Clinicopathological and genetic study of early-onset demyelinating neuropathy

Yeşim Parman,1,* Esra Battaloğlu,2,* Ibrahim Barış,2 Birdal Bilir,2 Mürüvvet Poyraz,1 Nisrine Bissar-Tadmouri,2 Anna Williams,3 Nadia Ammar,4 Eva Nelis,4 Vincent Timmerman,4 Peter De Jonghe,4 Ayaz Necefov,2 Feza Deymeer,1 Piraye Serdaroglu,1 Peter J. Brophy3 and G. Said5

1Department of Neurology, Istanbul University, Istanbul Medical Faculty, 2Department of Molecular Biology and Genetics, Boğaziçi University, Istanbul, Turkey, 3Department of Preclinical Veterinary Sciences, University of Edinburgh, Edinburgh, UK, 4Department of Molecular Genetics, Flanders Interuniversity Institute for Biotechnology, University of Antwerp, Belgium and 5Department of Neurology, Centre Hospitalier Universitaire de Bicêtre, Paris, France

Correspondence to: Yeşim Parman, Department of Neurology, Istanbul Medical Faculty, Istanbul University, Millet Cad., Capa, 34390 Istanbul, Turkey
E-mail: parman@tnn.net
* These two authors contributed equally to this work

Summary
Autosomal recessive demyelinating Charcot–Marie–Tooth disease (CMT4), Dejerine–Sottas disease and congenital hypomyelinating neuropathy are variants of hereditary demyelinating neuropathy of infancy, a genetically heterogeneous group of disorders. To explore the spectrum of early-onset demyelinating neuropathies further, we studied the clinicopathological and genetic aspects of 20 patients born to unaffected parents. In 19 families out of 20, consanguinity between the parents or presence of an affected sib suggested autosomal recessive transmission. Screening of various genes known to be involved in CMT4 revealed six mutations of which five are novel. Four of these novel mutations occurred in the homozygous state and include: one in GDAP1, one in MTMR2, one in PRX and one in NDRG1. One patient was heterozygous for a novel MTMR2 mutation and still another was homozygous for the founder mutation, R148X, in NDRG1. All patients tested negative for mutations in EGR2. Histopathological examination of nerve biopsy specimens showed a severe, chronic demyelinating neuropathy, with onion bulb formation, extensive demyelination of isolated fibres and axon loss. We did not discern a specific pattern of histopathology that could be correlated to mutations in a particular gene.

Keywords: early-onset demyelinating neuropathy; PRX; GDAP1; NDRG1; MTMR2

Abbreviations: AR = autosomal recessive; CMT = Charcot–Marie–Tooth disease; DSD = Dejerine–Sottas disease; EGR2 = early growth response gene 2; GDAP1 = ganglioside-induced differentiation-associated protein 1; HMSN = hereditary motor and sensory neuropathy; L-PRX = L-periaxin; MBP = myelin basic protein; MNCV = motor nerve conduction velocity; MTMR2 = myotubularin-related protein 2; NDRG1 = N-myc downstream-regulated gene 1; SSCP = single strand conformation polymorphism

