

Analyzing Histopathological Features of Rare Charcot-Marie-Tooth Neuropathies to Unravel Their Pathogenesis

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Background: Charcot-Marie-Tooth (CMT) neuropathies are very heterogeneous disorders from both a clinical and genetic point of view. The CMT genes identified so far encode different proteins that are variably involved in regulating Schwann cells and/or axonal functions. However, the function of most of these proteins still remains to be elucidated.

Objective: To characterize a large cohort of patients with demyelinating, axonal, and intermediate forms of CMT neuropathy.

Design: A cohort of 131 unrelated patients were screened for mutations in 12 genes responsible for CMT neuropathies. Demyelinating, axonal, and intermediate forms of CMT neuropathy were initially distinguished as usual on the basis of electrophysiological criteria and clinical evaluation. A sural nerve biopsy was also performed for selected cases. Accordingly, patients underwent first-level

analysis of the genes most frequently mutated in each clinical form of CMT neuropathy.

Results: Although our cohort had a particularly high percentage of cases of rare axonal and intermediate CMT neuropathies, we found mutations in 40% of patients. Among identified changes, 7 represented new mutations occurring in the *MPZ*, *GJB1*, *EGR2*, *MFN2*, *NEFL*, and *HSBP1/HSP27* genes. Histopathological analysis performed in selected cases revealed morphological features, which correlated with the molecular diagnosis and provided evidence of the underlying pathogenetic mechanism.

Conclusion: Clinical and pathological analysis of patients with CMT neuropathies contributes to our understanding of the molecular mechanisms of CMT neuropathies.

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CHARCOU-MARIE-TOOTH (CMT) disorders are inherited motor and sensory neuropathies with a general prevalence of 1:2500. Although CMT disorders represent a clinically heterogeneous group of neuropathies, their main features include progressive distal muscular atrophy, weakness, and sensory loss in 4 limbs with onset usually around the first and second decades of life. On the basis of nerve conduction studies, CMT disorders have been divided into primary demyelinating CMT1 and primary axonal CMT2 neuropathies. A third intermediate form has also been introduced in clinical practice, CMT-I, which has intermediate values of nerve conduction velocity.^{1,2}

The CMT disorders are genetically heterogeneous, with more than 40 loci that have been mapped on different chromosomes and at least 27 responsible genes isolated thus far (<http://www.molgen.ua.ac.be/CMTMutations/default.cfm>). This broad

genetic heterogeneity suggests that distinct pathways are disrupted in the pathogenesis of various forms of CMT neuropathy. The CMT genes identified so far encode different proteins that are variably involved in regulating Schwann cells and/or axonal functions (ie, Schwann cell-axon or Schwann cell-extracellular matrix relationships).² The exact function of many of these genes is still obscure, and the pathogenetic mechanism that causes the neuropathy is far from being identified.

Herein, we report the results of the molecular screening of 131 unrelated patients with CMT neuropathies for mutations in 12 responsible genes. Demyelinating, axonal, and intermediate forms of neuropathies were distinguished as usual on the basis of electrophysiological criteria and clinical examination, and the most frequently mutated genes responsible for each clinical form of CMT neuropathy were analyzed accordingly. We identified mutations in 52 of the 131 patients (40%), 7 of whom had a CMT neuropathy caused by a previously unre-

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ported mutation. Histopathological analysis of 3 patients also revealed morphological features that provided further evidence of the molecular mechanisms that underlie the CMT neuropathies.

METHODS

PATIENTS AND DIAGNOSIS

A cohort of 131 unrelated patients (median age, 45 years; range, 7-83 years; sex ratio, 81 males to 50 females) with clinical criteria of CMT neuropathy³ were recruited between January 2005 and December 2008 at the Department of Neurology of the San Raffaele Institute or were referred to the Institute for Molecular Diagnosis (both in Milan, Italy). All patients were of Italian origin, with the exception of patient 15, who was from Syria. Patients were interviewed for family and personal history and underwent a standard neurological examination, including evaluation for pyramidal or cerebellar signs, cranial nerve involvement, as well as the presence of bone abnormalities, joint retractions, and skin lesions. Acquired forms of inflammatory, infectious, metabolic, and toxic neuropathies were first excluded as sporadic cases. Whenever necessary in sporadic cases, a sural nerve biopsy was performed during the diagnostic procedure to exclude cases of acquired neuropathy. Patients were then determined to have the demyelinating, axonal, or intermediate subtype of CMT neuropathy by neurophysiological analysis, according to international guidelines^{3,4} and on the basis of the nerve conduction velocity of the median or ulnar nerves. Thus, of 131 unrelated patients with CMT neuropathy, 34 (26%) had demyelinating CMT1, 37 (28%) had HNPP (hereditary neuropathy with liability to pressure palsies), 34 (26%) had axonal CMT2, and 26 (20%) had an intermediate form of CMT neuropathy. In this cohort, both sporadic (60%) and familial (40%) cases were represented. Mode of inheritance was the subsequent criterion of classification in familial forms of the disease. Genetic analysis was performed by first analyzing those genes most frequently mutated in the various CMT subtypes. For example, patients with clear demyelinating neuropathy or signs of repetitive nerve palsy were investigated for 17p11.2 duplication/deletion using the MLPA (multiplex ligation-dependent probe amplification) technique.⁵ Subsequent evaluation included the analysis of *MPZ* (myelin protein zero) and *GJB1* (connexin 32) in CMT1 and the detection of *PMP22* (peripheral myelin protein 22) point mutations in the HNPP neuropathy.

