported not only by ortholog analysis of *GJB1* among species, but also by the other 2 mutations that have been identified at this position, p.V177E (20) and p.V177A (21). Although these mutations do not result in any change in the 3-D structure of the *GJB1* gene, the c.528_530delAGT and the p.V177A mutations lead to the formation of new hydrogen bonds in the neighbor amino acid residues. A number of potential mechanisms have been suggested for the pathological effects of changes in the extracellular loop where the amino acid residue 177 is located, as demonstrated in Fig. 2E. *In vitro* studies have suggested that certain Cx32 mutants affecting the extracellular loop,

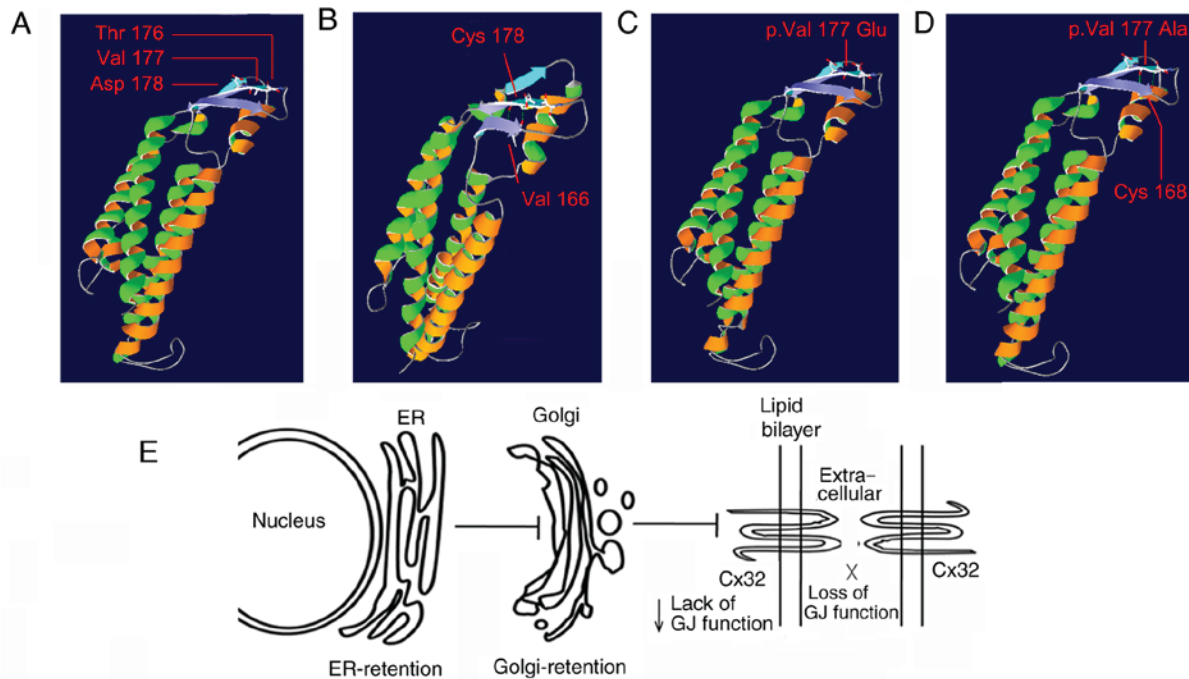


Figure 2. Structural model of the Cx32 protein. (A) 3-D model of wild-type Cx32, and the amino acid residue 177 of Val and its neighboring amino acids. (B) 3-D model of Cx32 with p.176_177del, and the new hydrogen bond formed between amino acid residues 178 and 166 (green dotted line). (C) 3-D model of Cx32 with p.Val177Glu. (D) 3-D model of Cx32 with p.Val177Ala, and the new hydrogen bond formed between amino acid residues 177 and 168 (green dotted line). (E) Potential mechanisms underlying the effects of mutations of the amino acid residue 177. Cx32, protein connexin 32; 3-D,3-dimensional.

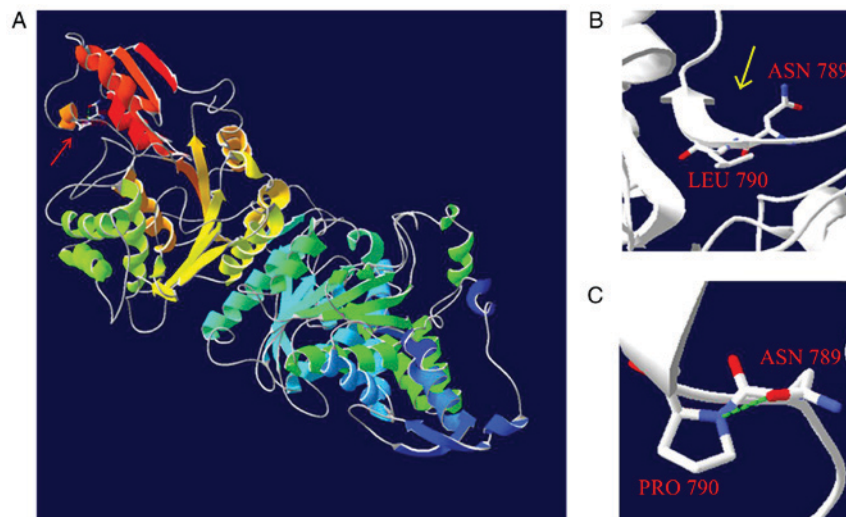


Figure 3. Structural model of the DHTKD1 protein. (A) 3-dimensional model of the DHTKD1 protein with the structure of the α -helix at position 790 (red arrow). (B) Structure alteration of β -fold affected by the mutation of p.P790L (yellow arrow). (C) Hydrogen bond (green dotted line) between amino acid residues 790 and 789. DHTKD1, dehydrogenase E1 and transketolase domain containing 1.

