Inherited demyelinating neuropathies with micromutations of peripheral myelin protein 22 gene

Federica Taioli, Ilaria Cabrini, Tiziana Cavallaro, Michele Acler and Gian Maria Fabrizi

Department of Neurological, Neuropsychological, Morphological and Movement Sciences, University of Verona, 37134 Verona, Italy

Correspondence to: Dr Gian Maria Fabrizi,
Department of Neurology,
University Hospital G. B. Rossi,
P. le L. A. Scuro 10,
37134 Verona,
Italy
E-mail: gianmaria.fabrizi@univr.it

The peripheral myelin protein 22 gene (PMP22) encodes an intrinsic membrane protein of compact myelin. Duplication or deletion of PMP22 causes the most common autosomal dominant neuropathies, Charcot-Marie-Tooth disease type 1A or hereditary neuropathy with liability to pressure palsies. Charcot-Marie-Tooth disease type 1A is a hypertrophic de-remyelinating neuropathy manifesting with peroneal muscular atrophy and uniform, marked, slowing of nerve conduction velocities. Hereditary neuropathy with liability to pressure palsies is a recurrent focal neuropathy with sausage-like myelin thickening (tomacula) and non-uniform nerve conduction velocity changes. Missense or nonsense mutations also cause more severe Charcot-Marie-Tooth disease type 1A forms of infancy or hereditary neuropathy with liability to pressure palsies, but they are presumably very rare. We performed a mutational scanning of PMP22 in 229 index patients (46 familial, 183 isolated) referred for suspected inherited neuropathy. The series included 125 cases with hereditary neuropathy with liability to pressure palsies (mean age 42.5 years), 47 cases with Charcot-Marie-Tooth disease type 1A (motor nerve conduction velocities at median nerve below 38 m/s) (mean age 40.7 years) and 57 cases with Charcot-Marie-Tooth with unknown nerve conduction velocities (mean age 43 years). Preliminary molecular studies ruled out PMP22 duplication or deletion or mutations in a comprehensive panel of Charcot-Marie-Tooth genes. Mutational scanning of PMP22 was done by denaturing high performance liquid chromatography and automated nucleotide sequencing. To investigate the molecular basis of phenotype-to-genotype correlations, we performed a transcriptional analysis of PMP22 using reverse-transcriptase polymerase chain reaction and quantitative real-time polymerase chain reaction in two phenotypically divergent nerve biopsies. Ten patients harboured eight micromutations of PMP22 including four novel changes. In six familial and three sporadic cases, detected mutations caused premature or delayed stop codons and were associated with hereditary neuropathy with liability to pressure palsies; the related pathological pictures ranged from classical tomaculous neuropathy to a mild demyelinating neuropathy with atypical non-tomaculous myelin thickenings. In a single family a c.179-2A > G mutation affecting the splice acceptor site of intron 2 cosegregated with a Charcot-Marie-Tooth disease type 1A-like syndrome and a peculiar pathological picture of demyelinating neuropathy without Charcot-Marie-Tooth disease type 1A-like classical onion bulbs or tomacula. Transcriptional analysis of a novel c.174_178 + 7delAAACGGTGAGGC deletion involving exon 2 and intron 2 demonstrated an unstable mutant transcript leading to a p.Asn59GlyfsX12 change; the mutation represented a null allele and caused a typical tomaculous hereditary neuropathy with liability to pressure palsies. The Charcot-Marie-Tooth disease type 1-like c.179-2A > G allele led to a stable transcript with an in-frame deletion of exon 3.
Introduction

The peripheral myelin protein 22-kDa (PMP22) gene encodes an intrinsic, tetraspan membrane glycoprotein that is expressed mainly in Schwann cells and represents a crucial, albeit minor, component of the compact myelin in peripheral nerves (Scherer and Wrabetz, 2008). The heterozygous duplication or deletion of chromosome 17p12, which includes PMP22, causes alternatively Charcot-Marie-Tooth type 1A (CMT1A) or hereditary neuropathy with liability to pressure palsies (HNPP) (Reilly and Shy, 2009). CMT1A is the most frequent hereditary motor-sensory polyneuropathy manifesting in late childhood or adolescence with peroneal muscular atrophy, mild to moderate distal sensory loss, pes cavus and uniform, slowed nerve conduction velocities; the pathological process is a hypertrophic neuropathy characterized by segmental demyelination, loss of large diameter fibres and complex onion bulbs (Schwann cell cytoplasmic processes surrounding residual fibres). HNPP manifests as an acute mononeuropathy usually provoked by compressions or trivial traumas variably affecting the brachial plexus or common peroneal, ulnar, brachial plexus, radial or median nerves; a rarer presentation includes chronic sensory polyneuropathy. Neurophysiology discloses focal slowing of nerve conduction velocities at common entrapment sites, diffuse mild slowing of nerve conduction velocities with prolonged distal motor latencies. The pathological counterpart of HNPP is tomaculous neuropathy, characterized by sausage-like myelin thickenings (tomacula), with minor features of de-myelination and relative preservation of fibre densities (Reilly and Shy, 2009).

