

PAPER

A novel mutation of myelin protein zero associated with an axonal form of Charcot–Marie–Tooth disease

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Objective: To report a new mutation in the *MPZ* gene which encodes myelin protein zero (P0), associated with an axonal form of Charcot–Marie–Tooth disease (CMT).

Methods: Three patients from an Italian family with a mild, late onset axonal peripheral neuropathy are described clinically and electrophysiologically. To detect point mutation in *MPZ* gene the whole coding sequence was examined. The structure of the mutated protein was investigated using the three dimensional model of P0.

Results: All patients showed a relatively mild CMT phenotype characterised by late onset and heterogeneity of the clinical and electrophysiological features. Molecular analysis demonstrated a novel heterozygous T/A transversion in the exon 3 of *MPZ* gene that predicts an Asp109Glu amino acid substitution in the extracellular domain of the P0. Asp109 is found at the protein surface, on β strand E, in the interior of the P0 tetramer.

Conclusions: The identification of Asp109Glu mutation confirms the pivotal role of P0 in axonal neuropathies and stresses the phenotypic heterogeneity associated with *MPZ* mutations. This study suggests the value of screening for *MPZ* mutations in CMT family members with minor clinical and electrophysiological signs of peripheral neuropathy.

Charcot–Marie–Tooth disease (CMT), or hereditary motor and sensory neuropathy (HMSN), is the most common inherited disorder of the peripheral nervous system.¹ On the basis of electrophysiological and neuropathological features, CMT has been divided into primary peripheral demyelinating and primary peripheral axonal neuropathies.² Several genetic loci and genes have been associated with peripheral demyelinating neuropathies which include CMT type 1 (CMT1), Dejerine–Sottas syndrome, congenital hypomyelinating neuropathy, and hereditary neuropathy with liability to pressure palsies (HNPP). Both dominant and recessive mutant alleles have been described.³ Peripheral axonal neuropathies include CMT type 2 (CMT2) and giant axonal neuropathy. At present, the recognised autosomal dominant CMT2 genotypes include: CMT2A, related to mutations in the kinesin-like protein *KIF1B* gene (1p36.2)⁴; CMT2B (3q13-q22), related to mutations in the small GTPase late endosomal protein *RAB7* gene⁵; CMT2C (with vocal cord paresis) (12q23-24)^{6,7}; CMT2D, related to mutations in the glycyl tRNA synthetase gene (7p14)⁸; CMT2E, related to mutations in the neurofilament light gene (*NF-L*) (8p21)⁹; CMT2F (7q11-q21)¹⁰; and proximal CMT2, or HMSN P (CMT2G) (3q13.1).¹¹ The CMT2 phenotype can also occur in families with mutations in the *GJB1* gene which encodes connexin-32.^{3,12} Besides these loci and genes, defects in *MPZ* gene which encodes myelin protein zero have also been reported¹²⁻²¹ (see also the European CMT Consortium database, <http://molgen-www.uia.ac.be/CMT/>).

Protein zero (P0) is a major structural component of peripheral nerve myelin and was initially associated with demyelinating CMT forms such as CMT1B, Dejerine–Sottas syndrome, and congenital hypomyelinating neuropathy.²² We report a novel point mutation in the extracellular domain of the *MPZ* gene (Asp109Glu) in a family with an axonal form of CMT.

METHODS

Clinical studies

The present study is based on three members of a family originating from southern Italy (Campania), affected by a

late onset peripheral neuropathy. The three patients are first cousins (fig 1). All affected individuals were examined at the department of neurological sciences of the University of Napoli Federico II, Italy, where a complete neurological examination was carried out by the same neurologist. Clinical classification of the patients was done according to Dyck's diagnostic criteria.¹ Examination of the upper and lower extremities included standard testing of all motor and sensory modes.

Electrophysiological studies

A battery of neurophysiological tests was applied in all the patients. These included needle electromyography (EMG) (biceps brachialis, tibialis anterior, and abductor digiti minimi muscles), antidromic (sural nerve) or orthodromic (median nerve) sensory conduction velocities, and motor nerve conduction velocities (median and peroneal nerves).

