doi:10.1093/brain/awl126 *Brain* (2006), **129**, 2093–2102

MFN2 mutation distribution and genotype/phenotype correlation in Charcot–Marie–Tooth type 2

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Mutations in mitofusin 2 (*MFN2*) have been reported in Charcot–Marie–Tooth type 2 (CMT2) families. To study the distribution of mutations in *MFN2* we screened 323 families and isolated patients with distinct CMT phenotypes. In 29 probands, we identified 22 distinct *MFN2* mutations, and 14 of these mutations have not been reported before. All mutations were located in the cytoplasmic domains of the MFN2 protein. Patients

presented with a classical but rather severe CMT phenotype, since 28% of them were wheelchair-dependent. Some had additional features as optic atrophy. Most patients had an early onset and severe disease status, whereas a smaller group experienced a later onset and milder disease course. Electrophysiological data showed in the majority of patients normal to slightly reduced nerve conduction velocities with often severely reduced amplitudes of the compound motor and sensory nerve action potentials. Examination of sural nerve specimens showed loss of large myelinated fibres and degenerative mitochondrial changes. In patients with a documented family history of CMT2 the frequency of *MFN2* mutations was 33% indicating that *MFN2* mutations are a major cause in this population.

Keywords: Charcot–Marie–Tooth type 2; mitofusin 2; genotype–phenotype correlation

Abbreviations: CMAP = compound motor action potentials; CMT = Charcot–Marie–Tooth disease type 1

Received February 7, 2006. Revised April 7, 2006. Accepted April 10, 2006. Advance Access publication May 19, 2006

Introduction

HMSN are a clinically and genetically heterogeneous group of disorders of the peripheral nervous system. The clinical features of HMSN include progressive distal muscle weakness and atrophy, starting in the lower limbs and later on also affecting the upper extremities, steppage gait, foot deformities, distal sensory loss, and decreased or absent tendon reflexes (Harding and Thomas, 1980). According to electrophysiological criteria, HMSN is divided into two main subtypes: HMSN I, also called Charcot–Marie–Tooth disease type 1 (CMT1), is a primarily demyelinating neuropathy with severely reduced motor nerve conduction velocities (NCV < 38 m/s), and HMSN II (CMT2) with primarily axonal loss characterized by normal or slightly reduced NCV but decreased amplitudes. Most CMT patients can be classified as either CMT1 or CMT2 using the NCV cut-off value of 38 m/s (Dyck *et al.*, 1993). However, in some CMT families, patients have been difficult to classify using these strict electrophysiological criteria, leading to the concept of intermediate CMT with median motor NCVs between 25 and 45 m/s (Davis *et al.*, 1978). In fact, some families show an even broader range of NCVs with the highest values within the normal range and the lowest values usually around 25 m/s. The neuropathological hallmark of CMT1 is segmental de- and remyelination, and onion bulb formation. In CMT2 patients, nerve biopsies typically show axonal loss and regenerative sprouting (Schröder, 2001).

Molecular genetic studies have shown extensive genetic heterogeneity within CMT1 and CMT2. However, in contrast to the well-known molecular genetic defects causing CMT1 (Suter and Scherer, 2003), genes underlying CMT2 have only recently begun to be identified (Inherited Peripheral Neuropathy Mutation Database, http://www.molgen.ua.ac.be/CMTMutations/). Initially, it was shown that mutations in a few genes that are usually associated with a CMT1 phenotype occasionally give rise to a CMT2 phenotype. In fact, female patients carrying a gap junction beta 1 (*GJB1, connexin 32*) mutation often have NCVs within the CMT2 range, while many male patients have intermediate NCVs (Birouk *et al.*, 1998). Subsequently, particular myelin protein zero (*MPZ*) mutations were also shown to result in an axonal phenotype (Marrosu *et al.*, 1998; De Jonghe *et al.*, 1999; Seeman *et al.*, 2004). However *GJB*1 mutations were not observed in male patients with normal NCVs and *MPZ* mutations only account for a small fraction of CMT2 patients. In addition, in multi-generational families with these particular *MPZ* mutations NCVs often show a broad range varying from 25 m/s to normal (De Jonghe *et al.*, 1999). Mutations in the neurofilament light gene (*NEFL*) were initially reported in families with CMT2 (CMT2E) (Mersiyanova *et al.*, 2000; De Jonghe *et al.*, 2001). Later on, *NEFL* mutations were also detected in patients with slow NCVs (CMT1) more appropriately defining the electrophysiological phenotype (Jordanova *et al.*, 2003). Recently, mutations in mitofusin 2 (*MFN2*) were reported in CMT2A families, previously linked to chromosome 1p35-36 (Züchner *et al.*, 2004). With the additional report of seven *MFN2* mutations in Japanese CMT2 patients (Kijima *et al.*, 2005) and three *MFN2* mutations in American CMT2 families (Lawson *et al.*, 2005), a total of 19 different *MFN2* mutations have been identified thus far.

In the current study, we screened a large cohort of patients (n = 323) with distinct CMT subtypes and identified 30 *MFN2* mutations in 29 probands. The aim of the present study was to analyse the frequency of *MFN2* mutations in axonal CMT, and to study the related clinical, electrophysiological and pathological phenotypes.

Patients and methods

Patients

The study included 323 unrelated individuals. Based upon clinical and electrophysiological data, 249 patients were diagnosed as CMT2 (77%), 20 with intermediate CMT (6%) and six patients with CMT1 (2%). Three patients with distal hereditary motor neuropathy (distal HMN; 1%) and one patient with hereditary sensory and autonomic neuropathy (HSAN; <1%) were also included in the study. The phenotype of 44 patients was not known (14%). In addition, 170 clinically healthy Europeans were screened as

controls for sequence variations. The study was approved by each collaborator's institutional review board or equivalent, and informed consent was obtained from all CMT patients and control persons, according to the Declaration of Helsinki.