Other mutations of PMP22 may also cause CMT1A or HNPP as well as more severe neuropathies of infancy namely, Dejerine–Sottas syndrome type 1A and congenital hypomyelinating neuroopathy type 1A. Whereas missense mutations lead to CMT1A, Dejerine–Sottas syndrome type 1A or congenital hypomyelinating neuropathy type 1A, and only exceptionally to HNPP (Sahenk et al., 1998; Shy et al., 2006), nonsense or frameshifting nucleotide changes introducing aberrant stop codons cause HNPP (Reilly and Shy, 2009). CMT1A, Dejerine–Sottas syndrome type 1A and congenital hypomyelinating neuropathy type 1A reflect various gain-of-function mechanisms exerted by the mutant alleles; conversely, HNPP subredits a loss of the wild-type PMP22 expression or protein function (Scherer and Wrabetz, 2008).

Micromutations of PMP22 are believed to be very rare, having a poor impact on neurological practice outside the typical phenotypical range (England et al., 2009). We identified 10 cases harbouring eight different mutations of PMP22 including nonsense nucleotide substitutions, frameshifting deletions/insertions or splicing mutations. The related phenotypes included HNPP as well as Charcot-Marie-Tooth disease-like, pathologically atypical, demyelinating neuropathies that correlated with variable alterations of PMP22 transcription in nerve biopsies.

Patients and methods

Patients

PMP22 was analysed in a cohort of 229 index patients with possible inherited neuropathy referred to us for molecular investigations. According to family history, 183 patients (80%) were apparently isolated and 46 were familial (with at least one relative symptomatic or affected) (20%). One hundred and twenty-five patients had suspected HNPP (age range 8–77 years; mean age 42.5 years), without the 17p12 deletion or point mutations in the myelin protein zero (MPZ) and gap-junction protein beta-1 (GJB1) genes; 47 patients had suspected CMT1A (age range 6–73 years; mean age 40.7 years) with motor nerve conduction velocities at the median nerve <38 m/s, without the 17p12 duplication or point mutations in MPZ, GJB1 or early growth response gene-2 (EGR2); 57 patients had suspected Charcot-Marie-Tooth disease (age range 9–80 years; mean age 43 years) with unknown values of median motor nerve conduction velocities without the 17p12 duplication or mutations in MPZ, GJB1, EGR2, mitofusin 2 (MFN2) and neurofilament light chain (NEFL) genes. PMP22 mutations detected in the probands were investigated in available relatives after obtaining informed consent.

All patients had undergone standard clinical and neurophysiological examinations at the referring centres or at our Institution.

Nerve biopsy studies

Archive sural nerve biopsies were performed in Proband 2 at age 36 years, Patient 5 at age 39 years, Patient 6 at age 14 years and Proband 8 at age 22 years. Nerves were processed for light and electron microscopy investigations, according to standard procedures. Teased fibres analysis was performed on three fascicles: 30 fibres were isolated from each fascicle and the percentage distribution of focal myelin thickenings was calculated on approximately 600–800 internodes, as described (Rizzuto et al., 1993).

Mutational analysis of PMP22

Genomic DNA was extracted from venous blood, following standard procedures. Major rearrangements at chromosome 17p12 were investigated by pulsed field gel electrophoresis or by multiplex ligation-dependent probe assay (MLPA) (MRC Holland).

After polymerase chain reaction (PCR) amplification, the four coding exons of PMP22 and related exon-intron boundaries (NCBI accession
number NM_000304) were analysed by denaturing high-performance liquid chromatography (Wave® System 3500 HT Transgenomic). Amplicons with abnormal elution profiles were sequenced on both strands by the GenomeLab™ DTCS Quick-start kit (Beckman Coulter) on a CEQ 8800 Beckman sequencer. DNA regions containing a frameshifting mutation were sequenced after subcloning into the AccelTaq™ Vector (Novagen). Primers sequences are available in Supplementary Table 1.

Transcriptional analysis

Transcriptional analysis was done on frozen sural nerve biopsies, available for Probands 2 and 5, carrying a heterozygous c.174_178+7delAAACCGGTGAGGC and a heterozygous c.179-2A > G mutation, respectively (Table 1). Total RNA was extracted from frozen biopsies using the TRI Reagent kit® (Ambion) and subjected to first-strand complementary DNA synthesis (reverse-transcriptase PCR) using the random hexamer primers and GeneAmp® RNA PCR kit (Applied Biosystem).

