Clinically Distinct Codon 69 Mutations in Major Myelin Protein Zero in Demyelinating Neuropathies

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Mutations in the major peripheral myelin protein zero (P0) gene on chromosome 1q21-q23 have been found with the hereditary demyelinating polyneuropathy Charcot-Marie-Tooth type 1B. Here, we describe 2 patients with distinct neurological characteristics, carrying different substitutions at the same codon—Arg69His and Arg69Cys. The patients were heterozygous for the mutation, which in both appeared to be de novo. Histological examination of sural nerve biopsy specimens revealed defective myelin as well as marked differences, confirming the importance of P0 in the compaction of myelin.


Charcot-Marie-Tooth type 1 (CMT1 or hereditary motor and sensory neuropathy [HMSN] type I) is the most frequently inherited, autosomal dominant, demyelinating polyneuropathy and appears to be genetically heterogeneous [1]. The locus for CMT1A, the most common form, maps to chromosome 17p11.2 and is usually associated with a DNA duplication of 1.5 Mb encompassing the peripheral myelin protein 22 (PMP22) gene. Single-base mutations in the PMP22 gene have been reported in a few nonduplicated CMT1A cases [1, 2]. Alterations in the major peripheral myelin protein zero (P0) gene, localized on chromosome 1q21-q23, have been shown to cause CMT1B [1, 3] and have also been found in patients with sporadic early-onset disease with a more severe phenotype, also known as Dejerine-Sottas syndrome [4]. P0 is a 28-kd integral membrane glycoprotein encoded by 6 exons. It belongs to the immunoglobulin superfamily, having a single extracellular domain resembling the immunoglobulin variable domain. In addition, P0 contains single transmembrane and cytoplasmic domains [5]. A role for P0 in myelin formation has been demonstrated by the analysis of mice lacking P0 expression due to targeted mutagenesis [6]. Since the extracellular domain (encoded by exons 2 and 3) is capable of homophilic interaction, P0 presumably plays a role in the compaction of peripheral myelin [7].

We performed a mutation screening of the P0 gene in unrelated CMT patients, already characterized as non-CMT1A, by single-stranded conformational analysis (SSCA) followed by sequencing of the relevant DNA region. To study the effect of P0 mutations on myelin compaction, we also performed electron microscopic examinations on sural nerve biopsy specimens from the patients.

Materials and Methods

Patients
CMT1 patients were identified on the basis of neurological abnormalities and markedly reduced nerve conduction. Patients had already been analyzed for the presence of the CMT1A duplication and screened for mutations in the PMP22 gene. With informed consent, sural nerve biopsy samples were taken from the patients and examined by light and electron microscopy.

Patient 1, a 28-year-old woman, had normal early development. From the age of 2 years onward, motor performances slowly deteriorated. Neurological examination at age 12 years revealed pes cavus, distal muscular weakness and atrophy of the lower legs and the hands, slight distal sensory loss, and nearly absent tendon reflexes. Motor nerve conduction velocity (MNCV) of the median nerve was 19 m/sec. Sural nerve biopsy was performed. In the following years her condition hardly deteriorated. The patient carried a Arg69His substitution in exon 3 of the P0 gene (see Results).

Patient 2, a girl, had normal development until the age of 6 months. Motor development then stopped and she deteriorated after the age of 10 months. At 14 months, she had an extreme head lag, had difficulties coughing, choked, and cried without sound. She did not roll over nor did she push herself up anymore. The arms and legs were weak and flaccid with areflexia. Sural nerve biopsy was performed. She died at the age of 22 months. MNCV in the ulnar nerve was 8.5 m/sec. She carried a Arg69Cys substitution in exon 3 of the P0 gene (see Results).

