Charcot-Marie-Tooth disease and related peripheral neuropathies: the role of genetic testing

Ph.D. Thesis

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INTRODUCTION

Hereditary motor and sensory neuropathies or Charcot-Marie-Tooth (CMT) disease and related neuropathies represent a heterogeneous group of hereditary disorders (Nelis, et al., 1999) (Boerkoel and Lupski, 2002) of the peripheral nervous system (PNS) with an estimated frequency of 1 in 2500 individuals (Skre, 1974). CMT is characterized by slowly progressive length dependent neuropathy manifesting as distal weakness of the legs progressing proximally, followed, in some cases, by hand involvement. Based on motor nerve conduction velocities (NCVs), two major types can be distinguished – type 1 or the demyelinating (CMT1) form (note: refers to all demyelinating forms independent of inheritance pattern), that is characterized by symmetrically slowed NCV (usually < 38 m/s; normal is > 45 m/s) and type 2 or the axonal (CMT2) (all axonal independent of inheritance pattern) form associated with normal or subnormal NCVs and reduced compound muscle action potential (Dyck and Gomez, 1968). Pathologic studies of patients with myelinopathies show segmental demyelination and remyelination, i.e., "onion bulb" formation (Chou, 1992) whereas studies of patients with axonopathies show normal myelin but fewer nerve fibers. Both CMT1 and CMT2 can be inherited as either an autosomal dominant (AD), autosomal recessive (AR) or X-linked (XL) trait, but often present as sporadic neuropathy (Figure 1).

During the last decade an enormous amount of information regarding peripheral nerve function and dysfunction has been obtained through the identification of genes responsible for disease in patients manifesting inherited peripheral neuropathies generating a complex classification based on the locus or gene involved (Table 1). However, in some instances mutations in the same genes can cause both demyelinating and axonal neuropathy, the same mutations can have variable disease onset even in the same family, and the inheritance pattern varies whether the same gene has a dominant negative or a loss of function mutation (Warner, et al., 1999b). Thus this classification will need revision in the near future, in the text CMT1 and CMT2 refers to demyelinating and axonal CMT, respectively, independent of inheritance pattern. Some of these genes/mutations contribute to a significant fraction of inherited peripheral neuropathy cases and thus molecular analyses can play a substantial role in establishing a precise and accurate etiological diagnosis, while other genes may be involved in only a small minority of patients.

DISEASES PHENOTYPES

Charcot-Marie-Tooth disease (CMT; MIM 118200, 118220): Clinical symptoms most frequently appear in the first or second decade of life, including tripping, followed by difficulties with heel-walking. Weakness of the peroneal muscles impairs foot dorsiflexion and eversion during gait, for which patients compensate by flexing the hip and knee with each step producing the steppage gait. To achieve better stroke patients flex their toes, which with time become rigid (*hammer toes).* Foot deformities such as *pes cavus* and *heel varus* occur late. In most cases weakness and wasting of the intrinsic hand muscles occurs late in the course of the disease but is not usually related to the degree of leg weakness or atrophy and is also not related to the age of the patient. The thumb lies flat in the plane of the hand instead of opposing the other fingers giving the appearance of *claw hand.* Decreased or absent ankle reflexes are virtually universal and most patients are areflexic. Mild sensory loss can be detected in approximately 70% of the cases. Patients rarely loose the ability to ambulate. Slow nerve conduction velocities (NCVs), as seen in the demyelinating form or CMT1, differentiate this form from the axonal or neuronal type or CMT2 in which the NCVs are normal or slightly slow, with reduced amplitudes. Approximately 60-70% of patients have CMT1 while about 20-40% is diagnosed with CMT2. Restless leg syndrome occurs in nearly 40% of CMT2 patients (Gemignani, et al., 1999) but is rare in CMT1 cases. Pathologic studies of patients with myelinopathies show segmental demyelination and myelin sheath hypertrophy, i.e., "onion bulb" formation, whereas studies of patients with axonopathies show normal myelin but fewer nerve fibers (Chou, 1992) (Low, et al., 1978).

Hereditary neuropathy with liability to pressure palsies (HNPP, MIM 162500): The clinical phenotype is characterized by recurrent episodes of nerve palsies at compression sites (Davies, 1954) (Windebank, 1993). The symptoms are transient numbness or weakness lasting hours to weeks. Approximately 64-78 % of patients present with acute painless mononeuropathy or brachial plexopathy (Pareyson, et al., 1996). After multiple attacks the recovery may be incomplete due to permanent nerve damage. The repeated demyelination and remyelination results in sausage-like thickening of the myelin sheath (tomacula) (Behse, et al., 1972). The neurological impairment is usually milder than in CMT1 but can become similar after the fifth decade. Electrophysiological findings include conduction blocks and mildly slow motor conduction velocities during exacerbations (Behse, et al., 1972).

Dejerine-Sottas neuropathy (DSN, MIM 145900): DSN was originally described as an interstitial hypertrophic neuropathy of infancy. The patients present with motor developmental delay, hypotonia and areflexia (Benstead, et al., 1990; Ouvrier, et al., 1987). Many patients walk eventually, although walking is often delayed beyond the second year. Probably because of proprioceptive deficits, all patients have ataxia and abnormal coordination. Virtually all patients are areflexic. Beginning with the distal musculature of the lower extremities, patients develop progressive muscular atrophy and weakness. By the second decade of life, nearly all patients have developed proximal weakness in addition to distal weakness and moderate to severe sensory deficits. Although some patients deteriorate over the first two decades of life and eventually die from respiratory complications, others have a relatively mild disease course except for progression of limb deformities and scoliosis (Ouvrier, et al., 1987) (Tyson, et al., 1997). Nerve conduction velocities show marked slowing (< 6-12 m/s) in a uniform pattern affecting all nerves and nerve segments (Ouvrier, et al., 1987) (Benstead, et al., 1990). The hypertrophic superficial nerves may be visible and palpable. Patients with DSN have the neuropathological features of both de-remyelination (onion bulb) and hypomyelination (thin myelin sheets) (Gabreels-Festen and Gabreels, 1993) (Ouvrier, et al., 1987).

Congenital hypomyelinating neuropathy (CHN, MIM 605253): CHN is the most severe form of the hereditary motor and sensory neuropathies. The clinical features are present at birth with marked hypotonia, areflexia and distal muscle weakness (Kasman, et al., 1976) (Boylan, et al., 1992). In some cases decreased fetal movements and arthrogryposis multiplex congenita have been described (Seitz, et al., 1986) (Boylan, et al., 1992). The NCVs are usually less than 6-12 m/s. Clinical distinction between CHN and DSN is often difficult, as frequently the first measurable clinical sign is motor development delay. The diagnosis of CHN is established by nerve biopsy showing hypomyelination with only a few thin myelin lamellas left without active myelin breakdown products and early onion bulb formations.

Roussy-Levy Syndrome (RLS, MIM 180800): RLS combines the CMT1 phenotype with marked tremor and sensory ataxia (Warner, et al., 1999a) (Harding and Thomas, 1980b). Symptoms include early *pes cavus*, distal leg muscle weakness and atrophy, distal sensory loss and gait ataxia. As the full phenotypic spectrum of CMT has been defined, however, tremor and sensory ataxia have been clearly identified as common, though variable features of CMT (Garcia, 1999; Harding and Thomas, 1980a). Therefore, although initially considered a 'forme

fruste' of Friedreich ataxia, RLS is a CMT variant. Genetic testing confirms this clinical impression. The original family described by Roussy and Lévy segregates an *MPZ* mutation (Plante-Bordeneuve, et al., 1999), and RLS has also been associated with the *PMP22* duplication and point mutations in *GJB1* (Thomas, et al., 1997) (Senderek, et al., 1999). RLS has never been associated with mutations in *FRDA*, the gene associated with Friedreich ataxia.

MODES OF INHERITENCE

CMT and related neuropathies exhibit all forms of mendelian inheritance – autosomal dominant (AD), autosomal recessive (AR) and X-linked (XL). AD-CMT1 is the most frequent pattern observed (Lupski and Garcia, 2001). Thirty-five linked loci (14 autosomal dominant, 12 autosomal recessive and 3 X-linked; although rarely some mutations in a gene isolated as a dominant locus may behave as a recessive allele in a given family) and twenty-five CMT associated genes have been identified (Figure 1, Table 1). HNPP and RLS show autosomal dominant inheritance whereas CHN is autosomal recessive or sporadic. DSN shows both AD and AR forms. Sporadic disease is often a result of new mutation and thus the absence of a family history does not preclude molecular genetic testing.

GENETICS

Mutations in genes encoding proteins required for proper development, maintenance or function of the peripheral nerve may result in neuropathy. Several of the disease genes initially identified encode (i) structural proteins that are important in myelination (e.g. *PMP22*, *MPZ*), (ii) proteins involved with radial transport through the myelin membrane sheath (e.g. *Cx32*), (iii) proteins involved with axonal transport (e.g. *NEFL*, *GAN1*), (iv) transcription factors associated with onset of myelination (*EGR2*), (v) members of signal transduction pathways (e.g. *PRX*, *MTMR2*, *SBF2*, *NDGR1*, *GDAP1*), (vi) mitochondrial function related protein (e.g. *MFN2*), (vii) endosome related proteins (*RAB7, SIMPLE*) and (viii) chaperones (*HSP22, HSP27*), (ix) a gene involved in DNA single strand break repair (*TDP1*) and (x) other genes (e.g. *LMNA*, *KCC3*, *GARS, DNM2*) whose peripheral nerve specific functions are less clearly established.

