ANALYSIS OF THE Cx32, MPZ AND PMP22 MUTATIONS IN THE TURKISH CHARCOT-MARIE-TOOTH PATIENTS

by

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ABSTRACT

ANALYSIS OF THE Cx32, MPZ AND PMP 22 MUTATIONS IN THE TURKISH CHARCOT-MARIE-TOOTH PATIENTS

Charcot-Marie-Tooth disease is the most common hereditary peripheral neuropathy with a prevalence rate of one in 2500. Although the disease is classified as demyelinating and axonal CMT based on clinical investigations, genetic studies revealed more than 28 genes and subtypes for both axonal and demyelinating forms.

In a total of 161 unrelated CMT patients were analyzed in this study. The incidence of CMT1A duplication in the Turkish population was found to be significantly lower (41.6 per cent) compared to that of other populations (70 per cent). The frequency of the HNPP deletion was found to be 66.6 per cent. Although this rate is lower than that of other populations (86 per cent), the difference was not statistically significant.

In this study, five nucleotide variations were identified; a novel and a previously reported mutation in *Cx32*, a previously reported polymorphism in *MPZ* and two novel mutations in *PMP22*. Phenotype/genotype correlations were performed for the identified mutations and the observed phenotype of the patients was found to be compatible with the mutations they carry.

Identification of causative mutations in the patients verifies the clinical diagnosis and leads the clinicians for choosing the therapeutic approach. However, for the patients that could not be genetically diagnosed further research is required to identify the pathogenic mutations in other CMT genes. On the other hand, identification of further mutations and genotype/phenotype correlations are of critical importance and will help elucidation of the pathogenic mechanisms leading to the CMT disease.

ÖZET

TÜRKİYE'DEKİ CHARCOT-MARIE-TOOTH HASTALARINDA Cx32, MPZ, PMP22 GENLERİNDE MUTASYON ANALİZİ

Her 2500 kişiden birinde görülebilen Charcot-Marie-Tooth hastalığı en yaygın herediter periferik nöropatidir. Klinik bulgulara göre demiyelizan ve aksonal olarak sınıflandırılan hastalık için genetik çalışmalarla 28'den fazla gen ve alt tip belirlenmiştir.

Toplumumuzda CMT hastalığının temelini aydınlatmak amacıyla laboratuvarımızda sürdürülen incelemelerin bir devamı olan bu çalışmada toplam 161 CMT ve HNPP hastası incelenmiştir. Diğer toplumlarda CMT1 hastalarının yüzde 70'inde görülen CMT1A duplikasyonu hastalarımızın ancak yüzde 41.7'sında saptanmıştır. Bu bulgu, önceki çalışmalarımızda elde ettiğimiz bulgularla tutarlılık gösterse de istatistiksel olarak Avrupa ve Amerika topluluklarına oranla oldukça düşüktür. HNPP hastalarında yapılan delesyon incelemesinde ise diğer toplumlarda HNPP hastalarının yüzde 86'sında görülen HNPP delesyonların hastalarımızın ancak yüzde 66.6'sını etkilediği tespit edilmiştir. Bu sıklık Avrupa ve Amerika toplumlarına oranla düşük olmakla birlikte aradaki fark istatistiksel açıdan önemli değildir.

Bu çalışmada, SSCP ve DNA dizi analizi ile Cx32 geninde biri yeni biri önceden bildirilmiş iki mutasyon, MPZ geninde önceden bildirilmiş bir polimorfizim ve PMP22 geninde iki yeni mutasyon olmak üzere toplamda beş nükleotid değişimi saptanmıştır. Belirlenen mutasyonlar için genotip/fenotip karşılaştırması yapılarak sözkonusu değişimlerin beklenen fenotiplere neden olduğu gösterilmiştir.

Hastalığa neden olan mutasyonların tanımlanması bu hastalarda klinik tanıyı doğrulayarak klinisyenlerin uygun tedavi yöntemlerini seçmelerinde yardımcı olacaktır. Genetik tanısı gerçekleştirilemeyen hastalarda diğer CMT genlerinin incelenmesi ve patojenik mutasyonların belirlenmesi gerekmektedir. Ayrıca, mutasyonların belirlenmesi ve genotip/fenotip karşılaştırmaları CMT hastalığına neden olan mekanizmaların belirlenmesine ışık tutması açısından son derece önemlidir.

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A	Adenine
APS	Ammonium persulfate
ARMS	Amplification Refractory Mutation System
Bp	Base pair
C	Cytosine
CMAP	Compound muscle action potential
CMT	Charcot-Marie-Tooth Disease
CMT1A-REP	CMT1A Repetitive Extragenic Palindromic Sequences
CNS	Central Nervous System
Cx29	Connexin 29
Cx32	Connexin 32
DNA	Deoxyribonucleic acid
DSS	Dejerine Sottas Syndrome
dNTP	Deoxynucleosidetriphosphate
EDTA	Ethylenediaminetetraacetic acid
EGR2	Early growth response-2 gene
ER	Endoplasmic Reticulum
EtBr	Ethidium Bromide
G	Guanine
GARS	glcyl-tRNA synthetase
Gas-3	Growth arrest specific gene-3
GDAP1	Ganglioside-induced differentiation-associated protein
GJB1	Gap Junction Beta
HMSN	Hereditary Motor and Sensory Neuropathy
HNPP	Hereditary Neuropathies with Liability to Pressure Palsies
HSPB1	Heat-shock 27-kD protein-1
IPL	Intraperiod Line
Kb	Kilo base
KDa	Kilo Dalton

KIF1B	Kinesin family member 1B
LITAF	Lipopolysaccharide-inducedtumor necrosis factor-alpha
LMNA	Lamin A/C
MAG	Myelin Associated Glycoprotein
Mb	Mega base
MBP	Myelin Basic Protein
MDL	Major Dense Line
MFN2	Mitofusin 2
Min	Minute
MITE	Mariner insect transposon-like element
MPZ	Myelin Protein Zero
mRNA	Messenger ribonucleic acid
NCV	Nerve Conduction Velocity
NEFL	Neurofilament-light gene
OD_{260}	Optical density at 260 nm
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PM	Plasma membrane
PMP22	Peripheral Myelin Protein
PNS	Peripheral Nervous System
pUB(n)	polyubiquitinated
RAB7	Small GTPase late endosomal protein
RBC	Red Blood Cell
RPM	revolution per minute
SC	Schwann cell
SDS	Sodiumdodecylsulphate
SSCP	Single Strand Conformation Polymorphism
STR	Short Tandem Repeats
T	Thymine
TBE	Tris-EDTA- Boric acid
TE	Tris-EDTA
TEMED	N, N, N, N’-Tetramethylethylenediamine
Tr	Trembler

Tr-J	Trembler-J
Tr-Ncnp	Trembler Ncnp
U	Unit
UTR	Untranslated region
UV	Ultraviolet
WF	Wild-type forward
WR	Wild-type reverse

1. INTRODUCTION

Charcot-Marie-Tooth disease (CMT), also known as hereditary motor and sensory neuropathy (HMSN), is the most common form of hereditary peripheral neuropathies with a prevalence rate of one in 2500 (Skre, 1974). The disease was first described by Jean Martin Charcot, Pierre Marie and Howard Henry Tooth in 1886 (Charcot and Marie, 1886, Tooth 1886) and comprises a heterogeneous group of disorders affecting both motor and sensory nerves.

The clinical features of CMT includes, slowly progressive muscle weakness, atrophy especially in the lower extremities, diminished or absent deep tendon reflexes and skeletal deformities such as pes cavus (Harding and Thomas 1980; Charcot and Marie, 1886). However, the clinical severity of the disease ranges in a broad spectrum, -from asymptomatic individuals to very severe cases with distal atrophy.

Based on clinical investigation and electrophysiological data, CMT was classified as demyelinating and axonal according to whether axons or myelinating Schwann cells are affected primarily. Markedly reduced NCVs and onion bulb formation are the main clinical indicators of demyelinating CMT. On the other hand, axonal form of CMT is characterized by normal or near normal NCV values and reduced compound muscle action potentials (CMAP). However, NCV and clinical evaluation can be inadequate in many cases since these indicators can overlap. Furthermore, even within the same subtype, the phenotypic expression of the disease is heterogeneous. Thus, molecular tools are very important to define the types of CMT. Today, 28 different loci and 21 genes are known to be responsible for CMT phenotype (Table 1.1.).

The disease may segregate in autosomal dominant/recessive or X-linked dominant/recessive fashion (McKusick, 1990).

Table 1.1. Genetical heterogeneity of CMT

Clinical Type	Genetic Subtype	Mode of Inheritance	Locus	Gene
Demyelinating	CMT1A	AD	17p11.2-p12	*PMP22*
	CMT1B	AD	1q22	*MPZ*
	CMT1C	AD	16p13.3-p12	*LITAF*
	CMT1D	AD/AR	10q21.1-q22.1	*EGR2*
	CMT1X	X-linked D	Xq13.1	*Cx32*
	CMT4A	AR	8q21.1	*GDAP1*
	CMT4B1	AR	11q22	*MTMR2*
	CMT4B2	AR	11p15	*SBF2*
	CMT4C	AR	5q32	*SH3TC2*
	CMT4D	AR	8q24.3	*NDRG1*
	CMT4E	AR	10q21-q22	*EGR2*
	CMT4F	AR	19q13.1-q13.2	*PRX*
	CMT DIA	AD	10q24.1-q25.1	Unknown
	CMT DIB	AD	19p12-p13.2	*DNM2*
Axonal	CMT DIC	AD	1p34	*YARS*
	CMT2A1	AD	1p36.2	*KIF1Bβ*
	CMT2A2	AD	1p36-p35	*MFN2*
	CMT2B	AD	3q13-q22	*RAB7*
	CMT2C	AD	12q23-24	Unknown
	CMT2D	AD	7p14	*GARS*
	CMT2E	AD	8p21	*NEFL*
	CMT2F	AD	7q11-21	*HSPB1*
	CMT2G	AD	12q12-q13.3	Unknown
	CMT2I	AD	1q22-23	*MPZ*
	CMT2J	AD	1q22-23	*MPZ*
	CMT2L	AD	12q24	*HSPB8*
	AR-CMT2A	AR	1q21.2	*LMNA*
	AR-CMT2B	AR	19q13.3	Unknown
	CMT2K	AR	8q21.1	*GDAP1*
	CMT2H	AR	8q21.1	*GDAP1*
	CMTX2	X-linked R	Xq24.2	Unknown
	CMTX3	X-linked R	Xq26	Unknown
	CMTX4	X-linked R	Xq24-q26.1	Unknown
	CMTX5	X-linked R	Xq21.32-q24	Unknown

1.1. Charcot-Marie-Tooth Disease Type 1 (CMT1)

The demyelinating CMT, CMT type 1 (CMT1), is the most common type of CMT (Dyck, 1984). Electrophysiological studies show reduced NCVs, usually smaller than 38 m/s. Hypertrophic myelin alterations and onion bulb formations on biopsy samples are the other features of CMT1 (Baxter *et al*., 2002). Although clinical severity of CMT1 is variable, reduced NCV is a common feature suggesting an intrinsic Schwann cell defect resulting in abnormal myelination.

Myelin is highly specific to higher vertebrates, and is made up by Schwann cells in the peripheral nervous system (PNS) and oligodendrocytes in the central nervous system (CNS) (Bronstein, 2000). Myelinating Schwann cells originate from a multipotent cell population in embryonic neural crest. After cell divisions, these multipotent cells migrate and form Schwann cell progenitors. The progenitor cells contact developing peripheral axons and form immature Schwann cells. Some of the promyelinating cells establish one-to-one association with axons in a special stage termed as 'promyelinating stage' (Webster 1993). The Schwann cells that receive promyelinogenic signals from the axons differentiate into myelinating phenotype and cover the developing axons while the others differentiate into non-myelinating phenotype (Kamholz *et al*., 2000 and Scherer, 1997).

During differentiation into myelinating phenotype, large amounts of membrane is produced, myelin associated genes are upregulated and cytoskeleton is modified (Scherer, 1997, Rummler *et al*., 2004). Schwann cells wrap around the axon until the axon is tightly covered with membrane (Figure 1.1). Non-myelinating Schwann cells do not express myelin related genes.

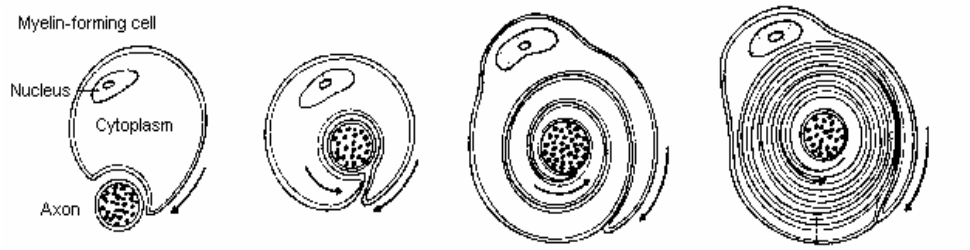


Figure 1.1. Myelination of axons

Myelin sheath is composed of two domains; compact and non-compact myelin containing different sets of proteins. The main component of compact myelin, which constitutes bulk of myelin sheath, is lipids such as cholesterol and sphingolipids (Wrabetz *et al*., 2004). In addition, this domain contains Myelin Protein Zero (MPZ), Peripheral Myelin Protein 22 (PMP22) and Myelin Basic Protein (MBP). Non-compact myelin contains mainly Connexin32 (Cx32), Connexin29 (Cx29), and Myelin Associated Glycoprotein (MAG) (Figure 1.2). The molecular architecture of myelin is highly specific and depends on accurately controlled synthesis and trafficking of myelin related proteins (Wrabetz *et al*., 2000).

