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Report

Mapping of a New Locus for Autosomal Recessive Demyelinating Charcot-Marie-Tooth Disease to 19q13.1-13.3 in a Large Consanguineous Lebanese Family: Exclusion of *MAG* as a Candidate Gene

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Autosomal recessive Charcot-Marie-Tooth disease (CMT) type 4 (CMT4) is a complex group of demyelinating hereditary motor and sensory neuropathies presenting genetic heterogeneity. Five different subtypes that correspond to six different chromosomal locations have been described. We hereby report a large inbred Lebanese family affected with autosomal recessive CMT4, in whom we have excluded linkage to the already-known loci. The results of a genomewide search demonstrated linkage to a locus on chromosome 19q13.1-13.3, over an 8.5-cM interval between markers D19S220 and D19S412. A maximum pairwise LOD score of 5.37 for marker D19S420, at recombination fraction [θ] .00, and a multipoint LOD score of 10.3 for marker D19S881, at θ = .00, strongly supported linkage to this locus. Clinical features and the results of histopathologic studies confirm that the disease affecting this family constitutes a previously unknown demyelinating autosomal recessive CMT subtype known as "CMT4F." The myelin-associated glycoprotein (*MAG*) gene, located on 19q13.1 and specifically expressed in the CNS and the peripheral nervous system, was ruled out as being the gene responsible for this form of CMT.

Hereditary motor and sensory neuropathy (HMSN), also known as "Charcot-Marie-Tooth disease" (CMT [MIM 118300]), is a heterogeneous group of disorders characterized by chronic distal weakness with progressive muscular atrophy and sensory loss in the distal extremities (Dyck et al. 1993). With a frequency of ~1/2,500, CMT is the most common inherited neurological disorder (Skre et al. 1974). Clinically, two major groups are defined; they are differentiated on the basis of peripheral nerve pathology and the results of nerve conduction velocity (NCV) studies. CMT type 1 (CMT1) is characterized by low NCVs resulting from demyelination in the motor and sensory nerves, whereas CMT type 2 (CMT2) refers to neuropathies with normal NCVs and no evidence of demyelination (Bell 1997). Modes of inheritance could be either autosomal dominant, autosomal recessive, or X-linked. Autosomal recessive demyelinating CMT type 4 (CMT4) is less frequent and more severe than the two other modes of inheritance. Five different CMT4 subtypes, each of which presents particular ethnic, pathological, or clinical characteristics, are known. They are CMT4A (MIM 214400) on 8q13-21.1 (Ben Othmane et al. 1993); CMT4B, which includes CMT4B1 (MIM 601382) on 11q22 (Bolino et al. 1996), caused by mutations in the *MTMR2* gene (MIM 603557) encoding myotubularin-related protein-2 (Bolino et al. 2000), and CMT4B2 (MIM 604563) on 11p15 (Ben Othmane et al. 1999); CMT4C (MIM 601596) on 5q23-33 (Leguern et al. 1996); CMT4D (MIM 601455) on 8q24 (Kalaydjieva et al. 1996); and CMT4E on

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10q21-22. The fifth subtype, CMT4E, corresponds to a congenital hypomyelinating neuropathy in which a homozygous missense mutation has been identified in an early-growth-response gene (*EGR2;* Warner et al. 1998).

We now report the assignment of a new locus for an autosomal recessive CMT4 subtype on chromosome 19q13.1-13.3 in a large inbred Lebanese family, using homozygosity mapping (Lander and Botstein 1987) and DNA pooling. The family presented with clinical features that may define a new CMT4F subtype. Involvement of the myelin-associated glycoprotein (*MAG*) gene, which is located on 19q13.1 and is specifically expressed in Schwann cells in the peripheral nervous system or in oligodendrocytes in the CNS, was ruled out in this study.

Thirteen members of a Shiite Muslim family originating from a village in the south of Lebanon were investigated (fig. 1). All affected members, with the exception of patient VI-6, who was too young to present all the clinical features, had nearly identical clinical, radiological, and laboratory findings and were therefore described together.