Genes associated with peripheral nerve structure:

Peripheral Myelin Protein 22 (PMP22): The first molecular event discovered, responsible for the majority of CMT, was the duplication of the chromosomal segment harboring *PMP22* (Lupski, et al., 1991) . This discovery introduced a novel molecular mechanism in human mutagenesis, non-allelic homologous recombination and defined a new group of disorders, the genomic disorders (Lupski, 1998) (Stankiewicz and Lupski, 2002). The reciprocal molecular event, deletion of the same fragment was looked for and found in HNPP (Chance, et al., 1993) (Chance, et al., 1994) . This molecular mechanism and the diseases gave substantial evidence for the presence of dosage sensitive genes in the human genome. PMP22 is an integral membrane protein of 160 amino acids with four transmembrane domains. The function of PMP22 is still unknown, however recent evidence shows, that PMP22 and P0 are involved in both trans-homophilic and trans-heterophilic interactions in cell culture based assay systems using retrovirally transduced HeLa cells (Hasse, et al., 2004). An extra copy of *PMP22*, due to the CMT1A duplication, is associated with CMT1 (Lupski, et al., 1991) (Raeymaekers, et al., 1991) (Patel, et al., 1992) and this single molecular event accounts for 70% of families with dominant CMT1 (Wise, et al., 1993) (Nelis, et al., 1996) and 76-90% of sporadic CMT1 (Nelis, et al., 1996) (Hoogendijk, et al., 1992). The CMT1A duplication is also associated with neuropathy in patients manifesting wide variations in clinical phenotypes such as DSN, RLS, calf hypertrophy and scapuloperoneal atrophy or Davidenkow syndrome (Lupski and Garcia, 2001). Deletion of *PMP22* leads to HNPP (Chance, et al., 1993). In one study (Tyson, et al., 1996), 50% of patients diagnosed with a multifocal neuropathy had the 17p11.2 deletion associated with HNPP. Thus, *PMP22* is a dosage-sensitive gene that when duplicated or deleted gives rise to demyelinating neuropathies. Point mutations in *PMP22* have been seen in CMT1, DSN and CHN phenotypes. (Valentijn, et al., 1992) (Simonati, et al., 1999) As anticipated, loss of function mutations (Nicholson, et al., 1994) including frameshift, nonsense and splice site mutant alleles result in HNPP because like the HNPP deletion, they effectively result in *PMP22* haploinsufficiency. Most of the missense mutations occur in the four predicted transmembrane domains.

Heterozygous *Pmp22* knockout mice display clinical manifestations comparable to HNPP (Adlkofer, et al., 1997). Transgenic mice and rats that overexpress PMP22 mimic the human disorder (Magyar, et al., 1996) (Sereda, et al., 1996). The finding that such mice also develop a pronounced distal axonopathy (Sancho, et al., 1999) led to a reappraisal of disease manifestation

in CMT1A and a careful examination of a cohort of such cases revealed that reduced compound motor and sensory nerve action potentials correlate with clinical disability, while motor NCV does not (Krajewski, et al., 2000). Consistent with this finding is an earlier observation that (Killian, et al., 1996) motor NCVs and clinical motor exam did not change significantly over a period of 22 years in eight CMT1A duplication cases.

PMP22 is expressed predominantly in the peripheral nervous system (Patel, et al., 1992) (Suter, et al., 1994). Most of the newly synthesized PMP22 is retained in the endoplasmic reticulum (ER), where it is degraded (Sancho, et al., 1999). Only a small percentage of PMP22 is transported from the ER to the Golgi where it undergoes complex glycosylation and becomes more stable. Axonal contact appears to stimulate the redistribution of PMP22 to the Schwann cell plasma membrane as myelination occurs (Pareek, et al., 1993). The ultrastructural pathology of the HNPP phenotype, tomacula formation and reduced myelin compaction (Yoshikawa and Dyck, 1991), suggests that *PMP22* plays a structural role in myelin formation and/or maintenance.

Strategies aimed at normalizing Pmp22 expression in transgenic mice have been encouraging (Perea, et al., 2001). When overexpression of *Pmp22* is switched off in adult mice, correction begins within 1 week and myelination is well advanced by 3 months (although the myelin sheaths are still thinner than normal), indicating that the Schwann cells are poised to start myelination.

***Myelin protein zero* (*MPZ*)**: The gene for *MPZ* maps to 1q22. MPZ protein contains a single membrane spanning region, a large hydrophobic glycosylated immunoglobin-like extracellular domain and a smaller basic intracellular domain (Lemke and Axel, 1985). MPZ is normally expressed exclusively by myelinating Schwann cells and accounts for 50% of the total PNS myelin protein (Lemke, 1988). About 85-90 different myelinopathy-associated *MPZ* mutations have been described (Fig. 2; http://molgen-www.uia.ac.be/CMTMutations/). These are mostly associated with CMT1 and sometimes DSN and CMT2 phenotypes. A few cases of CHN have also been reported to be associated with *MPZ* mutations (Warner, et al., 1996a) (Szigeti, et al., 2003). *In vitro* functional studies were able to demonstrate, that the *MPZ* truncating mutations associated with a more severe form of peripheral neuropathy result in premature stop codons

within the terminal or penultimate exons, thus escape nonsense mediated decay and are stably translated into mutant proteins (Inoue, et al., 2004).

The original Roussy-Levy family reported in 1926 has been shown to harbor a point mutation causing a missense amino acid substitution in the extracellular domain of *MPZ* (Plante-Bordeneuve, et al., 1999). Thus, mutations in *MPZ* show a clinically diverse spectrum of CMT and related phenotypes. A recent observation elegantly demonstrated how various truncating mutations in *MPZ* convey distinct neurological phemotypes, depending upon whether the truncated mRNA undergoes or escapes nonsense-mediated decay (Inoue, et al., 2004).

Knockout mice heterozygous for *Mpz* mimic the human CMT1 phenotype caused by *MPZ* mutations (Martini, et al., 1995). Homozygous *Mpz* knockout mice show severe hypomyelination with signs of non-compact myelin (Giese, et al., 1992) and pronounced loss of distal axons (Frei, et al., 1999).

Gene associated with transport through myelin:

***Connexin 32* (*Cx32*)**: The *Cx32* (*Gap junction B1*; *GJB1*) gene maps to Xq13.1. It consists of two exons and encodes a gap junction protein containing four transmembrane domains. A connexon (hemichannel) consists of six connexin subunits, and two connexons, each from one of the two apposing membranes, which form a functional channel that allows rapid transport of ions and small molecules (Bruzzone and Ressot, 1997). *Cx32* is expressed in myelinating Schwann cells and is localized to noncompact myelin in the paranode and Schmitt-Lanterman incisures consistent with its role in providing a radial diffusion pathway between the adaxonal and perinuclear cytoplasm of the Schwann cell (Neuhaus, et al., 1996) (Bergoffen, et al., 1993).

Over 250 different mutations have been described (http://molgen-www.uia.ac.be/CMTMutations/). These mutations occur throughout the entire *Cx32* protein and, unlike the *PMP22* and P_0 mutations, are not concentrated in transmembrane or extracellular domains. Mutations in *Cx32* account for nearly 10% of all CMT cases and are the most frequent cause of CMT after *PMP22* duplication. These mutations behave in a dominant fashion and represent 90% of CMTX. The nature of the neuropathy in CMTX, whether primarily axonal, demyelinating, or whether a mixed neuropathy, has been debated, but no consensus has yet been reached (Dubourg, et al., 2001) (Tabaraud, et al., 1999).

Cx32-deficient mice mimic the human CMT1X phenotype (Anzini, et al., 1997). These mice develop a slowly progressing demyelinating neuropathy, with enlarged periaxonal collars, abnormal non-compacted myelin domains and axonal sprouts (Kobsar, et al., 2002). This suggests that reflexive gap junctions may be required for myelin compaction; or, alternatively, *Cx32* may play a structural role in myelin compaction. Mice lacking *Cx32* show a distinct pattern of gene dysregulation in Schwann cells (Nicholson, et al., 2001) indicating that Schwann cell homeostasis is critically dependent on the correct expression of *Cx32*.

Genes associated with axonal transport:

***Neurofilament-light* (*NEFL*)**: *NEFL* encodes for one of the three subunits of neurofilaments which are the major type of intermediate filaments found in neurons. Mutations in *NEFL* have been identified in two independent families associated with autosomal dominant CMT2 (Mersiyanova, et al., 2000b) (De Jonghe, et al., 2001). Recent studies (Yoshihara, et al., 2002) (Jordanova, et al., 2003) have identified additional mutations in *NEFL* among CMT and DSN cases. In the latter study (Jordanova, et al., 2003), individuals harboring *NEFL* mutation had an early onset, severe CMT or DSN phenotype with moderate to severely reduced slowed NCVs. *In vitro* functional studies with mutated neurofilament light chain demonstrated defects in the assembly of intermediate filament networks, defective targeting of neurofilaments into processes and altered intracellular distribution of mitochondria, suggesting defective axonal transport as an underlying pathomechanism (Perez-Olle, et al., 2004). *Nefl* knockout mice develop normally and show no overt phenotype (Zhu, et al., 1997). In another transgenic model, a point mutation (Leu394Pro) causes massive, selective degeneration of spinal motor neurons accompanied by abnormal accumulations of neurofilaments and severe neurogenic atrophy of skeletal muscles (Lee, et al., 1994). Thus, both mouse mutants are not suitable models for human CMT2.