Molecular genetic studies

We isolated genomic DNA from total blood samples obtained from CMT patients and control persons using standard extraction protocols. In the patients, the *MFN2* mutation screening was performed by PCR amplifying all 17 coding exons of *MFN2* using intronic primers (Züchner *et al.*, 2004). PCR products were sequenced on an ABI3730 DNA Analyzer (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit 3.1 (Applied Biosystems). The DNA sequence data were collected and analysed using the ABI DNA Sequencing Analysis software 5.0 and Seqman II 5.07 (DNASTAR Inc., Madison, USA). When a mutation was detected in an index person, additional family members, if available, were analysed in order to determine co-segregation of the observed sequence variation with the disease. In families in which a *de novo* mutation was suspected, paternity was confirmed using 11 STR markers (D1S249, D2S367, D3S1614, D4S406, D5S407, D9S1677, D10S1686, D11S937, D13S170, D14S288, D21S1880).

The numbering of the *MFN2* codons was based upon the published amino acid sequence (National Center for Biotechnology Information accession numbers NP_055689 for the protein sequence and NM_014874 for the mRNA sequence). The *MFN2* mutations were defined according to the guidelines as described before (den Dunnen and Antonarakis, 2001).

Results

Molecular genetic analysis

We performed a mutation screening of the entire coding region and intron/exon boundaries of *MFN2* in a cohort of 323 unrelated CMT patients. Using direct DNA sequencing we found 30 *MFN2* mutations (29 single nucleotide substitutions and one deletion) in 29 patients. All sequence variations are summarized in Table 1, and the location of the pathogenic mutations in the MFN2 protein is shown in Fig. 1. The number of mutations does not agree with the number of probands because in CMT-529, two base pair substitutions transmitting in *trans* c.(748→T)+(1198C→T) lead to two different amino acid changes

Table 1 Sequence variations found in the coding region of the *MFN2* gene

Nucleotide change	Exon	Amino acid change	Domain	Index patient	Age at onset (years)	Transmission	Origin	Reported
c.227T→C	4	L76P		BAB1792	Unknown	Dominant	USA	Yes (1)
c.275T→C	4	L92P		PN-142	1	Unknown	Belgium	No
c.280C→T	4	R94W		CMT-472.1	2	*De novo*	Czech Republic	Yes (1,3)
c.280C→T	4	R94W		CMT-67	3	*De novo*	Belgium	Yes (1,3)
c.281G→A	4	R94Q		PN-198.1	7; 5	Dominant	Belgium	Yes (1,2)
c.281G→A	4	R94Q		CMT-306	7	*De novo*	Bulgaria	Yes (1,2)
c.281G→A	4	R94Q		PN-1511.1	3; 2	Dominant	Portugal	Yes (1,2)
c.281G→A	4	R94Q		PN-1700.1	3	*De novo*	Belgium	Yes (1,2)
c.299C→G	4	A100G		CMT-297	10	Dominant	Bulgaria	No
c.368C→T	5	P123L	GTP	PN-930.1	2	Dominant	Germany	No
c.494A→G	6	H165R	GTP	PN-807.3	6	Dominant	Serbia	No
c.493C→T	6	H165Y	GTP	CMT-469.1	12	Dominant	Czech Republic	No
c.617C→T	7	T206I	GTP	CMT-52.02	3; 4	*De novo*	Belgium	Yes (3)
c.617C→T	7	T206I	GTP	CMT-489	2	*De novo*	Czech Republic	Yes (3)
c.617C→T	7	T206I	GTP	CMT-499	3	*De novo*	Czech Republic	Yes (3)
c.748C→T*	8	R250W	GTP	CMT-529	4	Unknown	Poland	No
c.749G→A	8	R250Q	GTP	PN-1457.1	21	Unknown	Belgium	No
c.827A→G	9	Q276R		CMT-273	10	Dominant	Bulgaria	Yes (3)
c.830A→G	9	H277R		CMT-83.2	10; 15	Dominant	Germany	No
c.839G→A	9	R280H		PN-1263.1	45; 24; 21	Dominant	Belgium	Yes (1)
c.839G→A	9	R280H		CMT-404.1	5; 6; 7; 55	Unknown	Italy	Yes (1)
c.1081C→T	11	H361Y		CMT-507	2	*De novo*	USA	Yes (3)
c.1128G→A	11	M376I		CMT-479.1	35	Dominant	Czech Republic	No
c.1134_1142del9	11	L379_M381del		BAB1135	3	Dominant	USA	No
c.[1157A→C;1158G→T]	11	Q386P		PN-1497.1	1.5	*De novo*	Israel	No
c.1198C→T*	12	R400X		CMT-529	4	Unknown	Poland	No
c.2129T→C	18	L710P	CC2	CMT-291	6	Unknown	Bulgaria	No
c.2219G→C	19	W740S		BAB1359	Unknown	Dominant	USA	Yes (1)
c.2251C→T	19	Q751X		CMT-466.1	4	*De novo*	Czech Republic	No
c.2251C→T	19	Q751X		PN-270.4	5	Dominant	Belgium	No

Nucleotide positions are relative to the translation initiation site of MFN2 cDNA (NM_014874). GTP = GTP binding domain; CC2 = coiled-coil domain 2. Age at onset in years for index patient and family members. (1), Züchner *et al.* (2004); (2), Kijima *et al.* (2005); (3), Züchner *et al.* (2006).

*Two nucleotide changes in the same patient.

