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RESEARCH ARTICLE

Charcot-Marie-Tooth Neuropathy Type 2 and P0 Point Mutations: Two Novel Amino Acid Substitutions (Asp61Gly; Tyr119Cys) and a Possible "Hotspot" on Thr124Met

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Mutations in the gene for the major protein component of peripheral nerve myelin, myelin protein zero (MPZ, P0), cause hereditary disorders of Schwann cell myelin such as Charcot-Marie-Tooth neuropathy type 1B (CMT1B), Dejerine-Sottas syndrome (DSS), and congenital hypomyelinating neuropathy (CHN). More recently, P0 mutations were identified in the axonal type of CMT neuropathy, CMT2, which is different from the demyelinating variants with respect to electroneurography and nerve pathology. We screened 49 patients with a clinical and histopathological diagnosis of CMT2 for mutations in the P0 gene. Three heterozygous single nucleotide changes were detected: two novel missense mutations, Asp61Gly and Tyr119Cys, and the known Thr124Met substitution, that has already been reported in several CMT patients from different European countries. Haplotype analysis for the P0 locus proved that our patients with the 124Met allele were not related to a cohort of patients with the same mutation, all of Belgian descent and all found to share a common ancestor (7). Our data suggest that P0 mutations account for a detectable proportion of CMT2 cases with virtually every patient harbouring a different mutation but recurrence of the Thr124Met amino acid substitution. The high frequency of this peculiar genotype in the European CMT population is presumably not only due to a founder effect but Thr124Met might constitute a mutation hotspot in the P0 gene as well.

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Introduction

Charcot-Marie-Tooth neuropathy (CMT) or hereditary motor and sensory neuropathy (HMSN) comprises a heterogeneous group of inherited diseases of the peripheral nervous system. About 1 in 2,500 persons has a form of CMT, making it the most common inherited neuromuscular disorder (45). The clinical features of CMT include progressive distal muscle weakness and atrophy, foot deformities, steppage gait, distal sensory loss, and decreased or absent tendon reflexes. Variation in age of onset and clinical presentation in CMT is wide, ranging from severe distal atrophy and foot deformity starting in early childhood to minimal adult-onset pes cavus (14). CMT falls into two large subtypes, the demyelinating form, CMT1, and the axonal type, CMT2 (10). Electrophysiologically, CMT1 presents with severely reduced nerve conduction velocities (NCVs) in the motor and sensory nerves, usually less than 38 m/s for the motor median nerve. CMT2 patients have normal or subnormal NCVs. Histopathologically, CMT1 is associated with segmental demyelination and classical onion bulb formation in nerve biopsies. CMT2 shows depletion of large myelinated fibers with regenerative clusters of small axons while the myelin sheaths remain relatively preserved. In most pedigrees, the CMT is transmitted in an autosomal dominant manner. However, in some families the disease is linked to the X-chromosome, and even more seldomly it displays an autosomal-recessive mode of inheritance.

Three genes responsible for autosomal dominant CMT1 have been identified: mutations occur in the

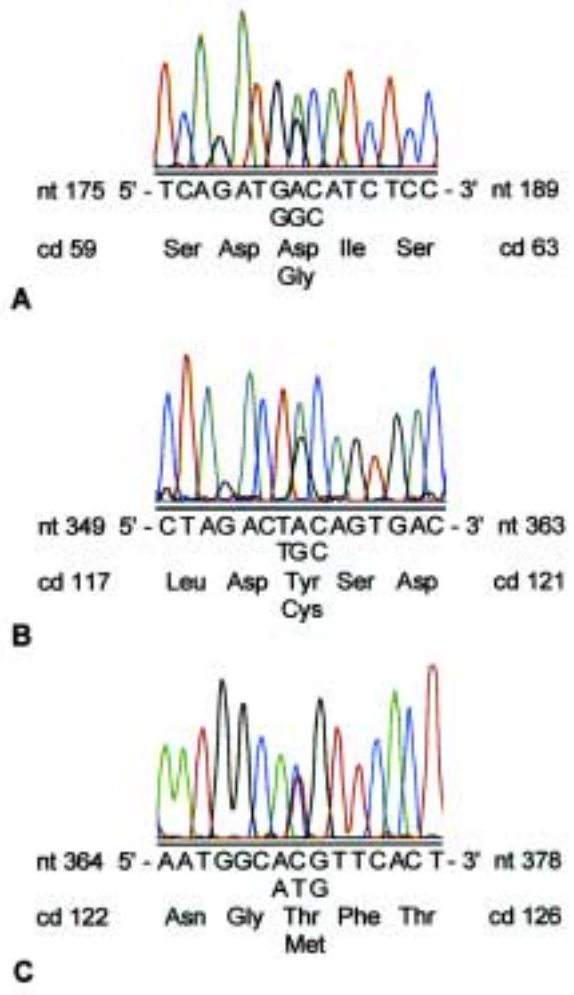


Figure 1. Identification of P0 mutations by automated sequencing analysis. The individual sense strand electropherograms from (**A**) patient AC09.V-6, (**B**) patient AC44.III-2, and (**C**) patient AC37.III-2 are shown at the top, and a limited reading frame indicating the corresponding amino acid substitutions is shown below.

peripheral myelin protein 22 gene (PMP22) on chromosome 17p11.2 (CMT1A) (24, 30, 49), the myelin protein zero gene (MPZ, P0) on chromosome 1q22-q23 (CMT1B) (15), and in the early growth response 2 gene (EGR2) on chromosome 10q21-q22 (51). In about 70% of cases, CMT1 is associated with a heterozygous 1.5 Mb tandem duplication of band 17p11.2 encompassing the PMP22 gene (27). Point mutations in PMP22, P0, and EGR2 can also cause two more severe, early onset forms of peripheral myelinopathy, Dejerine-Sottas syndrome (DSS) (16, 32, 47) and congenital hypomyelinating neuropathy (CHN) (44, 50, 51). Patients with the dominant X-linked type of CMT1 harbour mutations in the connexin32 (Cx32) gene located on chromosome Xq13.1 (3).

