

MYOTONIC DYSTROPHY: APPLICATION OF A NOVEL
TRIPLET-REPEAT-PRIMED-PCR APPROACH TO TURKISH DM PATIENTS
AND
MOLECULAR ANALYSIS OF A LARGE TURKISH KINDRED WITH
VON HIPPEL-LINDAU DISEASE

by

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to all Turkish DM patients

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ABSTRACT

MYOTONIC DYSTROPHY: APPLICATION OF A NOVEL TRIPLET-REPEAT-PRIMED-PCR APPROACH TO TURKISH DM PATIENTS AND MOLECULAR ANALYSIS OF A LARGE TURKISH KINDRED WITH VON HIPPEL-LINDAU DISEASE

Myotonic Dystrophy (DM) is an autosomal dominant neuromuscular disease with multisystemic effects. DM1 and DM2 are two clinically similar types of DM that are caused by different mutations on different genes. DM1, the more common form, is caused by an unstable CTG repeat expansion in the 3' UTR of the Myotonic Dystrophy Protein Kinase (DMPK) gene. In DM1, as a multisystemic disorder, several different systems are affected. The CTG region on the DMPK gene is highly polymorphic with 5-37 CTG repeats in the healthy population. Since there are several clinical similarities between DM1 and DM2, routine molecular diagnosis becomes very important in DM. In the framework of this study, a new Triplet-Repeat-Primed-PCR (TP-PCR) approach was established and applied to 117 suspected DM1 cases. Although the exact number of CTG repeats of highly expanded alleles cannot be determined by TP-PCR, we have proven that TP-PCR is a fast and reliable molecular diagnostic method that can also be used in future for other triplet repeat disorders.

Von Hippel-Lindau (VHL) disease is an autosomal dominant condition that is characterized by the development of various benign and malignant tumors in different parts of the organisms. In this study, we investigated a large VHL family and identified the disease-causing mutation in exon 3 of the VHL gene, leading to a premature stop codon in the protein.

ÖZET

MYOTONİK DİSTROFİ: YENİ BİR TRIPLET-REPEAT-PRIMED-PCR YÖNTEMİNİN TÜRK DM HASTALARINA UYGULANMASI VE VON HIPPEL-LINDAU HASTALIKLI GENİŞ BİR AİLENİN MOLEKÜLER ANALİZİ

Miyotonik Distrofi mültisistemik etkili, otozomal dominant geçiş gösteren nöromusküler bir hastalıktır. DM1 ve DM2, DM'in klinik olarak birbirine benzeyen, ama farklı genlerdeki mutasyonlardan oluşan iki alt tipidir. Daha sık görülen DM1, Miyotonik Distrofi Protein Kinaz (DMPK) genininin 3' UTR bölgesindeki instabil CTG tekrar artışından kaynaklanır. DM1'in multisistemik etkilerinden dolayı bir çok organ sistemi etkilenir. DMPK genindeki CTG bölgesi polimorfiktir ve sağlıklı bireylerde 5-37 CTG arasında değişir. DM1 ve DM2 arasındaki klinik benzerliklerden dolayı, DM'in moleküler tanısı büyük önem taşır. Bu tez çerçevesinde, yeni bir yöntem olan "Triplet-Repeat-Primed-PCR" yöntemi kurulmuş ve DM şüphesi olan 117 olguya uygulanmıştır. Yüksek CTG sayısı içeren allelerin CTG sayıları tam olarak belirlenemese de, bu tez çerçevesinde TP-PCR'ın hızlı ve güvenilir bir moleküler yöntem olduğunu ve gelecekte diğer üçlü-tekrar hastalıklarında kullanılabileceğini de ilk defa gösterdik.

Von Hippel-Lindau (VHL) hastalığı otozomal dominant geçiş gösteren ve vücudun farklı kısımlarında çeşitli iyi huylu ve habis tümör oluşumlarıyla kendini belli eden bir kanser türüdür. Bu çalışmada incelenen büyük VHL ailesinde görülen hastalığa, VHL geninin 3. eksonundaki bir değişimin, proteinde erken sonlanmaya neden olduğu tanımlanmıştır.

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LIST OF SYMBOLS/ABBREVIATIONS

A	Adenine
C	Cytosine
C _{av}	Voltage gated Calcium channels
g	Gram
G	Guanine
ID#	Identification number
m	Meter
M	Molar
mg	Milli gram
ml	Milli liter
mM	Milli molar
ng	Nano gram
nm	Nano meter
T	Thymine
μl	Micro liter
μm	Micro meter
aa	Amino acid
Akt/GSK3β	Protein Kinase B/glycogen synthase kinase 3β
APKC	Atypical protein kinase C
APP	Amyloid Precursor Protein
Arg	Arginine
ATM	Ataxia telangiectasia mutated gene
ATP	Adenosine Triphosphate
Bcl10-Malt1	B-cell lymphoma 10- Mucosa-associated lymphoid tissue 1
Bp	Base Pair
°C	Degree Celcius
CARD9	Caspase recruitment domain-containing-scaffold protein
CcRCC	Clear Cell Renal Cell Carcinoma
CKII	Casein Kinase II

ClC-1	Muscle specific chloride channel
CMT1	Charcot-Marie-Tooth Neuropathy Type 1
CNS	Central Nervous System
CTG	Cytosine Thymine Guanine
CUG-BP/hNab50	CUG binding protein
DM	Myotonic Dystrophy
DM1	Myotonic Dystrophy Type 1
DM2	Myotonic Dystrophy Type 2
DMF	DM-Flanking-PCR
DMPK	Myotonic Dystrophy Protein Kinase
DMSO	Dimethylsulphoxide
DMTP	DM-Triplet-Repeat-Primed-PCR
DMWD	Dystrophia myotonica-containing WD repeat motif
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxynucleoside 5'-triphosphate
ECM	Extracellular Matrix
EDTA	Ethylenedinitrilo-tetraacetate
EMG	Electromyography
EtBr	Ethidium Bromide
EtOH	Ethanol
FAM	Carboxyfluorescein
FISH	Fluorescence <i>in situ</i> hybridization
F-PCR	Flanking Polymerase Chain Reaction
FRDA	Friedreich's Ataxia
GFP	Green Fluorescent Protein
GLUT-1	Glucose transporter 1
GPCR	G-Protein Coupled Receptor
HB	Hemangioblastoma
HD	Huntington's Disease
HIF	Hypoxia-inducible factor
IGF-1R	Insulin-like growth factor-1 receptor
IgG	Immunoglobulin G
IR	Insulin receptor

IQ	Intelligence Quotient
Jade-1	PHD finger protein 17
JunB	jun B proto-oncogene
Kb	Kilo base
KDa	Kilo Dalton
MAPT	Microtubule-Associated Protein Tau
Mb	Mega base
MBNL	Muscle-blind-like-protein
MMP	Metalloproteinases
MP	Myosin Phosphatase
MRMR1	Myotubularin-related protein 1
mRNA	Messenger RNA
MYPT1	Myosin phosphatase target subunit
NF-KB	Nuclear Factor KB
NMDAR1	N-methyl-D-aspartate receptor
OD	Optical Density
OD ₂₆₀	Optical Density at 260 nm
PCR	Polymerase Chain Reaction
PLM	Phospholemman
PLN	Phospholamban
pVHL	Von Hippel Lindau Protein
RA	Retinal Angioma
RAN	Ras-related Nuclear Protein
RAR γ	Retinoic Acid Receptor Gamma Subunit
Rbp1	Retinol binding protein 1
Rbx1	Ring-box 1
RCC	Renal Cell Carcinoma
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RyR	Ryanodine receptor
SCA	Spinocerebellar Ataxia
SDS	Sodiumdodecylsulphate

