Arginine-164-tryptophan substitution in connexin32 associated with X linked dominant Charcot-Marie-Tooth disease

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Abstract

A Spanish family with X linked dominant Charcot-Marie-Tooth (CMTX1) neuropathy was screened for point mutations in the connexin32 gene (GJβ1). The patients showed a C-T transition at position 552 which predicts arginine to tryptophan substitution at amino acid 164 (R164K). This mutation destroys an *Aci*I restriction site at position 552 and creates a *Pfl*MI restriction site.

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Key words: X linked Charcot-Marie-Tooth neuropathy; connexin32; gap junction.

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CMTX1 disease is a form of demyelinating peripheral neuropathy in which males are generally more severely affected than females. Dominant (CMTX1) and recessive forms have been reported. This locus has been mapped within an approximately 1 cM region surrounding the DXS453 marker.[1] From a total of 34 CMTX1 families analysed to date, 27 show complete cosegregation between the disease phenotype and different frameshift, missense, and nonsense point mutations in the coding region of the GJβ1 gene.[2–6] We have analysed the coding region of this gene in a large Spanish family with CMTX1 neuropathy and report here complete linkage between the disease and a missense gene mutation.

Methods

Most of the family members were serially examined at the Neurology Unit of "La Candelaria" Hospital, Tenerife, Canary Islands. Neurophysiological studies were carried out on six affected males, four obligate females, and four unaffected sibs. Genomic DNA was isolated from peripheral blood cells or from fingernail clippings.[7,8] Linkage studies were performed by PCR amplification of the (CA)n tandem repeats from PGKP1 and DXS453 using primers and conditions as previously described.[9–11] The GJβ1 coding region was amplified using primer sets and thermal cycling conditions as previously reported,[2] then sequenced for both strands using the Promega fmol sequencing system. PCR products were digested with *Pfl*MI or *Aci*I according to the manufacturer's instructions. To perform a DP-SSCP analysis we designed a set of primers spanning the mutation (5′-GGTGTTCCG-GCTGTTGTTTGAGGC-3′, bases 479–502; and 5′-AGACGGTTTTCTCGGTGGGGC-GGG-3′, bases 604 to 628). Amplification reactions were performed in 100 µl volumes containing 500 ng of genomic DNA, 10 pmol of each primer, 2·5 units of *Taq* polymerase, and 10 µl of buffer supplied by the manufacturer (Promega). Thermal cycling conditions were: 94°C, two minutes; 35 cycles of 94°C for 45 seconds, 58°C for 30 seconds, 72°C for 30 seconds; and final extension at 72°C for five minutes. A 150 bp fragment was obtained and one fifth was loaded in a double phase polyacrylamide gel (upper denaturing gel: 2 cm of 6% polyacrylamide; bottom non-denaturing gel: 20% polyacrylamide) as described elsewhere,[12] but 8 mol/l urea was used instead of formamide in the denaturing stacking gel. It was then electrophoresed, stained with ethidium bromide, and visualised by UV.

Results

The pedigree shows an X linked dominant disorder (fig 1). The clinical phenotype was consistent with a non-hypertrophic demyelinating neuropathy. The onset of symptoms was in the first or second decade in males, but in the third decade in females. Clinical features and neurological findings were similar to those described for CMT1A patients in males, but milder in females. Only two males aged 2 years remain asymptomatic but areflexic. Median and peroneal nerve conduction velocities (NCV) were slowed (mean: 24·2 m/sec (range: 0–40) and 23 m/sec (range: 0–45 m/sec), respectively). Females showed normal or mildly reduced NCV (37–43 m/sec in peroneal nerve). Compound motor evoked potential amplitude was reduced in both males and females. Positive waves and fibrillations were recorded in four out of six males and in two out of four females. Linkage study with DXS453 and PGKP1 markers was uninformative. Afterwards, aware of the role that