Introduction
Charcot–Marie–Tooth disease (CMT) is the most common inherited peripheral neuropathy with a prevalence of 1 in 2500 (Skre, 1974). Distal muscle weakness and atrophy predominating in the lower extremities, diminished or absent deep tendon reflexes, distal sensory loss and skeletal deformities such as pes cavus are the clinical hallmarks of this disorder (Charcot and Marie, 1886; Dyck and Lambert, 1968a,b). CMT is a clinically and genetically heterogeneous group of disorders. CMT type 1 (CMT1), the demyelinating form, usually transmits as an autosomal dominant or X-linked trait (Dyck et al., 1993; Harding, 1995). However, autosomal recessive (AR) inheritance is frequently observed in populations with a high rate of consanguineous marriages, and the incidence of ARCMT is estimated to be 30–50% of all CMT forms in these populations (Martin et al., 1999).
ARCMT is often more severe and has an earlier onset than the dominant forms (Planté-Bordeneuve and Said, 2002). Demyelinating ARCMT, designated as CMT4, is genetically heterogeneous with eight loci and seven genes identified so far. Mutations in the following genes were identified in different CMT4 variants: ganglioside-induced differentiation-associated gene 1 (GDAP1) (Baxter et al., 2002; Cuesta et al., 2002) on chromosome 8q13–21 (CMT4A) (Ben Othmane et al., 1993), myotubularin-related protein 2 gene (MTMR2) (Bolino et al., 2000) on chromosome 11q23 (CMT4B1) (Bolino et al., 1996), CMT neuropathy 4B2 (AR, with myelin outfoiling) gene (CMT4B2/5SBF2/MTMR13) (Azzedine et al., 2003; Senderek et al., 2003a) on chromosome 11p15 (CMT4B2) (Ben Othmane et al., 1996), an uncharacterized transcript encoding a novel SH3/TPR protein domain (KIAA1985) (Senderek et al., 2003b) on chromosome 5q23–q33 (CMT4C) (Le Guern et al., 1996), N-myc downstream-regulated gene 1 (NDRG1) (Kalaydjieva et al., 2000) on chromosome 8q24 [hereditary motor and sensory neuropathy (HMSN)-Lom] (Kalaydjieva et al., 2000), early growth response gene 2 (EGR2) on chromosome 10q (Warner et al., 1998) or periaxin (PRX) (Boerkoel et al., 2001; Guilbot et al., 2001) on chromosome 19q (CMT4F) (Delague et al., 2000). The gene responsible for CMT Russe (HMSN-R), mapped to chromosome 10q23, still remains to be found (Thomas et al., 2001).

In this study, we report a clinicopathological and genetic survey of 20 patients with early-onset demyelinating neuropathy. Nineteen of them were likely to have AR inheritance.

**Patients and methods**

**Patients**

All patients signed an informed consent and the study was approved by Istanbul University, Istanbul Medical Faculty Ethical Committee.

Twenty unrelated patients aged 4–63 years, born to asymptomatic parents were studied. A neurological examination and EMG were performed in all patients. Nineteen families were likely to display AR transmission based on the presence of consanguinity and/or affected siblings. Of the 15 consanguineous families, three had at least two affected children. Three patients had an affected sibling and one was of Gypsy origin, thus belonging to a highly inbred population. One isolated patient with non-consanguineous parents was included in the study based on clinical and electrophysiological data (Table 1). Parents of 19 patients had no clinical signs and symptoms of peripheral neuropathy. EMGs were normal in 12 parent pairs (parents of patients P03, P03, P02, P06, P111-CMT225, P117, P128, P135, P147, P155, P174 and case 7). The father of case 6 showed a sensorimotor axonal neuropathy on EMG testing. Parents of patients F17.3, P14, P29, P58, P67, P110 and case 10 were normal by history but could not be examined. Affected siblings were also clinically examined. Cases 6, 7 and 10 have been described previously (Planté-Bordeneuve et al., 2001).

**Mutation screening**

DNA was isolated from peripheral blood samples of patients using the salting out method (Miller et al., 1988). The patients had previously tested negative for the CMT1A duplication and mutations in the peripheral myelin protein 22, myelin protein zero and gap junction protein β1 (PMP22, MPZ and GJB1) genes.

The coding regions of GDAP1 (Cuesta et al., 2002), EGR2 (Timmerman et al., 1999) and PRX (Boerkoel et al., 2001) were screened by polymerase chain reaction (PCR) and single strand conformation polymorphism (SSCP) analysis according to previously described protocols, except that the SSCP conditions for the PRX gene were 6% polyacrylamide gel electrophoresis (PAGE) for exon 7 fragments (a, b, c, d, e, f and h), 8% for exon 6 and 10% for exons 4, 5 and 7 fragments. Patient PCR products with abnormal gel mobility and those of controls were purified using the Qiagen PCR purification kit (Qiagen) and sequenced with the automated sequencer ABI 3700 PRISM and the Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems). The sequences were aligned with the CLUSTAL W program (http://work.bench.sdsc.edu). The MTMR2 gene was directly sequenced using the same primers as for exon amplification (Bolino et al., 2000). For screening this gene, PCR products, prepared for sequencing using the PCR Product Pre-Sequencing Kit (USB Corporation, Cleveland, OH), were sequenced using the BigDye Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase FS (Applied Biosystems). Unincorporated dye terminators were removed using MultiScreen 96-well filtration plates (Millipore, Bedford, MA). Electrophoresis was performed on an ABI PRISM 3730 DNA analyser (Applied Biosystems). Sequencing data were collected using the ABI DNA Sequencing Analysis Software, version 3.6 and analysed with the SeqMan 4.00 program (DNASTAR Inc.). Patients P14 and P65, of Gypsy origin, were also screened for the mutations in the entire NDRG1 gene, as described previously (Kalaydjieva et al., 2000).