Gestation and delivery were unremarkable, with no history of exposure to pre- or perinatal environmental toxins in the family. All patients had a delay in motor development. They started sitting by age 12–18 mo and began walking and talking at age ~2 years. From the beginning, gait was described as wide-based and mildly ataxic. At age 9–10 years, lower-limb distal muscle weakness and atrophy with pes cavus deformity were evident. By age 14–15 years, weakness of the upper limbs—in particular, of the hands—and finger retraction appeared. Upon examination, head circumferences and statures were normal. The neurological examination showed important sensory ataxia, bilateral steppage gait, mild kyphoscoliosis, symmetrical atrophy of intrinsic hand muscles and of muscles below the knees, pes cavus deformity, slightly diminished distal muscle strength of the upper and lower limbs, with paresthesia

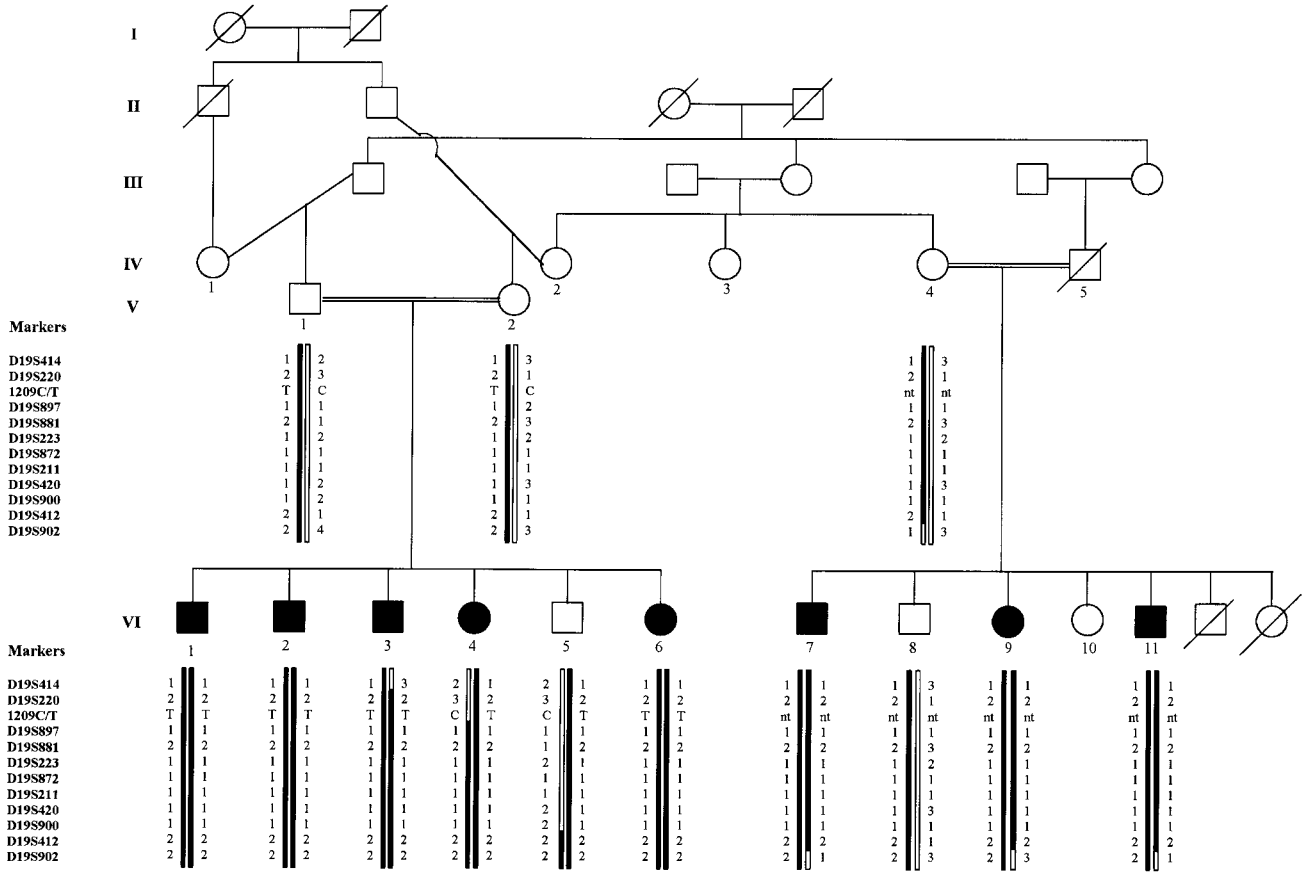

Figure 1 Haplotype reconstruction in a Lebanese family affected with autosomal recessive demyelinating CMT at 19q13.1-13.3. Markers are reported from centromere (*top*) to telomere (*bottom*). 1209C/T denotes a polymorphism in *MAG*. Blackened symbols represent affected individuals. The disease-bearing chromosome is blackened. nt = not tested.

