DETECTION OF DNA DUPLICATIONS IN CHARCOT-MARIE-TOOTH TYPE 1 (CMT1) PATIENTS BY DNA ANALYSIS

by

Nisrine Bissar-Tadmouri

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TO MY MOTHER AND FATHER

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ABSTRACT

Charcot-Marie-Tooth neuropathy (CMT), also known as Hereditary Motor and Sensory Neuropathy (HMSN), is a heterogeneous group of inherited diseases of peripheral nerves. It is estimated that 1/2500 persons have a form of CMT. CMT1, which typically shows dominant inheritance, has been associated with at least 4 distinct loci: The CMT1A locus on chromosome 17, the CMT1B locus on chromosome 1, the CMT1C locus, yet unmapped, and the CMTX locus on the X chromosome.

CMT1A appears to be the most prevalent form of CMT1 and is associated with a 1.5 Mb tandem DNA duplication of p11.2-p12 locus on chromosome 17. The duplications in various ethnic groups have similar frequencies suggesting that it is the most prevalent cause of CMT1A. Unequal crossing over during male gametogenesis is thought to be the mechanism leading to the CMT1A duplication. Evidence showed the responsibility of dosage effect of the PMP22 gene, which maps within the CMT1A duplication interval, for the disease phenotype.

The aim of this study was to accomplish molecular analysis of CMT in Turkey. Since CMT1A is the most common type of CMT for which the genetic defect has already been identified, the analysis was started by detection of duplication in CMT1 patients. Detection of the duplication is an important requirement for the differential diagnosis of CMT1A, especially for patients with clinical symptoms similar to those typical for other peripheral neuropathies.

In this study, sixty nine per cent of CMT1 patients were found to carry the duplication by using a CA repeat polymorphism. Presence of the duplications in these patients was confirmed by Southern analysis using two different markers. These markers were shown to be highly informative for the detection of duplications, correlating with the previously published data for other populations.

ÖZET

Charcot-Marie-Tooth Nöropatisi (CMT), diğer adıyla Kalıtsal Motor ve Duysal Nöropati (HMSN), periferal sinirleri etkileyen kalıtsal hastalıklar içinde heterojen bir grup oluşturur. Her 2500 kişiden birinin CMT tiplerinden birini taşıdığı tahmin edilmektedir. Genellikle otozomal baskın seyreden CMT1'in en az dört ayrı kromozom bölgesi ile ilgili olduğu bilinmektedir. CMT1A 17. kromozoma, CMT1B 1. kromozoma, CMTX X kromozomuna haritalanmış, CMT1C bölgesi ise henüz haritalanamamıştır.

CMT1A hastalığın en sık görülen tipidir ve 17. kromozomun p11.2-12 bölgesinin 1.5 Mb'lık duplikasyonu sonucu ortaya çıkmaktadır. Duplikasyonların değişik etnik gruplarda benzer çoklukta görülmesi duplikasyonların CMT1A'nın en sık rastlanan nedeni olduğunu düşündürmektedir. CMT1A duplikasyonuna yolaçan mekanizmanın erkek gametogenezinde eş olmayan 'crossing-over' olduğu düşünülmektedir. Bulgular duplikasyon bölgesi içine haritalanan PMP22 geninin yoğunluk etkisinin hastalık fenotipinden sorumlu olduğunu göstermiştir.

Bu çalışmanın amacı CMT'nin moleküler analizini Türkiye'de gerçekleştirmektir. CMT1A'nın CMT'nin en yaygın görülen tipi olması ve hastalığa neden olan genetik bozukluğun tanımlanmış olması nedeniyle, analiz CMT1 hastalarında duplikasyonların belirlenmesi ile başlatılmıştır. Duplikasyonların belirlenmesi, özellikle klinik semptomları diğer periferal nöropatilerle benzerlik gösteren hastalarda, CMT1A'nın ayırıcı tanısı için önemli bir gerekliliktir.

Bu çalışmada CA yineli dizi uzunluk polimorfizmi kullanılarak CMT1 hastalarının yüzde altmış dokuzunun duplikasyon taşıdığı bulunmuştur. Southern analiz metodu ile iki değişik markör kullanılarak duplikasyonların varlığı kanıtlanmıştır. Bu markörlerin duplikasyonların tanımlanmasında yüksek oranda belirleyici olduğu ve bu markörlerle elde edilen değerlerin diğer toplumlar için yayınlanmış değerler ile uyumlu olduğu gösterilmiştir.

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ABBREVIATIONS

A260	absorption at 260 nm
A280	absorption at 280 nm
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
cM	centimorgans
dNTP	2'-deoxynucleoside 5'-triphosphate
EDTA	ethylenedinitrilo tetraacetate
EtBr	ethidium bromide
hr	hour
Kb	kilobase
Kda	kilodaltons
M	molar
Mb	megabase
MCV	motor nerve conduction velocity
min	minute
MPZ	myelin protein zero
o/n	overnight
ORF	open reading frame
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
pmole	picomole
rpm	revolution per minute
SDS	sodium dodecyl sulphate
Tris	tris (hydroxymethyl)-aminomethan
UV	ultraviolet

I. INTRODUCTION

Charcot-Marie-Tooth neuropathy (CMT), also known as hereditary motor and sensory neuropathy (HMSN), is a clinically and genetically heterogeneous group of inherited diseases of peripheral nerves in which patients develop a progressive distal muscular atrophy and sensory neuropathy of the distal extremities (Chance and Fischbeck, 1994). A prevalence rate of 1 in 2500 has been documented for all forms of CMT combined (Skre, 1974).

Like many inherited neurological disorders, Charcot-Marie-Tooth is marked by variable expressivity. Some patients may have only minimal symptoms and their diagnosis may depend on nerve conduction studies or obligate carrier status, while others may have severe distal atrophy and marked hand and foot deformities (Vance, 1991).

Although CMT may be detected by electrophysiological methods based on measurements of nerve conduction velocity (NCV) in infancy, the onset of the disease is usually during the first or second decade of life. Ninety seven per cent of individuals who have inherited Charcot-Marie-Tooth will manifest clinical symptoms by age 27 (Chance and Fischbeck, 1994).

The advent of neurophysiological techniques made the classification of this group of disorders easier. In 1968, Dyck and Lambert divided the autosomal dominant forms of CMT into two types based on physiological and pathological criteria :

1.CMT1, the demyelinating form (onion bulbs), with severely decreased nerve conduction velocity and constituting the majority of CMT cases, and

2.CMT2, the axonal form, with normal or mildly decreased nerve conduction velocity.

In the majority of CMT1 and CMT2 pedigrees, the mode of inheritance is autosomal dominant. However, studies on NCV have suggested that autosomal recessive forms of both demyelinating and axonal types exist. In addition to that, many reports showed the existence of an X-linked form of CMT1 (Lupski et al., 1991).

A. Linkage Studies

Bird et al. (1980) initially suggested linkage of CMT to the Duffy blood group locus (Fy) on chromosome 1. However, in further studies, Bird et al. (1983) and Dyck et al. (1983) reported families of typical CMT1 for which linkage to Duffy blood group was excluded. Whereas Dyck et al. (1983) could discern no phenotypic differences between the linked and unlinked forms, Bird et al. (1983) suggested that slowing of nerve conduction is less marked and onion bulb formation on sural (referring to the calf of the leg) nerve biopsy less conspicuous in the Duffy unlinked form. This suggested that there are at least two clinically indistinguishable disorders within the category of CMT1. On that basis, CMT families not linked to Duffy were designated as having CMT1A while those which showed linkage to Duffy were designated as having CMT1B.

In 1983, Bird et al. detected linkage to two markers (D17S7 and D17S38) on the proximal short arm of chromosome 17 in six non-Duffy linked CMT1 families. This has since been confirmed by other investigators (Raemaekers et al., 1989). Vance et al. (1991) refined the localization of CMT1A between two markers D17S122 and D17S61, both mapping to band p11.2-12 on the pericentromeric region of chromosome 17.

In 1990, Chance et al. reported two clinically typical autosomal dominant CMT1 pedigrees which neither mapped to the region of the Duffy locus on chromosome 1q (CMT1B) nor to the proximal chromosome 17p (CMT1A). Confirmation of this exclusion suggested the existence of a third autosomal dominant locus for this already heterogeneous disorder. Nowadays, pedigrees with autosomal dominant CMT1 not mapping to chromosome 1q or to chromosome 17p are designated as CMT1C for which the locus (or loci) remain unassigned.

On the other hand, a second type of demyelinating neuropathy was reported and designated as HMSN type III or Dejerine Sottas Disease (DSD). Recent molecular genetic studies have revealed that DSD may be associated with loci either on chromosome 17p (DSDA) or on chromosome 1q (DSDB) (Hayasaka et al., 1993b).

Hereditary neuropathy with liability to pressure palsies (HNPP), another type of demyelinating neuropathy, has been found to be linked also to chromosome 17p11.2-p12 (Chance et al., 1993).

CMT2 appears to be genetically distinct from all mapped forms of CMT1. Loprest et al. (1992) excluded linkage of the neuropathy in three CMT2 pedigrees to the CMT1A region in 17p11.3-12 and the CMT1B region in 1q. A CMT2 locus was assigned by linkage studies to the short arm of chromosome 1 (1p36) and designated as CMT2A (Ben Othmane et al., 1993). Additional families fulfilling the diagnostic criteria for CMT2 did not have

evidence of linkage to this locus, thus, two other forms of CMT2, CMT2B and CMT2C were described, but their loci remain unassigned (Ben Othmane et al., 1993).

B. Demyelinating Disorders

Myelin is one of the fundamental adaptations of vertebrates. Composed of a spiral of cellular membrane that forms a multilamellar sheath around axons, myelin serves to speed electrical transmission by increasing axonal conduction velocity. Peripheral nervous system (PNS) and central nervous system (CNS) myelin is composed of Schwann cells and oligodendrocytes, respectively, and consists of a partially overlapping set of myelin related proteins and lipids (Suter and Snipes, 1995).

PNS myelin contains Myelin Protein Zero (P_0), Peripheral Myelin Protein 22 (PMP22), myelin basic protein (MBP), myelin associated glycoprotein (MAG) and gap junction protein connexin 32 (Cx32) (Scherer and Chance, 1995) (Figure I.1).

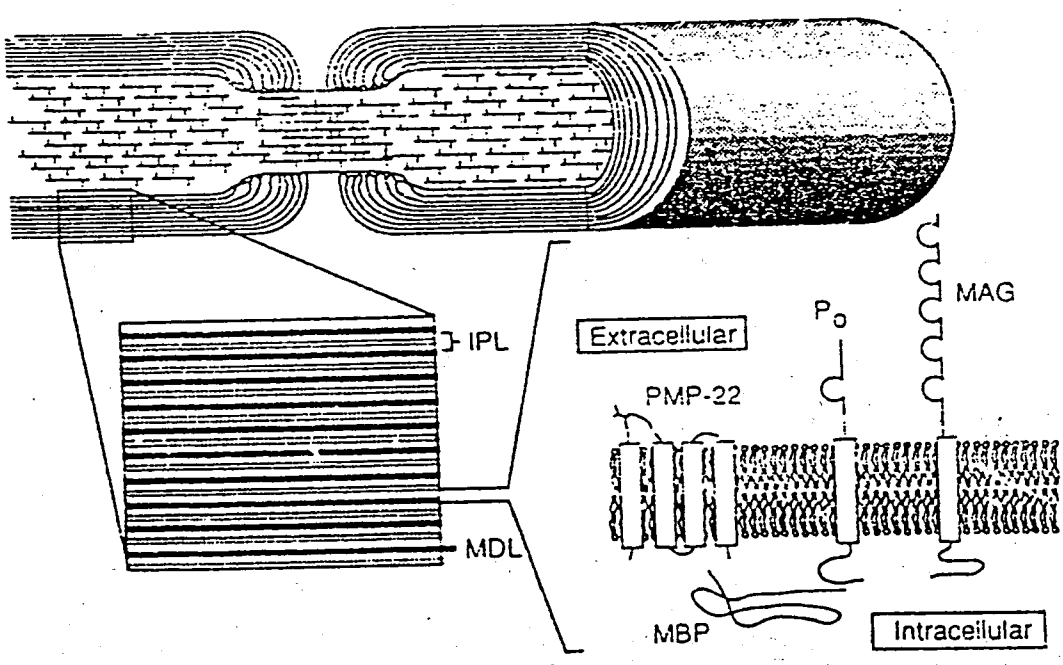


Figure I.1. Myelin structure and associated proteins (Suter et al., 1993).

Inherited demyelinating neuropathies of the peripheral nervous system are defined as the group of diseases that affect the formation and/or the maintenance of myelin and have been clinically classified as CMT typel and Dejerine Sottas Disease (Patel and Lupski, 1994).

Although the clinical manifestations of CMT1 may not present until adolescence or adulthood, the electrophysiological abnormalities are evident by an early age and usually by the age of two (Lupski et al., 1991).

1. CMT1

CMT1 constitutes 70 per cent of CMT cases and is characterized by atrophy in the peroneal muscle, leg muscle and distal arm. Weak or absent deep tendon reflexes and sensory defect are also detectable. In addition to this, hyperhidrosis, penetrating foot ulcers, progressive pes cavus and trophic limb changes are characteristics of this disease. Weakness of the intrinsic hand muscles usually occurs late in the course of the disease, but may not be related to the degree of leg weakness or atrophy. In severe cases, the wasting of the intrinsic hand muscles may give the appearance of claw hands. Patients may show symptoms of chronic diarrhea, nausea and vomiting. Heart block can be one of the consequences of the disease. NCV values are usually less than 40 m/sec.

Findings on CMT1 based mainly on sural nerve biopsies revealed a marked loss of myelinated fibers and abundant onion bulb formations which consist of circumpherencially directed Schwann cells and their processes around myelinated and demyelinated internodes (Dyck et al., 1993).

a. CMT1A

CMT1A is by far the most common form of CMT1 which constitutes more than 70 per cent of CMT1 cases, as estimated by a European collaborative study in 1996, and half of the familial cases of hereditary neuropathy (Wise et al., 1993).Molecular genetic analysis in CMT1A revealed a novel disease causing mechanism in humans that involves a large inherited DNA arrangement. It has been shown that CMT1A is completely linked and associated with a 1.5 Mb tandem DNA duplication in the chromosome 17p11.2-p12 region due to unequal crossing over during meiosis (Lupski et al., 1991). The duplication results

in the overproduction of the protein PMP22, which in turn exerts a deleterious effect on CMT1A phenotype.

b. CMT1B

CMT1B is another subtype of the demyelinating CMT type 1 neuropathies the symptoms of which are the same as those characterizing CMT1A. It is dominantly inherited and has a clinical onset from late childhood to adolescence. Mild to moderately reduced nerve conduction velocities (10-40 m/s) are present with moderate demyelination on nerve biopsies (Dyck et al., 1993).

The abundant peripheral myelin protein zero (MPZ) gene was mapped 130 Kb centromeric to the Fc receptor immunoglobulin gene cluster in band 1q22-q23. The gene is about 7 Kb long and consists of six exons corresponding to the functional domains. The 5' flanking region of the gene has a TA rich element (TATA-like box), two CAAT boxes, and a single defined transcription initiation site.

The integral membrane glycoprotein Protein Zero (P_0) is the major protein component (60 per cent of total protein) of peripheral nervous system myelin (Greenfield et al., 1973). P_0 expression is restricted to myelinating Schwann cells (Brockes et al., 1980) and the protein is a member of the immunoglobulin (Ig) gene superfamily. The P_0 precursor protein consists of 248 amino acids including a 29 amino acid long N-terminal signal sequence which is absent in the mature protein (Lemke, 1993). It has been proposed and later experimentally confirmed that P_0 is involved in homophilic interactions via the Ig domain and carbohydrate structures. It is thought that the function of P_0 as an adhesion molecule is essential for myelin compaction. This hypothesis is supported by the phenotype of mutant mice where the P_0 gene has been disrupted (Geise et al., 1992). Homozygous P_0^- mice exhibit defects in myelin compaction, hypomyelination, and degradation of myelin and axons. Behaviorally, these mice are deficient in motor coordination and exhibit tremors and occasional convulsions.

To determine if the P_0 gene is altered in CMT1B patients, genomic DNA from patients of one CMT1B family has been analyzed for mutations in P_0. A 3 bp deletion in exon 2 of the P_0 gene, that results in the deletion of serine at position 63 of the P_0 precursor protein, has been identified in all affected members of this family (Kullens et al., 1993). Sequencing of the P_0 gene in another CMT1B family showed a C to A point mutation at position 446 in exon 3 resulting in an Asp136 Glu substitution (Nelis et al., 1994). A major MPZ point mutation was found to co-segregate with CMT1B in a large CMT1B family. This point mutation converts a positively charged lysine in codon 96 to a negatively charged

glutamate. The same MPZ locus co-segregates with the CMT1B disease gene in a second CMT1B family with a splice junction mutation. Hayasaka et al. (1993a) found a mutation in another CMT1B family. The mutation, a methionine substitution for isoleucine at amino acid position 30, is located in the extracellular domain, which constitutes an immunoglobulin domain responsible for the function of the P_0 gene as an adhesion molecule (Figure I.2).

c. CMTX

Linkage studies and analysis of recombinants were initially used to map CMTX to the proximal long arm of the X chromosome and subsequently to refine the localization to band Xq13 (Bergoffen et al., 1993). Analysis of additional recombinants in CMTX families placed CMTX in a small interval between the markers DXS106 and DXS559 in Xq13.1 (Bergoffen et al., 1993). The gene for connexin32 (Cx32) is known to map to this interval (Corcos et al., 1992) and therefore, it was a candidate gene for CMTX. Northern analysis showed that the expression of the Cx32 gene in peripheral nerve is greater than that present in most other tissues (Bergoffen et al., 1993). Sequencing of the translated portion of the gene in samples from CMTX patients revealed seven variations from the control sequence in eight CMTX families.