Mutation Detection
Genomic DNA was isolated from leukocytes according to standard procedures. Primers were chosen according to pub-
lished P0 exonic and intronic sequences [5]: primers (5'-3')—P0ex2R1 CTGAGACCCACTCAGGAG; P0ex2F2 AGGTCCATGGTGCGTGG; P0ex2R2 TTATCCAACCCAGGATTCC; P0ex3F1 AGGTCCATGCTCAGATCC; P0ex3R1 AGTTGTGTATGCAATGCG; and P0ex3F2 TGGCTCATTGCTCAGACA. Primers A to F described by Hayasaka and colleagues [8] were also used. SSCAs were carried out essentially as described by Nicholson and coauthors [9]. For analysis of the separate alleles, the SSCA fragments were cut out of the gel and eluted in 100 μl of sterile water. After three cycles of freezing (liquid nitrogen) and thawing (65°C), 5 μl was used in a 50-μl standard polymerase chain reaction (PCR) for sequencing. PCR products for sequencing were also generated from genomic DNA (100 ng). Products were column purified (Qiagen) and 50 ng was directly sequenced using the dideoxy chain termination method with the Sequitherm Cycle Sequencing Kit (Epicentre Technologies) and 32P-end-labeled P0 primers. Samples were electrophoresed on 7% denaturing polyacrylamide gels.

Nucleotide numbering of P0 starts at the initiation site of translation. Amino acid numbering starts at the first amino acid (Ile) of the mature P0 protein (without the 29 amino acids from the signal peptide) [10].

**Light and Electron Microscopy**

Sural nerve biopsy specimens were prepared for light and electron microscopic examination including morphometric analysis. For ultrastructural analysis nerve specimens were fixed immediately in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4°C for 3 hours and postfixed in 2% osmium tetroxide, pH 7.4, for 1 hour, dehydrated in alcohol, and embedded in epoxy resin (Epon). More than 1,000 nerve biopsy specimens were analyzed this way, including from more than 100 patients with HMSN [11].

**Results**

**Mutations**

We analyzed the P0 gene for the presence of mutations using SSCA and direct sequencing. Patient 1 carried a G-to-A mutation at position 293, leading to an amino acid substitution of Arg to His at codon 69 (the same mutation was found in another CMT1B patient [12]). The Arg69His mutation appeared to be absent from exon 3 of both clinically normal parents, demonstrating the de novo origin of the substitution. Another de novo base change (C to T at position 292) was detected in the severely affected patient (Patient 2), resulting in an amino acid substitution of Arg by Cys at the same codon 69. The patients were heterozygous for the mutant and the normal allele. Paternity was confirmed for both patients. Furthermore, the entire coding region of the P0 gene of the patients was sequenced and revealed no further mutations.

**Light and Electron Microscopy**

Sural nerve biopsy specimens from both patients showed a marked reduction in density of myelinated fibers. Patient 1 (Fig 1) had 6,010 and Patient 2 7,200 fibers/mm², corresponding to 57% and 46% of the age-related values, respectively. In Patient 1, large-diameter fibers (> 6.5 μm) were totally lacking; most fibers were thinly myelinated and surrounded by onion bulbs of concentrically arranged Schwann cell lamellae. By ultrastructural examination of cross sections, the vast majority of fibers (80%) showed uncompacted myelin lamellae, which in some cases (> 12%) could not be discerned from a Schmidt-Lanterman incisure. The uncompacted structure was present over the whole or part of the circumference of the fiber and commonly involved the inner lamellae, but occasionally the outer lamellae as well. Dilatation of the major dense line was most pronounced. At some places, fusion of intraperiod lines had occurred (arrow). Bar = 1 μm.

![Electron microscopy of sural nerve specimen from Patient 1 (Arg69His), age 12 years. Uncompaction of myelin is seen at the inner and outer parts of the myelin sheath. Dilatation of major dense lines is most pronounced. At some places, fusion of intraperiod lines has occurred (arrow). Bar = 1 μm.](image)
Discussion

PO, the major structural component of peripheral nervous system myelin, is an integral membrane protein and its expression is confined to myelinating Schwann cells. We screened genomic DNA of CMT1 patients for mutations in the coding part of the P0 gene and demonstrated mutations in both patients. In contrast to Patient 1, Patient 2 was diagnosed as severely affected with CMT1. Surprisingly, the same exon 3 codon in both patients was substituted with different amino acids—Arg69His (Patient 1) and Arg69Cys (Patient 2). This amino acid is highly conserved among many species [10], suggesting that Arg69 plays an important role in formation and maintenance of the myelin sheath. Interestingly, the severely affected CMT1B patient described by Hayasaka and colleagues [4] carried a Ser34Cys substitution, suggesting a more dramatic effect on the function of P0 when cysteine is substituted at certain amino acid positions. Thus far, the majority of disease-associated amino acid substitutions in CMT1B patients have been found in the extracellular domain of P0 [1].

