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ORIGINAL ARTICLE

Gap junction beta 1 (*GJB1*) gene mutations in Italian patients with X-linked Charcot-Marie-Tooth disease

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Abstract X-linked Charcot-Marie-Tooth disease (CMT1X) is a peripheral neuropathy transmitted in a dominant manner and caused by mutations in the Connexin 32 (Cx32) gene (*GJB1*, gap junction beta 1). Here we report the mutation analysis of the *GJB1* gene in 76 subjects with possible CMT1 and absence of 17p11.2 duplication, and in 38 CMT2 patients without mutations in CMT2-associated-genes, selected from a cohort of 684 patients with peripheral sensory-motor neuropathy. The analysis was performed by direct sequencing of the coding sequence and exon/intron boundaries of the *GJB1* gene. The mutation screening identified 22 mutations in *GJB1*, eight of which have not been previously published: six point mutations (c.50C > G, c.107T > A, c.545C > T, c.545C > G, c.548G > C, c.791G > T) and two deletions (c.84delC, c.573_581delCGTCTTCAT). The *GJB1* mutation frequency (19.3%) and the clinical heterogeneity of our patients suggest searching for *GJB1* mutations in all CMT cases without the 17p11.2 duplication, regardless of the gender of the proband, as well as in CMT2 patients with possible X-linked inheritance.

Keywords X-linked Charcot-Marie-Tooth disease · Connexin32 (Cx32) · *GJB1* · Mutation · Italian

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Introduction

Charcot-Marie-Tooth disease (CMT) represents a genetically heterogeneous group of inherited motor and sensory neuropathies characterised by slowly progressive weakness, muscle wasting and sensory loss, primarily affecting distal leg muscles. Current classification of CMT continues to be based on forearm motor nerve conduction velocity (MNCV) that divides CMT into type 1 (demyelinating; MNCV < 38 m/s) and type 2 (axonal; MNCV > 38 m/s) and on the mode of inheritance. However, patients with X-linked CMT (CMT1X) typically have "intermediate" slowing of nerve conduction velocities, which are faster than in most CMT1 patients and slower than in most CMT2 patients (Nicholson and Nash 1993; Kleopa and Scherer 2006).

The X-linked form of CMT (CMTX) is the second most frequent form of CMT (Ionasescu et al. 1995; Nelis et al. 1996; Mersiyanova et al. 2000; Mostacciuolo et al. 2001; Hattori et al. 2003; Casasnovas et al. 2006) and is associated with a large number of mutations in the gap junction beta 1 (*GJB1*) gene on chromosome Xq13 encoding the gap junction protein Connexin 32 (Cx32) (Bergoffen et al. 1993; Shy et al. 2007). Cx32 is expressed by Schwann cells and oligodendrocytes. This protein belongs to a family of homologous integral membrane proteins that form functional channels allowing rapid transport of ions and small nutrients between coupled cells. In Schwann cells, Cx32 forms intracellular gap junctions between paranodal loops and Schmidt–Lanterman incisures that allow diffusion of small molecules through the myelin sheath (Balice-Gordon et al. 1998). Normal structure of Cx32 reveals the presence of two extra cellular (EC) loops, four transmembrane (TM) regions, one intracellular (IC) loop and two IC ends: carboxyl terminus (C) and amino terminus (N). Cx32 is also expressed in the central nervous system (CNS), in cell bodies

and in processes of oligodendrocytes (Scherer et al. 1995). This is of interest because subclinical CNS involvement shown by changes in visual evoked potentials (VEPs) and brainstem auditory evoked potentials (BAEPs) has been described (Nicholson and Corbett 1996; Bahr et al. 1999; Seeman et al. 2001; Taylor et al. 2003). The hypothesis is that *GJB1* mutations lead to a loss of normal cellular communication, which in turn may lead to myelinating Schwann cell dysfunction and peripheral neuropathy.

The clinical manifestations of CMTX vary. Onset may be congenital or delayed until the third decade of life (Ionasescu et al. 1996). Muscle weakness and wasting of the hand muscles may be severe. Clinical electrophysiological abnormalities are well characterised. Nerve conduction velocities are reduced, but the reductions are less severe than those seen in other forms of demyelinating CMT (30–40 m/s in males; 30–50 m/s in females) (Nicholson and Nash 1993; Kleopa and Scherer 2006). Men usually show an earlier onset than women and are more severely affected. Manifestations in female heterozygotes are variable, probably because of random X-chromosome inactivation (Lyonisation), as directly demonstrated in mice (Scherer et al. 1998). In some cases, involvement of female hemizygotes is severe, but overall, they are affected to a degree that lies between normal and affected male subjects (Nicholson and Nash 1993; Gutierrez et al. 2000; Lewis and Shy 1999; Lewis et al. 2000).

More than 294 different mutations in *GJB1* have been reported in CMTX patients, which affect both the 5′-untranslated region (UTR) region as well as the coding region of *GJB1*. These include missense, frameshift, deletion and nonsense mutations. However, missense mutations predominate by far (Inherited Peripheral Neuropathies Mutation Database, http://www.molgen.ua.ac.be/CMTMutations).

