Mutation Analysis of Gap Junction Protein Beta 1 and Genotype–Phenotype Correlation in X-linked Charcot–Marie–Tooth Disease in Chinese Patients

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Abstract

Background: Among patients with Charcot–Marie–Tooth disease (CMT), the X-linked variant (CMTX) caused by gap junction protein beta 1 (*GJB1*) gene mutation is the second most frequent type, accounting for approximately 90% of all CMTX. More than 400 mutations have been identified in the *GJB1* gene that encodes connexin 32 (CX32). CX32 is thought to form gap junctions that promote the diffusion pathway between cells. *GJB1* mutations interfere with the formation of the functional channel and impair the maintenance of peripheral myelin, and novel mutations are continually discovered.

Methods: We included 79 unrelated patients clinically diagnosed with CMT at the Department of Neurology of the Chinese People's Liberation Army General Hospital from December 20, 2012, to December 31, 2015. Clinical examination, nerve conduction studies, and molecular and bioinformatics analyses were performed to identify patients with CMTX1.

Results: Nine *GJB1* mutations (c.283G>A, c.77C>T, c.643C>T, c.515C>T, c.191G>A, c.610C>T, c.490C>T, c.491G>A, and c.44G>A) were discovered in nine patients. Median motor nerve conduction velocities of all nine patients were < 38 m/s, resembling CMT Type 1. Three novel mutations, c.643C>T, c.191G>A, and c.610C>T, were revealed and bioinformatics analyses indicated high pathogenicity.

Conclusions: The three novel missense mutations within the *GJB1* gene broaden the mutational diversity of CMT1X. Molecular analysis of family members and bioinformatics analyses of the afflicted patients confirmed the pathogenicity of these mutations.

Key words: Connexin 32; Electrophysiology; Gap Junction Protein Beta 1; Genetic Mutation; X-linked Charcot–Marie–Tooth Disease

INTRODUCTION

Charcot–Marie–Tooth disease (CMT), with a worldwide incidence of 1 in 2500, is the most common hereditary sensorimotor neuropathy, comprising a group of clinically and genetically heterogeneous peripheral neuropathies.[1,2] CMT is characterized by progressive distal muscle atrophy and weakness, sensory disturbance, the absence of deep tendon reflexes, and pes cavus deformity of the foot.[2,3] On the basis of median motor nerve conduction velocity (MCV), CMT is classified into Type 1 (CMT1; demyelinating, MCV <38 m/s; slower nerve conduction) and Type 2 (CMT2; axonal, MCV >38 m/s; slower nerve conduction). On the other hand, genetic testing provides an exact diagnosis of a specific subtype of CMT, including CMT1A, CMT1B, CMT1C, CMT1D, CMT1F, CMTX1, CMT2A1, CMT2A2, CMT2B, and CMT2O. X-linked CMT (CMTX) is an intermediate form of CMT and patients typically have "intermediate" slowing of nerve conduction velocities, faster than in CMT1 and slower than in most CMT2 patients.[4,5]

CMTX, accounting for 10–20% of all CMT patients, is the second most frequent type of the disease.[6] So far, five loci have been reported for CMTX: CMTX1 (OMIM 304040), CMTX2 (OMIM 302801), CMTX3 (OMIM 302802), CMTX4 (OMIM 310490), and CMTX5 (OMIM

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311070).[7] CMTX1, accounting for approximately 90% of all reported cases of CMTX, is caused by mutations in the gap junction beta 1 (*GJB1*) gene (>400 mutations; http://www.molgen.ua.ac.be/CMTMutations/) that encodes connexin 32 (CX32), a myelin-related protein in the peripheral nervous system (PNS) and central nervous system (CNS) that is considered to form gap junctions (GJs) that shorten the diffusion pathway of ions and small molecules between cells.[8] *GJB1* mutations are thought to interfere with the formation of the functional channel and impair maintenance of peripheral myelin. Here, we reported nine Chinese families with CMTX and identified three novel *GJB1* point mutations in three unrelated families.

METHODS

Patients

The study included 79 unrelated patients with a clinical diagnosis of CMT from the Department of Neurology in the Chinese People's Liberation Army (PLA) General Hospital between December 20, 2012, and December 31, 2015, and they underwent detailed history-taking, neurologic examination, laboratory investigations, nerve conduction studies (NCS), and genomic tests. This research was approved by the Chinese PLA General Hospital Ethics Committee and was conducted in accordance with the principles of *the Declaration of Helsinki*. All participants provided written informed consent before study participation.