***Kinesin family member 1B* (*KIF1B*)**: *KIF1B*, a member of the kinesin superfamily, encodes a molecular motor that transports specific organelles, as cargo, along microtubules. It has two isoforms – long (beta) and short (alpha). The alpha form is expressed in a variety of tissues and is responsible for the transport of mitochondria. The beta form is expressed specifically in the neurons. Knockout mice heterozygous for *KIF1B* have a defect in transporting synaptic vesicle precursors and develop progressive weakness similar to human neuropathies (Zhao, et al., 2001).

A loss-of-function mutation in the motor domain of human *KIF1B* was identified in a pedigree with autosomal dominant CMT2, however subsequently an *MFN2* mutation was segregating with disease phenotype in the same family (Zuchner, et al., 2004). Thus far no other mutations of *KIF1B* have been found in human peripheral neuropathy.

Transcription factors associated with myelination:

***Early growth response 2* (*EGR2*)**: *EGR2*, also known as *KROX20*, maps to 10q21 and consists of two exons which encode for a Cys_2His_2 type zinc-finger containing protein. Mouse *Egr2* is implicated in the establishment of myelination and thereafter its expression is restricted to myelinating Schwann cells (Zorick, et al., 1996) (Kioussi and Gruss, 1996). Homozygous knockout mice for *Egr2* show disruption in hindbrain segmentation (Swiatek and Gridley, 1993) (Schneider-Maunoury, et al., 1993) and the Schwann cells are blocked at an early stage of differentiation (Topilko, et al., 1994). Mutations in human *EGR2* are detected in patients with CMT1, DSN and CHN (Warner, et al., 1998) (Timmerman, et al., 1999). Most mutations occur in the zinc-finger domain. Functional studies have shown that most *EGR2* mutations affect the DNA binding and the amount of residual binding directly correlates with disease severity (Warner, et al., 1999b). In the same study, it also has been shown that a mutation in the R1 domain of EGR2, that is known to bind with NAB co-repressors and prevent its interaction with NAB proteins, leads to an increase in the transcriptional activity of EGR2. Thus, failure to activate or inactivate downstream genes or deregulation of EGR2 activity could be a possible pathological mechanism for the development of the disease phenotype.

Genes associated with signaling:

***Periaxin* (*PRX*)**: Human *PRX* maps to 19q13. It encodes a protein consisting of an N-terminal PDZ domain followed by a basic domain, a nuclear localization signal domain, 57 imperfect tandem pentameric repeats that may have a tripeptide spacer and an acidic domain. Mutations in *PRX* are associated with AR-DSN and CMT4F (Boerkoel, et al., 2001) (Guilbot, et al., 2001) (Takashima, et al., 2002a). PRX mutations cause an early-onset, but slowly progressive neuropathy with marked sensory component (Kijima, et al., 2004b). Alternative splicing results in two forms – L-periaxin and S-periaxin (Dytrych, et al., 1998). S-periaxin is restricted to the

cytoplasm. L-periaxin is initially seen in the nuclei of embryonic Schwann cells and subsequently in the plasma membrane of myelinating Schwann cells (Sherman and Brophy, 2000). It is expressed in the first uncompacted lamellae of the Schwann cell membrane that ensheathe the axon, and further synthesis of the protein in the rat sciatic nerve parallels the deposition of myelin (Gillespie, et al., 1994). In the mature myelin, periaxin is found in the cytoplasm-filled periaxonal regions of the sheath but is excluded from the compact myelin. Mice disrupted for *Prx* develop PNS compact myelin that degenerates as the animals age (Gillespie, et al., 2000) consistent with the requirement of periaxin in myelin stability. These mice are an important model to study neuropathic pain in late onset demyelinating disease.

Myotubularin related protein 2 (MTMR2): *MTMR2* maps to 11q22 and encodes for a dual specificity phosphatase. It also contains a GRAM domain, a SET-interacting domain and a PDZ-binding domain. Mutations in *MTMR2* cause a type of AR-CMT1 (CMT4B1) and CHN (Bolino, et al., 2000) (Bolino, et al., 2001). CMT4B1 is characterized by focally folded myelin. The mutations are distributed throughout the open reading frame. MTMR2 utilizes the lipid second messenger, phosphoinositol 3-phosphate, (PI(3)P), as a physiologic substrate. The known (Kim, et al., 2002) disease-associated *MTMR2* mutations show reduced phosphatase activity (Berger, et al., 2002), indicating that the phosphatase activity of MTMR2 is crucial for its proper function in the peripheral nervous system.

SET binding factor 2 (SBF2): It maps to 11p15 and encodes for an *MTMR2* related protein. *SBF2* is a member of the pseudo-phosphatase branch of myotubularins (it is also known as MTMR13). A homozygous inframe deletion encompassing exons 11 and 12 was detected in a consangious Turkish family (Senderek, et al., 2003). Recently, the Japanese family from one of the clinical reports of CMT and glaucoma (Kiwaki, et al., 2000) was found to have a nonsense mutation in SBF2, that segregated with a phenotype of markedly decreased MCV, myelin folding and juvenile onset glaucoma (Hirano, et al., 2004). It is expressed in various tissues including spinal cord and peripheral nerve. The histopathological hallmarks of the disease phenotype are focal outfoldings of myelin in nerve biopsies.

N-myc downstream regulated gene 1 (NDRG1): *NDRG1* maps to 8q24 and encodes for a phosphatase. A homozygous C-to-T transition in exon 7 (R148X) was identified in 60 individuals affected with hereditary motor and sensory neuropathy, Lom type (HMSNL)

(Kalaydjieva, et al., 2000). HMSNL is an AR CMT1 type of disorder with deafness and unusual neuropathological features (Kalaydjieva, et al., 1996). *NDRG1* is ubiquitously expressed and appears to play a role in cell growth and differentiation.

GDAP1: It encodes a ganglioside-induced differentiation associated protein originally isolated using a tetracycline-regulated expression system from differentiated Neuro2a cells (Liu, et al., 1999). It contains a glutathione-S-transferase domain. It is expressed at high levels in the brain and spinal cord and at lower levels in human sural and mouse sciatic nerves (Cuesta, et al., 2002). The *GDAP1* gene may be involved in a signal transduction pathway in neuronal development. Mutations in *GDAP1* are associated with autosomal recessive axonal neuropathies (Baxter, et al., 2002) (Boerkoel, et al., 2003) and AR-CMT2 with vocal cord paresis and hoarseness (Cuesta, et al., 2002). In one of these studies (Boerkoel, et al., 2003) , the pathological allele (487C>T, Q163X) was observed in three unrelated Hispanic families that had the same haplotype suggesting a founder mutation that probably arose among the Spanish and thereby entered the American Hispanic population (Claramunt, et al., 2005).

Genes associated with endosomes

RAB7: It encodes a GTP-binding protein that is a member of the RAB family of small GTPases which are important regulators of vesicular transport and are located in specific intracellular compartments. RAB7 has been localized to late endosomes and shown to be important in the late endocytic pathway (Vitelli, et al., 1996). It is universally expressed. Mutations in *RAB7* have been associated with CMT2B, an axonopathy (Verhoeven, et al., 2003).

SIMPLE: This gene encodes for an unglycosylated small integral membrane protein of the lysosome/late endosome (Moriwaki, et al., 2001). Two families having individuals with CMT1 (Street, et al., 2002) showed genetic linkage to markers in the 16p13.2 region where the *SIMPLE* gene also known as *LITAF* or *PIG7* maps.

Mitochondrial gene:

Mitofusin 2 (MFN2): Recently mutations in *MFN2* were found in 7 CMT2A pedigrees and in a significant number of sporadic cases (Zuchner, et al., 2004). Most patients had an axonal

neuropathy, moderately severe, with onset in childhood. In a Japanese study 8.6 % of CMT2 and unclassified patients had a mutation in *MFN2*, making this gene the most commonly involved in CMT2 (Kijima, et al., 2004a). Mitochondria are dynamic organelles and highly motile with frequent fusion and fission. MFN2 is localized to the outer mitochondrial membrane, and regulates mitochondrial network architecture by fusion of mitochondria. Although MFN2 is ubiquitously expressed, in the peripheral nerve the mitochondrial network has to be maintained for long distances from the cell body, this may explain the length dependent axonal neuropathy developing in patients with *MFN2* mutations (Kijima, et al., 2004a).

Chaperones

Heat-shock protein 27 (HSP27)* and *Heat-shock protein 22 (HSP22): Small heat-shock protein 27 mutations were found in distal motor neuropathy and in a family with CMT (Evgrafov, et al., 2004). In this case distal motor neuropathy and CMT are allelic, suggesting that these two groups of disorders are intimately related. While *HSP22* mutations were originally found in the distal motor neuropathy group (Evgrafov, et al., 2004), as anticipated, recently an *HSP22* mutation was identified in a family with CMT (Tang, et al., 2004). The pathomechanism of the development of neuropathy is less clear. In vitro data suggests, that neuronal cells transfected with mutant *HSP27* are less viable, than cells transfected with the wild-type protein. When the mutant *HSP27* is cotransfected with *NEFL*, neurofilament assembly is altered. In a yeast two-hybrid system HSP22 and HSP27 were found to interact (Benndorf, et al., 2001).

