

Homozygosity mapping of autosomal recessive demyelinating Charcot-Marie-Tooth neuropathy (CMT4H) to a novel locus on chromosome 12p11.21-q13.11

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Hereditary motor and sensory neuropathies, commonly referred to as Charcot-Marie-Tooth disease (CMT), are among the most common inherited neurological diseases, with an overall prevalence of about 1–4/10 000.¹ Clinically, the hereditary motor and sensory neuropathies are characterised by progressive muscular and sensory loss in the distal extremities with chronic distal weakness, deformation of the feet (pes cavus), and loss of deep tendon reflexes.² Two main subgroups have been defined on the basis of electrophysiological and histopathological characteristics: the demyelinating form (CMT1) and the axonal form (CMT2). CMT1 can be distinguished from CMT2 by measuring motor nerve conduction velocities in the median nerve: patients affected by CMT1 show reduced velocities (<38 m/s), whereas those affected by CMT2 show velocities of ≥38 m/s, the normal value being ≥48 m/s. Recently, a new group of CMT has been described, referred to as intermediate CMT^{3–4}; in this, nerve conduction velocities overlap CMT1 and CMT2, and nerve biopsies present characteristics of both demyelination and axonal loss.

CMT is also characterised by great genetic heterogeneity, with more than 30 loci and 19 genes identified to date^{5–6} (inherited peripheral neuropathy mutation database, IPNMD, <http://www.molgen.ua.ac.be/CMTMutations>). All modes of inheritance have been reported: autosomal dominant, autosomal recessive, and X linked. Autosomal recessive demyelinating forms (CMT4) are, in most cases, less frequent, of earlier onset, and more severe than the autosomal dominant CMT forms (CMT1), with a fast progression to severe disability leading to a higher frequency of wheelchair dependency early in life.⁷ To date, at least seven demyelinating forms with autosomal recessive inheritance have been identified:

- CMT4A (MIM 214400) at chromosome 8q13–q21.1^{8–9} caused by mutations in the ganglioside induced differentiation associated protein 1 gene (*GDAPI1*, MIM 606598),^{10–11} together with mixed demyelinating and axonal autosomal recessive phenotypes^{12–14};
- CMT4B1 (MIM 601382) at chromosome 11q22,¹⁵ caused by mutations in the myotubularin related protein 2 gene (*MTMR2*, MIM 603557)¹⁶;
- CMT4B2 (MIM 604563 and 607739)¹⁷ at chromosome 11p15, caused by mutations in the set binding factor 2/myotubularin related protein 13 gene (*SBF2/MTMR13*, MIM 607697)¹⁸;
- CMT4C (MIM 601596)^{19–20} at chromosome 5q23–q33, caused by mutations in *KIAA1985*,²¹ encoding a protein containing SH3 and TPR domains (MIM 608206);
- CMT4D (MIM 601455) or *HMSN-Lom*, at chromosome 8q24.3,²² caused by mutations in N-myc downstream regulated gene 1 (*NDRG1* MIM 605262)²³

Key points

- One Lebanese and one Algerian consanguineous family, comprising a total of 10 patients affected with autosomal recessive demyelinating Charcot-Marie-Tooth disease over three generations, were submitted to a genome-wide scan.
- The use of a homozygosity mapping approach showed linkage to a new locus (CMT4H) on chromosome 12p11.21–q13.11. Refinement of the linkage interval placed the CMT4H locus to a 11.5 cM region between markers D12S1648 and D12S1661, with a maximum LOD score of 6.97 ($\theta=0.001$) at marker D12S345. This interval spans about 15.8 Mb on the physical map and includes more than 90 genes.
- Mutation analysis of *PRPH* (a gene encoding a type III intermediate filament protein called peripherin) and *CNTN1* (encoding contactin 1, a neuronal cell adhesion molecule) did not show any pathogenic mutation.
- The autosomal recessive demyelinating peripheral neuropathy segregating in the two families described here represents a novel entity which we designated CMT4H.