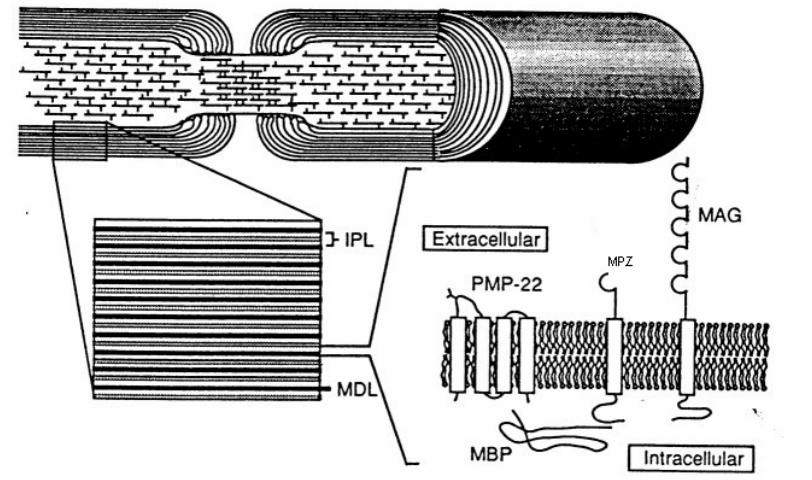


Figure 1.2. Diagram showing relationship of myelin proteins and structure of the myelinated axon in the peripheral nervous system (Suter *et al*., 1993).

Myelin sheaths function as an insulator around the axon and decrease the dispersion of the electrical current into the encircling medium while it is transmitted

through the axon. By this way, it provides faster and more efficient transmission of nerve impulses (Rummler et. al., 2004).

Integrity of axon-Schwann cell interactions is necessary for the maintenance of myelinating phenotype even after myelination has been completed (Scherer and Salzer, 1996). Altered communication between myelin and other tissue components such as fibroblasts, resident macrophages and extracellular matrix may lead to disruption of complex cellular organization of the peripheral nerve (Weinberg and Spencer, 1976). Axons and myelinating Schwann cells are vulnerable to the effects of mutations in many genes. Today, numerous animal models are available and wildly used to explain mutation dependent disease mechanisms of CMT.

The myelinating Schwann cells (SC) are primarily affected in the demyelinating form of CMT (Griffin and Hoffman 1993). Subsequent degeneration of axons can occur for reasons, which are not well-understood (Frei *et al*., 1999).

CMT1 is genetically heterogeneous and is inherited as an autosomal dominant/recessive or X-linked trait.

In early studies with CMT1 families, linkage to the Duffy blood group locus on chromosome 1 was shown (Bird *et al..,* 1980, 1982). The finding was later confirmed in a large Indian family, in which close linkage to the FC gamma R2 gene in the Duffy locus was identified. (Stebbins and Conneally, 1982; Lebo *et al.*. 1989). However, in some other studies linkage to the Duffy locus was excluded (Guiloff *et al*., 1982; Bird *et al*, 1983; Dyck *et al*. 1983; Marazita *et al*., 1985; Rossi *et al*., 1985; Ionasescu *et al*., 1987; Griffiths *et al.*., 1988). Subsequently, in two separate studies, linkage of CMT1A to 17p11.1.2-p12 locus was shown with two DNA markers, pEW301 and pA10-41 (Raeymaekers *et al*., 1989 and Vance *et al*., 1989) and linkage to Duffy locus was designated as CMT1B (Chance and Bird, 1989).

1.1.1. CMT Type 1A

CMT1A is the most common genetic subtype of CMT Type 1 and constitutes 70 per cent of all CMT cases (Nelis *et al*., 1996; Wise *et al*., 1993). In contrast to variability of clinical severity, it is characterized by slowed NCV (average around 20 m/s) (Kaku *et al*., 1993).

As an evidence of progressive nature of the disease, properties of nerve biopsy samples of CMT1A patients differ with age; demyelination is more common in children and hypomyelinated axons become more numerous with age (Wrabetz *et al.*, 2004).

Positional cloning studies to refine the chromosomal region of CMT1A led to identification of a tandem DNA duplication of 1.5 Mb on chromosome 17p11.2-12 responsible for the disease phenotype (Lupski *et al*., 1991). There are two homologous DNA sequences, defined as distal and proximal CMT1A-REP, flanking the 1.5 Mb DNA segment (Pentao *et al*., 1992 and Reiter *et al.*., 1997). Restriction enzyme endonuclease mapping indicated that each repeat element is approximately 24 kb in length (Kennerson *et al*, 1997). It is hypothesized that the high degree of homology between the repeats (more than 98 per cent) mediates misalignment and causes unequal crossing over during meiosis. This mechanism generates a duplicated and a deleted allele. Duplication and deletion result in CMT1A and Hereditary Neuropathies with Liability to Pressure Palsies (HNPP), respectively (Chance et.al., 1993). Homogeneity of size of the duplications and deletions in unrelated CMT1A and HNPP patients (Chance *et al*, 1993 and Wise *et al*, 1993) and high frequency of *de novo* duplications and deletions indicate that the mechanism is a precise and recurring one.

It was shown that all crossover break points in a series of CMT1A and HNPP patients are in CMT1A-REP region (Kiyosova *et al*, 1995). Seventy seven per cent of CMT1A and HNPP chromosomes contain breakpoints within a 7.9 kb interval of the CMT1A-REP repeats (Kiyosova and Chance, 1996). Presence of recombinational hotspot in this region was independently confirmed and a 700 bp length, mariner like

element was found in a 3.2 kb interval that is in the hotspot region (Reiter *et al*, 1996). This element, termed Mariner insect transposon-like element (MITE), was proposed to be cleaved by a transposase at or near the 3' end and so mediate strand exchange events.

The peripheral myelin protein gene (*PMP22*) is located within the CMT1A-REP region and duplication and deletion of *PMP22* is associated with CMT1A and HNPP, respectively (Lupski *et al*, 1992 and Pentao *et al* 1992). Most of the CMT1A patients carry three intact copies of *PMP22*; one copy on the normal chromosome and two copies on the mutated one (Warner *et al*., 1996). Patients who carry homozygous *PMP22* duplication were also reported (Lupski *et al*., 1991; Kaku *et al*., 1993 and LeGuern *et al*., 1997). Most cases of CMT1A are inherited from an affected parent indicating that the disorder does not affect the reproductive fitness. *De novo* duplications and deletions were reported to be frequent in all populations indicating absence of ethnic preference (Raeymaekers *et al*, 1991, Chance *et al*, 1993 and Wise *et al*, 1993). Most of the *de novo* cases result from an error in spermatogenesis and have paternal origin (Palau *et al*.., 1993). Approximately, ten per cent of the cases have maternal origin (Blair *et al*, 1996); suggesting that the misalignment of the CMT1A-REP repeats occur more frequently in male meiosis. Patients who are mosaic for the duplication in several somatic tissues were also reported (Sorour *et al*. 1996, Rautenstrauss *et al*., 1998). This indicates that reversion of the duplication can occur during mitosis (Liehr *et al*., 1996).

The 1.5 Mb tandem duplication is responsible for 71 per cent of inherited and 90 percent of sporadic cases of CMT1A (Wise *et al*., 1993).

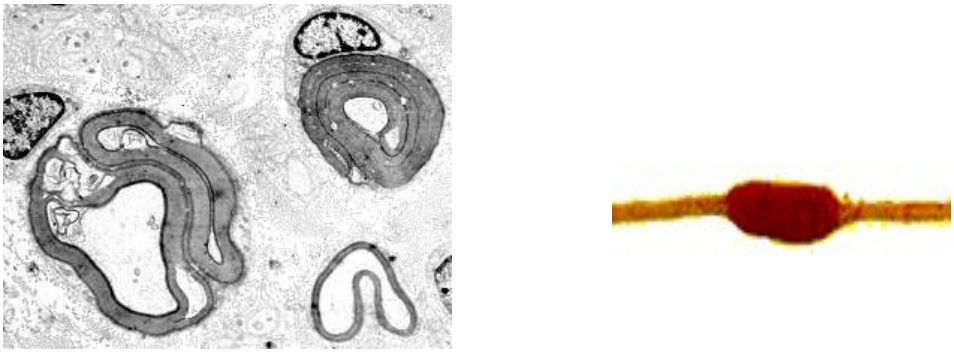
1.1.2 Hereditary Neuropathies with Liability to Pressure Palsies (HNPP)

Hereditary neuropathy with liability to pressure palsies (HNPP), also called familial recurrent polyneuropathy or tomaculous neuropathy, is an autosomal dominant disease which causes episodic, recurrent, demyelinating neuropathy (Windebank, 1992). First descriptions of disease were made by De Jong in 1947 and Davies in 1954 (De Jong, 1947

and Davies, 1954). HNPP usually has an onset during adolescence and cause attacks of numbness, muscular weakness and atrophy (Davies, 1954; Earl *et al*., 1964 and Staal *et al*., 1965).

Nerve biopsy samples of HNPP patients reveal presence of segmental demyelination (Figure 1.3.a) and tomaculae (Figure 1.3.b), that are sausage-shaped swellings of the myelin sheath in sensory and motor nerves (Madrid and Bradley, 1975 and Oda *et al*., 1975). NCVs are sometimes mildly reduced in HNPP patients and asymptomatic carriers.

HNPP was identified by using DNA markers spanning the duplicated region of 17p11.2 and a large interstitial deletion in three HNPP familes was investigated in this region (Chance *et al*., 1993). Subsequently, it was shown that the deletion spanned approximately 1.5 Mb and included all DNA markers that were known to be duplicated in CMT1A. Uniformity of the deleted region in all pedigrees was shown (Chance *et al*., 1993). This region includes the gene for peripheral myelin protein 22 (PMP22), the gene that is duplicated or the site of point mutation in CMT1A. The breakpoints in HNPP deletion and CMT1A duplication map to the same interval on chromosome 17p11.2 showing that these genetic disorders may be the result of reciprocal products of unequal crossover. More than 84 per cent of inherited and 86 per cent of sporadic cases of HNPP are associated with 1.5 Mb HNPP deletion (Nelis *et al*., 1996 and Reiter *et al*, 1997).



(a) (b)

Figure 1.3. Segmental demyelination (a) and tomaculae (b) in nerve biopsy samples of HNPP patients (Madrid and Bradley, 1975)

1.1.3. Peripheral Myelin Protein 22 and Molecular Mechanisms of CMT1A and HNPP

PMP22, also known as growth arrest specific gene-3 (*gas-3*) was first identified in degenerating distal stump of injured rat sciatic nerves, where its transcription is downregulated and NIH-3T3 fibroblast cell line, where its transcription is elevated (Jetten and Suter, 2000; Snipes *et al.*, 1999). The human *PMP22* spans approximately 40 kb and contains four coding exons and two 5' untranslated exons known as 1A and 1B (Suter at al. 1994, Nelis *et al*. 1999a). It is primarily expressed by myelinating Schwann cells in the PNS, especially during myelin formation (Snipes *et al*., 1992). Small amounts of *PMP22* are expressed in the CNS and other tissues (Snipes *et al*., 1992). In rats, mice and humans transcription of neuronal and non-neuronal forms of *PMP22* is regulated by two alternatively used promoters, promoter one and two, present in the upstream region of their corresponding exons 1A and 1B (Figure 1.4) (Suter *et al*., 1994). The relative expression of alternative *PMP22* transcripts is tissue specific. High levels of exon 1A transcripts are coupled to myelin formation and exon 1B transcripts as well as exon 1A transcripts are present in non-neural tissues including lung, heart, intestine and skeletal muscle (Suter *et al*. 1994 and Marier *et al*. 2003).

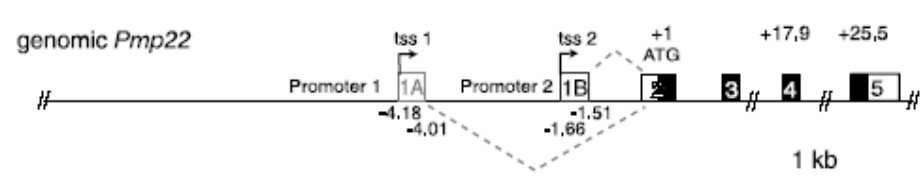


Figure 1.4. Diagram of *Pmp22* genomic locus with promoter 1 preceding the non-translated exon 1A and promoter 2 in the upstream exon 1B in mice.

The PMP22 gene encodes a 160-amino acid, membrane-bound polypeptide precursor with a molecular weight of 18 kilo Dalton (kDa). The polypeptide is post-translationally modified by N-linked glycosylation to form 22-kDa mature glycoprotein (Manfioletti *et al*., 1990). PMP22 protein has four transmembrane domains, two extracellular loops, one intracellular loop and cytoplasmic N and C

termini (D'Urso and Müller, 1997). The protein mainly localizes to the compact myelin sheath and constitutes two-five per cent of the total myelin protein (Pareek *et al*., 1993 and Haney *et al*., 1996).