Nerve conduction studies

All patients underwent NCS on the median, ulnar, tibial, peroneal, and sural nerves to assess motor and sensory conduction properties in the upper and lower limbs using the Keypoint electromyography system (Medoc Ltd., 1 Ha'dekel St. Ramat Yishai 30095, Israel), with skin temperature maintained at 37°C. MCVs, proximal and distal amplitudes of the compound muscle action potentials, and the proximal and distal latencies of the median, ulnar, tibial, and peroneal motor nerves were recorded. The sensory conduction velocities (SCVs), sensory nerve action potential, and the latency of the median, ulnar, and sural sensory nerves were also recorded. Normal limits from the laboratory were used as the standard reference for comparison.

Molecular analysis

Second-generation DNA sequencing technology was applied for gene analysis, and 72 genes (e.g., *PMP22*, *MPZ*, *LITAF*, and *EGR2*) related to CMT were examined. *GJB1* was included to identify patients with CMTX1. Genomic DNA was extracted from peripheral leukocytes of fresh blood samples of patients, and target genes were captured by the GenCap target region probe (MyGenostics, MD, USA) and amplified by polymerase chain reaction (PCR). The eluted DNA was finally amplified for 15 cycles using the following program: 98°C for 30 s (1 cycle), 98°C for 25 s, 65°C for 30 s, 72°C for 30 s (15 cycles), and 72°C for 5 min (1 cycle). The PCR product was purified using SPRI beads (Beckman Coulter, Inc.250 S.Kraemer Boulevard Brea, CA 92821,

USA) according to the manufacturer's instructions. The enriched libraries were sequenced on an Illumina HiSeq 2000 sequencer, which generated 100-bp paired reads. Sanger technology was applied to detect familial mutations among family members of these patients. Reference Genome is UCSC hg19 (http://genome.ucsc.edu/). Read Mapping is SOAP aligner (http://soap.genomics.org.cn/soapaligner.html) and Burrows–Wheeler Aligner (http://bio-bwa.sourceforge.net/bwa.shtml). Variant detection included identification of single-nucleotide polymorphisms and Indels using GATK and SOAPsnp (http://soap.genomics.org.cn/soapsnp.html). The database of Genomic Variants includes the 1000 Genomes Project (http://browser. 1000genomes.org/index.html) and the single nucleotide polymorphism database (dbSNP) (http://www.ncbi.nlm.nih.gov/projects/SNP/).

Bioinformatics analysis

Potential functional effects of *GJB1* mutations were predicted using Polymorphism Phenotyping 2 (PolyPhen-2) software (http://genetics.bwh.harvard.edu/pph 2/), sorting intolerant from tolerant (SIFT) (http://sift.jcvi.org/), and Mutation Taster (http://www.mutationtaster.org/). PolyPhen-2 classified the predicted effects of amino acid substitutions on the function of human proteins as "benign," "possibly damaging," "probably damaging," or "unknown." The functional impact of the mutation was predicted as "tolerated" or "damaging" by SIFT and as "polymorphism" or "disease-causing" by Mutation Taster.

RESULTS

Molecular analysis identified nine patients (five males and four females, mean age 32 ± 12 years, range 14–51 years) with *GJB1* mutations from 79 patients with a clinical diagnosis of CMT. Among patients carrying *GJB1* mutations, two had a positive family history compatible with X-linked inheritance.

Clinical findings

All patients with *GJB1* mutations showed varying degrees (mild to severe) of muscular atrophy, sensory disturbance, diminished reflexes, and foot deformities. Weakness and atrophy were marked in the bilateral distal lower extremities, such as in the anterior tibialis and gastrocnemius muscles, and without obvious involvement of the proximal muscles [Table 1].

Nerve conduction studies

MCVs and SCVs were reduced or not detected in patients with *GJB1* mutations. The amplitudes were reduced, and latencies were prolonged in accordance with slowed conduction velocities. MCVs in the median nerve of all nine patients were <38 m/s. Table 2 summarizes electrophysiological data from all patients with *GJB1* mutations.