- CMT4F at chromosome 19q13.3,²⁴ caused by mutations in the periaxin gene (*PRX*, MIM 605725)²⁵
- *HMSN-Russe*, at chromosome 10q22–q23,²⁶ for which the corresponding gene has not been cloned yet.

Finally, autosomal recessive mutations causing severe CMT1 or Dejerine-Sottas syndrome (MIM 145900) have been identified in genes most often causing autosomal dominant diseases—for example, *EGR2* (MIM 129010),²⁷ *PO* (MIM 159440),^{28–29} and *PMP22* (MIM 601097).³⁰ These are sometimes reported as CMT4E.

We report here the localisation of a new form of autosomal recessive demyelinating Charcot-Marie-Tooth neuropathy, which we named CMT4H, by using homozygosity mapping in two consanguineous families of Mediterranean origin.

METHODS

Two families, one Lebanese (family 500) and one Algerian (family 295), including 10 individuals presenting with a severe form of autosomal recessive demyelinating Charcot-Marie-Tooth disease, were included in this study (figs 1 and 3).

Abbreviations: CMT, Charcot-Marie-Tooth disease; LOD, log of odds ratio; STR, short tandem repeat

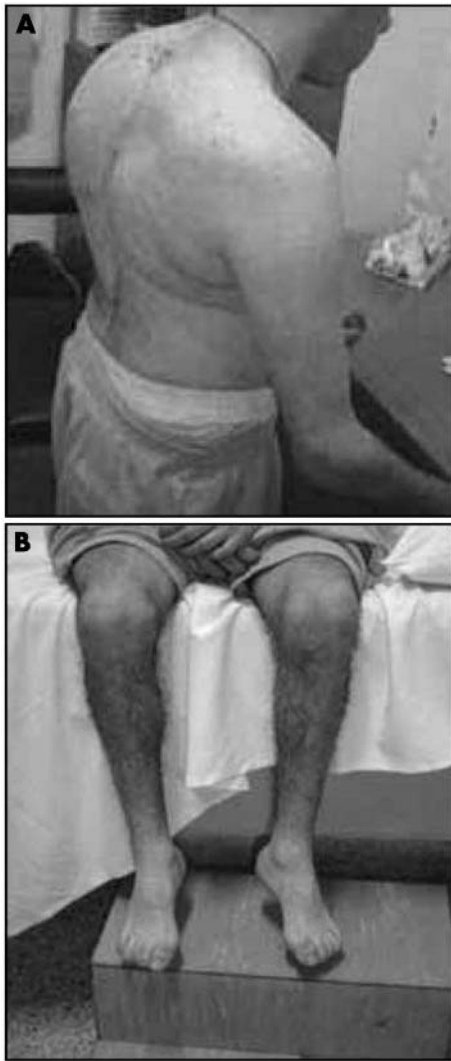


Figure 1 Severe Charcot-Marie-Tooth disease in patient 500.15 at the age of 21. (A) Deforming scoliosis is present despite spinal surgery. (B) Distal amyotrophy in the lower limbs. Reproduced with permission.

In the Lebanese family, patients 500.13, 500.14, 500.15, 500.17, and 500.21 had a clinical examination. The age at onset varied between 10 and 24 months. In all cases walking was delayed, to between 15 months to three years of age, gait was unsteady, deep tendon reflexes were absent, and mild symmetrical stocking-type hypoaesthesia was observed. All the patients examined except 500.21 presented with severe scoliosis and pes equinus with toe retraction. Patients 500.14 and 500.21 also had upper limb involvement consisting of thenar and hypothenar hypoplasia. Pupillary abnormalities, ataxia, nerve enlargement, tremor, deafness, and diaphragmatic or vocal cord paresis were absent in all patients. Progression of the disease was invariably slow.