Linkage to the CMT4C locus was tested by homozygosity mapping using markers DSS402, DSS436 and DSS210.

The mutations identified were described according to the guidelines of den Dunnen and Antonarakis (2000).

**Restriction enzyme digestion**

The PCR products of the PRX gene exon 7f fragment from P135, his relatives and the control chromosomes were subjected to Xhol digestion (3 h at 37°C) followed by 2% agarose gel analysis. TaqI and Rsal digestions were performed under the same conditions as for the PCR products of the NDRG1 gene exon 7 from P65 and the GDAP1 gene exon 3 from P58, respectively. The restriction fragments for TaqI digestion were visualized on a 4% agarose gel and those of Rsal on a 3% agarose gel.

Since the MTMR2 gene mutation in case 10 does not create or abolish any restriction enzyme recognition site, a new mismatched primer was designed to create an HphI restriction
<table>
<thead>
<tr>
<th>Patient no., current age-gender</th>
<th>Parental consanguinity/affected sibs</th>
<th>Mode of onset/first symptoms</th>
<th>Neurological examination</th>
<th>Skeletal deformities</th>
<th>CSF protein (mg/dl)</th>
<th>MNCV (m/s)</th>
<th>Biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>F17,3, 9-F</td>
<td>Parents distantly related</td>
<td>Walked at age 1.5 years. Unsteady gait</td>
<td>Severe distal weakness in all limbs, areflexia, sensory modalities abolished distally</td>
<td>PC, claw hands</td>
<td>ND</td>
<td>Med: 32</td>
<td>Demyelination, axon loss, occasional Ob, FD: 5200/mm²</td>
</tr>
<tr>
<td>P03, 24-F</td>
<td>Parents first cousins</td>
<td>Normal until age 8 years. Unsteady gait</td>
<td>Severe distal weakness in the LL, areflexia, all sensory modalities impaired</td>
<td>PC, hammer toes</td>
<td>57</td>
<td>Med: 36</td>
<td>ND</td>
</tr>
<tr>
<td>P14, 29-M (Gypsy)</td>
<td>Parents non-consanguineous, one affected brother</td>
<td>Normal until age 9 years. Unsteady gait</td>
<td>Severe distal weakness in all limbs, areflexia, all sensory modalities impaired, deafness</td>
<td>PC, hammer toes</td>
<td>36</td>
<td>Med: 27</td>
<td>Demyelination, severe axon loss, many Ob, FD: 2773/mm²</td>
</tr>
<tr>
<td>P29, 17-M</td>
<td>Parents first cousins</td>
<td>Walked at age 1.5 years. Unsteady gait since he walked</td>
<td>Severe distal weakness in all limbs, sensory ataxia</td>
<td>PC, hammer toes</td>
<td>ND</td>
<td>Med: 29</td>
<td>Demyelination, moderate axon loss, FD: 3120/mm²</td>
</tr>
<tr>
<td>P30, 32-M</td>
<td>Parents first cousins</td>
<td>Walked at age 1 year, unsteady gait</td>
<td>Severe distal weakness in all limbs, nystagmus, areflexia</td>
<td>PC, hammer toes</td>
<td>ND</td>
<td>Ul: 3</td>
<td>Demyelination, severe axon loss, many Ob, FD: 2200/mm²</td>
</tr>
<tr>
<td>P55, 4-F</td>
<td>Parents first cousins</td>
<td>Neonatal hypotonia, never walked</td>
<td>No skeletal deformities</td>
<td>Pes planus</td>
<td>ND</td>
<td>Inexcitable</td>
<td>Demyelination, severe axon loss, no Ob, FD: 2133/mm²</td>
</tr>
<tr>
<td>P58, 63-F</td>
<td>Parents first cousins, many affected members</td>
<td>Walked normally, unsteady gait at age 3 years</td>
<td>Severe proximal and distal weakness more marked in the LL, areflexia, died of respiratory failure at age 4 years</td>
<td>Pes planus</td>
<td>ND</td>
<td>Demyelination, severe axon loss, many Ob, some basal lamina type, occasional regenerating fibres, FD: 2106/mm²</td>
<td></td>
</tr>
<tr>
<td>P65, 13-F (Gypsy)</td>
<td>Parents non-consanguineous</td>
<td>DMM, walked at age 2 years</td>
<td>Severe distal weakness in the LL, areflexia, all sensory modalities impaired</td>
<td>PC</td>
<td>ND</td>
<td>Med: 14</td>
<td>ND</td>
</tr>
<tr>
<td>P67, 11-M</td>
<td>Parents first cousins</td>