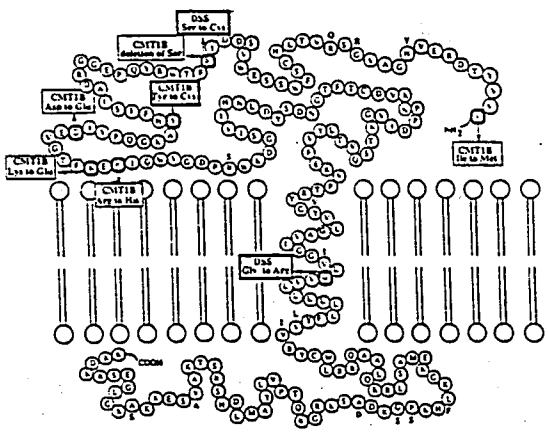


Figure I.2. P_0 Protein and sites affected by point mutations in CMT1B families (Patel et al., 1994).

Connexins are membrane-spanning proteins that assemble to form gap junctions, channels that facilitate the transfer of ions and small molecules from cell to cell. The connexin subunits are assembled into half channels, or connexons, that interact with their counterparts in neighboring cells to form complete intercellular channels (Dermeitzel et al., 1993).

Connexin32 is a member of the connexin family that was originally cloned in 1986. It has two extracellular loops, four transmembrane segments, and three cytoplasmic domains. The amino acid residues altered by the CMTX point mutations are generally conserved in vertebrates and are located in portions of the protein that are believed to be functionally important (Figure I.3).

The distribution of Cx32 protein at the nodes of Ranvier and Schmidt-Lanterman incisures suggests that Cx32 may form intracellular gap junctions that connect the folds of Schwann cell cytoplasm. This would allow transfer of ions, nutrients, and other small molecules around and across the compact myelin to the innermost myelin layers, and perhaps to the axon as well. This would explain the combination of myelin disruption and axonal degeneration that occurs with Cx32 mutations in CMTX (Hahn et al., 1990).

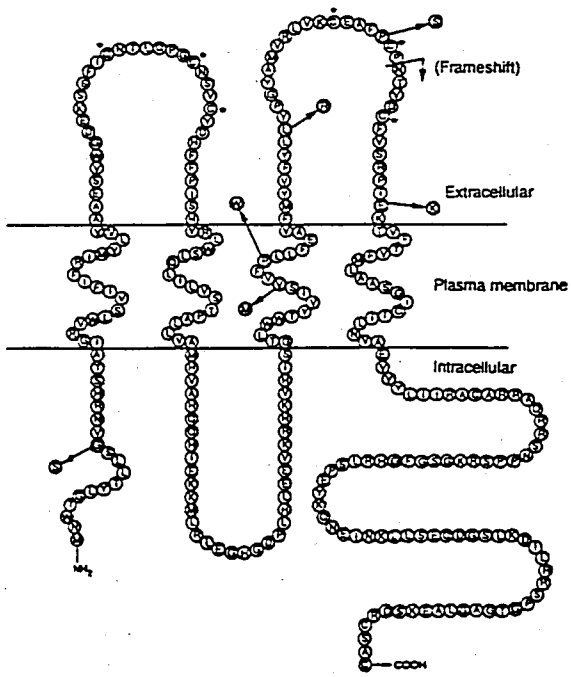


Figure I.3. Diagram of Cx32 showing transmembrane orientation and locations of the CMTX mutations (Bergoffen et al., 1993).

2. Dejerine Sottas Syndrome (CMT Type 3)

Dejerine Sottas syndrome is a hypertrophic, demyelinating neuropathy which appears to demonstrate autosomal recessive inheritance in most pedigrees, although autosomal dominant transmission has been documented (McCusick, 1992). Clinical symptoms are similar to, but more severe than those of CMT1A. NCV values are usually very low (less than 10m/s). By mutational analysis of the PMP22 coding region in two Dejerine Sottas patients, Roa et al. (1993a) identified individual missense point mutations present in the heterozygous state. One patient had a T-to-A transversion leading to a Met69-to-lys substitution, whereas the other had a C-to-T transition leading to a ser72-to-leu substitution. The patient with the met69-to-lys substitution had no detectable abnormality at birth but did not begin walking until the age of 15 months and did so with an abnormal gait. Bilateral pes cavus was noted at age six, and delayed nerve conduction velocity in the left ulnar nerve was measured at age seven. By age 18, she had severe lower limb weakness necessitating the use of a wheel chair and severe distal sensory loss in all four limbs. No other family member was known to be similarly affected. Electron microscopy of sural nerve biopsy demonstrated hypertrophy of the nerve with marked loss or abnormality of myelinated fibers. Valentijn et al. (1995) identified a *de novo* mutation in the PMP22 gene of another patient with Dejerine Sottas Disease. Sequence analysis showed a *de novo* C-to-A transversion at nucleotide 85 that resulted in an amino acid substitution his12-to-gln in the first transmembrane domain of PMP22.

These findings proved the hypothesis that mutations in the PMP22 gene may also be associated with DSD, and in this case it is designated as DSDA (Hayasaka et al., 1993b). Molecular genetic studies have revealed that DSD can be associated with point mutations in the P_0 gene as well, and thus it is designated as DSDB (Hayasaka et al., 1993b).

C. Axonal Neuropathies (CMT2)

CMT2 is a common inherited axonal neuropathy. Generally, it may have a later age of onset, less involvement of the hand muscles, and does not have palpably enlarged nerves. Extensive demyelination with "onion bulb" formation is not present in CMT2. Motor nerve conduction velocities are normal or only slightly abnormal in affected persons (Dyck et al.,

1993). CMT2 is genetically distinct from all mapped forms of CMT1. Ben Othmane et al. (1993) reported the localization of one form of CMT2 to chromosome 1p35-p36. This locus was designated as CMT2A. Additional families fulfilling the diagnostic criteria for CMT2 do not have evidence of linkage to this region on chromosome 1, suggesting genetic heterogeneity within CMT2 (Dyck et al., 1993). These pedigrees with axonal neuropathy unlinked to chromosome1p36 are designated as CMT2B. Further genetic heterogeneity within CMT2 is likely as there are kindreds with the features of axonal neuropathy with diaphragm weakness and vocal cord paralysis that are designated as having CMT2C (Dyck et al., 1994). Yoshioka et al. (1996) examined a CMT2C pedigree for evidence of linkage to chromosome 1p associated with CMT2A. The analysis included linkage studies with DNA markers mapping within the known CMT2A gene region on chromosome1, and all the results came out to be negative, which confirmed genetic heterogeneity that was previously described within axonal neuropathies.

D. CMT Type 4

Ben Othmane et al. (1993) demonstrated that one form of autosomal recessive CMT in Tunisian families represented a homogeneous form on clinical, electrophysiologic and pathologic criteria. The disorder, which they designated type A, is a severe neuropathy of childhood, characterized by early age of onset, rapidly progressive distal weakness, and atrophy of the limbs leading to an inability to walk in late childhood or adolescence, slow NCV, and hypomyelination. Linkage of this neuropathy for several markers from 8q13-q21.1 was demonstrated (Ben Othmane et al., 1993).

E. Hereditary Neuropathy with Liability to Pressure Palsies (HNPP)

Models of intermolecular crossing over would predict that beside the CMT1A duplication on one chromosome, the recombination event should also result in a reciprocal

deletion of the same sequences on the other homologous chromosome involved. Patients carrying such deletions have been found to suffer from the inherited neuropathy HNPP, also called tomaculous neuropathy (Chance et al., 1993).

HNPP is an autosomal dominant disease which presents as recurrent pressure palsies. The pressure palsy can be precipitated by apparently trivial trauma like sleeping on a limb and noticing that the resulting palsy, instead of clearing within seconds to minutes, remains for days to weeks (Wide bank et al., 1993). Clinically, HNPP patients exhibit moderately decreased NCV and the main pathological findings are segmental demyelination and remyelination with extreme focal myelin thickenings in some teased fibers of the peripheral nerves (referred to as tomacula i.e. sausage, based on the appearance of myelin in longitudinal sections). Interestingly, with respect to the function of a common disease gene (PMP-22 gene), the symptoms in HNPP appear to be reversible while, in striking contrast, CMT is typically a slowly progressive disease (Suter et al., 1994).

Since HNPP and CMT1A are reciprocal products of the same mechanism, the same methods used to detect CMT1A duplications are used in detecting HNPP deletions. The most common methods used to evaluate deletion of the 1.5 Mb region are analysis of RFLP loci and the polymorphic (CA)n repeat within the deletion region, Southern analysis to detect CMT1A-REP dosage differences, and pulsed field gel electrophoresis (PFGE). The European collaboration study presented in 1996 showed that the 17p11.2 deletion was present in 84 per cent of 156 unrelated HNPP patients (Nelis et al., 1996).

Lorenzetti et al. (1995) conducted a study on nine HNPP Italian families and found a deletion of the 17p11.2-p12 DNA markers in all of them. This lent further support to the hypothesis that haploinsufficiency plays a causative role in the disease.

F. Pathophysiology of CMT1A

1. The Duplication Region and its Origin

In an attempt to determine the size of the CMT1A duplication and to investigate the mechanism causing it, Pentao et al. (1992) constructed a 3.1 Mb restriction map of the CMT1A region. Physical mapping demonstrated that a 1.5 Mb tandem duplication of 17p11.2-p12 is present on the CMT1A chromosome. At least three CpG islands were

identified in the CMT1A monomer unit, with one near the 5' end of PMP-22. Several low copy repeat sequences were also detected in this region, one of which flanked the CMT1A monomer unit. The latter repeat, CMT1A-REP, is present in three copies on the duplicated chromosome. Furthermore, two duplicated probes VAW409 and EW401 were physically mapped within 1.0 Mb of each other, whereas the average genetic distance between the two probes is 4 cM (~ 4 Mb) in males and 14 cM (~14 Mb) in females (Figure I.4).

This large discrepancy between the genetic and physical distances of these duplicated markers suggests that the 17p11.2-p12 region may be extremely prone to meiotic recombination, a fact demonstrated previously by Greenberg (1991), who showed that a deletion in the 17p11.2 region is resulting in the Smith-Magenis syndrome (SMS) phenotype.

It was proposed by Pentao et al. (1992) that the CMT1A REP functions in the formation of the de novo CMT1A duplication by unequal crossing over during meiosis through misalignment of this repeat (Figure I.5). Analysis of segregation of the fully informative alleles showed that all 12 de novo duplications reported until 1993 were of paternal origin (Palau et al., 1993). On the other hand, Upadhyaya et al. (1993) reported the case of a male child with CMT1A duplication of maternal origin.

2. Trembler Phenotype Associated with Mutations in the PMP-22 Gene

The search for a similar mouse mutant that could be due to a PMP-22 defect identified the autosomal dominant Trembler mutation as a likely candidate (Falconer, 1951). Trembler (Tr) and TremblerJ (TrJ) animals show a marked decrease of axonal myelination in the PNS (Henry and Sidman, 1988) and continued Schwann cell proliferation throughout life (Henry et al., 1983). Grafting experiments of Tr sciatic nerve segments into normal mice and vice versa (Aguayo et al., 1977) have undoubtedly revealed that the Trembler mutation is due to an autonomous Schwann cell defect, which correlates with the expression of PMP-22 mainly in Schwann cells.

As a first step in finding candidate mutations, it was demonstrated that the allelic Tr (Falconer, 1951) and TrJ (Henry et al., 1983) mutations map to the genetically defined Tr locus on mouse chromosome 11 in a region of conserved synteny with human chromosome 17p (Davisson and Roderick, 1977). These mouse strains have been shown to carry point mutations in the first and fourth transmembrane domains of PMP-22 (Suter et al., 1992b).

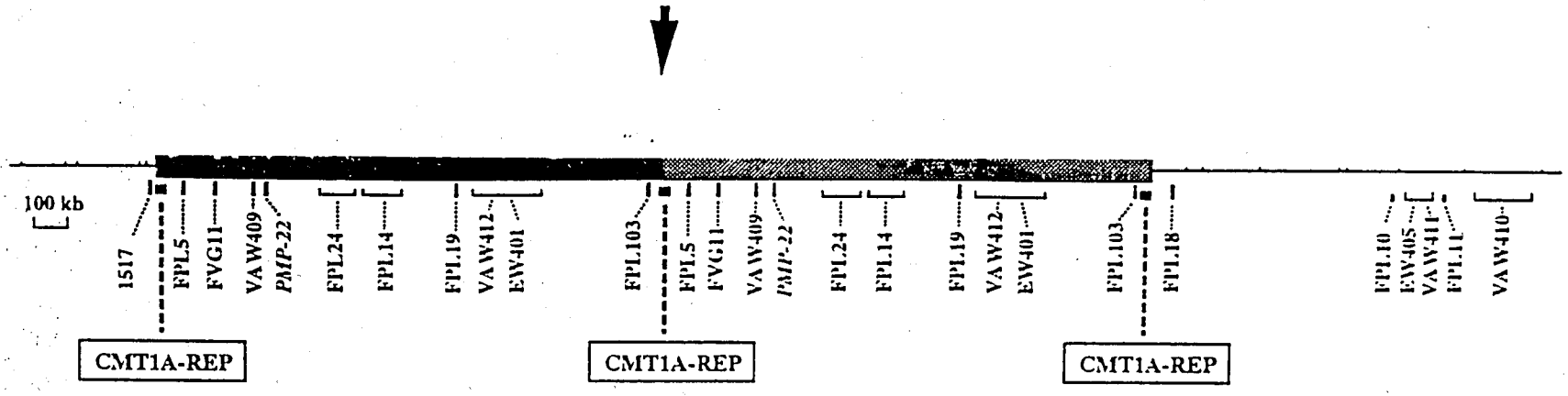


Figure I.4. The 1.5 Mb tandem CMT1A duplication in 17p11.2-p12. The arrow denotes the junction point. The marker locations are listed below (Pentao et al., 1992).

It is estimated that approximately 30 genes are present within the CMT1A duplication but only a few are expected to be responsible for a defect restricted to the PNS. It was proposed that mutations affecting the PMP-22 gene might be responsible for CMT1A and related peripheral neuropathies (Suter et al., 1992a) based on the following findings: Tr mice features resemble the neuropathy seen in CMT1A patients; defects in PMP-22 have been found to be responsible for hereditary peripheral neuropathies in mice; the group of genes on mouse chromosome 11 and the proximal arm of human chromosome 17 are of conserved synteny. Patel et al. (1992), Matsunami et al. (1992), and Timmerman et al. (1992) described the cloning of human PMP-22 cDNA and demonstrated that the PMP-22 gene maps within the duplication interval in CMT1A patients. Roa et al. (1993b) identified a case with a point mutation in PMP22 which resulted in the substitution of cysteine for serine in a putative transmembrane domain of PMP22. The point mutation arose spontaneously and segregated with the CMT1 phenotype in an autosomal dominant pattern. In another study, Roa et al. (1993a), identified a severely affected CMT1 patient who is a compound heterozygote for a recessive PMP22 point mutation and a 17p11.2-p12 deletion. The point mutation was detected by heteroduplex analysis of amplified PMP22 coding regions and by direct nucleotide sequencing. These results suggest that point mutations in PMP22 can result in both dominant and recessive alleles contributing to CMT1A and prove that the PMP22 gene has a causative role in CMT type1, since either a duplication of the

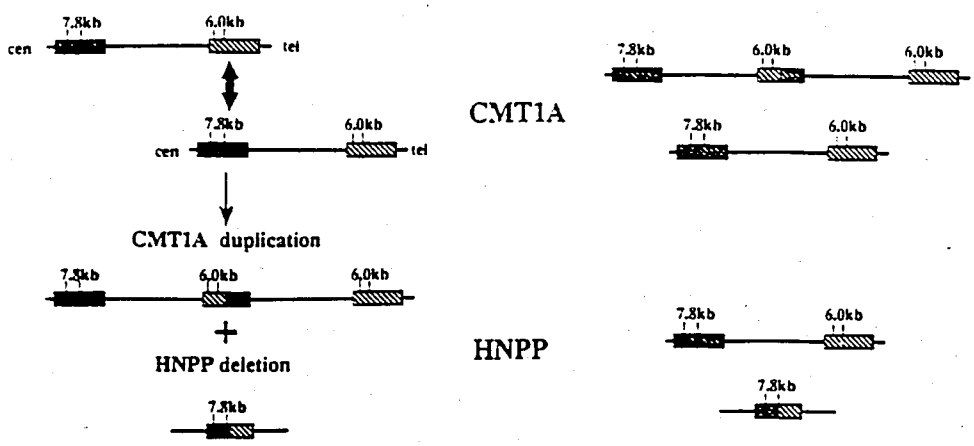


Figure I.5. Homologous recombination at flanking CMT1A REP elements leading to the CMT1A duplication and HNPP deletion (Lorenzetti et al., 1995).

region including the PMP22 gene or a point mutation in this gene can result in the disease phenotype. It was noticed that the partial loss of PMP22 in mice and humans (PMP22+\-) appear to be far less deleterious than the point mutations, which suggests that the majority of point mutations do not cause a simple loss of function, but have a toxic gain of function. The abnormal proteins could destabilize the myelin sheath, and thereby cause demyelination (Scherer et al., 1995).