Genes associated with DNA single strand break repair

Tyrosyl DNA phosphodiesterase 1 (TDP1): It maps to 14q32 and codes for a DNA repair enzyme, that repairs abortive single strand breaks (SSBs) created by topo1 (Pouliot, et al., 1999) and also repairs 3'-phosphoglycolated overhangs of DNA double strand breaks (DSBs) (Inamdar, et al., 2002). In the repair of SSBs, TDP1 cleaves the covalent bond formed between the tyrosine moiety of TopoI and the 3' end of the DNA and thus generates a 3' end compatible with ligation (Interthal, et al., 2001). In case of DSBs, that leave a 3'-phosphoglycolate overhang, TDP1 removes the glycolate to leave a 3' phosphate, which forms the substrate for

ligation. One familial homozygous *TDP1* mutation has been associated with autosomal recessive spinocerebellar ataxia and axonal neuropathy (SCAN1) (Takashima, et al., 2002b). *In vitro* functional studies revealed that mutations associated with the SCAN1 phenotype alter the sequestration of TDP1 into multi-protein single-strand break repair complexes making these complexes catalitically inactive (El-Khamisy, et al., 2005). Another set of *in vitro* functional studies showed that these mutations abolish the 3'-phosphoglycolate processing activity of the enzyme (Zhou, et al., 2005). The phenotype caused by mutations in *TDP1*does not quite classify as CMT, as CNS involvement is also present, rather it belongs to a new group of disorders affecting oculomotor praxis, the cerebellum, the spinal cord and the peripheral nerves in various combinations, all caused by alteration of the DNA repair pathways, with Ataxia-Oculomotor Apraxia (AOA1 and AOA2) (Moreira, et al., 2001) (Moreira, et al., 2004).

Genes associated with other PNS-specific functions:

***Lamin A/C* (*LMNA*)**: It maps to 1q21 and encodes a structural protein with similarity to cytoplasmic intermediate filament proteins. One familial mutation in *LMNA* is associated with AR-CMT2 (CMT2B1) (De Sandre-Giovannoli, et al., 2002). Other mutations in *LMNA* are associated with several different disorders including Emery-Dreifuss muscular dystrophy (EDMD) (Bonne, et al., 1999), limb-girdle muscular dystrophy (Muchir, et al., 2000), dilated cardiomyopathy (Fatkin, et al., 1999), familial partial lipodystrophy (Cao and Hegele, 2000) and mandibuloacral dysplasia (Novelli, et al., 2002). Thus, mutations in a single gene can cause different diseases affecting diverse tissues and organs including neurons, muscles, cardiovascular and skeletal systems as well as fat cells.

Lamins are the major structural proteins of the nuclear lamina underlying the nuclear membrane. They appear to play a role in DNA replication, chromatin organization, spatial arrangements of nuclear pore complexes, nuclear growth and anchorage of nuclear envelope proteins (Stuurman, et al., 1998). Mice lacking *Lmna* develop to term with no overt abnormalities (Sullivan, et al., 1999). However, their postnatal growth is severely retarded and is characterized by the appearance of muscle weakness.

Potassium chloride cotransporter 3 (KCC3): It maps to 15q13 and codes for a K^+-Cl^- cotransporter. Mutations in *KCC3* are associated with autosomal recessive peripheral neuropathy with agenesis of the corpus callosum (MIM 604878; 218000), also known as Andermann syndrome (Yan, et al., 2002). Heterozygous mice transgenic for deletion of the mouse homolog, *Slc12a6*, are indistinguishable from the wild type in appearance and gross behavior. However, homozygous animals exhibited weakness of the rear limbs beginning at two weeks. Hypomyelination, myelin decompaction, demyelination, axonal swelling and fiber degeneration were observed in the sciatic nerves of homozygous mice. KCC3 protein is detected in the brain and the spinal cord and, at low levels, in the dorsal root ganglion (Howard, et al., 2002) (Pearson, et al., 2001).

Glycyl tRNA synthetase (GARS): Mutations in *GARS* have been found in patients with autosomal dominant Charcot-Marie-Tooth axonal neuropathy type 2, designated CMT2D. Distal spinal muscular atrophy type V (DSMAV) is an allelic disorder with a similar phenotype. The clinical picture of patients with *GARS* mutations was different from other axonal CMT2 types in that weakness and atrophy were more severe in the hands than in the feet, and that sensory impairment had the same prevalence as the motor involvement (Antonellis, et al., 2003). The human GARS protein is encoded by a 17-exon gene that spans ~40 kb on chromosome 7p14 and is expressed in a ubiquitous fashion. The four CMT2D/dSMA-V–associated mutations occured in conserved amino acids. GARS is a member of the family of aminoacyl tRNA synthetases responsible for charging tRNAs with their cognate amino acids. The functional holoenzyme exists as a homodimer and contains three major functional domains: the WHEP-TRS domain for conjugation with other aminoacyl tRNA synthetases in enzyme complexes, the core catalytic domain for ligation; and the anticodon-binding domain for recognition of glycine-specific tRNAs (Freist, et al., 1996).

Dynamin 2 (DNM2): Mutations in *DNM2* on chromosome 19p12-13.2 were found in three unrelated families with CMT originating from Australia, Belgium and North America. DNM2 belongs to the family of large GTPases and is part of the cellular fusion-fission apparatus. In vitro experiments showed that mutations of DNM2 substantially diminished binding of DNM2 to membranes by altering the conformation of the beta3/beta4 loop of the pleckstrin homology domain. Additionally, two different mutations affecting the same amino acid, Lys558, segregated

with CMT and neutropenia, which has not previously been associated with CMT neuropathies (Zuchner, et al., 2005).

GENETIC TESTING

Recent advances in identifying specific mutations in various genes have led to extensive genotype-phenotype correlation studies, which have confirmed and elucidated further that genetic heterogeneity, age-dependent penetrance and variable expressivity are key characteristics of the hereditary motor-sensory neuropathies (HMSN). Molecular tools have increased the possibility of establishing a specific diagnosis. However, the expense associated with evaluating multiple genes for disease-causing mutation has also escalated. When deciding upon genetic testing one should consider multiple factors, including (i) availability of clinical testing, (ii) the yield of a specific molecular test, (iii) the aim of establishing a molecular diagnosis and (iv) in sporadic cases the frequency of *de novo* mutations.

Methods

To determine the frequency of the mutations in the various genes underlying CMT and related peripheral neuropathy we have performed a PubMed search with the headings *charcot* and *mutation* (734 hits) and after review of the abstracts identified 11 population based studies (Boerkoel, et al., 2002; Bort, et al., 1997; Choi, et al., 2004; Janssen, et al., 1997; Leonardis, et al., 1998; Marques, et al., 2005; Mersiyanova, et al., 2000a; Mostacciuolo, et al., 2001; Nelis, et al., 1996; Nicholson, 1999; Silander, et al., 1998; Wise, et al., 1993) reporting on genetic testing and frequencies of genes involved in CMT and related peripheral neuropathy.

A cohort of 153 unrelated CMT patients collected prior to the clinically availability of genetic testing (i.e. before circa 1993) was analyzed to estimate the contribution of the genes not reported in the population based studies. All patients referred to this study by their primary physician or neurologist received appropriate counseling and gave informed consent approved by the Institutional Review Board of Baylor College of Medicine. The clinical diagnosis was based on clinical examination, electrophysiological studies and, in a few cases, nerve biopsy and given by a neurologist (C.A.G. in most cases). CMT1 was defined as symmetrically decreased nerve conduction velocity (< 38 m/s in the lower extremities), and CMT2 as normal or subnormal NCV and reduced compound muscle action potential.

Preliminary mutation studies in this cohort for selected CMT associated genes have been reported (Boerkoel, et al., 2002). The cohort was screened for the CMT1A duplication, the HNPP deletion, and point mutations in *GJB1*, *MPZ*, *PMP22*, *EGR2*, *PRX*, *NEFL*, *SIMPLE*, *GDAP1*, *LMNA*, *TDP1*, and *MTMR2*. The CMT1A duplication /HNPP deletion testing (which tests for the duplication, or deletion of *PMP22*) was performed by pulsed-field gel electrophoresis (PFGE), whereas point mutations were detected by DHPLC followed by direct sequencing of the abnormal amplicons by protocols published earlier or by direct sequencing of all coding exons. The primers and conditions are available on the laboratory's website (http://www.imgen.bcm.tmc.edu/molgen/lupski/).

Results and discussion

Hereditary polyneuropathy is common and powerful diagnostic tests are clinically available. Thirty-five loci and 25 genes (Figure 1, Table 1) have been identified in CMT and related peripheral neuropathies generating a complicated molecular classification, making it difficult to apply the vast amount of information in clinical practice. In order to establish evidence-based guidelines we recently reviewed the medical literature and identified 11 population-based studies from various ethnic backgrounds (Boerkoel, et al., 2002; Bort, et al., 1997; Choi, et al., 2004; Janssen, et al., 1997; Leonardis, et al., 1998; Marques, et al., 2005; Mersiyanova, et al., 2000a; Mostacciuolo, et al., 2001; Nelis, et al., 1996; Nicholson, 1999; Silander, et al., 1998; Wise, et al., 1993). These studies reported results on 5 genes/genomic rearrangements: *PMP22* duplication/deletion; *MPZ*, *Cx32* and *PMP22* point mutations. The mutation frequencies of the individual genes in the total population and in phenotypic subgroups are depicted in Table 2. Similar mutation frequencies were detected in the various studies, revealing a uniform distribution of pathogenic mutations. Applying a simple clinical classification (demyelinating versus axonal neuropathy) and considering the inheritance pattern (Mostacciuolo, et al., 2001) markedly improved the diagnostic yield.