CMT2 is a much rarer disorder than CMT1 (all forms combined). Three loci for autosomal dominant CMT2 have been assigned to chromosomes 1p35-p36 (CMT2A) (2), 3q (CMT2B) (20), and 7p14 (CMT2D) (18). CMT2 with vocal cord paralysis and diaphragm weakness (CMT2C) has not yet been mapped to a chromosomal location (11). A locus for an axonal form of autosomal recessive CMT disease was found to map to chromosome 1q21.2-q21.3 (4). Most CMT2 families, however, do not link to any of these loci, and candidate genes remain to be identified. Interestingly, some patients with axonal CMT carry distinct point mutations in the Cx32 and P0 genes initially thought to be exclusively involved in the pathogenesis of demyelinating hereditary neuropathies (5, 7, 25, 28, 35, 41, 43, 48). The same Thr124Met amino acid substitution in the P0 gene segregated in several pedigrees of different geographic origin, but all European (5, 7, 39, 52). Genotype analysis with short tandem repeat (STR) markers flanking the P0 gene revealed that seven families and two isolated cases of Belgian ancestry all shared a common founder (7).

We have begun a regular screening of suspected CMT2 patients for P0 mutations by analysis of paraffin-embedded, archival nerve biopsy specimens. In this study, we report two novel P0 mutations associated with a CMT2 phenotype and the 13th family identified with the common Thr124Met P0 genotype. Furthermore, we performed haplotype analysis for polymorphic markers related to the P0 gene to test whether there is a unique ancestral 124Met allele not only in the Belgian but also in the general European population.

Materials and Methods

Patients. We previously identified four Cx32 mutations in a series of 45 patients that were diagnosed with CMT2 based on electrophysiologic and nerve biopsy studies (41). The remaining 41 cases and eight additional CMT2 cases that had been eliminated from the Cx32 study as male-to-male transmission occurred in the pedigrees were selected for mutation screening of the P0 gene. Twenty-two of the 49 patients were clinically sporadic, but in 13 of them no or incomplete information regarding family history was available so that a genetic transmission of the disease could not be excluded. Twenty-seven cases were familial with dominant inheritance (affected patients in more than one generation). Genomic DNA was extracted from nerve biopsy sam-

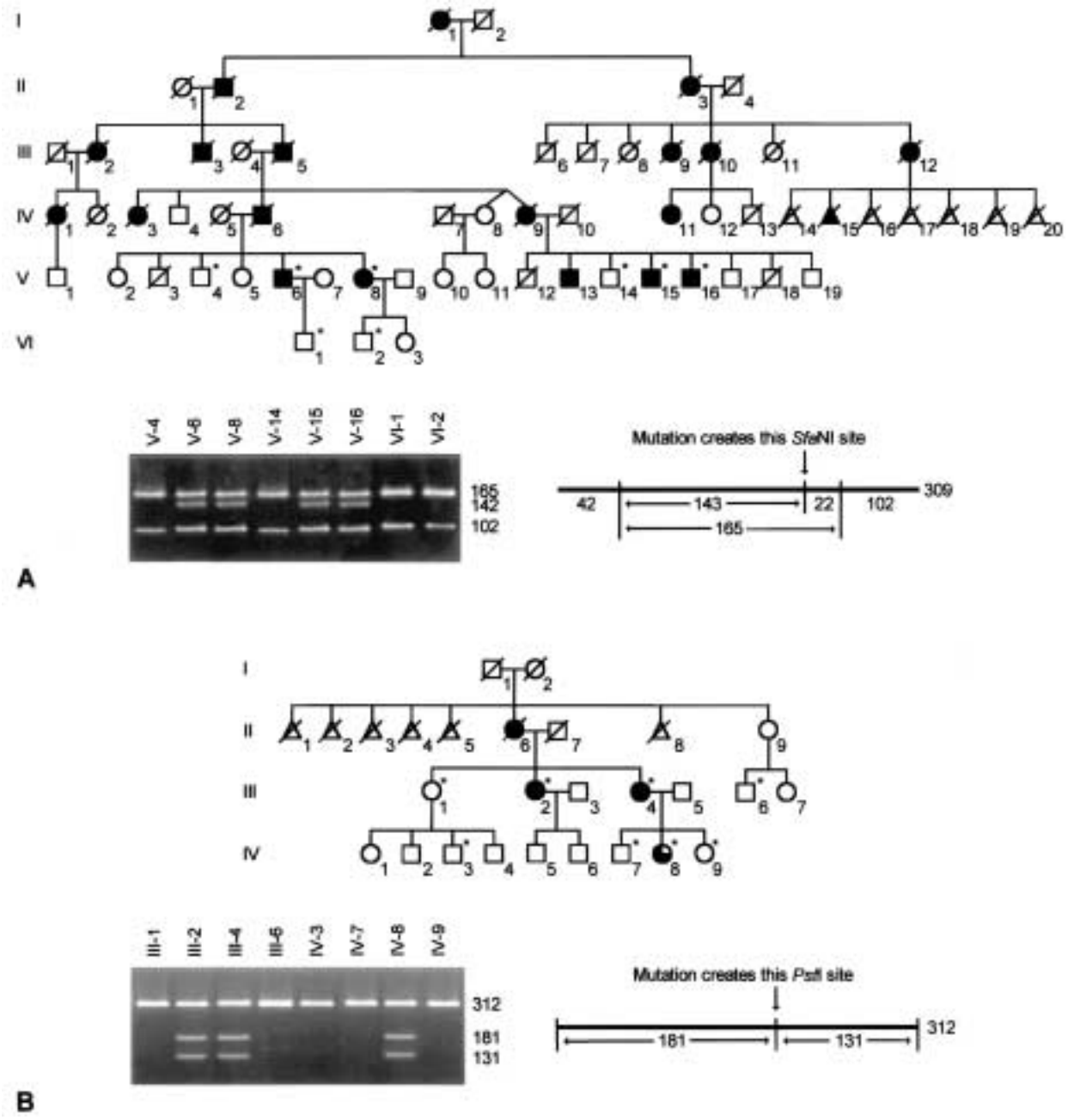
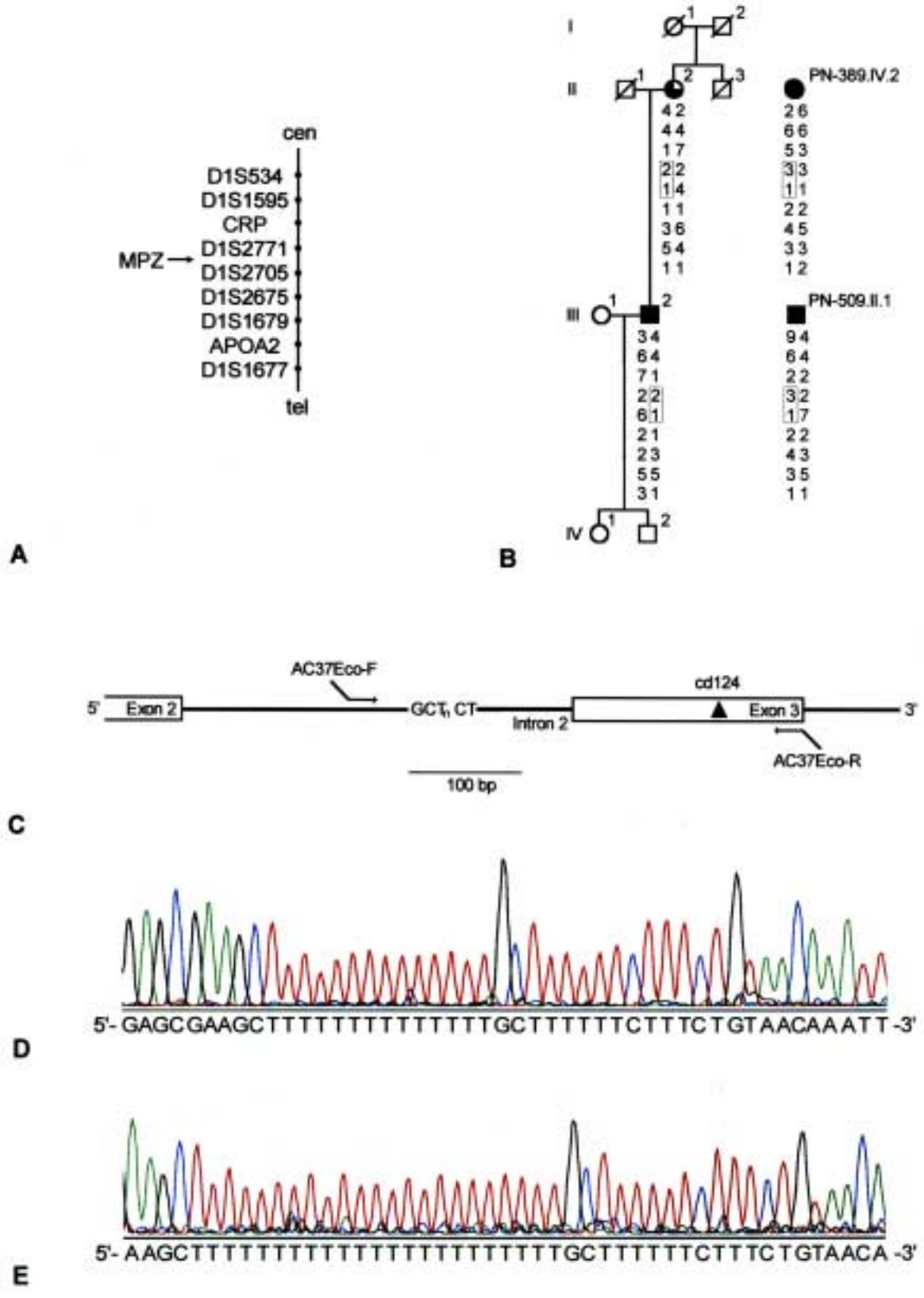