Ethics approval for this study was obtained by the San Raffaele Institute Ethics Committee. Patients signed an informed consent form during pretest genetic counseling and provided blood samples.

MOLECULAR ANALYSIS

Extraction of DNA from peripheral blood was performed using the BioRobot EZ1 extractor (Qiagen, Milan, Italy). Twelve genes responsible for CMT disease were analyzed: *PMP22*, *MPZ*, *GJB1*, *EGR2* (early growth response 2), *MFN2* (mitofusin 2), *NEFL* (neurofilament light chain), *HSPB1/HSP27* (heat shock protein 27), *HSPB8/HSP22* (heat shock protein 22), *GDAP1* (ganglioside-induced differentiation-associated protein 1), *LMNA* (lamin A/C), *MTMR2* (myotubularin-related protein 2), and *MTMR13* (myotubularin-related protein 13). To design primers for exon amplification, the presence of single-nucleotide polymorphisms and repetitive sequences was excluded. Direct sequencing was performed using ABI3130XL and ABI3730XL automated sequencers. As controls, at least 300 normal chromosomes were screened by denaturing high-pressure liquid chromatography (Transge-

nomics, Omaha, Nebraska) analysis and direct sequencing.⁶ For the identified changes, searches in mutation-specific databases (<http://www.molgen.ua.ac.be/CMTMutations>) and in single-nucleotide polymorphism databases (Ensembl and National Center for Biotechnology Information) were performed. The conservation of affected amino acid residues in different species was confirmed by *in silico* sequence alignment (National Center for Biotechnology Information). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on RNA extracted with Trizol (Invitrogen, Milan), according to the manufacturer's instruction (Roche, Milan).

RESULTS

MUTATION ANALYSIS

In our cohort, molecular analysis led to the identification of mutations in 53 of the 131 patients (40%); the percentage increases to 65% when only patients with a positive family history are considered. We could detect a genetic alteration in 19 of 34 patients (56%) with demyelinating CMT1 (the percentage increases to 100% when only patients with a positive family history are considered), in 15 of 37 patients (41%) with HNPP (the percentage increases to 75% when only familial cases are considered), in 13 of 34 patients (38%) with axonal CMT2 (the percentage increases to 63% when only familial cases are considered), and in 6 of 26 patients (23%) with an intermediate form of CMT neuropathy (the percentage increases to 31% when only familial cases are considered). Of the 34 patients with demyelinating CMT1, 14 (41%) showed *PMP22* duplication, 2 (6%) showed point mutations in *MPZ*, and 1 (3%) showed point mutations in each of the *GJB1*, *EGR2* and *NEFL* genes. These percentages increase when only patients with a positive family history are considered (data not shown). Moreover, of the 37 patients with HNPP, 12 (32%) displayed a *PMP22* deletion, and 3 (8%) had a point mutation in the same gene.

Of the 34 patients with axonal CMT2, 4 (12%) had mutations in *MPZ*, 4 (12%) had mutations in *MFN2*, and 2 (6%) displayed mutations in either the *GJB1* or *HSP27* gene. We identified only 1 mutation in *GDAP1* in a patient with autosomal recessive axonal CMT (**Table**). Of the 26 patients with CMT-I, 3 (12%) carried a mutation in the *GJB1* gene, 2 (8%) carried a mutation in the *MPZ* gene, and 1 (4%) carried a mutation in the *GDAP1* gene (Table).

We identified 7 changes in the *EGR2*, *NEFL*, *GJB1*, *MPZ*, *HSPB1/HSP27*, and *MFN2* genes that had not been previously reported (patients 1, 5, 9, 12, 20, 24, and 25, respectively) (Table). Hereafter, we describe in detail 4 probands. One is a male patient with intermediate CMT who, to our knowledge, carries the first splicing mutation reported so far in the *GJB1* gene. Moreover, histopathological analysis performed in 3 patients revealed peculiar morphological features that provided further evidence of the molecular mechanisms underlying the CMT neuropathies.

PATIENT 9

A male patient had progressive distal weakness at the lower limbs with onset at age 30 years, although he reported

