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DOI

[10.1007/s004390050396](https://doi.org/10.1007/s004390050396)

Publication date

1997

Published in

Human Genetics

[Link to publication](#)

Citation for published version (APA):

Janssen, E. A. M., Kemp, S., Hensels, G. W., Sie, O. G., de Die-Smulders, C. E. M., Hoogendijk, J. E., de Visser, M., & Bolhuis, P. A. (1997). Connexin 32 gene mutations in X-linked dominant Charcot-Marie-Tooth disease (CMTX1). *Human Genetics*, 99, 501-505. <https://doi.org/10.1007/s004390050396>

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ORIGINAL INVESTIGATION

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Connexin32 gene mutations in X-linked dominant Charcot-Marie-Tooth disease (CMTX1)

Received: 21 June 1996 / Revised: 29 November 1996

Abstract Single-strand conformational polymorphisms (SSCP) of the connexin32 gene were analyzed in 121 patients possibly affected by Charcot-Marie-Tooth (CMT) disease. The 121 patients were selected from 443 possible CMT/HNPP (hereditary neuropathy with liability to pressure palsies) patients based on genetic linkage to Xq13.1, absence of the 17p12 duplication and deletion, and absence of point mutations in PMP22 and P0. We found five new mutations at nucleotides 105 (C-T), 316 (C-G), 321 (C-T), 328 (T-C), and 657 (G-C), and three mutations at nucleotide 126 (C-T), 249 (G-A), and 477 (G-A) previously described in other unrelated families. The nucleotide changes resulted in seven amino-acid substitutions and one premature stop codon.

Introduction

Charcot-Marie-Tooth (CMT) disease is a peripheral neuropathy clinically characterized by distal muscle weakness, muscle atrophy, sensory loss, and areflexia. By means of electrophysiology, CMT can be divided into CMT1 with reduced nerve conduction velocities (NCVs) and CMT2 with NCVs in the near normal range. Genetically, CMT1 can be divided into autosomal dominant types (CMT1A

and CMT1B), accounting for 90% of the patients, and several distinct X-linked types. Most CMT1A patients carry a 1.5-Mb duplication at chromosome 17p12, a region containing the peripheral myelin protein (PMP22) gene. In a few patients, other mutations have been found in the PMP22 gene or in the P0 gene (CMT1B) located at chromosome 1q22–q23. In the X-linked dominant form (McKusick no. 302800) located at Xq13.1 (Gal et al. 1985; Pericak-Vance et al. 1995), mutations have been reported in the connexin32 gene (Bergoffen et al. 1993; Bone et al. 1995a; Cherryson et al. 1994; Fairweather et al. 1994; Ionasescu et al. 1994, 1995; Orth et al. 1994; Ressot et al. 1996; Tan and Ainsworth 1994; Tan et al. 1996).

The connexins comprise a family of structural proteins important for the formation of gap junctions. Hexameric assemblies of connexins (connexons) interact with their counterpart in adjacent cells to form gap junctions. These connections extend across the extracellular space and provide a pathway of direct communication between cells, thereby allowing diffusional or electrically driven exchange of small ions and second messenger molecules. To date, 13 connexins have been reported, all with different gating properties, molecular weights, and tissue specificity. A common feature is the presence, in each monomer, of two extracellular loops, four transmembrane regions, one intracellular loop, and two intracellular ends. The differences most commonly reside in the intracellular loop and the intracellular carboxyl terminus.

Following the localization of the connexin32 gene at Xq13.1 (Corcos et al. 1992), Bergoffen et al. (1993) described families in which a mutation of the connexin32 gene was linked to CMTX1. Presently, 40 different mutations have been reported in 58 different families, with 6 genotypes arising twice or more in presumably unrelated families. These mutations result in 27 amino-acid substitutions and 6 premature stop codons, in addition to 2 insertions and 5 deletions of 1–29 bp. In 11 families, no mutations have been described, despite positive linkage to Xq13.1.

Here, we describe a study in which 121 individuals were screened for mutations in the connexin32 gene. The

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individuals were selected from a group of 443 possible CMT/HNPP patients referred to us for molecular diagnosis. We made a selection based on genetic linkage to Xq13.1 (16 patients in 3 families) and on the absence of the 17p12 duplication, the 17p12 deletion, a PMP22-gene mutation, a P0-gene mutation (only exons 2 and 3 were screened), and male-to-male transmission.

Screening for point mutations was performed by single-strand conformation polymorphism (SSCP) analysis. We found a total of 8 point mutations of which 3 mutations have been previously reported (Bergoffen et al. 1993; Fairweather et al. 1994; Ionasescu et al. 1994; Bone et al. 1995a; Ressot et al. 1996).