Electrophysiological studies at the median nerve were carried out on Lebanese patients 500.14, 500.15, 500.17, and 500.21. Sensory action potentials could not be obtained, either in the median or in the sural or peroneal nerves. Severely reduced motor nerve conduction velocities at the median and cubital nerves, as well as low amplitude action potentials with prolonged distal latencies, indicated a peripheral nerve demyelinating process. A histological study of a sural nerve biopsy specimen was done in Lebanese patient 500.14 and showed a severe loss of myelinated fibres,

probably secondary to a demyelination–remyelination process (fig 2). The remaining fibres had features of congenital hypomyelination as well as a small proportion of typical onion bulbs. Other signs of altered myelination were observed, such as circumscribed myelin swellings and proliferation, with or without myelin outfoldings (fig 2).

In the Algerian family, clinical and electrophysiological investigations were carried out in patient 295.6, but all patients had the same characteristics. The age of onset was two years. Neurological examination showed muscle weakness and amyotrophy in the distal extremities, marked feet abnormalities (pes cavus), absent tendon reflexes in the four limbs, ataxia, and a waddling gait. No cranial nerve abnormalities were noted. Dysmorphic features such as scoliosis and a short neck were present. Progression was slow until the age of 15. Electrophysiological results showed a severe demyelinating motor and sensory neuropathy, with similar results to family 500. Histological studies of a sural nerve biopsy were undertaken in Algerian patient 295.6, and the results were very similar to those obtained in the Lebanese patients (data not shown).

We investigated 29 individuals at the genetic level. After informed consent had been obtained from all affected individuals and parents of the children, EDTA blood samples were collected and genomic DNA was extracted from lymphocytes using standard methods. Exploration protocols were in accordance with the ethics guidelines of the institutions involved. After exclusion of linkage to loci CMT4A⁹ and CMT4F,²⁴ a genome-wide screen was subsequently undertaken at the Centre National de Génotypage (CNG, Evry, France), using 400 polymorphic microsatellite markers with an average intermarker distance of 10 cM, as previously described.²⁴

In order to refine the size of the shared homozygous region, the following additional fluorescently labelled STR markers—chosen from the Généthon linkage map³¹—were tested on all individuals collected in the two families: D12S1643 (AFMb013yb1), D12S1631 (AFMa288wd5), D12S1698 (AFM337tf5), D12S1648 (AFMb041xb9), D12S1653 (AFMb283xh5), D12S1661 (AFMb314yh5), D12S339 (AFM294wc5), D12S1635 (AFM196xa3), and D12S1677 (AFMb347vb9). Markers were amplified by polymerase chain reaction (PCR) under standard conditions and amplified fragments were separated by capillary electrophoresis on a Beckman CEQ 8800 genetic analyser (Beckman Coulter).

Parametric linkage analyses were carried out with an optimised version of the LINKAGE package, version 5.2,³² available free at Infobiogen (<http://www.infobiogen.fr>). Pairwise LOD scores were calculated using the MLINK program, assuming equal male–female recombination rates, autosomal recessive inheritance with complete penetrance, and a gene frequency of 0.002. Allele frequencies were chosen from the Genome Database (<http://www.qdb.org>), assuming that they were the same as those defined by Généthon in the white population.³³

The coding sequence and exon–intron junctions of two candidate genes (*PRPH* and *CNTN1*) were explored. Intronic primers were designed using the Primer3 software, available at the Massachusetts Institute of Technology (MIT, <http://frodo.wi.mit.edu/>) and DNA sequences obtained at the University of California Santa Cruz Human Genome Browser (UCSC, July 2003 freeze, <http://genome.ucsc.edu/>), by comparing genomic DNA with cDNA sequence (GenBank Accession Numbers: NM_001843 and NM_006262 for *CNTN1* and *PRPH* respectively). Genomic DNA was amplified for one patient in each family under standard PCR conditions (primer sequences and amplification conditions are available on request). Amplified PCR fragments were fluorescently sequenced in both directions using sequencing facilities