<td>DMM, walked at age 3 years. Frequent falls</td>
<td>Distal weakness in all limbs, all sensory modalities impaired</td>
<td>PC, hammer toes, scoliosis</td>
<td>125</td>
<td>Med: 16</td>
<td>ND</td>
</tr>
<tr>
<td>P110, 43-M</td>
<td>Parents first cousins</td>
<td>Walked normally, unsteady gait at age 9 years, cramps</td>
<td>Severe distal weakness in the LL, all sensory modalities impaired</td>
<td>PC</td>
<td>ND</td>
<td>Med: 24</td>
<td>ND</td>
</tr>
<tr>
<td>P111 (CMT225), 24-M</td>
<td>Parents first cousins, two affected brothers</td>
<td>Walked normally, unsteady gait at age 9 years</td>
<td>Severe proximal and distal weakness in all limbs, areflexia (sensory testing cannot be done)</td>
<td>PC</td>
<td>ND</td>
<td>Inexcitable</td>
<td>Demyelinating, mild axon loss, FD: 5800/mm²</td>
</tr>
<tr>
<td>P117, 4-M</td>
<td>Parents first cousins</td>
<td>DMM, cannot walk independently</td>
<td>Severe proximal and distal weakness in all limbs, areflexia</td>
<td>PC</td>
<td>ND</td>
<td>Inexcitable</td>
<td>Demyelinating, moderate axon loss, Ob, FD: ND</td>
</tr>
<tr>
<td>P128, 21-F</td>
<td>Parents non-consanguineous, affected brother</td>
<td>DMM, walked at age 2.5 years</td>
<td>Mild proximal and severe distal weakness in all limbs, all sensory modalities impaired, tremor</td>
<td>PC, scoliosis</td>
<td>ND</td>
<td>Med: 14</td>
<td>Demyelinating, moderate axon loss, Ob, FD: ND</td>
</tr>
<tr>
<td>Case</td>
<td>Age</td>
<td>Parents</td>
<td>DMM, walked at age</td>
<td>2 years, frequent falls, unsteady gait</td>
<td>Mild distal weakness in the LL, DTRs absent in the LL, normal sensitivity to touch, pinprick, and temperature, sensory ataxia</td>
<td>PC, scoliosis</td>
<td>ND</td>
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<tr>
<td>P135</td>
<td>4-M</td>
<td>Parents first cousins</td>
<td>DMM, walked at age 2 years</td>
<td>Frequent falls, unsteady gait</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P147</td>
<td>6-M</td>
<td>Parents non-consanguineous</td>
<td>Walked at age 1 year</td>
<td>started to have unsteady gait at age 3.5 years</td>
<td>Severe distal weakness in all 4 limbs, proximal weakness in the LL, all sensory modalities impaired</td>
<td>PC, claw hands, scoliosis</td>
<td>161</td>
</tr>
<tr>
<td>P155</td>
<td>20-M</td>
<td>Parents first cousins, affected brother</td>
<td>DMM, walked at age 4 years</td>
<td>Frequent falls</td>
<td>Severe proximal and distal weakness more marked in the LL, areflexia, all sensory modalities impaired, in wheelchair</td>
<td>PC, pes equinovarus</td>
<td>ND</td>
</tr>
<tr>
<td>P174</td>
<td>28-M</td>
<td>Parents distantly related</td>
<td>Walked at age 1 year</td>
<td>gait was unsteady, cramps</td>
<td>Mild distal weakness of all limbs, areflexia, all sensory modalities impaired, tremor</td>
<td>PC</td>
<td>55</td>
</tr>
<tr>
<td>Case 6, 21-F</td>
<td></td>
<td>Parents non-consanguineous, affected sister</td>
<td>DMM</td>
<td>Severe distal weakness in all limbs, all sensory modalities impaired, hypophonia, in wheelchair</td>
<td>PC, claw hands, scoliosis</td>
<td>ND</td>
<td>Uln: 10</td>
</tr>
<tr>
<td>Case 7, 44-M</td>
<td></td>
<td>Parents distantly related</td>
<td>Walked at 13 months, unsteady gait at 3.5 years</td>
<td>Severe proximal and distal weakness in all limbs, hypophonia, areflexia, all sensory modalities impaired</td>
<td>PC</td>
<td>ND</td>
<td>Med: 6</td>
</tr>
<tr>
<td>Case 10, 24-F</td>
<td></td>
<td>Parents first cousins</td>
<td>DMM</td>
<td>Severe proximal and distal weakness in all limbs, M.O.O weak, hypophonia, areflexia, all sensory modalities impaired, tremor, in wheelchair</td>
<td>PC, hammer toes</td>
<td>18</td>
<td>Med: 17</td>
</tr>
</tbody>
</table>