In order to estimate the relative frequencies of the genes for which population based studies are not available, we used our own cohort of 153 consecutive unrelated CMT cases collected prior to the availability of genetic testing in commercial laboratories (precommercial era). We have screened 14 genes/genomic rearrangements (*PMP22* dup/del, point mutations in *Cx32*, *MPZ*, *PMP22*, *EGR2*, *PRX*, *NEFL*, *SIMPLE*, *GDAP1*, *LMNA*, *TDP1*, *MTMR2*) in this

cohort to estimate the relative frequency of pathogenic mutations in these genes (Table 3). The frequencies of the 5 mutations screened in the population based studies were similar, suggesting that estimates from this cohort is adequate. Seemingly mutations in the genes for which population studies are not available contribute to only a small minority (<1-2%) of patients with the CMT phenotype. Molecular testing in commercial laboratories confirmed the relative frequencies of the various genes harboring pathogenic mutations.

Duplication of a chromosomal segment harboring *PMP22* (i.e. the CMT1A duplication) (Lupski, et al., 1991) represents 43% of the total CMT cases, whereas the yield of duplication detection rises to 70% in CMT1. This group of patients is the potential beneficiaries of the novel therapeutic interventions and being the most common mutation it is likely that drug toxicity studies will address this population initially, just like data of idiosyncratic vincristine toxicity has emerged from patients with this mutation (Chauvenet, et al., 2003) (Graf, et al., 1996). Thus the aim is to identify all subject with the CMT1A duplication, therefore the test should be utilized as a screening test.

The deletion of the same chromosomal segment results in hereditary neuropathy with liability to pressure palsies, HNPP (Chance, et al., 1993). HNPP can mimic multifocal neuropathy (Tyson, et al., 1996), a frequently inflammatory disorder that requires immunosuppressant therapy. The individuals with HNPP amongst this group of patients need to be identified in order to do no harm. Although detection of deletion has a low yield in the CMT population, the clinical picture is distinctive and the deletion is specific for HNPP, thus testing of this phenotype identifies deletion mutations in > 90 % of patients. Deletion testing should serve as a confirmatory test. Importantly, essentially all commercially available DNA diagnostic tests for the common CMT1A duplication and HNPP deletion rearrangments can detect either mutation using a single assay.

After the CMT1A duplication and HNPP deletion, *Cx32* mutations are the next most common culprits in inherited neuropathy. A dominant inheritance pattern and lack of male to male transmission points to this gene on the X chromosome. Identification of a *Cx32* mutation determines an X-linked dominant inheritance pattern, enabling both genetic counseling and accurate estimation of recurrence risk. As the phenotype is intermediate, molecular testing for *Cx32* is appropriate in both CMT1 (after duplication testing) and in CMT2.

The population-based studies suggest that in patients with the CMT1 phenotype, *MPZ* and *PMP22* mutations are the next most common after *PMP22* duplication and *Cx32* mutations. In the CMT2 group, *Cx32* mutations are followed by *MPZ* mutations in frequency, however recent data, though not population based, suggests, that *MFN2* mutations may be one of the most common causes of CMT2 (Zuchner, et al., 2004)' (Kijima, et al., 2004a).

Mutations in other genes are responsible for the CMT phenotype in only a small minority of patients, however unique clinical features may point to a specific gene (e.g. *EGR2* external ophthalmoplegia, respiratory compromise) (Table 4). In the absence of a clinical clue the likelihood of establishing a molecular diagnosis is low.

The high frequency of *de novo* mutations in duplication/deletion (37-90%) (Hoogendijk, et al., 1992)' (Warner, et al., 1996b) illustrates that genetic disease is commonly sporadic in presentation, lacking a family history. Our data suggests that point mutations also occur frequently *de novo* (Boerkoel, et al., 2002). The frequently occurring *de novo* event necessitates having an index of suspicion for genetic disease even in the absence of a family history. In fact, in a patient presenting with chronic polyneuropathy in the absence of other signs and symptoms, after the most common systemic and treatable causes, such as diabetes, uremia and nutritional deficiency, genetic causes are more common, than autoimmune or paraneoplastic neuropathy. A rational diagnostic approach is presented as Figure 2.

Finally when performing genetic testing, one must consider the specific question posed and the likelihood whether the result alters medical management. In adults with the CMT phenotype *PMP22* duplication and *Cx32* mutation analysis establishes the molecular diagnosis in 65% of patients. The combination of *PMP22* duplication and *Cx32* mutation testing identifies the candidates for the clinical trials, and in the near future those, who may benefit from treatment with gene expression modifiers (selective progesterone antagonist (Sereda, et al., 2003), vitamin C (Passage, et al., 2004)), and identifies families whose members are potentially at risk for idiosyncratic drug reactions and determines inheritance pattern establishing grounds for accurate genetic counseling and prenatal diagnosis. If patients with the demyelinating form are tested as a group, the diagnostic yield increases to over 80% by performing *PMP22* duplication and *Cx32* mutation testing.

In the pediatric population the aim of testing has a different emphasis. The parents have a frequently devastated child with severe weakness and normal intellect. The parents major

concerns are prognosis and recurrence risk. For either of their questions the answer will depend on an accurate molecular diagnosis. Thus in children after testing for the common causes of peripheral neuropathy, *PMP22* duplication and *Cx32* mutations, the physician should proceed to panel testing for all the genes implicated in that phenotype.

NOVEL MUTATIONS IN SIMPLE

Recently, an autosomal dominant demyelinating form, CMT1C, was associated with mutations in *SIMPLE/LITAF* (MIM#603795) (Street, et al., 2003). SIMPLE/LITAF was initially identified as a p53-induced gene, *PIG7*. Independantly it was isolated as a lipopolysaccharide-induced transcription factor, LITAF, regulating tumor necrosis factor alpha gene expression. Both LITAF and PIG7 encode the identical 228 amino acid protein. A product encoded by the same gene was also found during investigation of genes induced by the Mycobacterium bovis cell wall complex-this identified a 161 amino acid protein, SIMPLE (Moriwaki, et al., 2001). Both SIMPLE and LITAGF/PIG7 share an identical stretch of 126 amino acids at their N-termini. SIMPLE maps to 16p13.3-p12 in an interval in which historical recombination delimit the CMT1C locus for an autosomal dominant form of demyelinating CMT (Street et al, 2002). Three SIMPLE mutations have been reported in CMT1C families (Street, et al., 2003).

Materials and methods

Subjects

Medical records of patients with CMT and related neuropathies was requested and reviewed to ascertain clinical diagnosis. These studies were approved by the Institutional Review Board of Baylor College of Medicine, Houston (Texas).

Mutation screening

Blood was obtained after informed consent and DNA isolated using the QIAamp DNA Blood Mini Kit (Qiagen, CA) and Puregene DNA isolation kit (Gentra Systems, MN). Primers flanking the coding exons were designed using Primer3. Purification of the PCR products and their sequencing was carried out as above. The chromatograms were analyzed using Sequencher (Gene Codes, MI). Loss or creation of restriction enzyme sites (*Hpa*II, *Nla*IV, *Scr*FI and *Tsp*45I; New England Biolabs, MA) in mutant chromosomes, identified using NEB Cutter, enabled independent confirmation of the mutations (Figure 3).

Sequence analysis

Numbering of mutations is according to widely accepted criteria (den Dunnen and Antonarakis, 2000). The DNA mutation numbering is based on the cDNA sequence with a 'c.' Symbol before the number, where +1 corresponds to the A of the ATG translation initiation codon in the reference sequence (AB034747.1) and the initiation codon is codon 1. All nucleotide changes were analyzed using the NNSplice program for prediction in alteration of splicing junctions and the exonic splicing enhancers program ESEfinder. The clinical relevance of missense mutations was examined using Granthams' chemical difference matrix. Further, the importance of a particular amino acid in the overall function is also relevant to clinical outcome. We accessed the relative importance to protein function of a specific aminoacid residue in a given context by its conservation across species- the regions containing these mutations were analyzed by BLASTP and multiple alignments were created using Clustatx and NJPlot.

Results

SIMPLE mutations

In our cohort of 192 unrelated CMT and related peripheral neuropathy cases we identified twelve different nucleotide changes in *SIMPLE* (Table 5). Nine of these were not identified in at least 180 control chromosomes and the remaining three, based on their high frequencies both in patients and control samples, were identified as polymorphisms. Each of the nine disease associated nucleotide changes were identified in the heterozygous state consistent with dominant alleles.

Pathogenicity of missense mutations

Three of the nine mutations in SIMPLE are missense (Table 5). Two of these three (p.Thr49Met and p.Leu122Val) are novel; p.Gly112Ser has been reported (Street, et al., 2003). The functional consequences of such mutations that lead to amino acid replacements can be analyzed by gauging the biochemical severity of missense changes and the location and/or context of the altered amino acid in the protein sequence. Our analysis using the Grantham scale, which categorizes codon replacements based on chemical dissimilarity between the encoding amino acids, suggests that each of the three missense mutations in SIMPLE is nonconservative. Since disease causing mutations are significantly more likely to occur at amino acid residues that

are conserved across species, we studied cross-species conservation of regions surrounding the mutation. Our analysis of the three missense mutations shows that the corresponding amino acids are conserved across a wide range of species (Figure 4). Leucine 122 and glycine 112 are conserved in all species examined-human, mouse, rat, chicken, horse and hen. Threonine 49 is also conserved in all except hen (Figure 4).

The p.Gly112Ser and p.Leu122Val segregate with the disease phenotype (Figure 3). The p.Gly112ser change was found in two families, Hou 537 and Hou 521. In the first family, the CMT1 phenotype segregates as an autosomal dominant trait (Figure 3), whereas in the second family, HOU 521 (Figure 3), it is associated with a sporadic neuropathy (BAB 1339).