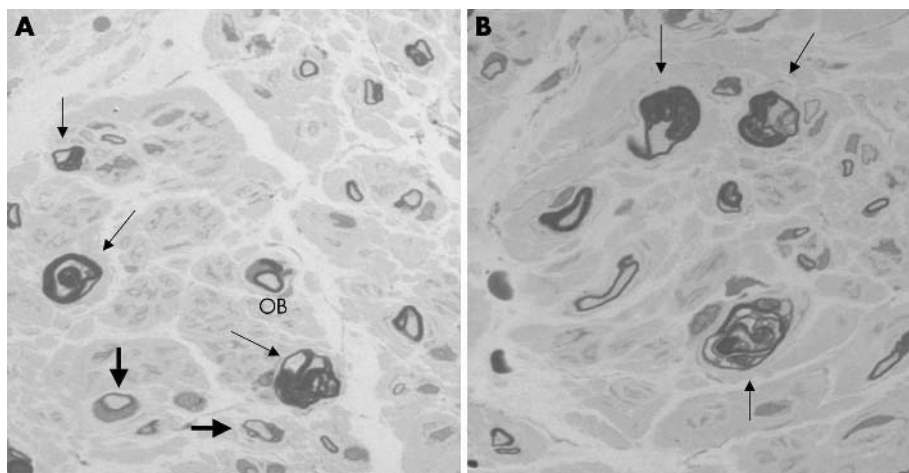


Figure 2 Histopathology of sural nerve from Lebanese patient 500.14. Semithin transverse section included in epon epoxy and coloured with blue toluidine. Original magnification $\times 540$. (A) Myelinated fibres are rare and generally surrounded by small onion bulbs (OB). The remaining fibres have small diameter hypomyelinated axons (thick arrows), or abnormal proliferation of myelin sheath with outfoldings (thin arrows). (B) Details of myelin outfoldings, typical of an initial demyelination-remyelination process.

(MWG Biotech, Ebersberg, Germany). Chromatograms were compared with reference sequences using Sequencher v4.2 (Gene Codes Corporation, Michigan, USA).

RESULTS

We used a homozygosity mapping strategy, based on the assumption that in consanguineous families affected subjects of the same kindred are homozygous by descent for the disease causing mutation.³⁴ Haplotypes were constructed manually from genotyping data and phases were assigned on the basis of the smallest number of recombinants. Analysis of the initial genome-wide screen data showed homozygosity in all affected individuals at markers D12S345 (AFM296yg5) and D12S85 (AFM122xf6) on chromosome 12q13.11. Significant linkage was obtained with a cumulative LOD score value of 6.97 and 5.09 at $\theta = 0.001$ respectively (table 1), although D12S85 was not fully informative in the Lebanese family (fig 3).

The minimum candidate interval at this point was restricted by D12S1617 (AFMa223yg1) on 12p12.1 and D12S368 (AFM128yd5) on 12q13.13, covering a 25.8 cM genetic region.

Additional genotyping of the nine STR markers allowed refinement of the candidate homozygous region in Lebanese patient 500.21 to a critical interval of approximately 11.5 cM between markers D12S1648 (AFMb041xb9) on the short arm of chromosome 12 (12p11.21) and D12S1661 (AFMb314yh5)

on the long arm of chromosome 12 (12q13.11) (figs 3 and 4). The size of the interval is approximate because it overlaps the centromeric region of chromosome 12. In the candidate region defined above, all affected individuals were homozygous for the disease allele, and markers were informative in all matings, with the exception of marker D12S85, which was not fully informative in the Lebanese family (fig 3). All markers in the homozygous region show positive LOD score values with a maximum pairwise LOD score of 6.97 at $\theta = 0.001$ for marker D12S345 (table 1). More than 90 genes are described in the databases (GENATLAS, <http://www.genatlas.org>; UCSC, July 2003 freeze, <http://genome.ucsc.edu/>) within the candidate homozygous region.