Table. Clinical and Genetic Features of Patients With Identified Mutations

Patient No.	Type of CMT ^a	Sex/ Age, y	Gene ^b	Mutation, cDNA; Protein	Inheritance	Age at Onset, y	Muscle Atrophy, UL/LL	Muscle Weakness, UL/LL	DTR, Proximal-Distal	Aid for Walking	Sensory Deficit	Other Features	CMT Score
1	CMT1	F/46	<i>EGR2</i>	c.1147G>C; p.Asp383His	AD	23	UL<LL	UL<<LL	Absent distal LL	Ankle supports	Superficial touch/pain and deep joint/vibration in LL	Pes cavus, trigeminal neuralgia	13
Son of 1	CMT1	M/19	<i>EGR2</i>	c.1147G>C; p.Asp383His	AD	16	UL<LL	UL-LL	Absent proximal-distal	Ankle supports	Superficial touch/pain and deep joint/vibration in LL	Pes cavus	17
2	CMT1	M/53	<i>GJB1</i>	c.171G>T; p.Gln57His	AD	6	UL<LL	UL<LL	Absent proximal-distal	Ankle supports	Superficial touch/pain and deep joint/vibration in LL	Pes cavus, scoliosis, nystagmus	15
3	CMT1	F/39	<i>MPZ</i>	c.233C>T; p.Ser78Leu	AD	10	UL<<LL	UL<<LL	Absent proximal-distal	Ankle supports	Superficial touch/pain and deep joint/vibration in LL	Pes cavus	13
Father of 3	CMT1	M/64	<i>MPZ</i>	c.233C>T; p.Ser78Leu	AD	ND	ND	ND	ND	ND	ND		
Daughter of 3	CMT1	F/13	<i>MPZ</i>	c.233C>T; p.Ser78Leu	AD	9	Absent	LL	Absent LL	None	None	Pes cavus	2
4	CMT1	F/47	<i>MPZ</i>	c.670 G>T; p.Asp224Tyr	S	43	Absent	Absent	Absent distal	None	Deep joint/vibration in UL/LL	Pes cavus	8
5	CMT1	F/37	<i>NEFL</i>	c.1319C>T; p.Pro440Leu	S	28	UL-LL	Absent	Absent proximal-distal	None	Superficial touch/pain in UL	Pes cavus	3
6	HNPP	M/34	<i>PMP22</i>	c.434delT; p.Leu145ArgfsX9	AD	7	LL	LL	Absent distal	None	Deep joint/vibration in LL	Pes cavus	4
Father of 6	HNPP	M/65	<i>PMP22</i>	c.434delT; p.Leu145ArgfsX9	AD	ND	ND	ND	ND	ND	ND		
7	HNPP	M/47	<i>PMP22</i>	c.434delT; p.Leu145ArgfsX9	AD	ND	Absent	Absent	Normal	None	Deep joint/vibration in LL	Pes cavus	4
8	HNPP	F/34	<i>PMP22</i>	c.434delT; p.Leu145ArgfsX9	AD	24	Absent	Absent	Absent distal	None	Superficial touch/pain in UL/LL	Pes cavus, onset with pain and edema	4
Father of 8	HNPP	M/70	<i>PMP22</i>	c.434delT; p.Leu145ArgfsX9	AD	ND	Absent	Absent	Absent distal	None	Normal	Pes cavus, diabetes type II	2
9	CMT-1	M/38	<i>GJB1</i>	c.-19 C>G	S	30	UL<LL	UL<LL	Absent distal UL/LL	Ankle supports	Superficial touch/pain and deep joint/vibration in UL/LL	Pes cavus, pyramidal signs, tremor	8
10	CMT-1	M/38	<i>GJB1</i>	c.7T>C; p.Trp3Arg	AD	10	UL<LL	UL<<LL	Absent UL/LL	Ankle supports	Superficial touch/pain and deep joint/vibration in UL/LL	Pes cavus	17
11	CMT-1	M/39	<i>GJB1</i>	c.224G>A; p.Arg75Glu	AD	10	UL<<LL	LL	Absent proximal-distal	Ankle supports	Superficial touch/pain and deep joint/vibration in UL/LL	Pes cavus, scoliosis	12
Sister of 11	CMT-1	F/43	<i>GJB1</i>	c.224G>A; p.Arg75Glu	AD	ND	ND	ND	ND	ND	ND		
12	CMT-1	M/72	<i>MPZ</i>	c.241C>T; p.His81Tyr	AD	50	LL	LL	Absent distal	None	Deep joint/vibration in LL	Pes cavus, hearing loss, cataract	9
Sister of 12	CMT-1	F/67	<i>MPZ</i>	c.241C>T; p.His81Tyr	AD	52	LL	LL	Absent distal	None	Deep joint/vibration in LL	Pes cavus	9
Nephew of 12	CMT-1	M/52	<i>MPZ</i>	c.241C>T; p.His81Tyr	AD	ND	Absent	Absent	Normal	None	None		0
13	CMT-1	M/49	<i>MPZ</i>	c.306delA; p.Val102fsX15	S	40	LL	Absent	Absent proximal-distal	None	Deep joint/vibration in LL		3
14	CMT2	F/72	<i>GDAP1</i>	c.-89_-88delTC	S	55	LL	LL	Absent distal	None	Deep joint/vibration in LL	Pes cavus, pyramidal signs	11
15	CMT2	F/7	<i>GDAP1</i>	c.174_176delGCCinsTGTG; p.P59fsX4	AR	1	UL<<LL	UL<<LL	Absent proximal-distal	Wheelchair	Deep joint/vibration in UL/LL	Pes cavus, scoliosis, tendon retractions, pes equinovarus	25

(continued)

Table. Clinical and Genetic Features of Patients With Identified Mutations (continued)