3. Characterization and Structure of PMP22

Efforts to identify a candidate gene that plays a primary role in the CMT1A phenotype revealed the importance of peripheral myelin protein PMP-22. Northern blot and in situ hybridization analysis revealed that the PMP-22 mRNA is mainly found in the peripheral nervous system PNS, especially in Schwann cells (Snipes et al., 1992). Snipes et al. (1992) demonstrated that PMP-22 is present as a 22-Kda protein in essentially all myelinated fibers of the PNS and is localized to compact myelin. Furthermore, expression of PMP-22 correlates with myelin formation during sciatic nerve development, and with myelin degradation and remyelination during sciatic nerve regeneration.

The encoded amino acid sequence of PMP-22 (160 amino acid residues in all species examined) predicts a molecular weight of 18 Kda for the PMP-22 core protein. One conserved consensus sequence for N-linked glycosylation was found (Welcher et al., 1991). The PMP-22 protein is highly conserved between species at the level of its primary structure (Patel et al., 1992). Studies on the protein structure revealed the presence of four hydrophobic, possibly membrane spanning domains (Suter et al., 1992b). This predicted secondary structure of PMP-22 is supported by experiments indicating that PMP-22 is likely to be a true integral membrane protein with four membrane spanning domains, since it is completely protected against proteinases after insertion into microsomal membranes (Manfioletti et al., 1990) (Figure I.6).

4. From Duplication to CMT1A Phenotype

The exact mechanism by which the duplication could result in the CMT phenotype is unknown, but Lupski et al. (1991) suggested two possible mechanisms for the disease

phenotype: first, overexpression of one or more genes in the region (dosage effect); second, interruption of a candidate gene at the duplication junction leading either to an altered gene product with a dominant deleterious effect or to an absence of the gene product, thus resulting in decreased levels of this product. Pentao et al. (1992) demonstrated that the PMP22 gene maps at some distance from the breakpoint junction of the duplication and is not likely to be disrupted.

The identification of rare patients with larger cytogenetically visible duplications of 17p harboring the region duplicated in CMT1A provided the unique opportunity to test gene dosage as a mechanism in CMT1A (Lupski et al., 1992). Further support to the gene dosage mechanism came from molecular studies of a patient who was homozygous for the CMT1A duplication (Lupski et al., 1991) and similar in severity to the Dejerine Sottas syndrome. Additional proof for gene dosage effect came from a case report showing a patient diagnosed as a CMT1A patient based on electrophysiologic and molecular studies (Figure I.7). Cytogenetic analysis for the patient and her parents showed that the father carries an apparently balanced reciprocal translocation between chromosomes 14 and 17 where the breakpoints are in 17p11 and 14p11. The patient inherited a normal chromosome 17 from each parent and the derivative chromosome 14 with the short arm of chromosome 17 from her father, resulting in trisomy of 17p which proved that her disease phenotype is not the result of disruption of a specific gene (Chance et al., 1992) (Figure I.7).

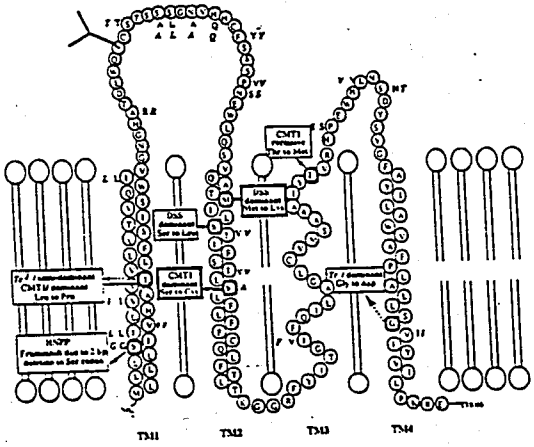


Figure I.6. Diagram of PMP22 with the four integral membrane structure. Point mutations associated with peripheral neuropathies are given on the diagram (Patel et al., 1994).

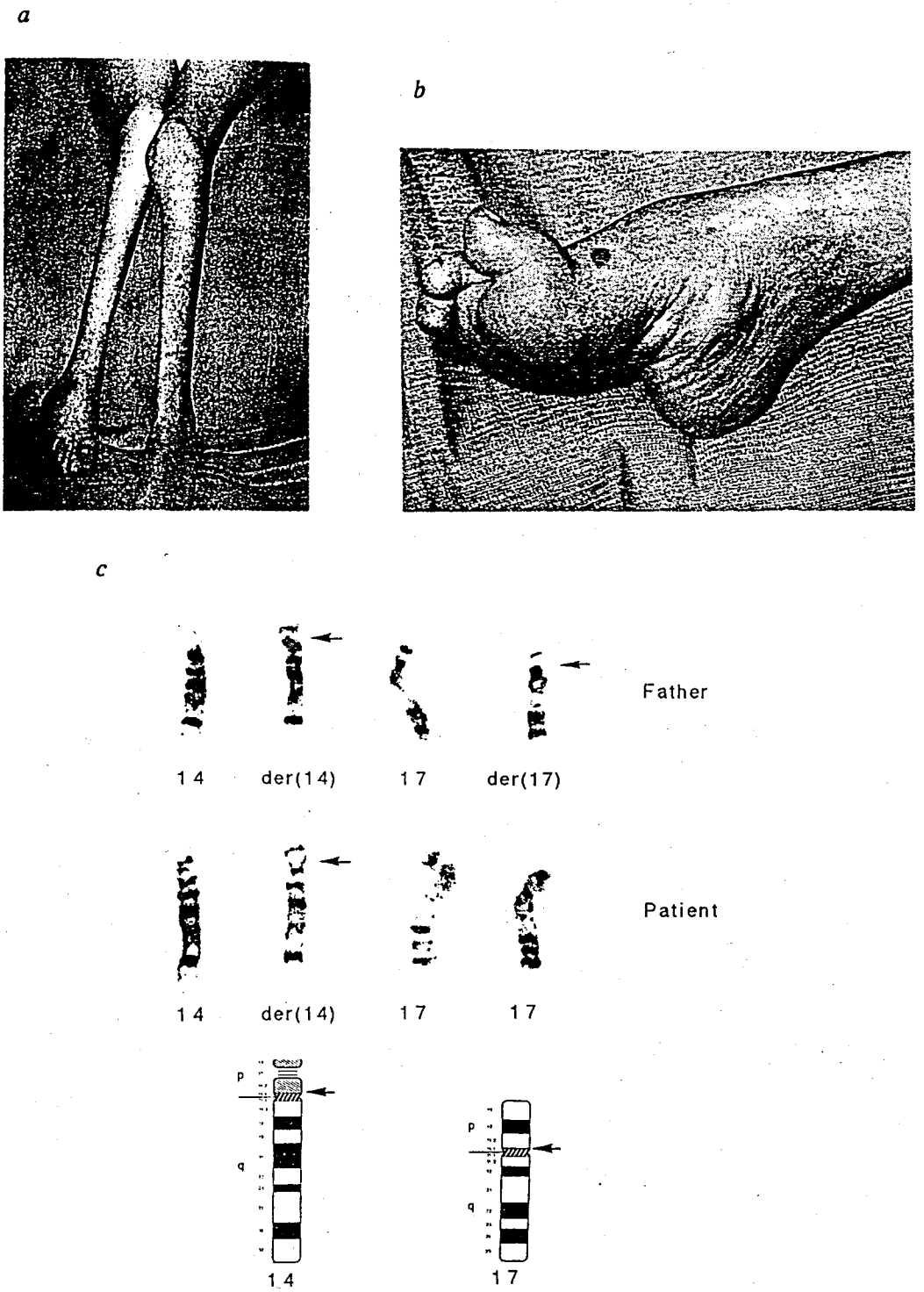


Figure I.7. Clinical and cytogenetic analyses for the CMT1A patient with no duplication (Chance et al., 1992).

a. Marked lower leg atrophy in the patient with trisomy 17p.

b. View of the right pes cavus foot deformity of the patient.

c. Giemsa-stained metaphase chromosomes of the 17p trisomy patient and father depicting the der (14) and der (17) chromosomes. Translocation breakpoints are shown by arrows.

Experimental evidence that CMT1A is caused by increased expression of the gene for PMP-22 was provided by a transgenic rat model of the disease. PMP-22 transgenic rats develop gait abnormalities caused by a peripheral hypomyelination, Schwann cell hypertrophy (onion bulb formation), and muscle weakness (when righting up, they fail to hold their body weight on the stretched hind limbs). Reduced nerve conduction velocities closely resemble those recorded in human CMT1A patients. When bred to homozygosity, transgenic animals completely failed to elaborate myelin, retarded in growth, and never learned to control limb movements (Sereda et al., 1996).

5. Methods Available for the Detection of the Duplication

Several methods are used for the detection of the duplication. These are:

a. Pulsed Field Gel Electrophoresis

To obtain an estimate of the size of the duplication, long range restriction mapping is performed using PFGE. Restriction enzymes Not1, Mlu1, SacII, and Nru1 are used to digest DNA from affected and control individuals to identify altered and/or novel fragments in CMT1A patients. Two SacII fragments of 600 Kb and 550 Kb, which are polymorphic alleles or variants arising as a result of methylation differences, can be detected in control individuals using VAW409R3 as a probe. However, a novel 500 Kb SacII fragment that shows Mendelian inheritance is observed in CMT1A patients (Lupski et al., 1991) (Figure I.8).

b. Dosage Difference by RFLP Analysis

Probe VAW409R3a (D17S122) detects polymorphic Msp1 alleles of 2.8 Kb, 2.7 Kb, and 1.9 Kb on Southern blots (Raeymaekers et al., 1992). Either a dosage difference between two different bands or, in rare cases, the presence of three bands can be detected in patients with the duplication. This probe is the most informative marker, having an average heterozygosity of 70 per cent. Dosage can also be scored between polymorphic

EcoR1\HincII alleles (11 Kb+9.6 Kb) detected by probe p132-G8R1 (a genomic subclone of the CMT1A candidate gene PMP22) due to a HincII site polymorphism (Patel et al., 1992). This marker is the second most informative one with 62 per cent heterozygosity in the patients. In addition, dosage difference can be scored between polymorphic Msp1 alleles (5.3Kb, 2.7Kb, 2.6Kb long) detected by probe pVAW409R1b (D17S122) and between alleles (5.5Kb, 4.4Kb long) detected by probe pEW401HE (D17S61). These probes detect heterozygous alleles in 44 per cent and 41 per cent of the cases respectively (Wise et al., 1993). In this study, DNA duplications in CMT1A patients were detected by RFLP analysis using probes p132-G8R1 and pEW401HE.

c. (GT)n Polymorphism at the D17S122 Locus

(GT)n sequences, which are known to be highly polymorphic and can be rapidly analyzed by the polymerase chain reaction (PCR), were identified in several probes. One of these, RM11-GT, was identified from VAW409R1 located at the D17S122 locus. This marker maps to 17p11.2-p12 locus and is also closely linked to CMT1A (Vance et al., 1991). Genotype data demonstrate the presence of three (GT)n alleles in 46 per cent of CMT1A individuals while all unaffected individuals are either homozygous or heterozygous for (GT)n alleles. In certain matings, only two (GT)n alleles can be detected in the affected

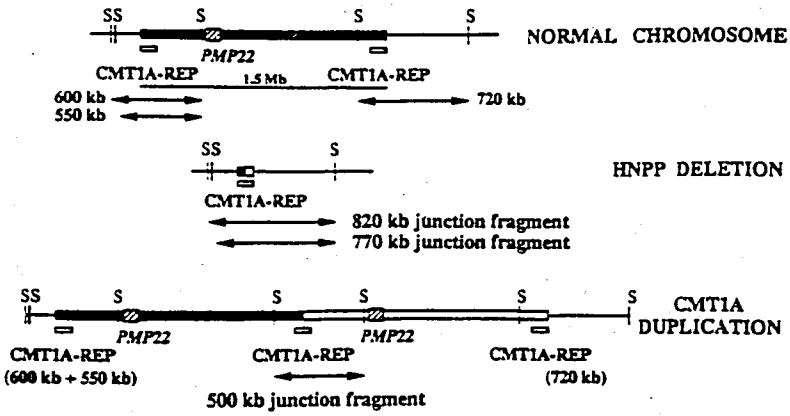


Figure I.8. PFGE analysis to detect HNPP deletion and CMT1A duplication junction fragments. DNA is digested with SacII and fractionated by PFGE followed by Southern hybridization with a probe that maps within the CMT1A-REP flanking the duplication/deletion region. A novel 500 Kb junction fragment is observed in CMT1A patients (Roa et al., 1993).

child, however, careful examination of the autoradiogram often shows that one of the two alleles is present in two copies. Schiavon et al. (1994) presented a study focusing on a non radioactive test for the RM11-GT polymorphism based duplication detection. Visualization of the alleles was accomplished by silver staining of the PCR products resolved on polyacrylamide gels (Schiavon et al., 1994). This non-radioactive method was also used in this study, and it proved to be fast and reliable.

d. FISH Analysis on CMT1A Patient Cells

Two-color fluorescence in situ hybridization (FISH) in interphase nuclei (Lupski et al., 1992) provide direct visualization of duplication of the VAW409 locus in CMT1A patients. A total of three VAW409 hybridization sites are observed in the majority of these cells from the CMT1A patients but in few cells from unaffected individuals (Lupski et al., 1991). Valentijn et al. (1992) used the two color fluorescence in situ hybridization to demonstrate that the duplication is a direct tandem repeat: they observed red-green for normal chromosomes and red-green red-green for duplicated chromosomes. In none of the nuclei analyzed was the order red-green green-red or green-red green-red compatible with an inverted repeat.

6. New Findings in the Field of CMT1A

a. Longitudinal Conduction Studies

A very recent study was conducted on eight patients from two nuclear families with CMT1A with proven duplication but with no appreciable change in their MCVs over 22 years. The findings that 22-year longitudinal conduction studies remain fairly constant in patients with CMT1A support the hypothesis stating that the disturbance of myelination expresses itself in childhood and is non progressive after childhood by MCV measurements. However, the mild progression of leg weakness in some patients may be due to a very slowly progressive distal axonal degeneration which is either secondary to chronic loss of myelin or an underlying independent axonopathy (Killian et al., 1996).

b. Effect of Vincristine Treatment on Asymptomatic CMT Disease

Vincristine sulfate is a vinca alkaloid frequently used in the chemotherapy for various types of cancers. It exerts both cytotoxic and neurotoxic activities by the binding and inactivation of tubulin, which causes disruption of the mitotic spindle apparatus leading to an axonal neuropathy (Sahenk et al., 1987). Graf et al. (1996) described three families with autosomal dominant CMT1, among whom a family member with a type of cancer suffered rapid onset severe neuropathy after receiving initial doses of vincristine. All three families had at least one affected family member with a 17p11.2-12 duplication.

Although mild neuropathy is commonly associated with vincristine therapy, most oncology patients who receive vincristine do not have acute onset, severe peripheral neuropathy. The clinical situation of CMT1A patients predicts that the 17p11.2-12 duplication predisposes to more severe neurotoxicity due to the deleterious effect of vincristine- tubulin interaction caused by the degeneration of peripheral myelin. It was demanded then that vincristine should not be given to any person at risk for or known to have a hereditary peripheral neuropathy since it is possible that other hereditary neuropathies like CMT1B and X-linked CMT can predispose patients to vincristine-related or other neurotoxic reactions (Graf et al., 1996).

c. A Recombination Hotspot Responsible for CMT1A and HNPP

Reiter et al. (1996) conducted a study on the CMT1A REPs to determine the mechanism by which the CMT1A duplication and the HNPP deletion are generated. Because of the high degree of homology between the proximal and distal ~30 Kb CMT1A-REPs, a homologous recombination event between misaligned REP sequences could occur anywhere within the ~30Kb region. However, most of the cross over events were suspected to occur within a 7.8 Kb fragment in each of the REPs (Figure I.9). Junction fragments of 1.7 Kb and 7.8 Kb length in CMT1A patients and HNPP patients respectively, were determined by hybridization of the 7.8 Kb suspected fragment to Southern blot panels of DNA from patients and control individuals (Figure I.9). These fragments were found to be stably inherited with the disease phenotypes of unrelated patients, which indicated a precise recombination mechanism in that small region of the repeat. Furthermore, sequencing the entire 7.8 Kb regions from the proximal and distal REPs, followed by alignment of the recombination regions in each of them lead to interesting findings. These aligned sequences were found to be AT rich in character and to have a percentage of

similarity not exceeding that found throughout the 7.8 Kb region. Longer stretches of identity were found outside the hotspot region. These data argued against the hypothesis stating that the increased identity between two homologous regions is the fact responsible for unequal crossing over and suggested that some other signal must be present at or near the site of strand exchange.