Figure 2. Pedigrees of (**A**) family AC09 and (**B**) family AC44. Males are represented as squares, females as circles, and triangles represent subjects whose sex was not known. Patients have black symbols, unaffected subjects open symbols. One individual with a mutation but without clinical symptoms (AC44.IV-8) is shown as a three-quarter-filled symbol. Deceased individuals are shown as cross-hatched symbols. An asterisk (*) marks probands clinically examined and sampled for DNA analysis. The results of restriction enzyme analysis for detection of the respective mutations are given below the pedigrees. The line drawings schematically represent the restriction patterns of normal and mutated PCR fragments. Numbers on the right of the gels and in the drawings indicate the size of digestion products in bp. Small 22 and 42 bp fragments in (A) are not visible under these electrophoretic conditions.


cen
D1S534
D1S1595
CRP
MPZ
D1S2771
D1S2705
D1S2675
D1S1679
APOA2
D1S1677
tel
A
I
II
III
IV
PN-389.IV.2
PN-509.II.1
B
AC37Eco-F
5'
Exon 2
GCT, CT
Intron 2
cd124
Exon 3
3'
AC37Eco-R
100 bp
C
5'- GAGCGAAGCTTTTTTTTTTTTTTGCTTTTTTCTTTCTGTAACAAATT -3'
D
5'- AAGCTTTTTTTTTTTTTTTTTTTTTTTTGCTTTTTTCTTTCTGTAACA -3'
E

ples that had been prepared by formalin fixation and paraffin embedding for routine histopathological procedures and stored in the archives of one of us (J.M.S.). Once a mutation was detected, leukocyte DNA from further available family members was screened for the specific mutation. DNA samples from 50 apparently unrelated normal control subjects (28 females and 22 males) were also tested under the same SSCP conditions that allowed the detection of P0 mutations and polymorphisms.

Mutation analysis. Primer sets for polymerase chain reaction (PCR) amplification of exons 1, 2, 4, 5, and 6 of the P0 gene were as described by Nelis et al. (26) while exon 3 was amplified with the primers reported by Roa et al. (33). PCR products were screened for nucleotide changes by single-strand conformation polymorphism analysis (SSCP). Fragments derived from exons 1, 5, and 6 were electrophoresed on a 1 × Mutation detection enhancement (MDE) gel (FMC Bioproducts, Rockland, ME, USA) at 15 W for 20 h, exon 2 on a 8% polyacrylamide gel with 10% glycerol at 8 W for 18.5 h, and exons 3 and 4 on a 0.5 × MDE gel with 10% glycerol at 10 W for 18 h. After electrophoresis, gels were silver stained. Fragments revealing abnormal SSCP patterns were directly sequenced using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Samples were run and analysed on an ABI PRISM 373 fluorescent DNA sequencer (Applied Biosystems). Restriction enzyme analysis was done as recommended by the manufacturer (New England Biolabs, Beverly, MA, USA). The resulting restriction fragments were fractionated on 2.5% agarose gels.

Haplotype analysis. The following STR markers flanking the P0 gene were analysed: D1S534 (GATA12A07), D1S1595 (GATA25B02), CRP (Mfd57), D1S2771 (AFMb334xb1), D1S2705 (AFMa323xd9), D1S2675 (AFMa244wh5), D1S1679 (GGAA5F09), APOA2 (Mfd3), and D1S1677 (GGAA22G10) (Figure 3A). Primers for PCR amplification were as published by the Genome Data Base (http://gdbwww.gdb.org) and sense primers were labelled with FAM fluorophores (Pharmacia Biotech, Uppsala, Sweden). The electrophoresis and analysis were performed on an ABI PRISM 310 genetic analyser (Applied Biosystems). Allele sizes were compared to samples from patients PN-389.IV.2 and PN-509.II.1 included in the study by De Jonghe *et al*. (1999) and numbered according to the Genome Data Base (GDB).