SERCA1	Sarcoplasmic/endoplasmic reticulum Ca^{+2} ATPase 1
Six5	sine oculis-related homeobox 5 homologue
SNP	Single Nucleotide Polymorphism
SP1	Specific protein 1
SSCP	Single Strand Conformational Polymorphism
STAT	Signal transducer and activator of transcription
TBE	Tris-Boric acid-EDTA
TE	Tris-EDTA
TNNT2	Cardiac Troponin T
TP-PCR	Triplet-Repeat-Primed Polymerase Chain Reaction
TSG	Tumor Suppressor Protein
U	Unit
UTR	Untranslated Region
UV	Ultraviolet
VCB-Cul2 E3	a protein complex (VCB-Cul2) with elongin C, elongin B, Cul-2, and Rbx1
VEGF	Vascular Endothelial Growth Factor
VHL	Von Hippel Lindau Disease

1. INTRODUCTION

In the framework of this thesis, the genetic analyses of two autosomal dominant multisystemic disorders, DM a neurological disease and VHL, a cancer syndrome, were investigated. Both of these diseases were rather neglected in Turkey, regarding their molecular analysis, and diagnostic procedures did not exist at the time we started this study.

Myotonic Dystrophy, a neuromuscular disease, belongs to the large group of triplet repeat disorders. The disease is caused by the expansion of a triplet repeat (CTG) in the 3' untranslated region of the DMPK gene, which was isolated in 1992 (Brook *et al.*). Because of the huge expansion potential of the repeats in DM, diagnosis of DM is more complicated than all other triplet repeat disorders. Since the conventional PCR technology cannot discriminate between homozygous healthy individuals with two normal alleles and affected individuals with high repeats, Southern Blotting, which is an inconvenient method for routine molecular diagnosis, is the method of choice. A breakthrough in the diagnosis of the DM was the development of the Triplet-Repeat-Primed PCR (TP-PCR) by Warner *et al.* (1996). This method enables the detection of the expanded repeat regions with fragment length analysis. Although the technique cannot give the exact repeat number, it was proven to be a quick and safe diagnostic method, not only for DM, but also for other repeat disorders, so that it soon replaced Southern Blotting in many laboratories.

Von Hippel-Lindau Disease is an inherited cancer syndrome, characterized by the development of various benign and malignant tumors in different parts of the body. Its pattern of inheritance is autosomal dominant. The VHL gene was isolated by positional cloning in 1993 (Latif *et al.*) and subsequently several mutation types were described in the VHL gene.

In the framework of this study, the molecular methods for diagnosing these two disorders were established and applied to Turkish patients.

1.1. DM as a Triplet Repeat Disorder

1.1.1. Triplet Repeat Disorders

The concept that considers the human genome as a stable structure has changed long time ago. Scientists discovered that the human genome contains many unstable elements, and that the genome itself has a very dynamic structure (Bermudez de Leon and Cisneros, 2008). In the human genome, there are approximately 3 billion of DNA basepairs, containing different recurring motives of repetitive DNA, such as microsatellite repeats and repetitive DNA sequences in the heterogeneous DNA regions, centromeres and telomeres of the chromosomes (Lutz, 2007).

The importance of triplet repeats was first discovered in 1991, when the Fragile X syndrome, which is the most common inherited mental retardation, was found to be caused by a triplet repeat (CGG) (Fu, 1991). Fragile X was the first disease model, to point out, that some mutations can be transmitted dynamically. The anticipation phenomenon, seen in this disease, explained the variability of expression, disease severity and age of onset. Today, there are 16 human neuromuscular and neurodegenerative diseases known, which are caused by trinucleotide expansions (Orr and Zoghbi, 2007). Another theory implicates that 40 neurologic, neurodegenerative or neuromuscular diseases are possibly linked to triplet repeats (Pearson *et al.*, 2005). The most common triplet repeats, associated with known diseases are: (CUG)_n, (CCG)_n, (GAA)_n, and (GAG)_n (Bermudez de Leon and Cisneros, 2008). Myotonic Dystrophy (DM), Friedreich ataxia (FRDA), spinocerebellar ataxias (SCA) and Huntington's disease (HD) are some of the neuromuscular and neurodegenerative diseases, that are caused by triplet repeats, but have absolutely different mechanisms. On the other hand, all of these diseases have the common feature of having a negative correlation between their triplet repeat size and the age of onset; besides, as the age of onset decreases, the disease severity increases. The Tables 1.1. and 1.2. classify the known triplet repeat disorders according to their mechanisms (Orr and Zoghbi, 2007).

Most of the trinucleotide expansion diseases were thought to fit into a model, in which the noncoding-site mutations result in a loss-of-function type of mechanisms, like FRDA, whereas, in the dominant diseases, the coding-site mutations cause a gain-of-

function mechanism, like SCAs and HD. Most interestingly, DM was the first disorder that does not fit into this model, showing always an autosomal dominant pattern, with a repeat in the noncoding region. (Ranum, & Cooper, 2006)

Table 1.1. List of the unstable repeat disorders, which have different clinical features, sorted by a loss-of-function, RNA-mediated or unknown mechanism (Orr and Zoghbi, 2007)

Disease	MIM Number	Repeat unit	Gene product	Normal repeat	Expanded repeat	Main clinical features length
<i>Loss of function mechanism</i>						
FRAXA	309550	(CGC) _n	FMRP	6–60	>200 (full mutation)	Mental retardation, macroorchidism, connective tissue defects, behavioral abnormalities
FRAXE	309548	(CCG) _n	FMR2	4–39	200–900	Mental retardation
FRDA	229300	(GAA) _n	Frataxin	6–32	200–1700	Sensory ataxia, cardiomyopathy, diabetes
<i>RNA-mediated pathogenesis</i>						
DM1	160900	(CTG) _n	DMPK	5–37	50–10,000	Myotonia, weakness, cardiac conduction defects, insulin resistance, cataracts, testicular atrophy, and mental retardation in congenital form
DM2	602668	(CCTG) _n	ZNF9	10–26	75–11,000	Similar to DM1 but no congenital form
FXTAS	309550	(CGG) _n	FMR1 RNA	6–60	60–200 (premutation)	Ataxia, tremor, Parkinsonism, and dementia
<i>Unknown pathogenic mechanism</i>						
SCA8	608768	(CTG) _n	SCA8 RNA	16–34	>74	Ataxia, slurred speech, nystagmus
SCA10	603516	(ATTCT) _n		10–20	500–4500	Ataxia, tremor, dementia
SCA12	604326	(CAG) _n	PPP2R2B	7–45	55–78	Ataxia and seizures
HDL2	606438	(CTG) _n	Junctophilin	7–28	66–78	Similar to HD

Table 1.2. List of gain-of-function diseases which encode polyglutamine (Orr and Zoghbi, 2007)