Immunofluorescence labeling and confocal microscopy showed that, PMP22 is synthesized in Endoplasmic Reticulum (ER) (D'Urso and Müller, 1997). To ensure folding properly, PMP22 interacts with a set of chaperon proteins and folding enzymes during biosynthesis. In normal Schwann cells, a small fraction of newly synthesized PMP22 traverses the Golgi, acquires a complex N-linked sugar moiety and traffics to the Schwann cell's membrane. PMP22, like the other membrane proteins, is subjected to the quality control mechanisms in ER or Golgi. The proteins that can not pass the quality control mechanisms are refolded in the ER or polyubiquitinated and rapidly degraded by proteasome in the cytosol (Fortun *et al*., 2006). It has been shown that, approximately 80 per cent of newly synthesized PMP22 is degraded within 30 minutes by the ubiquitin-proteasome pathway probably because of improper glycosylation or folding (Pareek *et al*., 1997 and Ryan *et al*, 2002).

The exact function of PMP22 still remains elusive. Based on its secondary structure and cellular localization, it is proposed to act as a channel protein connecting the outer surface of the myelin sheath with the periaxonal space and function in transportation of small nutrients, metabolites and ions (Nelis *et al*., 1999). It might also have an adhesive role and interact with other adhesion molecules located in the compact myelin (Suter and Snipes, 1995). It is known that the protein forms a complex with MPZ and this complex has been proposed to stabilize myelin by holding Schwann cell membranes together. PMP22 is most likely involved in controlling myelin thickness and maintenance. It also affects cell shape, proliferation and apoptosis of Schwann cell (Fabbretti *et al*., 1995; Karlsson *et al*., 1999). After myelination starts, Schwann cells stop dividing and PMP22 might have a role in suppressing Schwann cells mitosis besides it functions in membrane trafficking and organizing the myelin membrane layers (Chies *et al*., 2003 and Dicson *et al*., 2002). PMP22 is shown to colocalize with tight junctional complex in non-neural tissues suggesting that, it acts in formation of intracellular junctions of epithelial cells

(Notterpek *et al*, 2001; Roux *et al*., 2004). Since that finding, PMP22 is grouped with the claudin family of tight junction proteins because of its amino acid sequence similarities.

Molecular genetic studies with naturally occurring and genetically engineered rodents reveal that *PMP22* is a highly dosage sensitive gene. Overexpression studies in rat demonstrated that high levels of PMP22 do not impair with the early stages of myelination and do not affect the expression of other myelin genes. However, these rats show similar clinical and histological features as CMT1A patients. $PMP22^{tg}$ is a transgenic mouse model carrying additional copies of *PMP22* (Maygar el al, 1996). These mice show hypomyelination, reduced NCVs, increased number of non-myelinating Schwann cells in the affected nerves. Furthermore, it was shown that increase in copy number of *PMP22* results in a more severe phenotype in animal models and human (Huxley *et al*, 1996; Lupski *et al*. 1991 and Kaku *et al*., 1993). All of these findings supports the idea that the functioning of the protein is dosage dependent and the amount of PMP22 protein that is produced, transported and inserted into the plasma membrane must be tightly controlled (Brugnoli *et al*., 2005). Regulatory mechanisms functioning in these control steps are likely to be affected in the disease status. Gene dosage alteration is proposed as a disease mechanism and it was hypothesized that elevated expression of *PMP22* alters Schwann cell differentiation (Hannemann *et al*., 1994). On the other hand, interactions of PMP22 with other myelin proteins may require a well-defined stoichiometry and altered dosage of PMP22 may disrupt this stoichiometry (Nelis *et al*., 1999).

To date, approximately 50 different mutations in *PMP22* have been identified associated with CMT1A, HNPP or a more severe condition, Dejerine Sottas Syndrome (DSS). Most of them are found in the transmembrane domains of PMP22 protein indicating functional importance of these domains. Some of these mutations may result in production of nonfunctional proteins. However, most of the PMP22 point mutations cause phenotypes more severe than HNPP, and duplication related CMT1A (Valentijn *et al*., 1993 and Roa *et al*., 1993). It was hypothesized that point mutations in the gene that cause loss of function are responsible for HNPP and the ones leading to gain of function are responsible for CMT type 1. These mutations

must produce a gain of abnormal function due to either a dominant negative effect or a toxic effect. Most of the *PMP22* point mutations are associated with trafficking impairment (Isaacs *et al*., 2002; Naef *et al*., 1997 and Sanders *et al*., 2001) and mutated proteins can not reach to the membrane and accumulate in aggresomes within the cells in animals. So, altered degradation of *PMP22* along the secretory pathway has been suggested as a disease mechanism. The Trembler (Tr) mice naturally carry a dominant point mutation which results in glycine to aspartate substitution in a membrane associated region of *PMP22* (Suter *et al*., 1992b). The mice exhibit severe hypomyelination in early development, aberrant continuous Schwann cell proliferation and reduced NCVs. The same mutation was found in two DSS patients in the same family (Ionasescu *et al*., 1997). Homozygous Tr mice are devoid of myelin, while heterozygous Tr mice have about 5-30 per cent of the normal number of myelinated fibers (Henry and Sidman, 1988). Comparison of the heterozygous and homozygous phenotypes suggests that Tr point mutation leads to a toxic gain of function (Adolkofer *et al., 19*97). The trembler protein is not transported to the plasma membrane and unlike the wild type PMP22 protein, does not promote cell death (Brancolini *et al.,* 1997; Naef *et al*., 1997; Zoidl *et al*, 1997). In addition, the Trembler protein can form heterodimers with the wild type protein and prevents its normal trafficking in the cell by this direct interaction (Tobler *et al*., 1999). It is a potential explanation for dominant inheritance. Tembler-J (Tr-J) is another naturally occurring mouse model, which carries a proline to leucine mutation in the first transmembrane domain of PMP22 (Suter *et al*. 1992a; 1992b and Valentijn *et al*., 1992). The Tr-J mouse shows similar pathological features as CMT1A patients with identical mutation (Valentijn *et al*., 1992), including thinly myelinated axons, demyelination and remyelination, onion bulb formation and altered Schwann cell proliferation (Notterpek *et al*. 1997). The third naturally occurring mouse model is Trembler Ncnp (Tr-Ncnp), which is associated with an in-frame deletion of *PMP22* exon four resulting in loss of 47 amino acids in transmembrane domains 2 and 3 (Suh *et al*., 1997). Homozygous Tr-Ncnp animals show amyelination with huge vacuolar aggregates in Schwann cells, suggesting absence of PMP22 these transmembrane domains disrupts the function of protein and cause its aggregation in cytoplasm (Suh *et al*., 1997). Furthermore, coexpression of

Tr and Tr-J mutation in Hela and Schwann cells causes an overload of PMP22 protein in the ER while the wild-type protein can still reach to the cell surface.

To understand the molecular mechanism of HNPP, *PMP22* deficient mice were constructed (Adlkofer *et al*., 1995). Homozygous *PMP22* null mice show tomaculae at an early onset, onion bulbs and myelin degeneration at an older onset. Heterozygous *PMP22* deficient mice developed much milder phenotype confirming dosage sensitivity of *PMP22*. The components of compact myelin proposed to have a set of stoichiometry and as well as overexpression of *PMP22*, underexpression of the gene may perturb stoichiometry of myelin sheath and results in destabilization of the myelin sheath (Scherer *et al*., 1997).

In addition to deletion of *PMP22* gene, frame-shift mutations were shown to cause HNPP phenotype. This is consistent with the idea that loss of function is the disease mechanism underlying HNPP.

According to all of these findings, it is likely that, the mutated proteins accumulate in the cell and cause a toxic gain of function. Based on animal model studies, Fortun and collegues offered a compensatory mechanism for both point mutation and duplication related CMT1A (Fortun *et al*., 2006). In normal cells, most of PMP22 is polyubiquitinated (pUb(n)) and rapidly degraded by the proteasome, whereas a small fraction traffics to Golgi and is incorporated into the plasma membrane (PM). Mutations in PMP22, such as the L16P in the TrJ mouse, or overexpression, like in the C22 mice, overwhelm the degradative capacity of the proteasome, leading to the formation of protein aggregates. The amount of PMP22 targeted for degradation is increased in the gene duplications and point mutations. In this situation, turnover rate of the abnormal protein reduces and its accumulation, together with other ubiquitinated substrates occurs. These aggregates form prior to the medial Golgi and associated with autophagosomes/autolysosomes and lysosomes, indicating that Schwann cells activate autophagy to cope with accumulation of abnormal aggregates and maintain cellular homeostasis (Figure 1.5.) (Fortun *et al*., 2006). Up to date, such a compensatory mechanism was not reported in human. It

was hypothesized that aggresome formation may contribute to the cellular alterations and subsequent demyelination in human (Notterpek *et al.,* 1999).

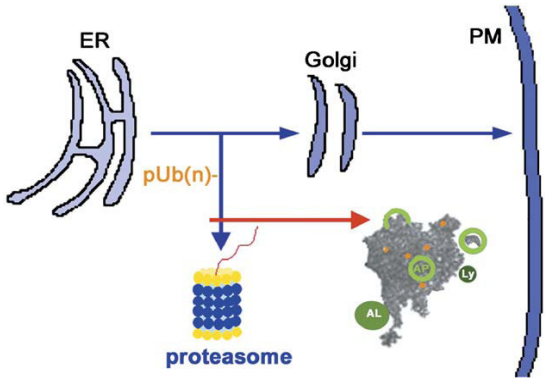


Figure 1.5. Model for protein aggregation in PMP22-associated neuropathies.

In contrast to dominant mutations, in recessive mutations and polymorphisms, PMP22 proteins can reach the cell membrane although not as efficiently as wild-type PMP22 and do not form large aggregates indicating less effected trafficking (Liu *et al*., 2004).

1.1.4. CMT Type 1B:

Genetic analysis indicated that CMT1B is linked to 1q21.3-q23 locus, where the myelin protein zero (*MPZ* or P_0) gene is located (Bird *et al*., 1997).

MPZ gene was initially cloned from rat (Lemke and Axel, 1985 and Lemke *et al*., 1988), and has six exons in rats, mice and humans (Lemke *et al*, 1988 and Hayasaka *et al*., 1993). The gene spans 7 kb and is expressed mainly by myelinating Schwann cells.

The MPZ gene encodes a protein of 248 amino acids; the 29 amino acid signal peptide is cleaved before the insertion of final 219 amino acid protein into the myelin sheath (Lemke *et al*., 1988). MPZ is a single pass transmembrane protein, which has an extracellular immunoglobulin-like domain (amino acids one through 124), a transmembrane domain (amino acids 125 through 150) and a cytoplasmic domain

(amino acids 151 through 219) enriched with basic residues (Lemke and Axel, 1985). The amino acid sequence of the protein is evolutionary conserved and shows 95 per cent similarity in human, rat and cow (Hayasaka *et al*., 1993).

MPZ is the most abundant protein of the peripheral nervous system (Greenfield *et al*., 1973). The protein is normally targeted to compact region of the myelin sheath along with PMP22 and MBP. MPZ probably interact with these proteins in stringent stoichiometry and participate in myelin formation and maintenance. Cytoplasmic domain of the protein is very important in compaction of the Schwann cell cytoplasm and formation of major dense line. Furthermore, MPZ plays an adhesive role in myelination by holding adjacent wraps of membrane through homophilic interactions of extracellular domain.

Cell culture and animal model studies showed that MPZ gene dosage must be tightly controlled. A mouse model homozygous for *MPZ* deletion developed a DSS phenotype with severely hypomyelinated nerves, onion bulbs and reduced NCVs (Zielasek *et al*., 1996 and Martini *et al*., 1995). On the other hand, heterozygous mice have a rather mild phenotype and develop de- and re-myelination and formation of onion bulbs. Their NCVs were slightly reduced and they have CMT1 phenotype. These pathological effects can be explained by a loss of function mechanism.

Overexpression of *MPZ* results in dysmyelinating neuropathy in mice (Wrabetz *et al*., 2000) and as the copy number and the level of transgenic mRNA rise, the phenotype worsens. Increased dosage of MPZ may alter trafficking in the promyelin forming Schwann cells (Yin *et al*., 2000) or the protein may perturb stoichiometry of the myelin sheath.

More than 70 point mutations were detected in *MPZ* gene and these mutations are associated with CMT1B, DSS and congenital hypomyelination (CH). Shy and colleagues found that CMT1B associated *MPZ* mutations has later onset compared to DSS. They hypothesized that early onset *MPZ* mutations disrupt the tertiary structure of the MPZ protein and interfere with MPZ mediated adhesion during myelination,

whereas late onset neuropathy was thought to stem from mutations, which allow myelination but disrupt Schwann cell axonal interactions (Shy *et al*., 2004).

Most of the *MPZ* mutations were found in the cytoplasmic domain of the protein indicating functional importance of this region (Wong and Filbin, 1994). They might prevent insertion of the protein within the plasma membrane or result in loss of adhesion function of MPZ. However, humans with heterozygous missense mutations are more severely affected than the heterozygous *MPZ* knockout mice. Although this may stem from the difference of the two organisms, it is possible that missense mutations exert their effect via a gain of function or dominant negative effect. These mutations may lead to retention and aggregation of the protein in the ER and altered trafficking of the mutant protein may induce apoptosis in the Schwann cells (Khajavi *et al*., 2005).