A 451 base pair complementary DNA corresponding to the full-length PMP22 was amplified using primers Ex1-forward (Fw) 5'-CTCCTCGGTGCTGAGTATA-3' plus Ex4-reverse (Rv) 5'-ATAGAT GACACCCTGCTGAA-3'. Aberrantly spliced complementary DNA from Patient 2 was amplified and sequenced using primers Ex1-Fw plus IVS (intervening sequence) 2-Rv 5'-CTCCCAGTGTCGATT TT-3'.

Quantitative real-time PCR of PMP22 complementary DNA was performed by the relative standard curve method, using messenger RNA from a surgical sample of acoustic Schwannoma to prepare the standard curve. PMP22 expression was analysed in Probands 2 and 5 and in two control patients including a 40-year-old male with spastic paraparesis and normal sural nerve biopsy (Control 1), and a 45-year-old male suffering from multifocal motor neuropathy with conduction block and normal sural nerve biopsy (Control 2). Two target regions of PMP22 were amplified: one amplicon spanned exons 1–2, the other spanned exon 3. Because both mutations removed exon 3, in Probands 2 and 5 the 3Fw-3Rv amplicon represented only the wild-type transcript, whereas the 1Fw-2Rv amplicon represented both the wild-type and mutant transcripts. Therefore, the exon 1-2/exon 3 ratio (wild-type + mutant/wild-type) was assumed as an indicator of the transcriptional levels of the mutant versus the wild-type alleles. Expression levels of PMP22 complementary DNAs were also normalized to exon 8 of the glyceraldehyde-3-phosphate dehydrogenase complementary DNA. Reactions were replicated four times, using the iTaq™ SYBR® Green Supermix with ROX (Bio-Rad), and analysed on a 7300 real-time PCR System (Applied Biosystems). Pairs of primers were as follows: Ex1-Fw 5'-ATCATCGTCTCCCAC GTCG-3' and Ex-2Rv 5'-CAGTGCCTGTCCATTGCG-3'; Ex3-Fw 5'-GCCACCATGATCCTGCTGAT-3' and Ex3-Rv 5'-CCCTTGTTGAG GTGAAGAGT-3'; GAPDH Ex-8Fw 5'-CCACAAGTCCATGCGCATAC TG-3' and Ex-8Rv 5'-ATCAGGGCAGTTTCCCG-3'.

Results

Detected mutations

In 10 apparently unrelated probands we identified eight different heterozygous PMP22 mutations (Table 1).


A singleton splicing mutation (c.179-2A > G) affected the 3'-splice site of intronic intervening sequence 2 (IVS2). In silico analysis by the NNSplice 0.9 software (www.fruitfly.org) suggested a skipping of exon 3, confirmed by transcriptional analysis, and an in-frame p.Glu60_Ala106del protein change.

<p>| Table 1 Mutations and phenotypes in 10 patients with micromutations of PMP22 |
|-----------------------------|-----------------|----------------|----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Patient, sex, age (years), phenotype</th>
<th>Familiarity</th>
<th>Nucleotide change</th>
<th>EX/IVS</th>
<th>Protein change</th>
<th>Nerve biopsy</th>
<th>References</th>
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<tbody>
<tr>
<td>1, F, 16 HNPP</td>
<td>+</td>
<td>c.130_131delAC</td>
<td>EX2</td>
<td>pThr44LeufsX177</td>
<td>tomacular n.</td>
<td>-</td>
</tr>
<tr>
<td>2, M, 35 HNPP</td>
<td>-</td>
<td>c.174_178 + 7delAAACCGGTGAGGC (g.3918762_g.3918773del)</td>
<td>EX2/IVS2</td>
<td>pAsn99GlyfsX12</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>3, M, 17 HNPP</td>
<td>+</td>
<td>c.174_178 + 7delAAACCGGTGAGGC (g.3918762_g.3918773del)</td>
<td>EX2/IVS2</td>
<td>pAsn99GlyfsX12</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>4, M, 15 HNPP</td>
<td>+</td>
<td>c.174_178 + 7delAAACCGGTGAGGC (g.3918762_g.3918773del)</td>
<td>EX2/IVS2</td>
<td>pAsn99GlyfsX12</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>5, M, 41 CMT1</td>
<td>+</td>
<td>c.179-2A &gt; G</td>
<td>IVS2</td>
<td>pGlu60_Ala106del</td>
<td>demyelinating n.</td>
<td>Ekici et al., 2000***; Park et al., 2006*; Young et al., 1997**; Lenssen et al., 1998**</td>
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<tr>
<td>6, F, 14 HNPP</td>
<td>(de novo)</td>
<td>c.281_282insG</td>
<td>EX3</td>
<td>pArg95GlnfsX127</td>
<td>tomacular n.</td>
<td>-</td>
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<td>c.346_347insCC</td>
<td>EX4</td>
<td>p.Ile116ThrfsX4</td>
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<td>-</td>
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<tr>
<td>8, M, 22 HNPP</td>
<td>+</td>
<td>c.371G &gt; A</td>
<td>EX4</td>
<td>p.Trp124X</td>
<td>tomacular n.</td>
<td>-</td>
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<td>9, F, 19 HNPP</td>
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<td>c.434delT</td>
<td>EX4</td>
<td>p.Leu145ArgfsX9</td>
<td>ND</td>
<td>Pareyon et al., 1996***</td>
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<tr>
<td>10, M, 28 HNPP</td>
<td>-</td>
<td>c.443_444delTC</td>
<td>EX4</td>
<td>p.Leu148GlnfsX72</td>
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<td>-</td>
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</table>