including p.E186K, p.T55I, p.A40V and p.R183S, interfered with their trafficking between the endoplasmic reticulum (ER) and Golgi apparatus (22-24). As a result, these abnormal proteins were retained in the ER and/or Golgi and degraded by proteasomal or lysosomal pathways (25). Although a fraction of certain mutants, including R183S, may reach the cell membrane, the amount of protein is too low to allow sufficient gap junction formation (3). In addition, they are not able to induce functional gap junction formation for normal communication between cells (26). Therefore, the present

study confirmed that the c.528_530delAGT mutation is the pathogenic mutation in this family.

Allelic heterogeneity and phenotypic variability complicate CMT genetic analysis. In the study by Williams *et al* (20), there was a mutation of p.V177E detected in a 51-year-old European male with the peripheral hallmarks of CMT, optic atrophy and cerebellar ataxia. In the study by Ikegami *et al* (21), a mutation of p.V177A was identified in a 15-year-old Japanese boy with CMTX, and in his mother and maternal uncle. The patient presented with foot pain and exhibited distal muscle

weakness and atrophy of the lower limbs, with pes cavus and hammer toe deformities, without detection of his motor NCV. His maternal uncle had a similar presentation. His mother only had a mild pes cavus deformity, but with decreased motor NCVs. The clinical presentations of the patients in the present study were also different from these previous cases. The proband in the present study presented with more severe symptoms compared with his mother. Therefore, there may be a particular genotype-phenotype association with respect to the *GJB1* gene.

The *GJB1* gene encodes the gap junction beta 1 protein Cx32, a member of the large connexin family that encodes homologous proteins and forms gap junctions (27). Cx32 is highly expressed in Schwann cells, and cannot be functionally compensated by other connexin family proteins as they are not expressed in peripheral nerves. A total of 6 connexins compose a hemichannel, 2 of which form a gap junction channel allowing the diffusion of ions and small molecules (28). CMTX with *GJB1* mutations has complex and diverse mechanisms of action, including failure to synthesize Cx32 protein, Cx32 protein transport failure to the plasma membrane and formation of gap junctions with abnormal biophysical properties (29). Certain amino acid changes, including p.E208K (30,31) and p.R75Q (24,32), are associated with >1 of these mechanisms. Loss of function is hypothesized to be a common factor across *GJB1* mutations. This may be the reason why the type of mutation and the domain wherein the mutation was located were not identified to be associated with clinical severity in previous statistical analyses (33,34). However, patients carrying the same mutation, including p.S26L and p.M34T, may present with severe or mild clinical symptoms (33). Therefore, the precise phenotype-genotype association in this disease requires additional investigation.

There was a rare variation, c.2369C>T, in the *DHTKDI* gene identified in this family. *DHTKDI* was demonstrated to be the pathogenic gene in a Chinese CMT disease type 2Q (CMT2Q) pedigree with the c.1455T>G mutation (35). These patients presented with progressive and severe weakness and atrophy of the distal muscles. The electrophysiological and morphological results were characteristic of axonal features without demyelinating damage. There has been no other CMT2Q patient or pedigree described, to the best of our knowledge. Indeed, in the small pedigree in the present study, an autosomal-dominant linked inheritance mode may be a possibility. However, the electrophysiological and clinical presentations of the patients were not consistent with CMT2Q. In addition, the *DHTKDI* mutation has been more commonly identified in alpha-aminoacidic and alpha-ketoadipic aciduria, an autosomal-recessive linked inherited disease whose symptoms exhibit apparent heterogeneity, ranging from psychomotor retardation, hypotonia, ataxia, epilepsy and failure to thrive, to no clinical phenotype at all. Therefore, we hypothesized that the c.2369C>T variation in the *DHTKDI* gene was not the pathogenic mutation in this pedigree for CMT2Q. The fact that the same protein may be affected in 2 inherited diseases indicates that there may be a particular genotype-phenotype association with respect to the *DHTKDI* gene (36).

In summary, the present study described a novel mutation in the *GJB1* gene in a Chinese CMTX pedigree with a rare variation in the *DHTKDI* gene. The results provided

additional information regarding the range of mutations of the *GJB1* gene, which may facilitate the understanding of the genotype-phenotype association of CMTX.

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Availability of data and materials

All data generated and analyzed during the present study are included in this published article.

Authors' contributions

ZHZ drafted the manuscript. ZHZ and ZTC performed the genetic experiments. RLZ and YZW made significant contributions towards the design of the study, collected clinical samples and obtained the clinical data of patients.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Fujian Provincial Hospital, Fujian Medical University (Fuzhou, China). Informed consent was obtained from all individuals included in the study.

Patient consent for publication

Informed consent was obtained from all individuals included in the study.

Competing interests

The authors declare that they have no competing interests.

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