Patient No.	Type of CMT ^a	Sex/ Age, y	Gene ^b	Mutation, cDNA; Protein	Inheritance	Age at Onset, y	Muscle Atrophy, UL/LL	Muscle Weakness, UL/LL	DTR, Proximal-Distal	Aid for Walking	Sensory Deficit	Other Features	CMT Score
Sister of 15	CMT2	F/10	<i>GDAP1</i>	c.174_176delGCCinsTGTG; p.P59fsX4	AR	1	UL<<LL	UL<<LL	Absent proximal-distal	Wheelchair	Deep joint/vibration in UL/LL	Pes cavus and equinovarus, scoliosis, tendon retractions	27
16	CMT2	M/24	<i>GDAP1</i>	c.715C>T; p.Leu239Phe	AR	1	UL<<LL	UL<<LL	Absent proximal-distal	Wheelchair	Deep joint/vibration in LL	Pes cavus and equinovarus, scoliosis, tendon retractions	23
Cousin of 16	CMT2	F/34	<i>GDAP1</i>	c.715C>T; p.Leu239Phe	AR	3	UL<<LL	UL<<LL	Absent distal	Ankle supports	Superficial touch/pain and deep joint/vibration in UL/LL	Pes cavus, tremor, hand deformities	17
17	CMT2	M/64	<i>GJB1</i>	c.43C>T; p.Arg15Trp	AD	51	UL<LL	UL<LL	Absent distal LL	Ankle supports	Deep joint/vibration in UL/LL	Pes cavus	11
Daughter of 17	CMT2	F/38	<i>GJB1</i>	c.43C>T; p.Arg15Trp	AD	30	Absent	UL	Absent distal LL	None	Normal	Pes cavus, scoliosis, trophic skin alterations	1
18	CMT2	F/61	<i>GJB1</i>	c.689G>T; p.Arg230Leu	AD	50	UL<<LL	UL<<LL	Absent proximal-distal	None	Deep joint/vibration in LL	Pes cavus	9
Sister of 18	CMT2	F/59	<i>GJB1</i>	c.689G>T; p.Arg230Leu	AD	50	UL-LL	UL-LL	Absent proximal-distal	None	Deep joint/vibration in LL	Pes cavus	9
Daughter of 18	CMT2	F/30	<i>GJB1</i>	c.689G>T; p.Arg230Leu	AD	ND	Absent	Absent	Absent proximal-distal	Plantar	Normal	Pes cavus	0
19	CMT2	M/47	<i>HSP27</i>	c.379C>T; p.Arg127Trp	AD	20	UL<<LL	UL<<LL	Absent LL	Walking frame	Normal	Pes cavus, tremor and trophic skin alterations	8
20	CMT2	F/58	<i>HSP27</i>	c.404C>G; p.Ser135Cys	AD	20	UL<<LL	UL<<LL	Absent LL	None	Superficial touch/pain and deep joint/vibration in LL	Pes cavus, cerebellar signs, constipation	11
Mother of 20	CMT2	F/80	<i>HSP27</i>	c.404C>G; p.Ser135Cys	AD	50	ND	ND	ND	Cane	ND		
21	CMT2	F/52	<i>HSP27</i>	c.365-13C>T	S	45	UL<<LL	UL<<LL	Absent LL	Cane	Deep joint/vibration in UL/LL	Pes cavus	17
22	CMT2	M/27	<i>MFN2</i>	c.839G>A; p.Arg280His	AD	6	UL<<LL	UL<<LL	Absent LL	Ankle support	Deep joint/vibration in LL	Pes cavus and equinovarus	12
23	CMT2	M/55	<i>MFN2</i>	c.2113G>A; p.Val705Ile	S	50	LL	LL	Absent distal	Ankle support	Deep joint/vibration in LL		9
24	CMT2	F/37	<i>MFN2</i>	c.2140A>G; p.Ile714Val	S	9	UL-LL	UL-LL	Absent LL	Ankle support	Deep joint/vibration in LL	Pes cavus, scoliosis, proximal muscle weakness in LL, hand deformities	15
25	CMT2	F/71	<i>MFN2</i>	c.2213C>T; p.Ala738Val	AD	6	UL<<LL	UL<<LL	Absent proximal-distal	Ankle support, cane	Superficial touch/pain and deep joint/vibration in LL	Pes cavus, scoliosis, proximal muscle weakness in LL, cataract	18
26	CMT2	M/65	<i>MPZ</i>	c.131C>T; p.Ser44Phe	AD	55	LL	LL	Absent LL	Ankle support and cane	Deep joint/vibration in LL	Pes cavus	10
Daughter of 26	CMT2	F/25	<i>MPZ</i>	c.131C>T; p.Ser44Phe	AD	25	Absent	Absent	Normal	None	Normal	Pes cavus	0
Cousin of 26	CMT2	F/52	<i>MPZ</i>	c.131C>T; p.Ser44Phe	AD	46	LL	LL	Reduced	None	Deep joint/vibration in LL	Pes cavus, trophic skin alterations	3
27	CMT2	F/60	<i>MPZ</i>	c.166G>A; p.Glu56Lys	AD	44	LL	LL	Absent LL	None	Deep joint/vibration in LL		6
Brother of 27	CMT2	M/57	<i>MPZ</i>	c.166G>A; p.Glu56Lys	AD	46	UL>>LL	UL-LL	Absent LL	None	Superficial touch/pain and deep joint/vibration in LL		6
28	CMT2	M/67	<i>MPZ</i>	c.208C>T; p.Pro70Ser	AD	50	UL<<LL	UL<<LL	Absent LL	None	Normal	Pes cavus, scoliosis	7