Disease	MIM number	Gene product	Normal repeat length	Expanded repeat length	Main clinical features
HD	143100	Huntingtin	6–34	36–121	Chorea, dystonia, cognitive deficits, psychiatric problems
SCA1	164400	Ataxin 1	6–44 ^b	39–82	Ataxia, slurred speech, spasticity, cognitive impairments
SCA2	183090	Ataxin2	15–24	32–200	Ataxia, polyneuropathy, decreased reflexes, infantile variant with retinopathy
SCA3	109150	Ataxin3	13–36	61–84	Ataxia, parkinsonism, spasticity
SCA6	183086	CACNA1A	4–19	10–33	Ataxia, dysarthria, nystagmus, tremors
SCA7	164500	Ataxin7	4–35	37–306	Ataxia, blindness, cardiac failure in infantile form
SCA17	607136	TBP	25–42	47–63	Ataxia, cognitive decline, seizures, and psychiatric problems
SBMA	313200	Androgen receptor	9–36	38–62	Motor weakness, swallowing, gynecomastia, decreased fertility
DRPLA	125370	Atrophin	7–34	49–88	Ataxia, seizures, choreoathetosis, dementia

1.1.2. History of Myotonic Dystrophy

In 1909, Hans Steinert first identified DM as a distinct disease, involving myotonia, muscle atrophy, muscle weakness and cardiovascular features (Philips and Harper, 1996). 83 years later, the first molecular knowledge came with the discovery of the DMPK gene on chromosome 19q.13.3. Cause of the disease was an unstable CTG repeat in the 3' untranslated region of the gene (Brook *et al.*, 1992; Fu *et al.*, 1992; Mahadevan *et al.*, 1992). In 1994, a subtype of the disease with similar symptoms, but with no CTG repeat, was reported (Ricker, *et al.*, 1994; Thornton *et al.*, 1994; Schara and Schoser, 2006). Four years later, a new locus on chromosome 3 was described and this form of the disease was called DM2 (Ranum *et al.*, 1998). Since then, several studies have been conducted to unravel the mechanisms of DM. Genetically engineered mice, lacking the DMPK gene, were first constructed in 1996 (Reddy *et al.*, 1996). Further, knockouts of the neighboring genes were developed in 2000 (Klesert *et al.*, 2000). Different approaches with mRNA, containing CTG repeats, were used, to identify the reasons for the multisystemic nature of the disease (Mankodi and Thornton, 2002).

1.1.3. Clinical Correlates of DM1

Myotonic Dystrophy Type 1 is a neuromuscular disease with multisystemic effects, and follows an autosomal dominant pattern of inheritance. The anticipation of the disease, seen between intergenerations, can be huge. Although, no absolute distinction exists inbetween, the disease can be divided into four categories; a) congenital b) childhood-onset c) adult-onset d) late-onset or asymptomatic. For less than 400 repeats, as the CTG expansion size increases, the severity of the disease also increases and usually the higher number of CTG indicates an earlier age of onset. However, this correlation becomes less significant for the longer repeats (>400 CTG) (Machuca- Tzili *et al.*, 2005; Schara & Schoser, 2006).

Congenital patients usually inherit the disease from their mothers. The theory implies that oogenesis can generate larger repeat expansions than spermatogenesis (Jansen *et al.*, 1994, Mankodi & Thornton, 2002).

DM1 patients have a specific appearance, because of the weakness and atrophy of the facial muscles and ptosis. As DM1 is a multisystemic disorder, several different systems are affected. Skeletal and cardiac muscle, gastrointestinal tract, central nervous system, endocrine system involvements and cataract are observed in DM1 patients (Machuca-Tzili *et al.*, 2005).

Skeletal muscles: The best recognized feature of DM1 is myotonia, which is the delay of the muscle relaxation. Myotonia is most commonly seen in the peripheral muscle, such as hands and forearm. Progressive muscle wasting and weakness are also observed in the skeletal muscle. A theory suggests that muscle wasting in DM1 is very different from the muscle wasting seen in other muscular dystrophies. A defect in the muscle anabolism causes this muscle wasting, rather than necrosis or fibrosis of muscles (Halliday *et al.*, 1985; Grigs *et al.*, 1990; Wheeler, 2008).

Cardiac abnormalities: Cardiac conduction abnormalities are commonly observed in DM1 patients. Myocardial disease is also observed, but in a very rare fashion, implying that the disease cannot be associated with DM1 and can be coincidental. Conduction delay can be at any section, but is mostly observed in His-Purkinje system. Atrial flutter and fibrillation are the most common reasons of arrhythmia, observed in one in every four patients (Phillips and Harper, 1996).

Gastrointestinal tract abnormalities: DM1 patients often suffer from dysphagia, which is the difficulty in swallowing. Heartburn, chestpain, regurgitation, coughing while eating, are also observed in some patients. Dyspeptic symptoms, like vomiting, nausea and early satiety and epigastric pain are often seen in DM1 patients. Also there are some intestinal disorders like diarrhea -associated with malabsorption- and constipation (Bellini *et al.*, 2006).

Endocrine System Abnormalities: There are several types of endocrine system abnormalities, like testicular atrophy with reduced fertility, frontal balding in males, which is a common feature of the typical facial appearance and insulin resistance. Lastly, hyperhidrosis is a rare abnormality seen in endocrine system of DM1 patients (Machuca-Tzili *et al.*, 2005).

Cataracts: Iridescent, posterior capsular, multicolored opacities are observed in DM1 patients. Cataract is the easiest treatable symptom of the disease. (Machuca-Tzili *et al.*, 2005).

Central Nervous System (CNS) Abnormalities: Visual-spatial defects, behavioral problems and personality changes are commonly observed in juvenile and adult-onset forms of DM1. Facial impressions -resulting from facial muscle weakness and ptosis- as well as delayed motor responses, give the impression of reduced IQ. Although the IQ of patients is in normal range, the average is found to be lower than the normal controls. Moreover, in severe cases with a high number of CTG repeats, reduced IQ values are found in one third of DM patients. Apathy, which is the absence of emotion or enthusiasm, and increased daytime sleepiness are the most common behavioral changes, observed in these patients. Recent studies hypothesize that there is also involvement of neurodegeneration, which is supported by the finding of ribonuclear inclusions at hippocampus, dentate gyrus, thalamus, substantia nigra and the brain stem tegmentum. Cell loss in specific areas of the brain, like in dorsal raphe nucleus, superial central nucleus, dorsal and ventral medullary nuclei and subtrigeminal medullary nucleus was also found (Oyanagi *et al.*, 1994; Ono *et al.*, 1996; Jiang *et al.*, 2004; Meola and Sansole; 2007).

The prevalence of DM1 is 2 to 5 in 100,000 with the congenital form, seen in 1 in 3500. Disease incidence is 13 in 100,000 (Harper *et al.*, 2001, Harper *et al.*, 2004, Schara and Schoser, 2006).

1.1.4. Molecular Genetics of DM1

1.1.4.1 The DM1 Mutation Locus and the DMPK Protein. The mutation that causes Myotonic Dystrophy Type 1 was identified in 1992 as expansion of a CTG repeat in the 3' untranslated region (3' UTR) of a protein kinase gene. The gene, named Myotonic Dystrophy Protein Kinase (DMPK), was localized to chromosome 19q13.3, which is a gene-dense area. This finding made DM1, the first dominantly inherited disease, which has

a mutation in the uncoding region of a gene (Brook *et al.*, 1992; Buxton *et al.*, 1992; Fu *et al.* 1992; Mahadevan *et al.*, 1992)

The CTG region on the DMPK gene is highly polymorphic with 5 to 37 CTG repeats in the healthy population. Premutation state is between 38 and 50, which can be counted as hidden disease allele with the potential of becoming unstable. Congenital patients can have more than 1500 repeats (Brook *et al.*, 1992; Gharehbaghi-Schnell *et al.*, 1998). In different tissues of the patients, somatic instability is observed; besides, repeat size can increase from 50 to 80 per year. (Martorell *et al.*, 1998; Day and Ranum, 2005)

The DMPK cDNA has 15 exons and is predicted to code for a protein of about 70 kDa. The neighboring genes, affected from the CTG expansions, are Six5 (sine oculis-related homeobox 5 homologue), which is at 3' end of the DMPK, and the DMWD, which is at the 5' of the gene (Lam *et al.*, 2000).

