Mutational analysis and genotype/phenotype correlation in Turkish Charcot–Marie–Tooth Type 1 and HNPP patients


The major Charcot–Marie–Tooth Type 1 (CMT1) locus, CMT1A, and Hereditary neuropathy with liability to pressure palsies (HNPP) cosegregate with a 1.5-Mb duplication and a 1.5-Mb deletion, respectively, in band 17p11.2. Point mutations in peripheral myelin gene 22 (PMP22), myelin protein zero (MPZ), and connexin 32 (Cx32) have been reported in CMT1, and in PMP22 in HNPP patients without deletion. We have screened 54 CMT1 patients, of variable clinical severity, and 25 HNPP patients from Turkey, with no duplication or deletion, for mutations in the PMP22 and Cx32 genes. A novel frameshift mutation affecting the second extracellular domain of PMP22 was found in an HNPP patient, while a point mutation in the second transmembrane domain of the protein was detected in a CMT1 patient. Two point mutations affecting different domains of Cx32 were identified in two CMTX patients. Another patient was found to carry a polymorphism in a non-conserved codon of the Cx32 gene. The clinical phenotypes of the patients correlate well with the effect of the mutation on the protein.

Charcot–Marie–Tooth disease (CMT) is the most common inherited peripheral neuropathy, with a total prevalence rate of 1 in 2500 (1). Clinical features of CMT1, the major form of the disease, include progressive distal muscle weakness and atrophy, often with pes cavus deformity. These signs are accompanied by mild sensory loss and absence of deep tendon reflexes. All patients have considerably reduced nerve conduction velocities (NCVs) (2) and segmental de- and re-myelination with typical onion-bulb formation are evident on nerve biopsies (3). Clinical onset is usually during the second or third decade of life. There is a wide range of variation in clinical severity among unrelated individuals and even among family members (4), including identical twins (5). CMT1 is a genetically heterogeneous disorder that exhibits mostly autosomal dominant inheritance (2). The first locus for autosomal dominant CMT1, CMT1B, was mapped to chromosome 1q21-23 (6–8). The gene responsible for CMT1B codes for the major peripheral myelin glycoprotein myelin protein zero (MPZ) (9) that functions as a double adhesion molecule to hold together the myelin sheaths (10). The second locus, CMT1A, was mapped to chromosome 17p (11). CMT1A constitutes more than 70% of CMT1 cases (12). It is most often associated with a 1.5-Mb tandem duplication on chromosome 17p11.2-p12 (13), a region that contains the peripheral myelin protein 22 (PMP22) gene (14–17), or rarely with point mutations in the PMP22 gene, as reviewed by Nelis et al. (18).
An X-linked dominant form of CMT1 (CMTX) was found to be associated with point mutations in connexin 32 (Cx32), a gap junction protein gene (19).

Hereditary neuropathy with liability to pressure palsies (HNPP) is an autosomal dominant disorder causing variously located recurrent nerve palsies, precipitated by minor trauma.

HNPP is associated with decreased motor velocities, prolonged distal latencies, and altered sensory nerve action potentials, even in clinically non-affected areas (20). An interstitial deletion of the 1.5-Mb region, which is duplicated in the majority of CMT1A patients, was demonstrated in 84% of HNPP patients (12, 21). The description of mutations in the PMP22 gene of some HNPP patients, as reviewed in Nelis et al. (18), demonstrated the involvement of this gene in the pathogenesis of HNPP.

In this study, we report mutations in PMP22 and Cx32 genes in Turkish patients with inherited peripheral neuropathy. Five sequence alterations were identified, two of which represent novel mutations, and one represents a novel polymorphism.

Patients and methods

Patients

Blood samples were obtained from 64 unrelated CMT1 patients and 39 HNPP patients diagnosed according to well-established criteria (2, 3, 22, 23). Informed consent was obtained.

Duplication/deletion analysis

Patients were screened first for the presence of the CMT1A duplication or HNPP deletion on chromosome 17p11.2. Ten duplications and 14 deletions were detected by EcoRI/Sacl Southern blots hybridized with pNEA 102, a subclone of C20G2, which maps to the distal CMT1A-REP element (24). The presence of duplications or deletions was assumed by the detection of the 3.2- and 7.8-kb junction fragments in CMT1A and HNPP patients, respectively. Recombinations outside the hot-spot region were detected by visual comparison of the intensity of the hybridization signals of the EcoRI/Sacl fragments from the proximal and distal CMT1A-REPs (25). These results were confirmed by dosage analysis using restriction fragment length polymorphism probes pVAW401HE and pVAW409R3a (26).