(continued)

Table. Clinical and Genetic Features of Patients With Identified Mutations (continued)

Patient No.	Type of CMT ^a	Sex/ Age, y	Gene ^b	Mutation, cDNA; Protein	Inheritance	Age at Onset, y	Muscle Atrophy, UL/LL	Muscle Weakness, UL/LL	DTR, Proximal-Distal	Aid for Walking	Sensory Deficit	Other Features	CMT Score
Daughter of 28	CMT2	F/41	<i>MPZ</i>	c.208C>T; p.Pro70Ser	AD	ND	Absent	Absent	Normal	None	Normal	Pes cavus	0
Sister of 28	CMT2	F/74	<i>MPZ</i>	c.208C>T; p.Pro70Ser	AD	50	LL	LL	Absent LL	Ankle support	Superficial touch/pain and deep joint/vibration in LL	Pes cavus, scoliosis, trophic skin alterations	10
29	CMT2	F/67	<i>MPZ</i>	c.208C>T; p.Pro70Ser	S	60	UL<LL	UL<<LL	Absent distal LL	None	Superficial touch/pain and deep joint/vibration in LL	Pes cavus, pyramidal signs, proximal muscle weakness in LL	11

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; CMT, Charcot-Marie-Tooth; DTR, deep tendon reflexes; HNPP, hereditary neuropathy with liability to pressure palsies; LL, lower limbs; ND, not determined; S, sporadic; UL, upper limbs.

^aOn the basis of nerve conduction studies, CMT disorders have been divided into primary demyelinating CMT1 and primary axonal CMT2 neuropathies. A third intermediate form has also been introduced in clinical practice, CMT-I, which has intermediate values of nerve conduction velocity. Hereditary neuropathy with liability to pressure palsies is a disorder that affects peripheral nerves.

^bGenes in bold indicate new mutations identified in this study.

an inability to run and jump since infancy. Clinical examination revealed pes cavus, distal areflexia, ataxia, severe peroneal muscle weakness, and pyramidal signs. Nerve conduction studies demonstrated an intermediate form of CMT disease in this patient. In this proband, we identified a new c.-19 C>G substitution in intron 1 of the *GJB1* gene. The mutation was de novo because it was not present in the proband's mother. The availability of the nerve biopsy of this patient allowed us to assess the effect of the nucleotide change at the messenger RNA (mRNA) level. Analysis by RT-PCR performed on RNA extracted from the nerve biopsy revealed the presence of a smaller band of 161 base pairs (bp) in the patient, compared with the wild type of 429 bp (the band of 785 bp corresponds to genomic DNA contamination). Direct sequencing suggested that the mutation leads to the activation of a cryptic splicing site in exon 2 and to a deletion of 278 bp of exon 2, which contains the physiological ATG initiation site of CX32 (**Figure 1A and B**). A different in-frame ATG may be used, leading to a shorter protein lacking 92 amino acids at the N-terminus (**Figure 1C**). To our knowledge, this represents the first splicing mutation reported in the *GJB1* gene.

In this patient, the analysis of semithin sections of the sural nerve biopsy was also consistent with intermediate CMT, with signs of axonal and demyelinating neuropathy. In fact, we observed a mild degree of endoneurial fibrosis with evident reduction of large myelinated nerve fibers (**Figure 1D and E**) and many clusters of small myelinated nerve fibers, indicating axonal regeneration. Moreover, we detected some naked axons, indicating demyelination, and many fibers with thin myelin sheaths and typical onion bulbs, suggesting chronic remyelination. We did not observe signs of acute axonal degeneration (**Figure 1F and G**).

PATIENT 5

A 35-year-old woman had numbness and moderate weakness in the upper limbs starting from age 27 years. Nerve conduction studies revealed a demyelinating neuropathy.

Pes cavus and proprioceptive deficit were present at examination. We first excluded a duplication of the *PMP22* gene as well as point mutations in the *MPZ* and *PMP22* genes. In this proband, we identified a new mutation in the tail domain of the *NEFL* gene (p.Pro440Leu) that was not found in the unaffected mother. The morphological analysis revealed interesting features that were consistent with the putative function of the NEFL protein in axons. Semithin sections showed loss of large and small myelinated fibers, a mild degree of endoneurial fibrosis, and signs of both demyelinating and axonal neuropathy. We observed thinly myelinated fibers and onion bulbs, indicating chronic demyelination and remyelination (**Figure 2A and B**). In addition, we found myelin ovoids, suggesting active axonal degeneration, and a few clusters of small myelinated fibers, indicating axonal regeneration (**Figure 2B and C**). A characteristic change was the presence of some massively dilated axons, surrounded by thinned myelin with increased neurofilament (NF) density and loss of microtubules (**Figure 2D-F**), which is consistent with the role of NEFL in regulating axonal diameter and the assembly of a normal network of filaments in the axon. These aspects were also reminiscent of a previously described patient with a p.Pro22Ser mutation in the head domain of NEFL.⁷