Nucleotide and protein changes followed the nomenclature of the Human Genome Variation Society (www.hgvs.org/mutnomen). An X indicated a stop codon; a number after an X indicated the number of residues occurring from the mutated codon to the novel stop codon. *The nucleotide change was reported previously (Ekici et al., 2000; Park et al., 2006); here we provide the protein change predicted by transcriptional analysis. **The same mutation was already reported as Gly99fsX122 (Young et al., 1997; Lenssen et al., 1998). The frameshifting G-insertion is predicted to cause a mutant PMP22 longer than the wild-type (222 instead of 160 amino acid residues).

***The Trp124X nonsense mutation was associated previously to a different nucleotide change (C.372G > A) (Pareyon et al., 1996). c. = complementary DNA sequence; EX = exon; F = female; fs = frameshift; g. = genomic DNA sequence; IVS = intervening sequence; M = male; n = neuropathy; ND = not done.
Transcriptional analysis of c.174_178 + 7delAAACGGTGAGGC and c.179-2A > G

In Patient 2 with the c.174_178 + 7delAAACGGTGAGGC, PCR amplification of PMP22 complementary DNA using primers EX1Fw and IVS2Rv produced a 467 base pair DNA band; direct sequencing of the fragment demonstrated a retention of IVS2 resulting in a p.Asn59GlyfsX12 mutation (Fig. 1).

In Proband 5 with the c.179-2A > G splice site mutation, PCR amplification of PMP22 complementary DNA using primers EX1Fw and EX4Rv produced the wild-type 451 base pair band plus a 313 base pair extra band; direct sequencing of the smaller fragment demonstrated the skipping of the entire exon 3, leading to an in-frame p.Glu60_Ala106del (Fig. 2). The predicted mutant protein would be shorter (113 amino acids) than the wild-type (160 amino acids). Kyte-Doolittle hydropathy plots suggested the deletion of the second transmembrane domain, the intracellular loop and the third transmembrane domain (Supplementary Fig. 1).

With quantitative reverse-transcriptase PCR, the fold-difference in the levels of the normalized PMP22 exons 1-2 relative to Control 1 was 0.49 ± 0.05 (median ± standard deviation) in Proband 2 and 1.39 ± 0.09 in Proband 5. The mean ratio between the levels of PMP22 exons 1-2 and exon 3 (wild-type + mutant/wild-type) was similar in controls and Patient 2, whereas it was significantly increased in Patient 5 (P < 0.01 Student's t-test) (Fig. 3). These experiments indicated that PMP22 transcripts from nerve biopsies contained essentially the wild-type messenger RNA in Proband 2 and both the wild-type and mutant messenger RNA in Proband 5.

Clinical phenotypes

Clinical findings in the probands and examined relatives are reported below. Table 2 summarizes the available neurophysiological records in the probands.

Patient 1, p.Thr44LeufsX177

A 16-year-old female with positive family history suffered from bilateral positional acroparesthesias in the fourth and fifth fingers. At 14 years of age, following appendicectomy, she presented drop of right foot and toe, which cleared up in 3 months. Examination revealed mild bilateral pes cavus, difficult gait on heels, mild right
The 49-year-old mother, carrying the same p.Thr44LeufsX177 mutation, had undergone surgical decompression for bilateral carpal tunnel syndrome at age 45 years.