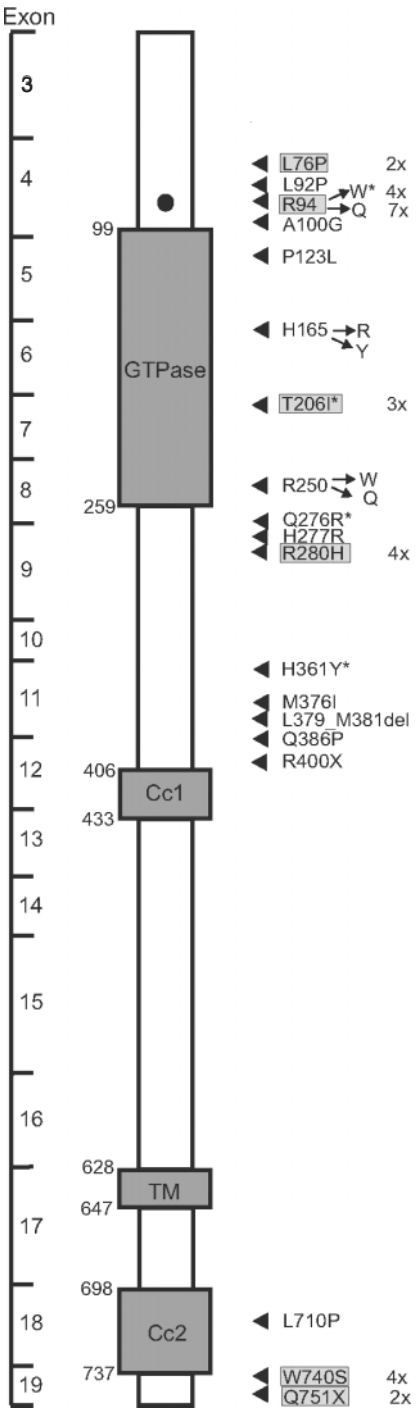

Fig. 1 Distribution of disease-associated mutations in the MFN2 protein. The size of the MFN2 protein domains corresponds to the number of amino acids involved (NP_055689). The dark grey boxes represent predicted domains of MFN2: GTPase, GTPase domain; Cc1, coiled-coil domain 1; TM, transmembrane domain; Cc2, coiled-coil domain 2. Numbers on the left indicate amino acid residue positions at the beginning and the end of each protein domain (according to Human Protein Reference Database (HPRD: http://www.hprd.org/protein/). Symbols: *mutations associated with CMT2 and optic atrophy (Züchner *et al.*, 2006), (filled circle) mutation hot spot. Boxed mutations indicate that the respective mutation was found more than once in our patient sample or reported before, and the frequency of mutations is shown.

were found. The c.748C→T substitution changes the arginine at position 250 to tryptophane, while the c.1198C→T mutation changes the arginine at position 400 into a stop codon. In patient PN-1497.1, we also found a dinucleotide change (c.1157A→C; 1158G→T) occurring in *cis* and mutating the glutamine residue at position 386 into proline (Q386P). In CMT-466.1 and PN-270.4, we detected a nucleotide substitution (c.2251C→T; p.Q751X) resulting in a nonsense mutation. In patient BAB1135, we found a deletion of nine nucleotides (c.1134_1142del9) in exon 11, resulting in an in frame elimination of three amino acids (p.L379_M381del). All remaining probands had a single nucleotide substitution leading to a missense mutation (Table 1). None of these mutations was found in 340 control chromosomes of healthy European individuals.

Segregation analysis of the respective *MFN2* mutations was confirmed in 14 CMT2 families suggesting autosomal dominant inheritance. In 10 probands, the *MFN2* mutations occurred *de novo*. For 5 index patients, pedigree information was lacking and therefore the mode of transmission of the mutation is defined as unknown (Table 1).

Of the 30 *MFN2* mutations identified, some occurred more than once. The R94W (c.280C→T) mutation segregated in a Czech family (CMT-472.1) and occurred as a *de novo* mutation in a Belgian patient (CMT-67). This arginine at position 94 seems to be prone to mutation as it is also altered to a glutamine (R94Q; c.281G→A) in a Bulgarian (CMT-306), a Portuguese (PN-1511.1) and two Belgian (PN-198.1, PN-1700.1) patients. The R280H (c.839G→A) mutation was found twice, in a Belgian (PN-1263.1) and an Italian family (CMT-404.1). These three missense mutations (R94W, R94Q, R280H) have been reported previously (Züchner *et al.*, 2004; Kijima *et al.*, 2005; Züchner *et al.*, 2006). The T206I (c.617C→T) mutation occurred as a *de novo* mutation in three probands of Belgian (CMT-52.2) and Czech (CMT-489, CMT-499) origin, while the nonsense mutation Q751X (c.2251C→T) was found in a Belgian (PN-270.4) and Czech patient (CMT-466.1). The repetitive finding of these 5 missense mutations (R94W, R94Q, T206I, R280H and Q751X) suggests that these amino acid changes might be hotspots for point mutations (Coulondre *et al.*, 1978; Bird, 1980). Both the L76P and W740S *MFN2* mutations, in the North American families BAB1792 and BAB1359 respectively, were also described previously (Züchner *et al.*, 2004).

In summary, we identified 30 *MFN2* mutations comprising 22 different mutations of which 14 have not been reported previously. Except for two nonsense mutations (R400X, Q751X) and one deletion (L379_M381del), all of the mutations were missense mutations (Table 1).

Clinical and electrophysiological findings

In the cohort of 323 unrelated index patients with distinct CMT subtypes, we identified a *MFN2* mutation in 29 probands. Twenty-eight of them were diagnosed with CMT2 on a total of 249 CMT2 patients (11%) and one patient

presented with an intermediate CMT phenotype (5%). Mutations in *MFN2* were not found in patients diagnosed as CMT1 or with a distal hereditary motor neuropathy or a hereditary sensory and autonomic neuropathy. The majority (29/30; 97%) of the *MFN2* mutations was observed in patients diagnosed as CMT2. Overall, the diagnostic yield of *MFN2* mutations in CMT2 patients was 11%. However, when considering only patients with a family history of CMT the diagnostic yield was 18%, and with a family history of CMT2 the yield increased to 33%.

The age at onset was available for 27 index patients, ranging from 1 to 45 years (Table 1). The mean age at onset was 8 years. The majority of index patients experienced their first symptoms before they were 10 years old (Fig. 2A). According to the age at onset, two groups of patients could be delineated, a large group with early onset (<10 years; mean age 3.5 years) and a smaller group with late onset (⩾10 years; mean age 20.5 years). Overall, the disease status was milder (grade 2 for lower limbs, Fig. 2A) and the disease course was more slowly progressive in the late-onset group compared to the early-onset group (grades 2–3–4 for lower limbs, Fig. 2A). Within the early-onset group, 33% of patients with age at onset before 5 years are wheelchair-dependent, whereas 17% of patients with age at onset between 5 and 10 years are wheelchair-bound, reflecting a more severe phenotype depending on earlier age at onset (Fig. 2A).