In light of these findings, and in addition to presence of three exons in the hotspot region, one of which showing homology at the amino acid level to the conserved regions of several insect transposases, a mariner transposon-like element was suggested to be present near the hotspot for homologous recombination. This element was called MITE for Mariner Insect Transposon like Element. At the amino acid level, there was no divergence

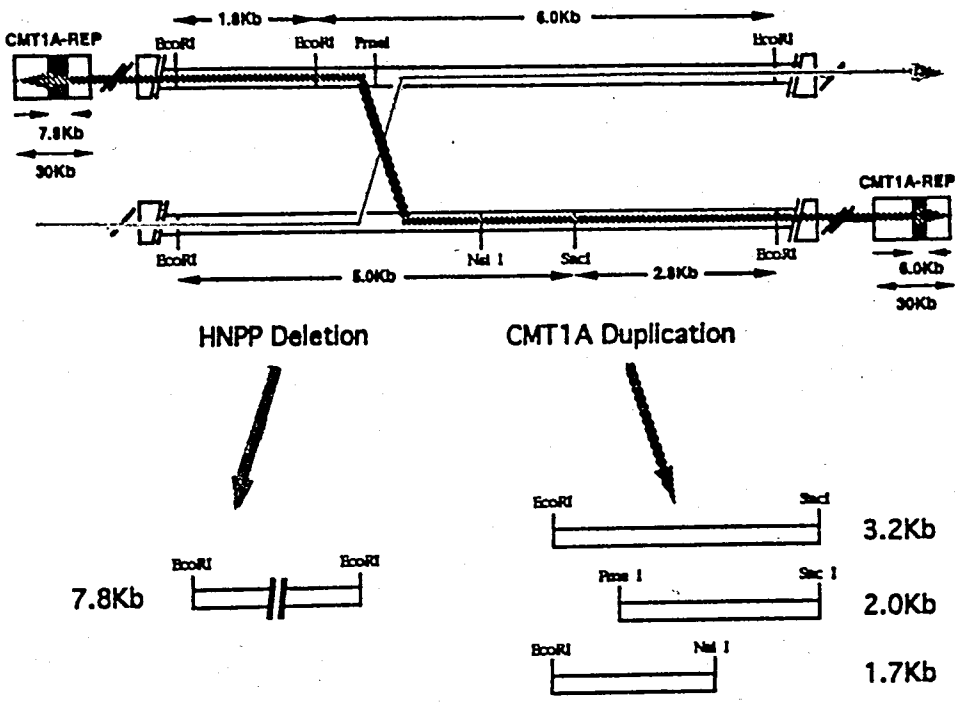


Figure I.9. Analyzing the hotspot of homologous recombination in CMT1A REPs. The 7.8 kb region on which the recombination occurs in the CMT1A REPs (Reiter et al., 1996).

in this mariner within the 116 amino acid transposase coding regions conserved in all transposases studied. This suggested that the functional groups of this transposase may be intact and that it can be transcribed into an RNA product. However, the two shifts in reading frame required for its alignment with other transposase coding regions indicate that it is probably not translated into a functional transposase. On the other hand, Northern blot analysis was performed using the fragment which harbors the mariner transposase as a probe. This analysis identified a low abundance ~7.5 Kb transcript apparently in human testis but not in ovaries. This finding explains the almost exclusive occurrence of the unequal crossing over during male meiosis. It also suggests the existence of an active source of mariner transposase somewhere else in the human genome, since the transcript expected from the ORF of MITE transposase is ~1 Kb in length. Such a hypothesis is possible in light of what is known of P-element transposase which can cause an increase in homologous recombination by DNA cleavage upon its addition, even in the absence of a transposable element or target.

Several ways with which MITE could mediate an increase in homologous recombination between the REPs were suggested. The most likely hypothesis states that the mariner-like transposase, translated from other sources detected in the human genome, could cleave its target site at any of the 88 TA dinucleotides, the preferred target site for mariner insertion, between the sites which flank the hotspot region. This double strand break could be the signal for initiating strand exchange which results in a hotspot of homologous recombination. Identifying cDNAs for a mariner-like transposase is a necessary initial step for the further characterization of MITE, and if a target site for a mariner-like transposase is detected in humans, it may provide an opportunity to increase site specific homologous recombination for the purposes of human gene therapy (Reiter et al., 1996).

II. AIM OF THE STUDY

The last few years have witnessed a great interest in the studies of all forms of Charcot-Marie-Tooth disease (CMT), which results in about 70% slow nerve conduction CMT1 and 30% normal nerve conduction CMT2. The fact that CMT1A represents about 70% of CMT1 cases allowed the molecular studies for the detection of the cause of this type of the disease to progress. This, in turn, has facilitated the diagnosis and the differentiation of CMT1A from other peripheral neuropathies.

The detection of the 17p11.2-p12 duplication in CMT1A patients is possible by the use of four different techniques. These techniques aimed at distinguishing three copies of certain markers which are known to map to the duplication interval in CMT1A patients. In addition to the sensitive radioactive methods, a fast and reliable non-radioactive method has been developed for this purpose which was the method of choice in recent studies conducted on CMT1A patients in the Italian population.

In light of these facts, we aim in this study at:

1- Investigating the most prevalent form of CMT, CMT1A, in Turkey and detecting the frequency of the CMT1A duplication in the Turkish population.

2- Establishing two different techniques for the detection of CMT1A duplications, which facilitates differential diagnosis of this type from other types of CMT. The techniques applied in this study are:

a- The radioactive RFLP analysis which is a sensitive and reliable method.

b- The fast non-radioactive method based on the detection of (GT)n polymorphisms; and the determination of the number of copies of this marker for the patients in question by densitometric analysis.

III. MATERIALS

A. Equipment

The equipment used in this study is listed in Table III.1.

Table III.1. Facilities of the laboratory.

Autoclaves:	Eyela Autoclave, MAC-601, JAPAN
Balances:	Electronic Balance Model VA124-1AAZM13AAE, Gec Avery U.K.
	Electronic Balance Model CC081-10ABAAGA, Gec Avery, U.K.
Cameras:	Polaroid, DS34, USA.
	BioDoc Video Documentation System, BIOMETRA, GERMANY
Centrifuges:	SORVALL RC-5B Refrigerated Superspeed Centrifuge, DuPont, SS34 rotor, USA
	Eppendorf, Centrifuge 5415C, GERMANY
Refrigerators:	4°C Sanyo Medicool, JAPAN
Deepfreezers:	-20°C, BOSCH, GERMANY
	-70°C, SANYO, JAPAN
Electrophoresis equipment:	DGGE system Model# DGGE-200, C.B.S. Scientific Co., USA
	EC 140 Mini vertical gel system, USA
	Horizon 58, Model 200, BRL, USA
	Horizon 1020, Model H1, BRL, USA
Incubators:	Shaking Incubator, HYBAID, UK
	Oven, EN400, Nuve, Turkey
	Incubator, Plus Series, GALLENKAMP, GERMANY
	Orbital Incubator, GALLENKAMP, GERMANY
Magnetic stirrers:	Chiltern Hotplate Magnetic Stirrer, HS31, U.K.
Ovens:	Microwave Oven, Vestel, TURKEY.
	65dC EN400, Nuve, TURKEY.
	56dC, LEEC, U.K.
Shaker:	VIB, InterMed, DENMARK.
Spectrophotometers:	UV/Visible Spectrophotometer, LKB Biochrom, U.K.
Thermocyclers:	UNO-Thermoblock, BIOMETRA, GERMANY
	Techne, Progene, U.K.
Transilluminators:	Chromato-Vue Transilluminator, Model 1TM-20UVP, USA.
Densitometer:	CD60, DESAGA SARSTEDT-GROPPE, GERMANY.

B. Buffers and Solutions

All chemicals and solutions used in this study were purchased from MERCK (GERMANY) and SIGMA (USA) unless stated otherwise in the text. Absolute ethanol was purchased from DELTA (TURKEY).

Probes were radioactively labeled by the random priming method using the Prime-a-Gene labeling system from Promega (USA). The alpha-^{32}P-dCTP (111 Tbq/mmol) was purchased from Izotop (Budapest). Sure blot nylon membranes (Oncor, USA) were used for Southern blotting.

Kodak (USA) X-ray film cassettes, amplifying screens and X-ray films (X-AR 5) were used for autoradiography. Developer and fixer (GBX) were also purchased from Kodak.

1. Solutions Used in DNA Extraction from Peripheral Blood

RBC Lysis Buffer:
155 mM NH_4Cl
10 mM $KHCO_3$
mM Na_2EDTA

Nuclei Lysis Buffer:
10 mM Tris (pH 8.0)
400 mM NaCl
2 mM Na_2EDTA

Proteinase K:
20 mg/ml in dH_2O

SDS:
10% stock solution

Ammonium acetate (NH_4Ac):
9.5 M saturated stock solution

TE Buffer:
20 mM Tris (pH 8.0)
0.1 mM Na_2EDTA

Alcohol:
Absolute ethanol

2. Solutions and Buffers used in Gel Electrophoresis

a. Common Components

10X Loading Buffer:	2.5 mg/ml BPB 1% SDS in 2 ml glycerol
6x Blue Orange Loading Dye:	Xylene cyanol, bromophenol blue, and orange G (from PROMEGA)
Ethidium Bromide:	10 mg/ml
10X TBE Buffer: (Tris-Borate)	1 M Tris-Base 900 mM Boric Acid 20 mM Na_2EDTA (pH 8.3)
20x TAE Buffer: (Tris-acetate)	gr Tris base 22.85 ml acetic acid, 40 ml 0.5 M EDTA (pH 8.0) in 1 L dH_2O

b. Agarose Gels

1-2% agarose (w/v) in 0.5x TBE buffer containing (0.5 μg/ml) ethidium bromide
2% NuSieve, 1% agarose in 0.5x TBE buffer containing 0.5 μg/ml ethidium bromide.

c. Non-denaturing Polyacrylamide Gels

8% acrylamide (29:1 acrylamide-bisacrylamide, in H_20)

3. Silver Staining Buffers

Buffer A:	10% Ethanol 0.5% Glacial acetic acid
Buffer B:	0.1% Silver nitrate ($AgNO_3$)
Buffer C: (Prepared fresh)	1.5% NaOH 0.01% $NaBH_4$ 0.015% Formaldehyde
Buffer D:	0.75% Na_2CO_3
Buffer E:	5% Glycerol

4. Solutions Used for Growing *E.coli*

Lauria Broth (LB):	10 g bactotryptone 5 g bacto-yeast extract 10 g NaCl 950 ml dH_2O
Agar:	10 g bactotryptone 5 g bacto-yeast extract 10 g NaCl 950 ml dH_2O 18 g agar
Ampicillin:	20 µg/ml
Rnase:	10 mg/ml

5. Buffers and Solutions Used in Extraction of Plasmid from *E.coli*

Solution 1:	50 mM Glucose 10 mM EDTA 25 mM Tris-HCl (pH 8)
Solution 2:	0.2 N NaOH, 1%SDS
Solution 3:	5 M Potassium acetate (pH 4.8)
STE Buffer:	0.1 M NaCl 10 mM Tris-HCl (pH 8.0) 1 mM EDTA

6. Buffers and Solutions Used for Southern Analysis

Denaturation Solution	0.4 N NaOH
DNA Transfer solutions:	0.5 N NaOH 0.6 M NaCl
Neutralization Buffer:	0.5 M Tris pH 7 1M NaCL
Hybridization Buffer:	350 mL 20% SDS 150 ml 20x SSPE 50 ml PEG, 5 g milk powder in 1 L
Washing Solutions:	W1 1x SSC, 0.1% SDS W2 0.5xSSC, 0.5% SDS W3 0.1x SSC, 0.5% SDS W4 0.1x SSC
Stripping Solution:	0.1x SSC, 0.5% SDS

C. Fine Chemicals

1. Primers

a. Primers Flanking the (GT)n Repeat

GT strand: 5'-CAGAACCACAAAATGTCTTGCATTC-3'

CA strand: 5'-GGCCAGACAGACCAGGCTCTGC-3'

This set of primers was purchased from Biometra.

b. β-Globin Primers

KM 29: 5'-GGTTGGCCAATCTACTCCCAGG-3'

RS 43: 5'-GCTCACTCAGTGTGGCAAAG-3'

This set of primers was kindly provided by Prof. A Nazlı Başak, Boğaziçi University, Department of Molecular Biology and Genetics.

2. Enzymes

The enzyme Taq DNA polymerase was purchased from MBI Fermentas (LITHUANIA). The restriction enzymes *EcoRI, HincII, MspI, BamHI, and HindIII* were purchased from Promega (USA).

3. DNA Molecular Weight Standards

ΦX 174 DNA / Bsu RI (Hinf I) marker (Promega, USA) with fragments of 726, 553, 500, 427, 417, 311, 249, 200, 151, 140, 118, 100, 82, 66, 48, 24 bp.

λDNA/HindIII marker, (PROMEGA USA) with fragments of 23.1, 9.4, 6.7, 4.4, 2.3, 2, 0.6, 0.13 Kb.

4. Probes

p132-G8R1:	11.0 Kb EcoR1 insert in PTZ19R
pVAW409R3a:	2.1 Kb EcoR1-BamH1 insert in PUC18
pVAW409R1b:	2.5 Kb EcoR1-BamH1 insert in PUC18
pEW401HE:	0.85 Kb EcoR1-HindIII insert in PUC18

These probes were kindly provided by Dr. James Lupski, Baylor College of Medicine, Houston, USA.

IV. METHODS

A. DNA Extraction from Peripheral Blood by Ammonium Acetate

DNA is extracted from white blood cells by the "Salting out" method which is simple and fast. Anti-coagulated blood, taken into EDTA tubes is transferred to 50 ml Sorvall tubes and mixed with ice-cold RBC lysis buffer (30 ml/ 10 ml of blood). The mixture is kept at 4°C for 15 minutes to lyse the erythrocyte membranes, and then centrifuged at 5000 rpm (5K), 4°C for 10 minutes. The resulting supernatant is discarded, and the pellet is resuspended in 10 ml of lysis buffer by vortexing. Another centrifugation is done at 5000 rpm (5K), 4°C for 10 minutes, the supernatant is discarded and the nuclear pellet is resuspended in 3 ml of nuclei lysis buffer to lyse the nuclear envelope of leukocytes. Thirty µl proteinase K and 50 µl of SDS are added consequently and the mixture is incubated at 37°C for overnight or at 56°C for three hours for digestion of proteins. One point seven milliliters of saturated 9.5 M ammonium acetate are added, and the tubes are shaked vigorously to precipitate the proteins. The tubes are centrifuged at 10,000 rpm (10 K), at room temperature for 20 minutes. The supernatant is transferred to a clean 50 ml Falcon tube and two volumes of absolute ethanol is added. The tube is inverted gently several times until the DNA is precipitated, and then the DNA is fished out, dried and dissolved in 500 µl of TE buffer.

1. Spectrophotometric Measurement

The concentration of the isolated DNA is determined by spectrophotometric analysis. This method is used in this study to determine the concentration of the genomic DNA to be used in PCR reaction. DNA is diluted in water in a ratio of 1:50 to 1:100 and its optical density is measured at 260 and 280 nm. Knowing that 50 µg of dsDNA has an absorbance of 1.0 at 260 nm (OD_{260}), the concentration of DNA is determined according to the following formula:

Concentration (μg/ml)=50μg/ml x OD_{260} x Dilution Factor

The ratio between the spectrophotometric measurements at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the DNA sample. Pure preperations have a value of 1.8. Values greater than 1.8 indicate RNA, and values less than 1.8 protein contamination.

2. Minigel

The approximate concentration of the isolated DNA is also determined by agarose gel electrophoresis. DNA fragments are separated on agarose gels by electrophoresis. DNA molecules run towards the positively charged electrode, due to the presence of negative charged phosphate groups at the DNA backbone. Visualization of DNA bands can be done by soaking the gel in Ethidium Bromide (EtBr) and observing it under UV. The amount of DNA is then estimated by comparing its intensity with known amounts of DNA. This method is used for determining the amount of genomic DNA to be used for Southern analysis, since it helps to estimate the amount of genomic DNA needed for digestion and to get, more or less, equal amounts of DNA on the blot.