Table 1

Pairwise LOD Scores between the CMT4F Locus and 11 Marker Loci on 19q13.1-13.3

	LOD Score at θ =							Maximum Pairwise	
Marker	.0001	.01	.05	.1	.2	.3	.4	LOD Score	Maximum θ
D19S414	$-\infty$	1.67	2.67	2.75	2.27	1.51	.69	2.77	.084
D19S220	$-\infty$	3.12	3.41	3.20	2.42	1.49	.57	3.42	.043
D19S897	2.81	2.76	2.54	2.25	1.66	1.04	.47	2.81	.001
D19S881	5.14	5.04	4.63	4.09	2.99	1.84	.75	5.14	.001
D19S223	4.88	4.78	4.37	3.86	2.80	1.71	.65	4.88	.001
D19S872	.19	.18	.16	.12	.07	.03	.01	.19	.001
D19S211	.38	.37	.31	.25	.15	.07	.02	.38	.001
D19S420	5.37	5.26	4.83	4.28	3.12	1.92	.77	5.37	.001
D19S900	2.05	2.01	1.84	1.62	1.18	.75	.33	2.05	.001
D19S412	$-\infty$	2.37	2.68	2.49	1.78	.92	.13	2.68	.045
D19S902	$-\infty$	−1.81	−.08	.40	.46	.17	−.06	.51	.154

on the distal portions of the lower limbs, absent osteotendinous reflexes in four limbs, and distal sensory abnormalities—including deficits of position, vibration, pain, and temperature—in both the upper and lower extremities. No seizures, cranial nerve abnormalities, or facial weakness were noted. Intelligence and speech were normal. The progression of the disease had been very slow. Only patient VI-7 could no longer walk, essentially because of a severe left pes equinovarus. Total body X-rays revealed the presence of scoliosis in all patients. Magnetic-resonance imaging (MRI), which was performed for patients VI-1 and VI-2, showed a large cisterna magna. The results of abdominal ultrasound, echocardiography, and electroencephalography; evoked potentials; auditory brain-stem responses; and the results of ophthalmologic evaluation performed in some of the patients were all unremarkable. The results of routine laboratory tests as well as levels of plasma very-long-chain fatty acids and results of white-blood-cell enzyme assays were within the normal range. The karyotype was normal (46,XY; patient VI-1). The results of NCV studies performed on five affected individuals revealed the total absence of any sensory or motor evoked response in the upper and lower limbs. Electromyograms (EMGs) showed normal insertional activity, absence of spontaneous activity, and moderately reduced recruitment of motor-unit potentials in the distal muscles of four limbs. The results of EMG and NCV studies performed on parents were completely normal.

Histopathologic examination of the whole sural nerve–biopsy specimen from patient VI-1 disclosed a severe depletion of myelinated fibers (estimated value of density <1,000 fibers/mm^2) and numerous extensive concentric Schwann-cell proliferation with multiple small onion bulbs isolated or surrounding the rare remaining myelinated fibers. These data indicated a severe axonal loss, following an initial process of chronic demyelination-remyelination.

After familial investigations, EDTA blood samples were collected for genetic studies. Informed consent was obtained from all adults and from parents of children included in the study. DNA was extracted from lymphocytes by use of standard methods (Miller et al. 1988). A pooling strategy (for review, see Sheffield et al. [1995]) was used in the first genomewide screening. Three separate DNA pools—the "affected" pool, the "obligate-carrier" pool, and the "unaffected" pool—were prepared by mixing equimolar quantities of individual DNAs. The screening set comprised 400 highly polymorphic fluorescently labeled markers from the ABI PRISM Linkage Map Set, version 2.0 (PE Biosystems), that had an average spacing of 10–20 cM and were chosen from the Généthon linkage map (Dib et al. 1996). Amplifications were performed in a 7.5-μl final volume on 40 ng pooled DNA. The cycling conditions used were those recommended by the manufacturer (PE Biosystems). Pooled PCR products were separated on a 5% Long Ranger denaturing urea-polyacrylamide gel and were analyzed on an ABI 377 DNA sequencer (PE Biosystems). Peaks were analyzed with GENESCAN, version 3.1, and GENOTYPER, version 2.1 (PE Biosystems).