All patients with an *MFN2* mutation experienced as their first symptoms difficulty with walking and frequent falls, muscle weakness in the distal legs and occasional muscle cramps in the legs. *Pes cavus* was sometimes observed. Muscle atrophy was observed in the feet and the anterior and lateral part of the lower legs. Muscle strength in the lower limbs was severely affected, with a large number of patients showing complete distal paralysis (29 out of 32, 91%), resulting in bilateral foot drop, proximal paresis (15 out of 32, 47%) and wheelchair-dependency (9 out of 32, 28%) (Fig. 2B). Deep tendon reflexes in the lower limbs were weak or absent. In the upper limbs many patients experienced severe paresis of the distal muscles (20 out of 30, 67%) and proximal paresis (6 out of 30, 20%), although some patients had no weakness in arms and hands (6 out of 30, 20%) (Fig. 2C). Pyramidal signs were absent in all patients. In some patients muscle atrophy was present in the intrinsic hand muscles, including thenar and hypothenar. In the upper limbs reflexes were either normal, weak or absent. Sensory testing for all modalities (touch, pain, vibration, position and temperature) was normal to decreased, especially in the lower limbs.

In some probands additional features were present, such as tremor of fingers and arms or scoliosis. Around 20 years, three probands (CMT-52, CMT-273, CMT-507) experienced an optic atrophy with a subacute onset and partial or full recovery (Züchner *et al.*, 2006). The affected father and son of family CMT-52 also had mild hearing loss. In one patient (CMT-83.2) parkinsonism occurred at older age, and in

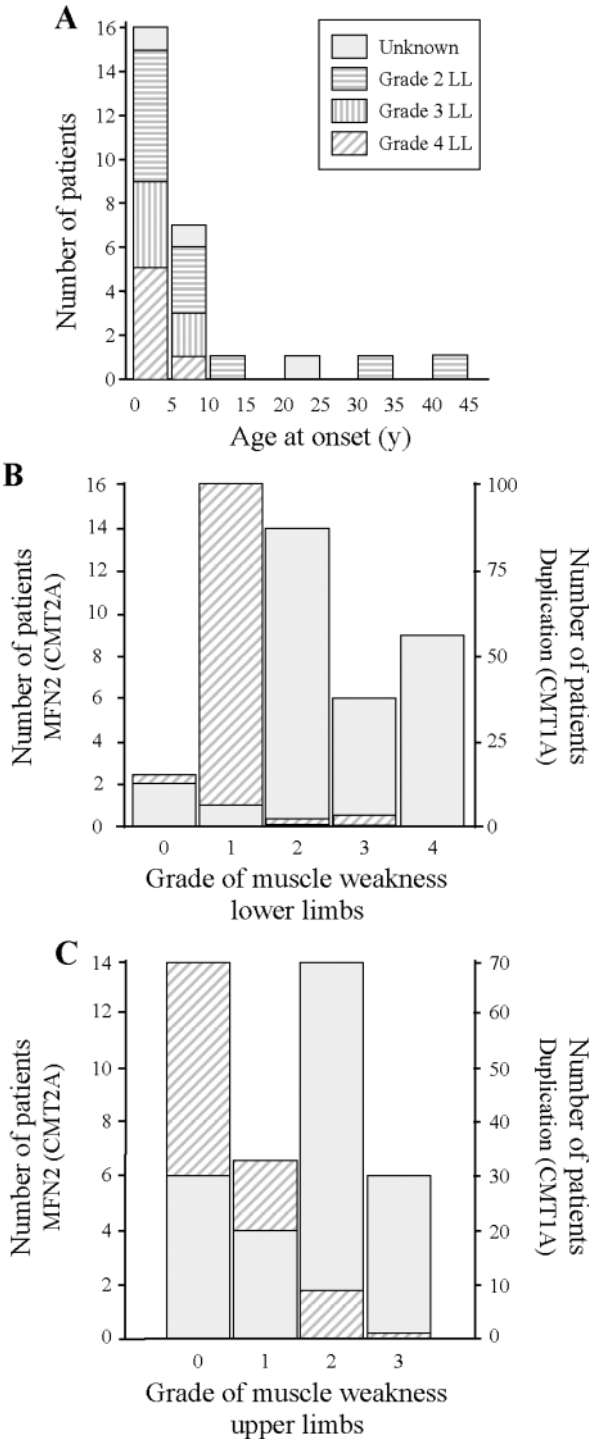

Fig. 2 Phenotypic spectrum of *MFN2* mutations. (**A**) Number of probands with an *MFN2* mutation in relation to age at onset in years. Gradation in lower limbs: 0, no weakness; 1, paresis of distal muscles; 2, paralysis of distal muscles; 3, proximal weakness; 4, wheelchair-dependent. Gradation in upper limbs: 0, no weakness; 1, mild paresis of distal muscles; 2, severe paresis of distal muscles; 3, proximal weakness. (**B**) Number of patients shown for each grade of muscle weakness in lower limbs. (**C**) Number of patients shown for each grade of muscle weakness in upper limbs. The number of patients with an *MFN2* mutation (CMT2A) is indicated by grey bars, and compared to CMT1A duplication patients (hatched bars).