The DMPK protein is a serine/threonine kinase. It has a hydrophobic carboxy-terminal domain, a coiled-coil region about 12 kDa and a catalytic domain at the N-terminal, which is about 46 kDa. Six different conserved isoforms of the protein are found, resulting from three possible alternative splicing events. DMPK is most abundant at skeletal and heart muscles. Some of the theoretical targets of the DMPK are: CUG binding protein, (CUG-BP/hNab50), phospholemman (PLM), phospholambon (PLN), myosin phosphatase target subunit (MYPT1). (Lam *et al.*, 2000; Kaliman & Llagostera, 2008).

Functions of the DMPK protein are thought to be skeletal muscle integrity, cytoskeleton modification, cardiac muscle atrioventricular conduction, ion channel gating and cell metabolism. Through cytoskeleton modification, DMPK can be also involved in synaptic plasticity. Fail in this function may be the reason of the cognitive dysfunction seen in DM1 patient. Together with this data, some correlation between phenotypes and the molecular function of the protein has been hypothesized (Figure 1.1).

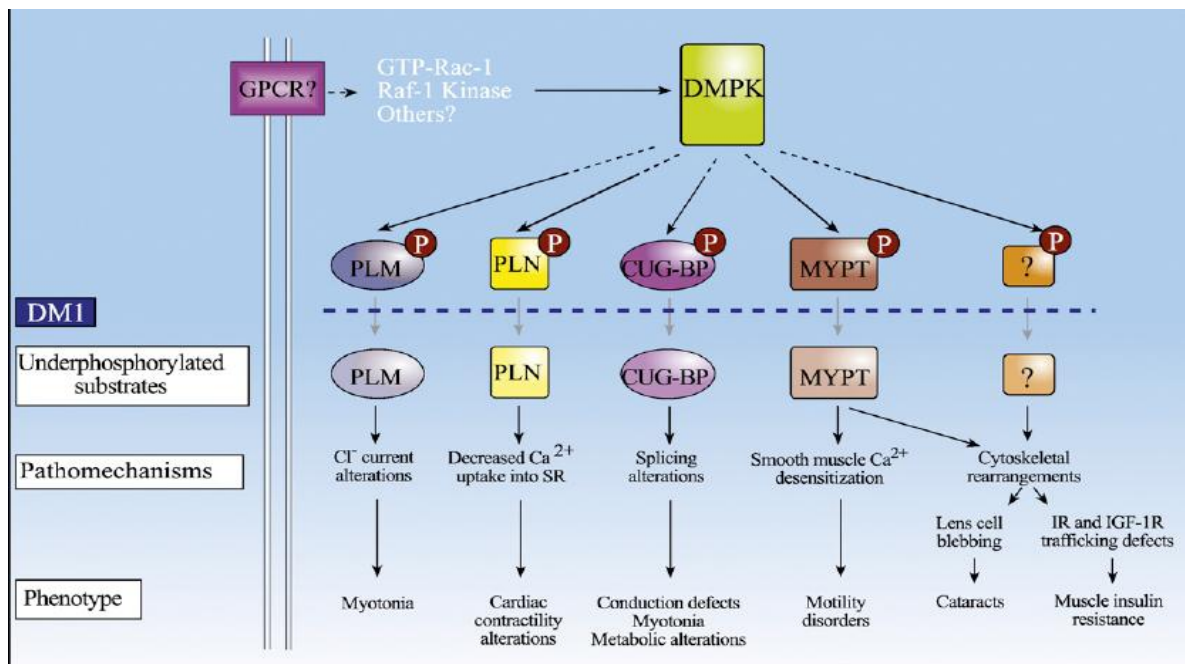


Figure 1.1. Hypothetical model for the pathophysiological role of DMPK in DM1.

MP: Myosin Phosphatase; GPCR: G-Protein Coupled Receptor; IR: insulin receptor;

IGF-1R: insulin-like growth factor-1 receptor (Kaliman& Llagostera, 2008)

1.1.5. Molecular Mechanism of DM

Three main theories and an additive model exist to explain the molecular mechanisms of DM.

1.1.5.1. Haploinsufficiency of the DMPK Protein. The first model to be developed was the haploinsufficiency of the DMPK protein. Early findings showed that the mRNA and the protein levels of the DMPK, were lowered in the patients. A study, conducted on the fibroblasts and muscle biopsies of DM1 patients, showed that when the CUG repeat was labeled with fluorochromo-conjugated probe with 10 repeats of CAG, DMPK mRNAs were observed as bright foci, whereas, foci forming was not present in control cases (Taneja *et al.*, 1995).

When DMPK mRNA is transcribed, large-sized CUG repeats are also transcribed at the 3' UTR. This mRNA, with a long repeat, somehow fails to go out from the nucleus, whereas the mRNA of the normal allele can (Figure 1.2). *In vitro* studies showed that long

repeats of CUGs tend to form long hairpin loops that have double-stranded RNA structure. On the other hand, there is no information about how this mRNA can behave *in vivo*. Therefore, the reason why these mRNAs retain in the nucleus is unknown (Napierala & Krzyosiak, 1997; Tian *et al.* 2000 Tapscot and Thornton, 2001).

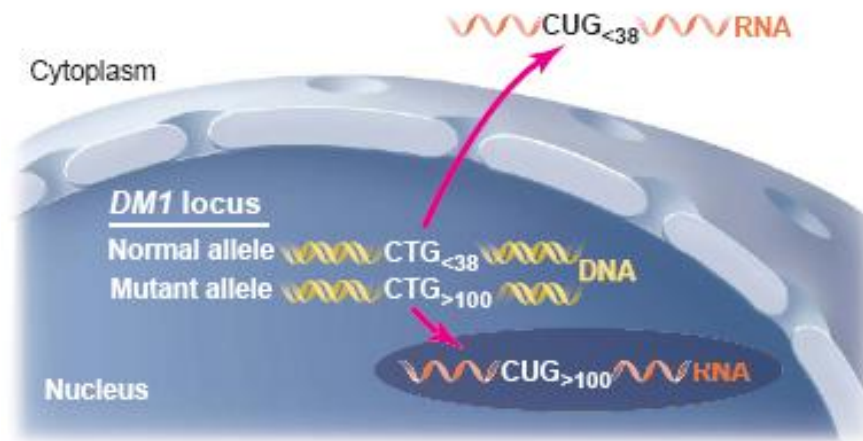


Figure 1.2. Schematic representation of nucleus retention in DM1: mutant alleles produce long mutant mRNA with more than 100 CUG repeats that retains in the nucleus (Tapscot and Thornton, 2001)

To test this model, DMPK knockout mice were developed. In these mice, only mild late-onset myopathy and cardiac conduction abnormalities were observed, whereas other multisystematic defects, such as progressive muscle weakness and myopathy, were absent (Reddy *et al.*, 1996; Jansen *et al.*, 1996). Moreover, although the disease prevalence is high, no other mutations like point mutations, were reported to cause DM1. Therefore, haploinsufficiency of the DMPK is not sufficient to understand the mechanism, although it may explain some features of the disease (Gatchel and Zoghbi, 2005).