1.1.5. CMT Type 1C and 1D

In two families with CMT1 phenotype no linkage to CMT1A or CMT1B was found suggesting further genetic heterogeneity (Chance *et al*., 1990). Street and collegues mapped the CMT1C locus to chromosome 16p13.1-p12.3 (Street *et al*., 2002). Subsequently, lipopolysaccharide-induced tumor necrosis factor-alpha factor (LITAF) gene was identified as the causative gene for CMT1C (Street *et al*., 2003). Another subtype of CMT1 was found to be caused by mutations in the early growth response-2 gene (*EGR2*) located on 10q21.1-q22.1 and designated as CMT1D (Warner *et al*., 1998). EGR2 is a Schwann cell transcription factor and is thought to regulate the expression of late myelin genes such as *MPZ* and MBP, thus playing a key role in myelogenesis (Nagarajan *et al*., 2001).

1.1.6. X-LINKED CMT1

CMT1X is the second most common cause of CMT1 after CMT1A duplication. Initial linkage studies mapped CMTX gene to the proximal long arm of X chromosome (Xq13.1), where the Connexin32 (*Cx32*)/Gap Junction Beta 1 (*GJB1*) gene is known to be

located (Bergoffen *et al*., 1993a and Corcos *et al*., 1992). Subsequent direct sequencing of the Cx32 gene in CMTX patients revealed that CMTX is associated with *Cx32* mutations (Bergoffen *et al*., 1993b). CMT1X is considered to be an X-linked dominant disorder because it affects female carriers. Clinical severity of the disorder varies among affected females, probably owing depending on the proportion of the inactivation X chromosome carrying the mutant *Cx32* allele (Scherer *et al*., 1998). These patients usually have a later onset (after the end of second decade) and milder symptoms, while the affected males have an earlier age of onset and more severe clinical features. The clinical manifestation of CMT1X is nearly indistinguishable from those seen in patients with CMT1A or CMT1B. However, pattern of inheritance and faster NCVs (25-40m/s) than in typical CMT1A and CMT1B help to discriminate CMT1X from other CMT1 subtypes. In addition, there are more evidence of de- and remyelination in biopsies of male CMT1X patients (Sander *et al*., 1998).

1.1.5.1. Connexin32. Cx32 belongs to connexin protein super family. Like the other members of the family, the protein has four transmembrane domains, two extracellular loops, an intracellular loop and cytoplasmic N- and C- termini (Buruzzone *et al*., 1996 and Simon and Goodenough, 1998). In the plasma membrane, six connexins oligomerize to form a highly ordered hemichannel, termed connexon. A connexon can interact with another one located on the apposing membranes to form a gap junction. These gap junctions permit quick transport of small molecules and ions across membranes (Bennet *et al*., 1991). Cx32 protein is synthesized in liver, pancreas and peripheral nerve (Paul, 1986) and localized to incisures and paranodal loops of the myelinating Schwann cells and probably forms gap junctions between adjacent layers of the myelin sheath. This system could provide fast communication between the outer myelin layers and the adaxonal region (Nelis *et al*., 1999b).

Up to date, more than 250 CMTX-related mutations have been identified within coding and noncoding regions of the Cx32 gene (Krutovskikh and Yamasaki, 2000). More than 150 of these mutations are missense mutations, emphasizing the importance of each amino acid. The clinical presentation of the patients with missense mutations appears to be relatively uniform, including those with a deleted gene. Many of the mutant proteins do not reach cell membrane and do not form

functional channels in Xenopus oocytes and the ones that reach the cell membrane form functional channels with altered biophysical characteristics, indicating loss of function mechanism (Burzzone *et al*., 1994). On the other hand, altered trafficking of mutant Cx32 has been observed in rat pheochromocytoma cells leading to a potentially toxic cytoplasmic accumulation of mutated protein (Deschénes *et al*., 1997) indicating gain of function mechanism.

1.2. AXONAL CMT (CMT2)

Axonal CMT is less common than demyelinating CMT and designated as CMT type 2 (CMT2). It is characterized by normal or slightly reduced NCVs and reduced compound muscle action potential (CMAP). Clinical features of the disorder are distal muscle weakness, atrophy, pes cavus and sensory impairment (Dyck *et al*., 1993) resembling to that of CMT1. However, detailed comparative observations revealed that CMT2 patients have slightly reduced NCVs. Nerve biopsy samples of CMT2 patients reveal thinly myelinated fibers, chronic axonal atrophy and regeneration. However, some of the neurons remain normal allowing normal or only slightly reduced NCVs.

In the axonal form of CMT, the axon suffers from the primary injury and subsequent degeneration of myelin sheaths is termed as Wallerian degeneration rather than demyelination (Griffin and Hoffman 1993, Scherer and Salzer 2001). Secondary demyelination in axonal CMT can be attributable to axonal atrophy (Dyck *et al*. 1993b).

Linkage studies indicate genetic heterogeneity of CMT2. Eight loci and five genes, including *MPZ*, have been identified. In addition, *Cx32* mutations are believed to be involved in CMT2 (Birouk *et al*., 1998 and Marrosu *et al*. 1998). There are autosomal dominant, autosomal recessive and X-linked forms of axonal CMT (Table 1.1).

1.2.1. Autosomal Dominant CMT2

Autosomal dominant CMT2 was reported to be less common with a frequency of 20 per cent than autosomal dominant CMT1 that has a prevalence rate of 60 per cent (Ionasescu *et al*., 1993).

Zao and collegues identified a loss of function mutation in the kinesin family member 1B gene (*KIF1B*) located on 1p36.2 in all affected individuals of a Japanese CMT2 family (CMT2A1) (Zhao *et al*., 2001). Another genetic subtype of CMT2 was shown to result from mutations in the mitofusin 2 gene (*MFN2*) on chromosome 1p36.2 (CMT2A2) (Zuncher *et al*., 2004). CMT2B was shown to be associated with mutations in the small GTPase late endosomal protein gene (*RAB7*) located on 3q13-q22 (Auer-Grumbach *et al*., 2000). CMT2C locus has been assigned to chromosome 12q23-24 (Dyck *et al*., 1994 and Klein *et al*., 2003). In families with CMT2 which were designated as CMT2D independent groups identified a mutation in the glcyl-tRNA synthetase gene (*GARS*) located on 7p15 (Ionasescu *et al*., 1996; Pericak-Vance *et al*., 1997 and Antonellis *et al*., 2003). Another genetic subtype of CMT2, CMT2E was identified and shown to be associated with neurofilament-light gene (*NEFL*) located on 8p21 (Mersiyanova *et al*., 2000; Georgiou *et al*., 2002 and Leung *et al*., 2006). CMT2F was found to be caused by mutations in the gene encoding heat-shock 27-kD protein-1 (*HSPB1*) located on 7q11.23 (Ismailov *et al*., 2001). Another autosomal dominant CMT2 locus has been assigned to chromosome 12q12-q13.3 for CMT2G (Berciano *et al*., 1986 and Nelis *et al*., 2004) but yet no genes have been identified. Tang *et al*. identified a mutation in HSPB8 gene located on 12q24 in Japanese family having axonal CMT phenotype (CMT2L).

1.2.1.1. CMT2 with *MPZ* Mutations. CMT2I and CMT2J were reported in association with missense mutations in the *MPZ*. Marruosu and colleagues found a missense mutation in the extracellular domain of the *MPZ* in a large Sardinian family with CMT2 (Marruosu *et al*., 1998). Subsequently, involvement of *MPZ* mutations in CMT2 was confirmed by independent research groups (Chapon *et al*., 1999; De Jonghe *et al*., 1999; Senderek *et al*., 2000; Boerkoel *et al*., 2002 and Auer-Grumbach *et al*., 2004). The phenotype of affected patients was consistent with CMT2 with prominent sensory involvement. Therefore, De

Jonghe concluded that patients with late onset and nearly normal NCVs should be screened for *MPZ* mutations especially in the presence of additional clinical features such as marked sensory disturbances, pupillary abnormalities, or deafness (De Jonghe *et al*., 1999).

1.2.2. Autosomal Recessive CMT2

Autosomal recessive CMT2 is a very rare form of CMT. The first autosomal recessive CMT2 case was reported in a large consanguineous Moroccan family and genomewide search indicated linkage of the disorder to markers on 1q21.2-q21.3 in this family (Bouhouche *et al*., 1999). Subsequently, three consanguineous Algerian families with autosomal recessive CMT2 were identified and the disease was linked to chromosome 1q21. De Sandre-Giovannoli *et al*. identified a homozygous mutation in the Lamin A/C gene (*LMNA*) located on this region (De Sandre-Giovannoli *et al*., 2002). This form was designated as AR-CMT2A.

The second locus, 19q13.3, for axonal form of autosomal recessive CMT was identified in a large Costa Rican family with CMT2 phenotype and designated as AR-CMT2B (Leal *et al*., 2001). In a large Tunisian family with autosomal recessive axonal CMT with pyramidal features was reported. The disease was linked to 8q13-q21.1 region containing the gene coding for ganglioside-induced differentiation-associated protein 1(*GDAP1*). The gene was known to be mutated also in CMT2 families in which there is autosomal dominant inheritance (Barhoumi *et al*., 2001).

1.2.3. X-linked CMT2

In a study with French families diagnosed as CMT2 based on intermediate NCVs, it was observed that Cx32 mutations are responsible for the observed phenotype in 40 per cent of the cases (Rouger *et al*., 1997). Females carrying heterozygous *Cx32* mutations may have normal or slightly reduced NCV values and these patients are often diagnosed as CMT2. For this reason it was suggested to screen CMT2 patients even if no male to male transmission is observed in the pedigree (Birouk *et al*., 1998 and Marrosu *et al*. 1998).

Apart from Cx32 mutations four further loci are known to cause X-linked CMT. Ionasescu and collegues. identified two chromosomal regions, Xp22.2 and Xq26 associated with CMTX2 and CMTX3, respectively (Ionasescu *et al*., 1991; 1992). One other X-linked form of CMT2 has been identified that is linked to Xq24-q26 (Cowchock *et al*., 1985) and designated as CMTX4 (or Cowchock Syndrome). Last X-linked form was found liked to Xq21.32-q24 in a Korean family and designated as CMTX5 (*Kim et al*., 2005). Electrophysiological data of CMTX2, CMTX3 and CMTX5 patients were consistent with both demyelination and axonal involvement. Up to date no gene has been identified responsible for these subtypes.

2. AIM OF THE STUDY

The aim of the study is to genetically diagnose CMT and HNPP patients and confirm the clinical findings. The mechanisms leading to this most common peripheral neuropathy will be addressed by genotype/phenotype correlations for the patients with mutations. The overall aim of this study is to complement our previous studies that were performed to unravel the molecular bases of CMT and HNPP diseases in the Turkish population.

For this purpose, we have investigated presence of CMT1A duplication and HNPP deletion in respective families by PCR-STR analysis.

CMT1 patients that were negative for CMT1A duplication were analyzed for mutations in the genes *Cx32*, *MPZ* and *PMP22* that are known to be responsible for CMT1 phenotype. The PMP22 gene was further screened for point mutations in HNPP patients negative for the deletion. Cx32 and MPZ genes were screened in CMT2 patients since mutations in these genes have been reported previously in several CMT2 families.

3. MATERIALS

3.1. Subjects

Peripheral blood samples of Turkish CMT and HNPP patients were provided by neurology departments of different hospitals especially Istanbul University Medical School, Department of Neurology.

3.2. Chemicals

All solid and liquid chemicals used in this study were purchased from Merck (Germany), Sigma (USA), Riedel de-Häen (Germany), and Carlo Erba (Germany), unless stated otherwise in the text.

3.3. Buffers and Solutions

3.3.1.DNA Extraction from Peripheral Blood

Buffers and solutions used in DNA extraction are shown in Table 3.1

Table 3.1. Buffers and solutions used in DNA extraction

Solution	Content
Cell lysis buffer	155 mM NH_4Cl 10 mM $KHCO_3$ 1 mM Na_2EDTA (pH 7.4)
Nucleus Lysis Buffer	10 mM Tris-HCl (pH 8.0) 400 mM NaCl 2 mM Na_2EDTA (pH 7.4)
Sodiumdodecylsulphate (SDS)	10 per cent SDS (w/v) (pH 7.2)
Proteinase K	20 mg/ml
5 M NaCl solution	292.2 g NaCl in 1 l dH_2O
TE Buffer	20 mM Tris-HCl (pH 8.0) 0.1 mM Na_2EDTA (pH 8.0)

3.3.2. Polymerase Chain Reaction (PCR)

Buffers and solutions used in PCR reaction are shown in Table 3.2.

Table 3.2. Buffers and solutions used in PCR reaction

Solution	Content
10 X PCR Buffer with $(NH_4)_2SO_4$	200 mM $(NH_4)_2SO_4$ 0.1 per cent Tween (Fermentas,Lithuania)
$MgCl_2$	25 mM $MgCl_2$ (Promega, USA)

3.3.3. Agarose Gel Electrophoresis

Solutions used in agarose gel electropheresis are shown in Table 3.3.