Effect of SIMPLE mutations on splicing

Six mutations do not result in amino acid changes (Table 5). However, they could result in changes in DNA motifs that directly affect splicing or regulate splicing (exonic and intronic splicing enhancers and silencers) and thus potentially result in aberrant splicing patterns leading to a change in the open reading frame or, alterations in the levels of mRNA expression. We analyzed all nine mutations for their effect on splicing. Splice-site predictions (NNSplice) do not detect any changes in splicing per se in these nine mutations. However, we find that these nucleotide changes result in loss of seven binding motifs for ESE proteins and in the creation of five such sites. In addition there is a predicted change in the binding affinity of four ESE motifs.

Potential pathogenic mutations

We identified two potentially pathogenic mutations, c.146C>T (Thr49Met) and c.671T>C, which lies in the 3' UTR of *SIMPLE*. These nucleotide changes were also not detected in at least 180 control chromosomes. The segregation status of c.146C>T (Figure 3) could not be unequivocally established because the mother, who also has this mutation, is suspected to have CMT-she has absent ankle jerks. However, reliable information on her NCS could not be obtained. The segregation status of c.671T>C could not be determined due to lack of samples from other family members. Further, exonic splicing enhancer predictions indicate that the c.671T>C change may lead to creation of a SRp40 site.

Disease associated rare variants

Five silent mutations, c.330C>T (p.Asn110), c.447G>A (p.Lys159), c.539C>G (3'UTR), c.585C>T (3'UTR) and c.629C>T (3'UTR), did not segregate with the disease phenotype. However, these nucleotide changes were not detected in at least 180 control chromosomes (Table 5) and are predicted to modify ESE motifs.

SIMPLE mutations may cause both demyelinating and axonal neuropathy

The clinical features of patients manifesting neuropathy in association with SIMPLE mutations are summarized in Table 6. All patients with SIMPLE mutations, except one (BAB1399), presented with a uniform clinical pattern typical of CMT1, marked by slow nerve conduction velocity (17-26 m/s). Both sensory and motor nerves were affected. In one patient (BAB 2104) nerve biopsy was available and revealed chronic demyelination and remyelination presenting as onion bulb formations. One patient (BAB 1399) had a diagnosis of CMT2. The diagnosis was based on relatively preserved motor and sensory velocities (43m/s) and neuropathology revealing axonal loss.

The age of presentation was 6-34 years of age. All patients had a slowly progressive course and no evidence of cranial neuropathy except for one patient with hearing loss from age 61. Phrenic nerve conduction studies revealed delayed latency in one proband and she was symptomatic with recurrent pneumonias.

Discussion

We identified nine different heterozygous nucleotide changes of *SIMPLE* in neuropathy patients. However, five rare variants, although not identified in greater than 180 control chromosomes, did not co segregate with disease. Thus their pathogenicity could not be established. Nevertheless, potential roles for modifying mutations at another locus remain to be explored. Three mutations were not found in greater than 180 control chromosomes, affected a conserved amino acid or regulatory sequence and co segregated with disease consistent with their representing pathogenic mutations. The Gly112Ser allele was identified in both a family segregating CMT1 as a dominant trait and in a sporadic CMT1 case as a potential *de novo* mutation. Of the three pathogenic mutations/potentially pathogenic mutations identified in four families (Figure 3), only family HOU 537 shows autosomal dominant inheritance, whereas the

remaining three families represent sporadic cases. The non-availability of one or both parental sample in sporadic cases precludes unequivocally establishing these as *de novo* mutations. Thus, it appears that *de novo SIMPLE* mutations may be a contributing factor to sporadic demyelinating neuropathy.

Five families, HOU 537, HOU 521, K1551, PN282 and K1552 (Bennett, et al., 2004), (Street, et al., 2003) suggest that the mutation c.334G>A (p.Gly112Ser) may represent a mutation hotspot as a result of a CpG dinucleotide or, be the result of a founder effect. However, the presence of different haplotypes in the region around *SIMPLE* in families PN282, K1551 and K1552, makes it unlikely that the mutation is due to a founder effect (Bennett, et al., 2004). Similarly, the c.630C>T change found in four families (Table 5) may also represent a mutation hotspot due to a CpG dinucleotide. All four families with disease associated *SIMPLE* mutations have been diagnosed with CMT1.

The novel missense mutation, p.Leu122Val, forms part of a 38 amino acid stretch that is highly conserved among all species examined (Figure 4). This 38 amino acid stretch contains two $CX_{2\text{-}3}C$ motifs that are known as CXXC knuckels and are characteristic of Zn2+ binding domains. This 38 amino acid stretch is part of an approximately 70 amino acid sequence, known as the *LITAF* domain (Interpro AC IPR006629 and SMART AC SM00714), which is conserved to a lesser degree than the 38 amino acid stretch albeit in a wider range of species extending from humans to *Caenorhabditis elegans* and even to plants such as *Arabidopsis thaliana*. The Leu122Val mutation forms part of the second CXXC motif. The Gly112Ser change lies in a 19 amino acid hydrophobic stretch between the two CXXC motifs and this hydrophobic stretch is thought to insert, but not traverse, into the membrane and bring the two knuckles together to form a compact Zn2+ binding structure. These two missense mutations, p.Leu122Val and p.Gly112Ser, as well as the two other reported missense mutations, p.Thr115asn and p.Trp116Gly (Street, et al., 2003), all lie in this conserved 38 amino acid stretch. Clustering of mutations (Figure 4) and the high level of conservation suggest functional significance of this part of the protein. We hypothesize that the stereochemical alterations in this region may abrogate normal interaction of the 19 amino acid stretch with the membrane which in turn may affect its binding to Zn2+.

SIMPLE mutations can be disease associated pathogenic alleles (N=5) often found in association with sporadic CMT1 or rare variants (N=11) representing polymorphisms whose

clinical significance is undetermined. The clinical phenotype of neuropathy associated with mutations in *SIMPLE* may convey both demyelinating and axonal CMT.

EGR2 MUTATIONS AND THEIR FUNCTIONAL SIGNIFICANCE

Myelination of the peripheral nervous system by Schwann cells involves a complex temporal pattern of gene activation. One of the most important transcription factors marking the transversion from the promyelinating to the myelinating state in Schwann cell development is EGR2, which activates the transcription of several myelin-associated genes, including *MPZ*, *PMP22*, *MAG*, *CX32* and *PRX*.

The early growth response 2 gene (*EGR2*) is a member of a family of early growth response (EGR) genes which includes *EGR1* (*NGFI-A, Krox24* and *Zif268*), *EGR3* and *EGR4* (*NGFI-C*). Experimental evidence has shown that the *EGR* genes encode transcription factors which contain Cys_2His_2 zinc fingers which bind a GC-rich consensus binding site. The EGR proteins mediate a variety of signaling pathways that are important for cellular growth and differentiation and responses to hormonal stimuli.

Studies of Egr2-null, and recently Egr2 hypomorphic mice confirmed that this transcription factor is a prime regulator of Schwann cell myelination and hindbrain segmentation. In these animals Schwann cells were associated with individual axons and expressed SCIP, suggesting that they could progress to the promyelinating stage, but not beyond.

Subjects and Methods

Subjects

After IRB approval informed consent was obtained. The patient population consisted of previously identified subjects with *EGR2* mutations in a cohort with CMT and related neuropathies referred to our laboratory for genetic testing and subjects with identified sequence variations of unknown significance in *EGR2* by commercial laboratories.

Sequencing

DNA was isolated from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Ca) and PCR of all amplicons was carried out using primers as described previously. The PCR

products were treated with SAP and exonuclease (Amersham). These products were then used for sequencing by dye-primer chemistry (Applied Biosystems) and electrophoresed on an ABI 377 automated sequencing machine. The resulting chromatograms were analyzed using the Sequencher software package (ACGT Codes).

In vitro mutagenesis

All expression constructs were made with the pcDNA3.1(+) vector from Invitrogen (Carlsbad, CA). Wild-type *EGR2* coding sequence was PCR amplified from EST 362693 (GenBank accession no. aa018140) using the primers BamHIF (5(prime)-CGCGGATCCCCACCATGGTGACCGCCAAGGCCGTAGAC-3[prime]) and XbaIR (5[prime]-GCTCTAGATCAAGGTGTCCGGGTCCGAG-3[prime]). The wild-type PCR fragment was digested with *Bam*HI and *Xba*I and cloned into the pcDNA3.1(+) vector. Using the wild-type construct as a template, the mutant constructs I268N, S382R, R409W, R359W and E412K were made using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The DNA sequences of all constructs were confirmed by fluorescent automated sequencing. The luciferase reporter construct containing the two EGR2 consensus binding sites and the CMV-driven*lacZ* reporter were described previously.

Cell culture conditions and transient transfection assays

HeLa cells were cultured in DMEM supplemented with 10% FBS. Cells were plated in 12-well plates (Corning, Boston, MA) at 3.5×10^4 cells/ml and grown for 1 day before performing transfections. All transfections were performed using 250 ng of the luciferase reporter, 50 ng of a CMV-driven *lacZ* reporter and the indicated amounts of the expression plasmids. Bluescript plasmid (Stratagene) was added as required to make a total of 2 μg DNA/transfection. Harvesting of the cells 48 h after transfection and the luciferase assays were done according to standard protocol The average luciferase activity of duplicate samples was normalized to the [beta]-galactosidase activity from the transfected *lacZ* reporter.

Results

We have identified 10 patients with pathogenic mutations in the *EGR2* gene, including a novel *de novo* mutation E412K. Nine patients were reported previously. Clinical data is

summarized in Table 7. We have preformed *in vitro* functional assays in a luciferase reporter system to evaluate whether these novel mutations alter transcriptional activity. The E412K mutation is in the third zinc-finger domain and reduces transcriptional activity by 65 percent (Figure 5).