F = female; M = male; DMM = delayed motor milestones; UL = upper limbs; LL = lower limbs; PC = pes cavus; OB = onion bulbs; DTR = deep tendon reflexes; TR = triceps, M.O.O = m. orbicularis oculi; ND = not done; FD = fibre density (cases 6, 7 and 10 were described by Plante-Bordeneuve et al., 2001).
site with the wild-type allele. Exon 9 of the gene was amplified with 5'-TTCTAGGCAAGGTTTG-3' and 5'-GGCTGGCTACACCGGC-3' primers. The resulting 170 bp PCR product was digested with HphI that produces 144 and 26 bp fragments for the wild-type allele and remains uncut for the mutant allele. The digestion products were separated on a 4% agarose gel.

Morphological study
The sural nerve was biopsied at the ankle level under local anaesthesia in 13 patients and processed for light and electron microscopy. The nerve samples were fixed at 4°C in 3.6% glutaraldehyde, buffered at pH 7.4. Thionin-stained 1 μm thick transverse sections were used for light microscopy and 0.1 μm thick sections stained with uranyl acetate and lead citrate for electron microscopy. After post-fixation in osmium tetroxide, another nerve fragment was macerated in 66% glycerin for 48 h, following post-osmification for the teased fibre study.

Immunohistochemistry
Frozen sections (5 μm) of sural nerve biopsy of P135 embedded at an optimal cutting temperature were collected on 3-aminopropyltriethoxysilane-subbed slides. The sections were fixed in a 4% paraformaldehyde solution. Immunofluorescence for l-periaxin (l-PRX) and myelin basic protein (MBP) was performed as previously described (Dytrych et al., 1998; Gillespie et al., 2000).

Western blotting
l-PRX and myelin protein zero were detected by western blotting of SDS-solubilized sural nerve as previously described (Dytrych et al., 1998).

Results
Clinical findings
The clinical and electrophysiological findings are summarized in Table 1. There were eight female and 12 male patients. Motor milestones were delayed in eight patients, one patient never walked, and 11 patients had early-onset neuropathy (first symptoms starting between age 3 and 9 years). Pes cavus along with distal muscle atrophy was present in 18 patients. Six patients had hammer toes and three patients had claw hands. Scoliosis was noted in six. Two patients had tremor. Motor deficit was more pronounced distally and more severe in the lower limbs in all patients. Sensory loss, affecting all four extremities, mainly affected position and vibratory senses, resulting in sensory ataxia in four patients. Areflexia was present in 13 patients. Cranial nerves were affected in four patients; upper facial weakness and hypophonia in three, and deafness in one. CSF protein level was measured in six patients and was elevated in three patients. Motor nerve conduction velocity (MNCV) was markedly reduced (<12 m/s) in six patients and no responses were obtained in three patients. In 11 patients, median or ulnar MNCVs were between 12 and 36 m/s. Compound muscle action potential amplitudes were decreased in all patients. Needle EMG was normal in one patient but otherwise showed chronic neurogenic alterations.