Molecular and bioinformatics analysis

On molecular analysis, nine *GJB1* missense mutations, including c.283G>A, c.77C>T, c.643C>T, c.515C>T, c.191G>A, c.610C>T, c.490C>T, c.491G>A, and

Table 1: Clinical characteristics of patients with *GJB1* mutations

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9
Sex	Female	Male	Male	Female	Male	Male	Female	Male	Female
Age 1	22	27	3	15	31	4	18	23	16
Age 2	29	43	27	25	45	14	26	51	26
FH	−	−	−	−	−	−	+	−	+
MS (LL)	IV+	III−	I	III	II	II	IV	I	III
Atr (LL)	+	+	+	+	+	+	+	+	+
Ref (LL)	↓	↓	−	↓	−	↓	↓	−	↓
Foot-de	+	+	+	+	+	+	+	+	+
Bottle	−	+	+	−	+	−	−	+	−

Age 1: Age of onset; Age 2: Age of inspection; FH: Family history; MS (LL): Muscle strength (lower limbs); Atr (LL): Atrophy (lower limbs); Ref (LL): Reflex (lower limbs); Foot-de: Foot deformity; Bottle: "Inverted champagne bottle" shape of the leg; *GJB1*: Gap junction protein beta 1; FH: + positive, − negative; Atr: + yes; Foot-de: + yes, − no Bottle: + yes, − no; ↓: reduced.

Table 2: Electrophysiological findings of patients with *GJB1* mutations

| Nerve	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9
Median motor									
dCMAP (mV)	4.3	0.1	3.7	2.1	4.5	1.7	2.6	7.5	5.2
MCV (m/s)	36.5	29.0	34.9	37.2	32.4	34.2	35.8	36.7	35.6
Median sensory									
SNAP (μV)	4.5	ND	ND	1	ND	0.8	2.5	4.2	4.1
SCV (m/s)	46	ND	ND	39.5	ND	31.7	36.0	50.0	42.0
Ulnar motor									
dCMAP (mV)	6.5	2.9	4.7	4.1	4	2.1	6.4	5.7	5.7
MCV (m/s)	40.2	26.7	36.8	38.2	29.5	32.2	41.2	41.1	42.1
Ulnar sensory									
SNAP (μV)	2.3	ND	ND	1.9	ND	1.1	ND	3.3	3.7
SCV (m/s)	41.2	ND	ND	33.3	ND	29.8	ND	40.0	40.0
Tibial motor									
dCMAP (mV)	0.9	0.1	0.1	ND	0.1	ND	ND	0.3	1.1
MCV (m/s)	35.4	26.4	33.3	ND	22.4	ND	ND	34.3	32.3
Peroneal motor									
dCMAP (mV)	1.5	ND	ND	1.7	ND	ND	ND	ND	0.9
MCV (m/s)	42.7	ND	ND	44.9	ND	ND	ND	ND	38.9
Sural sensory									
SNAP (μV)	ND	ND	ND	ND	ND	ND	ND	7	2.4
SCV (m/s)	ND	ND	ND	ND	ND	ND	ND	48.4	43.1

MCV: Motor nerve conduction velocity; SCV: Sensory nerve conduction velocity; SNAP: Sensory nerve action potential; *GJB1*: Gap junction protein beta 1; dCMAP: Distal compound muscle action potential; ND: Not detectable.

c.44G>A, were revealed in nine unrelated patients. Of these, c.643C>T, c.191G>A, and c.610C>T had not been reported previously and were absent in the 1000 Genomes Project and the dbSNP, suggesting that they were not polymorphisms.

The novel c.643C>T, c.191G>A, and c.610C>T missense mutations were detected in patients #3, #5, and #6, respectively, and were all associated with no positive family history (absent in asymptomatic parents, information unavailable, and asymptomatic mother, respectively). Molecular analysis performed on the asymptomatic mother of patient #6 revealed a c.610C>T mutation in exon 2 [Figure 1], and NCS revealed subclinical peripheral nervous damage in the mother. The c.643C>T, c.191G>A, and c.610C>T mutations would result in amino acid changes from arginine (R) to tryptophan (W) at codon

215 (p.R215W), cysteine (C) to tyrosine (Y) at codon 64 (p.C64Y), and leucine (L) to phenylalanine (F) at codon 204 (p.L204F), respectively, and were predicted by the PolyPhen-2 software (score 0.999, sensitivity 0.14, specificity 0.99; score 1.000; sensitivity 0.00, specificity 1.00; and score 1.000; sensitivity 0.00, specificity 1.00, respectively) to be "probably damaging" and capable of disrupting the function of *GJB1*. Mutation Taster and SIFT, respectively, predicted the three mutations as being functionally "disease-causing" and "damaging" (SIFT score of 0).

