Mutations in the 5′ region of the myotubularin-related protein 2 (MTMR2) gene in autosomal recessive hereditary neuropathy with focally folded myelin

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Summary
Focally folded myelin has been recognized as a distinctive feature in some individuals with severe inherited demyelinating neuropathy, with an onset in childhood. Such cases have been shown to be genetically heterogeneous. Alterations in the myotubularin-related protein 2 (MTMR2) gene on chromosome 11q22 have recently been shown to give rise to this phenotype. Mutations have been identified in the 3′ region of the MTMR2 gene in four unrelated families, in two of whom the disorder had been mapped to chromosome 11q22 by genetic linkage analysis. We have sequenced the entire coding region and flanking intronic regions of the MTMR2 gene in eight families with early onset autosomal recessive neuropathies. Two novel mutations were identified in exon 4 at the 5′ end of the MTMR2 gene in an English and an Indian family. The clinical phenotype and sural nerve pathology in these two families differs in severity, with the proband in the English family having an earlier onset and more severe neuropathy with prominent cranial nerve involvement. This is probably due to mutation type and possible involvement of small nucleotide polymorphisms in phenotype modulation. Detailed sural nerve pathology is presented in both cases. Mutations in the MTMR2 gene are thus an important cause of autosomal recessive demyelinating neuropathy. Identifying further mutations and defining their phenotype will help to clarify the genetic classification of this group of disorders.

Keywords: Charcot–Marie–Tooth disease; dysmyelination; hereditary neuropathy

Abbreviations: CMT = Charcot–Marie–Tooth disease; HMSN = hereditary motor and sensory neuropathy; MPZ = myelin protein zero; MTMR2 = myotubularin-related protein 2; PCR = polymerase chain reaction; SNP = small nucleotide polymorphism

Introduction
Considerable advances been made in recent years into the molecular genetics of the hereditary motor and sensory neuropathies (HMSN), especially with autosomal dominant and X-linked recessive inheritance (Reilly, 2000), and this is now extending to autosomal recessive inheritance (Thomas, 2000). For clinical purposes it is still useful to distinguish those with a demyelinating neuropathy (HMSN I) from those with an axonal neuropathy (HMSN II) (Dyck and Lambert, 1968a, b), although the disability in both is primarily related to axon loss. The demyelinating neuropathies can show distinctive structural features, one of which is the presence of focal myelin enlargements (tomacula) that are encountered in hereditary neuropathy with liability to pressure palsies (Behse et al., 1972; Madrid and Bradley, 1975) and other disorders. Tyson et al. (1997) analysed a series of cases of severe hereditary demyelinating neuropathy with an onset in childhood and showed that they were clinically heterogeneous. Two unrelated cases in this series, both with autosomal recessive inheritance, showed focally folded myelin sheaths on nerve biopsy.

The first recognition of neuropathy with focally folded myelin was by Ohnishi et al. (1989), and subsequently by Gabreëls-Festen et al. (1990) and others, in families showing autosomal recessive inheritance. The condition is now known to be genetically heterogeneous. Families have been mapped to chromosome 11q22, referred to as Charcot–Marie–Tooth
demonstrated focally folded myelin (see Table 1).

In two families, sural nerve biopsy had a total volume of 50 μl, which contained 20 ng of DNA, 0.2 mM dNTPs, 1 U TaqGold polymerase, 1.5 mM MgCl₂, 75 mM Tris–HCl pH 9.0, 20 mM (NH₄)₂SO₄, 0.01% Tween 20 and 50 pmol of each primer. PCR was performed on a Perkin-Elmer 9700 thermal Cycler (Perkin-Elmer, Applied Biosystems, Foster City, Calif., USA). The cycling consisted of denaturation at 94°C for 15 min, followed by 25 cycles of 94°C for 30 s, 60°C to 50°C touching down protocol for 30 s and 72°C for 30 s. After that, 12 cycles of constant annealing temperature at 50°C and a final amplification at 72°C for 10 min were carried out. PCR fragments were checked on a 1% agarose gel. The PCR products were purified by using a Qiaquick purification kit (Qiagen, Hilden, Germany) and resuspended in 50 μl of deionized water. For each exon, 100 ng of amplified product was sequenced using forward and reverse primers and the BigDye Terminator cycle sequencing kit (Perkin-Elmer). Sequencing was performed on an ABI377 automated sequencer. Sequence alignment and analysis was carried out using a Sequence Navigator (Perkin-Elmer).