1.1.5.2. Haploinsufficiency of the Neighboring Genes. This hypothesis claims that the expansion of the CTG repeat region affects the expression of other neighboring genes, like Six5. As it is well established now, DNA sequence has an affect on nucleosome formation. Particular sequences make a strong binding site for nucleosomes. The changes in the DNA sequences are known to be causing position effect variation on the chromatin binding sites. Studies demonstrate that CTG is a strong nucleosome-binding site and alters the chromatin

structure (Wang *et al.*, 1994; Otten and Tapscott, 1995). The exact mechanisms, how the repeats cause alteration is unknown, however it has been theorized that they can adopt non-B conformations, toroids, hairpin loops and single stranded regions (Groenen and Wieringa, 1998).

Six5 is located at the 3' end of the DMPK gene and the expansion also covers its promoter region. Six5 acts as a transcription factor and it is very similar to sine oculis, the fruit fly gene, which is essential for the eye development. It is also similar to a mouse gene family, that plays a role in regulating the development of distal limb muscles (Boucher *et al.*, 1995; Ranum and Day, 2004; Gatchel and Zoghbi, 2005). Because the wasting of distal muscles and cataract are the cardinal clinical signs of DM1, the Six5 protein was thought to be affected in DM1, too. The theory suggests that under normal conditions, the open region of the promoter becomes closed by the chromatin alteration and as a result, expression levels drop. More CTG repeats cause more closure, resulting in an increase in disease severity. Other neighboring genes, affected by altered chromatin structure are DMWD and IgG receptor gene. DMWD, expressed in testes, is located upstream of DMPK. DMWD was thought to be involved in DM1 disease mechanism, because infertility is a symptom of DM1. Furthermore, the IgG receptor is located in 4 Mb distance to the expansion site, and this may explain the low IgG levels of DM patients (Junghans *et al.*, 2001; Ranum and Day, 2004).

In order to prove these theories, expression studies were conducted. There was no decrease in the expression levels of DMPK (Krahe *et al.*, 1995), however, conflicting results were published for the neighboring genes. An allele specific RT-PCR was used to identify the expression levels of Six5. The mutated allele was found to have a decreased expression, whereas the wild-type allele had normal levels (Thornton *et al.*, 1997). In another study, however, there was no decrease in the cytoplasmic expression levels of Six5 (Hamshire *et al.*, 1997). In knockout mice studies of the Six5 gene, cataract development, which is one of the clinical features of DM1, was observed (Klesert *et al.*, 2000). On the other hand, this cataract had not the property of iridescent opacity or posterior location, like the one observed in DM1. Although the haploinsufficiency model explained some of the clinical features of DM1, it was not sufficient to explain all symptoms seen in DM patients (Ranum and Day, 2004).

1.1.5.3. RNA Pathogenesis Model. The current favorite model is RNA pathogenesis model. According to this model, the mutant RNA, containing high levels of CUG repeats, has a toxic effect on the cells. This model explains, how two different DM types, caused by different mutations on different chromosomes, can have similar clinical features. In DM2, the toxic cause of mRNA is the CCUG tetranucleotide repeat. Toxic-gain-of-function of mRNAs is a very new subject, because no similar effect was observed in other disorders, prior to DM. To support this model, transgenic mice, containing high CUG repeat expansions, were tried to be developed. However, the attempt was unsuccessful, because the mice became infertile (Monckton *et al.*, 1998; Ranum and Day, 2004). Another study overcame this problem by inserting a large number of CUG repeats (~ 250) at the 3' end of the skeletal actin gene, which is transcribed only at skeletal muscles. The resulting mice developed myotonia and myopathic features. This was an expected result, because the toxic mRNAs were only created in the muscle cells, therefore a multisystemic effect would not be observed (Mankodi *et al.*, 2000). In a similar study, conducted by Seznec and coworkers (2001), transgenic mice (DM300), which contained a 300 CTG-repeat DMPK gene and the neighboring genes, were constructed. The mRNA levels transcribed were lower, and it was not specific to skeletal muscles. Apart from myotonia, skeletal muscle histological abnormalities and altered expression of tau isoforms were found in these animals (Ranum and Cooper, 2006).

The molecular mechanism assumed to be acting in the RNA pathogenesis model can be classified in three events: formation of nuclear foci, splicing alteration of specific genes and consequent impairment of muscle differentiation (Bermudez de Leon & Cisneros, 2008).

Nuclear foci formation: Mutant DMPK mRNAs were found to form a discrete ribonuclear foci in the nuclei of DM1 patients' fibroblasts by fluorescence *in situ* hybridization (FISH) experiments (Taneja *et al.*, 1995). Another experiment used Northern Blot analysis on fractionated RNA to show the accumulation of the mutant DMPK transcriptions in the nuclei in muscle tissues. Another important finding of this experiment was that the longer CUG repeats form more nuclear retention, pointing to the role of the expansion size on foci formation (Davis *et al.*, 1997). Ribonuclear foci formation was also found in cerebral cortex, hippocampus, dentate gyrus, thalamus, substantia nigra and brain

stem tegmentum in DM1 patients, whereas control samples, with or without a neurological disease, did not show these inclusions. Severity of the foci formation was again in correlation with the CUG repeat size. These foci were not co-localized with the PML bodies, nucleolus, perinuclear compartment or speckles (Jiang *et al.*, 2004; Bermudez de Leon and Cisneros; 2008).

Studies revealed that *in vitro*, mutant DMPK-mRNA can bind to CUG-binding protein (CUG-BP1) and ETR3-like factor (CELF) families, which take place in alternative splicing reactions in the foci. It was previously shown that CUG-binding proteins bind to short repeats of (CUG)₈ oligonucleotides (Timchenko *et al.*, 1996). However, so far, *in vivo* studies failed to show co-localization of the mutant mRNA with this protein (Fardaei *et al.*, 2001; Bermudez de Leon and Cisneros, 2008).

Another protein family, that is present in ribonuclear foci with DMPK, is the muscle-blind-like-protein (MBNL) family, which has a role in alternative splicing. MBNL family members bind to double stranded RNA hairpins, composed of CUG repeats (Miller *et al.* 2000). MBNL1, MBNL2 and MBNL3 are the three isoforms of this protein family, and they all are found to co-localize with the ribonuclear foci in the muscle cell cultures and skeletal muscle sample cells (Fardaei *et al.*, 2001; 2002; Mankodi *et al.*, 2001). In the cortical neurons, MBNL1 and MBNL2 were associated with nuclear foci, whereas CUGBP1 ETR3 and CEFL4, which are other members of CEFL family, were not (Jiang *et al.*, 2004; Bermudez de Leon and Cisneros, 2008).

Transcription factors were found to be bound and sequestered by mutant DMPK-RNA in the muscle cells. The composition of the nuclear foci, changes in different tissues. For instance, specific protein 1 (Sp1), the signal transducer and activator of transcription family members (STAT1 and STAT3) and the retinoic acid receptor gamma subunit (RAR γ) were found in co-localization with DMPK-mRNA in muscle cells, whereas the co-localization of these proteins were not confirmed in cortical neurons (Ebrilidze *et al.*, 2004; Jiang *et al.*, 2004; Bermudez de Leon and Cisneros, 2008).