PATIENT 20

A 58-year-old woman had weakness and cramps in 4 limbs since age 20 years. Clinical examination confirmed muscle weakness (more evident at lower limbs), ataxia, pes cavus, reduced tendon reflexes, and cerebellar signs. Nerve conduction studies revealed an axonal CMT. When she was interviewed, it was found that she had a positive family history for CMT diseases. The disease gene in this pedigree was likely transmitted as either an autosomal dominant or X-linked dominant trait. We first excluded mutations in the *MFN2*, *MPZ*, and *GJB1* genes most frequently mutated in autosomal dominant or X-linked axonal CMT. We identified a new p.Ser135Cys mutation in the *HSPB1* gene, which was also present in the affected

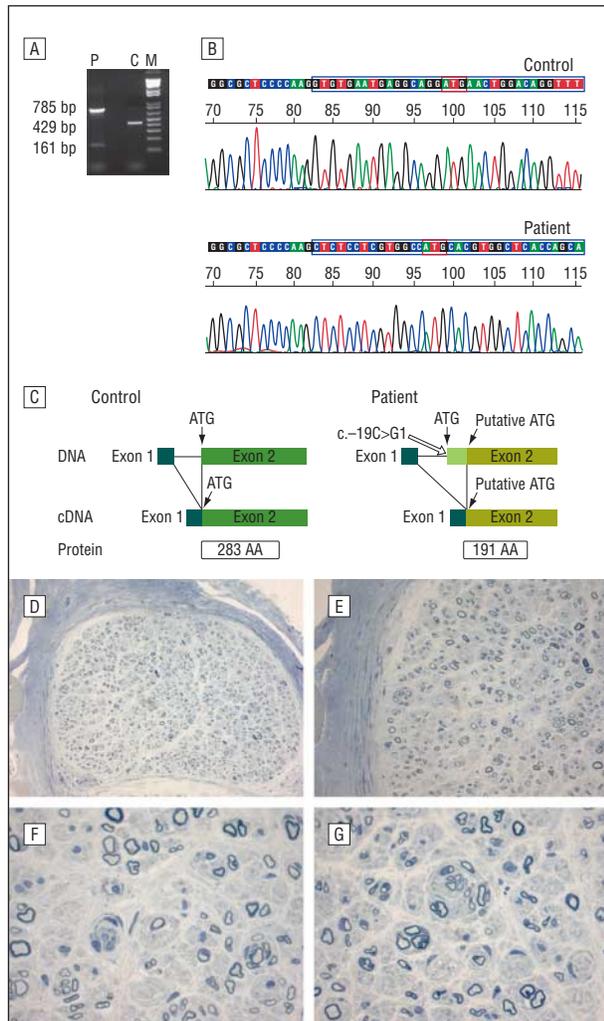


Figure 1. A new mutation in the *GJB1* gene leading to a putative loss of function of CX32 (patient 9). A, Reverse transcriptase–polymerase chain reaction revealed a smaller band in the patient’s messenger RNA. bp indicates base pairs. B, Direct sequencing of reverse transcriptase–polymerase chain reaction products; the blue boxes correspond to exon 2, and the red boxes indicate the start codon for translation. C, The dark green boxes indicate the complementary DNA (cDNA) deletion, and the light green box represents the portion of exon 2 that is deleted in the patient cDNA. D (original magnification $\times 100$) and E (original magnification $\times 250$), A reduction of large myelinated fibers in the semithin sections of the patient sural nerve biopsy. F and G, Clusters of small myelinated axons, suggesting axonal regeneration (original magnification $\times 600$); onion bulbs are observed. C indicates control; M, marker; P, patient.

mother of the proband. Morphological analysis of the sural nerve biopsy revealed a normal density of myelinated fibers in transverse semithin sections (**Figure 3A**). Only 1 small fascicle had myelinated fiber loss (**Figure 3B**). We observed a few clusters of regenerated fibers (**Figure 3A**), and many axons were clearly inappropriately small for their myelin sheaths, indicating axonal atrophy (**Figure 3C-E**). Moreover, we did not observe signs of acute axonal degeneration and demyelination, whereas myelin remodeling was evident with excessively thick myelin and fissures (**Figure 3C-F**). Ultrastructural analysis revealed atrophic axons with increased microtubule density (**Figure 3G-J**). This finding is consistent with the role of the HSP27 protein, encoded by the *HSPB1* gene, which