Patient 2, p.Asn59GlyfsX12

A 35-year-old male clerk without apparent family history for neuromuscular disorders presented an acute right radial neuritis after knee arthroscopy and walking on crutches. He had a drop of right fingers and wrist, mild sensory loss over the dorsum of thumb and index and absent brachioradialis reflex. Neurophysiology disclosed a diffuse mild decrease of sensory nerve conduction velocities and bilateral slowing of motor nerve conduction velocities of median nerves at wrists and ulnar nerves at elbows (records not available).

Figure 2 The c.179-2A > G mutation detected in Patient 5 causes the skipping of PMP22 exon 3. (A) Diagrams of normal (wild-type) and aberrant RNA splicing. Lowercase letters indicate nucleotides at the end of intron 2; capital letters indicate nucleotides of exon 3; the wild-type ag and mutant gg splice acceptor site are bold typed. (B) Direct nucleotide sequencing of the mutant complementary DNA demonstrated the skipping of exon 3 resulting into the in-frame deletion of 47 amino acids (p.Glu60_Ala106del); Gly60 in the mutant protein corresponds to Gly107 in the wild-type protein.

Figure 3 Expression levels of PMP22 exons 1-2 relative to PMP22 exon 3 in nerve biopsies from Controls 1 and 2 and Patients 2 and 5. Values were calculated using the relative standard curve method (*P < 0.01 versus controls; Student’s t-test).
Table 2 Records of electroneurography

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at examination (years)</th>
<th>Age at onset (years)</th>
<th>Ulnar</th>
<th>Median</th>
<th>Peroneal</th>
<th>Sensory Median</th>
<th>Sural</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>DML</td>
<td>CMAP</td>
<td>CV</td>
<td>CB DML CMAP</td>
<td>CV</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Below elbow</td>
<td>Above elbow</td>
<td>Below elbow</td>
<td>Above elbow</td>
<td>Below</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>14</td>
<td>3.4</td>
<td>16.8</td>
<td>44.6</td>
<td>30.5</td>
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<tr>
<td>3</td>
<td>17</td>
<td>12</td>
<td>3.7</td>
<td>1.8</td>
<td>41</td>
<td>21</td>
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<td>14</td>
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<td>16.7</td>
<td>56.4</td>
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</tr>
<tr>
<td>5</td>
<td>41</td>
<td>31</td>
<td>4.3</td>
<td>4.7</td>
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<td>35</td>
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<td>14</td>
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<td>6</td>
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<td>7</td>
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<td>26</td>
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<td>12.8</td>
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<tr>
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<td>22</td>
<td>14</td>
<td>3.4</td>
<td>N.A.</td>
<td>30</td>
<td>28</td>
<td>– 7.5</td>
</tr>
</tbody>
</table>

Reported data refers to the most affected limb. Patients 2, 9 and 10 had no records available. Conduction block was defined by 50% reduction in amplitude of CMAP obtained by proximal stimulation compared with CMAP obtained from distal stimulation. Pathological records are highlighted in bold. CB = conduction block; CMAP = compound motor action potential; CV = conduction velocity; DL = distal latency; DML = distal motor latency; N.A. = records not available; SAP = sensory action potential.

Patient 3, p.Asn59GlyfsX12
A 17-year-old male with negative family history, after weight lifting, presented an acute painless drop of left wrist lasting 2 weeks. At 12 years of age, on awakening, he had experienced a right arm palsy lasting for 3 days. The proband presented winged scapula, mild weakness of left plantar dorsiflexion (with Achilles tendon hyporeflexia) and of right deltoid, supraspinatus and infraspinatus muscles. Parents were healthy and clinically normal but the 48-year-old father, carrier of the p.Asn59GlyfsX12 mutation, had mild bilateral slowing of peroneal motor nerve conduction velocities.

Patient 4, p.Asn59GlyfsX12
A 15-year-old male suffered from an acute painless left foot drop at age 14 years. He had difficult gait on heels and mild weakness of feet dorsiflexion with preservation of all deep tendon reflexes. The proband’s mother, carrier of the p.Asn59GlyfsX12, reported positional acroparesthesias in the legs and peroneal atrophy, mildly ataxic and step-and-glove distribution; deep tendon areflexia in the lower limbs.

Patient 5, p.Glu60_Ala106del
A 41-year-old male lorry driver with negative family history suffered from distal paresthesias in all four limbs. Since 31 years of age he complained of recurrent right sciatica and cervical pain with radiation to the arm; MRI demonstrated disc protrusions at the C5-C6 level. Examination disclosed mild bilateral pes cavus, mildly ataxic gait impossible on heels; bilateral weakness (MRC scale = 4) in fingers flexion and extension, in toes extension and plantar dorsiflexion (MRC scale = 3); mild weakness of hand interosseous muscles; multimodal sensory loss with stock-and-glove distribution; deep tendon areflexia in the lower limbs.

