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GJB1/Connexin 32 whole gene deletions in patients with X-linked Charcot–Marie–Tooth disease

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Abstract

The X-linked form of Charcot–Marie–Tooth disease (CMTX) is the second most common form of this genetically heterogeneous inherited peripheral neuropathy. CMT1X is caused by mutations in the *GJB1* gene. Most of the mutations causative for CMT1X are missense mutations. In addition, a few disease causative nonsense mutations and frameshift deletions that lead to truncated forms of the protein have also been reported to be associated with CMT1X. Previously, there have been reports of patients with deletions of the coding sequence of *GJB1*; however, the size and breakpoints of these deletions were not assessed. Here, we report five patients with deletions that range in size from 12.2 to 48.3 kb and that completely eliminate the entire coding sequence of the *GJB1* gene, resulting in a null allele for this locus. Analyses of the breakpoints of these deletions showed that they are nonrecurrent and that they can be generated by different mechanisms. In addition to *PMP22*, *GJB1* is the second CMT gene for which both point mutations and genomic

rearrangements can cause a neuropathy phenotype, stressing the importance of CMT as a genomic disorder.

Keywords

Charcot–Marie–Tooth disease; GJB1; Connexin32; Gene deletion; Genomic rearrangement

Introduction

Charcot–Marie–Tooth (CMT) disease is represented by a group of inherited peripheral neuropathies characterized by distal symmetric polyneuropathy [1] with progressive muscle weakness and atrophy with sensory loss [2]. CMT can be broadly divided into demyelinating (CMT1) and axonal (CMT2) forms based upon primarily affecting glia (Schwann cells) or axons (neurons) as evidenced by electrophysiological and neuropathological features; genetically, there are autosomal dominant, autosomal recessive, and X-linked forms. It is a genetically heterogeneous disease as mutations in 31 different genes have been described to cause the different clinical forms of CMT and related hereditary neuropathies. The most common form of CMT, CMT1A [MIM #118220], is caused by the recurrent 1.4-Mb duplication of chromosome 17p12 that includes the dosage-sensitive gene *PMP22* (peripheral myelin protein 22) [3, 4]. The reciprocal deletion of the same region is the cause of hereditary neuropathy with liability to pressure palsies (HNPP) [MIM #162500] [5, 6]. The second most common form of CMT is the X-linked form (CMT1X, [MIM #302800]) caused by mutations in the *GJB1* gene, also known as Connexin 32 (Cx32) [7].

GJB1 maps to chromosome Xq13.1 and is 10 kb in length. It is comprised of one coding exon (exon 2) and three non-coding exons (exons 1, 1A, and 1B) that can be differentially spliced to form different 5' UTRs. This gene encodes the 238-amino-acid gap junction, beta 1/connexin 32 protein that is involved in multimeric interactions to form connexons. Hemichannel connexons from two membranes interact to form a gap junction used for creating a radial diffusion pathway that enables communication between the Schwann cell nuclei and axon. Connexin 32 can be expressed as three transcript isoforms that code for the same protein but differ only in the nucleotide sequence of their 5' UTR and are apparently regulated in a cell typespecific manner [8].

Although males are generally more affected ranging from a moderate to severe phenotype, CMT1X is considered a dominant disease as females can be mildly affected whereas in some cases they can remain asymptomatic. Some reports have also noted the potential involvement of the central nervous system in some patients with CMT1X as well as reduced audition, apparently due to slowed acoustic nerve conduction [9].

CMT1X is caused predominantly by missense mutations that may change almost every amino acid in the protein and which are believed to cause a dominant negative effect of the protein when it interacts with other connexins to form connexons at gap junctions. Some mutations allow the protein to form functional channels, but these have altered gating and biophysical properties [10]. However, some nonsense mutations, small deletions, and a few

small insertions that cause premature termination codons or frameshifts in the *GJB1* protein have also been reported, suggesting that loss of function of connexin 32 is also causative for the CMT1X phenotype [10, 11]. There have only been a few reports of patients with complete deletion of the *GJB1* gene, where deletion was suspected due to lack of amplification of the exons of the gene by polymerase chain reaction (PCR) and further confirmed by Southern blot [12–16]. In the first report, the deletion was mapped between the polymorphic markers *DXS106* (Xq11.2–12) and *DXS559* (Xq13.1) [12]. However the size, genomic content, and breakpoint sequence have not been determined for any reported *GJB1* deletions; the latter absence of breakpoint sequences prevents one from surmising the mechanisms for rearrangement.

Here, we report five patients with CMT1X that carry three different deletions spanning 12 to 48 kb and comprising the *GJB1* gene. The gene deletions were initially ascertained in an MLPA-based diagnostic service and further characterized by array comparative genomic hybridization (aCGH), having the breakpoint junctions refined by PCR and sequencing. We describe these deletions and their breakpoint junctions and discuss the possible mechanisms that generate rearrangements in this region leading to CMT1X.