Genetic analysis

After informed consent, peripheral blood samples were obtained from the three affected individuals. DNA was isolated from leucocytes using standard methods. Duplication of the 17p11.2-12 region was tested with pVAW409R3 and pVAW412HEC probes, as previously described.²³ To detect point mutations in the *MPZ* gene, the whole coding sequence was examined. Genomic fragments containing exons and intron-exon boundaries were amplified by polymerase chain reaction (PCR) using published primer sets.²⁴ PCR products were screened by single strand conformation polymorphism (SSCP) on a horizontal polyacrylamide gradient gel at two different temperatures, as elsewhere described.²⁵ Fragments revealing abnormal SSCP

Abbreviations: CMT, Charcot–Marie–Tooth disease; HMSN, hereditary motor and sensory neuropathy; HNPP, hereditary neuropathy with liability to pressure palsies; P0, myelin protein zero; SSCP, single strand conformation polymorphism

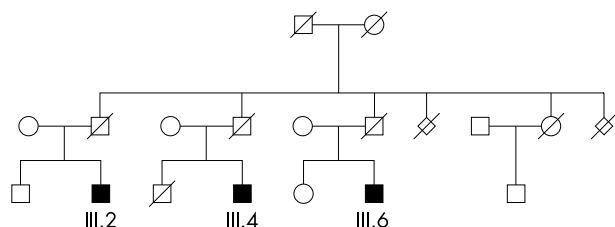


Figure 1 Pedigree of Italian family with Asp109Glu mutation. Squares, male; circles, female; solid black symbols, clinically and electrophysiologically affected; white symbols, referred unaffected and nor clinically or electrophysiologically examined; diagonal slash, deceased.

pattern were directly sequenced by current methods on an ABI310 automated sequencer (Applied Biosystems, Foster City, California, USA).

The P0 protein three dimensional structure²⁶ was obtained from the Protein Data Bank database.

RESULTS

Clinical studies

The three patients, henceforth referred to as case III.4, III.6, and III.2, respectively, had a mean age of disease onset of 53 years. Current ages are given in table 1.

Case III.4—The index patient was a 68 year old man who had complained of weakness and cramps in the lower limbs since he was 60. Neurological examination showed bilateral “steppage,” marked distal muscle weakness and wasting in the lower limbs and mild in the hands, bilateral pes cavus, calf enlargement, reduction of vibration sense, and absence of distal deep tendon reflexes in the four limbs. Pinprick sensation was moderately reduced distally in the lower limbs.

Case III.6—This patient was a 53 year old man. He complained of fasciculations in the gastrocnemius muscles since he was 50 years old. Neurological examination showed impairment of gait, mild distal muscle weakness and wasting in the lower limbs, bilateral pes cavus, reduction of vibration sense, and absence of distal deep tendon reflexes in the lower limbs. Pinprick sensation was normal.

Case III.2—This patient was a 52 year old man. Since the age of 50 he had complained of distal paraesthesiae. Neurological examination showed mild reduction of vibration sense in the lower limbs, bilateral pes cavus, and clawing of the toes. Pinprick sensation was normal and deep tendon reflexes were present.

None of the patients had cranial nerve involvement.

Electrophysiological studies

The electrophysiological findings are summarised in table 1 and described in more detail as follows.

In patient III.4, EMG examination showed a clear neurogenic pattern. The mean duration of motor unit action potentials was markedly increased in all the muscles explored but fibrillation was present only in the tibialis anterior. Compound muscle action potentials were unrecordable in the extensor digitorum brevis by stimulation of the peroneal nerve, and within the range of normality in the median nerve. Motor and sensory nerve conduction velocities were moderately reduced. Nerve sensory action potential was unrecordable in the sural nerve and moderately reduced in the median nerve.

In patient III.6 the EMG findings were very similar to those of patient III.4, but he did not have fibrillation in any of the muscles explored. Compound muscle action potentials were markedly reduced in the peroneal nerve and within the range of normality in the median nerve. Motor and sensory nerve conduction velocities were moderately reduced in all the nerves explored. Sural nerve sensory action potential amplitude was severely reduced, and median nerve SAP was moderately reduced.

In patient III.2, EMG examination showed a moderate increase in mean motor unit action potential duration (+36%) and no fibrillation. The recruitment pattern was of intermediate type with an increase in firing frequency. Compound muscle action potential and sensory action potential amplitudes and nerve conduction velocities were within the range of normality in all the nerves explored.

Molecular analysis

Duplication of the 17p11.2 region was excluded in all patients. SSCP analysis of *MPZ* exon 3 revealed aberrant profiles of migration in all patients compared with normal controls. Direct sequencing showed a heterozygous T→A transversion at nucleotide 327 of the coding sequence. This base substitution predicts the replacement of aspartic acid with glutamic acid at codon 109 (Asp109Glu). The variant was found in all three affected subjects, and it was not detected in 304 control chromosomes, indicating that this sequence variation is not a rare polymorphism.