Patient 6, p.Arg95GlnfsX127
A 14-year-old female presented a palsy of left suprascapular nerve with impossible abduction of the arm, lasting for 3 weeks. Examination also revealed mild bilateral pes cavus, difficult gait on heels and diffusely decreased deep tendon reflexes. Both parents were clinically and neurophysiologically normal and did not carry the p.Arg95GlnfsX127 mutation. Paternity test was confirmatory.

Patient 7, p.Ile116ThrfsX4
A 41-year-old male cook, a heterozygous carrier of haemochromatosis without overt family history of neuromuscular disorders, complained of recurrent and transient numbness of right upper limb and diffuse acroparesthesias. At 26 years of age, while driving, he had presented an acute palsy in left arm abduction lasting for 3 weeks; a brain CT scan had been negative. At age 41 years, after exclusion of the 17p12 PMP22 deletion, he was diagnosed with chronic inflammatory demyelinating polyradiculoneuropathy because of neurophysiological findings and mildly elevated protein levels in cerebrospinal fluid (60 mg/dl); he was treated unsuccessfully with intravenous immunoglobulins and oral prednisone. Cervical MRI revealed a disc herniation at the C5-C6 level. Examination disclosed mild bilateral pes cavus, mildly ataxic gait impossible on heels; bilateral weakness (MRC scale = 4) in fingers flexion and extension, in toes extension and plantar dorsiflexion (MRC scale = 4); multimodal sensory loss with stock-and-glove distribution; deep tendon areflexia in the lower limbs.

After molecular diagnosis, the proband’s brother, a 37-year-old male prison officer was also shown to carry the p.Arg95GlnfsX127 mutation. Examination also revealed mild bilateral pes cavus, difficult gait on heels and diffusely decreased deep tendon reflexes. Both parents were clinically and neurophysiologically normal and did not carry the p.Arg95GlnfsX127 mutation. Paternity test was confirmatory.
mutation. He referred a 3-year history of paresthesias and episodic numbness in his right hand especially when driving. Neurological examination was normal. Motor and sensory nerve conduction velocity studies in the four limbs were consistent only with an entrapment neuropathy of right median nerve at wrist. At right median nerve motor nerve conduction velocities were 42.5 m/s, distal motor latencies 6.2 ms and compound motor action potential 13.5 mV. At left ulnar nerve motor nerve conduction velocity was 57.5 m/s below elbow and 45.8 above elbow; compound motor action potential was 15.7 mV and distal motor latencies 2.6 mV.

**Patient 8, p.Trp124X**

A 22-year-old male mason with positive family history (four relatives in the paternal line reported with similar disorders) complained of stumbling gait as an outcome of an acute right foot drop presented at age 21 and only partially recovered. At 14 years of age he suffered from acute painless weakness in abduction and external rotation of right shoulder and recovered in ~6 weeks; at age 18 he had had a prolonged (3 months) right foot drop, experienced after a prolonged squatting, and recovered after 3 months. Examination revealed bilateral pes cavus with hammer-toes; weakness of right supraspinatus and infraspinatus muscles, as well as decreased light touch sense in the fourth and fifth fingers in the left side and diffuse areflexia. Neurophysiological tests were reported as consistent with a multifocal demyelinating motor-sensory polyneuropathy (data not available). The 50-year-old mother had decreased vibration sense with stocking and glove distribution and diffuse hyporeflexia in the upper limbs.

**Pedigree 9, p.Leu145ArgfsX9**

A 19-year-old female with positive family history presented an acute painless right wrist drop. After 1 month from onset she disclosed severe weakness of fingers’ extension, pes cavus, decreased vibration sense with stock and glove distribution and generalized areflexia. Neurophysiological tests were reported as consistent with a multifocal demyelinating motor-sensory polyneuropathy (data not available). The 50-year-old mother had experienced similar disturbances; the 24-year-old brother was affected by left carpal tunnel and Guyon’s canal syndromes.

**Patient 10, p.Leu148GlnfsX72**

A 28-year-old male with negative family history presented an acute palsy of left axillary nerve with weakness in arm abduction lasting several months. At 27 years of age he experienced a left hand drop, which resolved in a few days, and at age 15 prolonged weakness of left forearm flexion. Neurological examination revealed weakness of finger extensors (MRC scale = 4) and of right foot and toe dorsiflexion (MRC scale = 4) with mildly stepping gait; Achilles tendons areflexia and diffuse hyporeflexia in the upper limbs.