Materials and methods

Genomic DNAs from five anonymous samples provided by Athena Diagnostics were interrogated by aCGH using custom-designed 4×44 K Agilent high-density oligonucleotide microarrays containing probes for 29 known CMT genes and their 10-kb upstream and downstream regions. Sex-matched controls' DNAs (NA10851 and NA15510) obtained from cell lines from Coriell Cell Repositories (<http://ccr.coriell.org>) were used for the comparative genomic hybridization. Following digestion with the enzymes *AluI* and *RsaI*, the sample DNAs were labeled with Cy5-dCTP and the control DNAs were labeled with Cy3-dCTP using the BioPrime Array CGH genomic labeling kit (Invitrogen Corporation, Carlsbad, CA, USA). Purification of the labeled products, array hybridization, washing, scanning, and data analysis were conducted according to the manufacturer's protocol.

To analyze the breakpoint junctions of the deletions, different combinations of primers were designed for each of the three (A31, A32, and A33) different deletions (A31-F1: 5' ATG GAT TTA CAT TGT TCG CT 3'; A31-R2: 5' ATA AAC AAT GAA AGC GAT GAG 3'; A31-R4: 5' CAC CCT TGT GTT GTA GTG AGA 3'; A32-Fwd: 5' CCT GAA GCATTT CTT TAT GGC 3'; A32-Rev: 5' TCC AAG TTC AGT CTC GTC CAC 3'; A33-F7: 5' GAG TGC ATG CCC TCT AAC TGT AC 3'; A33-R1: 5' ACA CAC AAG TTT GAA GGT ATC ACC 3'). Breakpoints for each of the deletions were determined by long-range PCR using TaKaRa *LA Taq* polymerase and/or standard PCR using Qiagen HotStar *Taq* polymerase. PCR products of the predicted size containing the breakpoints were subsequently sequenced using standard Sanger dideoxy sequencing. DNA sequences were analyzed and aligned against the human genome reference sequence build 36 (hg18) using the UCSC alignment tool Blat (<http://genome.ucsc.edu/cgi-bin/hgBlat>) and BioEdit Sequence Alignment Editor.

Results

Array CGH results of the five samples showed three different types of deletions that included the entire coding region of the *GJB1* gene. Further PCR amplification and sequencing refined the deletion regions and sequence breakpoints. The first deletion, spanning almost 27 kb in a male patient (A31; Fig. 1), comprised the entire *GJB1* gene until just a few bases before the beginning of the adjacent gene *ZMYM3*. A 12-base microhomology at the breakpoint junction (Fig. 2a) suggests a fork stalling and template switching (FoSTeS) event [17]. The presence of divergent *Alu* repeated elements from different subfamilies (*AluY* and *AluJb*) at the breakpoint might also lead to the interpretation that the deletion occurred by an *Alu*–*Alu* non-allelic homologous recombination (NAHR) event [18]. The second and smallest deletion spanning 12.2 kb was observed in three different patients (A32, A34, A36; Fig. 1). This deletion eliminates most of the *GJB1* gene, except for exon 1 that transcribes into the 5' UTR of isoform 1; however, the coding exon is completely deleted, resulting effectively in a null allele. Although the possible regulatory upstream sequences and the first 5' UTR noncoding exon of the *GJB1* gene are not eliminated by this deletion, we cannot assess if this remaining sequence can affect in some way the expression of the adjacent or nearby genes. In the three cases with the same apparent deletion, the breakpoint junction sequence showed an adenine insertion (Fig. 2b), this 'information scar' at the breakpoint suggests a non-homologous end joining (NHEJ) event as the mechanism for this deletion [19, 20]. Consistent with finding the identical deletion, these three subjects (A32, A34, and A36) are reportedly from the same family, based on the limited information that we have from these anonymized samples. The third and largest deletion spans 48.3 kb and completely eliminates the *GJB1* gene in the female patient tested (A33; Fig. 1). The breakpoint region showed a high percent identity between the upstream and downstream sequences which include *AluSx* repetitive elements, with a 24-bp segment of 100% identity (Fig. 2c). The high identity between the *AluSx* elements and possible polymorphisms in the sequences make it difficult to accurately define the exact position where the breakpoint occurred. However, the presence of these *Alu* elements in the breakpoint region suggests either a FoSTeS event, where the microhomologies of the *Alu* sequences were utilized for a template switch and priming DNA replication [21], or a NAHR event between these *AluSx* elements.

Discussion

Since the discovery that *GJB1* is the gene mutated in Xlinked Charcot–Marie–Tooth disease, more than 270 mutations have been reported, mostly missense mutations (Inherited Peripheral Neuropathies Mutation Database: <http://www.molgen.ua.ac.be/CMTMutations>). Although there have been previous reports of patients with *GJB1* deletions, to our knowledge this is the first time that the genomic span and breakpoints of *GJB1* deletions have been comprehensively assessed and studied. Analyses of these breakpoints showed that *GJB1* deletions are nonrecurrent in the majority of cases and can be produced by different mechanisms (Table 1). The breakpoint of the smallest deletion (12.2 kb) appears to have been produced by a NHEJ event and therefore it is probably a nonrecurrent deletion private to the family studied.