### Material and methods

**Genetic sequencing**

Ethical approval was obtained from the National Hospital for Neurology and Neurosurgery Ethics Committee for research into neuropathy. DNA was extracted from blood samples obtained with informed consent from affected and unaffected individuals from families with autosomal recessive neuropathies (Table 1). Clinical details, the results of nerve conduction studies and those for nerve biopsy if carried out were obtained in all cases included in the study. Chromosome 17 duplication was excluded in all families. The MPZ gene was sequenced in the two families with focally folded myelin and was found not to carry mutations. The 18 exons and flanking intronic regions of the MTMR2 gene were amplified by the polymerase chain reaction (PCR) using primers reported by Bolino et al. (2000a, b). PCR was carried out in a PCR with cycling consisting of denaturation at 94°C for 15 min, followed by 25 cycles of 94°C for 30 s, 60°C to 50°C touching down protocol for 30 s and 72°C for 30 s. After that, 12 cycles of constant annealing temperature at 50°C and a final amplification at 72°C for 10 min were carried out. PCR fragments were checked on a 1% agarose gel. The PCR products were purified by using a Qiaquick purification kit (Qiagen, Hilden, Germany) and resuspended in 50 μl of deionized water. For each exon, 100 ng of amplified product was sequenced using forward and reverse primers and the BigDye Terminator cycle sequencing kit (Perkin-Elmer). Sequencing was performed on an ABI377 automated sequencer. Sequence alignment and analysis was carried out using a Sequence Navigator (Perkin-Elmer).

<table>
<thead>
<tr>
<th>Family</th>
<th>Affected</th>
<th>Consanguinity</th>
<th>Origin</th>
<th>Sural nerve biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>1</td>
<td>Yes</td>
<td>India</td>
<td>Focally folded myelin</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>Yes</td>
<td>UK</td>
<td>Focally folded myelin</td>
</tr>
<tr>
<td>VA</td>
<td>3</td>
<td>No</td>
<td>North Africa</td>
<td>Hypomyelination</td>
</tr>
<tr>
<td>BH</td>
<td>2</td>
<td>No</td>
<td>North Africa</td>
<td>Hypomyelination</td>
</tr>
<tr>
<td>H</td>
<td>1</td>
<td>Yes</td>
<td>Pakistan</td>
<td>N/A</td>
</tr>
<tr>
<td>S</td>
<td>2</td>
<td>No</td>
<td>UK</td>
<td>N/A</td>
</tr>
<tr>
<td>MAG</td>
<td>1</td>
<td>Yes</td>
<td>Ireland</td>
<td>N/A</td>
</tr>
<tr>
<td>BU</td>
<td>2</td>
<td>No</td>
<td>Malta</td>
<td>Axonal loss</td>
</tr>
</tbody>
</table>

N/A = not available.
MTMR2 gene mutations in hereditary neuropathy

Fig. 1 (A) MTMR2 exon 4 in case 8 from the study of Tyson et al. (1997) and an unaffected individual control. The arrow indicates the deletion of a G at base 324. This creates a frameshift in the mRNA and leads to an abnormally truncated protein. (B) MTMR2 exon 4 in case 9 (Tyson et al., 1997) and family. The arrows indicate the missense mutation of a G to an A at position 308, changing glycine to glutamic acid at codon 103. Case 9 is homozygous AA, and his unaffected parents and a number of other family members are heterozygous AG.
RTT gene mutations and polymorphisms

<table>
<thead>
<tr>
<th>Exon</th>
<th>Amino acid change</th>
<th>Base</th>
<th>Pathogenic mutation</th>
<th>Present in controls</th>
<th>Segregation in family</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lys 3 Thr</td>
<td>Base 8, A→C</td>
<td>No</td>
<td>A = 42, 70%, C = 18, 30%</td>
<td>No</td>
</tr>
<tr>
<td>1</td>
<td>No</td>
<td>Exon 1, 3′ +13 bp C→T</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2A</td>
<td>No</td>
<td>Exon 2A, 3′ +31 bp G→A</td>
<td>Unlikely</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2B</td>
<td>No</td>
<td>Pentanucleotide repeat</td>
<td>No</td>
<td>6 repeats = 58%</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon 2B, 5′−2 bp ATTTT</td>
<td></td>
<td>7 repeats = 34%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8 repeats = 8%</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Gly 103 Glu</td>
<td>Base 308, CGA-CAA</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>Frameshift</td>
<td>Del G, base 324</td>
<td>Yes</td>
<td>No</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
<td>No</td>
<td>Codon 377, base 1131 C→T</td>
<td>No</td>
<td>C = 40, 66%, T = 20, 34%</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>No</td>
<td>Codon 411, base 1233 G→T</td>
<td>No</td>
<td>G = 58, 94%, T =2, 3%</td>
<td>No</td>
</tr>
</tbody>
</table>

N/A = only one family individual available. Number of control chromosomes = 60. For Exon 4, 160 English and 40 Indian chromosomes sequenced.