Of the previously reported mutations, c.490C>T mutation was detected in patient #7, whose father had similar symptoms. This mutation would result in an amino acid change from arginine (R) to tryptophan (W) at codon 164 (p. R164W). Both parents underwent molecular analysis and the patient's father tested positive for the same mutation;
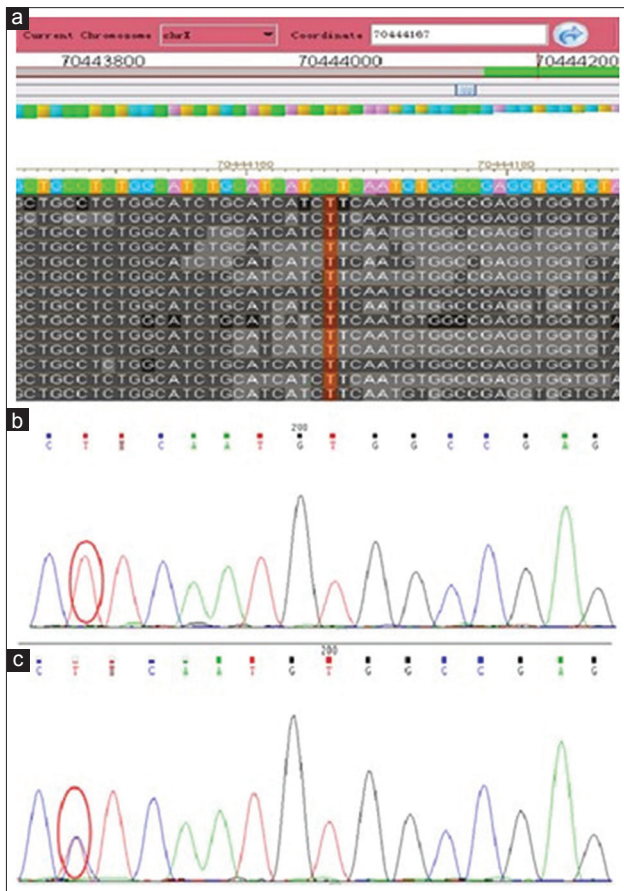
Figure 1: (a) Next-generation DNA sequencing of patient #6 and a c.610C>T mutation was revealed; (b) molecular analysis (Sanger technology) of patient #6 and a c.610C>T mutation was revealed; (c) molecular analysis of patient #6's mother revealed the same mutation.

however, mutation information was unavailable for other paternal relatives. In addition, c.44G>A mutation was detected in patient #9 whose grandmother, father, and father's sister had similar symptoms [Figure 2]. The mutation would result in an amino acid change from arginine (R) to glutamine (Q) at codon 15 (p.R15Q). Molecular analysis of the patient's aunt and patient's 3-year-old asymptomatic son revealed that they had the same mutation.

Discussion

In this study, nine missense mutations within *GJB1* were identified, of which three were novel missense mutations discovered using molecular analysis. *In silico* analysis indicated the disease-causing features of the three novel mutations. It is well-known that there are more than 400 mutations identified for the *GJB1* gene including missense, deletion, and frame-shift mutations.[9] So far, mutations of p.I71S, p.R15W, p.R75W, p.R183C, p.N205S, p.N2K, p.I127F, p.D178G, p.R15Q, p.R164Q, p.R183H, p.T188A, p.R75W, p.L131P, and p.R164W have been reported from China,[9-12] of which mutations of p.I71S, p.N2K, p.I127F, and p.D178G are novel[10-12] and have not been reported from other countries. Mutations of p.R15W, p.R75W, p.R183C, and p.R164Q cause CNS damage, which is consistent with
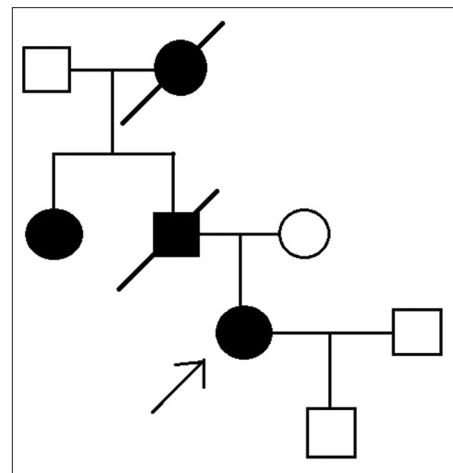
Figure 2: Pedigree analysis of patient #9.