Materials and methods

Patients

The families and patients were identified in neurology and genetics clinics in The Netherlands and Germany. The samples were sent to us for molecular diagnosis of CMT/HNPP after obtaining informed consent. One of the families investigated (family 1) has been described previously (Heimans et al. 1982).

The 121 persons used in this investigation had no 17p12 duplication/deletion, and no mutations were found in PMP22 or P0. No male-to-male transmission was known. DNA was isolated from white blood cells by using the Genomics whole-blood kit (Talent SRL, Trieste, Italy).

Linkage

Two CA repeats were used for linkage analysis, i.e., DXS453 within the candidate region for CMTX1, and DXS441 flanking the candidate region for CMTX1. The genetic distance between the two markers is 2.7 cM. Two-point linkage analyses were performed using the MLINK section of the 5.1b version of the LINKAGE program (Lathrop et al. 1984).

Polymerase chain reaction amplification and SSCP analysis

For SSCP analysis, the connexin32 gene sequence (Kumar and Gilula 1986; Paul 1986) was split into seven overlapping polymerase chain reaction (PCR) fragments using the following primer sets: 1A nt 52–72 (forward), 1B nt 179–198 (reverse); 2A nt 175–194 (forward), 2B nt 293–313 (reverse); 3A nt 293–313 (forward), 3B nt 423–443 (reverse); 4A nt 424–444 (forward), 4B nt 554–574 (reverse); 5A nt 555–575 (forward), 5B nt 682–703 (reverse); 6A nt 678–696 (forward), 6B nt 793–815 (reverse); 7A nt 795–817 (forward), 7B nt 916–935 (reverse). All primers were based on the human liver gap junction (GJB1) cDNA sequence (Kumar and Gilula 1986; Paul 1986) generated by the computer program Primer (Version 0.5) written by S. E. Lincoln, M. J. Daly, and E. S. Lander (Whitehead Institute).

PCR amplifications were processed in the presence of α -32P-dATP through 35 cycles of 1 min at 93°C, 1 min at 63°C, and 1 min at 72°C. After PCR, 40 μ l 0.1% SDS/10 mM EDTA/bromophenol blue was added. An aliquot of 3 μ l from this sample was added to 18 μ l SSCP-layer mix (95% formamide/0.1% xylene cyanol/0.1% bromophenol blue/2 mM EDTA) and denatured at 94°C for 3.5 min.

From these denatured samples, 4 μ l were loaded onto a 8% polyacrylamide gel containing 10% (v/v) glycerol. Samples were electrophoresed for 20 h at 6 W at room temperature. Gels were exposed to Kodak X-omat AR films at –70°C with intensifying screens.

Sequencing

After SSCP, shifted bands that were different from the control samples were cut out by using a razor blade. DNA was isolated by freezing/thawing in water and amplified as described above with the appropriate SSCP primers. Sequencing was performed by means of the SequiTherm cycle sequencing kit (Epicenter Technologies, Madison, Wis.) according to the manufacturer's instructions.

Restriction analysis

The mutations found by SSCP analysis were confirmed by restriction analysis of the PCR products. The mutation at nucleotide 105 could be detected with *Msp*I, the mutation at nucleotide 126 with *Hae*III, the mutation at nucleotide 321 with *Dde*I, and the mutation at nucleotide 477 with *Nla*III. The mutations at nucleotides 249, 316, 328, and 657 could not be detected by restriction analysis.

Results

Clinical findings

All 121 patients were sent to us with a suspected clinical diagnosis of CMT1. Affected males had pes cavus, distal leg weakness, atrophy of tibialis anterior and peroneal muscles, areflexia, and distal sensory loss starting in childhood or adolescence. Weakness and sensory loss of intrinsic hand muscles and fore arms were present in most patients of 30 years and older. Among obligate carriers, the clinical picture varied from asymptomatic to almost as severe as in affected males. Electrophysiological investigations showed NCVs varying from normal to severely decreased (20–50 m/s, see Table 1.)

Molecular genetics

Analysis of markers at Xq13.1 showed significant linkage in family 1 (3.91 with DXS441) and positive LOD scores in families 2 and 3 (1.81 and 1.08, respectively, with DXS453). SSCP analysis of genomic DNA by means of 7 SSCP-primer sets gave 9 band shifts. Sequence analysis of the fragments isolated from the acrylamide gels showed 8 point mutations. One resulted in a premature stop codon and the other seven in amino-acid substitutions. No band shifts were found in family 1 and, in one case, a band shift did not result in a mutation within the coding region. The mutations are summarized in Table 1. Three mutations at nucleotides 126, 249, and 477 have previously been described in presumably unrelated families (Bergoffen et al. 1993; Fairweather et al. 1994; Ionasescu et al. 1994; Bone et al. 1995a; Ressot et al. 1996).