The remaining patients, without duplication (54) and deletion (25), were further screened for the presence of point mutations in the coding and exon-flanking regions of Cx32 and PMP22 genes. We had previously identified two novel mutations in the MPZ gene in this patient group (27).

Amplification and SSCP

The coding region of Cx32 and the four coding exons of the PMP22 gene were amplified using the primer sequences described by Bergoffen et al. (19) and Roa et al. (28), respectively. Polymerase chain reaction (PCR) products were analyzed for the presence of SSCP on two different 0.5 × HydroLink MDE (FMC, USA) gels, one of which contained 4% glycerol. Products were visualized by silver staining.

Sequence analysis

Sequence analysis of exons 3 and 4 of PMP22, and amplicons 1, 2 and 3 of exon 2 of Cx32, was performed on purified PCR products using the same primers as for the SSCP analysis. For the automated sequencing, we used the ABI PRISM dRhodamine Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Restriction analysis

Ten microliters of the amplified products were digested with five units of the corresponding restriction enzyme in a final volume of 15 μl. Digestions were incubated at 37°C overnight. Except for the Nla III digestion in P34, restriction fragments were resolved on 2% agarose gels, stained with ethidium bromide, and visualized under UV. Nla III restriction fragments were resolved on an 8% polyacrylamide gel (19 acrylamide:1 bisacrylamide), followed by silver staining.

Results

SSCP and subsequent sequencing analysis identified point mutations in 5 patients. Two patients, P23 and P28, were found to carry mutations in the PMP22 gene, while 2 others, P10 and P34, had point mutations in the Cx32 gene. In addition to these, the fifth patient was found to have a polymorphism in the Cx32 gene (Table 1). The mutations detected were confirmed by restriction analysis. Restriction analysis was extended to 50 unrelated healthy controls. None had the variation, and thus we concluded that the mutations identified are not common polymorphisms.
Table 1. List of the PMP22 and Cx32 mutations presented in this study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Phenotype</th>
<th>Gene affected</th>
<th>Mutation type</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>*P23</td>
<td>HNPP</td>
<td>PMP22</td>
<td>Frameshift</td>
<td>364-365delCC</td>
<td>P122Late stop</td>
</tr>
<tr>
<td>P28</td>
<td>Severe CMT1</td>
<td>PMP22</td>
<td>Missense</td>
<td>215C&gt;T</td>
<td>S72L</td>
</tr>
<tr>
<td>P34</td>
<td>Moderate CMT1</td>
<td>Cx32</td>
<td>Missense</td>
<td>271G&gt;A</td>
<td>V91M</td>
</tr>
<tr>
<td>*P10</td>
<td>Moderate CMT1</td>
<td>Cx32</td>
<td>Missense</td>
<td>631T&gt;C</td>
<td>Y211H</td>
</tr>
<tr>
<td>P27</td>
<td>CMT1</td>
<td>Cx32</td>
<td>Silent</td>
<td>507C&gt;T</td>
<td>D169D</td>
</tr>
</tbody>
</table>

*Patient with novel mutation case.

Clinical findings

Patient P23 is a 20-year-old female who complained of 4th- and 5th-finger paresthesia of the right hand that regressed spontaneously in 6 months, at age 15. She had the same complaint at age 17, but paresthesia spread to other fingers of the same hand. Since then, she had night paresthesia of feet. She also had paresthesia when leaning on her elbows and crossing legs. At the age of 17, she was found to have bilateral pes causus, mild distal muscle weakness of the upper extremities with hyporeflexia and diminished vibratory sensation in the lower extremities. She also had bilateral median, ulnar and peroneal, and right radial hypoesthesia. Electrophysiology showed slowed ulnar motor NCVs (30 m/s), and prolonged distal latencies with multiple entrapment neuropathies (bilateral ulnar, median and peroneal). Radial nerve biopsy revealed a demyelinating neuropathy with tomacula in teasing.

Patient P28 is a 7-year-old male whose mother had pes causus without any clinical signs. He was affected since birth and motor milestones were delayed. His examination showed pes causus, distal wasting, weakness of all limbs, and areflexia. He was very ataxic and could not walk alone. Electrophysiology showed inexcitable nerves.