Table 3.3. Solutions used in agarose gel electrophoresis

Solution	Content
10 X TBE (Tris-Borate) Buffer	0.89 M Tris-Base 0.89 M Boric Acid 20 mM Na_2EDTA (pH 8.3)
1 or 2 per cent Agarose Gel	1 or 2 per cent (w/v) Agarose in 0.5 X TBE Buffer
Ethidium Bromide (EtBr)	10 mg/ml
10 X Loading Buffer	2.5 mg/ml Bromophenol Blue 1 per cent SDS in 2 ml glycerol
Stop Loading Dye	1 X Bromophenol Blue 40 per cent glycerol 25 mM EDTA

3.3.4. Polyacrylamide Gel Electrophoresis (PAGE)

Table 3.4. Solutions used in PAGE

Solution	Content
10 X TBE Buffer	0.89 M Tris-Base 0.89 M Boric Acid 20 mM Na_2EDTA (pH 8.3)
30 per cent Acrylamide Stock	29 per cent Acrylamide (29:1) 1 per cent N, N'-methylenebisacrylamide
Ammonium Peroxodisulfate	10 per cent APS (w/v) (APS)
10 X Stop Loading Buffer (denaturing)	95 per cent formamide 20 mM EDTA 0.05 per cent Xylene Cyanol 0.05 per cent Bromophenol Blue

3.3.5. Silver Staining

Buffers and solutions used in silver staining are shown in Table 3.5.

Table 3.5. Solutions used in silver staining

Solution	Content
Buffer A	10 per cent Ethanol 0.5 per cent Glacial Acetic Acid
Buffer B	0.1 per cent $AgNO_3$ in dH_2O
Buffer C	1.5 per cent NaOH 0.1 per cent $NaBH_4$ 0.015 per cent Formaldehyde

3.4. Fine Chemicals

3.4.1. Enzymes

Taq DNA Polymerase and the restriction enzyme HpaII were purchased from Promega (USA). The restriction enzymes Esp3I and Bsp143II (Hae II) were obtained from Fermentas. The other restriction enzymes Hpy188I was purchased from New England Biolabs.

3.4.2. Oligonucleotide Primers

The sets of primers used for STR analysis for which the sequences are given in Table 3.6. were purchased from Genset (France) or GibcoBRL (Germany) and previously used in our laboratory by İbrahim Barış (Barış, 2002).

The sequences of the primers used for Amplification Refractory Mutation System (ARMS) are given in table 3.7. These primers were purchased from Elips (Turkey).

The sequences of the primers to amplify exons of the Cx32, MPZ, and PMP22 genes are given in Table 3.8. These primers purchased from Operon (USA) and Iontek (Turkey) and previously used in our laboratory by Birdal Bilir (Bilir, 2001). In the tables, F and R indicate forward and reverse primer, respectively.

Table 3.6. Sequences of the primers used for amplification of STR loci

GenBank Accesion No	Primer Name	Primers	PCR Product Length (bp)	PCR Annealing Temp (°C)
AC005703	4AF	CTACTTGCATATGCACTTTC	116-136	59
	4AR	GCACTAAAGTAGCTTGTAAC		
AC0013248	9AF	CAACCATCAGTGATTTGATGGTTTA	137-187	60
	9AR	CTGTTCTTCTTAATCCTTAACCAGT		
AC0013248	9BF	TCTCAGTCCTGATTTCTTGATTTTG	95-135	61
	9BR	CCAGAGCTAACACCACATTCA		

Table 3.7. Sequences of the primers used for ARMS

Primer Name		Sequence	PCR Product Length (bp)	PCR AnnealingTemp
Cx32 Wild Type	WF	GTGGTGGCTGCAGAGAGT	189	64
	C3	TTGCTGGTGAGCCACGTGCATGGC		
Cx32 Mutated	MF	GTGGTGGCTGCAGAGAGA	189	64
	C3	TTGCTGGTGAGCCACGTGCATGGC		
Cx32 C1-C3	C1	TGAGGCAGGATGAACTGGACAGGT	306	64
	C3	TTGCTGGTGAGCCACGTGCATGGC		

Table 3.8. Sequences of the primers used for exon amplification of Cx32, MPZ, and PMP22 genes

Gene	Exon	Primer	Primer Sequence (5′→3′)	PCR Product Length (bp)
Cx32	1	1F	TGAGGCAGGATGAACTGGACAGGT	306
		1R	TTGCTGGTGAGCCACGTGCATGGC	
	1	2F	ATCTCCCATGTGCGGCTGTGGTCC	432
		2R	GATGATGAGGTACACCACCT	
	1	3F	CGTCTTCATGCTAGCTGCCTCTGG	304
		3R	TGGCAGGTTGCCTGGTATGT	
MPZ	1	1F	CCCGTTCAGTTCCTGGT	172
		1R	GTCCCAAGACTCCCAGAGTA	
	2	2F	CTTCCTCTGTATCCCTTACTG	290
		2R	CTCCTTAGCCCAAGTTATCT	
	3	3F	TCATTAGGGTCCTCTCACATGC	370
		3R	GCCTGAATAAAGGTCCTTAGGC	
	4	4F	GGAGTCCTACATCCTCAATGCAG	310
		4R	CCCACCCACTGGAGTAGTCTCCG	
	5	5F	GAAGAGGAAGCTGTGTCCGC	251
		5R	CACATCAGTCACCGAGCGACT	
	6	6F	AGTCGCTCGGTGACTGAT	225
		6R	TAGCTCCATCTCGATGACCA	
PMP22	1	1F	CTCCTCGCAGGCAGAAACTC	166
		1R	CTGAACCAGCAGGAGCACGGG	
	2	2F	TCAGGATATCTATCTGATTCTC	194
		2R	AAGCTCATGGAGCACAAAACC	
	3	3F	TGGCCAGCTCTCCTAAC	200
		3R	CACCCCGCTTCCACATG	
	4	4F	GCCATGGACTCTCCGTC	250
		4R	CCTATGTACGCTCAGAG	

3.4.3. DNA Size Markers

Size markers were 100-bp DNA ladder between sizes 100 bp to 3000 bp (Promega, USA and Fermentas, Lithuania) (Table 3.9).

3.4.4. Other Fine Chemicals

Deoxyribonucleoside triphosphates (dNTPs) were purchased from Promega (USA). PCR products were purified by using QIAquick PCR Purification Kit (Qiagen, Germany).

Table 3.9. DNA size markers.

100-bp DNA Ladder (Promega)	100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500 bp
100-bp DNA Ladder (Fermentas)	100, 200, 300, 400, 500, 600, 700, 800, 900, 1031, 1200, 1500, 2000, 3000 bp

3.5. Equipment

Autoclave	:	Model MAC-601 (Eyela, Japan)
Balances	:	Electronic Balance Model VA124 (Gec Avery, UK)
		Electronic Balance Model CC081 (Gec Avery, UK)
Centrifuges	:	Centrifuge 5415C (Eppendorf, Germany)
		Universal 16R (Hettich, Germany)
Deep Freezers	:	-20°C (Bosch, Germany)
		-70°C (GFL, Germany)

Documentation System	:	BioDoc Video Documentation System (Biometra, Germany)
Electrophoretic Equipments	:	Horizon 58, Model 200 (BRL, USA) Sequi-Gen Sequencing Cell (Bio-Rad,USA) DGGE System Model # DGGE-200 (C.B.S. Scientific Co., USA) Horizon 1020, Model H1 (BRL, USA)
Incubators	:	Oven EN400 (Nuve, Turkey)
Magnetic Stirrer	:	Chiltern Hotplate Magnetic Stirrer, HS31 (UK)
Ovens	:	Microwave Oven (Vestel, Turkey) 65dC EN400 (Nuve, Turkey) 56 dC (LEEC, UK)
Power Supplies	:	Power Pac Model 3000 (Bio-Rad, USA) PSU 400/200 (Scie-Plus, UK)
Refrigerator	:	4°C Medicool (Sanyo, Japan)
Spectrophotometer	:	CE 5502 Scanning Double Beam 5000 Series (CECIL Elegant Technology, UK)
Thermocyclers	:	Thermal Reactor TR1 (Hybaid, UK) PTC-200 (MJ Research, USA) Techne (Progene, UK) iCycler (Bio-Rad, USA)

Vortex	:	Nuvemix (Nuve, Turkey)
Water Purification System	:	Millipore Elix 3 (Millipore, France)

4. METHODS

4.1. DNA Extraction from Peripheral Blood Samples

Ten ml peripheral blood was collected from each patient into a tube containing K_3EDTA to prevent coagulation. Blood sample was transferred into a 50-ml falcon tube and 30 ml of ice-cold red blood cell (RBC) lysis buffer was added. The contents were mixed thoroughly and the mixture was kept at 4°C for at least 20 minutes (min) to lyse the cell membranes. Centrifugation was performed at 5000 revolution per minute (rpm) at 4°C for ten min. The supernatant containing the RBC debris was removed and the pellet containing the leukocyte nuclei was washed with three ml RBC lysis buffer to remove the cell debris. The pellet was suspended in 10 ml of cold RBC lysis buffer by vortexing. After centrifugation at 4°C for ten min, the supernatant was discarded and pellet was cleaned with three ml RBC lysis buffer. Five ml nuclei lysis buffer was added and the pellet was dissolved by vortexing. In order to digest nuclear proteins thirty µl proteinase K (20 mg per ml) and 50 µl of ten per cent SDS were added, mixed gently and incubated at 37°C over night or at 56°C for three hours. After the incubation step, 10 ml 2.5 M NaCl solution was added to the mixture, shaken thoroughly and centrifuged at 5000 rpm at 20°C for 30 min. Supernatant was transferred into a new 50-ml falcon tube and approximately 30 ml absolute EtOH was added into mix. The tube was inverted gently until the DNA threads become visible. DNA was fished out with the aid of a micropipette, and was left to dry for 2 hours. It was dissolved in 300µl TE, and stored at -20°C.

4.2. Spectrophotometric Analysis

Spectrophotometry was used to determine the concentration of the DNA extract. The DNA sample was diluted in a ratio of 1:100 with dH_2O. Optical density of the diluted sample at 260 nm (OD_{260}) was measured and the concentration of DNA sample was calculated using the formula given below;

Concentration (µg/ml) = 50 µg/ml × OD_{260} × dilution factor

4.3. STR-PCR ANALYSIS

CMT1A duplications and HNPP deletions on chromosomal region of 17p11.2-p12 were screened by short tandem repeat PCR (STR-PCR) analysis.

4.3.1. PCR for STR Analysis

AC005703 (A4), AC0013248 (9A) and AC0013248 (9B) markers were used in STR analysis (Table 3.1) (Badano *et al*., 2001 and Latour *et al*.2001). These markers cover 0.5 Mb of the CMT1A duplication region containing the *PMP22* gene. PCR reactions were performed in a 50 µl volume containing approximately 500 ng genomic DNA, 1X Mg^{2+}-free buffer, 1.5 mM of $MgCl_2$, 0.4µM of each primer, 0.25 mM of each dNTP and 1 U of Taq DNA polymerase. The PCR program was as follows: an initial denaturation step at 94°C, followed by 35 cycles of 30 second (sec) at 94°C, 30 sec at appropriate annealing temperature, 30 sec at 72°C and 8 min at 72°C.

4.3.2. Agarose Gel Electrophoresis

Thirty ml, one per cent agarose gel was prepared by boiling 0.3 g agarose in 30 ml of 0.5 X TBE buffer. To visualize the DNA fragments under ultraviolet (UV) light, 3 µl ethidium bromide was added. The gel was poured onto an electrophoresis plate with a plastic comb and left at room temperature until it was solidified. The plate was placed into an electrophoresis tank and 0.5 X TBE buffer was added onto the gel. Five µl PCR product was mixed with 1µl of 10X loading buffer. Zero point six µl of 100 bp-DNA ladder was mixed with 1µl of 10X loading buffer. These mixtures were loaded on agarose gel and run at 150 Volt for about 15 min. Then, the DNA bands were visualized under UV light and their lengths were compared with that of 100 bp DNA ladder.

4.3.3. Polyacrylamide Gel Electrophoresis (PAGE) for STR Analysis

Two 20 cm×20 cm glass plates were placed on the table with the inside surfaces of the plates facing up. The surfaces of the plates and 0.75-mm plastic spacers were cleaned with ethanol to remove oily fingerprints and dust. The spacers were placed on the two edges of the plates and plates were tightened with clamps. Twelve per cent acrylamide gel solution was prepared as described in section 3.3.4 and poured into the area in between the two plates. A plastic comb with 20 well was inserted. The gel was left for at least one hour at room temperature until it was polymerized.

The gels were inserted into the electrophoresis tank containing 0.6 X TBE buffer. The comb was removed gently and the wells were cleaned using distilled H_2O. Three µl of each PCR product was mixed with 5µl of 1X loading buffer and loaded onto the wells. The gel was run for 3.5 hours at 325 Volts.

4.3.4. Silver Staining

To visualize the separated alleles on polyacrylamide gel, silver-staining method was used, as described in section 3.3.5. For this purpose, first the DNA fragments were fixed in buffer A for 3 min. Buffer A was removed and the gel was left in one per cent (w/v) silver nitrate solution for ten mimutes. In order to prevent non-specific staining, it was washed with distilled dH_2O and then with freshly prepared buffer C solution. Incubation proceeded for approximately ten min, until the bands appeared. Then buffer C was replaced with buffer D to terminate color reaction. After 5 min, the gel was transferred to a transparent folder and all sides of the folder were heat sealed.

4.4. MUTATION ANALYSIS

In the patients without CMT1A duplication, point mutations in Cx32, MPZ and PMP22 genes were screened. The patients negative for HNPP deletion were screened for mutations in the PMP22 gene.