To evaluate the frequency of mutations of the *GJB1* gene in a series of Italian patients, we tested 114 patients affected by sensory and motor peripheral neuropathy with a possible X-linked transmission or sporadic pattern and absence of the 17p11.2 duplication and CMT2 most commonly associated mutations.

Subjects and methods

Between 1997 and 2006, 684 consecutive blood samples from patients with peripheral motor and sensory neuropathy were seen at our Service of Medical Genetics for molecular diagnosis of CMT. The clinical phenotype was retrospectively defined based on the clinical and electrophysiological data and, when available, on sural nerve biopsy evaluation, reported by highly specialised Italian neurological centres. A CMT1 was diagnosed when the MNCV, recorded from median/ulnar nerves, was <38 m/s, whereas CMT2 was diagnosed when upper-limb MNCV was >38 m/s with reduced compound muscle action potential (CMAP).

The presence of the 17p11.2 duplication was previously ruled out in 359 patients with possible CMT1 by means of pulse-field gel electrophoresis analysis and microsatellite analysis of seven polymorphic markers (http://www.molgen.ua.ac.be/CMTMutations; Latour et al. 2001). One hundred and fifty-eight patients (158/359, 44%) had 17p11.2 duplication. Mutational analysis of the most common genes involved in axonal neuropathies (mitofusin-2, myelin protein zero, neurofilament light gene and heat-shock proteins) was performed in 325 CMT2 patients. Seventy-six CMT1 patients who did not carry the CMT1A duplication and 38 CMT2 patients without mutations in the above-mentioned genes, with a possible X-linked transmission or sporadic pattern, were analysed for mutations in the *GJB1* gene. Two hundred unrelated healthy Italian subjects were used as normal controls. Informed consent was obtained from all subjects included in the study.

Genomic DNA was extracted from peripheral blood samples according to a standard protocol. The coding sequence of *GJB1*, including exon–intron boundaries and the 5′-UTR region, was amplified by polymerase chain reaction (PCR). PCR products were analysed for mutations by direct sequencing on automated sequencer 3100—Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Results

Molecular analysis identified 22 *GJB1* mutations in 114 patients affected by sensory-motor peripheral neuropathy. Among patients carrying *GJB1* mutations, 20 had a positive family history compatible with X-linked inheritance. In 13 families, mutation occurrence was confirmed in other family members by molecular analysis. Out of 22 patients with mutations, one had a mutation in the 5′-UTR region (−459C > T), 16 had a missense mutation, three carried a frame-shift mutation that leads to a premature stop codon, one had a small deletion and another a small insertion (Table 1). Moreover, the c.490C > T mutation was found in three families, and the c.64C > T was detected in two families. Two different mutations (c.545C > T, c.545C > G) affected the same nucleotide in two families. Eight out of these 22 mutations were not previously described: six point mutations (c.50C > G, c.107T > A, c.545C > T, c.545C > G, c.548G > C, c.791G > T) and two deletions (c.84delC, c.573_581delCGTCTTCAT). Further sequencing analysis did not identified any of these new mutations in 200 normal controls.

Table 1 Gap junction beta 1 (*GJB1*) gene mutations in Italian patients with X-linked Charcot-Marie-Tooth (CMT) disease

Patient	Mutation	Aminoacidic change	Type	Domain	Reference	Phenotype	Inheritance	Gender
CMT-88	−459 C > T			5′UTR	Flagiello et al. (1998) Ionasescu et al. (1996)	Axonal	XL	F
CMT-106	c.20 A > G	Tyr7Cys	Missense	N-term	Schiavon et al. (1996)	Demyelinating	XL	M
CMT-6	c.22 A > C	Thr8Pro	Missense	N-term	Haites et al. (1998)	Demyelinating	XL	M
CMT-14	c.44 G > A	Arg15Gln	Missense	N-term	Fairweather et al. (1994)	Axonal	XL	F
CMT-47	c.50 C > G	Ser17Cys	Missense	N-term	This study	Demyelinating	XL	M
CMT-37	c.64 C > T	Arg22stop	Stop	N-term	Ionasescu et al. (1996)	Demyelinating	XL	F
CMT-42	c.64 C > T	Arg22stop	Stop	N-term	Ionasescu et al. (1996)	Demyelinating	XL	M
CMT-87	c.84 delC	Ile28fs	Stop	TM1	This study	Demyelinating	XL	M
CMT-114	c.107 T > A	Leu36Pro	Missense	TM1	This study	Demyelinating	XL	M
CMT-24	c.282 C > G	His94Gln	Missense	TM2	Mostacciuolo et al. (2001)	Demyelinating	XL	M
CMT-113	c.329 G > A	Gly110Asp	Missense	EC1	Kochanski et al. (2004)	Demyelinating	XL	M
CMT-9	c.424 C > T	Arg142Trp	Missense	TM3	Bergoffen et al. (1993)	Demyelinating	XL	M
CMT-18	c.478 T > C	Tyr160His	Missense	EC2	Bone et al. (1997)	Axonal	XL	F
CMT-46	c.490 C > T	Arg164Trp	Missense	EC2	Ionasescu et al. (1996)	Demyelinating	XL	M
CMT-59	c.490 C > T	Arg164Trp	Missense	EC2	Ionasescu et al. (1996)	Axonal	NDA	F
CMT-98	c.490 C > T	Arg164Trp	Missense	EC2	Ionasescu et al. (1996)	Demyelinating	XL	M
CMT-7	c.545 C > T	Ser182Phe	Missense	EC2	This study	Axonal	XL	F
CMT-51	c.545 C > G	Ser182Cys	Missense	EC2	This study	Axonal	XL	F
CMT-4	c.548 G > C	Arg183Pro	Missense	EC2	This study	Demyelinating	NDA	M
CMT-91	c.572_580insCCGTCTTCA	Phe193_Met19insTVF	Insertion	TM4	Vazza et al. (2006)	Demyelinating	XL	M
CMT-72	c.573_581delCGTCTTCAT	Val192Met194del	Deletion	TM4	This study	Demyelinating	XL	M
CMT-15	c.791 G > T	Arg264Leu	Missense	C-term	This study	Demyelinating	XL	M