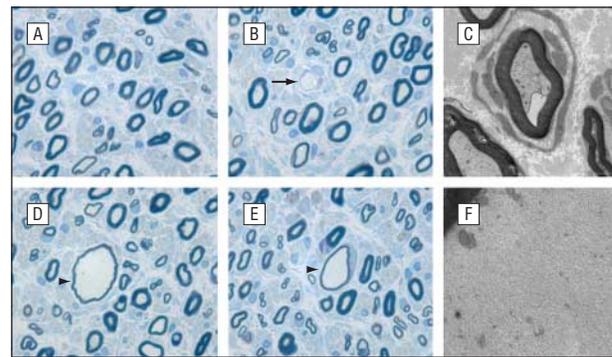


Figure 2. Sural nerve biopsy of patient 5. A, Loss of small and large myelinated fibers and signs of acute axonal degeneration (original magnification $\times 600$). B (original magnification $\times 600$) and C (original magnification $\times 18\,000$), Acute demyelination and chronic remyelination, such as onion bulbs (arrow). D and E, Dilated axons with thin myelin (arrowheads) (original magnification $\times 600$). F, Dilated axons with increased neurofilament density and loss of microtubules (original magnification $\times 40\,000$).

regulates NF assembly. As a consequence, loss of NFs results in an increased density of microtubules.^{8,9} A different substitution at the same codon, p.Ser135Phe, had already been reported in CMT patients with axonopathy. In vitro, the p.Ser135Phe provoked disruption of NF assembly and aggregation, which was not reverted by co-expression of the wild-type HSP27 protein. This finding suggests that the p.Ser135Phe acts through a dominant negative effect likely because the mutant HSP27 protein has a higher affinity for NFs than does the wild-type HSP27 protein.¹⁰⁻¹² The new p.Ser135Cys mutation that we identified might act through a similar mechanism. To our knowledge, the morphological features that we have described in the nerve biopsy sample of this patient provide for the first time in vivo evidence of the role of HSP27 in the regulation of NF assembly.

PATIENT 15

Two sisters displayed early-onset severe neuropathy. The proband, a 7-year-old girl, had delayed motor milestones, an unsteady gait throughout infancy, bilateral pes equinovarus with Achilles tendon retraction, and kyphoscoliosis. Peroneal muscle atrophy and weakness progressed rapidly with length-dependent distribution, and the patient was unable to walk independently since age 4 years. Sensory ataxia was present. The older sister had a very similar clinical history. Neurophysiological analysis showed a motor nerve conduction velocity of 38 m/s and an amplitude of 0.08 mV, suggesting an intermediate form of CMT neuropathy. Since the parents were first cousins and healthy, the disease in the family was likely to be inherited as an autosomal recessive trait. Therefore, we first screened the *GDAP1* gene, and we detected in the 2 affected sisters a homozygous mutation, c.174_176delGCCinsTGTG, p.P59fsX4 (already reported as L58LfsX4).¹³ The parents were healthy carriers as they were both heterozygous for the mutation. The nerve biopsy displayed morphological features consistent with the molecular diagnosis. Semithin section analysis of the sural nerve biopsy of the proband demonstrated a mild degree of endoneurial fibrosis. We also

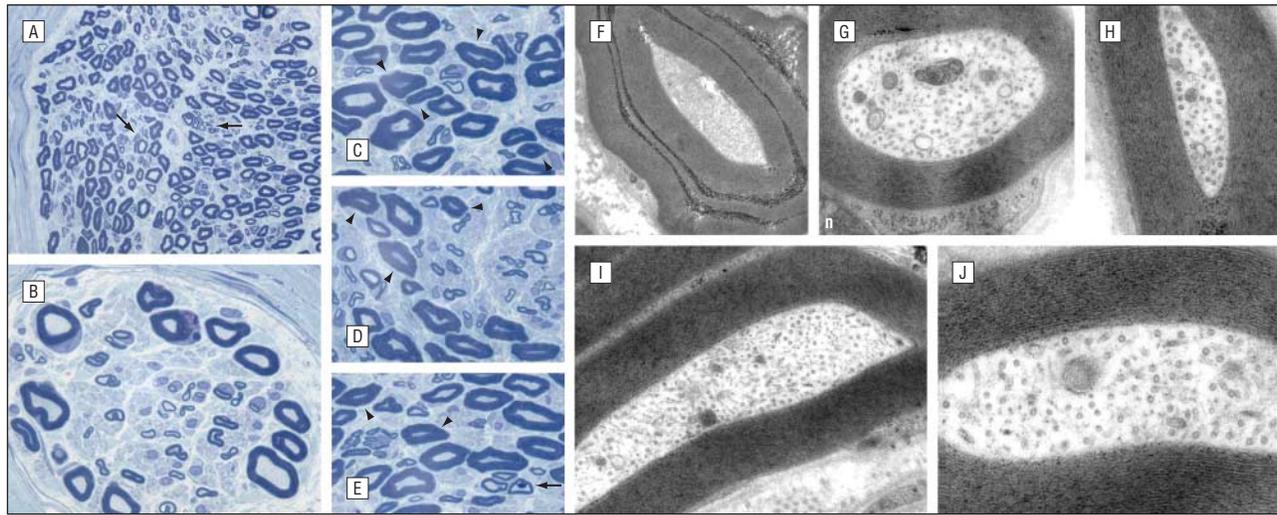


Figure 3. Sural nerve biopsy of patient 20. Normal density of myelinated fibers (A) (original magnification $\times 250$), with the exception of 1 small fascicle (B) (original magnification $\times 600$). A, Arrows show clusters of regenerated fibers. C, D, and E, Thick myelin and fissures (arrowheads) (original magnification $\times 600$). F, Signs of myelin remodeling (original magnification $\times 18\,000$). G–J, Axonal atrophy with increased microtubule density and loss of neurofilaments (original magnification $\times 40\,000$); n indicates the Schwann cell nucleus (G).