Discussion

Connexin32 is highly expressed in liver, kidney, and the peripheral nervous system where it is solely found at the nodes of Ranvier and Schmidt-Lantermann incisures (Bergoffen et al. 1993; Spray and Dermietzel 1995). In the

Table 1 Patients, age, age of onset, mutations, and clinical signs (*n.a.* not available, *n.r.* not recordable, *n.p.* not performed)

Patients	M/F (age in years)	Age of onset (years)	Mutation at	NCV (m/s) median nerve, peroneal nerve	Motor sign
SP 33	F (54)	8–12	arg105trp	33/22	Pes cavus, atrophy and paresis of distal arm and leg muscles
SP 35	F (89)	Asymptomatic	arg105trp	42/n.r.	Pes cavus, paresis of intrinsic hand and tibialis anterior muscles
SP 11	F (33)	Asymptomatic	arg126stop	44/50	Pes cavus, slight paresis and atrophy of foot muscles
SP 511	M (30)	10	arg126stop	24/n.r.	Pes cavus, atrophy and paresis of distal arm and leg muscles
2x-1	F (88)	Asymptomatic	val249ile	50 ^a /32	Pes cavus, atrophy of peroneal muscles
2x-2	F (60)	?	val249ile	56 ^a /49	Pes cavus, slight paresis of intrinsic hand and peroneal muscles
2x-3	M (55)	18	val249ile	42 ^a /31	Pes cavus, severe paresis and atrophy of tibialis anterior muscles
2x-6	M (50)	15	val249ile	55 ^a /46	Pes cavus, severe paresis of tibialis anterior muscles
2x-7	F (48)	Asymptomatic	val249ile	54 ^a /45	Pes cavus, paresis of tibialis anterior muscles
SP 302	F (30)	24	ser316cys	49/51	Paresis of tibialis anterior and peroneal muscles
3x-1	F (91)	60	pro321ser	49/28	Pes cavus, severe paresis of peroneal muscles, and slight paresis of intrinsic hand muscles
3x-3	M (59)	5	pro321ser	n.r./n.r.	Pes cavus, severe atrophy and paresis of distal arm and leg muscles
3x-4	M (54)	5	pro321ser	30/n.r.	Pes cavus, severe atrophy and paresis of peroneal and intrinsic hand muscles
3x-5	M (62)	19	pro321ser	n.r./n.r.	Pes cavus, severe atrophy and paresis of intrinsic hand and distal leg muscles
3x-7	F (33)	Asymptomatic	pro321ser	48/41	None
3x-8	F (27)	Asymptomatic	pro321ser	46/42	None
3x-10	F (39)	18	pro321ser	n.p.	Slight paresis of tibialis anterior muscles
4x-1	F (48)	10–14	leu328pro	29/29	Slight atrophy and paresis of peroneal and intrinsic hand muscles
4x-2	F (77)	10	leu328pro	n.a.	Pes cavus, atrophy and paresis of peroneal and intrinsic hand muscles
4x-3	F (72)	?	leu328pro	n.r./n.p.	Slight atrophy and paresis of distal arm and leg muscles
4x-4	F (67)	Asymptomatic	leu328pro	n.a.	Patient never visited neurologist
4x-5	M (38)	21	leu328pro	30 ^a /35	Pes cavus, atrophy of leg muscles
SP 156	F (36)	10–14	val477met	24/n.r.	Pes cavus, atrophy of peroneal and intrinsic hand muscles, sporadic patient
SP 51	M (35)	5	gly657arg	31/n.p.	Pes cavus, slight atrophy of peroneal and intrinsic hand muscles, sporadic patient

^aAssessment of NCV of ulnar nerve instead of median nerve

central nervous system, it is expressed throughout the internodal region and in oligodendrocytes. The reason for this difference in regional expression in the peripheral and central nervous systems is unknown. The assembly of gap junctions in the peripheral nervous system might be important for paranodal capacitance, for the guidance of myelination, or for the exchange of nutrients between the soma and cytoplasmic processes. In vitro tests with oocytes have shown that various mutations in the connexin32 gene can give rise to a reduction in the number of gap junctions formed, a change in voltage-gating response, or a decrease in the function of the gap junctions (Bruzzone et al. 1994; Dahl et al. 1992; Werner et al. 1991).