data reported in literature from other countries.[13] The newly discovered three missense mutations have broadened the mutation diversity of the *GJB1* gene. Molecular analysis was conducted on some of the family members of the patients. The asymptomatic mother of patient #6 (male) was confirmed as carrying c.610C>T mutation which was identical to that in patient #6; although she had no symptoms, NCS revealed subclinical PNS damage. This phenomenon indicates that female carriers are less affected or may be asymptomatic, probably due to X-inactivation.[14,15] Both patient #7 and her father presented with similar symptoms and carried c.490C>T mutation, which was not detected in the mother, suggesting dominant inheritance.[13] In addition, both patient #9 and her paternal aunt carried c.44G>A mutation; however, her 3-year-old son carried the same mutation but remained asymptomatic. This is probably because CMT onset usually occurs after adolescence and most males are clinically affected starting from 10 years of age;[16,17] it is likely that clinical symptoms had not yet manifested in this carrier. In addition, patient #4 (female) with c.515C>T mutation (het) was reported to have X-linked recessive CMT.[18] Although some cases have been reported to have "recessive" CMTX1, some obligate carriers among these have electrophysiological evidence of peripheral neuropathy.[13]

In accordance with CMT Type 1 presentation, MCVs in the median nerve of the nine patients were <38 m/s. A study indicates that in CMTX1, demyelination occurs initially, followed by chronic remyelination; when demyelination has advanced to a certain extent, axonal loss begins.[4] There is a gender-based predisposition, and males are usually more affected than females in CMTX1. Men with CMT1X typically have "intermediate" slowing of NCV and mildly prolonged distal motor latencies. Forearm MCVs are typically 30–40 m/s in affected males and 30–50 m/s in affected females,[4,16,17] which are reportedly faster than in most CMT1 and slower than in most CMT2 patients. Accurately, this phenotype should be classified as intermediate CMT. In our study, patients 2, 3, 5, 6, and 8 are male, and male patients were more severely afflicted than

females with regard to electrophysiological manifestations, and this is in concordance with reports from previous studies.

The reported *GJB1* mutations are predicted to affect all regions of the CX32 protein. Many CX32 mutations result in absence of functional channels; other mutants form functional channels with altered biophysical characteristics, for example, some mutations have reduced pore diameter which may prevent the diffusion of second messengers such as inositol triphosphate, cyclic adenosine monophosphate, and Ca^{2+}.[19] Mutants in the C-terminal domain form functional GJs, but some channels may have lesser stability.[20]

CNS involvement has been reported in CMTX1 patients.[13] Apparently many, but not all, patients with CMT1X show abnormalities of evoked potentials.[21] CX32 is expressed by Schwann cells in the PNS and oligodendrocytes in CNS. However, in our study, patients did not report symptoms associated with CNS, and clinical examination did not detect increased muscle tone, hyperreflexia, and Babinski sign. In Schwann cells, CX32 forms the intracellular GJs between paranodal loops and Schmidt–Lanterman incisures that allow diffusion of small molecules.[22] Loss-of-function mutations mainly lead to abnormalities in PNS whereas gain-of-function mutations lead to CNS manifestations.[23-25] All *GJB1* mutations likely cause loss-of-function and seldom cause gain-of-function,[17,24,25] and this may explain the results of our study. Moreover, R164Q and R164W mutations have been reported to have striking presentation of an acute, transient encephalopathy associated with magnetic resonance imaging (MRI) changes in CNS myelin, often "triggered" by travel to high altitudes, intense physical activity, or acute infections.[13] However, patients with these two mutations in our study did not manifest CNS symptoms, which indicate that CNS involvement can be subclinical. Our study limitation lies in lack of MRI, visual evoked potential, and brainstem auditory evoked potential to confirm CNS involvement.

In conclusion, we identified three novel missense mutations of the *GJB1* gene associated with CMTX1, which broadened the mutation diversity of CMT1X. Molecular analysis of family members and bioinformatics analysis confirmed the pathogenicity of these mutations. However, studies with larger sample size are necessary to study the electrophysiological characteristics of CMTX1 between different genders and the mode of inheritance. We recommend that auxiliary examinations be applied to evaluate CNS involvement in patients with CMTX1.

Conflicts of interest
There are no conflicts of interest.

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