Several zones of homozygosity, which showed reduction to homozygosity in the affected pool, were selected for genotyping of individual samples. Nevertheless, only marker D19S420 (AFM326xh9) on 19q13.2 was found to be informative in the parents and homozygous in all the affected individuals and was selected for further genotyping. To determine the size of the shared homozygous region, 10 new microsatellite markers surrounding D19S420 were selected from the Généthon human linkage map and were genotyped in all individuals. Recombination events restricted the homozygous candidate region to an 8.5-cM interval distal to D19S220 (AFM214yf12) and proximal to D19S412 (AFM284yg) (fig. 1).

Parametric linkage analysis was performed with an

optimized version of the LINKAGE package, version 5.2 (Lathrop et al. 1985). Pairwise LOD scores and multipoint LOD scores were calculated using programs from FASTLINK, version 3.0P. The disease was assumed to be caused by a fully penetrant autosomal recessive gene with a frequency of .002. Allele frequencies for microsatellite markers were chosen from the Genome Database and were assumed to be the same as those defined by Généthon for the Caucasian population (Lefranc et al. 1978).

A maximal pairwise LOD score of 5.37 was obtained for D19S420 (table 1), and multipoint LOD-score analysis gave a maximum of 10.3 for D19S881, at θ = .00 (fig. 2). Markers D19S872 and D19S211 showed poor LOD-score values (table 1) as a result of their lack of informativity. The other markers were informative in all matings, and all affected individuals were homozygous for the disease allele.

Of all the different genes already mapped to 19q13.1-13.3, the most likely candidate gene for this particular form of CMT was the *MAG* gene on 19q13.1. Sequencing of its coding sequence was consequently performed in two affected children (patients VI-2 and VI-4) and a control individual. Total RNA was extracted from lymphocytes by use of the total RNA isolation kit (PROMEGA), and the *MAG* cDNA was sequenced after reverse transcription (M-MLV Reverse Transcriptase [Gibco BRL]) in illegitimate transcripts (Chelly et al. 1991). Fragments used for sequencing were obtained by means of a two-step PCR amplification process using reverse-transcribed cDNA as a template. However, as a result of the high GC