The breakpoints of the two larger deletions (26.7 and 48.3 kb) were shown to be embedded in *Alu* sequences. *Alu* repetitive elements can potentially mediate NAHR events. However, closely related *Alu* family members are likely required to provide the minimal efficient processing segment necessary to support a homologous recombination event. The *Alu* sequences that we observed at the breakpoints of our deletions (A31 and A33) are divergent (73% identity) and unlikely to support homologous recombination. Alternatively, these could be nonrecurrent deletions produced by a replication-based mechanism that uses microhomology regions from *Alu* elements to switch DNA templates and prime DNA replication.

Nonrecurrent rearrangements thought to be driven by replication-based mechanisms such as FoSTeS/microhomology-mediated break-induced replication (MMBIR) have been previously reported to change the copy number and dosage of genes. The MMBIR model proposes that single-stranded 3' ends, generated from a collapsed replication fork that occurs as the replisome proceeds through a nicked DNA template, can invade another replication fork and anneal with single-stranded DNA by means of microhomology. This primes low processivity replication that can allow template switching and generate complex DNA rearrangements [22]. Nonrecurrent complex rearrangements in genomic disorders like Pelizaeus–Merzbacher disease [17], Potocki–Lupski syndrome [23], and *MECP2* duplication syndrome [24] underline the importance of microhomology and replicationbased mechanisms in *de novo* generation of pathogenic rearrangements.

The fact that the entire *GJB1* gene deletion is causative of CMT1X in these and previously reported affected individuals is consistent with some studies in which *GJB1* null mice with complete absence of the connexin 32 protein develop demyelinating peripheral neuropathy and axonal degeneration [25]. Transgenic expression of human *GJB1* in Schwann cells of a *GJB1* knockout mouse model of CMTX has been shown to rescue the CMT phenotype, decreasing demyelination [26]. These findings suggest that CMT1X, due to loss of function alleles produced by nonsense or frameshift mutations and gene deletions, is a disease in which gene therapy may be a feasible treatment option.

In addition to the well known and common 1.4-Mb duplication in chromosome 17p12 that causes CMT1A and whose reciprocal deletion causes HNPP, most of the CMT-associated mutations are either point mutations or small deletions. *GJB1* is the second CMT gene, in addition to *PMP22*, for which entire gene deletions have been reported, stressing the importance of CMT as a genomic disorder and the occurrence of genomic rearrangements as the cause of common Mendelian diseases.

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Fig. 1. *GJB1* deletions region. **a** Genomic region including *GJB1* and bordered by the genes neuroligin 3 (*NLGN3*) and zinc finger, MYM-type 3 (*ZMYM3*). None of the deletions described here involve any of these flanking genes. *GJB1* has two predominant transcript isoforms (depicted in the figure) that differ in the exons that form their 5' UTR. All of the deletions eliminate the *GJB1* coding exon 2, which is the only coding exon in this gene. **b** Array CGH results for patients with *GJB1* deletions. The x-axis denotes the genomic location with each dot representing an interrogating oligonucleotide probe: black dots

represent no change in hybridization signal intensity between subject and control; green dots represent loss of signal intensity, consistent with deletion in the subject. Horizontal solid blue bars represent the genes *GJB1* and *ZMYM3*. The y-axis represents the log₂ ratio of relative hybridization signal intensity in subject versus control. Note the difference in the dynamic range of deletions in aCGH from females (A34 and A33) versus males

Fig. 2.

Breakpoints of *GJB1* deletions. The proximal and distal sequences refer to the reference sequence and to their position from the centromere. The patient breakpoint sequences (A31, A32, A34, A36, A33) are given with matches to the proximal reference sequence shown in blue and matches to the distal reference sequence shown in red. Boxed sequences (purple) correspond to the breakpoint junction. **a** Breakpoint sequence of the deletion in sample A31 with 12 bp of microhomology at the junction. **b** Breakpoint sequences of the deletions in subjects A32, A34, and A36; note that an adenine (A) is inserted at the breakpoint junction. **c** Breakpoint sequence of the deletion in subject A33. It is challenging to define the breakpoint junction because of the extent of sequence similarity shared between the *AluSx* sequences at the breakpoint. The breakpoint occurs somewhere within the 70-bp region of similarity in which the patient breakpoint sequence switches from matching the proximal reference sequence to matching the distal reference sequence.

Table 1Genomic coordinates and sizes of the *GJB1/Cx32* deletions

Sample	Gender	Deletion coordinates (hg18)	Deletion size	Possible mechanism
A31	Male	chrX: 70,348,979–70,375,735	26,756 bp	FoSTeS/NAHR
A32	Male	chrX: 70,358,360–70,370,631	12,270 bp	NHEJ
A33	Female	chrX: 70,317,443–70,365,808	48,365 bp	FoSTeS/NAHR
A34	Female	chrX: 70,358,360–70,370,631	12,270 bp	NHEJ
A36	Male	chrX: 70,358,360–70,370,631	12,270 bp	NHEJ