**Pathological phenotypes**

In Probands 2 and 8 (Fig. 4A and B), sural nerve biopsies disclosed focal myelin thickenings with 40–250 μm length and a 10–40 μm diameter that occurred in ~25% of internodes; the results fitted the criteria of typical tomaculous neuropathy (Behse et al., 1972). The myelinated fibre density was 6157/mm² in Patient 2 and 6493/mm² in Patient 8 (range in four age-matched controls = 6300–7100/mm² of fascicular area). Histograms showed a relatively preserved bimodal spectrum and percentages of myelinated fibres with diameters >8 μm were 18.3 and 21.1%, respectively. No onion bulbs were detectable in either case. On teased fibres from Patients 2 and 8, tomacula were present in 23 and 27% of internodes.

In Proband 6 (Fig. 4C and D), the myelinated fibres density was slightly reduced (5568/mm²); 10.7% of fibres had a diameter >8 μm with a unimodal histogram. Several fibres had thin myelin sheaths and few were surrounded by simple onion bulbs. On teased fibres, tomacula occurred in 2.8% of internodes whereas non-tomaculous globoid or fusiform myelin thickenings were present in 7% of internodes.

Proband 5 (Fig. 4E and F) disclosed a mild loss of fibres (4204/mm²; 6.57% with diameter >8 μm) and prevalently simple onion bulbs; most fibres had thin myelin sheaths and some had no myelin. On teased fibres, many internodes were thinly remyelinated and 14.5% of them disclosed paranodal or internodal segmental demyelination; 0.43% of internodes had non-tomaculous globoid or fusiform myelin thickenings.

**Discussion**

By reporting 10 unrelated patients with eight different micromutations of PMP22 we emphasized the aetiological role of PMP22 in determining a variable spectrum of demyelinating neuropathies that may include clinically and pathologically atypical hereditary demyelinating neuropathies. Transcriptional analysis in selected sural nerve biopsies provided molecular clues to phenotypical variability.

Detected mutations caused alternatively premature stop codons, delayed stop codons or aberrant splicing; to our knowledge, four variants were previously unreported (Table 1). Lack of missense mutations in the present series may be related to the relative advanced age of patients (mean age 42 years), absence of Dejerine–Sottas syndrome and prevalence of HNPP cases in the series analysed. Seven cases were familial whereas three were sporadic; among the sporadic cases, the singleton Patient 6 with a c.281_282insG mutation (p.Arg95GlnfsX127) was definitely a de novo case. Nine probands (Patients 1–4, 6–10) had HNPP presenting acute, sometimes recurrent, focal neuropathies; one proband (Patient 5) manifested a CMT1-like syndrome of peroneal muscular atrophy.

With regard to HNPP probands, neurophysiology fitted to the clinical phenotype showing mild slowing of nerve conduction, prolonged distal motor latencies and focal slowing of nerve conduction velocities at common entrapment sites. Sural nerve biopsies were performed in three patients: whereas two had a tomaculous neuropathy indistinguishable from the one associated with the common PMP22 deletion, the third proband, Patient 6 with p.Arg95GlnfsX127, had an atypical pathology with a prevalence of irregular myelin thickenings that did not fit the criteria of tomacula. The HNPP phenotype was conveyed by mutations that introduced premature stop codons as well as delayed stop codons without any obvious differences in clinical or neurophysiological presentations.

Among mutations causing a delayed stop codon, p.Arg95GlnfsX127 was reported previously as p.Gly94fsX222 in
several families of German and Dutch ancestry (Young et al., 1997; Lenssen et al., 1998), and correlated with a more severe HNPP/CMT1 overlap disease characterized by accentuated involvement of peroneal muscles, marked slowing of nerve conduction velocities and by numerous *tomacula* with reduced fibre densities and loss of large diameter fibres and frequent onion bulbs (Lenssen et al., 1998). On the other hand, Patient 6 with p.Arg95GlnfsX127 disclosed a typical clinical and electrophysiological HNPP associated with very few *tomacula* and a relatively preserved fibre density. By conceptual translation, p.Arg95GlnfsX127 is 61 residues longer than the wild-type and lacks the normal C-terminal part; when expressed *in vitro* it does not target the plasma membrane, interferes with the trafficking of wild-type *PMP22* and forms perinuclear aggregates (Johnson et al., 2005). Although such a toxic gain-of-function *in vitro* correlated tentatively with a particularly severe HNPP (Lenssen et al., 1998; Johnson et al., 2005), no transcriptional studies were performed in nerve biopsies to check whether the mutant is stable and possibly translated or, vice versa, unstable and degraded by mechanisms of messenger RNA quality control (Isken and Maquat, 2007). Such studies would be necessary to understand the functional consequences of other mutations with premature stop codons that were similarly associated with more severe CMT1-like (Abe et al., 2004), or even Dejerine–Sottas syndrome-like phenotypes (Ionasescu et al., 1997).