Discussion

EGR2 is a zinc-finger transcription factor, in which mutations are responsible for a small number of patients with Charcot-Marie-Tooth disease and related peripheral neuropathies. We have identified two novel *de novo* mutations and established pathogenicity by *in vitro* functional and DNA binding assays. The E412K mutation is located in the third zinc-finger domain and reduces transcritptional activity. Predicted pathogenicity was confirmed in an *in vitro* functional assay.

NATURAL HISTORY OF CMT CAUSED BY EGR2 MUTATIONS

Mutations in EGR2 were identified in patients with congenital hypomyelinating neuropathy (CHN), Charcot-Marie-Tooth disease (CMT) and Dejerine-Sottas neuropathy (DSN). Most EGR2 mutations occur in the Zinc-finger domain and act as dominant-negative alleles. A recessive form of CHN was described in a family with a mutation in the R1 domain, a site of interaction for the Nab suppressor proteins. While *EGR2* mutations are rare, case reports of severe respiratory compromise and death makes physicians uneasy when treating patients with this disorder. We have ascertained 10 consecutive patients with various *EGR2* mutations and collected follow-up information regarding disease progression and respiratory compromise, to better characterize the natural history of the peripheral neuropathy.

Subjects and Methods

Subjects

After IRB approval informed consent was obtained. The patient population consisted of previously identified subjects with *EGR2* mutations in a cohort with CMT and related neuropathies referred to our laboratory for genetic testing and subjects with identified sequence variations of unknown significance in *EGR2* by commercial laboratories. Medical records were reviewed and records of follow-up visits obtained.

Disease progression

Clinically significant progression was defined as a change in a measurable parameter in motor function (measured as change in assisting device with ambulation or loss of ambulation), progression in respiratory status (measured by the development of restrictive pulmonary disease or respiratory failure) and death.

Results

We collected follow-up data on ten patients (Table 8). The mean follow-up was 17.5+/-10 years. These included 3 patients from a recessive family, 3 patients from an autosomal dominant family and 4 sporadic cases. Clinically significant progression defined as change in assistive device for ambulation, restrictive pulmonary disease or respiratory failure, and death was apparent in 60, 50 and 10% of the patients, respectively. Within families the phenotype and progression rate showed little variation (Figure 6). However, the same mutation was identified in two of the sporadic cases, and the phenotype varied from mild childhood onset disease to a very severe, lethal condition.

Discussion

We analyzed clinical data in a cross-sectional cohort of 11 patients (Table 7), and confirmed that respiratory compromise in the form of restrictive pulmonary disease, recurrent pneumonias or respiratory failure are common, occurring in 45 % of cases, and correlates with disease severity. EGR2 knockout and hypomorphic mice confirmed that EGR2 is an important transcription factor in hindbrain development, mainly in separation of rhombomeres 5 and 6. Cranial nerve findings were common in our cases (55%), but interestingly other cranial nerves were affected as well (Table 7). Whether this is due to a cranial neuropathy or lower motor neuron lesion due to altered hindbrain development is hard to elucidate, as imaging does not have a resolution high enough and autopsy specimens were not available.

Case reports of severe, occasionally lethal cases in the literature make genetic counseling difficult. We performed a small follow-up study on 10 patients to establish the natural history of the disease (Table 8). While within families the clinical phenotype and the clinically significant progression did not show marked variability, the same mutation in two sporadic cases showed striking differences in presentation and outcome, from a mild, barely progressive, childhood

onset case to a severe, lethal phenotype, pointing out that the genetic background plays an important role in determining the phenotype (Figure 6).

IMPAIRED INNERVATION CAUSES DISTURBANCE OF MUSCLE FIBER TYPE DIFFERENTIATION

Congenital hypomyelinating neuropathy (CHN) is a hereditary polyneuropathy clinically characterized by early infantile onset, hypotonia, areflexia and paresis with a distal to proximal gradient (Lupski and Garcia, 2001). Prenatal onset may lead to arthrogryposis multiplex congenita. Nerve conduction velocities are severely slowed or absent. Although the clinical and electrophysiological characteristics overlap, pathology can assist in the differentiation of CHN from Dejerine-Sottas Neuropathy (DSN) with markedly reduced myelination and absence or paucity of onion bulb formation in CHN. However, in the absence of nerve pathology CHN can be difficult to differentiate from DSN on a clinical basis alone and this has resulted in some inconsistency of nosology in the literature. CHN is a genetically heterogeneous disorder associated with mutations in *MPZ, EGR2, PMP22* and *MTMR2* (Lupski and Garcia, 2001).

Myelin protein zero (P_0) is a transmembrane protein of the immunoglobulin superfamily. It is the major myelin structural protein in the peripheral nervous system, expressed exclusively by myelinating Schwann cells (Yoshida and Colman, 1996). P_0 functions as a homophilic adhesion molecule. An intact cytoplasmic domain is necessary for the P_0 mediated adhesion (Wong and Filbin, 1994). An adhesion assay combined with *in vitro* mutagenesis in S2 cells showed that the mutation causing Charcot-Marie-Tooth disease type 1B (CMT1B) in humans lowers adhesion capacity less then two distinct mutations conveying the DSN phenotype (Ekici, et al., 1998). In mice, absence (Giese, et al., 1992) or overexpression (Wrabetz, et al., 2000) of P_0 results in hypomyelination and peripheral neuropathy. Mutations in the human *MPZ* gene cause CMT1B, DSN, CHN, Charcot-Marie-Tooth disease type 2 (CMT2) and Roussy-Levy Syndrome (RLS) (Warner, et al., 1996a).

Muscle pathology is infrequently available in cases of CHN (13 reports) (Anderson, et al., 1973) (Nara, et al., 1995) (Bornemann, et al., 1996) as on one hand molecular diagnosis offers a non-invasive way of concluding to a specific diagnosis and, on the other hand nerve biopsy is performed if the index of suspicion for a peripheral neuropathy is higher than for

congenital myopathy. If myopathy is suspected, muscle biopsy is performed, which can obfuscate neuropathy, as in the case reported here.

Case report

The patient presented at birth with hypotonia, arthrogryposis of the left hand and neuromuscular respiratory failure. She was ventilator dependent until approximately one month of age and required nocturnal ventilatory support until 31 months of age. At six months of age an echocardiogram showed marginal septal hypertrophy, this has improved subsequently. She had normal cognitive development with profound motor development delay. At 31 months of age she has a head lag, can sit unsupported for only a limited time, is able to crawl and kneel. She is unable to pull to a stand. She has marked deficits in fine motor skills.

Neurological examination revealed facial diplegia, decreased bulk of the tongue, palatal weakness and difficulty producing lingual sounds. She has axial and appendicular hypotonia, diffuse muscle weakness and areflexia.

Muscle biopsy performed at six weeks of age exhibited peripherally located nuclei, mild fiber size variation and lack of group atrophy. Histochemical analysis revealed poor fiber type differentiation on ATPase (Figure 7A), NADH, SDH (Figure 7B) and COX stainings. Ultrastructural analysis showed streaming of the Z and A bands and disruption of the sarcomeres (Figure 7C and D). The pathology was suggestive of broad A-band.

Subsequent clinical evaluations noted the persistence of areflexia and the cranial nerve involvement became apparent. Neurophysiological studies showed lack of sensory and motor responses except for right median nerve motor response with very small amplitude (0.1 mV) and marked conduction delay (11 m/s) consistent with severe peripheral sensory-motor neuropathy. Sural nerve biopsy at age 18 months exhibited large axons with little to no compact myelin and few basal lamina onion bulbs (Figure 8). The remarkable myelin deficiency was more consistent with CHN than with DSN.

Molecular methods

Mutation analyses for *PMP22*, *MPZ*, *EGR2* and *PRX* were performed by Athena diagnostics. Our laboratory performed sequencing of *MPZ* exon 4 of the proband and her parents. Briefly, DNA was isolated (Puregene DNA isolation kit) and PCR of exon 4 was carried

out using primers as described previously. The PCR products were treated with SAP and exonuclease (Amersham). These products were then used for sequencing by dye-primer chemistry (Applied Biosystems) and electrophoresed on an ABI 377 automated sequencing machine. The resulting chromatograms were analyzed using the Sequencher software package (ACGT Codes). Digestion of PCR fragment with *Hha* I (Amersham) was performed by standard methods.

Results and discussion

Molecular analysis of the proband's DNA excluded mutations in *PMP22*, *EGR2* and *PRX* genes. Analysis of *MPZ* revealed a 3 bp deletion of CTA from nucleotide position 550-552 and insertion of G at 550 in exon 4 (550_552delCTAinsG) (den Dunnen and Antonarakis, 2001) of the gene (Figure 9A and B). By conceptual translation the frameshift mutation derives a mutant protein replacing the last 65 amino acids of the wildtype protein by a 49 amino acid novel sequence (Figure 9C). This mutation was not identified in either parent and DNA marker analysis confirmed parentity, results consistent with a *de novo* mutation. The second allelic change (684C>T) in the proband is a benign polymorphism, and identified in the father.

With the exception of two mutations, all other *MPZ* mutations (http://molgen-www.uia.ac.be/CMTMutations/) are heterozygous and behave as dominant alleles. The two exceptions, deletion of Phe64 and Val102fs, are seen in both heterozygous and homozygous states in different individuals of the same family. The heterozygotes have a CMT1 phenotype whereas the homozygotes have DSN. Having the more severe phenotype in the homozygous state indicates that these mutations are not truly dominant and like most other human disease causing mutations represent semi-dominant alleles.