No sequence variation was found in the coding sequence or in the exon-intron junctions of two genes, *CNTN1* and *PRPH*. This probably excludes them as the genes responsible for CMT4H. However, further genotyping mapped *PRPH* outside the candidate interval (fig 4).

DISCUSSION

We describe the assignment of a new locus for autosomal recessive demyelinating Charcot-Marie-Tooth disease (CMT4H) to a genetic interval of approximately 11.5 cM at chromosome 12p11.1–q13.11 in an Algerian family and a large consanguineous Lebanese family. Disease haplotypes were not shared by the two families, excluding a founder origin of the disease. As this locus is the eighth one that has

Table 1 Two point LOD scores between the CMT4H locus and informative STR markers on chromosome 12p11.1–q13.11

Marker	LOD score at recombination fraction $\theta =$						
	0.001	0.01	0.05	0.1	0.2	0.3	0.4
D12S1643	0.71	2.01	2.80	2.77	2.11	1.24	0.45
D12S1631	-0.54	0.93	1.86	1.99	1.60	0.94	0.30
D12S1698	-0.51	0.89	1.76	1.79	1.24	0.56	0.09
D12S1648	5.64	6.28	6.27	5.71	4.20	2.52	0.96
D12S345	6.97	6.82	6.20	5.39	3.72	2.07	0.70
D12S1653	5.81	5.68	5.12	4.42	2.99	1.63	0.56
D12S85	5.09	4.97	4.51	3.92	2.70	1.50	0.54
D12S1661	3.35	3.92	4.02	3.63	2.51	1.31	0.36
D12S339	-∞	1.93	2.85	2.88	2.26	1.37	0.52
D12S1635	-∞	1.12	2.49	2.85	2.55	1.75	0.80
D12S1677	-∞	1.01	1.98	2.14	1.79	1.15	0.46