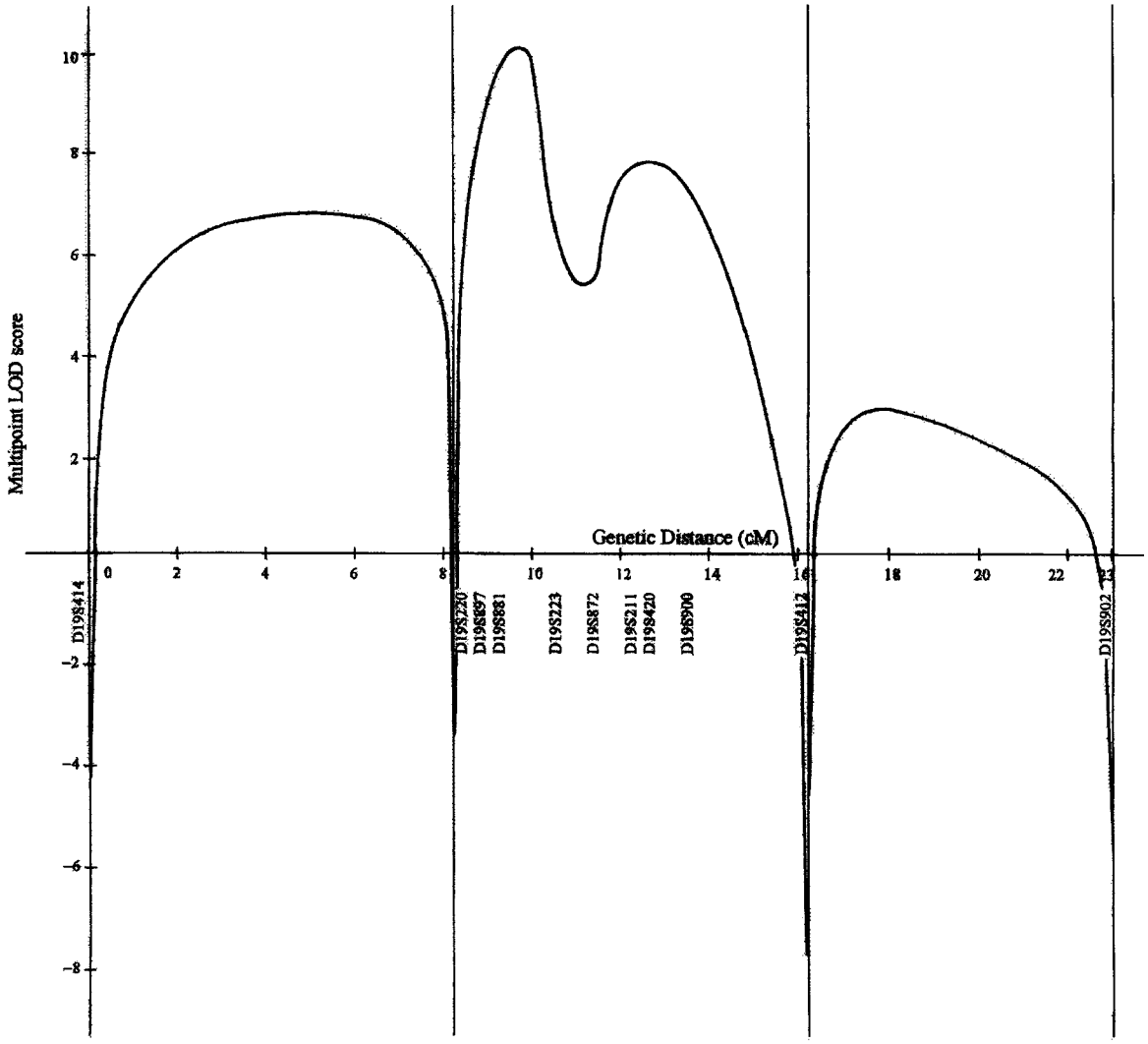

Figure 2 Multipoint LOD-score analysis of the CMT4F locus in the interval from D19S414 (centromeric) to D19S902 (telomeric). The *Y*-axis represents the multipoint LOD score ($\log_{10}$ value), and the *X*-axis represents the genetic distance (in cM) along the interval.

content of the amplified products (55%–75%), amplification was obtained only when the PCRx Enhancer System (Gibco BRL) was used, and the 5′ extremity of the cDNA fragment (684 bp) could not be synthesized. Exons 5–7, which corresponded to the unamplified 5′ cDNA sequence, were consequently amplified from genomic DNA. PCR primers and annealing conditions are described in table 2.

Amplification products were sequenced in both directions, with use of the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) on an ABI 377 sequencer (PE Biosystems). Electrophoregrams were analyzed using Sequence Analysis software, version 3.3 (PE Biosystems). Sequences were compared with reference sequences, by use of Sequence Navigator, version 1.0.1 (PE Biosystems). The reference sequences for cDNA (GenBank accession number M29273) and genomic DNA (GenBank accession number AC002132) were chosen from the GenBank sequence library.

The results of sequencing of the *MAG* coding region in two affected patients and a control individual showed neither a base change leading to an amino acid change nor a deletion, but some discrepancies were observed between the originally published cDNA sequence (Sato et al. 1989) and the cDNA and genomic DNA sequences used as references. A 399C→T change in exon 5, a 1140C→G change in exon 8, and a 1981C→G change in the 3′ UTR region were not taken into consideration, considering that 399T, 1140G, and 1981G were correct in any of the three sequences. Finally, 1989insG was observed in the 3′ UTR region in the affected patients as well as in the control individual, suggesting a polymorphism or an error in the originally published cDNA sequence. Alternative splicing of the 45-bp exon 12

Table 2

Primer Sequences for PCR Amplification and Sequencing of the *MAG* Coding Sequence