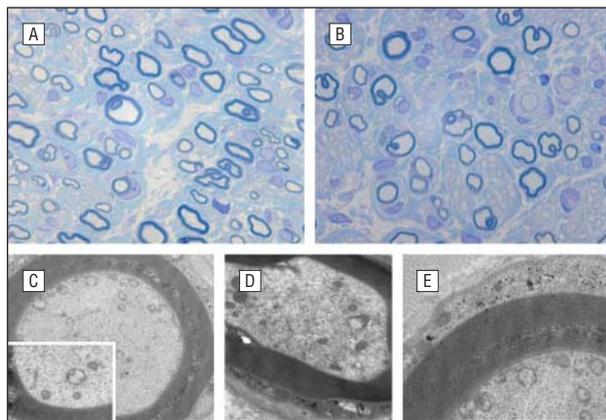


Figure 4. Sural nerve biopsy of patient 15. A, Semithin analysis revealed reduction of large myelinated fibers (original magnification $\times 600$). B, Onion bulbs indicating chronic remyelination were also observed (original magnification $\times 600$). C and D (original magnification $\times 18\,000$) and E (original magnification $\times 30\,000$). Electron microscopy showed mitochondrial abnormalities in many axons with focal accumulation, enlargements, and disorganized cristae (C, inset) (original magnification $\times 30\,000$).

observed a reduction of large myelinated nerve fibers together with signs of axonal degeneration and some denuded axons, indicating demyelination (**Figure 4A**). The myelin sheaths of several fibers were thin with respect to the axon size, suggesting hypomyelination, while the presence of onion bulbs indicated chronic remyelination (**Figure 4B**). By electron microscopy, we observed clear hypomyelination and mitochondrial abnormalities in many axons, including focal accumulations, enlargement, and disorganized mitochondrial cristae (**Figure 4C–E**). These findings are in agreement with the putative involvement of the *GDAP1* protein, which has been suggested to regulate mitochondrial fission in vitro.¹⁴ Although the mutation that we identified in this pedigree has already been reported, to our knowledge, the morphological analysis confirmed for the first time in human nerves the role of *GDAP1* in the regulation of mitochondrial fission.

COMMENT

In this study, we identified mutations in 52 of 131 patients (40%), 7 of whom had CMT neuropathy caused by a previously unreported mutation. The distribution of the identified mutations was consistent with that reported in other studies.^{15,16} Moreover, we describe 4 probands: 1 patient who, to our knowledge, carried the first splicing mutation identified in the *GJB1* gene affecting mRNA expression and 3 patients whose morphological analysis was helpful for further assessing in vivo the mechanism underlying CMT neuropathies. In addition, to our knowledge, for 3 of the 4 probands, the identified mutations had not been previously reported.

In patient 5, who had demyelinating CMT, we identified a new mutation in the *NEFL* gene. Genetic analysis in this case was in agreement with the observation of dilated axons in the nerve biopsy, which was also reminiscent of an already described patient with a different mutation in the *NEFL* gene.⁷ A routine neurophysiological examination identified a classical case of demyelinating neuropathy, and only by analyzing the sural nerve biopsy did we find concomitant axonal involvement with the morphological characteristic of the *NEFL* mutation. In patient 20, we identified a new mutation in the *HSPB1/HSP27* gene, which correlated well with the observation of an increased microtubule density in the nerve biopsy specimen. The function of the *HSPB1/HSP27* gene has been suggested mainly by features described by in vitro studies, and therefore our morphological findings represent the first demonstration in vivo of the role of HSP27 in the regulation of NF assembly. Similarly, the pathological features observed in the nerve biopsy of patient 15 were consistent with the role of *GDAP1* in mitochondrial dynamics as mainly suggested by in vitro studies. The presence of enlarged aberrant mitochondria in the axon of this patient's nerves further supports the role of *GDAP1* in the regulation of mitochondrial fission in vivo.

Finally, a new mutation in the *GJB1* gene was demonstrated in patient 9 with an intermediate form of CMT neu-

ropathy. In this case, the availability of the nerve biopsy allowed us to predict the effect of the mutation on the corresponding protein. We provided evidence that this mutation affects the splicing of *GJB1* mRNA, leading to loss of CX32.

Overall, although we report individual cases, the biological features described in each nerve biopsy are likely to reflect a common disease mechanism for each CMT subgroup. In fact, similar findings have been reported in other studies from human samples, transgenic mice, and in vitro experiments.^{17,18} The integration and cross-contamination of all the available findings would constitute a suitable archive to facilitate clinical-pathological correlations. In particular, the histopathological analysis performed using sural nerve or skin biopsies¹⁹ in selected cases may reveal morphological features that can contribute to advancing our understanding of CMT neuropathies. In conclusion, we provide evidence that histopathology may play an important role in revealing features that can predict or further assess the mechanisms underlying CMT neuropathies.

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