DISCUSSION

In this study we describe a family displaying a mild axonal phenotype. In patients III.4 and III.6 electrophysiological examination showed slightly reduced nerve conduction velocities, severely reduced or absent compound muscle action potentials in the distal muscles of the legs, and clear signs of chronic denervation on EMG in all the muscles

Table 1 Motor and sensory conduction studies

| Patient | Age (years) | Median | | | Peroneal | | | Sural | | | |
|---------|-------------|-------------------------|----------------------------|------------------------|-------------------------|------------------------|-------------------------|---------------------------|-------------------------|-------------------------|-------------------------|
| | | Elbow-wrist | | Wrist-III digit | Knee-ankle | | Sura-lateral malleolus | | | | |
| | | MCV (m/s) | DML (ms) | CMAP amplitude (mV) | SCV (m/s) | SAP amplitude (µV) | MCV (m/s) | DML (ms) | CMAP amplitude (mV) | SCV (m/s) | SAP amplitude (µV) |
| III.4 | 68 | 41 (50 to 69) | 4.1 (2.8 to 4.2) | 11 (6 to 34) | 40 (46 to 64) | 5 (10 to 20) | | | NR | | NR |
| III.6 | 53 | 42 (52 to 72) | 4.1 (2.6 to 4.0) | 7 (7 to 40) | 48 (49 to 67) | 7 (10 to 25) | 34 (42 to 59) | 10 (3.4 to 5.0) | 0.7 (3 to 30) | 41 (47 to 61) | 0.6 (4 to 27) |
| III.2 | 52 | 52 (52 to 72) | 3.1 (2.6 to 4.0) | 8.3 (7 to 40) | 52 (49 to 67) | 14 (10 to 25) | 42 (42 to 59) | 3.9 (3.4 to 5.0) | 8.8 (3 to 30) | 53 (47 to 61) | 10 (4 to 27) |

Values are mean (range); abnormal data in bold. CMAP, compound muscle action potential; DML, distal motor latency; MCV, motor conduction velocity; NR, not recordable; SAP, sensory action potential; SCV, sensory conduction velocity.

explored. Thus, on the basis of clinical and electrophysiological features, a diagnosis of mainly axonal neuropathy was postulated. For patient III.2, who instead presented with marginal clinical signs and a neurophysiological pattern of neurogenic type in a single distal leg muscle, molecular analysis was necessary to establish the CMT diagnosis.

The disease transmission in the present family is suggestive of an autosomal dominant inheritance, though parents and siblings—who were not evaluated either clinically or electrophysiologically—were said to be asymptomatic. The patients' offspring were not examined. Molecular analysis demonstrated a novel heterozygous T/A transversion in exon 3 of the *MPZ* gene, corresponding to an Asp109Glu amino acid substitution in the extracellular domain of the protein P0. Protein P0 accounts for 50–60% of the protein content in peripheral myelin. It is an integral membrane protein exclusively expressed by myelinating Schwann cells and plays a central role in the regulation of myelination. P0 is composed of three structural domains: an immunoglobulin-like extracellular domain, a membrane ring shaped spanning domain, and a cytoplasmic C-terminal domain.^{27–29} P0 monomers self assemble to form a tetramer.²⁶

Patients carrying *MPZ* mutations usually have clinical and electrophysiological features of a peripheral demyelinating neuropathy including phenotypes of CMT1B, Dejerine–Sottas syndrome, and congenital hypomyelinating neuropathy. However, cases with axonal features have also been reported^{12–21} (table 2). The clinical and electrophysiological features of the present family are clearly different from the classic demyelinating phenotypes of HMSN, while similarities can be found with phenotypes described as mainly axonal. The most frequent mutation reported in axonal CMT is the Thr124Met substitution, which is associated with a clinically distinct CMT phenotype characterised by late onset peripheral neuropathy, Argyll-Robertson pupils, marked sensory abnormalities, dysphagia, and deafness.^{13–15–19} Misu *et al* described three families with an axonal form of CMT associated with Asp75Val substitution.¹⁸ In these families the major clinical features were indistinguishable from those associated with the Thr124Met mutation.