**Mutation segregation**

To look for segregation of the disease in an autosomal recessive pattern, four polymorphic repeat markers close to the MTMR2 gene were analysed in the two families thought to have mutations (Table 1). These fluorescence-labelled markers were D11S937, D11S4175, D11S898 and D11S908. The protocol for analysis of these markers is the same as that for the MTMR2 pentanucleotide repeat detailed above.

**Sural nerve biopsy**

Sural nerve fascicular biopsies were obtained from a standard retromalleolar site. Following routine aldehyde fixation, post-osmication, dehydration through an ascending alcohol series and resin embedding, semi-thin sections were stained with thionin and acridine orange (Sievers, 1971). Ultrathin sections for electron microscopy were contrasted with uranyl acetate and lead citrate. Teased fibre preparations were obtained by the method described by Spencer and Thomas (1970).

**Results**

Two novel MTMR2 mutations were identified; these were not present in 80 English and 20 Indian controls (Fig. 1A and B). No pathogenic mutations were detected in the other six families although a number of novel polymorphisms were identified (Table 2). Two of the polymorphisms were not present in controls. The 3′ +12 bp C→T change in exon 1 did not segregate with the disease within the family, indicating a non-pathogenic change. The 3′ +31 bp G→A change in exon 2A found in family C is discussed further below.

**Family C**

This family was of English origin and the proband’s parents were second cousins (Fig. 2A). A homozygous G→A mutation was detected in exon 4 of the MTMR2 gene, changing glycine to glutamic acid at codon 103. The mutation and polymorphic markers close to the gene segregated with the disease in family C (Figs 1B and 2A). A sequence variant changing a G to an A in exon 2a +31 bp 3′ also segregated with the disease in this family; this change is likely to be in linkage disequilibrium with the exon 4 mutation, but may modulate the phenotype. The variant was not present in 20 controls. This change did not affect the splicing of this exon or create an aberrant splice site when analysed using the splice prediction program RNAfold, although this does not rule out the possibility that the variant induces abnormal splicing.

The clinical features of this patient have been reported previously by Tyson and colleagues (Tyson et al., 1997) (Case 9), therefore only a summary is given here. Case 9 was noticed to have an abnormal cry at birth but his early developmental milestones were normal. Difficulty with gait was evident when he began walking at the age of 13 months, but did not become prominent until the age of 3 years when he was found to have hypotonic muscle weakness in the limbs and to be areflexic. Vocal cord paralysis with stridor then appeared, as did difficulty with phonation, chewing and swallowing. The limb weakness increased and he has been confined to a wheelchair since the age of 16 years. Diaphragmatic weakness has also developed so that he requires NIPPV (non-invasive positive pressure ventilation) at night. Examination showed bilateral facial and jaw weakness, tongue wasting, mild stridor and dysarthric speech. There was diffuse upper limb weakness, diaphragmatic weakness and total hypotonic paralysis in the legs. He was areflexic and showed distal sensory impairment in the limbs. Electromyography demonstrated widespread denervation, absent sensory action potentials and a motor conduction velocity of 16 m/s in the ulnar nerve.

**Family V**

This family was of Indian origin. Both the parents and the paternal grandparents were first cousins (Fig. 2B). A homozygous deletion of an A at base 324 causing a frameshift mutation was detected. This mutation created a premature stop site in mRNA and hence a truncated protein (Fig. 1A).

The clinical features of this patient have also been reported
Fig. 2 (A) Pedigree of case 9 (Tyson et al., 1997) and family. The arrow indicates proband Case 9. (B) Pedigree of Case 8 (Tyson et al., 1997) and family. The arrow indicates proband Case 8. Square = male, circle = female, filled symbol = affected individual.
Fig. 3 Longitudinal electron micrograph through a myelinated nerve fibre from Case 8 (Tyson et al., 1997) showing multiple myelin outpouchings from the adaxonal aspect of the myelin sheath (between arrows) and another (open arrow) protruding into the axon. A = axon. Bar = 5 µm.

by Tyson and colleagues (Tyson et al., 1997) (Case 8) and therefore only a summary is given here. Case 8 began to walk at 12 months of age but he progressively developed bilateral footdrop; manual dexterity was observed to be poor and his speech was slightly indistinct. Examination showed mild dysarthria, distal upper limb and anterolateral lower leg muscle wasting, weakness and tendon areflexia. No sensory loss was detected. Sensory nerve action potentials were absent. Motor nerve conduction velocity was 15 and 17 m/s in the ulnar and median nerves, respectively.