Reaction and Primer Sequence	Position (5′)[a]	Amplification-Product Size (bp)	Annealing Temperature (°C)
RT+PCR1[b]:			
1:			
Forward: 5′-AGGTGCAGAAGCAACTGAGT-3′	−108	1,212	58
Reverse: 5′-CGTGGACAGGATCTGCTTCT-3′	1225		
2:			
Forward: 5′-GGGACAATGGTGGCCGTA-3′	996	1,084	64
Reverse: 5′-AAGGAGGTTAGAGAGGAGCC-3′	2100		
Nested PCR2s[b]:			
1a:			
Forward: 5′-ATGGTGCCGGACAACTGC-3′	477	684	56
Reverse: 5′-GCTTCTCCTTGAAGATGGTGA-3′	1090		
2a:			
Forward: 5′-GGGGGAGACGGTCTCTAT-3′	1016	568	56
Reverse: 5′-GGCAATCAGGATGGCAAAGG-3′	1584		
2b:			
Forward: 5′-TTCCAGGGAGCCCATCGA-3′	1506	505	64
Reverse: 5′-CCTCACTGTCACCTGCCT-3′	2011		
2b′:			
Forward: 5′-AGAAGTACGAGAGAGCGAGAGG-3′	1705	305	60
Reverse: 5′-CCTCACTGTCACCTGCCT-3′	2011		
Genomic PCRs[c]:			
Exon 4:			
Forward: 5′-GTGTCACTGCATTCCAGCCC-3′	IVS3-207	375	56
Reverse: 5′-GTCCTACCCGATCACCCC-3′	IVS4+120		
Exon 5:			
Forward: 5′-CTGAGCAGGTTGGAGGTGGG-3′	IVS4-117	578	66
Reverse: 5′-TGCTGGGGAAGGTGCGGG-3′	IVS5+92		
Exon 6:			
Forward: 5′-GGAGGCGATACCGTTGAGGAG-3′	IVS5-128	502	56
Reverse: 5′-CCGCATCCCCAGGTCCAC-3′	IVS6+77		
Exon 7:			
Forward: 5′-GACAGTGTTGGGGGCGGG-3′	IVS6-91	558	56
Reverse: 5′-AAGGAGGATGAGGCAGAGCAGCAGG-3′	IVS7+208		

[a] Numbering is according to the cDNA sequence published by Sato et al. (1989)

[b] Designed according to the cDNA sequences (GenBank accession number M29273).

[c] Designed according to the genomic DNA sequences (GenBank accession number AC002132).

Table 3

Major Clinical Features of Autosomal Recessive Demyelinating CMT4

CMT4 Subtype (Location)	Clinical Signs[a]							MNCV[b] (m/s)	Pathological Characteristics[c]
	Age at Onset	Muscle Weakness	Tendon Reflexes	Sensory Loss	Disease Progression	KS	Foot Deformity		
CMT4A (8q13-21)	Early childhood	Distal	−	Mild	Incapacity, 1st decade	+	+	Reduced, ~30	Hypomyelination; loss of MF; presence of basal laminal onion bulbs
CMT4B1 (11q23)	2–4 years	Distal and proximal	−	Moderate	Death, 4th decade	+	+	Slow, 15–17	Loss of MF; presence of focally folded myelin sheaths
CMT4B2 (11p15)	<20 years	Distal and proximal	−	Moderate	Incapacity, 3d–4th decade	+	+	Reduced to slow, 15–30	Loss of MF; presence of focally folded myelin sheaths
CMT4C (5q32)	Childhood and adolescence	Delayed walking	Reduced	Mild	Increased disability	+	+	Reduced, ~24	Loss of MF; presence of few classical onion bulbs
CMT4D (8q24)	Childhood	Distal	−	Moderate, deafness	Incapacity, 5th decade	+	+	Slow, 10–15	Loss of MF; presence of onion bulbs
CMT4E (10q21-22)	Birth	Distal	−	Moderate	Increased disability	−	+	Slow, 9–20	Thin or absent myelin sheaths; presence of onion bulbs
CMT4F (19q13.1-13.3)	Early childhood	Distal	−	Severe	Slow progression	+	+	Not detectable	Severe loss of MF; presence of onion bulbs

[a] A plus sign (+) denotes presence; a minus sign (−) denotes absence. KS = kyphoscoliosis.
[b] MNCV = motor NCV.
[c] MF = myelinated fibers.