Seeman *et al* reported a Czech family with an axonal CMT associated with Glu97Val mutation and characterised by deafness and abnormal pupillary reaction.²⁰ In addition, Bienfait *et al* described a female heterozygous for two mutations in *MPZ* and affected by an intermediate phenotype associated with pupillary light–near dissociation.²¹ In contrast, none of the cases described here had pupillary abnormalities, dysphagia, deafness, trophic changes, or lancinating pains in the legs. Moreover, disease progression in our patients seemed to be slower than in patients affected by Thr124Met substitution. The cases described here are also clearly different from others characterised by Asp6Tyr

substitution, showing an electrophysiological and pathological pattern of “intermediate” HMSN.³⁰ More clinical and neurophysiological similarities can be found in Sardinian patients carrying a Ser44Phe substitution,¹⁴ even though some of these have more severe disease progression. The clinical phenotype of our patients also overlaps with that associated with Asp61Gly and Tyr119Cys substitutions,¹⁷ which are characterised by a moderate late onset axonal peripheral neuropathy.

The present case study also emphasises the clinical and electrophysiological variability already reported for other *MPZ* mutations. While patients III.4 and III.6 showed typical clinical CMT features and diffuse electrophysiological findings suggestive of a mainly axonal neuropathy, in patient III.2, where pes caves and clawing of the toes were the only stigmata suggesting a hereditary neuropathy, molecular analysis showed the same mutation.

The mechanism underlying the expression of a predominantly axonopathic versus a predominantly demyelinating phenotype for a given *MPZ* mutation remains to be elucidated. In both cases, however, demyelination is likely to be the primary cause of the two phenotypes.¹⁹ Most *MPZ* mutations result in decompaction of the myelin sheath and subsequent demyelination, probably caused by disturbance of the P0 structural role in the compaction of the sheath. However, we did not do nerve biopsies and cannot confirm myelin alterations.

Analysis of the three dimensional structure of the extracellular domain of P0 (PDB entry code, 1NEU²⁶) allowed us to identify the position of the Asp109Glu mutation described in this study. In P0, Asp109 is found at the protein surface on β strand E (secondary structural elements are labelled as proposed by Shapiro *et al*²⁶). In the protein structure, the Asp109 side chain is hydrogen bonded to the main chain and the side chain atoms of Ser111. Substitution of the Asp with the longer Glu side chain may lead to the disruption of these two stabilising intramolecular interactions. Furthermore, with respect to the tetrameric assembly of the P0 extracellular domain observed in the crystal structure,²⁶ Asp109 is found in the interior of the doughnut shaped P0 tetramer and is not involved in intermolecular contacts. Thus the influence of the mutation described in this study on the ternary/quaternary structure of P0 is difficult to determine on a structural basis. However, it is possible that the mutation Asp109Glu may destabilise the DE loop of P0, or also influence the interaction of the P0 tetramer with other myelin proteins.

Conclusions

We describe a novel *MPZ* point mutation in a CMT2 family with a mild phenotype and late symptom onset, characterised by clinical and electrophysiological heterogeneity and requir-

Table 2 Axonal Carcot–Marie–Tooth disease caused by *MPZ* mutations

| Mutation | Phenotype | Reference |
|------------------------|---|---|
| Thr124Met or Asp75Val | Late onset, pupillary abnormalities, dysphagia, deafness, marked sensory disturbances | De Jonghe, ¹⁵ Chapon, ¹⁶ Senderek, ¹⁷ Misu, ¹⁸ Hanemann ¹⁹ |
| Glu97Val | Pupillary abnormalities, deafness | Seeman ²⁰ |
| Asp6Tyr | Intermediate nerve conduction velocity | Mastaglia ³⁰ |
| Ser44Phe | Some patients with severe progression | Marrosu ¹⁴ |
| Asp61Gly or Tyr119Cys | Late onset, some patients with severe progression | Senderek ¹⁷ |
| His81Tyr and Val113Phe | Intermediate phenotype, pupillary abnormalities | Bienfait ²¹ |
| Asp109Glu | Late onset, mild neuropathy | This study |

ing medical attention in the fifth or sixth decade of life. As intrafamilial phenotype variation is common in CMT,² the phenotypic variability caused by myelin gene mutations cannot be ascribed to genotype alone but could reflect other genetic factors such as modulator genes, or endogenous or environmental factors. The identification of the Asp109Glu mutation confirms the central role of P0 in axonal neuropathies and stresses the phenotypic heterogeneity associated with *MPZ* mutations. Our study further suggests the value of *MPZ* screening in members of CMT families who present with minor clinical and electrophysiological signs of peripheral neuropathy.

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