Table 2 Electrophysiological data of patients with an *MFN2* mutation

Patient	Amino acids	Age	R/L	Median motor		Ulnar motor		Peroneal motor		Tibial motor		Median sensory		Ulnar sensory		Sural sensory	
Normal values →				Amp 4.0	CV 49.0	Amp 4.0	CV 49.0	Amp 3.0	CV 41.0	Amp 3.0	CV 41.0	Amp 17.0	CV 46.0	Amp 17.0	CV 47.0	Amp 12.0	CV 44.0
CMT-472.1	R94W	15	R	**0.3**	**39.0**	**A**	**A**	–	–	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**
CMT-472.2	R94W	52	R	**A**	**A**	**A**	**A**	–	–	**A**	**A**	**2.0**	**35.0**	**2.0**	**28.0**	**A**	**A**
CMT-67	R94W	34	L	**A**	**A**	**0.2**	**42.0**	–	–	–	–	**A**	**A**	**A**	**A**	–	–
PN-198.1	R94Q	33	R	–	49.7	–	53.7	**0.1**	**32.6**	–	–	**5.0**	**45.6**	**A**	**A**	**A**	**A**
			L	**3.0**	54.3	**3.0**	49.7	–	–	–	–	–	–	–	–	–	–
PN-198.3	R94Q	11	R	**1.0**	55.1	**3.0**	61.1	**A**	**A**	**A**	**A**	**2.0**	**45.5**	**2.0**	**44.2**	**A**	**A**
			L	**0.5**	57.2	**1.0**	54.5	–	–	**A**	**A**	–	–	–	–	–	–
CMT-306	R94Q	11	R	14.1	**45.1**	10.0	55.5	**1.5**	**30.8**	11.7	**32.7**	**7.1**	–	**3.7**	–	**A**	**A**
PN-1511.1	R94Q	4	R	**1.4**	**37.0**	–	–	**A**	**A**	**1.0**	**37.0**	**3.1**	**32.0**	–	–	**A**	**A**
PN-1511.2	R94Q	29	R	**2.4**	**46.9**	**1.7**	**48.9**	**A**	**A**	–	–	**1.8**	48.6	**2.8**	47.3	**4.8**	**37.5**
			L	–	–	–	–	–	–	–	–	–	–	–	–	**4.2**	**41.7**
CMT-297	A100G	29	R	5.0	**48.4**	7.9	50.0	3.0	**22.0**	–	–	**3.3**	–	**12.0**	–	**A**	**A**
PN-930.1	P123L	9	R	–	**44.8**	–	–	–	–	–	–	–	–	–	–	–	–
PN-807.3	H165R	36	R	**1.0**	54.3	–	–	**A**	**A**	**A**	**A**	–	–	–	–	–	–
CMT-469.1	H165Y	52	R	5.25	51.1	–	–	**0.2**	**34.0**	**A**	**A**	**3.6**	53.2	**4.6**	**45.6**	**A**	**A**
			L	7.6	51.7	–	–	–	–	**A**	**A**	**2.2**	47.5	–	–	**A**	**A**
CMT-469.2	H165Y	22	L	–	–	–	–	10.6	45.3	14.9	51.5	–	–	–	–	17.0	**40.1**
CMT-489	T206I	16	L	**A**	**A**	**0.05**	**41.0**	–	–	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**
CMT-499	T206I	21	R	**A**	**A**	**A**	**A**	–	–	**A**	**A**	**A**	**A**	**A**	**A**	**A**	A
CMT-529	(R250W)		R	**2.7**	53.8	5.1	54.0	–	–	**0.7**	48.0	**9.6**	50.0	**10.0**	52.0	18.0	45.2
CMT-273	Q276R	28	R	7.5	**45.6**	8.5	51.4	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**
CMT-83.2	H277R	54	R	–	–	–	–	–	**35.0**	–	–	–	–	–	–	–	–
CMT-83.7	H277R	17	R	–	–	–	–	–	42.0	–	**34.0**	–	–	–	–	–	–
PN-1263.1	R280H	77	R	10.0	61.0	11.1	50.0	–	–	–	–	**2.8**	–	**1.9**	–	–	–
PN-1263.3	R280H	25	R	–	–	–	–	–	–	8.0	**34.7**	–	–	–	–	**4.0**	**38.0**
			L	–	–	–	–	–	–	4.9	**34.1**	–		–	–	**3.9**	**34.8**
CMT-404.1	R280H	39	R	**0.64**	**42.5**	**1.16**	**37.8**	–	–	–	–	**7.4**	**42.1**	**3.5**	**39.0**	**1.52**	**35.2**
CMT-479.1	M376I	65	L	4.5	53.0	8.1	61.0	**A**	**A**	**0.1**	**39.0**	**14.0**	**39.0**	**12.0**	**41.0**	**A**	**A**
BAB1135	L379_M381del	21	R	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**
			L	**A**	**A**	–	–	–	–	**A**	**A**	–	–	–	–	–	–
PN-1497.1	Q386P	13	R	**A**	**A**	**A**	**A**	–	–	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**
			L	**A**	**A**	**A**	**A**	–	–	**0.3**	54.7	**A**	**A**	**A**	**A**	**A**	**A**
CMT-291	L710P	28	R	**2.5**	49.8	**2.7**	**42.9**	**A**	**A**	**A**	**A**	**2.9**	**19**	**2.8**	**17**	**A**	**A**
BAB1359	W740S	12	R	–	–	↓	nl	–	–	**A**	**A**	–	–	–	–	↓	nl

A, absent evoked response; Age, age at examination; Amp, amplitude (motor: in mV; sensory: in μV); CV, conduction velocity (in m/s); –, not measured; nl, normal (no precise data); ↓, decreased (no precise data). Bold = abnormal values. R/L = right/left.

another patient (CMT-83.7) schizophrenic psychosis was diagnosed.

Electrophysiological data were available for 21 probands and six secondary patients of six families (Table 2). The NCVs of the motor median nerve ranged from 37 to 61 m/s; in only one nerve the NCV was lower than 38 m/s, in seven nerves the NCV was reduced within the range of 38–49 m/s, and in the remaining 11 nerves the NCV was normal. In eight motor median nerves the compound motor action potentials (CMAPs) could not be elicited. Reduced NCVs were always associated with severely reduced CMAPs. However, severely reduced CMAPs were also often observed in nerves with normal NCVs. In some patients both NCVs and CMAPs were normal. One patient (PN-1511.1) had NCVs lower than 38 m/s in all nerves tested and could therefore be diagnosed as CMT1. However, his affected father (PN-1511.2) had NCVs higher than 38 m/s suggesting an intermediate CMT phenotype in this family.

Pathological findings

We investigated the sural nerve biopsy of a 6.5 year-old boy (PN-930.1; *MFN2* mutation P123L) and the sural nerve and a muscle biopsy of a 2.6 year-old girl (PN-142; *MFN2* mutation L92P) using both conventional light microscopy, including morphometry, and electron microscopy, with special emphasis on mitochondrial changes. A fine structural study of axonal mitochondria has thus far not been performed although a few other biopsies of cases with *MFN2* mutations have recently been described at the light microscopic level (Kijima *et al.*, 2005).