Genetic findings
Pathogenic mutations in the homozygous condition were identified in five patients. A novel G to T transversion at nucleotide position 445 of GDAP1 was identified in P58 in the homozygous condition. Her mother was found to be heterozygous for the mutation (Fig. 1A). By TaqI restriction analysis, the unaffected father and two unaffected sibs were identified as heterozygous carriers while the index patient and two affected sibs were homozygous for the mutation (data not shown). The mutation was absent in 100 control chromosomes. This nucleotide change causes the substitution of aspartic acid at codon 149 by tyrosine (D149Y).

An NDRG1 mutation was identified in one of the two Gypsy patients. Patient P65 had the founder R148X nonsense mutation in the homozygous condition while her mother was heterozygous for the same mutation (Fig. 1B). The unaffected father was also identified to be a carrier of the mutation by Rsal restriction analysis (data not shown).

A novel mutation in PRX was identified in patient P135. The patient was homozygous and her mother was heterozygous for a C to T transition at nucleotide position 3208 causing the nonsense mutation R1070X (Fig. 1C). The mutation creates a premature translational stop codon at 28 amino acids upstream of the acidic domain of l-PRX. Digestion analysis with the restriction enzyme XhoI confirmed the segregation of the mutated allele in the proband and his consanguineous parents, whereas 50 controls tested negative for the mutation.

In case 6, we detected a PCR fragment of MTMR2 exon 8 that was ~450 bp longer than the wild-type fragment. Sequence analysis of MTMR2 revealed a homozygous 446 bp insertion in exon 8. The insertion was flanked by a 15 bp direct repeat (5'-GAAAGCTGGAGAATA-3'). Repeat-Masker analysis (A. F. A. Smith and P. Green, RepeatMasker at http://ftp.genome.washington.edu/RM/RepeatMasker.html) showed that the sequence of the insertion was 100% homologous to that of the 3' end of the human long interspersed nuclear element (LINE) L1Hs and had a poly(A) stretch of 87 bp (Fig. 2). By conceptual translation, this mutation causes an insertion of 47 amino acids and a premature stop.

A second novel MTMR2 mutation was identified in case 10 in the heterozygous condition. The mutation (841_844 delATCA), causing a frameshift and a premature translation stop at codon 290, was absent in 60 control chromosomes (data not shown). However, no mutation could be identified in the second allele by direct sequencing. Samples from other family members were not available for further analysis.
By homozygosity mapping, the disease in 19 patients was found not to be linked to the novel SH3/TPR domain-coding gene responsible for CMT4C. Patient P111 (CMT225), found to be homozygous for the locus, was analysed further and was identified to have a mutation in this gene in another study (Senderek et al., 2003b).

All patients tested negative for mutations in EGR2. The mutations are presented in Table 2.

**Nerve biopsy findings**

Semi-thin sections showed a depletion of myelinated fibres in the biopsy specimens of patients F17.3, P14, P29, P30, P55, P58, P117, P128, P135, P174, and cases 6, 7 and 10. Hypo/demyelinating neuropathy with onion bulbs was the prominent feature in all. Teased fibre preparations of myelinated fibres displayed extensive demyelination or myelin thickness irregularities with segmental demyelination. Patient P58, who carries a GDAP1 mutation, had a severe reduction of large myelinated fibres; most of the fibres were thinly myelinated and Schwann cell proliferation was prominent. Occasional clusters of regenerated fibres were also seen. Electron microscopy showed basal lamina and ‘classical’ onion bulbs. Details of the morphological study of the patients with the MTMR2 mutation (cases 6 and 10) have been described previously elsewhere. Both had a severe loss of myelinated fibres, but only case 6 had focal myelin thickening (Planté-Bordeneuve et al., 2001). Histopathological aspects of the patient with the PRX mutation (P135) were not distinct in semi-thin sections. He had a moderate reduction of myelinated fibres. Demyelination and remyelination with many fibres displaying myelin irregularities, decompacted lamellae and onion bulbs were prominent features. Electron microscopy showed Schwann cell proliferation forming onion

![Fig. 1](image)
bulbs, occasional focal myelin thickening, abnormalities of the paranodal myelin loops and Schmidt–Lantermann incisures with absence of paranodal septate-like attachments (Fig. 3).