4.4.1. Amplification of Exons

The coding exons and flanking intronic sequences of the Cx32, MPZ and PMP22 genes were amplified by PCR method. The primer sets used in amplification of the exons were shown in Table 3.3. PCR reactions were performed in a 50 µl volume containing approximately 500 ng genomic DNA, 1 X Mg^{2+}-free buffer, 1.5 mM of $MgCl_2$, 0.4µM of each primer, 0.25 mM of each dNTP and 1 U of Taq DNA polymerase. The steps of PCR program was:

Initial denaturation at 94 °C for 5 min

Denaturation at 94 °C for 1 min

Annealing at appropriate temperature (Table 4.1.) for 1 min — 30 cycles

Extension at 72 °C for 1 min

Final extension at 72 °C for 10 min

After the program was completed, amplification of exons was confirmed by running the samples on one per cent (w/v) agarose gel.

4.4.3. Single Strand Conformation Polymorphism (SSCP) Analysis

Single strand conformation polymorphisms in the amplified exon were determined by SSCP analysis. The plates were prepared as described in section 4.3.3. Thirty-five ml eight per cent (w/v) acrylamide gel with and without four per cent glycerol (v/v) were prepared. For this purpose, 9.3 ml of 30 per cent (29:1 acrylamide-bisacrylamide in dH_2O) stock acrylamide solution was mixed with 2.1 ml of 10X TBE buffer. Total volume was adjusted to 35 ml with dH_2O. After adding 350 µl of 10 per cent APS (w/v) and 35 µl

TEMED the solution was poured in between the two plates. A comb with 20 well was inserted between two plates and the gel was allowed to polymerize at room temperature.

After the polymerization was completed, the comb was removed gently, the wells were cleaned with dH_2O, and the gels were placed into electrophoresis tanks containing 0.6X TBE buffer. Ten µl of each PCR product was mixed with 10 µl of denaturing loading dye prepared as described in section 3.3.4. Before loading, the mixture was denatured at 94˚C for 5 min and chilled on ice for five min. and eight µl of denatured sample was loaded onto the gels. Electrophoresis of the samples was carried out at 150-250 Volts depending on the length of the fragment for 16 hours and the DNA fragment were visualized by silver-staining method as described in section 4.3.4.

4.4.4. DNA Sequence Analysis

PCR products that showed altered migration patterns in SSCP analysis were cleaned using QIAquick purification kit (Qiagen, UK.). Sequencing of both sense and antisense strands of the samples were performed by Iontek (Turkey).

4.4.5. Restriction Endonuclease Analysis

The presence of the sequence alterations were confirmed by restriction endonuclease digestions. For this purpose, ten µl of PCR product, amplified with appropriate primer set, was mixed with 3U of restriction enzyme and 2 µl of corresponding buffer. Total volume was adjusted to 20 µl with dH_2O. The mixture was incubated at 37°C over night. The resulting digestion products were run on two per cent agarose gel (w/v) and visualized by ethidium bromide staining under UV light. The alteration in the sequence either created a restriction site or abolished a previously existing one.

4.4.6. Screening for Novel Mutations in Cx32 Gene with Amplification Refractory Mutation System

A novel mutation that does not create or abolish a restriction enzyme recognition site was identified in *Cx32* in patient P278. This variation was in the 306 bp length region that was amplified by using C1 and C3 primers. To verify this alteration, ARMS method was used and two new forward primers were designed to be allele specific at the last 3'-nucleotide. The last nucleotide of the first primer (MF) was the mutated nucleotide and this primer can only amplify the mutated sequence. Last nucleotide of the other primer (WF) was wild-type nucleotide and this primer can only amplify the wild-type sequence (Table 3.2).

Two multiplex PCR reactions were performed. In the first reaction, the WF primer and C1 was used as forward primers and C3 was used as reverse primer. In the second reaction, MF primer, C1 the C3 were used. Each reaction was performed in two separate tubes containing the DNA samples of the patient, who thought to carry this novel mutation, or DNA sample of a healthy control. The PCR mixtures were prepared as described in section 4.4.1. The PCR program was as follows: an initial denaturation step at 94°C, followed by 35 cycles of 30 second (sec) at 94°C, 1 min at appropriate annealing temperature, one min at 72°C and 10 min at 72°C. The experiment was performed three times.

Amplification of the PCR fragments was checked on 1.5 per cent agarose gel.

5. RESULTS

The molecular bases of CMT and HNPP diseases in our population were investigated in a total 161 patients clinically diagnosed as CMT1, CMT2 or unclassified CMT (Table 5.1).

Table 5.1. Number of patients investigated in this study

Clinical diagnose	Total number of patients	The number of patients investigated by STR-PCR analysis	The number of patients investigated by mutation analysis
CMT1	40	12	28
CMT2	65	10	55
Unclassified CMT	12	12	0
HNPP	44	12	22

5.1. STR Analysis

Detection of CMT1A duplication and HNPP deletion was the preliminary step in our studies since these mutations are known to account for 70 per cent of CMT1 and 86 per cent of HNPP cases, respectively (Nelis *et al*., 1996; Wise *et al*., 1993). Forty six unrelated CMT and HNPP patients were investigated for the presence of CMT1A duplication or HNPP deletion by highly informative STR markers, 4A, 9A and 9B. Observation of three alleles or 2:1 dosage difference between two alleles was used as the criteria for the presence of the CMT1A duplication. Genetic diagnosis of the HNPP deletion was based on detection of a single allele for all three markers tested. When all these three markers yielded a single allele an additional marker, STR20, was used to confirm presence of the deletion in the patient. Examples of STR gels were shown in figure 5.1. Presence of two allels without a dosage difference for all tested STRs indicated absence of CMT1A duplication/HNPP deletion in patient P483.s For STR 4A, patients P460 and P495 showed 1:2 dosage difference and patient P456, revealed three bands that directly diagnose the presence of the CMT1A duplication. Observation of one band for this marker in patients P468, P452 and P480 indicated presence of the HNPP deletion (Figure 5.1.a) that was later verified by other STRs. Detection of three bands in patient P456 for STR9A confirms the

data that was obtained from STR4A (Figure 5.1.b). Patients P495 and P497 revealed three bands for STR9B. In the same gel, only one band was observed in patients P496 and P498 (Figure 5.1.c). In this figure and in proceeding ones, P indicates patient, M indicates size marker and C indicates control.

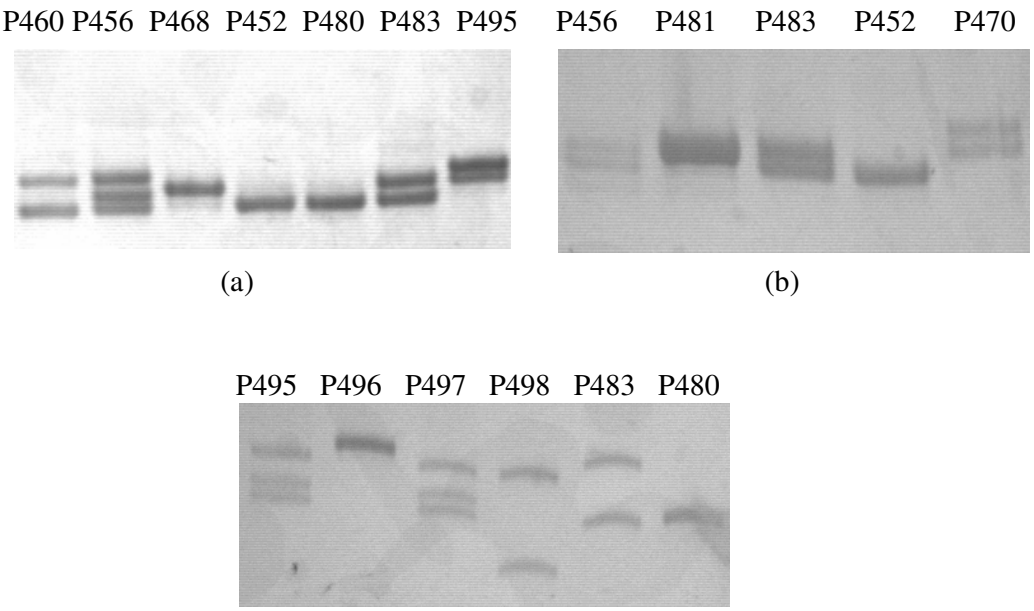


Figure 5.1. Twelve per cent STR gel of 4A (a), 9A (b) and 9B (c) markers.

CMT1A duplication and HNPP deletion were identified in five of 12 (41.6 per cent) CMT1 patients and eight of 12 (66.6 per cent) HNPP patients, respectively. The difference of the duplication and deletion rates from the values reported for other populations was checked by chi-square test. The difference was found to be significant for CMT1A duplication (χ^2=3.88, p≤0,05, df=1). However, the difference for HNPP deletion was insignificant (χ^2=2,96, p≤0,10, df=1). CMT1A duplication or HNPP deletion was not observed in any of the 10 CMT2 or 12 CMT patients. The results of STR-PCR analysis are summarized in table 5.2.

Table 5.2. Results of STR-PCR analyses

Clinical Diagnosis	Number of patients analysed	CMT1A Duplication	HNPP deletion	Chi-square values
CMT1	12	5	-	3.88
HNPP	12	-	8	2.96
CMT2	10	0	-	ND*
CMT	12	0	-	ND*

*ND: Not done

5.2. Mutation Analyses

Twenty eight non duplicated CMT1 patients were further analyzed for mutations in Cx32, MPZ and PMP22 genes. PMP22 gene was also screened in thirty-two HNPP patients that were negative for HNPP deletion. The study was extended to screening of CMT1 genes, Cx32 and MPZ, in 55 CMT2 patients since CMT2 phenotype may also result from mutations in these genes. Mutation screening was performed by SSCP analysis, followed by sequencing of sense and antisense strands of the exons that show single strand conformation polymorphisms on the gels.

5.2.1. Sequence Variations in the Cx32 Gene

Altered migration patterns for the only exon of Cx32 gene were observed in two male patients: patients P278 and P385 (Figure 5.2 a and b).

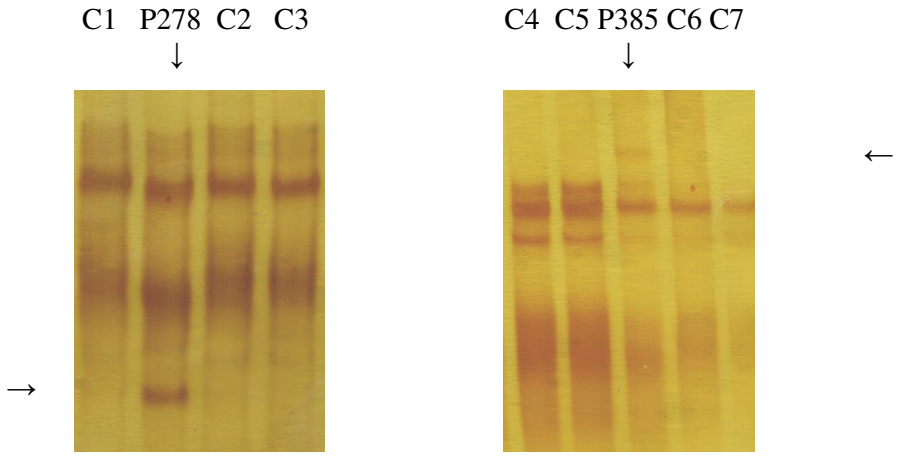
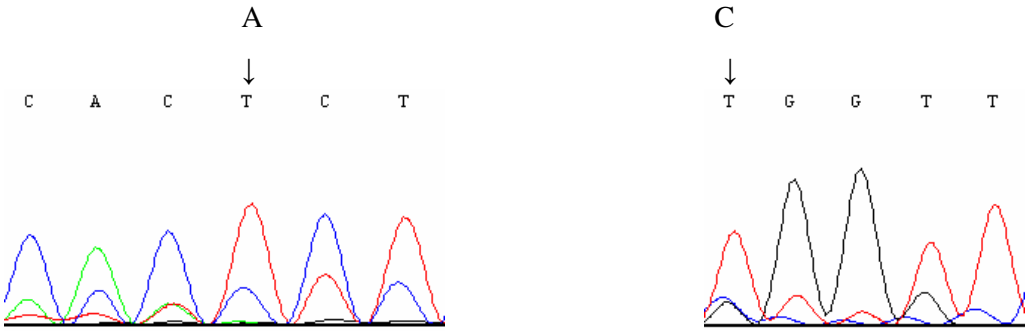


Figure 5.2. Eight per cent SSCP gels showing altered migration patterns for patient P278 (a) and P385 (b) in the only exon of *Cx32*.

Sequencing analysis of DNA sample of patient P278 revealed a T to A transition at position 126 (c.126T>A), resulting in a serine to arginine substitution at amino acid position 42 (p.S42R) in this X-linked gene (Figure 5.3.a). Since this sequence variation did not create a restriction site or did not abolish a pre-existing one, ARMS method was used to confirm the nucleotide change. For this purpose, a 189 bp region spanning the nucleotide change was amplified using DNA samples of patient P278 and healthy individuals with wild-type or mutated primer pairs. In these multiplex PCR reactions, a 306 bp sequence of the Cx32 gene was also amplified as an internal control. The wild-type primer (WF) was refractory to PCR and amplification of the 189 bp fragment was not observed in the patient. On the other hand, the wild-type primers could amplify the region on DNA samples of the healthy individuals. As expected, mutated primer (MF) was refractory to PCR in control individuals and amplified the region only on the DNA sample of patient P278 (Figure 5.4). The amplification of 306 bp fragment was observed in all samples. To prove that the sequence change detected is a novel mutation and not a common polymorphism, ARMS test was extended to 50 healthy individuals. The PCR products were observed in all of the samples when PCR was performed using the wild-type primer set. Amplification with mutated primer was not observed in normal individuals.