N-term amino terminus, *TM1*, *TM2*, *TM3* and *TM4* transmembrane 1, 2, 3 and 4, *EC1* and *EC2* extra cellular 1 and 2, *C-term* carboxyl terminus, demyelinating, *MNCV* median/ulnar nerves <38 m/s, axonal, normal or subnormal motor motor nerve conduction velocity (>38 m/s) and reduced compound muscle action potential, *XL* X-linked, *NDA* no data available, Gender of proband: *F* female, *M* male

Discussion

Here we report the results of a mutation screening in the *GJB1* gene involved in CMTX carried out on 114 patients referred to our centre with a diagnosis of motor and sensory peripheral neuropathy in which a male-to-male inheritance and the 17p11.2 duplication were previously ruled out. Twenty-two mutations have been identified, including eight novel mutations. The EC2 domain was the most affected, as also shown in a large study that described 34 Spanish families with mutations in the *GJB1* gene (31.8 vs. 36.8%) (Casasnovas et al. 2006).

In two families, the probands (CMT-37 and CMT-51) were female subjects with severe neuropathy with distal paresis requiring ankle–foot orthesis in the second decade of life. In the CMT-37 patient, the ulnar nerve MNCVs was 35 m/s with normal CMAP and distal latency, whereas in the CMT-51 patient, MNCVs were in the axonal range.

In CMT-7 and CMT-51 pedigrees, in which the same aminoacidic residue was differently mutated (Ser182Cys and Ser182Phe), all were female subjects affected by axonal neuropathy (CMT-7: mother and daughter; CMT-51: mother and two daughters). However, a significant

intrafamiliar phenotypic variability, ranging from a severe, early onset neuropathy to a mild, late-onset peripheral nervous disease, was present.

The clinical variability, particularly in female subjects, has already been reported and may be explained by the process of Lyonisation, which leads to a mosaic of wild-type and mutant myelin in heterozygous female subjects (Gutierrez et al. 2000). The likelihood that a female patient with a severe neuropathy could be affected by CMT1X must be therefore taken into account. These findings suggest that *GJB1* mutation analysis should be performed in female subjects with axonal neuropathy, regardless the severity of peripheral neuropathy. Furthermore, this gene should be investigated in female subjects with demyelinating neuropathy with possible X-linked inheritance or sporadic cases.

The *GJB1* mutation frequency in our series of CMT1 patients was 6.1% (22/359) and increased to 19.3% (22/114) in patients without 17p11.2 duplication and autosomal dominant inheritance, therefore representing the second most frequent cause of CMT. Mostacciuolo et al. (2001) described 12 mutations in the *GJB1* gene and reported a similar mutation frequency (20.3%). Four of the mutations identified (Tyr7Cys, Thr8Pro, Arg164Trp and Phe193_Met19insTVF) were already reported in our population (Mostacciuolo et al. 2001; Vazza et al. 2006). Three aminoacidic residues (Arg22, Ser182 and Arg164) were affected in more than one family, confirming that they are hot spots for mutations.

To our knowledge, this is the largest cohort of CMT patients with *GJB1* mutations reported in the Italian population. The high frequency of *GJB1* mutations and the variability of clinical phenotype suggest that all CMT1 patients, negative for 17p11.2 duplication/deletion and without male-to-male inheritance, regardless of the gender of the proband, should be tested for mutations of the *GJB1* gene, as should CMT2 patients with possible X-linked inheritance. A careful collection of the family history is therefore relevant for addressing molecular tests, offering accurate genetic counselling and predicting recurrence risk within the family.

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