**Periaxin immunofluorescence and western analyses**

Immunofluorescence analysis of the sural nerve biopsy specimen of P135 revealed staining with N-terminal and repeat region antibodies but not with a C-terminal antibody for L-PRX, even though there was MBP-positive staining indicating the presence of some residual myelin (Fig. 4). The L-PRX (anti-N-terminal, anti-repeat and anti-C-terminal) and MBP antibodies reacted positively on normal human sural nerve biopsy. The presence of a truncated L-PRX in the patient was confirmed by western analysis (Fig. 5).

**Discussion**

We have identified disease-associated mutations in six of 20 early-onset demyelinating neuropathy patients. Four novel mutations, one in GDAP1, one in MTMR2 and one in PRX, were found in the homozygous condition in three patients, and one KIAA1985 mutation was reported elsewhere (Senderek et al., 2003b). The fifth mutation was a 4 bp deletion in exon 9 in MTMR2 in the heterozygous condition that leads to a frameshift. However, we were not able to find the second allele. A patient of Gypsy origin had the R148X founder mutation in NDRG1.

The non-conservative GDAP1 mutation (D149Y) in patient P58 changed a positively charged aspartic acid to a polar uncharged tyrosine. Aspartic acid at that position is conserved in many other species (mouse, rat, cow, pig, chicken and frog) and the mutation is segregating with the disease phenotype in the family, indicating that it is a pathogenic mutation. The substitution of this essential amino acid might lead to loss of
function of the GDAP1. GDAP1 encodes a 358 amino acid protein but its function in peripheral nerves remains elusive. Patient P58 has a severe sensorimotor neuropathy with early onset. She had pes planus and was wheelchair-bound at age 60 years.

The insertion of an L1Hs in MTMR2 in case 6 leads to a severe demyelinating neuropathy. The patient is wheelchair-bound like her affected sibling. Her 70-year-old father probably has an acquired axonal neuropathy. There could be many causes for his neuropathy, but unfortunately the work-up could not be done since he did not keep his appointments. The c.841_844delATCA MTMR2 variant identified in case 10 could represent either a polymorphism or a causative mutation. Hypothetically, the truncated protein lacks a functional tyrosine phosphatase domain that is highly conserved among family members. Moreover, this variant could not be detected in healthy Turkish controls that were analysed by the appropriate PCR restriction enzyme assay. Consequently, the deletion leading to a premature stop codon is most probably a pathogenic mutation rather than a harmless variant.

One of the two Gypsy patients was homozygous for the R148X founder mutation in NDRG1 causing HMSN-Lom. She had delayed motor milestones with a severe sensorimotor neuropathy. The patient’s phenotype was similar to that of other patients carrying the same mutation, except for deafness. Deafness is known to be an invariant feature of the phenotype and usually develops in the third decade (Kalaydjieva et al., 1996). Thus, our patient, age 13 years, has to be followed for the development of hearing impairment. The other Gypsy patient (P14) who had a sensory neural deafness was born to consanguineous parents and has an affected sibling. He did not carry a mutation in NDRG1 and has to be analysed further for the CMT Russe locus because of his ethnic origin.

Recessive point mutations have been reported in PRX in patients with Dejerine–Sottas disease (DSD) and CMT4 (Boerkoel et al., 2001; Guilbot et al., 2001; Takashima et al., 2002). In patient P135 with a PRX mutation, the motor milestones were delayed and he had a mild motor neuropathy; sensory ataxia was more prominent. Although his phenotype was compatible with DSD, these findings are somewhat dissimilar to the clinical features of reported patients with PRX mutations. A marked sensory impairment along with severe motor neuropathy was found in all reported

Fig. 3 Patient 135. (A) Light microscopy: semithin section of the sural nerve biopsy shows loss of myelinated fibres, demyelination and remyelination, with many fibres with myelin irregularities (arrows) and decompacted lamellae (×40). (B) Electron microscopy: Schwann cell proliferation forming onion bulbs (arrows) and (C) abnormalities of the paranodal myelin loops and Schmidt–Lantermann incisures with absence of paranodal septate-like attachments (arrows). Scale 1 μm.
patients except for the patient with the C715X mutation who had a mild motor neuropathy. Interestingly, the patient with the most upstream mutation (R82fsX96) was reported to have no clinical and histopathological features distinct from those of other patients with mutations downstream of the gene. The R1070X mutation, identified in this study, is the most downstream mutation reported so far and provides evidence for the suggested pathological role of the acidic domain found at the C-terminus of the protein. The absence of this domain in our patient and probably in other reported patients with PRX mutations may block interaction of this cytoskeleton-associated Schwann cell-specific protein with