B. Analysis of the RM11-GT Genotype

Polymerase Chain Reaction (PCR): Genomic DNA of 0.1 μg is amplified in each reaction in a 25 μl reaction mixture containing 25 pmole of each of the primers flanking the (GT)n repeat, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP and 0.5 units of Taq polymerase in its 1x reaction buffer. For multiplex PCR the same mixture is used in addition to 25 pmole of each of the primers flanking a part of the β-globin gene. In both cases, the PCR program is as follows:

	Temperature(°C)	Time	
Initial denaturation	94	3 min	
Denaturation	94	30 sec	⎫
Annealing	55	30 sec	⎬ 24 cycles
Extension	72	30 sec	⎭
Final extension	72	10 min	

To check the quality and quantity of the amplification, 5 µl aliquot of the PCR product is mixed with loading buffer to a final concentration of 1X and electrophoresed on a 1 per cent agarose gel. Amplification products are run on 8% acrylamide gels (1mm thick, containing 10% glycerol, 0.5% amonium persulfate, 6 µl TEMED, and 1x TAE) at 100 V for 2- 2.5 hrs to resolve the alleles. The gel is then silver stained according to the following steps:

a) Incubate the gel in buffer A for three min., discard the buffer and repeat this step with a fresh aliquot of buffer A and discard it.
b) Incubate in buffer B for 10 min. and discard the buffer.
c) Wash twice with dH_2O for 10 sec.
d) Incubate the gel in buffer C for 20 min. and discard the buffer
e) Incubate the gel in buffer D for 5-10 min. then discard the buffer.
f) Incubate in 5% glycerol for five min., then seal the gel.

Densitometric analysis is then applied on the stained gel. The heights of the peaks representing the bands are recorded, and the ratio between them is calculated.

C. Southern Analysis

1. Electrophoresis and DNA Transfer

Ten μg of genomic DNA is digested with the suitable restriction enzyme(s) (either Msp1 digestion, or EcoRI-HincII double digestion) and the fragments are resolved on 1% agarose gels in 1x TAE buffer at 50 V in a long horizontal BRL H1 electrophoresis apparatus. Lambda phage DNA digested with RE HindIII is used as a marker.

The unnecessary parts of the gel are trimmed away, and the dimensions of the gel are measured. The gel is left in 0.4 N NaOH solution for 15 min. for denaturation of DNA molecules in alkali medium. A glass plate, placed on 4-cm high petridishes placed in a dish, is used to support the gel and two sheets of Whatman paper are put over the glass, the two ends touching the bottom of the dish. Then the dish is filled with the transfer solution poured to the level of the glass plate, wetting also the Whatman papers. Air bubbles between the sheets of Whatmann papers were removed by rolling a glass rod. The gel is placed onto the Whatman papers on the support. A membrane sheet of the same size with the gel, prewetted thoroughly in the transfer solution, is placed over the gel. Then, a Whatman paper is placed on the membrane. During stacking, trapping of air bubbles is avoided in each step. Paper towels, cut a little larger than the gel, are placed on the Whatman paper. A glass plate is put on the top of the stack and weighed down with a 500 g weight. The transfer solution passes upwards through the gel toward paper towels carrying with it the denatured DNA fragments, which bind to the membrane. The towels are changed often during the first two hours as they become wet, and DNA is allowed to blot overnight. The towels and the Whatmann papers are removed the next morning, and the membrane is marked on the side not containing the DNA and soaked in the neutralizing solution until the pH becomes 7.0. The membrane is allowed to dry, and the DNA is fixed on the membrane by baking the membrane at 65^{o}C for 15 min. The blot can be stored at room temperature for one week or at 4^{o}C indefinitely.

2. Preparation of Probes for Hybridization Reactions

Plasmids into which a cDNA probe has been cloned were obtained in DH5α *E. coli* cell strain in stab cultures.

a. Rapid Small Scale Plasmid Isolation

This process is done only to check the lengths of the plasmid vector and the probe inserted in it before going to the large scale isolation of the plasmid to gain the needed probe.

E.coli cells harboring the plasmid are inoculated in 5 ml of LB broth containing ampicillin. Since the plasmid carries the gene for ampicillin resistance, the bacterial cells containing this plasmid can be selected by growing in medium with the appropriate antibiotic. The cells are grown overnight at 37°C under vigorous shaking and harvested by centrifugation at 5000 rpm for five min at 4°C in a Sorvall superspeed centrifuge. The supernatant is discarded and the tube is inverted to drain. The bacterial pellet is resuspended by vortexing in 200 µl of ice cold solution I. The suspension is transferred into a 1.5 ml Eppendorf tube in order to carry out the subsequent centrifugations in an Eppendorf centrifuge. The pellet is left for five min at room temperature and then 400 µl freshly prepared solution II is added, mixed rapidly by hand and left on ice for five min in order to lyse the cells. The chromosomal DNA is pelleted by addition of 300 µl ice cold solution III, mixing, and centrifugation for five min at 4°C. The supernatant containing the plasmid DNA is transferred into a fresh tube, measuring the volume. An equal volume of phenol/chloroform (1:1) is added and mixed by vortexing. The solution is centrifuged for two min at room temperature. The aqueous phase is transferred to a clean tube. At this step, proteins partition to phenol/chloroform interphase. The plasmid DNA is precipitated by adding an equal volume of isopropanol, mixing by hand, and centrifuging for five min at room temperature. The supernatant is removed and the pellet is left to air-dry. The salts on the tube walls are washed off by adding 1 ml 70% ethanol, mixing, and recentrifuging. The supernatant is discarded and the pellet is left to air-dry. The DNA pellet is dissolved in 0.5 ml TE buffer.

Samples of 1 µl and 5 µl from the isolated plasmid DNA are run in a minigel to determine the purity and amount of DNA. When RNA concentration is high in the sample, it appears as a large band at the bottom of the minigel. In order to get rid of the

contaminating RNA, 20 mg/ml of RNase A is added and the digestion is allowed to proceed at 37°C for one hr.

b. Large Scale Isolation of Plasmid DNA

The purpose of this process is to isolate plasmid DNA in large quantities in order to subsequently isolate a large quantity of the probe that has been cloned into it.

Growing the bacteria to a high density: All incubations are performed in an orbital shaker under vigorous shaking at 37°C. LB broth (10 ml) is inoculated with a single bacterial colony and incubated overnight in the presence of ampicillin (20 μg/ml). Zero point one milliliters of the overnight culture is incubated for five to seven hours in 25 ml of LB containing ampicillin. Following the incubation, the cell culture is divided into two 500 ml ampicillin containing LB medium, and incubated overnight.

Harvesting: The bacterial cells are harvested by subsequent centrifugations in a common centrifuge bottle at 4°C, 5 K for 10 min. The pellet is washed in 100 ml ice-cold STE buffer.

Plasmid DNA isolation: The bacterial pellet is suspended in 30 ml solution I by vortexing. The suspension is left for five min, then 60 ml of freshly made solution II is added, mixed by inverting the tube several times and left on ice for 10 min. Fourty-five ml of solution III is added to the suspension, mixed by inverting the tube several times sharply and left on ice for 10 min. The precipitated chromosomal DNA is centrifuged at 4°C and 7 K for 30 min to obtain a compact pellet. The supernatant is transferred into smaller centrifuge tubes. Plasmid DNA is precipitated by adding isopropanol (0.6 volumes) into each tube, mixing and leaving at room temperature for 15 min. The precipitate is centrifuged at 10 K for 20 min at room temperature. The DNA pellet is washed with one ml 70% ethanol at room temperature to remove salts and air-dried. The pellet is dissolved in 1 ml TE buffer, and RNase (5 μg) is added and incubated for 30 min at room temperature. Phenol/chloroform (1:1) extraction is performed twice to remove protein components. Sodium acetate (final concentration of 0.3 M) and then 10 ml of isopropanol are added, mixed and left for 15 min at room temperature, to allow DNA precipitation. After centrifugation for 20 min at room temperature, the pellet is air dried for half an hour and dissolved in 0.5 ml TE.

A 1 μl sample of the purified plasmid DNA is mixed with 9 μl 1x loading buffer and a minigel is run to determine the purity and amount of DNA. Spectrophotometric analysis is also made to determine the concentration and purity of plasmid DNA.

c. cDNA Insert Isolation

The plasmid DNA (10 μg) into which the cDNA probe was cloned, is digested at 37°C for 2-5 hours with the appropriate restriction enzyme as follows:

Digest Mix: 10 μg plasmid DNA
5 μl 10X RE buffer
10 units RE
made up to 50 μl with sterile dH_2O

When digestion is complete, the reaction mix is combined with 5 μl 10x loading buffer, loaded into a long slot in a 1% agarose gel, and electrophoresed at 100 V for 30 min. The cDNA insert band is cut out under long UV light. DNA is eluted from agarose gel by electrophoresing in dialysis tubing that contains 500 μl 0.5x TBE buffer. After clamping one end of the dialysis tubing, the gel slice containing the insert DNA is put in and the free end of the tubing is clamped. Then it is placed in an electrophoresis tank containing 0.5x TBE. Elution is performed at 100 V for 20 min and efficiency is checked under UV light. Then the buffer containing the insert is transferred with a micropipette into a 1.5 ml Eppendorf tube. An equal volume of phenol/chloroform (1:1) is added, mixed and centrifuged for two min at 5000 rpm. The aqueous phase is extracted with chloroform. The insert DNA is then precipitated by addition of sodium acetate to a final concentration of 0.3 M and two volumes of ethanol at -20°C for two hours, and pelleted by centrifugation at 13 K for 10 min. The supernatant is drained and the pellet is air-dried for 30 min. The DNA is resuspended in 50 μl TE, checked on a minigel, and the concentration is measured using spectrophotometry.

Labeling the probes: DNA probes are labeled to a high specific activity using alpha-^{32}P-dCTP by the random priming method. The Prime-a-Gene labeling system, which is based on using a mixture of random hexanucleotides to prime DNA synthesis in vitro from any linear double-stranded DNA template, is applied.

The probe DNA is dissolved in deionized H_2O at 1-25μg/ml and denatured in boiling water for 10 minutes, and then rapidly chilled in an ice bath. One μl of each of the non-isotopically labeled dNTPs are mixed to yield 3 μl of a premix from which 2 μl are taken for

the labeling reaction. Then the components of the reaction mix are added in a tube on ice in the following order:

		Final concentration
Sterile dH_2O	to 50 µl	
Labeling 5X buffer	10 µl	1X
mixture of unlabeled dNTPs	2 µl	20 µM each
denatured DNA template	25 ng	500 ng/ml
Nuclease free BSA	2 µl	400 µg/ml
α-^{32}P dCTP	5 µl	333 nM
Large Klenow Fragment	5 units	100 U/ml

The reaction tube is incubated at room temperature overnight. The reaction is terminated by heating in boiling water for five minutes, subsequently chilling in an ice bath, and adding EDTA to 20 mM. The reaction mix is then either used directly in a hybridization reaction or stored at -20°C for later experiments.

3. Hybridization, Washing, and Autoradiography of Southern Blots

The two-hour prehybridization and the overnight hybridization of Southern blots are carried out in plastic boxes in the shaking incubator at 65°C. At the end of the prehybridization step, the labeled probe containing 1 µl of labeled λ, is pipetted into the hybridization solution in the plastic box and hybridization is allowed to proceed afterwards. After hybridization, blots are washed under stringent conditions to get rid of non-specific hybridization as follows:

a) The blot membrane is rinsed twice with 1X SSC and 0.1% SDS, and the liquid is dumped into liquid container.
b) The blot membrane is washed with 1XSSC and 0.1% SDS, in the shaking incubator at 65°C for >15 min.
c) The blot membrane is washed with 0.5X SSC and 0.5% SDS preheated to 50-60°C, for 15-60 min.

d) The blots are then washed with 0.1X SSC and 0.5% SDS preheated to 68-70^{o}C, at 65^{o}C for 30 min.
e) The blot membrane is then rinsed briefly in 500 ml 0.1X SSC.

After washing, the blot is dried briefly on Whatman filter paper for less than 10 min, wrapped in plastic wrap and placed in a film cassette. An X-ray film and an intensifying screen are placed over it successively. Autoradiography is performed for 1-3 days depending on the specific activity of the labeled probe.

4. Strip Wash

This step is performed before rehybridizing the blot with another probe. The stripping solution is heated to boiling, poured into the box containing the blot, and shaken at 65^{o}C for 10-20 min. The solution is discarded and the step is repeated until all traces of radioactivity are gone. The blot is then dried briefly on Whatman paper to be ready for another hybridization, or stored at 4^{o}C for later use.

V. RESULTS

A. Patients

This study focuses on the detection of the CMT1A DNA duplications in twenty six unrelated CMT1 patients from Turkey using both a radioactive and a non-radioactive method. Also two families were studied and the pattern of inheritance of the duplication was determined. Moreover, the presence of the DNA deletion was investigated in three HNPP patients using the same methods, in light of the fact that CMT1A and HNPP are reciprocal products of the same mechanism. The blood samples and NCV values of CMT1 patients were provided by Istanbul University Medical School Department of Neurology. The patients studied exhibited signs and symptoms consistent with a clinical diagnosis of either CMT1 or HNPP. Patients representing clinical evidence, as well as abnormal (<40 m/s) NCV values are diagnosed as having CMT1, whereas those with normal or near normal NCVs were diagnosed as CMT2. Genomic DNA was isolated from peripheral blood samples, and molecular analysis was done.

B. DNA Analysis Based on RM11-GT Marker

1. Optimization of the PCR

To detect the intensity differences between the two alleles for the RM11-GT locus, the PCR reaction is caught at the exponential phase at which the amount of amplified products of each allele reflects the number of copies of this allele in template DNA. When the PCR reaches the plateau, the relative intensities of the two alleles, detected in heterozygous individuals, drops and leads to false prediction of absence of a duplication in CMT1A patients. In addition to that, the slippage effect increases tremendously giving rise to numerous bands, and retarding the analysis (Figure V.1). The purpose of this

optimization test was to identify the number of amplification cycles that correspond to the late exponential phase of the PCR curve for amplification of RM11-GT locus.

Zero point one μg of genomic DNA from patient number 2, who was known to be heterozygous for this locus and to carry the duplication, was used in the optimization test. The genomic DNA was amplified in nine separate PCR tubes under the same conditions, except that each tube was taken out of the thermocycler after a different number of cycles was completed. Thus, tubes containing products of 20, 21, 22, 23, 24, 25, 26, and 27 cycles of PCR were obtained. Five μl of each product was run on a NuSieve gel to detect the quantity of the product in each tube (Figure V.2).

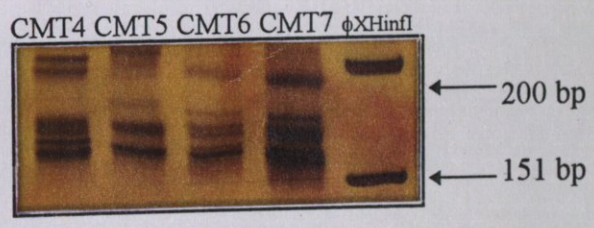
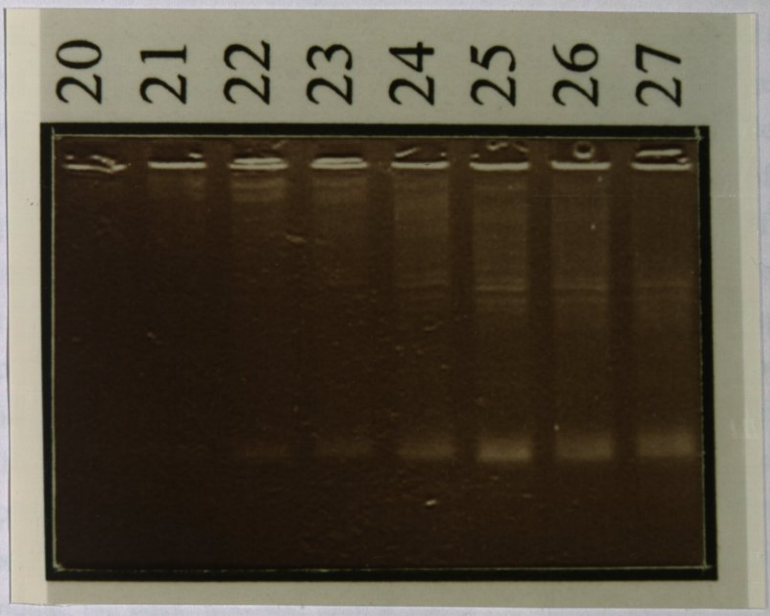


Figure V.1. Thirty cycle PCR products run on an 8% acrylamide gel. The slippage effect is obvious in patients CMT4, CMT6, and CMT7.

a



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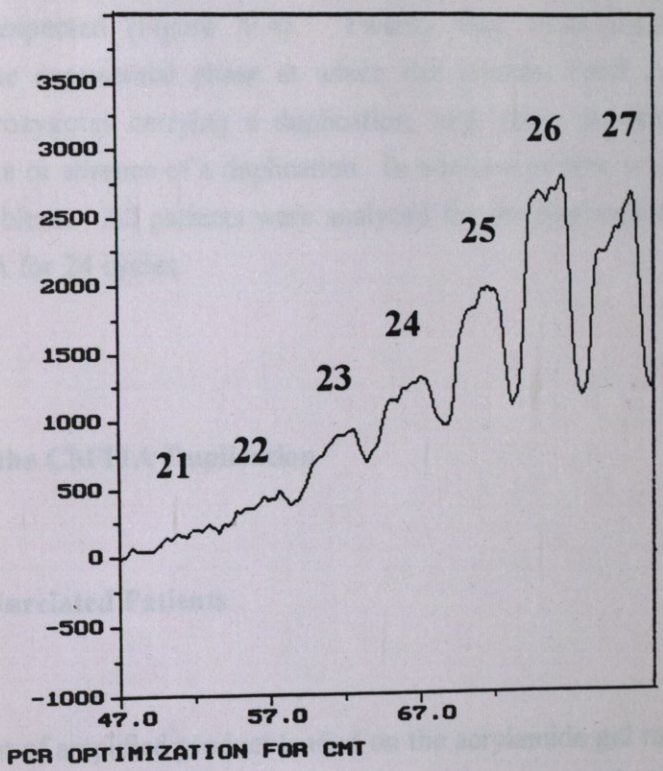


Figure V.2. PCR optimization for the marker RM11-GT.

a. The PCR bands for DNA of patient number 2 run on a NuSieve gel. No product was seen in the 20 cycle lane.

b. Densitometric analysis revealing the intensity of each band obtained at different cycle numbers. An exponential phase of the PCR curve with a sharp rise was detected between cycle numbers 22 and 26, and a plateau was obtained with 26 and 27-cycle products.