The human P0 gene contains an oligo-dT tract in intron 2 that is polymorphic with at least five alleles transmitted in a Mendelian fashion (29). We amplified a genomic segment encompassing this oligo-dT tract and part of exon 3 (including codon 124) by PCR through 35 cycles of 30 s at 95°C, 30 s at 60°C, and 90 s at 72°C. The two primers were AC37Eco-F (5′-CGCA-GAATTCCTCCCTTCTCCAGCACTA-3′) and AC37Eco-R (5′-AGCTGAATTCCTTTTTCAAAGA-CATACAGCG-3′) with 5′-*Eco*RI linkers (Figure 3C). *Eco*RI-digested PCR products were subcloned into the *Eco*RI site of the vector pGEM-3zf+ (Promega, Madison, WI, USA). For each patient, ten constructs were sequenced using T7 and SP6 primers to identify the length of the oligo-dT stretch residing on the mutant 124Met allele. All manipulations, including restriction digestion, cloning of PCR products, culture of bacterial cells, and isolation of plasmid DNA were done according to standard procedures.

Nerve biopsies. Sural nerve biopsies were studied in the index cases AC09.V-6, AC44.III-2, and AC37.III-2. The nerves were fixed in 3.9% phosphate-buffered glutaraldehyde, post-fixed in 2% phosphate-buffered solution of osmium tetroxide, and embedded in epoxy resin. For light microscopy, semi-thin sections were stained with toluidine blue and paraphenylenediamine. Quantitative studies on transverse semi-thin sections of the nerves were performed using the Kontron KS300 system (Kontron, Eching/Munich, Germany) with image evaluation software from the same manufacturer. Ultrathin sections were contrast enhanced with lead citrate and uranyl acetate and were examined with a Philips 400T electron microscope.

Results

Mutation analysis. SSCP analysis of P0 exons 1-6 revealed aberrant profiles of migration in five CMT2

Figure 3. (Opposing page) Haplotype analysis for the 124Met P0 allele. (**A**) Order of markers used for STR analysis. (**B**) Comparison of the 1q22-q23 STR haplotypes of family AC37 (on the left) and of patients PN-389.IV.2 and PN-509.II.1 included in the study by De Jonghe et al. (1999) (on the right). The respective disease haplotypes are boxed. (**C**) Localisation of the single nucleotide repeat polymorphism in P0 intron 2 and of codon 124 in exon 3. The positions of oligonucleotide primers for generation of amplimer AC37Eco are indicated. As heterozygosity for the oligo-dT tract obscured a clear reading of the sequence, the PCR products were clone purified. (**D**) This sequence of 14 thymine bases comes from patient AC37.III-2 and resides on the same chromosome that contains the C→T transition in codon 124. (**E**) In patient PN-389.IV.2, the mutated allele contained an oligo-dT tract of 23 thymines.

Family	Mutation	Patient	Sex	Age of onset	Age	Foot deformity[a]	Muscle atrophy[b]	Gait disturbance[c]	Reduction of reflexes[d]		Sensory loss[e]	Nerve conduction velocity (m/s)		
									Ankle	Knee		Median	Tibial	Peroneal
AC09	Asp61Gly	V-6	M	60	63	+	++	+	++	++	+	40	31	
		V-8	F	50	60	++	++	+++	++	++	+	43		
		V-15	M	57	61	+	+	+	+	+	+	42.7	40.6	40.9
		V-16	M	59	64	+	+	+	+	+	+			
AC44	Tyr119Cys	III-2	F	49	54	+	+	++	++	+	+	39	27	31
		III-4	F	47	60	+	+	+	++	++	+			
		IV-8	F	-	34	-	-	-	-	-	-			
AC37	Thr124Met	III-2[f]	M	45	52	+	++	+++	++	++	+	35.6	-[g]	-[g]

[a] Foot deformity: no foot deformity (-); foot deformity without impairment (+); requires orthopaedic shoes (++); requires surgery (+++)
[b] Muscle atrophy: no atrophy (-); mild atrophy (+); marked atrophy (++); severe atrophy (+++)
[c] Gait disturbance: no gait disturbance (-); difficulty in running (+); steppage gait (++); need for walking aids (+++)
[d] Reduction of reflexes: reflexes not reduced (-); reduced reflexes (+); absent reflexes (++)
[e] Distal sensory loss: no sensory loss (-); decreased sensibility (+); absent sensibility (++)
[f] The patient complained of pains and paraesthesias in the lower limbs. His phenotype included mild hearing loss and pupillary abnormalities.
[g] No motor potentials could be elicited with neither surface nor needle lectrodes.

Table 1. Clinical and electrophysical data.

Case	Sex	Age at Biopsy	Myelinated fibre density (1/mm²)[a]	Myelin area per endoneurial area (%)[b]
AC09.V-6	M	62	3,954	8.5
AC44.III-2	F	51	4,952	8.9
AC37.III-2	M	47	4,656	8.7
		51	3,400	5.5

[a] Normal range: 6,000-9,000/mm²
[b] Normal range: 20-30%

Table 2. Morphometric data.

patients out of 49 tested. Analysis of the PCR product generated with primers for exon 2 showed an altered migration pattern in patient AC09.V-6. Automated fluorescent sequencing disclosed an A→G transition at nucleotide position 182 (the first nucleotide of the ATG start codon is numbered 1). This base substitution predicts the replacement of aspartic acid with glycine at codon 61 (Figure 1A). The sequence change creates an additional *Sfa*NI restriction site in the PCR fragment yielding additional bands in mutation carriers (Figure 2A). Patients AC44.III-2 and AC37.III-2 produced different mobility shifts of single stranded fragments amplified from exon 3. Sequencing in patient AC44.III-2 revealed that the A normally present at the second position of codon 119 is substituted by a G altering wild type tyrosine to cysteine in the P0 protein (Figure 1B). The sequence change introduces a novel *Pst*I restriction site in the PCR product (Figure 2B). Patient AC37.III-2 had a heterozygous C→T transition at nucleotide 371 resulting in a Thr124Met aminoacid substitution (Figure 1C). This mutation creates a novel restriction site of *Nla*III enzyme. The mutations could neither be detected in healthy relatives nor in any of the 50 normal control individuals.