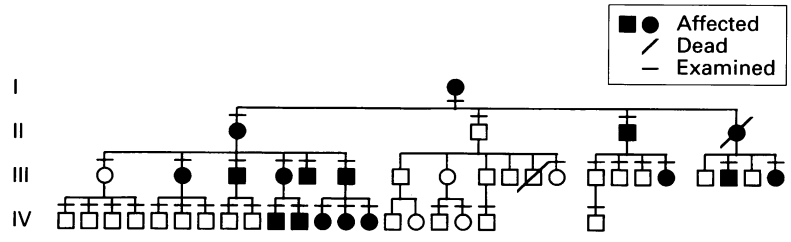

Figure 1 Pedigree of family CC1 showing an X linked dominant disease

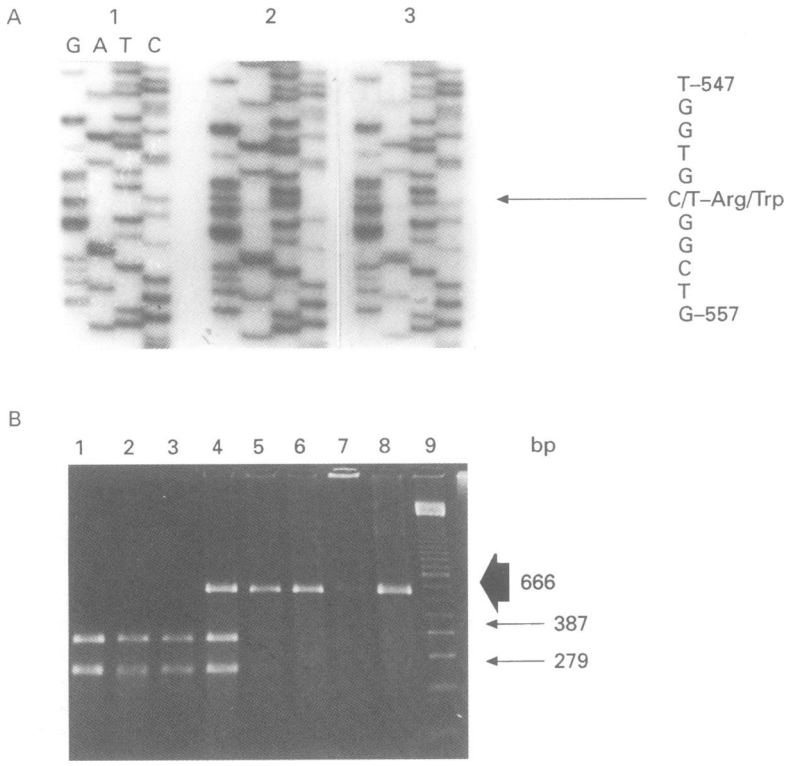

Figure 2 (A) Identification of the mutation in connexin32 (GJβ1). (1) Wild type sequence. (2) C^{552} to T transition in an affected male. (3) Sequence from an affected, heterozygote female carrying the mutation. The nucleotide numbering of the Cx32 cDNA is as reported.[13] *(B) PflMI restriction digestion of the C→T transition; lanes 1 to 3 affected males; lane 4 affected female; lanes 5 to 8 unaffected family members; lane 9 100 ladder marker. The 666 bp was digested in two fragments sized 387 bp and 279 bp respectively (arrows).*

Cx32 could play in CMTX1,[2] we directly sequenced the GJβ1 coding region in seven affected males, two obligate carriers, and six healthy subjects. All the patients and obligate carriers had a C-T transition at position 552 (fig 2A), which predicts an arginine to tryptophan substitution at amino acid 164. No additional mutations were found in the 15 subjects sequenced. As this mutation creates a *Pfl*MI restriction site and destroys an *Aci*I restriction site at position 552 (fig 2B), simplified analysis of the rest of the pedigree was carried out by restriction digestion with one or both of the endonucleases. *Pfl*MI does not digest wild type GJβ1 exons at any site of the sequence, so this enzyme was preferred to show the mutation. Moreover, GJβ1 exon 2 has many *Aci*I restriction sites and this enzyme does not digest the mutation; thus it made the scoring difficult. The mutated allele was also recognised by DP-SSCP analysis (fig 3). In addition, DNA samples from 20 unrelated females were *Pfl*MI digested to confirm that this mutation was not a polymorphism in the Canarian population. The complete cosegregration of the mutation with the disease disclosed a lod score of 10·84 at $\theta = 0$.

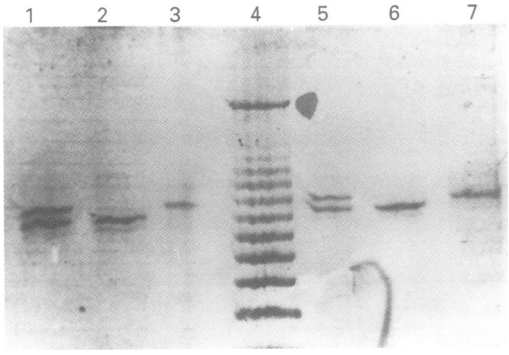

Figure 3 SSCP profiling of the 150 bp PCR fragment that includes C→T transition. Lanes 1 and 5 affected females; lanes 2 and 6 affected males; lanes 3 and 7 unaffected family members; lane 4 100 ladder marker.

Discussion

To date, this is the first reported Spanish CMTX1 family, in spite of a high prevalence of 28·2 CMT cases per 100 000 reported in the best population based study performed in our country, with no X linked pedigrees being found.[14] CMTX1 has been well established as being the result of a broad spectrum of mutations of the GJβ1 exon 2. The prevalence of dominant and recessive forms of CMTX has been established as being 3·1 per 100 000, and accounts for 10% of all CMT cases.[15] All of the molecularly analysed families have been American or European in origin. Clinical manifestations of the disease in males are qualitatively similar to those in CMT1A patients. Neurophysiological findings agree with those already found for CMTX1.[16] In fact, intermediate NCV were recorded in females and younger males carrying the mutation.

Connexin32 is a β member of the connexin family. These are membrane spanning proteins that aggregate circularly to form hexamers named connexons. Assemblage of connexons of neighbouring cells gives rise to intercellular channels named gap junctions which allow direct transfer ions and small molecules. From their amino acid sequences, two extracellular, four transmembrane, and three cytoplasmic domains have been predicted.[17] The R164K mutation has also been recently described in an American family (family 4).[6] The authors only reported *Aci*I as the enzyme used to recognise the mutation, but we preferred restriction digestion with *Pfl*MI because the mutation creates a unique restriction site in the translated region of GJβ1 gene. The R164K mutation is at the top of the second extracellular domain of the Cx32 polypeptide. The substitution of the basic arginine for the non-polar tryptophan reduces the hydrophilicity and the surface exposition probability, averaged over a window of seven amino acids encompassing the substitution, and may result in poor hemichannel interaction of pore integrity. Mutations have been reported affecting each domain and a few families shared the same mutation. This observation probably predicts geographical clustering of mutations, useful for the screening of new families. Two new Spanish families are now being studied as possible X linked dominant pedigrees.

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