These products were then run on an 8% acrylamide gel in order to determine the cycle number giving rise to the highest peak ratio between the two separated copies of the RM11-GT locus in this heterozygous patient. After visualizing the bands on the gel by silver staining, analysis was done with the use of the densitometer (Figure V.3). The densitometer is a very sensitive machine that gives an accurate measurement of the intensity of each band it encounters using different wavelengths selected by the user. The intensity of each band detected by the densitometer is represented by a peak on a graph. The relative difference between two peak hights reflects the relative intensity of each band with respect to the other. The ratio of the two peaks given by the densitometer for each cycle product was calculated, and found to be, more or less, stable for products between cycle number 21 and 24 (1.31-1.34). The ratio, then, dropped to a minimum after 24 cycles (1.14), and shadow bands started to appear (Figure V.4). To check the accuracy of this optimization, the same test was performed for a heterozygous individual who was normal for this locus (Figure V.3). In this individual, the peak ratio was almost stable between 21 and 27 cycle products (1.09-1.19), as expected (Figure V.4). Twenty four cycle amplification, therefore, represents the late exponential phase at which the greatest band intensity difference is detected in heterozygotes carrying a duplication, and, thus, provides accurate decision about the presence or absence of a duplication. In addition to that, it prevents slippage and shadow band problems. All patients were analyzed for the duplication by amplification of the genomic DNA for 24 cycles.

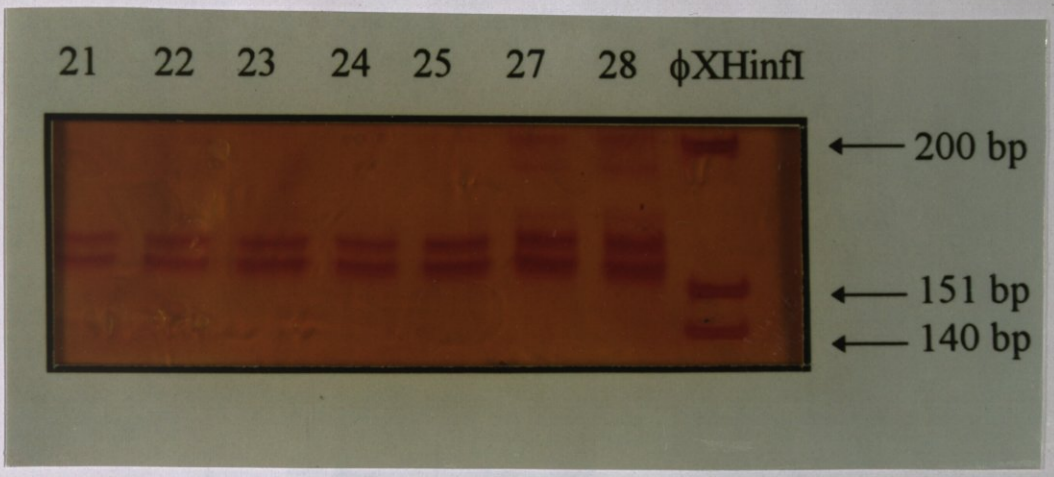
2. Detection of the CMT1A Duplication

a. Analysis of Unrelated Patients

The amount of amplified product loaded on the acrylamide gel ranged from 2 µl to 10 µl depending on the quality of the PCR product, and decided usually after running a sample of the product on a 1% agarose gel. Different banding patterns were detected on the gel (Figure V.5). Some patients were homozygous, thus uninformative for this microsatellite marker. On the other hand, patients heterozygous for the marker either showed:

1. Three bands each of which represented a copy of the marker with a number of GTs different from that of the others, and thus was a direct proof for the presence of a duplication, or

a



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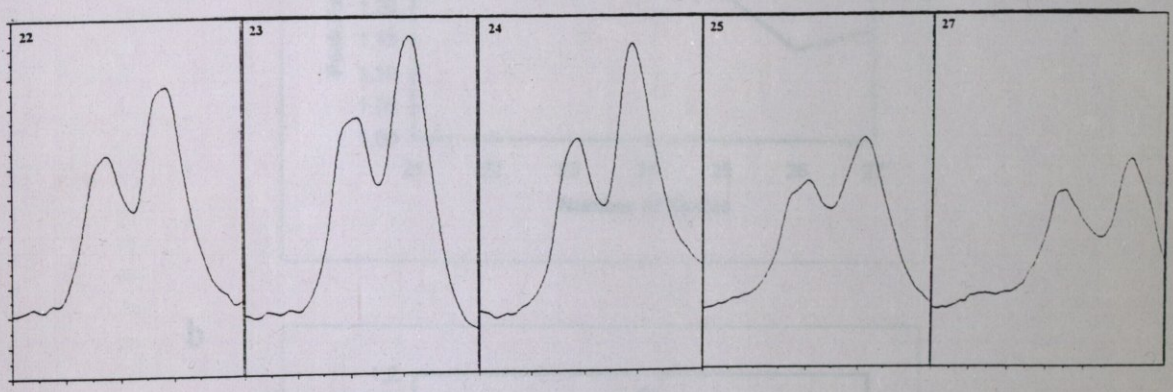

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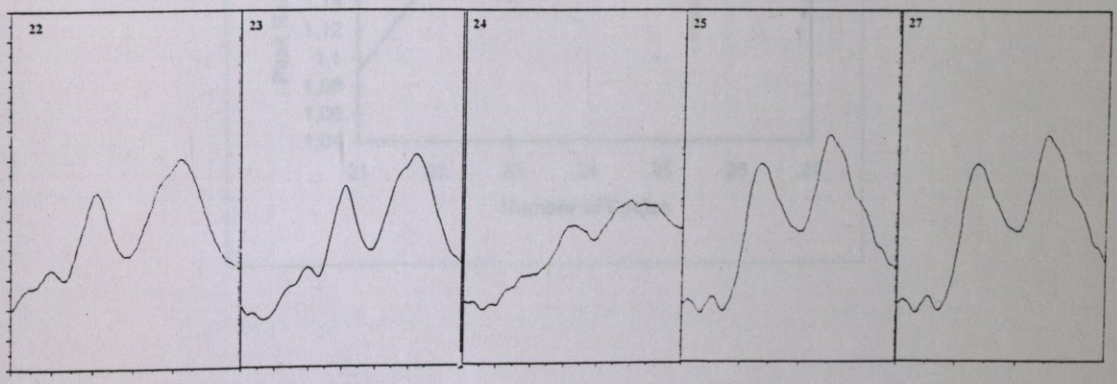


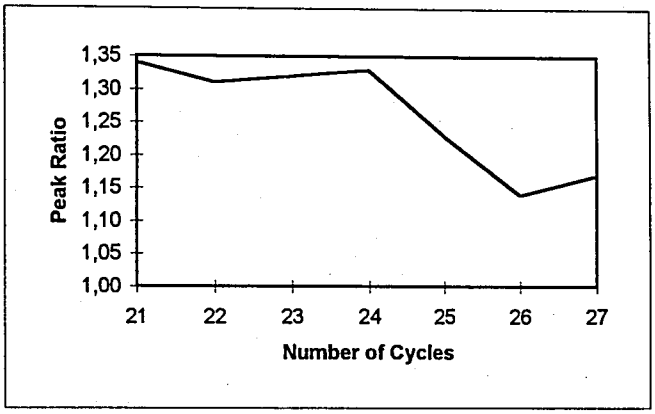
Figure V.3. Polyacrylamide gel showing the cycle products for patient number 2 and the densitometric analysis for patient number 2 and for a normal individual.

a. The alleles of the marker RM11-GT for patient number 2 were amplified for 21 to 27 cycles and resolved on an 8% polyacrylamide gel.

b. Densitometric analysis for some of the cycle products for patient number 2. The first peak always represents the upper band and the second represents the lower band as seen on the gel.

c. Densitometric analysis for some of the cycle products for a normal individual.

a



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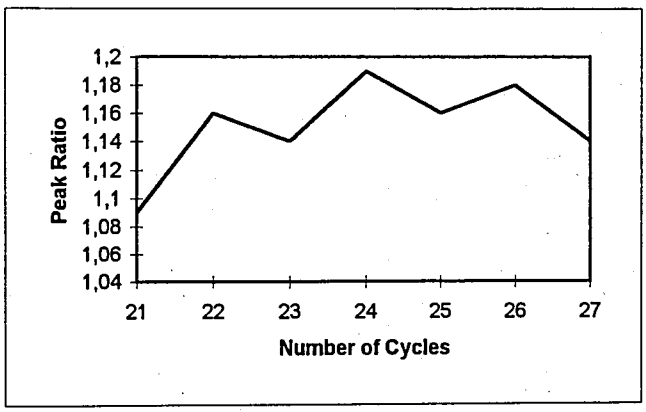
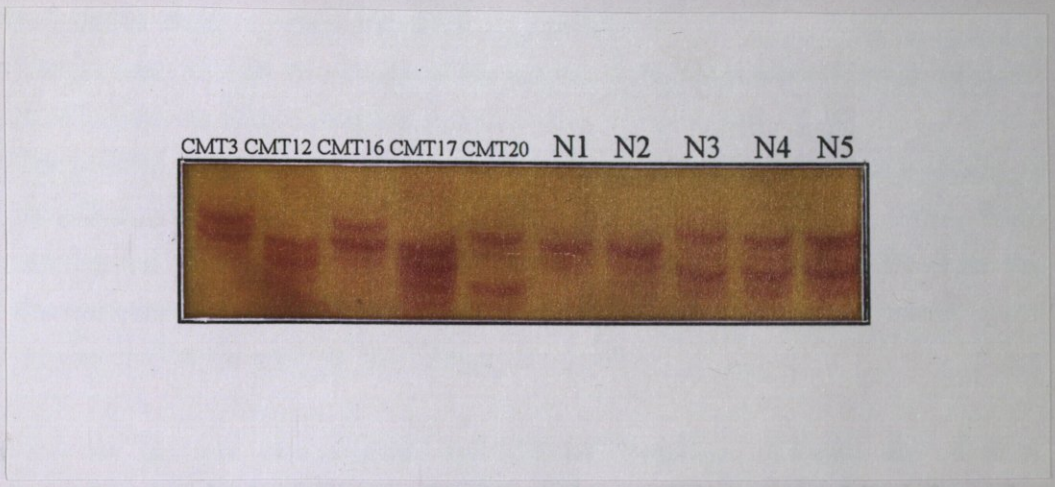


Figure V.4. Graphs representing number of cycles versus peak ratio for patient number 2 and for a normal individual.

a. For the patient, the peak ratio was stable between cycle number 21 and 24 and then dropped quickly between cycles 24 and 26.

b. For the normal individual, the ratio was almost stable between cycles 21 and 27.

a



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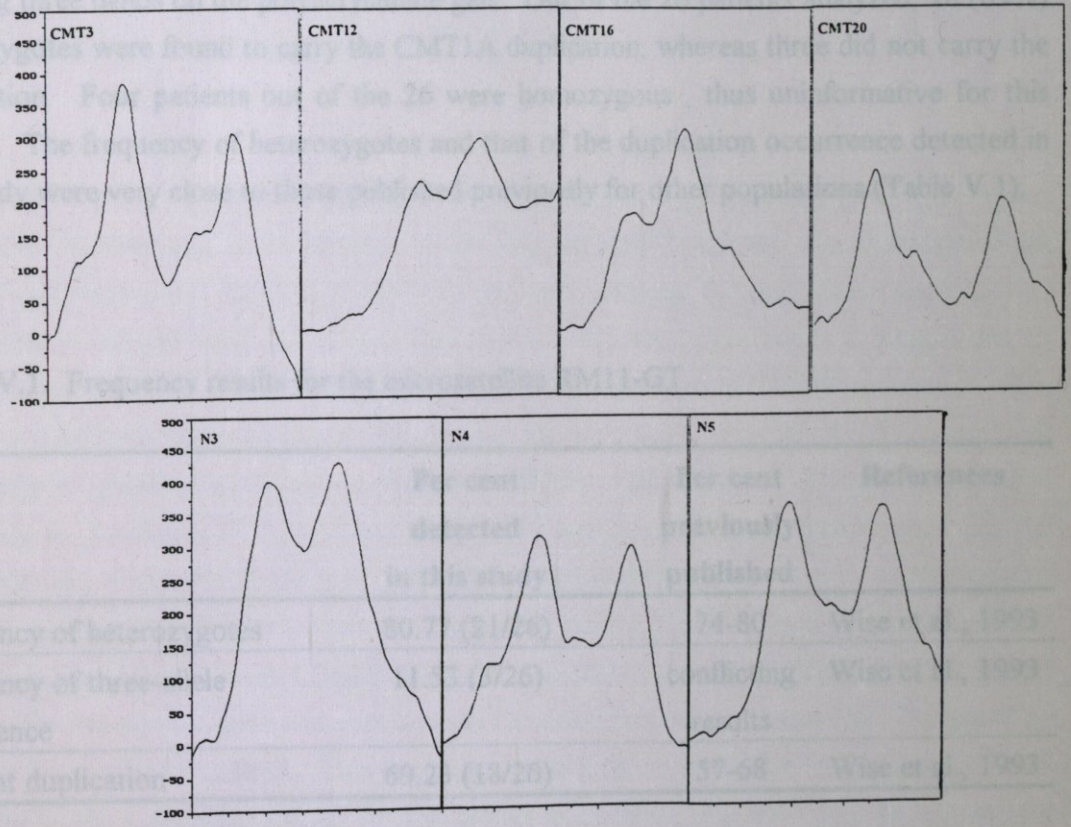


Figure V.5. Detection of duplications in different patients using the marker RM11-GT.

a. Patient CMT16 shows two bands one of which has double the intensity of the other, while two alleles of equal intensity are seen in patients CMT3, CMT12, CMT20 in whom no duplication is detected, and in normals N3, N4, N5. Three alleles are detected for patient CMT17. Patient CMT19 and normal N1 are homozygous and thus uninformative for this marker.

b. Densitometric analysis comparing difference in peak ratios between patients CMT3, CMT12, CMT16, CMT20, and normals N3, N4, N5.

2. two bands which represent two different possibilities:
a. The presence of only two copies of the marker if the bands were of equal intensity (CMT1 patients with no duplication).
b. The presence of three copies of the marker if one band was double the intensity of the other (patients carrying the duplication). In this case the more intense band represented two copies of the marker with equal number of GTs. At this stage, the densitometeric analysis was needed to give the decision about the presence of the duplication for the patients in question.

Twenty six patients were analyzed for RM11-GT marker. Twenty one of these (~81%) were found to be heterozygous for this marker, and three were fully informative showing three bands on the polyacrylamide gels. Out of the 26 patients analyzed, 18 (69%) heterozygotes were found to carry the CMT1A duplication, whereas three did not carry the duplication. Four patients out of the 26 were homozygous , thus uninformative for this marker. The frequency of heterozygotes and that of the duplication occurrence detected in this study were very close to those published previously for other populations (Table V.1).

Table V.1. Frequency results for the microsatellite RM11-GT.