SSCP analysis of the PCR fragment generated with the primer set for exon 6 revealed an altered mobility in two non-familial patients. Sequence analysis illustrated that one patient carried a known silent mutation affecting serine in codon 228 (26). The same SSCP pattern as for this patient was found in two out of 50 control individuals. The other patient had a single nucleotide substitution (G→A) in the 3′-untranslated portion of exon 6, 52 nucleotides downstream of the TAG stop codon. The SSCP pattern representing this mutation was not detected in any of the control samples, but in the healthy father.

Haplotype analysis. Subsequent to the report by De Jonghe *et al*. (1999) documenting that the high frequency of the Thr124Met mutation in a Belgian CMT2 cohort is due to a founder effect, we analysed patients from family AC37 for polymorphic markers from the chromosome 1q22-q23 region. Both affected individuals from family AC37 shared the allele at the closest telomeric marker D1S2705 with the Belgian families (allele 1: 150 bp, GDB allele frequency: 0.30). Allele sizes for the remaining telomeric marker were all different while some of the Belgian families had been found to share larger disease haplotypes at the telomeric side of P0. Moreover, the disease haplotypes differed for the closest centromeric marker D1S2771 (allele 3: 257 bp for the Belgian patients, GDB allele frequency: 0.39, but allele 2: 251 bp for family AC37, GDB allele frequency: 0.33) (Figure 3B).

Analysis of the single nucleotide repeat polymorphism in intron 2 of the P0 gene provided further evi-

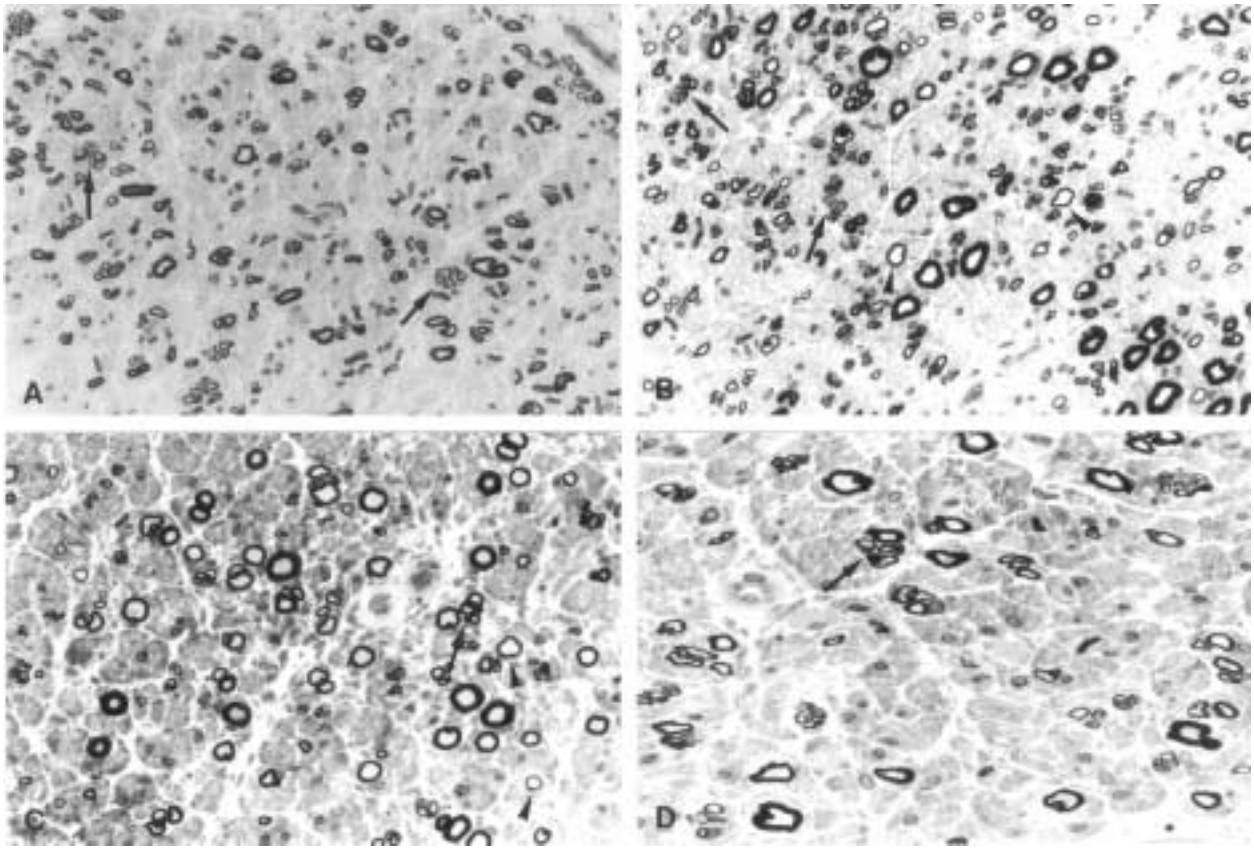


Figure 4. Transverse sections of sural nerve biopsy samples from (**A**) patient AC09.V-6 at age 62, (**B**) patient AC44.III-2 at age 51, (**C**, **D**) patient AC37.III-2 at age 47 and at age 51. The number of large myelinated fibres is reduced and numerous clusters of regenerated, thinly myelinated nerve fibres are encountered (arrows). Some isolated axons have disproportionately thin myelin sheaths (arrowheads). Comparison of the biopsy specimen in (**C**) with the specimen in (**D**) from the same patient (AC37.III-2), but sampled four years later, shows progressive fibre loss with age. Semi-thin sections, toluidine blue, ×350.

dence for a different genealogical origin of the 124Met P0 allele. Sequencing analysis of cloned, purified PCR products encompassing the intron 2 polymorphism and the mutated threonine site in codon 124 revealed different disease haplotypes. In mutation carriers from family AC37, the mutated P0 allele was found to contain an oligo-dT tract of 14 thymine bases (Figure 3D). Patient PN-389.IV.2 from one of the Belgian kindreds had 23 thymines associated with the C→T transition in codon 124 (Figure 3E). The same allele size of the oligo-dT stretch was observed in PN-509.II.1 (not shown).

The phenotype of CMT2 patients. Index cases AC09.V-6 and AC44.III-2 were part of families in which multiple patients were present in the pedigree. In family AC09, the disease was autosomal dominantly inherited. Family AC44 was compatible with autosomal dominant or X-linked inheritance. A mutation of the Cx32 gene was excluded. As patient AC37.III-2 was more severely affected than his mother the diagnosis of CMTX was initially favoured, but the Cx32 gene was normal. All patients investigated clinically had a progressive peripheral neuropathy with first disabling symptoms not until the fifth or sixth decade (Table 1). Electrophysiological examination showed slight to moderate slowing of NCVs except for subject AC37.III-2 who had MNCV <38 m/s (Table 1). Motor compound muscle action potential (cMAP) amplitudes and sensory nerve action potential (SNAP) amplitudes were decreased.