Direct binding experiments have shown a major role for the P0 extracellular domain in the compaction of peripheral myelin [7]. The P0 extracellular domain contains a nonasaccharide that is N-linked to Asn93 and two cysteines at positions 21 and 98. Both P0 molecules in the homophilic pair have to be glycosylated for adhesion to take place [13]. The cysteines form a disulfide bond that is essential to the functioning of P0 as an adhesion molecule [14]. The cytoplasmic domain of P0 is thought to interact with a component of the opposing membrane in compact myelin, thereby holding these membranes together at the major dense line [15].

Recently, a molecular model of the P0 extracellular domain was described [16]. According to this model, Arg69 is postulated to be on putative β-strand 6 of the P0 extracellular domain. This residue is predicted to be essential for complementary electrostatic interactions from apposed membranes and may also be involved in the protein-carbohydrate interaction at the base of the molecule [17]. Substitution of this residue with cysteine probably results in inappropriate formation of either intramolecular or intermolecular disulfide bonds, whereas Arg69His most likely leads to disrupted P0-P0 interactions through steric hindrance or changes in electrostatic interactions, or both, all leading to defective myelin [17]. On the other hand, both mutations also might lead to deleterious conformational changes or disrupt the P0 extracellular domain—carbohydrate link.

In support of this hypothesis, the histological examinations of sural nerve biopsy specimens showed defective compaction of myelin (see Fig). Although in several instances the uncompacted myelin structure might be ascribed to Schmidt-Lanterman incisures, the overall frequency on cross sections (80% in Patient 1, 43% in Patient 2) markedly surpassed the frequency of Schmidt-Lanterman incisures in normal sural nerves (0–5.5%, n = 3) or in nerves from patients with genetically unspecified, demyelinating HMSN (≤ 9%, n = 9) [18]. In both patients wide spacing of myelin layers was seen most frequently at the inner layers of the sheath. Patient 2 with Arg69Cys showed an increase in neurofilament density of demyelinated axons, a feature sometimes seen in demyelinated axons in human or animal pathology [11, 19]. Family analysis showed that both Arg69 substitutions were de novo mutations, underlining the role of P0 in the pathogenesis of CMT1B.


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Cognitive and Brain Magnetic Resonance Imaging Findings in Adrenomyeloneuropathy

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Neuropsychological functioning and brain magnetic resonance imaging (MRI) were evaluated in 84 men with adrenomyeloneuropathy (AMN). MRI was normal in 61%, the “pure AMN” group, while 39%, the “cerebral AMN” group, showed brain white matter abnormalities. Except for mild deficits in psychomotor speed and visual memory, neuropsychological function was normal in pure AMN. Most patients with cerebral AMN had normal IQ and language but evidenced impaired psychomotor speed, spatial cognition, memory, and executive functions. Patients with MRI evidence of very severe cerebral disease had global and language impairment as well, and deficits in all patients were highly correlated with degree of brain MRI involvement.

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X-linked adrenoleukodystrophy (ALD) [1] presents in childhood as a rapidly dementing illness with a characteristic pattern of demyelination demonstrable by magnetic resonance imaging (MRI) [2]. In adulthood, it presents most commonly as a slowly progressive paraparesis associated with degeneration of the long tracts in the spinal cord [2], which is referred to as adrenomyeloneuropathy (AMN). While, in comparison with childhood ALD cerebral function in AMN is well preserved, we have shown previously that some AMN patients develop subcortical dementia [3], and 45% have white matter abnormalities demonstrable by MRI [4]. This report correlates neuropsychological function and brain MRI in AMN patients.

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