	Per cent detected in this study	**Per cent previously published**	**References**
Frequency of heterozygotes	80.77 (21/26)	74-80	Wise et al., 1993
Frequency of three-allele occurrence	11.53 (3/26)	conflicting results	Wise et al., 1993
Per cent duplication	69.23 (18/26)	57-68	Wise et al., 1993

b. Family Analysis

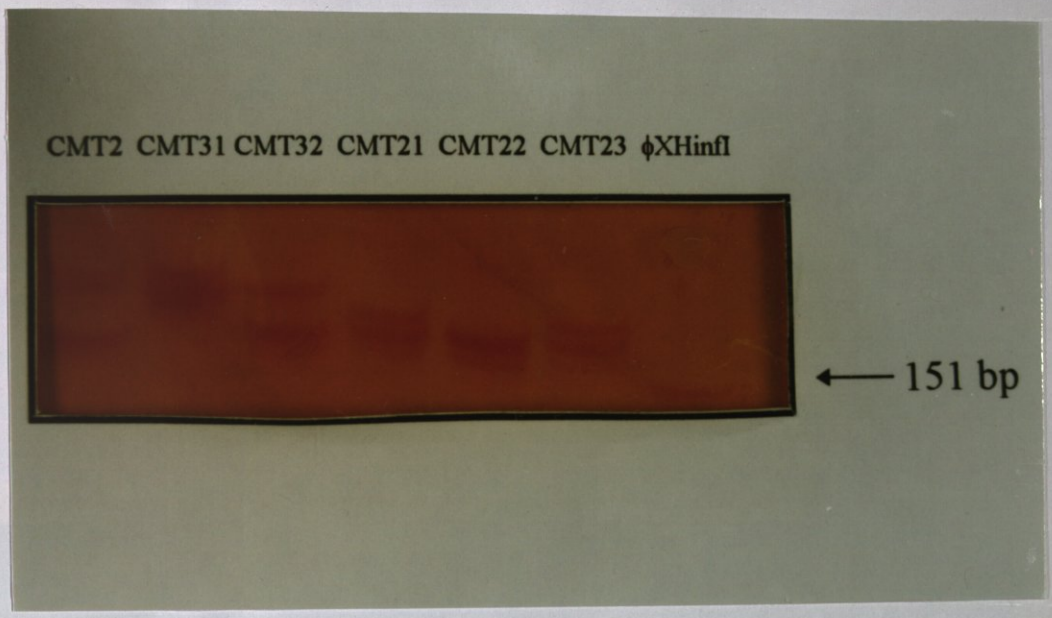
The two families investigated in this study presented the high advantage of a family based analysis. The analysis provides a further confirmation for the presence of the duplication by following the inheritance pattern of the duplicated allele. In addition to this,

it helps find out spontaneous mutations, if there is any, whenever the patient's parents are found to be normal. In the first family, patient number 2 was shown to inherit the duplicated allele from the mother (CMT32) and the normal allele from the father (CMT31). In the second family, the patient (CMT21) inherited a normal allele (upper allele) from the mother (CMT23). This mother shows a duplication in her lower allele. The father was uninformative for this marker but showed the same lower allele seen in the mother. These parents are first degree cousins and most probably they carried the same duplication segregating with this lower allele. The duplicated allele in the patient was then probably inherited from the father (Figure V.6).

3. Detection of the HNPP Deletion

The detection of only one band on the polyacrylamide gel may represent two copies of a marker in homozygous individuals, or one copy in HNPP patients. Confirmation of the HNPP deletion required the analysis of other markers spanning the duplication interval, and the detection of one band for all markers tested. On the other hand, a simple test (a comparative multiplex PCR) was performed for the detection of HNPP deletions. The multiplex PCR was performed using the primers spanning the marker RM11-GT along with other primers spanning a region of a gene on a different chromosome. The primers used in this study for multiplex PCR were spanning a 533 bp region of the β-globin gene and the band expected from the product of its amplification corresponded to two copies of this region. The multiplex PCR products for the HNPP patient (CMT27) and two other control individuals were then run on a Nusieve gel and the ratio between the band for CA repeat and that for the control gene was estimated in the control individuals and then compared to the ratio for the HNPP patient. This experiment was a direct proof for the presence of only one copy of the CA repeat marker for the patient in question (Figure V.7). In this patient only one band, as well, was detected in the analysis for the other two markers. Three patients were clinically HNPP in question. The HNPP deletion was found in one of them (CMT27) while the other two were found to carry the CMT1A duplication.

a



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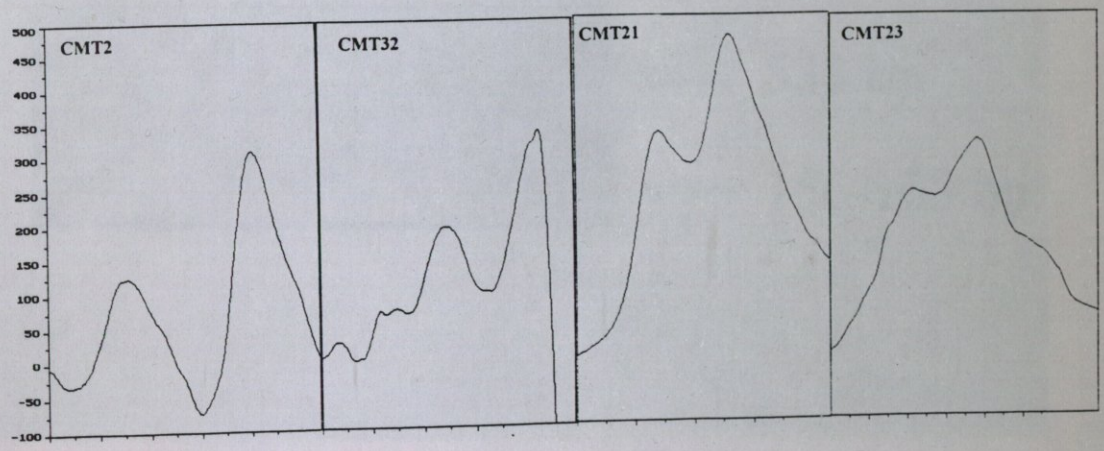

Figure V.6. Family analysis of the marker RM11-GT.

a. Separation of the copies of the marker RM11-GT on a polyacrylamide gel. In both families, the duplication was detected in patients (CMT2 and CMT21) and in their mothers (CMT32 and CMT23) listed simultaneously.

b. Densitometric results showing the duplication in CMT2 inherited from his mother (CMT32) and the duplication in CMT21 and his mother (CMT23).

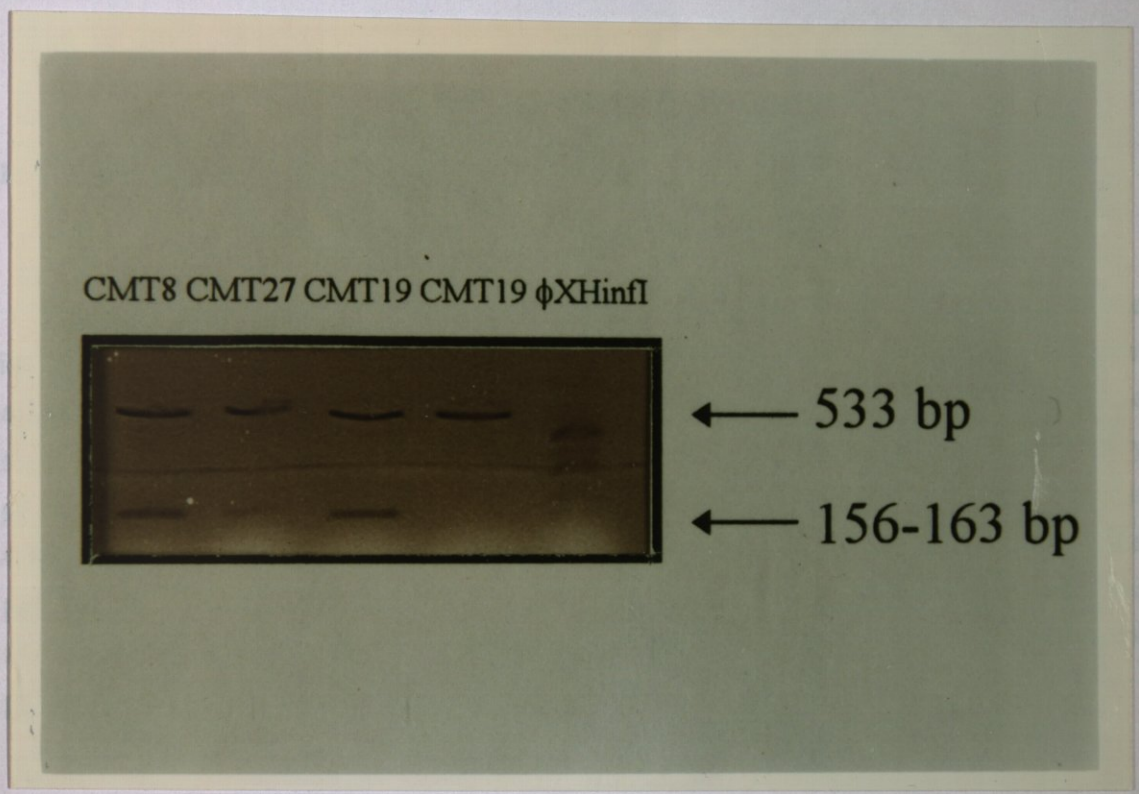

Figure V.7. Detection of HNPP deletion. This figure shows the suspected HNPP patient (CMT27) between two controls CMT8, and CMT19. These controls were found to be homozygous for the marker RM11-GT but heterozygous for other markers which meant that their amplified band represented two alleles. The intensity ratio of the CA repeat band (156-163 bp) relative to the β-globin band (533 bp) was found to be much weaker in the control individuals than in the HNPP patient. CMT19 was also amplified for the β-globin gene only and run next to the weight marker to confirm the position of the β-globin band.

C. RFLP Analysis

Ten μg of genomic DNA was digested with the restriction enzyme Msp1, and was analyzed with the probe pEW401HE, or double digested with EcoR1/HincII and analyzed with the probe p132-G8R1 (Figure V.8.a). The digested DNA was then run on a large 1% agarose gel for 18 hours at 50V, and the gel, soaked in alkali for denaturation of the DNA, was prepared for Southern blot to transfer the DNA to a nylon membrane hybridized later to the appropriate probes (Figure V.8.b).

1. Preparation of the Probes

The probes, inserted into plasmids, were provided in *E.coli* and the small scale isolation of the plasmids from *E.coli* followed by its digestion with the required restriction enzymes, was a test for the plasmid and insert quality and size (Figure V.9.a). The large scale isolation was then performed to isolate a large quantity of probe (Figure V.9.b). Fifty μl of digested plasmid DNA was run on a 1% agarose gel for the separation of the insert which was then cut from the gel, separated from the agarose in a dialysis tubing, and then purified to be labeled and used in hybridizations. The concentration of the probes was measured by spectrophotometric analysis and were found to be 0.565 μg/μl for pEW401HE, and 0.34 μg/μl for p132-G8R1.

2. Visual Detection of Duplication

The advantage of Southern blot analysis came from the possibility of visual detection of the duplication without the need for densitometric analysis. Dosage was scored in between polymorphic EcoR1/HincII alleles detected by probe p132-G8R1 which is a genomic subclone of PMP22, because of a HincII site polymorphism. This marker was highly informative with a good percentage of heterozygous individuals (~72%; Figure V.10). The second analyzed marker, pEW401HE (D17S61), was less informative and showed heterozygote frequency of ~39%. (Figure V.11).

a

b

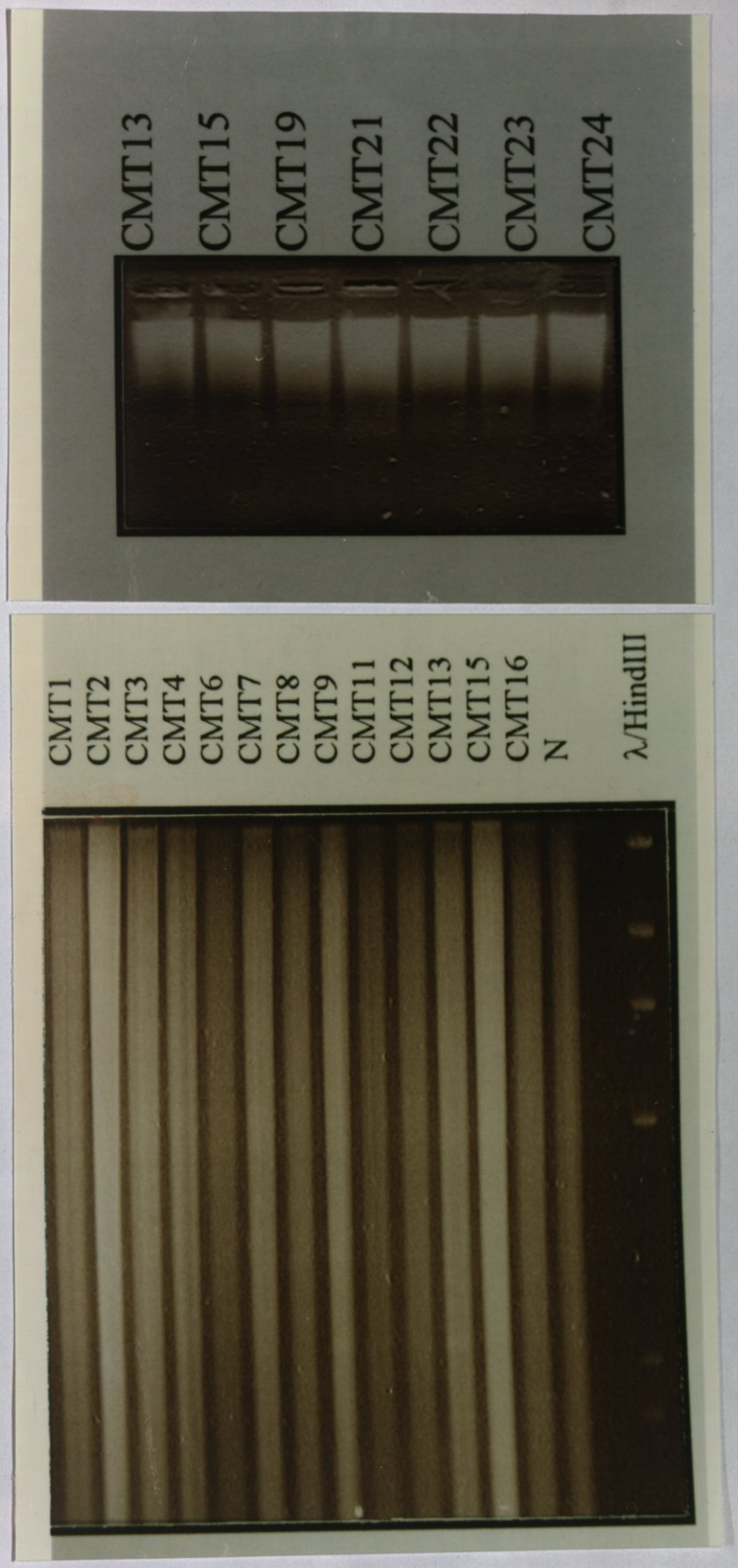

Figure V.8. EcoR1/HincII DNA double digest products.

a. Testing the extent of digestion on a 1% agarose gel.

b. Overnight run of some digested DNA samples on a 1% agarose gel.

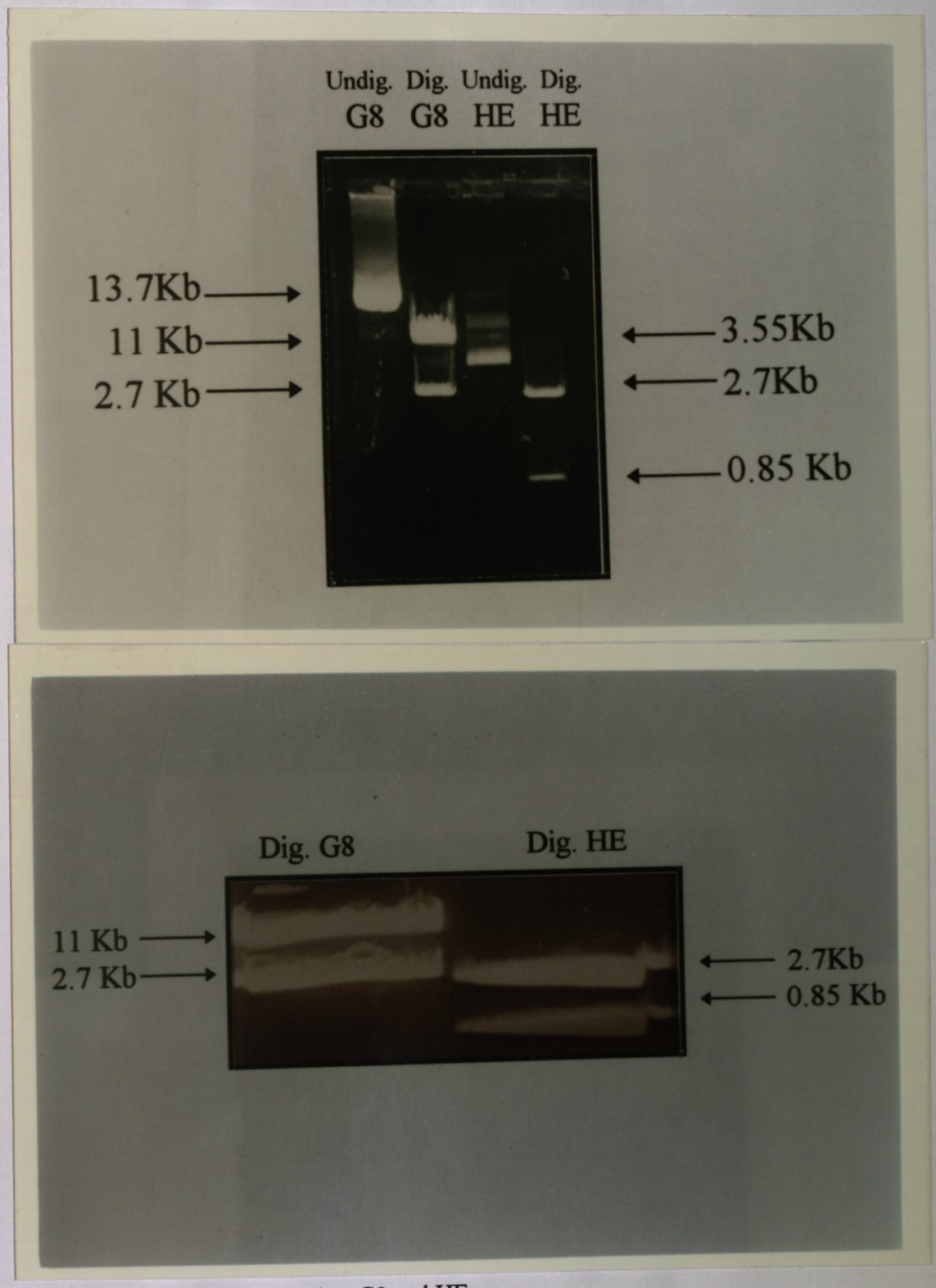

Figure V.9. Isolation of the probes G8 and HE.

a. Digestion of the small scale isolated plasmids showing the length of undigested (undig.) plasmid next to its digested (dig.) form.

b. Running the digested large scale isolated plasmids on a 1% agarose gel to separate the inserts.