The proband V-8 of family AC09 was markedly disabled and had to use braces to ambulate. Other mutation carriers from the same generation were only moderately affected. One of them (V-15), currently 61 years old, still worked as a scaffolder. Sixteen further family members from five different generations, most of them deceased at the time of the present study, were reported as being affected by a peripheral neuropathy.

Spectrum of structural abnormalities		Findings in the index patients[a] AC09.V-6	AC44.III-2	AC37.III-2
Axonal changes	Loss of axons	+++	++	+++
	Atrophy	++	++	+
	Acute degeneration	+	+	+
	Bands of Buengner (empty Schwann cells)	++	+	+
	Clusters of regenerated fibers	++	++	++
Myelin changes	Myelin degradation products	+	+	+
	Onion bulb formation	+	+	+
	Disproportionately thin myelin sheaths	+	+	++
	Changes of compact myelin			
	adaxonal vacuoles	+	++	+
	microvesicular complexes	++	+	+
	membranous whorls	++	++	++
	uncompacted second inner myelin loop	-	+	+
	abaxonal ringed inclusions	-	-	+
	dislocated terminal loops	+	-	+
	Changes at paranodes			
	vesicular/vacuolar changes	++	+	-
	membranous whorls	++	-	-
	abnormal axon/myelin relations	+	+	+
	node widened	+	-	-
	Changes at Schmidt-Lanterman clefts			
	membranous whorls	+	++	-
	abnormal granular inclusions	-	+	-
	vesicular disintegration	-	+	-

[a] Frequency of ultrastructural changes: not seen (-); rare (+); repeatedly seen (++); frequent (+++)

Table 3. Fine structural changes.

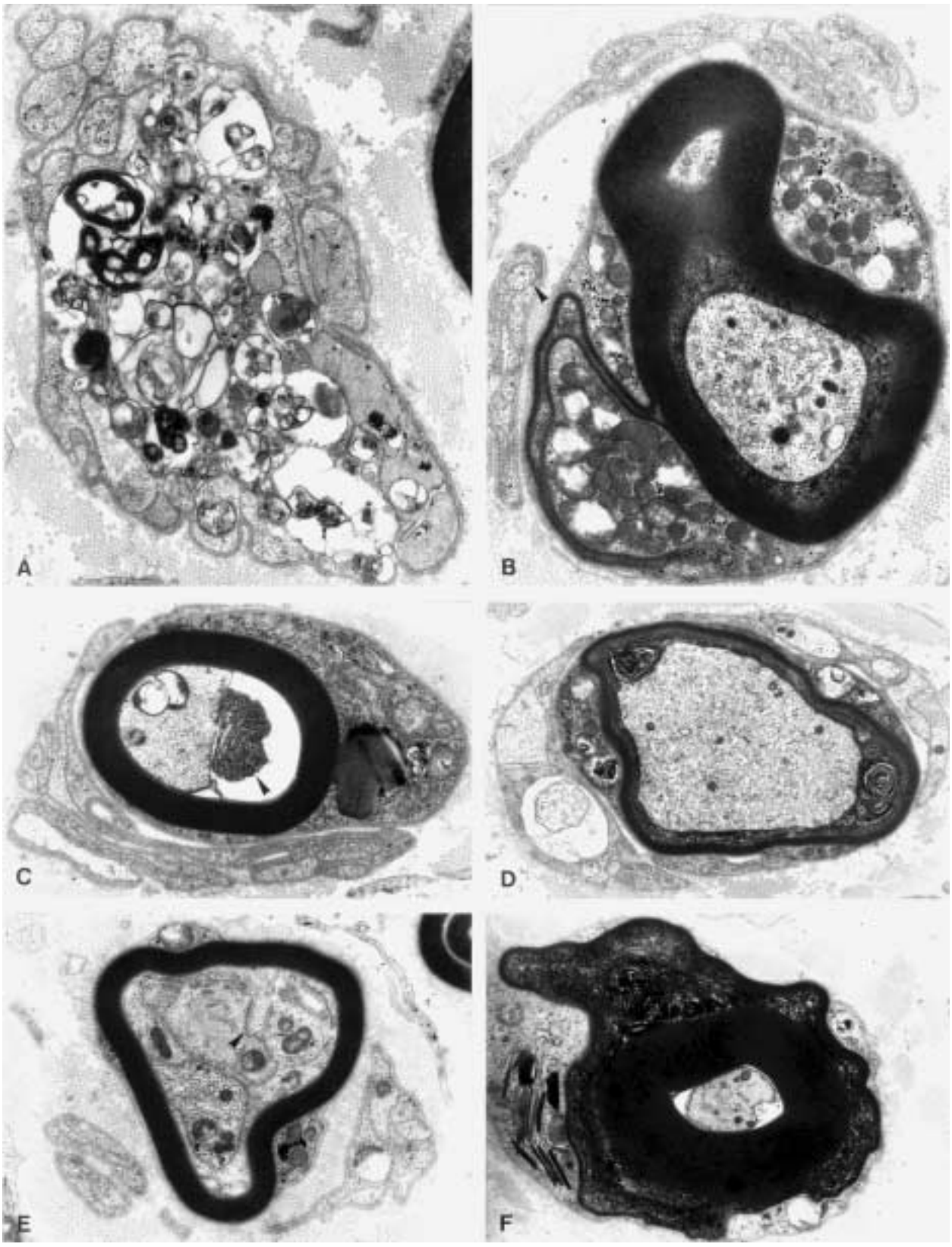
Patients III-2 and III-4 from family AC44 had minor neurological problems and one younger proband (IV-8) was asymptomatic. Deceased patient II-6 was reliably reported to have had foot deformity and problems with walking. Proband III-6 did not carry the codon 119 P0 mutation but had mild peripheral neuropathy. NCVs were slightly decreased with normal amplitudes, indicating mild demyelination. He had Hodgkin lymphoma in his medical history and had received chemotherapy for this condition. His phenotype may be explained by paraneoplasia or side effects of vincristine (40), rather than the codon 119 mutation is not disease related.