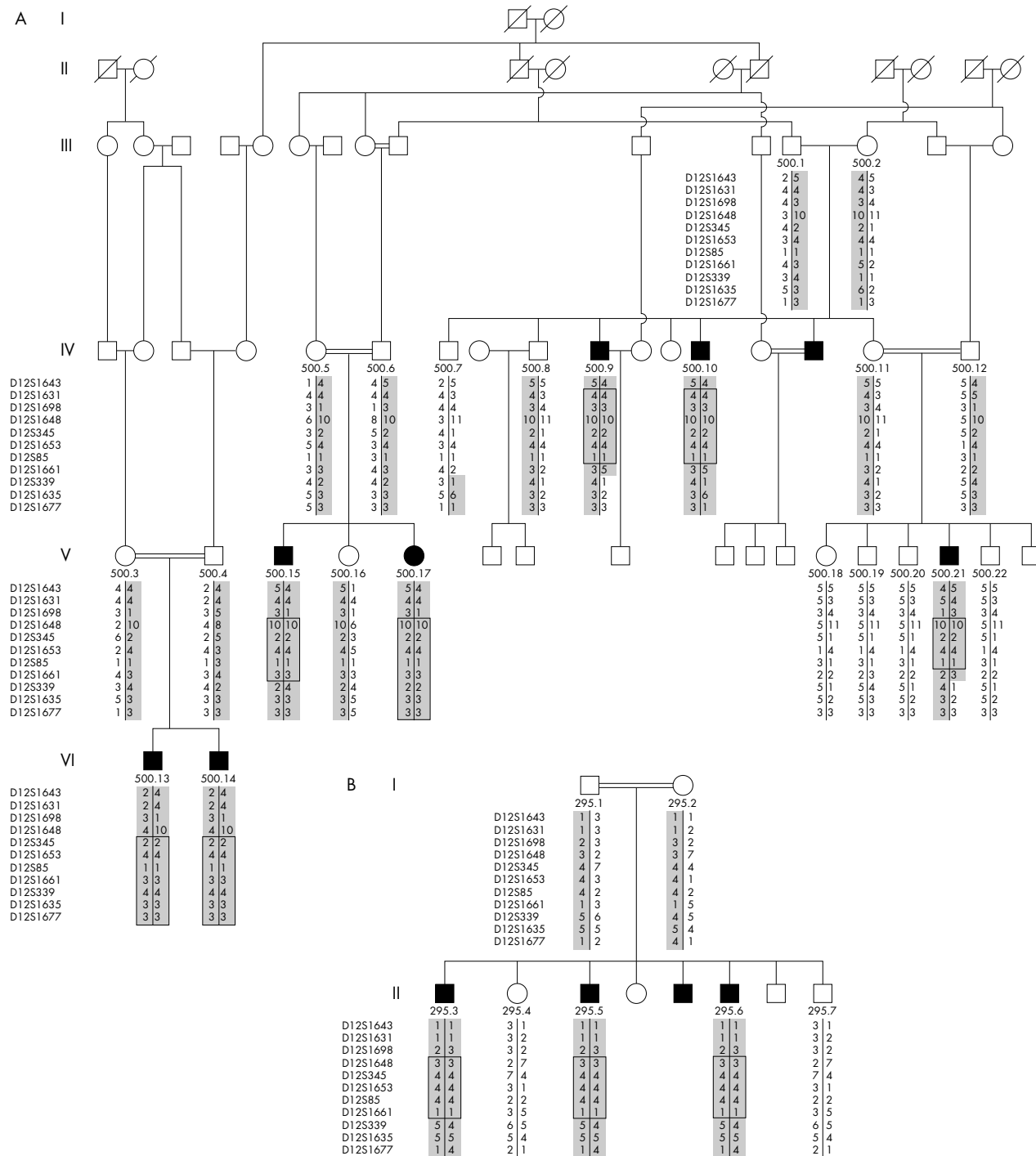


Figure 3 (A) Haplotype reconstruction in a Lebanese family (family 500) presenting with an autosomal recessive demyelinating form of Charcot-Marie-Tooth disease (CMT4H). (B) Haplotype reconstruction in an Algerian family (family 295) presenting with CMT4H. Short tandem repeat (STR) markers are reported from the telomere of the short arm (12p) to the telomere of the long arm (12q) of chromosome 12. Black symbols indicate affected individuals. The disease bearing chromosome is represented in grey. The homozygous candidate interval is boxed in each affected individual.

been mapped for autosomal recessive demyelinating CMT (CMT4), we named it CMT4H, considering that HMSN-Russe should be named CMT4G.

Recently, a locus for an autosomal dominant form of axonal CMT (CMT2G) has been mapped to the same chromosomal region,³⁵ in an interval which partially overlaps the candidate region for CMT4H (fig 4). The overlapping region is 7.6 cM long (4.33 Mb) and contains about 35 genes.

Although the modes of transmission and the phenotypes are different between CMT2G and CMT4H, the two diseases could still be allelic. Indeed, mutations in several genes responsible for demyelinating CMT also result in axonal CMT—that is, *GDAP1* mutations can result either in a demyelinating form of CMT (CMT4A)¹⁰ or in an axonal form (AR-CMT2C),¹¹ but both of these have an autosomal recessive mode of inheritance. However, mutations in the same gene can be found in a heterozygous or a homozygous state; this is