(a) (b)

Figure 5.3. Chromatogram showing nucleotide alterations in the Cx32 gene in hemizygous patients P278 (a), and P385 (b) in antisense strands.

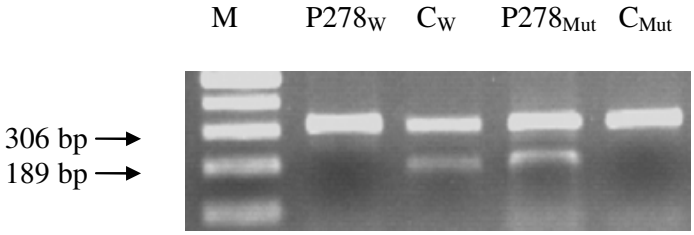


Figure 5.4. One per cent agarose gel showing the results of ARMS analysis. W: Wild-type, Mut: Mutated

A previously reported missense mutation, G to A conversion at nucleotide 44 (c.44G>A) was found in patient P385 (Figure 5.3. b). Hypothetically, the base change causes an arginine to glutamine substitution at amino acidic position 15 (p.R15Q). The mutation abolished a restriction site of HpaII that normally cuts the 306 bp long PCR fragment into 51 bp and 225bp fragments. The restriction results of P385 and two healthy controls are shown in figure 5.5.

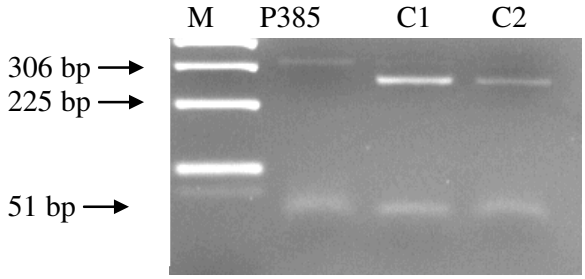


Figure 5.5. HpaII restriction results of P385

5.2.2. Variations in the MPZ Gene

SSCP results revealed an abnormal migration profile in the fifth exon of the MPZ gene in patient P356 (Figure 5.6).

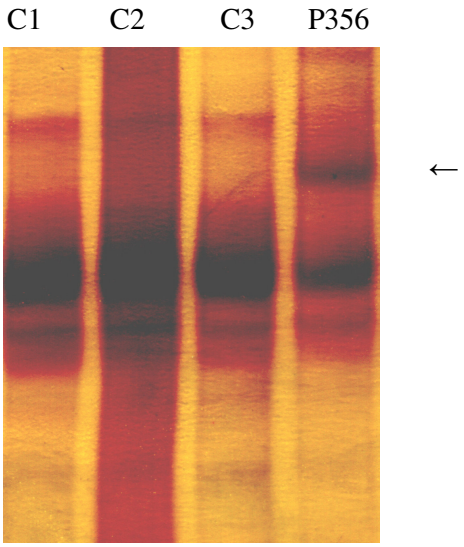


Figure 5.6. Eight per cent SSCP gel showing an abnormal migration profile for patient P356 in the MPZ gene

Sequencing chromatogram revealed a base substitution, c.637G>C, in patient P356 (Figure 5.7). Although the transition results in a glycine to arginine substitution at amino acidic position 213 (p.G213R), the same variation was previously reported as a polymorphism by P. Seeman (Personal information, 2004). Patient P356 was heterozygous for this base change, which created a restriction site for HaeII. The enzyme digested the 247 bp length PCR fragment into 123bp and 124 bp in the patient (Figure 5.8). To check the frequency of the polymorphism in the Turkish population, the restriction analysis was extended to 50 unrelated healthy controls and 25 CMT patients. The base change was observed in only one control and four CMT patients in heterozygous form. The difference of the percentages between patient group and control group is tested by chi-square test and found to be significant (χ^2=5.06, p≤0,025, df=1). The data for restriction analysis for c.637G>C variation are summarized in Table 5.3.

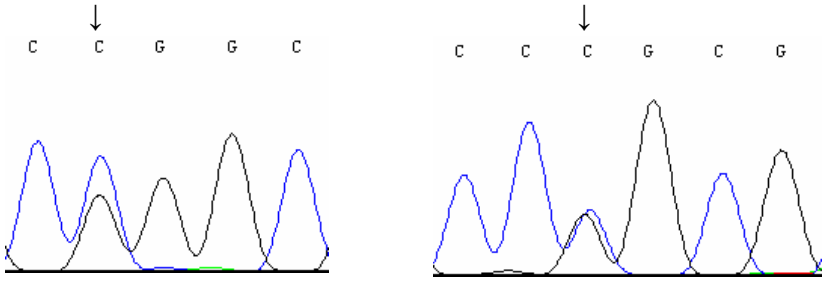


Figure 5.7. Chromatogram showing c.667G>C change in patient P356 in the MPZ gene in sense (a) and antisense (b) strands.

Table 5.3. The frequency of c.637G>C variation

Group	Total number of indviduals	Chromosomes positive for the variation	Chromosomes negative for the variation	Chi-square results
CMT patients	25	4	46	5.06
Healthy controls	50	1	99	5.06

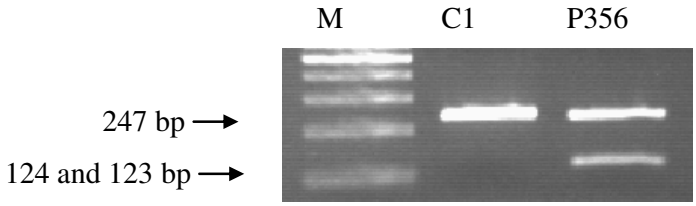
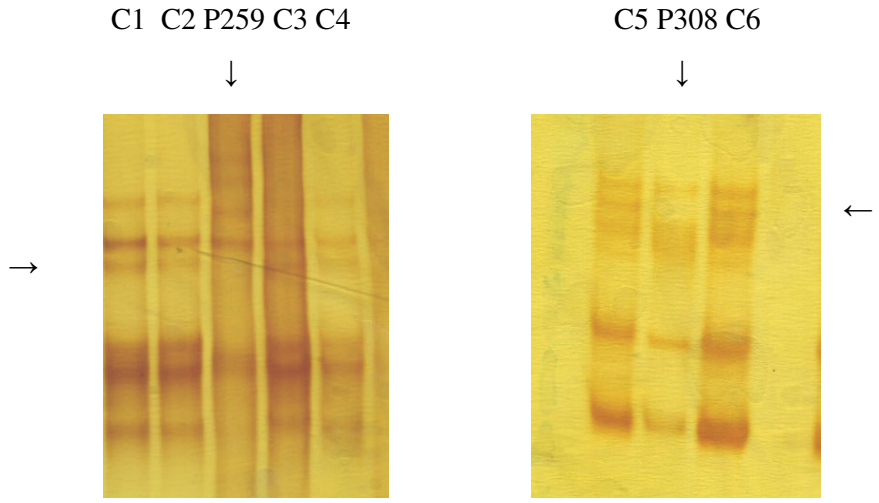


Figure 5.8: Agarose gel showing HaeII restriction for patient P356

5.2.3. Variations in the PMP22 Gene

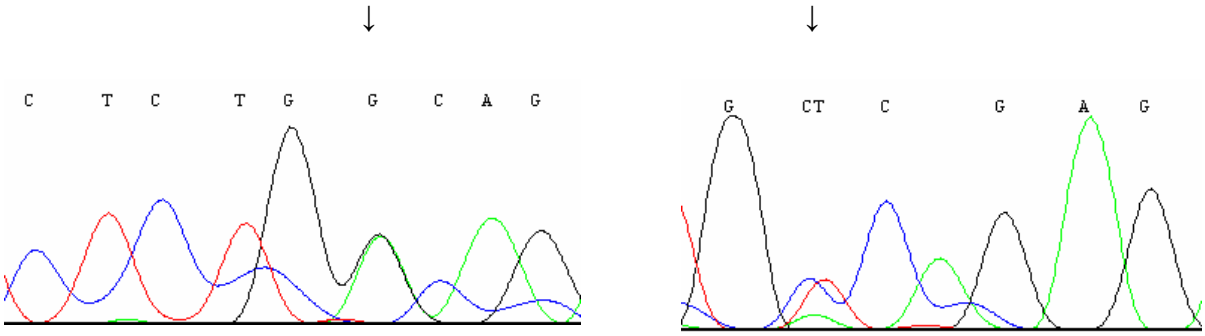
SSCP results of PMP22 exons revealed altered migration profiles in a CMT1 patient, P259 (Figure5.9) and an HNPP patient, P308 (Figure 5.9.b).



(a) (b)

Figure 5.9. SSCP gels showing altered migration of the PMP22 gene in patients P259 (a) and in P308 (b)

Sequencing analysis of the second exon of *PMP22* in patient P259 revealed a c.117G>A change laeding to a stop codon at position (p.W39X) in patient P259 (Figure 5.10). The alteration created an additional restriction site for Hpy188I, that digests the 156 bp length wild type PCR fragment into 15bp and 141bp. The patient's sample digestion gave 141bp, 92 bp, 49 bp and 15 bp bands indicating that the patient is heterozygous for the alteration (Figure 5.11). To prove that this alteration is not a polymorphism, restriction analysis was extended to 50 healthy individuals. Variation was not observed in this group.



(a) (b)

Figure 5.10. Chromatograms showing c.117G>A change in patient P259 in the second exon of *PMP22* in sense (a) and antisense (b) strands.

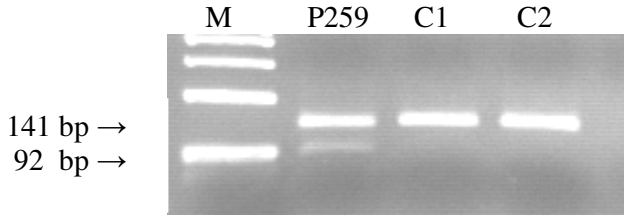


Figure 5.11. Hpy188I restriction results of P259

Another heterozygously inherited novel mutation, deletion of C at position 60 was (60delC) detected in the first exon of *PMP22* in patient P308 (Figure 5.12). The frame-shift mutation abolished the restriction site of the Esp3I, which normally cuts the 208 bp PCR fragment into 92 bp and 116 bp (Figure 5.13). Restriction analysis was extended to 50 controls and all of them found to be negative for the variation.

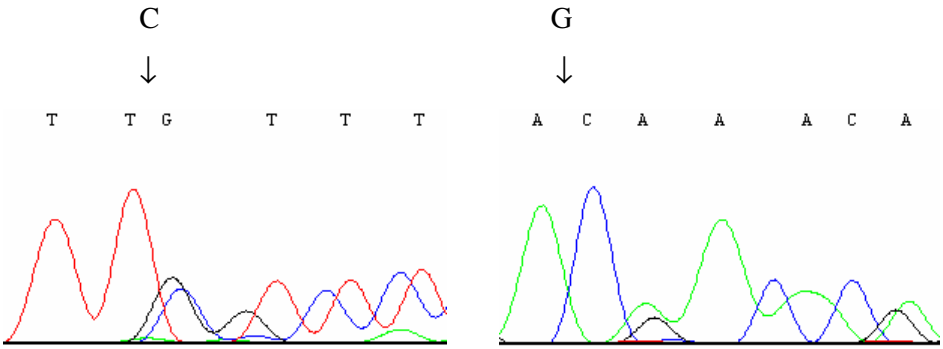


Figure 5.12. Chromatogram showing c.60delC deletion in the firs exon of *PMP22* in patient P308 in sense (a) and antisense (b) strands.

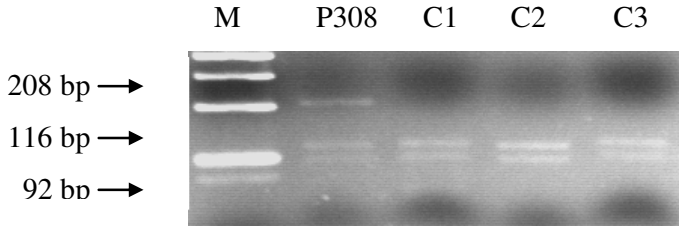


Figure 5.13. Two per cent agarose gel showing Esp3I digestion

The segregation of identified mutations could not be investigated in the family members since the DNA samples of them were not available. The sequence alterations identified in this study are summarized in Table 5.4.