Patient P10 is a 21-year-old male. He had an affected male cousin and his mother and grandmother complained of hand tremor. He had hand tremor with an unsteady gait since age 13. At age 20, he was found to have pes causus, horizontal nystagmus and distal muscle wasting and weakness at the lower extremities, milder distal weakness at the upper extremities, Achillean areflexia, and diminished vibratory sensation at the lower extremities with ataxia. Electromyography (EMG) showed a sensorimotor axonal neuropathy with demyelinating features. His median motor NCV was 39 m/s. His sural nerve biopsy revealed a demyelinating hypertrophic neuropathy with moderate axonal loss. His mother was found to have pes causus, Achillean areflexia, and diminished vibratory sensation in her lower extremities. Her EMG showed slowed NCVs.

Patient P34 is a 22-year-old male whose mother was affected. He was found to have pes causus, mild distal weakness of upper limbs, moderate distal wasting and weakness of the lower limbs, and areflexia of lower limbs. He was ataxic on walking. Electrophysiology showed slowed motor NCVs (median motor NCV is 29 m/s), prolonged distal latencies with low compound muscle action potential amplitudes, and inexcitable sensory nerves.

There was no consanguinity in any of the families, except in a parent of P28, who were distant relatives. All patients had healthy siblings, except P23.

PMP22 mutations

Patient P23, who shows an HNPP phenotype, was found to carry a novel frameshift mutation, 364-365delCC. The 2-nucleotide deletion causes a shift in the reading frame sequence, starting from codon P122, which is 39 codons before the stop codon, and results in a protein 87 amino acids longer than the wild-type protein. The mutation abolishes a restriction site of Msp I that normally digests the 230-bp fragment into 160 and 70 bp (Fig. 1).

A previously reported missense mutation in the PMP22 gene associated with a severe CMT1 phenotype was found in patient P28. A C to T transition at nucleotide 215 causes a shift in the reading frame sequence, starting from codon P122, which is 39 codons before the stop codon, and results in a protein 87 amino acids longer than the wild-type protein. The mutation abolishes a restriction site of Msp I that normally digests the 230-bp fragment into 160 and 70 bp (Fig. 1).

A previously reported missense mutation in the PMP22 gene associated with a severe CMT1 phenotype was found in patient P28. A C to T transition at nucleotide 215 causes substitution of leucine for serine at amino acid position 72 (S72L).

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**Fig. 1.** Restriction analysis for patient P23. UD represents an undigested PCR product, and M is a length marker. N1, N2, N3, and N4 are PCR products from normal controls digested with Msp I. The digestion produces 160- and 70-bp fragments in the normal controls. The patient is heterozygous for the frameshift mutation, which abolishes the Msp I restriction site and leaves an undigested 230-bp fragment.
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**Fig. 2.** Restriction analysis for patient P28. UD represents an undigested PCR product and M is a length marker. *Taq* I digestion in the patient, P, leaves an undigested 217-bp fragment representing the abolishment of the restriction site in the mutant allele. PCR fragments from the normal controls, N1, N2, N3, and N4, show complete digestion of the 217-bp fragment into 144- and 73-bp fragments.

in the second transmembrane domain of PMP22. Restriction analysis with *Taq* I leaves the 217-bp PCR fragment of the mutant allele undigested and produces 144- and 73-bp fragments in the normal allele (Fig. 2).

**Connexin 32 mutations**

Patient P34 was found to carry a G to A transition at nucleotide 271, leading to the substitution of methionine for valine at codon 91 (V91M). The mutation affects the second transmembrane domain of connexin 32 and creates an *Nla* III restriction site. Cutting the 438-bp long PCR fragment from the normal allele produces 116-, 96-, 66-, 59-, 55-, 36-, and 10-bp fragments. The mutation results in digestion of the 59-bp fragment into 53- and 6-bp fragments by the enzyme (Fig. 3).

Another novel mutation, a T to C transition at nucleotide 631 in the *Cx32* gene, was detected in patient P10. The mutation is predicted to result in a tyrosine to histidine substitution at codon 211 (Y211H). Digestion of the 320-bp-long PCR product with *Rsa* I in normal individuals produces 254- and 66-bp fragments. Restriction analysis with *Rsa* I enzyme in the patient and his mother revealed the abolishment of the restriction site by the mutation (Fig. 4).

Screening for mutations in the coding exon of connexin 32 revealed a novel polymorphism, a C to T transition at nucleotide 507, in a CMT1 patient (P27). This variant does not change the incorporation of the amino acid aspartic acid (D169D) into the protein. The 438-bp PCR fragment from the normal allele has two *Mae* II restriction sites producing three fragments of 225, 142, and 71 bp. The nucleotide change in the patient abolishes the second restriction site and produces two fragments sized 367 and 71 bp (Fig. 5). This polymorphism was not detected in 20 unrelated healthy controls.