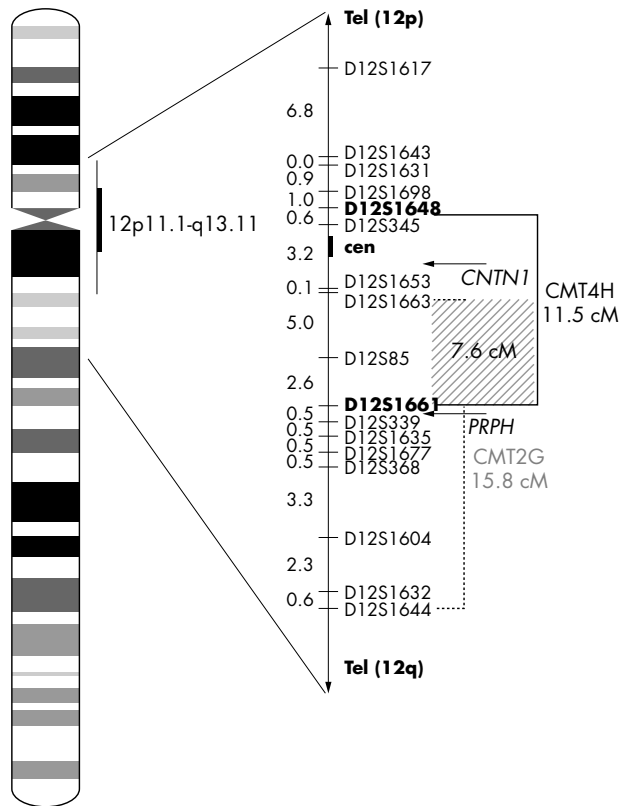


Figure 4 Schematic representation of the Charcot-Marie-Tooth disease CMT4H candidate region at chromosome 12p11.1-q13.11, indicating the partial overlap with the CMT2G locus. Candidate intervals for CMT4H and CMT2G are represented by bold and dotted lines, respectively. Marker order was obtained from the UCSC human genome browser (July 2003 Freeze) and genetic distances, indicated in cM at the left, were obtained from the Génethon human genetic linkage map.³¹ Markers in bold represent the upper and lower limits of the CMT4H interval. The CMT2G/CMT4H overlapping region (7.6 cM) is represented by a dashed box. The two candidate genes screened in our study are indicated by arrows.

the case for mutations in *PMP22*, which usually cause autosomal dominant CMT1A,^{30,36} but are sometimes associated with a recessive pattern of inheritance.³⁷ Nonetheless, fewer than half the CMT2G interval at the distal extremity overlap with the CMT4H critical region, suggesting that mutations in two distinct genes are likely to cause CMT2G and CMT4H. Further screening of candidate genes in families affected by CMT2G and in those presenting with CMT4H should definitely exclude the possibility that both diseases are allelic.

Of the 90 genes described in the databases (GENATLAS, <http://www.genatlas.org>) in the homozygous candidate, two candidate genes were tested for the presence of mutations in the coding sequence. The first, *PRPH*, encodes a type III intermediate filament protein called peripherin, important for neurite elongation both during development and during axonal regeneration in the peripheral nervous system.³⁸ Our choice is supported by the fact that mutations in genes encoding proteins from the intermediate filament family, *NEFL* and *LMNA*, have previously been identified in other forms of peripheral neuropathy: mutations in *NEFL* cause CMT2E³⁹ and CMT1F,⁴⁰ while mutations in *LMNA* are responsible for AR-CMT2A.⁴¹ Another gene, Contactin 1 (*CNTN1*), was then considered as a strong candidate gene. It encodes a neuronal cell adhesion molecule of the immunoglobulin superfamily, which mediates cell surface

interactions during nervous system development. More precisely, in association with other proteins it plays a role in the formation of paranodal axoglial junctions in myelinated peripheral nerve.⁴² Although these functions are suggestive of implication in CMT, no disease causing mutations were identified in either gene, and *PRPH* has definitely been excluded as the causative gene for CMT4H, as it has been mapped outside the candidate interval by further genotyping (fig 4).

Further investigations are being undertaken to reduce the linkage interval and identify the molecular defect responsible for CMT4H. As the two families described here are from different ethnic backgrounds, it will be of value to test linkage to the CMT4H locus in more families presenting with severe demyelinating autosomal recessive CMT. A candidate gene screening strategy is also in process.

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