Table 5.4. Sequence alterations identfied in this study

Index Patient	Clinical Diagnosis	Gene	Nucleotide Change	Mutation Type	Amino acid Change
P259	CMT1	*PMP22*	c.117G>A	Nonsense (Novel)	p.W39X
P278	CMT1/2?	*Cx32*	c.126T>A	Missense (Novel)	p.S42R
P308	HNPP	*PMP22*	c.60delC	Frame-shift (Novel)	p.F20fs
P356	CMT2	*MPZ*	c.637G>C	Polymorphism	p.G213R
P385	CMT1	*Cx32*	c.44G>A	Missense	p.R15Q

6. DISCUSSION

Charcot-Marie-Tooth disease comprises a clinically and genetically heterogeneous group of disorders. Because of the variations in phenotypic expression and overlapping clinical features different subtypes of CMT can not be distinguished by clinical examination or electrophysiological studies. In this respect, genetic diagnosis is of critical importance for differential diagnosis, genetic counseling, and presymptomatic diagnosis of the disease. Besides, genotype/phenotype correlations help understanding the disease mechanisms underlying CMT. Up to date, genetic linkage and subsequent molecular studies have revealed that more than 28 genes are responsible for the disorder (www.molgen.ua.ac.be./CMT/mutations)

CMT1A duplication and HNPP deletion are the most frequent mutations leading to CMT1 and HNPP phenotypes, respectively. Thus, identification of these mutations is the preliminary step of the genetic analysis for the disease. Several methods such as pulsed-fielded gel electrophoresis (Lupski *et al*., 1991 and Pentao *et al*., 1992) , southern hybridization (Reiter *et al*., 1996 and Lopes *et al*., 1996), fluorescence *in situ* hybridization (Rautenstrauss *et al*., 1997), PCR with poly (CA) markers (Chevillard *et al*., 1994 and Blair *et al*., 1995), real-time fluorescent PCR (Ruiz-Ponte *et al*., 2000) and PCR based methods using short tandem repeats (STR) have been used to detect CMT1A duplication and HNPP deletion. Because of its advantages in cost, labor, time and accuracy, STR method, was used for molecular diagnosis of CMT1A/HNPP in this study. The method has been developed by Latour *et al*. (Latour *et al*., 2001) and detects duplications/deletions with an accuracy of 100 per cent for CMT1A and 98 per cent for HNPP, respectively. It was used in our laboratory previously by İbrahim Barış (Barış, 2002).

In this study, using the STR-PCR method, five of 12 (41.6 per cent) CMT1 patients were found to carry the CMT1A duplication. The frequency of the duplication was reported as 70 per cent for the European and Northern American populations (Nelis *et al*., 1996; Wise *et al*., 1993). In our patient group, the duplication frequency is significantly lower than those reported for these populations. Although the sample size is comparably small in this study, the frequency is concordant with that of our previous studies performed with larger sample

sizes (Tadmouri, 2003; Barış, 2002 and Bilir, 2001). In fact, in more isolated populations, such as in Italy, this frequency was reported to be around 60 per cent (Guzzetta *et al*., 1995) and in Northern Sweden it was 37.5 per cent (Holmberg *et al*., 1994). The incidence of autosomal recessive cases is higher in the Turkish population than in European and Northern American populations because of high rate of consanguineous marriages. Thus, while the cohort of CMT1 patients were selected autosomal recessive cases were avoided. Except for one patient, negative for the duplication, apparently there are no consanguineous marriages in this group. However, because of the social reasons, incidence of marriages within the same district is common in our population that causes founder effect. For this reason, even though the families are seemingly unrelated, they may share common ancestors and may present autosomal recessive cases.

X-linked cases might have been not distinguished in our group due to unavailability of the pedigrees and this may also lower the duplication frequency. In addition, further analysis of the clinical data of the patients revealed that one of the patients who was diagnosed as CMT1 had NCV values very close to cut-off values (38 m/s), indicating that the patient may have axonal CMT. In one other patient, NCV value was not available. Two of the cases negative for the duplication were familial and should be screened further for point mutations in the MPZ, PMP22 and LITAF genes. The possibility of an acquired neuropathy cannot be excluded in the three sporadic cases.

It is known that CMT1A duplication is responsible for 71 per cent of inherited and 90 percent of sporadic cases of CMT1A (Wise *et al*., 1993). In our cohort, although two of the duplicated cases were inherited the pedigrees were not available for the other three cases and the frequency could not be determined.

Eight of 12 HNPP (66.6 per cent) patients were found to carry the HNPP deletion. The frequency of deletion in our patient group is lower than the values reported for other populations (86 per cent). However, the difference between frequencies of the two populations is not statistically significant for the HNPP deletion. Two of the deletion cases were inherited, four were sporadic and for two of the cases the pedigrees were not available. Thus, the frequency of the inherited and sporadic cases could not be calculated.

In this study, 28 non-duplicated CMT1 patients were screened for mutations in the Cx32, MPZ and PMP22 genes and three sequence variations, two in *Cx32* and one in *PMP22* were identified.

Cx32 mutations were detected in two male CMT patients. The first patient, P278, is a 42-year-old male that was referred to our lab as CMT1/CMT2 phenotype. He had abnormal mobility, distal weakness in upper limbs, pes cavus deformity and sensory ataxia. The EMG results were not recorded. He was found to carry a novel c.126T>A change in *Cx32* resulting in p.S42R substitution. Population screening for this variation revealed that it is the causative mutation rather than a common polymorphism. In addition, protein blast search results revealed that the amino acid at this position is highly conserved among Homo *sapiens*, Bos *taurus*, Ovis *aries*, Macaca *fascicularis*, Canis *familiaris*, Equus *caballus*, Mus *musculus*, and Rat species.

The second patient, P385, was referred to our laboratory with CMT1 diagnosis. Abnormal mobility, distal weakness in upper limbs and pes cavus were reported. His median motor NCV was 30 m/s. This patient was found to carry a previously reported c.44G>A base change resulting in p.R15Q in *Cx32* (Fairweather *et al*., 1994). This amino acid is evolutionary conserved among the species Homo *sapiens*, Bos *taurus*, Rat, Macaca *mulata*, Canis *familiaris*, Equus *caballus*, Pan *troglodyte*s and Gallus *gallus*.

Cx32 is a membrane-spanning protein that forms intracellular gap junctions. These channels connect to the folds of Schwann cell cytoplasm, allowing the transfer of ions, nutrients, and molecules to the innermost myelin layers. Up to date, more than 250 mutations were found in *Cx32*. Disease causing mutations were found to be scattered throughout the gene (Krutovskikh *et al*., 2000). However, they are known to affect the functioning of the Cx32 protein in different ways. Our findings are confirming this knowledge. In patient P278 the mutated nucleotide corresponds to the first extracellular loop of Cx32 protein. This domain is critical for docking of gap junctional hemichannels to each other in order to form a functional channel (Krutovskikh and Yamasaki, 2000). On the other hand, the mutation in patient P385 affects the amino terminal domain of the protein that has been shown to play a role in voltage-gaiting of gap junctional channels. Same mutation was previously reported by Fairweather *et al* (Fairweather *et al*., 1994) and predicted to interfere with gaiting sitimulus

control. The two *Cx32* mutations affect domains with different functions and this may explain the phenotypic variation between the patients. P278 with the mutation in the first extracellular loop has a more severe phenotype compared to that of P385. Patient P278 was suspected to have a CMT2 phenotype with sensory ataxia and the age of onset was in the second decade. However, the mutation affecting the amino terminal domain causes CMT1 without sensory ataxia and the age of onset was in the third decade. Interestingly, CMT1/CMT2 phenotypes are reported to have Cx32 mutations commonly in the extracellular or transmembrane domains of the protein. This is consistent with the CMT1/CMT2 phenotype that is reported in patient P278.

Patient P259 is a 40-year-old female who was referred to our laboratory with CMT1 diagnosis. She had delayed motor milestones and early onset. Clinical investigation revealed distal weakness in upper limbs, pes cavus deformity and sensory ataxia. Her median motor NCV was 21 m/s. She was found to carry a novel G to A base change at position 117 (c.117G>A) in the PMP22 gene. This change results in a premature stop codon (p.W39X) leading to formation an aberrant protein that is 121 amino acids smaller than the wild-type. This protein lacks a part of first extracellular domain, all transmembrane domains except the first, the intracellular loop and second extracelular domains. It is likely that the truncated protein is addressed to the membrane but cannot be functional completely and interfere with the normal functioning of the wild type protein via a dominant negative affect. On the other hand, it may cause a toxic gain of function by interfering with the normal trafficking of the protein to the membrane.

Among the thirty-two HNPP patients negative for deletion one patient, P308, was found to carry another causative mutation in the PMP22 gene. This patient was an 18-year-old male, referred to our laboratory with HNPP diagnosis. Based on familial information, symptoms like right sided distal weakness in upper limb and drop foot were appeared at the age of 14. His median motor NCV was 41.9 m/s. He was found to be heterozygous for a novel one base pair deletion at position 60 (c.60delC), resulting in a frame-shifting mutation (p.F20fs). This out of frame deletion leads to production of an aberrant PMP22 protein that is 92 amino acids smaller than the wild-type protein. Hypothetically, the polypeptide completely lacks the second extracellular domain, all transmembrane domains except the first one and the intracellular loop.

PMP22, a major component of myelin, has an adhesive role. It was proposed to act as a channel protein connecting the outer surface of the myelin sheath with the periaxonal space and function in transportation of small nutrients, metabolites and ions (Nelis *et al*., 1999). It might also affect Schwann cell growth and differentiation. Alterations in expression profile of PMP22 gene as in the CMT1A and HNPP may perturb stoichiometry of myelin sheath and results in destabilization of myelin sheath. Up to date, more than 60 mutations were identified in *PMP22* associated with CMT1, HNPP or DSS (http//www.molgen.ua.ac.be/CMTMutations/) most of which affect the transmembrane domains of the protein. Although, both *PMP22* mutations detected in this study results in very small aberrant proteins that lack the same domains, the phenotypes of the patients are distinct from each other. This may imply that the two aberrant proteins affect the individuals either via different pathogenic mechanisms or the genetic background and the modifier genes have a very dominant effect. The phenotypic expression of the patient 308 is consistent with the hypothesis that HNPP is caused either by deletion of complete PMP22 gene or by a loss-of-function mutation in PMP22, resulting in haploinsufficiency. However, the truncation of the protein in patient P259 probably leads to a gain of function mutation and CMT1 phenotype. In fact, frame-shifts starting from the same amino acid (G94fs) were reported previously to cause both CMT1/DSS and HNPP phenotypes (Ionasescu *et al*., 1995; Boerkoel *et al*., 2002; Young *et al*., 1997 and Lenssen *et al*.,1998). Further analysis needs to be performed to understand the exact disease mechanisms.

HNPP is known to be a genetically homogeneous disease resulting either from deletions or point mutations of *PMP22*. However, only one of 32 HNPP patients in our cohort was found to be positive for point mutations indicating at least one other unidentified locus responsible for the disease. In accordance with this suggestion, Nelis *et al*., excluded a large part of chromosome 17p in non-deleted families (Nelis *et al*., 1996).

Fifty-five CMT2 patients were screened for mutations in the Cx32 and PMP22 genes since these CMT1 genes are known to be involved in CMT2 phenotype. However, no sequence variations could be identified in these patients except for a polymorphism detected in patient P356.

Patient P356 is 11-year-old male who was referred to our laboratory with CMT2 diagnosis. Abnormal mobility and distal weakness were reported. His median motor NCV was 36 m/s. He was found to carry a G to C change at position 637 (c.637G>C) resulting in substitution of glycine to arginine (positively charged, basic amino acid) at amino acid position 213 (p.G213R) of the MPZ protein. Although the substitution is non-conservative and this amino acid is conserved among the species (Pan *troglodytes*, Equus *caballus*, Macaca *mulata*, Canis *familiaris*, Bos *taurus*, Mus *musculus* and Sus *scrofo*) it was previously reported as a polymorphism by P. Seeman (Seeman, 2004). We have found that the frequency of the variation among Turkish healthy individuals is one per cent (one in 100 chromosomes) and eight per cent (four in 50 chromosomes) in CMT patients. The difference of the percentages between the patient group and the control group is statistically significant. Thus, it can be suggested that the variation may have a modifier effect on the development of the disease. The amino acid is in the cytoplasmic major dense line of the protein and this domain is known to have a function in mediating compaction of the Schwann cell cytoplasm. It was hypothesized that basic residues in the cytoplasmic domain of MPZ may interact with phospholipids of adjacent cytoplasmic aspects of Schwann cell membranes (Kirschner and Ganser, 1980). Further functional analysis is required to prove the functional importance of the amino acid.

Because of overlapping clinical features between CMT1 and CMT2 phenotypes presence of the CMT1A duplication and mutations in *Cx32* and *MPZ* were also tested among the CMT2 patients. All were negative for the mutations except patient P278 that was clinically diagnosed as CMT1/CMT2 and was found to carry a Cx32 mutation.

Identification of causative mutations in the patients verifies the clinical diagnosis. However, for the patients that could not be genetically diagnosed further research is required to identify the pathogenic mutations in other CMT genes. On the other hand, identification of further mutations and genotype/phenotype correlations will help elucidation of the pathogenic mechanisms leading to the CMT disease.

7. CONCLUSION

This study emphasizes the importance of genetic analysis in differential diagnosis of the CMT disease. CMT1A duplication was detected in 41.6 per cent of the CMT1 cases and HNPP deletion was detected in 66.6 per cent of the HNPP cases. The frequency of CMT1A duplication found to be significantly low compared to those of other populations indicating either high incidence of autosomal recessive cases in the Turkish population or unavailability of clinical data. Three myelin genes, Cx32, MPZ, and PMP22 were also analysed for mutations in the study. One novel and one previously reported mutation were identified in the Cx32 gene resulting in CMT1 and CMT1/2 phenotype. Analysis of the MPZ gene revealed a previously reported polymorphism in a CMT2 patient. PMP22 gene was mutated in one CMT1 and one HNPP patient that were both novel mutations. In this study, phenotype/genotype correlations were performed for the identified mutations and the observed phenotype of all patients were found to be compatible with the mutations they carry. Further studies are required for identification of unknown genetic loci and elucidate the exact functions of the proteins that are responsible for the disease.

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