Myelin protein zero is a homotypic adhesion molecule (Wong and Filbin, 1994) (Ding and Brunden, 1994). It forms dimers and tetramers in intact myelin of isolated sciatic nerve of *Xenopus laevis* (Thompson, et al., 2002). Having mutant and wild type proteins concomitantly may compromise dimerization and tetramerization. Functional studies of P_0 revealed that mutations in the intracellular domain, which altered the protein kinase C target motif (RSTK), resulted in decreased adhesion (Xu, et al., 2001). The RSTK (arginine-serine-threonine-lysine) motif was destroyed by the frameshift mutation in our patient. Thus it is conceivable that the molecular mechanism is a dominant negative effect.

The muscle pathology was obtained in early infancy. In the neonatal period it can be challenging to differentiate between myopathy and neuropathy on a clinical basis, as both can present as a hypotonic infant with respiratory failure. The presence or absence of deep tendon reflexes can be helpful, though if the myopathy is severe enough, reflexes can be diminished also. In our case the presence of mild hypertrophic cardiomyopathy placed myopathy above neuropathy in the differential diagnosis. However, follow up examinations revealed cranial nerve involvement and sustained areflexia despite motor development prompting neuropathy evaluation.

Muscle biopsy features of CHN or DSN have been reported only sparsely and most prior to the era of molecular genetic testing. As the two clinical phenotypes overlap, it is difficult to differentiate between them in literature review because of the incompleteness of the available data. Muscle biopsies have only been studied in 13 patients (Anderson, et al., 1996) (Nara, et al., 1995) (Bornemann, et al., 1996). The muscle pathology was normal in three, signs of denervation were found in five, denervation in association with type II fiber predominance in one, type I fiber atrophy in three and type I fibers predominated in one. Electron microscopy revealed few atrophic fibers and sparse lipid inclusions. The inconsistency in morphological changes could be related to different ages at time of biopsy and heterogeneous molecular pathomechanism.

The muscle biopsy in our case was obtained at an early age. It showed absence of classic features of denervation, but it revealed poor fiber type differentiation. Fiber type differentiation is completed postnatally and has been studied in the mouse (Jansen and Fladby, 1990) (Redenbach, et al., 1988), which demonstrated that type I fiber differentiation is dependent upon innervation. In our case the inadequate nerve function may have altered or slowed fiber type differentiation, especially that of the type I fibers, as reflected by NADH, SDH and COX staining patterns, consistent with the mouse studies. This is the first reported evidence of altered muscle differentiation caused by abnormal innervation in humans.

The electron microscopic findings of streaming of the A and Z bands have not been reported in CHN or DSN. Interestingly, a study of the *trembler* mouse investigated the effect of hypomyelinating neuropathy on muscle (Gale, et al., 1982). *Trembler* has mutation of *Pmp22*, the most frequent gene affected in CMT. The earliest ultrastructural changes at 12 days of age were broadening of the Z-line and streaming of Z-line material into the region of the A-band. In

this study immunohistochemistry was not reported, rather innervation was the focus. In the mouse model of peripheral neuropathy caused by mutation in *Mpz* the muscle pathology has been studied at six months of age only, and by that time fiber grouping occurred, consistent with neuropathic changes (Frei, et al., 1999). Electronmicroscopic observations are unavailable from this mouse model. Ultrastructural studies of *trembler* and our case suggest, that streaming of the A and Z bands may be caused by altered innervation by unmyelinated axons and precede classic features of denervation.

In conclusion, we report CHN case caused by a novel frameshift mutation in the *MPZ* gene. We provide evidence that nonspecific muscle biopsy findings and ultrastructural changes can be consistent with the effects of neuropathy. Our observations support a role for innervation in postnatal skeletal muscle development.

SUMMARY

CMT represents a clinically and genetically heterogeneous group of disorders caused by aberration of the intimate relationship between the Schwann cell sheath and the neural axon. The clinical symptoms of weakness and muscle atrophy ultimately results from axonal death and muscular denervation, the final common pathway of nerve damage. A simple clinical classification of CMT (demyelinating versus axonal) improves the yield of genetic testing and determines which genes should be tested for. In the demyelinating form, the combination of CMT1A duplication and *Cx32* mutation testing has a yield of approximately 80%. *MPZ* and *PMP22* mutations are the next most common culprits. In cases of axonal CMT, *Cx32* mutations are followed by *MPZ* mutations in the population-based studies, and there is suggestion that *MFN2* mutations maybe quite common, though population based studies are not available for this gene yet. Recent discoveries of potential small molecular treatments for a specific subtype of CMT shifted the emphasis in genetic testing: instead of a molecular diagnosis for the few patients referred to tertiary care centers we need to find most patients with the potentially treatable molecular pathomechanism. While the robust amount of new information taught us about peripheral nerve function and dysfunction, it also made genetic testing for all known genes impractical in clinical practice. Today genetic testing should be utilized to address specific questions in a logical stepwise fashion based on evidence from population-based studies.

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Figure legends

Figure 1.

Genetics of CMT neuropathy. This figure depicts the delineation of the CMT phenotype into primarily myelin versus axon involvement, the modes of inheritance that can be observed, and the 35 different linked genetic loci. For 25 of these loci specific genes have been identified and are shown below. Note that at some of the loci either dominant or recessive inheritance may be observed depending on the specific mutation. Also mutations in *GJB1*, encoding the gap junction protein connexin-32, can present as either an axonal or demyelinating neuropathy, or have features of both. In other genes specific mutant alleles can cause either a CMT1 or CMT2 phenotype.

Figure 2.

Flow-diagram depicting a stepwise approach in the diagnosis of CMT and related peripheral neuropathies. In cases with a positive family history, electrophysiology establishes the objective diagnosis of demyelinating versus axonal disease. Taking into account the inheritance pattern helps limit the possible causative genes, then testing can proceed in the order from most common to least common. In sporadic cases of peripheral neuropathy, after exclusion of the most common causes of peripheral neuropathy, a sural nerve biopsy may indicate a specific non-genetic etiology or suggest genetic disorder. If the index of suspicion for CMT is high, genetic testing can follow the establishment of the demyelinating versus axonal phenotype by electrophysiology even preceding sural nerve biopsy.

Figure 3.

Segregation analysis of *SIMPLE* mutations: Individuals tested are shown with four digit identification numbers. Pedigrees are labeled as HOU followed by a three-digit identification number. (A) HOU 537 shows autosomal dominant mode of inheritance. BAB 1837 has not been clinically studied. However, it is believed that she has symptoms suggesting peripheral neuropathy. (B-E) Pedigrees of sporadic cases. Restriction analysis of mutations in different pedigrees: (A) and (B) loss of *Hpa*II results in an uncut band at 367 bp, (C) loss of *Nla*IV site results in a decrease in intensity by half of the 38 bp band and the appearance of a 285 bp

product which merges with the broad band at 247 bp; (D) loss of *Tsp*45I results in an uncut band at 367 bp and (E) creation of *Scr*FI site results in appearance of a 106 bp band.

Figure 4.

Conservation of missense mutations found in *SIMPLE* across various species: Amino acid number 1 is the first methionine of the open reading frame of *SIMPLE* (GenBank accession number AB034747.1) and its homologues (A) Gly112 and Leu122, marked with arrows, are conserved in human (Hs – homo sapiens), rat (Rn – *Rattus norvegicus*), mouse (Mm – *Mus musculus*), horse (Ec – *Equus caballus*) and hen (Gg – *Gallus gallus*). They are also conserved in the *LITAF*-like domain of murine *Nedd4*. CX(2-3)C motifs are boxed. (B) Thr49 is marked with an arrow and found to be conserved in human, mouse, rat and horse. It is also conserved in rat orthologue (Rn_2 – LOC363537).

Figure 5.

The effect of *EGR2* mutations on transcriptional activity in an *in vitro* system using luciferase assay. The values are normalized to beta-galactosidase activity.

Figure 6.

Diagram showing rate of progression of CMT in patients with *EGR2* mutations. Every line depicts one patient, color corresponds to the depicted mutation. Clinically significant progression was defined as a change in a measurable parameter in motor function (measured as change in assisting device with ambulation or loss of ambulation), progression in respiratory status (measured by the development of restrictive pulmonary disease or respiratory failure) and death.

Figure 7.

Light microscopic photomicrograph of the muscle biopsy demonstrating poor fiber type differentiation on ATPase (A) and succinate dehydrogenase (B) enzyme histochemistry. ATPase staining shows that type 1 fibers are poorly differentiated, whereas in the SDH stain type 1 and type 2 fibers cannot be distinguished at all.

Electronmicrograph demonstrating broadening of the A-bands, streaming of the A-bands into the Z-bands and disruption of the sarcomers (C and D).

Figure 8.

Light microscopic photomicrograph of the sural nerve reveals diffuse hypomyelination consistent with congenital hypomyelinating neuropathy.

Figure 9.

Detection of normal and mutant *MPZ* alleles: (A) the DNA sequence of a normal allele in the region around 550 nucleotides downstream of the predicted translation start site, (B) the DNA sequence of the patient demonstrating one normal allele and one allele with the mutation 550_552delCTAinsG. (C) confirmation of the mutation by *Hha*I digestion: lane 1 - 100 basepair marker; lane 2 - patient, lanes 3 & 4 - parents; lane 5 - undigested PCR fragment, (D) the last 65 amino acids in the wild type MPZ protein are replaced with a novel 45 amino acid sequence. The bold and underlined four amino acids represent the protein kinase C target.