![Immunofluorescence analysis of a sural nerve biopsy from patient with an R1070X mutation.](image)

(A, B and C) Nerve fibres from the patient show staining with N-terminal PRX antibody (A), MBP (A) and in the same fibres (C). (D, E and F) Nerve fibres from the patient show staining with repeat region PRX antibody (D), MBP (E) and in the same fibres (F). (G, H and I) Nerve fibres from the patient show no staining with C-terminal PRX antibody (G), but staining with MBP (H) and in the same fibres (I), indicating that a truncated PRX protein is formed. (J, K and L) Nerve fibres from a normal control show staining with the C-terminal PRX antibody (J), MBP (K) and in the same fibres (L). These fibres are much larger than those in the patient. Scale 5 μm.
albeit that there was less in the patient biopsy. Protein to demonstrate that myelin was present in both samples absent. Both blots were reprobed for the myelin protein zero size (43 kDa) of the C-terminal region that is predicted to be the truncated protein in the patient corresponds very closely to patient sural nerve by western blot. Note that the decrease in size (Fig. 5)

Comparison of the L-PRX content of normal and patient sural nerve by western blot. Note that the decrease in size of the truncated protein in the patient corresponds very closely to the size (43 kDa) of the C-terminal region that is predicted to be absent. Both blots were reprobed for the myelin protein zero protein to demonstrate that myelin was present in both samples albeit that there was less in the patient biopsy.

downstream proteins that are required to transmit the extracellular signals to the nucleus and cause destabilization of the peripheral nerve myelin (Sherman and Brophy, 2000).

Histopathological aspects of all the biopsies had the same characteristics. They all showed a severe, chronic demyelinating neuropathy, onion bulb formation, extensive demyelination of isolated fibres and axon loss. Both demyelinating and axonal nerve lesions are found to be associated with GDAP1 mutations. Recently, an intermediate form with both axonal degeneration and demyelination is also described (Senderek et al., 2003c). In the biopsy of our patient (P58) with the GDAP1 mutation, demyelinating features with thinly myelinated fibres and onion bulb formation were more prominent. Case 6 with the MTMR2 mutation also had a demyelinating neuropathy with focal thickening of myelin. On the other hand, case 10 with a mutation in the same gene did not show this finding. Histopathological characteristics of patient P135 with a PRX mutation were compatible with the reported data, showing demyelination, remyelination, onion bulb formation with occasional focal thickening of myelin and detachment of terminal loops. Most of these findings have already been described in chronic demyelinating neuropathies. Focally folded myelin is observed in CMT4B1 and CMT4B2. It is clear that this finding is not a specific morphological change as shown in patients with the MTMR2 and PRX mutations (cases 6, 10 and P135). The absence of septate-like attachments in P135 and in the patient reported previously with the PRX C715X mutation (Takashima et al., 2002) suggests that l-PRX might also play a role in the maintenance of axoglial junctions, but this suggestion has to be supported by further research.

The incidence of ARCMT is relatively common in populations with a high rate of consanguineous marriages. Although the majority of the patients included in the present study were born to consanguineous parents and were screened for mutations in six demyelinating ARCMT genes, we observed a low mutation detection rate (25%). This finding confirms further locus heterogeneity of demyelinating neuropathies of infancy. On the other hand, a minority of mutations might have been missed if they reside in the regulatory regions of the genes or are due to limited sensitivity of SSCP analysis used for screening of GDAP1, PRX and EGR2. Additionally, chronic inflammatory demyelinating polyneuropathy (CIDP) of infancy cannot be excluded in at least one of the patients (P147) since he was an isolated case and received corticosteroids and/or intravenous immunoglobulin with marked clinical benefit. Finally, the recently identified SBF2 gene should be screened in these patients; however, the frequency of mutations in this gene among demyelinating ARCMT patients is still unknown. According to our clinical and genotypic evaluation, there is no major locus responsible for ARCMT at least in the Turkish population. We did not observe a specific histopathological characteristic that can be attributed to early-onset demyelinating neuropathies.

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