According to family informants, proband AC37.II-2 carrying the Thr124Met mutation had mild gait disturbance and early fatiguing in the lower limbs. This had always been attributed to ageing and postthrombotic syndrome, and neuropathy had never been verified by clinical examination. Her son (III-2) worked in a waste incineration plant and was occupationally exposed to various toxins including arsenic, lead, and mercury. He had severe, rapidly progressive CMT with marked sensory abnormalities in the lower extremities. His pupils were irregular but pupillary reflexes to light and accommodation were normal. Bilateral mild hearing loss was noted. His daughter and son were subjectively healthy (at age 16 and 19), yet refused consent for genetic testing.

Nerve biopsy findings. Nerve biopsy samples were available for the three index cases. Histopathological and morphometric findings conformed with a predominantly axonal lesion (Figure 4, Table 2). In semi-thin sections, marked loss of large myelinated fibers and multiple clusters of regenerated axonal sprouts were noted. Typical, large onion bulb formations were not apparent. In general, myelin sheaths were of normal thickness. Occasionally, myelin sheaths were disproportionately thin and some axons had abnormally thickened myelin. There were no inflammatory infiltrates. Moderate endoneurial edema was present in several nerve fascicles. Patient AC37.III-2 was biopsied at age 47 and at age 51. Comparison of biopsy specimens indicated progression of the neuropathy towards further depletion of large myelinated fibers.

Electron microscopy (Figure 5, Table 3) revealed numerous degenerating axons, bands of Buengner, and groups of regenerated or regenerating axons. Some of these clusters were surrounded by Schwann cell processes forming onion bulb-like structures. Incipient onion bulbs with single layers of Schwann cells were occasionally detected. Some myelin sheaths formed abnormal loops. Paranodal and segmental demyelination was not observed. Analysis of myelin periodicity did not reveal pathological changes. In a few regenerated fibers, the innermost myelin loops were not yet properly packed. At non-compact parts of the myelin sheaths, vacuolar degeneration, membranous whorls, and microvesicular disintegration were frequent. Some

Figure 5. (Opposing page) Electron microscopic changes in the sural nerve of case AC44.III-2. (**A**) Typical band of Buengner with multiple Schwann cell processes and myelin degradation products. ×13,800. (**B**) Paranodal myelin loops are surrounded by several flattened Schwann cell processes with one unmyelinated axon (arrowhead). ×14,300. (**C**) Small onion bulb formation with flattened Schwann cell processes. A large adaxonal vacuole contains a condensed microvesicular degradation product (arrowhead). ×11,100. (**D**) Cluster of regenerated axonal sprouts consisting of several unmyelinated axons and a single myelinated fiber with a disproportionately thin myelin sheath. ×8,700. (**E**) Remyelinated fiber with abnormal adaxonal Schwann cell processes and a collagen pocket (arrowhead). Supernumerary, empty Schwann cell processes adjacent to this fiber indicate preceding demyelination and remyelination. ×14,000. (**F**) This atrophic axon is surrounded by a disproportionately thick myelin sheath. The fiber is sectioned at the site of a myelin loop that is extending at the level of a Schmidt-Lanterman incisure with membranous whorls. ×6,500.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adaxonal vacuoles included membranous whorls or microvesicular complexes.

Discussion

We screened genomic DNA of 49 unrelated CMT2 patients for mutations in the coding region of the P0 gene. Altered SSCP patterns were observed in five patients and sequencing of PCR products identified five single base changes: two novel missense mutations, Asp61Gly and Tyr119Cys, the recurrent Thr124Met substitution, and two conceivably harmless single nucleotide polymorphisms. We found evidence that our patients with the 124Met P0 allele were not related to a cohort of patients with the same mutation, all of Belgian descent and all found to share a common ancestor (47). We believe that the high frequency of the 124Met allele in European CMT populations is not only due to a founder effect but codon 124 might be a mutation hotspot as well.

Mutations and polymorphisms in the P0 gene. All three amino acid substitutions in the P0 gene were non-conservative and changed residues that are invariant in the P0 sequences of different vertebrates (1, 17, 22, 37, 53). Both novel mutations segregated with transmission and expression of the disease. Though we did not have conclusive family data for the Thr124Met mutation the pathogenic nature of this genotype is very likely as it has already been identified in several CMT pedigrees. Moreover, the mutations were not observed in the DNA samples from the 50 unrelated control individuals. In consens with previous observations of others (26), the silent mutation in the serine codon 228 occurred with a detectable frequency in the general population, which was represented by our healthy control cases. The novel single base substitution in the 3′-untranslated region is obviously not related to the disease but constitutes a rare familial polymorphism, as evidenced from its absence in the control samples and its presence in an unaffected parent.

P0 is a highly conserved transmembrane protein that is exclusively expressed by myelinating Schwann cells. It constitutes the major structural component of peripheral nervous system myelin and localises to the compact portion of the myelin sheaths. The structural model predicts an immunoglobulin-like extracellular domain, a single membrane spanning domain, and a cytoplasmic C-terminal domain (9, 21, 23). P0 monomers self-assemble to form a tetramer oriented around a central hole (42). P0 presumably functions as an adhesive element between the myelin layers helping to form and maintain the highly organised structure of the myelin sheath (8, 42). The Asp61Gly substitution in exon 2 (family AC09) is located in a region of the P0 extracellular domain that is supposedly responsible for tetramerisation. This interface is formed by interactions between sidechains of the respective amino acid residues. Consequently, the substitution of aspartic acid with glycine may impair tetramerisation as it removes an acidic sidechain. In wild type P0, cysteins at positions 50 and 127 build a disulphide bridge. Based on the crystal structure of the P0 extracellular domain, the novel Tyr119Cys substitution (family AC44) would produce an outwardly pointing thiol group that may form detrimental disulphide aggregates with the wild-type partner molecules. The Thr124Met substitution (family AC37) occurs in the middle of a β-strand. It presumably disrupts the in (hydrophobic)-out (hydrophilic) alternation resulting in a misfolded protein. Alternatively, the mutant proteins might be unstable or might fail to reach the cell membrane. The phenotype would then be due to a gene dosage effect as only wild-type molecules from the normal allele are expressed in the myelin wraps. Another possibility is that the altered proteins poison the Schwann cell when accumulating in the cytoplasm.