(GenBank accession number X98405) was observed in the two affected patients as well as in the control individual. A 1209C/T polymorphism was detected in patients VI-2 and VI-4 and in the control individual. Since patient VI-4 was heterozygous for the polymorphism, the affected sibs (patients VI-1, VI-3, and VI-6), the unaffected brother (patient VI-5), and the parents (patients V-1 and V-2) were also tested for the polymorphism. The other branch of the family was not tested for this polymorphism, since mRNA was not available. Patients VI-1–VI-3 and patient VI-6 were homozygous for the change, whereas the affected sister (patient VI-4), her unaffected brother (patient VI-5), and the parents were heterozygous for the change (fig. 1). Assuming identity by descent and considering that patient VI-4 presented a recombination at marker D19S220, we were able to place *MAG* outside the homozygous candidate region because of the heterozygosity for this polymorphism in patient VI-4.

In the present study, we report a large inbred Lebanese family in whom affected members present the heterogeneous neurological defects commonly seen in autosomal recessive CMT4. Five pathological forms, CMT4A–E, are now recognized (Ben Othmane et al. 1993; Bolino et al. 1996; Kalaydjieva et al. 1996; Leguern et al. 1996; Warner et al. 1998; Ben Othmane et al. 1999). Clinical descriptions of these different forms and of the form CMT4F presented here are summarized in table 3. Although the clinical symptoms of the affected sibs in this report are similar to —if somewhat more severe than—those in the other forms of CMT4, there is enough genetic evidence to suggest a new CMT4F subtype. Furthermore, the results of a genomewide search excluded linkage to previously described autosomal recessive CMT4 loci and placed the gene of interest distal to D19S220 and proximal to D19S412, over an 8.5-cM interval at 19q13.1-13.3. The positive pairwise LOD score of 5.37 for D19S420, at $\theta = .00$, and the maximum multipoint LOD score of 10.3 for D19S881, at $\theta = .00$, strongly supported linkage of the disease gene to the aforementioned region.

Of all the different genes already mapped to 19q13.1-13.3, the most likely candidate gene for this particular form of CMT was the *MAG* gene on 19q13.1. Indeed, although the role of *MAG* in myelination is still unclear (Attia et al. 1989; Li et al. 1994), its gene encodes a transmembrane glycoprotein of the CNS and the peripheral nervous system that is thought to play a role in the formation and maintenance of the myelin sheaths (Quarles et al. 1983–84). Furthermore, the presence of anti-*MAG* antibodies in the serum of patients affected with some demyelinating sensory or sensorimotor neuropathies and the overexpression of a shorter alternatively spliced *MAG* mRNA in dysmyelinating mutant mice have been reported (Frail et al. 1985; Sato et al. 1986). Nevertheless, none of the abnormalities usually observed in nerve-biopsy specimens from mice, which have a knocked-out *MAG* gene (Montag et al. 1994), were observed in the nerve-biopsy specimen from patient VI-1. Also, sequencing of the entire *MAG* gene-coding region in two affected patients revealed neither a base change nor a deletion, most probably excluding a point mutation in the coding region as the cause of the disease. Finally, heterozygosity for the 1209C/T polymorphism in one of the affected children placed the *MAG* gene outside the homozygous candidate region and definitely excluded this gene as the gene responsible for the disease (fig. 1).

Thus, refinement of the candidate interval and identification of the gene will need further investigations in this family and in families presenting the same new CMT4F subtype. Identification of the gene will improve understanding of the pathogenesis of autosomal recessive CMT4 disease.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/index.html (for reference sequences for cDNA [accession number M29273] and genomic DNA [accession number AC002132], and for exon 12 [accession number X98405])
Généthon, http://www.Genethon.fr/ (for human linkage map and markers' allele frequencies)
Geneclinics: Medical Genetics Knowledge Database, http://www.geneclinics.org (for clinical and genetic information about different CMT types)
Genome Database, http://gdbwww.gdb.org/ (for maps and markers' allele frequencies)
National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/ (for information on genes, ex-

pressed-sequence tags, or sequence-tagged sites assigned to a genetic locus, Genemap'99, information on genes, BLAST search, and retrieval of sequences)

Neuromuscular/Hereditary Motor Sensory Neuropathies, http://www.neuro.wustl.edu/neuromuscular/time/hmsn.html (for information on mapped loci associated with hereditary motor and sensory neuropathies)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CMT [MIM 118300]; CMT4A [MIM 214400]; CMT4B1 [MIM 601382]; CMT4B2 [MIM 604563], CMT4C, known as "CMT neuropathy, demyelinating" [MIM 601596]); CMT4D, known as "neuropathy, hereditary motor and sensory, Lom type" [MIM 601455]; and the *MTMR2* gene [MIM 603557])

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