Splicing Alterations: In the human genome, there are only about 25.000-30.000 genes, which express more than hundreds of thousands of proteins. This diversity, partly

results from alternative splicing. Isoforms of developmental stage- and tissue-specific proteins, are created by alternative splicing. In human organism, alternatively spliced mRNAs are observed in 74 per cent of the genes. Alternative splicing does not only create different isoforms, but it regulates on/off states of the genes. For instance, when mRNA is altered in a way that causes frameshift followed by premature termination, these terminated mRNAs are degraded by nonsense-mediated decay. It is well established now, that if the regulator proteins are affected somehow, alternative splicing can be altered, and inappropriate protein isoforms, in the wrong tissues, cause disease (Kuyumcu-Martinez and Cooper, 2006; Ranum & Cooper, 2006).

As mentioned earlier, in DM1, brain and muscle tissues contain ribonuclear foci, having mutant DMPK associated with some alternative splicing regulator proteins, like MBNL. However, this change in alternative splicing event only results in alteration of some particular pre-mRNAs, rather than disrupting all mRNAs in DM1. Today, 10 genes are known to be affected with a total of 14 alternative splicing events in three tissues (Table 1.3). Only two of the misregulated splicing events are associated directly with disease symptoms. These symptoms are mytonia, caused by muscle specific chloride channel, (ClC-1) and insulin resistance, caused by insulin receptor (Ho *et al.*, 2004; Ranum and Cooper, 2006; Bermudez de Leon and Cisneros, 2008).

In other muscle diseases, if there is a loss-of-function-causing mutation in the ClC-1 gene, a little decrease in the expression of this gene is observed and it is enough for mytonia to occur. In DM1 patients, ClC-1 protein is decreased in less than 10 per cent compared to normal levels, this also shows mytonia. The embryonic splicing pattern of ClC-1 fails to change into the adult splicing form, resulting in a shorter mRNA, causing the degradation of mRNAs by the nonsense mediated decay (Ranum and Cooper, 2006).

Another protein, that is affected and correlated directly with the symptoms of DM, is the IR gene. With an exon-inclusion alternative splicing defect, the fatal form, which has a lower signaling capacity, is formed. This phenomenon results in the increased insulin resistance of DM1 patients (Savkur *et al.*, 2001; Ranum and Cooper; 2006).

TNNT2 is the gene that codes for the cardiac troponin T, which is a thin-filament contractile protein. Fifteen per cent of hypertrophic cardiomyopathies result from a mutation in this gene. There are several isoforms of the TNNT2 protein: In the heart, exon 5 is excluded in adults; however there are few cases, in whom exon 5 is present in DM1 patients (Machuca-Tzili et al, 2005).

Myotubularin-related protein 1 MRMR-1 is one of the possible targets of missplicing. There are six isoforms of the protein and the C isoform, which is important in myogenesis, is observed to be reduced in the congenital DM1 patients' skeletal muscle biopsies (Machuca-Tzili et al, 2005).

Tau, also known as MAPT, is a member of the microtubule-associated protein family. Exon 2, which is excluded from the alternative protein in DM1 patients, has a role in binding axonal membrane. Therefore, when this part of the protein is absent, the microtubule stabilization can be disrupted. Exon 10 of the gene encodes the part of the protein, which binds to microtubules. Disruption of this domain may result in poor microtubule-tau binding (Bermudez de Leon and Cisneros, 2008).

NMDAR1 has a role in learning process and in normal long-term potentiation in the hippocampus (Tsien *et al.*, 1996). Exon 5, which has an affect on pharmacological behavior, gating and subcellular distributions of NMDAR1, is found to be included increasingly in temporal cortex of DM1. Although there is no direct data, memory impairment of DM1 patients can result from this inclusion (Traynelis *et al.*, 1995; Jiang *et al.*, 2004; Bermudez de Leon and Cisneros 2008).

The amyloid precursor protein, known as APP, has a role in transcription activation, by being sequestered by secretases and binding to acetyltransferase complex by these sequestered peptides. This protein's fatal isoform -lacking exon 7- is found in DM1 patients. On the other hand, the direct role of this protein in the mechanism of DM1 is unknown (Jiang et al, 2004; Bermudez de Leon and Cisneroz, 2008).

Table 1.3. Genes found to be alternatively spliced in DM patients
(adapted from Ranum and Cooper, 2006; Leroy *et al.*, 2006)

Gene	Exon/Intron	Tissue	DM pattern	References
Cardiac troponin T (TNNT2)	Exon 5	Heart	exon inclusion	Philips <i>et al.</i> , 1998
Insulin receptor (IR)	Exon 11	skeletal muscle	exon exclusion	Savkur <i>et al.</i> , 2001, 2004
Chloride channel (CLCN-1)	Intron 2	skeletal muscle	intron retention	Charlet <i>et al.</i> 2002, Mankodi <i>et al.</i> , 2002
Chloride channel (CLCN-1)	Exon 7a	skeletal muscle	exon inclusion	Charlet <i>et al.</i> 2002, Mankodi <i>et al.</i> , 2002
Tau (MAPT)	Exon 2 and 3	Brain	exon exclusion	Jiang <i>et al.</i> 2004
Tau (MAPT)	Exon 10	Brain	exon exclusion	Jiang <i>et al.</i> 2004
Tau (MAPT)	Exon 6c and 6d	Brain	exon exclusion and inclusion	Leroy <i>et al.</i> , 2006
Myotubularin-related protein 1 (MRMR1)	Exon 2.1 and exon 2.3	skeletal muscle	exon exclusion	Buj-bello <i>et al.</i> 2002, Ho <i>et al.</i> , 2005
Fast skeletal troponin T (TNNT3)	Fetal exon	skeletal muscle	exon inclusion	Kanadia <i>et al.</i> , 2003
N-methyl-D-aspartate receptor (NMDAR1)	Exon 5	Brain	exon inclusion	Jiang <i>et al.</i> 2004
Amyloid precursor protein (APP)	Exon 7	Brain	exon exclusion	Jiang <i>et al.</i> 2004
Ryanodine receptor (RyR)	Exon 70	skeletal muscle	exon exclusion	Kimura <i>et al.</i> , 2005
Sarcoplasmic/endoplasmic reticulum Ca ⁺² ATPase 1 (SERCA1)	Exon 22	skeletal muscle	exon exclusion	Kimura <i>et al.</i> , 2005
Sarcoplasmic/endoplasmic reticulum Ca ⁺² ATPase 2 (SERCA2)	Intron 19	skeletal muscle	intron retention	Kimura <i>et al.</i> , 2005

Impaired Cell Differentiation: Deficient muscle differentiation is found in DM1 skeletal muscle cell models with overexpressed mutant DMPK-mRNA. A reporter gene, such as LacZ or GFP, fused with the 3' UTR of the mutant mRNA and localized in the ribonuclear foci, cause differentiation disruption in C2C12 myoblasts, which is a model for muscle differentiation (Amack *et al.*, 1999 Amack and Mahadevan *et al.*, 2001). Similar findings are also observed in transgenic mice, overexpressing both mutant and wild-type DMPK-mRNA; they seem to have aberrant and delayed muscle differentiation. Because of the delayed upregulation of some genes, such as p21 and myogenin, the DM1 myoblasts are not able to withdraw from the cell cycle, causing impairment in differentiation. These genes (p21 and myogenin) are found to be regulated by the mutant DMPK-mRNA (Bermudez de Leon and Cisneros, 2008).