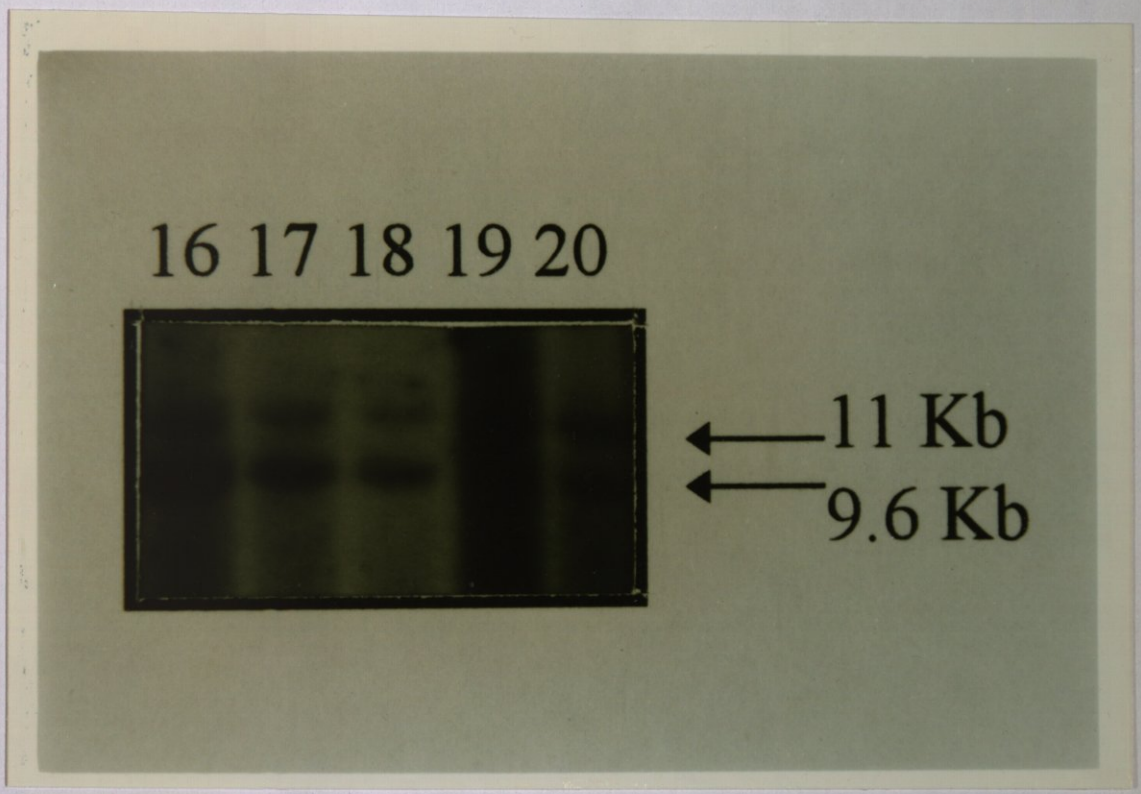

Figure V.10. Analysis of the marker p132-G8R1. The bands detected are 11 Kb and 9.6 Kb in length and the duplication can be easily seen in patients 16, 17 and 18, while patient 20 shows no duplication.

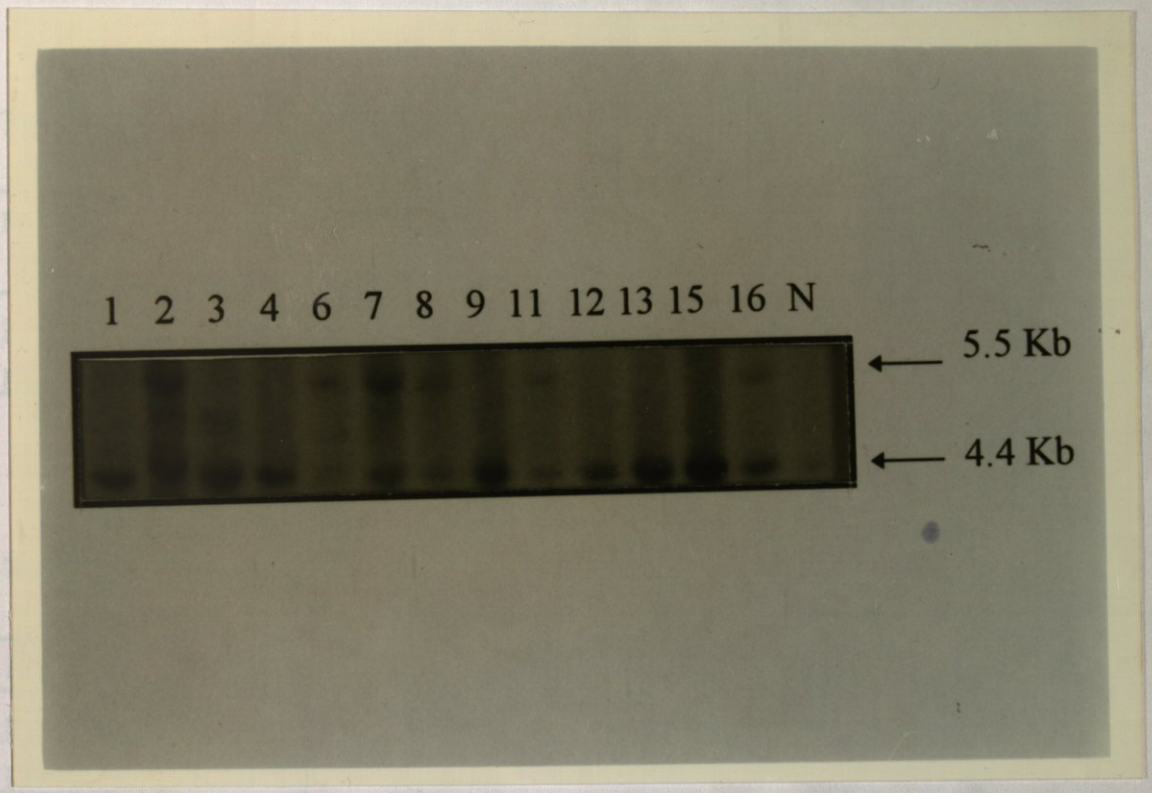

Figure V.11. Analysis of the marker pEW401HE. The bands detected are 5.5 and 4.4 Kb in length. The duplication can be seen in patients 2, 6, 7, and 16, and a high percentage of homozygosity is noticed.

A different number of the same patients was studied for each marker and the results were compared and found to be consistent with each other and with those of the marker RM11-GT concerning patients carrying the duplication. The percentage of heterozygosity for marker p132-G8R1 was ~79 (11/14), and ~71% (10/14) of the patients were found to carry the duplication with the use of this marker, whereas one did not carry the duplication. On the other hand, the percentage of heterozygosity for marker pEW401HE was ~38 (7/18), and ~27% (6/18) of the patients analyzed with this marker were found to carry the duplication. Patients homozygous for one marker were heterozygous for the other two and therefore the results completed each other. The variety of the number of patients analyzed with each marker arose because of an insert contamination which retarded our analysis on some of the patients. Tables V.2 and V.3. list these percentages and compare them with those published for other populations.

Table V.2. Percentage of heterozygosity for the markers used in Southern analysis.

Marker	Allele size	Per cent heterozygosity in this study	Previously published percentage
p132-G8R1	11 Kb and 9.6 Kb	78.57 (11/14)	64
pEW401HE	5.5&4.4 Kb	38.88 (7/18)	41

Table V.3. Percentage of duplication detected with each marker.

Marker	Number of patients studied	Duplication percentage
p132-G8R1	14	71.42
pEW401HE	18	27.77

D. Summary of the Results

Out of the 26 patients analyzed for the marker RM11-GT, 21 patients were informative, and 18 of these were found to carry the duplication. Fourteen patients were analyzed with marker p132-G8R1, and 10 of the 11 informative individuals were found to carry the duplication. Seven heterozygotes were detected among the 18 patients analyzed for PEW401HE marker, and five of these were found to carry the duplication (Table V.4).

On the other hand, many patients were informative for more than one marker at the same time, and this was an advantage that helped confirm the results. Ten cases were solved with the use of marker RM11-GT alone, and five of these carried the duplication. Five of the six patients who were informative for both RM11-GT and p132-G8R1 carried the duplication. Two patients were informative for all three markers and both of them were found to carry the duplication. All the three patients informative for both RM11-GT and pEW401HE, and all the three informative for only p132-G8R, carried the duplication. Two patients were informative for only pEW401HE, and both did not carry the duplication (Table V.5). Only one HNPP patient was detected among three individuals in question for HNPP.

Table V.4. List of the number of informative individuals and of the duplications detected with each of the three markers.

	RM11-GT	**p132-G8R1**	**pEW401HE**
Number of patients analyzed	26	14	18
Number of heterozygotes	21	10	7
Number of duplication carrying individuals	18	10	6

Table V.5. List of the number of patients informative for one or more markers, and of those carrying the duplication in each case. (+) refers to informative individuals, (-) refers to uninformative individuals.

Number of Patients	RM11-GT	p132-G8R1	pEW401HE	Duplication Carriers
10	+	-	-	5
6	+	+	-	6
2	+	+	+	2
3	+	-	+	3
3	-	+	-	3
0	-	+	+	0
2	-	-	+	2

VI. DISCUSSION

Peripheral neuropathies with their different types and origins have attracted the attention of researchers during the last decade. Interest in conducting studies on these neuropathies often arose from the lack of medical treatment for these diseases, which forced clinicians to rely on electrophysiological methods to help the affected individuals. Charcot-Marie-Tooth, in its different forms, is one of these neuropathies on which a great focus has been made, and for which different kinds of genetic causes have been discovered. The fact that CMT type 1A is the most prevalent demyelinating form of the disease encouraged investigators to concentrate on this type, for which interesting observations were made on genetic basis. The common problem of diagnosing CMT1A phenotype, especially in patients showing clinical features typical for other peripheral neuropathies or other forms of CMT, has encouraged researchers to look for the genetic causes of the disease.

Detection of DNA duplications in unrelated patients, which proved that this rearrangement is the primary cause of the disease, has made DNA analysis an important requirement for the differential diagnosis of CMT1A. On that basis, the idea of establishing methods capable of detecting this genetic defect crystallized. Researchers competed for developing fast, safe, and reliable methods which can be applied to confirm the diagnosis for affected individuals, and to reach the step of prenatal diagnosis. Since there were no previous studies on CMT in Turkey at the molecular level, we have started investigations on the most prevalent form, CMT1A in this study. We tried to detect its frequency in the Turkish population, and to establish the molecular methods used for the detection of duplications.

In this study, 69% of the 26 patients analyzed, who show decreased NCVs, as well as clinical symptoms of CMT1, were found to harbor the CMT1A duplication. This frequency falls near the range of 57% to 68% mentioned in the literature for other populations and ethnic groups.(Wise et al., 1993), which proved a nearly similar frequency of the disease in the Turkish population despite the small number of individuals analyzed so far.

The methods for duplication detection followed in this study can be divided into two categories: the radioactive, and the non-radioactive. Both methods rely on the analysis of markers localized in the duplicated 1.5 Mb interval, which ranged in their importance from very informative to the least informative depending on the percentage of heterozygosity of individuals for every marker. With the most informative marker in this study, p132-G8R1, a 71.42% duplication frequency has been detected relative to the 28% frequency detected with the marker pEW401HE. Heterozygosity for any marker is a very important

requirement without which analysis cannot be made, and the need for the study of other markers arises.

The frequency of Turkish pEW401HE heterozygotes (38.88%) was found to be close to that mentioned for other populations (41%) while that for G8 (78.57%) was far greater than the frequency calculated in the literature (62%). This was actually not very surprising due to the known heterogeneity in the Turkish population.

The non-radioactive method relied on the CA repeat marker in the RM11-GT locus. CA repeat markers are highly polymorphic markers scattered throughout the human genome and used in many different studies. The high degree of heterozygosity of this RM11-GT marker reflected the variation of the number of these CA's between different individuals. Although fully informative individuals showing three distinguishable alleles constituted only a small percentage of our patients, it did not pose an actual problem since full analysis could be made on two allele heterozygotes. In addition to that, conflicting results were mentioned in the literature for fully informative three allele percentage in different populations.

Silver staining, the technique used in this study for visualization of the RM11-GT alleles, was found to be highly sensitive. However, many problems may arise with the analysis of this marker, the most important of which is the appearance of shadow bands along with the real bands. This is because of the common mistake of slippage where the polymerase skips one or two repeats during the amplification process. These shadows affect the analysis of the results, and sometimes make it impossible , especially when densitometry is involved. In this study, after the optimization of the PCR conditions where a 24-cycle program was chosen for the best visualization of allele intensity differences, the problem of shadow bands was eliminated to a high extent allowing densitometric analysis to proceed smoothly.

The densitometric analysis adopted in this study has proved to be indispensable for the confirmation of the duplication result on silver stained gels. The sensitivity of the machine to every trace of color change on the previously set background provided a very accurate curve on the graph. The accuracy of the measurement was further enhanced by decreasing the width of the slit, the reading eye, which moves in a vertical direction. This obliged the eye to give a greater number of readings for every small distance it passed over the bands. The densitometer was set to read silver stained gels using a medium slit width and a range of wavelengths between 570 and 600 nm. Following the pattern of the curve, the nicks representing shadows could be distinguished, from the high peaks that represent bands, consistent with the banding pattern seen on the gel.

The heterozygosity frequency for the marker RM11-GT was estimated to be 81% in the Turkish population and ranging between 74% and 80% in previously published data. The high number of heterozygous individuals for this marker is very useful for monitoring

the origin of the duplication, as well as segregation of this chromosomal region in the CMT1A families. This proved the importance of family based analysis, not only with this marker but also with the other markers to follow the inheritance pattern of the duplicated allele. The CMT1A duplication was estimated in the literature to account for as much as 90% of sporadic CMT1 (Hoogendijc et al., 1992). The high heterozygosity of the marker RM11-GT has the advantage of allowing the detection of these sporadic cases.

The advantage of the radioactive method was its very high sensitivity to the extent that detection of the duplication was almost always possible, without the need for densitometric analysis. But it had the disadvantage of being time-consuming in addition to the use of the hazardous radioactive material.

The detection of the duplication in asymptomatic parents of patients was not surprising in view of the fact that only 10% of affected persons with CMT1 seek medical consultation for symptoms related to the disease either because they have few or no symptoms or they become accustomed to their symptoms (Dyck et al., 1992). This fact was proved by the discovery of the CMT1A duplication in asymptomatic patients, who seeked medical consultation only after developing very severe symptoms secondary to their treatment with vincristine.

Three patients analyzed in this study showed clinical symptoms typical for the HNPP phenotype and were considered to be in question for HNPP. Our analysis proved the presence of an HNPP deletion in only one of these patients, while the other two were found to carry a duplication. This fact, along with the problem of similarity between the clinical phenotype of CMT1A and that of other peripheral neuropathies stressed the importance of DNA analysis for differentiating CMT1A patients and reflected the need for fast and reliable methods for detecting the duplications.

This study forms the basis of research on CMT at the molecular level in Turkey. Two of the methods used for the detection of the DNA rearrangement causing CMT1A were established in our laboratory. This study demonstrates the importance of molecular analysis for the differential diagnosis of CMT1A that is hard to diagnose depending solely on clinical manifestations. Future efforts will be directed towards detecting the mutations responsible for the CMT1 phenotype in individuals for whom no duplication was determined.

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