The sural nerve of patient PN-930.1 showed an apparent loss of large myelinated nerve fibres compared to an age-matched control (Fig. 3A, B). These fibres were replaced by numerous large bands of Büngner with or without small regenerated myelinated or unmyelinated axons. The regenerated fibres were arranged in clusters of not more than 2–3 myelinated fibres. There was no formation of

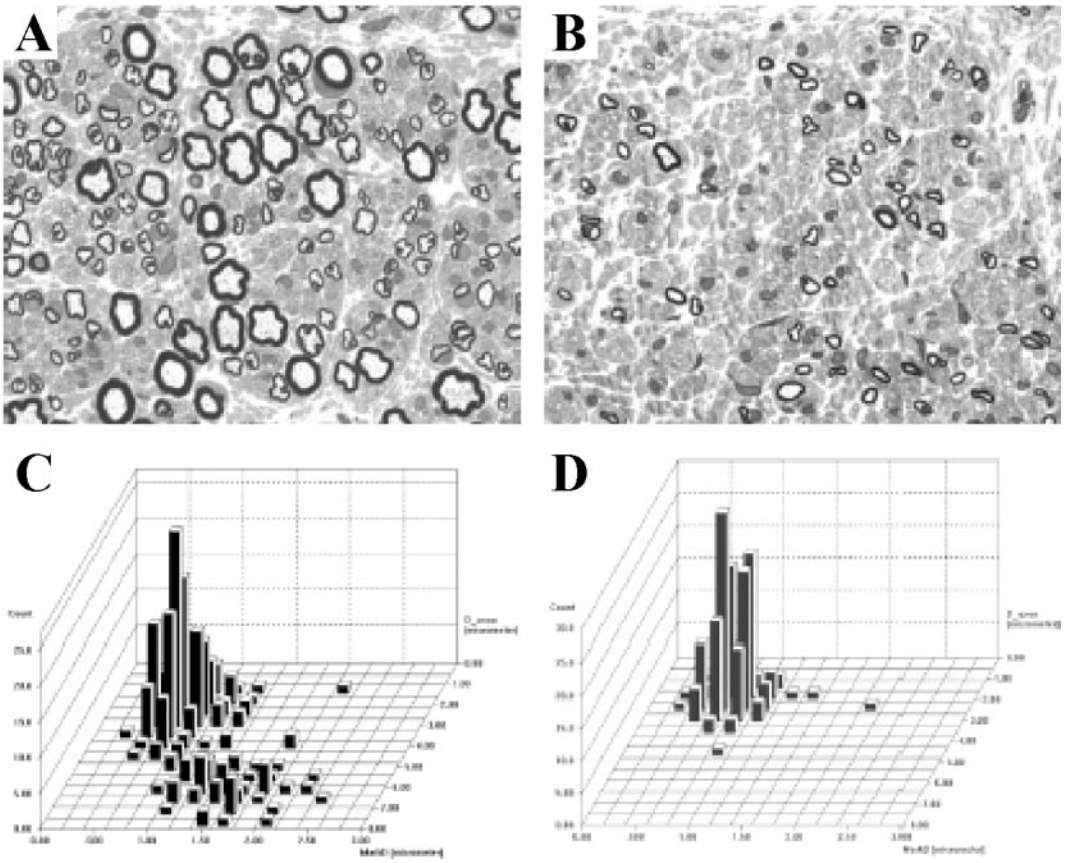

Fig. 3 Light microscopic findings in sural nerve biopsy (PN-930.1). Light microscopic comparison between semithin cross sections of sural nerves from a control at the age of 62 months (**A, C**), and from the patient at the age of 77 months (**B, D**) shown at the same magnification (×600) (**A, B**) and following optic-electronic evaluation of five representative areas of equal size (**C, D**). No large myelinated nerve fibres are present in the patient's nerve (**B, D**), instead there are numerous large bands of Büngner with or without small regenerated myelinated or unmyelinated axons. Several bands of Büngner are rather circular in shape. The number of Schwann cell nuclei (within the bands of Büngner) has clearly increased. No typical onion bulb formations are seen with several layers of supernumerary, flat Schwann cell processes around individual remyelinated axons and no myelin degradation products are detectable although there is some degree of endoneurial oedema.

onion bulbs, and no myelin degradation products were detected, although there was some degree of endoneurial oedema (Fig. 4A). The density of small myelinated fibres was similar to that of the control (Fig. 3C, D). The mitochondria showed no paracrystalline, membranous, globular or granular inclusions, although degenerative changes were repeatedly noted at sites of accumulation in paranodal extensions of axons into myelin loops and in unmyelinated axons, seemingly indicating abnormal fusion, fission, swelling, fading or condensation into 'dense bodies' (Fig. 4A, B, C). Close contacts between neighbouring mitochondria without fusion were repeatedly seen, sometimes with tapered tips. The outer membrane of occasional mitochondria appeared to be wrinkled or fused with the inner membrane. Most mitochondria were quite small and electron dense. Otherwise the mitochondria in axons of this patient appeared not to differ significantly from those seen in axons of patients with mitochondrial DNA mutations which have previously been biopsied and investigated in one of the co-author's (J.M.S.) laboratories.

The sural nerve biopsy in the younger girl (PN-142) revealed a similar loss of large myelinated fibres with even less evidence of regeneration (no clusters of regenerating fibres) (Fig. 4D, E). Some myelin sheaths were disproportionately thin. Few thin Schwann cell processes encircling small myelinated fibres resembled incipient onion bulb formations; yet they were associated with unmyelinated axons indicating degeneration and regeneration. Morphometry of myelinated fibres was not performed because of artificial tissue shrinkage. At the electron microscopic level, degenerating mitochondria, focally accumulated in axons (Fig. 4D, E), showed swelling, condensation, transformation into myelinoid, dense bodies, and irregularities of inner and outer mitochondrial membranes were similar to those in the older boy (PN-930.1). No paracrystalline, globular or other characteristic inclusions were noted in axonal mitochondria. Longitudinal sections revealed no apparent reduction of the length of mitochondria in axons when compared to controls. Occasionally, accumulation of small mitochondria was noted in the adaxonal compartment of