HNPP mutations reported here included a deletion of 12 nucleotides spanning the 5’-end of exon 2 and the splice donor site of intron 2 (c.174_178 + 7delAAACGGTGAGGC) that led to a premature termination codon (p.Asn59GlyfsX12) as indicated by the mutant transcript detected in the available nerve sample from Patient 2. However, because quantitative reverse-transcriptase PCR in the nerve biopsy from Patient 2 demonstrated that *PMP22* messenger RNA was represented mainly by the wild-type transcript, the c.174_178 + 7delAAACGGTGAGGC is probably a null allele whose messenger RNA is largely degraded by nonsense-mediated messenger RNA decay.

The syndrome of peroneal muscular atrophy in Patient 5 correlated to a c.179-2A > G splicing mutation. Indeed, both Proband 5 and his cousin were affected by peroneal muscular atrophy without recurrent palsies, associated with homogenously slowed nerve conduction velocities, in the CMT1 range, and prolonged distal motor latencies. The proband’s nerve biopsy disclosed a de-remyelinating neuropathy with prevalently simple onion bulbs of myelinated fibres. One *tomacula* is present (asterisk) and few fibres are surrounded by irregular thickenings of the myelin sheath (arrowheads). Occasional remyelinating fibres are surrounded by flattened processes of the Schwann cells (arrows). (D) Irregularly thickened myelin sheaths (arrowheads), one *tomacula* (asterisk) and segmental de-remyelination (greater than symbol). (E) Mild loss of fibres, prevalence of thinly myelinated fibres, occasional naked axons (dash), and simple onion bulbs around remyelinated fibres (arrows). (F) Diffuse segmental and paranodal de-remyelination; a non-tomaculous fusiform myelin thickening (arrowhead). Original magnification: A, C and E = × 40; B, D and F = × 10.
and without an increase of the total fascicular area or of the Schwann-cell nuclei number (Fig. 4E and F). This pathological picture was also unique differing from the hypertrophic de-remyelinating neuropathy of CMT1A as well as from the HNPP-associated tomaculous neuropathy. The correlation between c.178-2A > G and the detected phenotype is probably specific since the same mutation was already reported in two unrelated patients originating from different ethnic backgrounds and affected with a clinically and neurophysiologically defined CMT1 (Ekici et al., 2000; Park et al., 2006). Different splice-site mutations of PMP22 were previously associated with HNPP (Bort et al., 1997; Meuleman et al., 2001; Bellone et al., 2006) or CMT1A (Nelis et al., 1994); but, with the exception of a HNPP-associated c.179 + 1G > C that led to a premature stop codon (Bellone et al., 2006), they could not be verified at the transcriptional level. Here, sequencing of the mutant messenger RNA amplified in the proband’s nerve demonstrated that the A to G change in the acceptor splice site of IV2 caused the skipping of the entire exon 3 leading to an in-frame p.Glu60_Ala106del mutation. Interestingly, quantitative reverse-transcriptase PCR transcriptional analysis was consistent with a substantial stability of the mutant messenger RNA indicating that its level was similar to that of the wild-type messenger RNA in peripheral nerves. If translated, the p.Glu60_Ala106del protein would undergo the deletion of the second transmembrane, the intracellular and the third transmembrane domain as predicted by Kyte-Doolittle hydropathy plots. We hypothesize that the shorter and aberrant protein would not cause a mere loss-of-function; rather, it could behave similarly to CMT1A-associated missense mutants.

In conclusion, the report demonstrates that micromutations of PMP22 may have variable effects on the sequence and expression levels of mutant transcripts and on clinical and pathological expression. Furthermore, similarly to the 17p12 deletion (Reilly and Shy, 2009), PMP22 micromutations disclosed remarkable intrafamilial variability in the present series; indeed, affected pedigrees’ members could manifest acute palsies, present minor symptoms of entrapment neuropathies or signs of chronic polyneuropathy, or could be asymptomatic in adulthood with only minor changes of nerve conduction velocities. Lack of a positive family history due to de novo origin, reduced penetrance or phenotypical variability of PMP22 micromutations, together with the absence of the common 17p12 deletion or duplication, may lead to misdiagnosing HNPP with acquired neuropathies such as, for example, chronic inflammatory demyelinating polyradiculoneuropathy (Patient 7). Thus, mutational scanning of PMP22 should not be overlooked in selected demyelinating neuropathies.

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Supplementary material

Supplementary material is available at Brain online.

References