Nerve biopsy findings
Cases 9 (family C) and 8 (family V) appeared to be similar. There was a severe loss of myelinated fibres, the density in Case 9 being 910/mm² and 3096/mm² in Case 8 (normal range for this age group, 8000–11 000/mm²; Jacobs and Love, 1985). Surviving myelinated fibres were generally hypomyelinated, but showed focal regions where the myelin was thrown into irregular protrusions from the outer aspect of the myelin sheath or which occasionally arose from the adaxonal aspect of the sheath (Fig. 3). Transverse electron microscope sections through such regions demonstrated that some of these outpouchings contained axonal protrusions, whereas others contained Schwann cell cytoplasm (Fig. 4). The presence of circumferential Schwann cell processes and basal lamina around some fibres (Fig. 5) indicated the occurrence of repeated episodes of demyelination and remyelination. Longitudinal electron microscope sections showed that at the nodes of Ranvier the Schwann cell nodal processes were often deficient (Fig. 5). Teased fibre studies revealed the presence of multiple myelin thickenings superficially resembling tomacula, but which were smaller in diameter and more irregular in contour (Fig. 6). These were present on all large and medium calibre fibres but could not be detected on small diameter fibres because of poor myelin staining.

Discussion
We have identified two pathogenic mutations in the 5′ end of the MTMR2 gene in an English and an Indian family,
both with autosomal recessive inheritance, with hereditary neuropathy with focally folded myelin. We also sequenced the MTMR2 gene in six other families with autosomal recessive neuropathies, but no pathogenic mutations were identified in these kindreds, suggesting the presence of other genes for autosomal recessive neuropathy. This finding extends the spectrum of mutations in this gene to both 3′ and 5′ regions of the MTMR2 gene. The mutations severely disrupt the gene and are either deletions, insertions or complex rearrangements, although we identified a missense change in the English family. The mutations that disrupt the gene are likely to result in loss of function of the protein by significantly altering its structure or disrupting splicing. This occurs in the Indian family V, where the mutation causes a truncated protein. The missense mutation of a glycine to glutamic acid at codon 103 in family C causes an identical severe clinical and pathological phenotype to that of other CMT 4B families, and this mutation is also likely to cause loss of function by substituting a key amino acid in the protein, rendering it inactive. MTMR2 is a protein tyrosine phosphatase and may act to dephosphorylate its target substrate in a similar fashion to the myotubularin (MTM1) gene in X-linked myotubular myopathy (Laporte et al., 1996, 1998; Cui et al., 1998). The lack of MTMR2 is likely to lead to overactivity of the substrate, but how this results in demyelination and focal folding of the myelin sheaths is as yet uncertain. It is clear that focal folding of the myelin is not a specific morphological change. Apart from CMT4B1 and CMT4B2, it has been
observed in a number of other neuropathies. Comparable changes have been described in a patient with demyelinating neuropathy in association with type II Waardenberg’s syndrome and Hirschsprung’s disease (Jacobs and Wilson, 1992), and somewhat less florid findings have been noted in congenital hypomyelinating neuropathies by Vallat and colleagues (Vallat et al., 1987) and in a patient with a heterozygous MPZ point mutation (Nakagawa et al., 1999). It is also observable in transgenic mice, into the genome of which extra copies of the human PMP22 gene have been inserted (personal unpublished observations).

The families with MTMR2 mutations identified so far have originated from Italy, Saudi Arabia, India and England. All had consanguineous parents. The phenotypes reported have been very similar (Gambardella et al., 1997), with all patients affecting showing a severe early childhood-onset neuropathy with prominent weakness beginning in the lower limbs, spreading proximally and later affecting the upper limbs. In the Italian and English families, additional features have been weakness of the jaw, facial and bulbar muscles, deafness and diaphragmatic weakness. In our Indian family, the affected individual had a relatively milder clinical and pathological phenotype compared with the other kindreds. These data are provocative and suggest the presence of a significant genotype phenotype effect, the phenotype being dependent upon the severity of the mutation, interaction with SNPs and the resultant available active MTMR2 protein. No clinical data are available as yet from the Saudi Arabian families. Although these cases have been categorized as CMT disease, the phenotype is closer to that of Dejerine–Sottas disease, apart from the fact that the reduction in conduction velocity is less severe than usually accepted for the latter disorder, in which conduction velocities <6–10 m/s are included in the diagnostic characteristics (Dyck et al., 1993; Gabreëls-Festen et al., 1994). This calls into question the utility of these eponymous titles.

The present results characterize further the MTMR2 gene mutations in CMT4B1. They also extend the clinical phenotypes in these families. This was the second gene reported for autosomal recessive HMSN and it appears to account for the majority of autosomal recessive families where the peripheral nerve pathology is characterized by focally folded myelin. The other identified autosomal recessive mutations are those for the early growth response 2 (EGR2) gene (Warner et al., 1998) and the n-myc downstream regulated gene 1 (n-myc DRG1) in HMSN Lom (Kalaydjieva et al., 2000). The rapid progression of the human genome project will enable the cloning of other genes responsible for neuroopathies that will permit further genetic classification of this group of disorders.

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