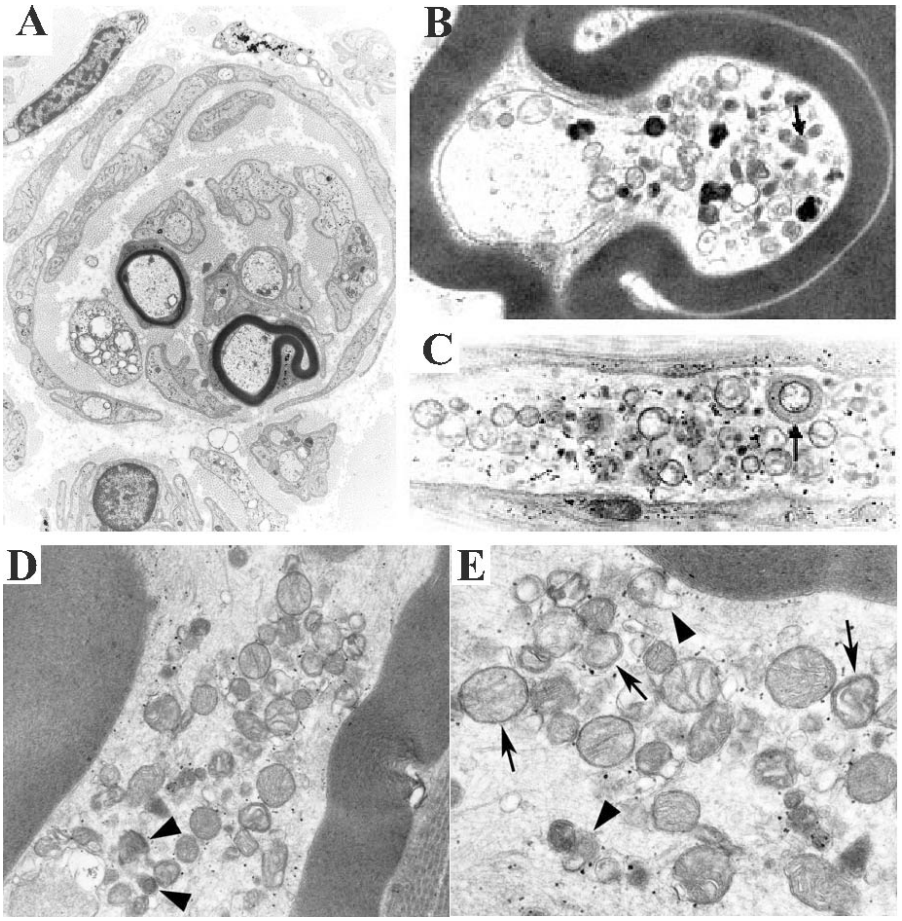

Fig. 4 Electron microscopic findings in sural nerve biopsy. (**A**) Electron micrograph of the patient's sural nerve (PN-930.1). Large reinnervated band of Büngner with 2 remyelinated and at least 6 unmyelinated sprouts at the site of a distally degenerated and regenerated nerve fibre. There is a large number of Schwann cell processes with no evidence of degeneration. The collagen filaments inside and outside this band of Büngner have similar diameters. Fibroblastic cells or macrophages are apparent inside and outside but only the inner one shows an increased number of (artificially swollen) mitochondria. Original magnification: ×13 000. (**B, C**) Mitochondrial abnormalities in the patient's nerve (PN-930.1). (**B**) This fortuitous section through an axon protruding into a paranodal myelin loop shows considerable accumulation of normal and abnormal mitochondria. There are various degenerative changes such as swelling, transformation into membranous whorls, suggestive splitting or fusion (arrow), or dissolution. Only some mitochondria appear to be well preserved. (**C**) This unmyelinated axon shows further mitochondrial changes such as uneven density of the inner and outer membranes, or a concentric inner component with somewhat collapsed intra-cristal space despite normal appearance of the outer 'double membrane' (arrow), or wavy mitochondrial membranes. In some small mitochondria, the matrix appears to be dissolved and the outer and the inner membrane fused. In between are neurosecretory granules, glycogen granules, components of the axoplasmic reticulum, neurofilaments and microtubules. Primary magnifications: (**B**) ×28 000, (**C**) ×35 000. (**D**, **E**) Electron microscopic findings in sural nerve of patient PN-142: intra-axonal accumulation of mitochondria showing on high magnification several irregular arrangements of cristae (arrows) and degenerative features (arrowheads). Furthermore, in panel (**E**), uncommon swollen intra-axonal mitochondria with ruptured cristae are visualized as well as normal appearing mitochondria. (**D**) Magnification: ×108 000; (**E**) Magnification: ×164 000

a myelinating Schwann cell. As in PN-930.1 a focal increase of the number of mitochondria was noted in endoneurial fibroblasts. The muscle biopsy revealed a moderate degree of neurogenic atrophy without fibre type grouping and without increase of connective tissue. The modified Gomori trichrome stain did not reveal any 'ragged red' fibres which would have indicated abnormal accumulation of mitochondria. At the electron microscopic level, abnormal mitochondria were noted in an occasional necrotic muscle fibre, but not in fibres of normal size, or in atrophic (or possibly hypotrophic) fibres.

Discussion

In the present study, we screened a cohort of 323 index patients with distinct CMT subtypes for mutations in the *MFN2* gene. In 29 probands, we identified an *MFN2* mutation. Patients with an *MFN2* mutation presented with a classical CMT phenotype but more severe (28% of our CMT2A patients were wheelchair-dependent) compared to e.g. the CMT1A-duplication phenotype (Fig. 2B, C). Furthermore, some patients developed additional features such as optic neuritis sometimes resulting in optic atrophy (Züchner *et al.*, 2006). Interestingly, *MFN2* mutations do not

only cause the clinically 'pure' form of CMT2 neuropathy. Recently, one CMT family with pyramidal signs was reported with a missense mutation in *MFN2*. The phenotype was referred to as HMSN V (Zhu *et al.*, 2005). Furthermore, Züchner *et al.* (2006) showed that five different missense mutations in *MFN2* led to axonal CMT associated with optic atrophy (referred to as HMSN VI) (Dyck *et al.*, 1993).