**Discussion**

In this study of 64 unrelated CMT1 patients and 39 unrelated HNPP patients, the frequencies of CMT1A duplication (15.6%) and HNPP deletion (35.9%) were found to be low compared to those...
reported for other populations (70.7 and 84%, respectively) (12). This can be attributed to the small number of our patients. Among the patients studied, 62.5% of CMT1 and 64% of HNPP patients were sporadic cases; thus, the possibility of an acquired neuropathy in some of them cannot be ruled out. In addition, four of the CMT1 cases without any mutation have NCV values very close to the cut-off value (38 m/s), indicating that they may have an axonal type of disease (CMT2).

In all the patients presented in this study, the severity of the phenotype seems to correlate with the predicted impact of the mutation detected. PMP22 is predicted to have four transmembrane domains in which the amino acid sequence is highly conserved across species (29). PMP22 has been hypothesized to act as an adhesion molecule interacting with other PMP22 proteins in the membrane or with other adhesion molecules located in the myelin sheath (30). Alterations in expression, as in CMT1A duplication and HNPP deletion patients, or in the structure of the protein, may impair the formation and/or the maintenance of peripheral myelin.

In patient P23, who exhibits an HNPP phenotype, the 2-nucleotide deletion affects the second extracellular domain of the protein. This frameshift starting from codon P122 results in an aberrant protein that is 87 amino acids longer than the wild type and is probably non-functional. This agrees with the hypothesis that HNPP is caused either by the loss of the complete PMP22 gene or a loss-of-function mutation in PMP22 (31–33).

Patient P28 was found to carry a missense mutation in codon 72 (S72L) of the PMP22 gene associated with a severe CMT1 phenotype. Interestingly, the same mutation was previously found to be associated with a severe CMT1 (previously known as Dejerine Sottas Syndrome) and congenital hypomyelination neuropathy in different patients (34–38). Similarly, two other mutations in the same codon, S72W (39) and S72P (40), were reported to cause a severe CMT1 phenotype. A change in this conserved amino acid residue in the transmembrane domain, therefore, might cause a dominant negative effect or a gain of function rather than a loss of function as in HNPP patients.

In fact, most of the PMP22 mutations are located in the putative transmembrane domains and tend to cause a more severe phenotype than that of the CMT1A duplication (18). Mutation analysis indicates the functional importance of the transmembrane domains of PMP22 and suggests that the protein acts as an anchor protein or as part of a channel, rather than as an adhesion molecule (41). However, further analysis needs to be done to shed light on the function of PMP22.

Cx32 belongs to the connexin family of channel-forming peptides. These channels are composed of two hemichannels or connexons, each composed of six identical connexin subunits arranged circularly in the plasma membrane. The four transmembrane domains of Cx32 (M1, M2, M3, and M4) are closely packed in a ring so that helix M3 forms the wall of the channel and helices M2 and M4 are on both sides of it (42).

The two Cx32 mutations detected in this study affect different domains of the protein and are associated with moderate CMT1 phenotypes. The Y211H mutation in patient P10 affects the third cytoplasmic domain of the protein towards the carboxy terminus and is very close to the membrane–cytoplasm boundary. This mutation introduces a positive charge and may interfere with gating stimulus control (43). A Y211X nonsense mutation associated with a moderate phenotype has been reported (44), causing the synthesis of a premature and therefore presumably non-functional protein.

The mutation in patient P34 affects the second transmembrane domain of Cx32 only slightly since a nonpolar amino acid is substituted by another nonpolar residue. The alteration may cause incorrect alignment of two exons, or lead to the inability to form a functional gap junction between two exons. Frameshift mutations in this domain were also reported to lead to a moderate phenotype because a truncated protein is not incorporated into the membrane; thus, formation of gap junctions by other connexins are allowed (45, 46).

Genotype/phenotype correlation in CMT1 is very important in assessing the function and the functional domains of the myelin proteins. Analysis of the mutations in the connexin 32 gene would reveal which parts of the molecule are important for the proper formation of functional gap junctions. Point mutations in PMP22 can result in CMT1 phenotypes of different severity, or HNPP phenotypes, depending on the effect of the mutation on the structure and function of the protein. Determination of the mutational spectrum of peripheral myelin genes, coupled with functional studies of the mutations, are required to ascertain the mechanism through which different abnormal myelin proteins and, consequently, myelin dysfunction can lead to variable expression and often to late-onset disease.

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