To observe the effect of neuronal differentiation in DM1 patients, the PC12 neuronal cell line, which is a widely used model for neuron differentiation, was constructed with a 90 CTG-repeat-containing DMPK 3' UTR region. In these cells, nerve growth factor-induced neuronal differentiation was found to be impaired. Also Akt/GSK3 β signaling pathway, regulating Tau phosphorylation was changed in these cells lines. Finally, current properties of C_{av} channels were altered towards more positive potential (Hernandez-Hernandez *et al.*, 2006; Bermudez de Leon, and Cisneros, 2008).

1.1.5.4. Additive Model. This model simply implies that each of the above pathogenic models contribute to DM1. Some of the features of DM1 are created by haploinsufficiency of DMPK, some others are created by haploinsufficiency of Six5 and other neighboring genes, like DMWD, and the remaining symptoms are created by the toxic gain-of-function and splicing alterations of mRNA (Ranum and Day, 2004).

1.1.6. TP-PCR: a Powerful New Tool for DM Diagnosis

Genetic testing of DM1 patients is of great importance, because of the clinical similarities between DM types and also because of the often difficult differential diagnosis in neurological disorders. DNA testing of DM1 and the DMPK gene is usually performed by Southern Blotting (Brooks *et al.*, 1992). However, besides being time-consuming, Southern Blotting is also unsuitable for automation, which makes it difficult for routine

clinical use. Polymerase Chain Reaction (PCR) is applied for rapid detection of CTG repeats. But simple PCR techniques are only sufficient for amplification of maximum 100 CTG repeats. Moreover, long CTG repeats form easily unusual secondary structures, which make them more difficult to amplify. Another major problem in genetic testing of DM1 is, when normal PCR conditions are applied to an affected person with an expansion >100 CTG, only the normal allele is obtained, since the expanded allele cannot be amplified. Also in healthy individuals, if the person is homozygous for the repeat, only a single normal allele is observed. In this case, for discriminating some healthy cases from affected patients, Southern Blot analysis is required. To replace the tedious Southern Blotting and to unambiguously identify large alleles, Warner and coworkers (1996) developed a fluorescent PCR system, which is also applicable to other triplet repeat disorders.

The PCR assay, called triplet-repeat-primed-PCR (TP-PCR), uses three (P1, P3 and P4) instead of two flanking (P1 and P2) primers. The first primer is the fluorescently labeled flanking primer (P1) and binds to the region just next to the CTG repeat locus. The P3 tail primer has a random DNA sequence that does not contain any homology to known human sequences; also it has little or no self-complementarity and should not be complementary to GCA or TGC repeats. Finally, P4 (paired primer) contains the exact sequence of the tail primer (P3) at its 5' terminus and a five repeat of GCA or TGC at its 3' terminus.

In the early reaction, P4 binds randomly to different locations of the repeat region, resulting in different length-sized products. The P4 primer is always used in small quantity, compared to other primers (usually 10:1 molar ratio), which ensures the primer to be exhausted early in the reaction. The early products are amplified by the remaining P3 and P1 primers (Figure 1.3. and 1.4.).

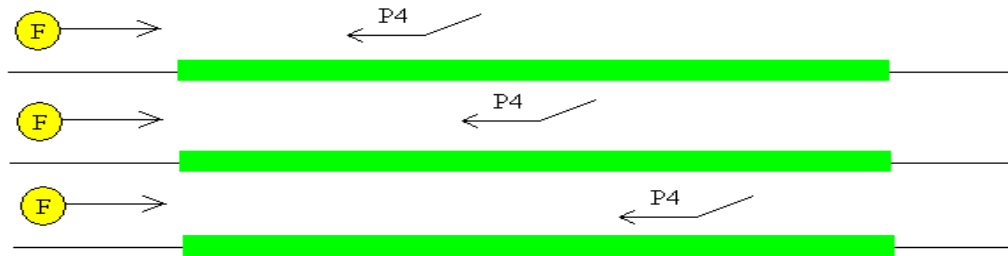


Figure 1.3. Binding of P4 primers at CTG repeat region: a mixture of products are formed with P4, binding at multiple sites of the repeat locus (adapted from Warner *et al.*, 1996)

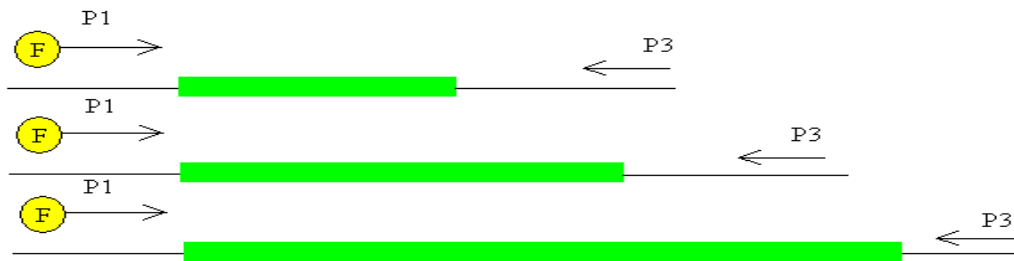


Figure 1.4. PCR amplification with P3 and P1 primers. The products are amplified from the ends by P3. For completing extension of the larger sized products within the PCR product mixture and conserving the representation of the longer products, a long extension time is required (adapted from Warner *et al.*, 1996).

Labeled PCR products of high CTG triplet repeats give a specific pattern on the automated laser fluorescence DNA sequencer. A characteristic ladder with a three base-pair periodicity is observed. In healthy individuals, two peaks are observed (Figure 1.5.). If the CTG repeat is not high (about 50), a single peak is observed (Figure 1.6.). If the repeat size is very large, no peak is observed and the height of the ladder peaks gradually diminish (Figure 1.7.). Thus, TP-PCR cannot give the exact number of the CTG repeats, but it can distinguish between the homozygous healthy individuals and the patients with a high repeat number (Warner *et al.*, 1996).

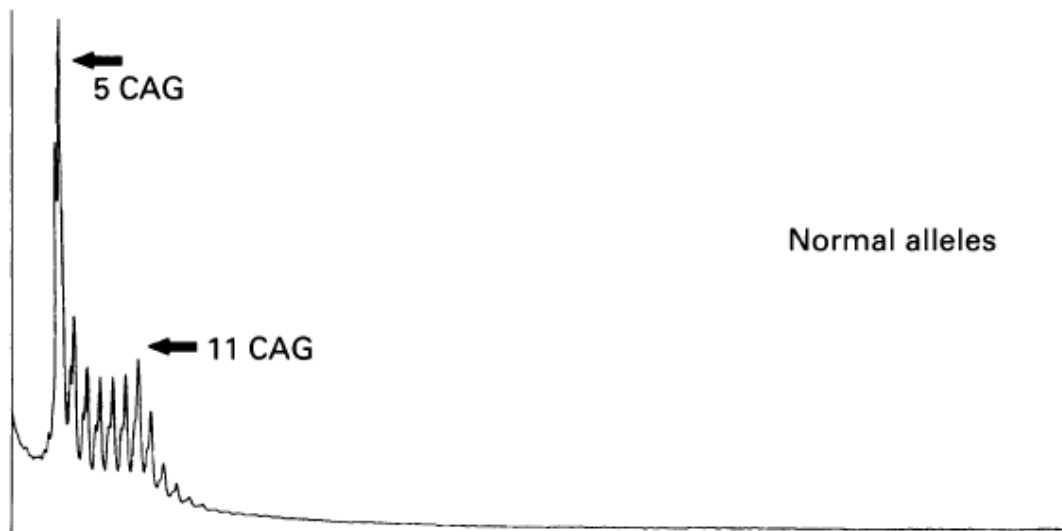


Figure 1.5. Fragment analysis result of a healthy individual: The peaks of both normal alleles are seen in healthy cases. Peaks stop to emerge after the large allele.