Two subgroups could be delineated based upon differences in age at onset and severity of the disease (Fig. 2A): (i) most patients had an early onset (<10 years) and severe disease status (grades 2–3–4 for lower limbs; legend to Fig. 2B); and (ii) a smaller group experienced a later onset and a milder disease course (grade 2 for lower limbs, legend to Fig. 2B). On average, the clinical phenotypes associated with *MFN2* mutations observed in this study are more severe than the phenotypes reported in previous studies. This is partly explained by a selection bias since the initial reports described large families suitable for linkage studies. This obviously implies that the phenotype can not be so severe as to compromise reproductive fitness. The cohort of CMT patients screened by Kijima *et al.* (2005) more closely resembles the population analysed in the current study. They observed one *de novo* mutation while we could prove that the *MFN2* mutation occurred *de novo* in at least 10 out of 29 probands (34%). However, this number could even be higher since in six patients no sufficient information was available to delineate the inheritance pattern. The high percentage of *de novo* mutations probably relates to our selection criteria that did not exclude patients with severe phenotypes or patients without a family history of CMT.

The motor median NCVs cluster within the normal range or are slightly to moderately reduced. The lowest value is 37 m/s, which is close to the cut-off value of 38 m/s separating CMT2 from CMT1. This pattern is strikingly different from the broad range of NCVs observed in families with *GJB1*, some *MPZ* and *NEFL* mutations. In addition CMAPs were often severely reduced. The electrophysiological data thus strongly suggest that the underlying pathology is primarily axonal. The pathological findings were mainly characterized by severe loss of large myelinated fibres and degenerative mitochondrial changes. The limited number of clusters of regenerating nerve fibres and their small size appears to be correlated to the severity of this axonal type of neuropathy in the two young individuals studied by biopsy. As previously noted in the Vizioli type of neuropathy (HMSN VI) with optic atrophy, there were no characteristic crystalline, paracrystalline, or globular mitochondrial inclusions, or proliferated cristae in mitochondria of axons such as typically seen in muscle fibres of cases with mitochondrial myopathies. Yet the generally small size of axonal mitochondria, irregularities of the outer mitochondrial membrane and closely adjacent mitochondria without evidence of fusion at sites of focal accumulations were conspicuous findings which might be of interest in a disorder such as the present one.

Except for two nonsense mutations (R400X, Q751X) and one deletion (L379_M381del), we found only missense mutations in *MFN2*. This finding concurs with previous studies in which, with the exception of a nonsense mutation (R418X), only missense mutations have been reported (Züchner *et al.*, 2004; Kijima *et al.*, 2005; Lawson *et al.*, 2005; Züchner *et al.*, 2006). Some of the *MFN2* mutations (L76P, R280H, T206I, W740S) were found more than once or were reported previously, suggesting that these amino acid positions might be prone to mutation. However, the arginine at amino acid position 94 was changed to a glutamine (R94Q) in four of our probands, while in one proband it changed to a tryptophan (R94W). The fact that both amino acid changes (R94Q and R94W) have been reported previously (Züchner *et al.*, 2004), and also in as genetically distinct a population as the Japanese (Kijima *et al.*, 2005), clearly indicates codon 94 as a hot spot for mutation.

MFN2 is a large mitochondrial transmembrane GTPase. In mitochondria the MFN2 protein spans the mitochondrial outer membrane with a large N-terminal and a relatively short C-terminal domain exposed to the cytosol (Rojo *et al.*, 2002). The domain structure of *MFN2* consists of a GTPase domain near the N-terminus, a coiled-coil domain, two transmembrane spans and a coiled-coil domain in the C-terminal tail (Fig. 1). Strikingly, all of our MFN2 mutations are located in the cytoplasmic domains of the MFN2 protein, more specifically before the N-terminal coiled-coil domain or in the last 50 amino acids. Neither of the transmembrane domains TM1 and TM2 or the region immediately upstream or downstream of the transmembrane domains is affected. Nine mutations were located immediately upstream of the GTPase domain, while eight of the 30 mutations are within the GTPase domain of the MFN2 protein (Fig. 1 and Table 1). Four mutations lie in or immediately after the C-terminal coiled-coil domain. It has been suggested that this terminal coiled-coil domain functions as a mitochondrial tether between adjacent mitochondria before mitochondrial fusion (Koshiba *et al.*, 2004). Missense mutations in the C-terminal coiled-coil domain of MFN1, which is structurally and functionally very similar to MFN2, indicated a significant reduction of the stability of the tether between mitochondria and a reduced tubular mitochondrial morphology (Koshiba *et al.*, 2004). In our study there was no correlation between the site or type of mutation (GTP, coiled-coil domain or surrounding sequences of the MFN2 protein) and the age at onset, clinical severity or additional features in the *MFN2* mutation patients (data not shown).

The diagnostic yield of *MFN2* mutations in CMT2 was 11%. This frequency is significantly lower than the 23 and 19% reported in recent studies (Kijima *et al.*, 2005; Lawson *et al.*, 2005). However, when we consider only our patients with a family history of CMT the diagnostic yield was 18%. A further selection of the patients with a family history of CMT2 increased the yield to 33%, suggesting that currently *MFN2* mutations are the most common cause of CMT2. We

would therefore suggest that a genetic testing of CMT2 patients should begin with the screening of *MFN2*. Furthermore, screening of *MFN2* is also useful in patients with a late-onset neuropathy with a family history and maybe also in isolated patients.

Acknowledgements

We gratefully acknowledge the participation of all patients and their relatives in this study. We appreciated the contribution of the VIB Genetic Service Facility (http://www.vibgeneticservicefacility.be/) for genotyping and sequencing. The study was supported by the Fund for Scientific Research (FWO-Flanders), the Medical Foundation Queen Elisabeth (GSKE), the University of Antwerp (UA), the Interuniversity Attraction Poles program of the Belgian Federal Science Policy Office (BELSPO) and the Association Belge contre les Maladies Neuromusculaires (ABMM) to V.T. and P.D.J. Support was provided by National Institute of Health (NIH grants NS 2P01-NS26630-14 and 2R01-NS29416-09) to J.M.V., the Czech Ministry of Health (VZ00064203/6506) to P.S., the Polish State Scientific Committee (KBN) No. 2P05E 112 28 to A.K., and the Austrian Science Fund (FWF, P17494-B14) to M.A.-G. K.V. is a postdoctoral fellow of the FWO-Flanders, Belgium. A.J. received visiting research fellowships from the BELSPO and NATO/FWO, Belgium. P.S. received a European Neurological Society (ENS) fellowship.

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