Clinical phenotype. The P0 mutations presented in this study were associated with an axonal type peripheral neuropathy with onset not until the fourth to sixth decade. Two patients had severe lower limb weakness that required walking aids to allow ambulation. These observations conform with what discriminates CMT2 from CMT1 in comparative studies in which CMT2 patients were found with more pronounced weakness and atrophy, but later onset of symptoms (14). In keeping with this, one younger mutation carrier (AC44.IV-8) was asymptomatic as she had not yet reached the age of onset of CMT in her family. In previous studies of others, the Thr124Met genotype was reported to cause a clinically distinct phenotype (5, 7). Analogously, patient AC37.III-2 had peripheral neuropathy with additional features such as painful sensory disturbances, bilateral sensorineural hearing loss, and pupillary abnormalities.

Phenotypic discordance in families AC09 and AC37 was apparently not merely age-related and could not be explained on the basis of the genotype alone. Environmental influences could be tracked down for patient AC37.III-2 who was occupationally exposed to lead, mercury, and arsenic which all have proven toxicity to the peripheral nervous system (40). He suffered from severe peripheral neuropathy while his mother was paucisymptomatic. However, the question whether toxic exposure aggravated the neuropathy could not be simply

settled. On the one hand, the patient had never experienced medical complications of chronic intoxication other than neuropathy, and no haematological abnormalities were reported. On the other hand, as observed in some of the Belgian patients with the 124Met allele, the presence of this peculiar allele alone may suffice to explain rapid progression of the disease. Nevertheless, we speculate that a P0 mutation renders the nerves more susceptible to neurotoxicity. Conclusively, low doses that normally do not damage the peripheral nerves and do not cause other classical symptoms of intoxication might have a devastating effect in the predisposed nerve.

Electrophysiology and nerve biopsies. Mutations of the P0 gene have been advocated to cause hereditary diseases of peripheral nerve myelination including CMT1B and two more severe, early-onset forms, DSS and CHN (50). However, recent reports demonstrated that the phenotypic spectrum of P0 mutations also covers the axonopathy CMT2 (5, 7, 25, 28). All mutation carriers in the present study had electrophysiologic abnormalities compatible with an axonal lesion. MNCVs were above the cut off value of 38m/s for CMT1, except for subject AC37.III-2. However, the value was still intermediate (between 30 to 40 m/s) while patients with P0 mutations associated with CMT1B usually have MNCVs at the lower extreme of the CMT1 range. It is likely that NCV slowing in patient AC37.III-2 was due to nearly complete loss of large, fast-conducting axons as seen in the nerve biopsies and reflected by low amplitudes of cMAPs and SNAPs.

In CMT1B patients with other P0 mutations, a demyelinating process is observed with onion bulb formation. Uncompacted myelin was found in some cases while others had focally folded myelin sheaths (12) or tomacula (46). Sural nerve biopsies from the present series displayed minor evidence of demyelination and myelin sheaths only occasionally showed incipient thickening and loop formations. Myelin periodicity was normal. The most prominent finding was depletion of large myelinated fibers and multiple clusters of small regenerating fibers indicating a principally axonal lesion. Interestingly, mutations in another myelin related protein, the gap junction protein Cx32, can set off a demyelinating process (19, 36) but can also cause axonal degeneration with relative preservation of the myelin sheaths (13, 38, 41). Our cases with Cx32 mutations showed various fine structural alterations of the axon-Schwann cell attachment zone which supposedly represent the electron microscopic correlates of disturbed axoglial interactions. In the present set of P0 mutations, however, we could not detect ultrastructural abnormalities that would corroborate the view that impairment of Schwann cell and axon relationships (31, 34), underlies axonal degeneration in patients with myelin protein mutations. The answer to this question may require autopsy studies of proximal nerve segments, or expression of mutant P0 genotypes in cultured cells and engineering of mutations into the mouse P0 gene.

Exon	Codon	Amino acid change	References
2	44	Ser → Phe	Marrosu et al., 1998
2	61	Asp → Gly	this work
3	102	frameshift	Pareyson et al., 1999[a]
3	119	Tyr → Cys	this work
3	124	Thr → Met	Schiavon et al., 1996[b]
			Wolf et al., 1997[b]
			De Jonghe et al., 1998
			Chapon et al., 1999
			this work

[a] Heterozygous individuals had mild CMT2 while the homozygous offsprings from a consanguineous marriage had DSS.

[b] Patients were reported to have CMT1 with onset only in the 4th decade.

Table 4. Reported P0 mutations associated with CMT2.

Recurrence of the Thr124Met genotype and frequency of P0 mutations in CMT2. Genotyping for STR markers flanking the P0 gene and for the polymorphic oligo-dT stretch within P0 intron 2 demonstrated that family AC37 and the Belgian patients with the Thr124Met mutation are unrelated. Our data show that at least two distinct ancestral Met124 P0 alleles exist in the European CMT population. In agreement with this observation, the Thr124Met mutation was independently reported by at least three further investigators from different European countries (5, 39, 52). It would be interesting to include these families in haplotype analysis of the P0 gene to test the hypothesis that the high frequency of the ACG to ATG transition in codon 124 is not only due to a founder effect but that Thr124 is a mutation hotspot. This seems reasonable to assume as most of the CpG dinucleotides are hypermutable, owing to the methylation of the cytosine originating a 5-methylcytosine, which is then easily deaminated to form thymine (6).

Except for the Met124 allele, which was also reported in CMT of type 1 yet with late onset, CMT2-causing P0 mutations were not observed in classical demyelinating neuropathies. The phenotypic variability of CMT1B, DSS, and CHN has been related to the position

and nature of P0 mutations (50). Analogously, an additional set of distinct P0 mutations may exclusively express CMT2 phenotypes (Table 4). However, the pathogenic models explaining genotype-phenotype correlations are still disputable. For example, mutations producing outwardly pointing thiol groups in the extracellular domain were advocated to cause severe demyelination/remyelination as in DSS due to a dominant negative effect (formation of abnormal disulphide bridged P0 aggregates) (50). This is not in line with our findings in family AC44 with such a mutation but expressing moderate, late-onset CMT2.

The frequency of P0 mutations in CMT2 is probably underestimated due either to the fact that the P0 gene is not routinely screened in patients with CMT2, or to an inaccurate diagnosis of late onset familial neuropathy. We assume that about 5% of CMT2 cases (3 out of 53) might be related to P0 mutations. The frequency of Cx32 mutations in CMT2 is even higher, ranging from about 8% in our own cohort up to 15% to 20% in the studies of others (43, 48). Hence, if the Cx32 and the P0 gene are analysed, a molecular diagnosis can be established in at least one out of six CMT2 cases without the co-operation of family members, and genetic counselling can be offered to individual patients. However, as the present data are limited to a small number of cases, further series of CMT2 patients need to be investigated to clarify how common P0 mutations are in the CMT2 population.

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