In this study, we have found 8 different mutations in 24 patients out of a group of 443 possible CMT/HNPP patients. Of these patients, 109 (24.6%) showed a duplication at chromosome 17p12, 47 (10.6%) had a deletion at chromosome 17p12, 12 (2.7%) had a mutation within the

PMP22 gene, 3 (0.7%) had a mutation in the P0 gene, and 24 (5.4%) had a mutation in the connexin32 gene. Cherryson et al. (1994) have reported that 10% of the CMT population can be accounted for by CMTX. The lower percentage may be explained by a difference in patient group composition. We have investigated CMT/HNPP patients and patients for whom molecular diagnostics have been used to exclude CMT. If we exclude the HNPP-suspected patients, a percentage of 6.8% CMTX1 patients is found. This is still less than 10%, which may be explained by the presence of different subtypes of CMTX and by the possibility that not all the CMTX1 mutations are found by SSCP analysis. Promotor and intron/exon splice-site mutations may also give rise to CMTX1. For example, no mutation was found in family 1, which had to be classified as CMTX1 based on the two-point LOD score with marker DXS441 at Xq13.1. Until now, 11 out of 58 families with positive LOD scores for Xq13.1 have been reported as

lacking mutations in the coding regions of the connexin32 gene. Investigation of the two promoters described by Neuhaus et al. (1995) and the DNA-binding region B2 described by Bai et al. (1995) is warranted in these families.

We have investigated the coding region of connexin32 and found the following mutations. (1) An Arg105Trp mutation causes a change from a hydrophilic to a hydrophobic amino acid in the NH₂-terminus and may influence the gating stimulus control. (2) An Arg126Stop mutation gives rise to an incomplete protein. (3) A Val249Ile mutation is located in the first extracellular loop and may result in poor interaction between the hemichannels of the connexon or reduced pore integrity. Amino acid 87 is a proline, which is present at the same position in all the various types of connexins in man, mouse, rat, and chicken. A mutation in this very strongly conserved amino acid has previously been described in connexin26, where it causes a reversal of the voltage gating (Suchyna et al. 1993); this is probably also the case for (4) the Pro321Ser mutation. This region of the connexins may function as a voltage sensor. (5) In the case of the Ser316Cys mutation, the extra possibility of forming a disulfide bond may interfere with the function of the voltage sensor region. (6) The Leu328Pro mutation probably alters the function of the voltage sensor region, because the extra proline will introduce an extra α -helix. Bruzzone et al. (1994) have shown that mutations in the third transmembrane region, which lines the aqueous pore, cause loss of intracellular transport. Connexons are formed but transport is deficient because of the incorrect alignment of two connexons or the inability to form a functional gap junction between two connexons. (7) The Val477Met mutation in the third transmembrane region may thus impair or block channel formation between two connexons. (8) The Gly657Arg mutation in the fourth transmembrane region introduces a positive charge, which may reduce membrane stability and/or modify channel formation.

The impact of connexin mutations may be different in cases where the incorporation of the connexin32 molecule into the membrane is affected and in cases where the channel formation between two connexons is disturbed. In the former cases, only non-mutated molecules will be incorporated, and the number of functional gap junctions will be reduced. This loss of gap junctions might be compensated for by the formation of gap junctions composed of other connexins (Bone et al. 1995b). In the latter cases, the mutations may have a dominant negative effect on the formation of gap junctions as described by Bruzzone et al. (1994). Table 1 gives an overview of the mutations and the clinical features. The most severely affected patient has the Pro321Ser mutation, which probably causes a reversal in voltage gating. A much later onset is found in patients carrying the Arg126Stop mutation. Apparently, the incorporation of a mutated connexin32 has more effect on the myelin sheath than the absence of connexin32.

One of the characteristics of CMTX1 is that female carriers of the mutation can be asymptomatic or only slightly affected and have intermediate to normal nerve conduction velocities. Variation of clinical signs among

female patients might be the result of different X-inactivation ratios.

Following the demonstration that mutations in connexin32 segregate with CMTX1 (Bergoffen et al. 1993), other mutations have been described (Bergoffen et al. 1993; Bone et al. 1995a; Bruzzone et al. 1994; Cherryson et al. 1994; Fairweather et al. 1994; Ionasescu et al. 1994, 1995; Orth et al. 1994; Ressot et al. 1996; Tan et al. 1994, 1996). These mutations reveal which parts of the connexin32 molecule are important for properly functioning gap junctions. Whether connexin32 is coexpressed with other connexins and whether other proteins are important for the function of connexin32 in myelin remain to be elucidated.

Acknowledgements The authors thank Drs C. Höweler, A. Gal, H. Kroes, E. Wesby-van Swaay, and Prof. M.F. Niermeijer for sending patient material and for additional cooperation.

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