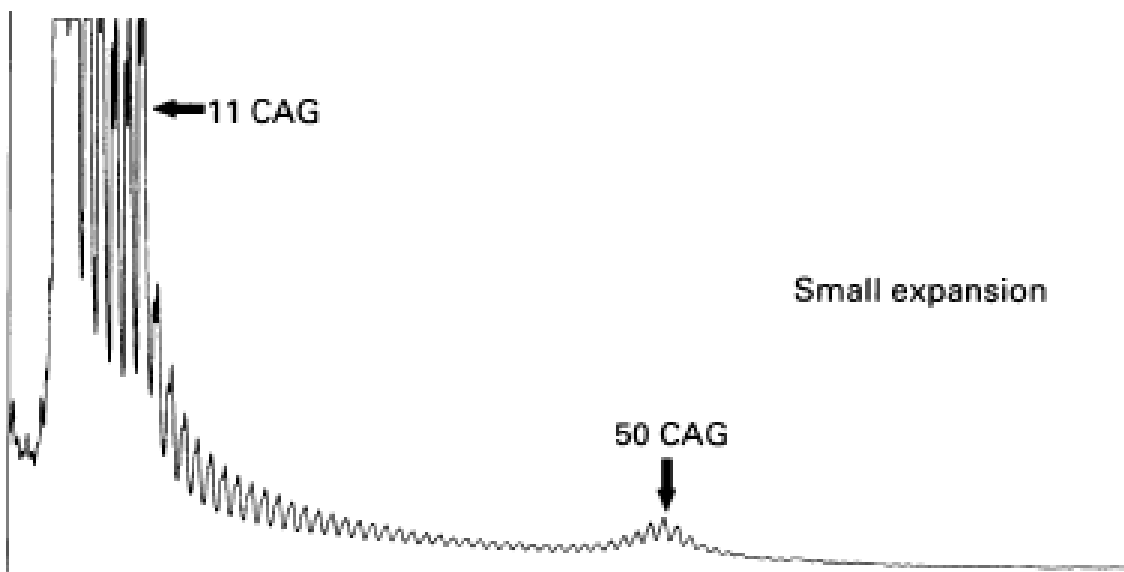


Figure 1.6. Fragment analysis result of small expansions. A ladder peak is observed in the small expansions up to ~50 CTG repeats with laser fluorescence automated sequencer.

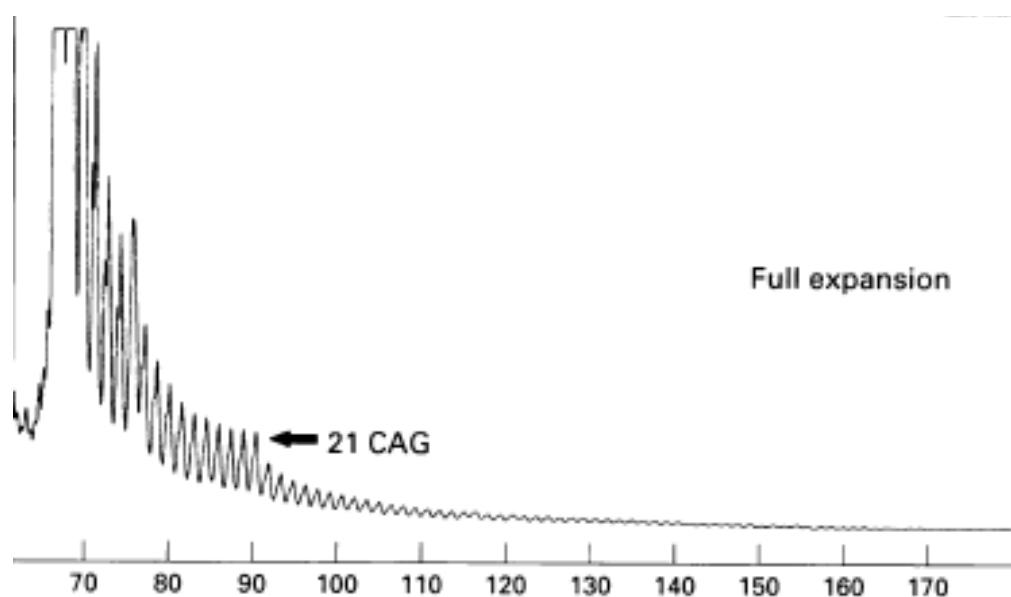


Figure 1.7. Fragment analysis result of a DM affected individual: In cases with large expansions only one of the allele can be observed, but the other gradually diminishing peaks show the presence of a high repeat allele.

1.1.7. DM in Turkey

The prevalence of DM1 in Turkey is unknown, and there are no epidemiological studies available to compare the disease prevalence to other countries. As a population with a high rate of consanguineous marriages, homozygous DM1 should not be rare in Turkey. Nevertheless, to the best of our knowledge, there are no professional web sites or civil organizations to collect, maintain, and update this information in Turkey. So far, there are only a few internationally cited papers on DM1 in Turkey. In these studies, most DM patients are diagnosed according to clinical and electrophysiological findings only, and two papers report Southern Blotting analysis results (Ertekin *et al.*, 2001; Kilic *et al.*, 2007; Kocaaga *et al.*, 2008 Akbas 2001; Erginel-Unaltuna N and Akbas F, 2004).

In the framework of this study, a new and powerful technique is established for the first time in Turkey, which can be routinely used in DM1 analysis.

1.2. Von Hippel-Lindau Disease

1.2.1. Background, History and Epidemiology of VHL Disease

Von Hippel-Lindau (VHL) disease is an autosomal dominant condition that is characterized by the development of various benign and malignant tumors in different parts of the organisms.

In 1864, several reports were published about the angiomas in the eye, however it was Eugen von Hippel, who first described the familial nature of retinal hemangioblastomas, naming them “*angiomatosis retinae*” in 1904. In 1927, Arvid Lindau described the angiomas, seen in the eye, as part of an “angiomatous lesion of the central nervous system” and found out that the disease was heritable. Thus, the disease was named “von Hippel-Lindau” by Charles Davinson in 1936. (Choyke *et al.*, 1995; Kim & Kaelin, 2004). The VHL tumor suppressor gene was isolated by positional cloning and shown to be on chromosome 3p25-26 by Latif and coworkers (1993).

The incidence of VHL is predicted to be 1 in 36.000 live births per year (Chan *et al.*, 2007). Different prevalences are seen and reported throughout Europe: the prevalence of VHL in East Anglia is estimated to be 1 in 53.000, with an incidence of 1 in 36.000, whereas, in Northwest England VHL prevalence is estimated to be 1 in 85.000 with an incidence of 1 in 45.500. In Freiburg, Germany, the estimated prevalence is 1 in 38.951 (Friedrich, 2001)

1.2.2. Clinical Features and Classification of VHL Disease

Different types of tumors and cysts are seen in VHL patients, such as spinal hemangioblastoma, cerebellar hemangioblastoma, pheochromocytoma, pancreatic and renal cysts, endolymphatic sac tumors, hemangiomas of the adrenals, liver and lungs and papillary cystadenoma of the epididymis or broad ligament (Figure 1.8.) (Friedrich, 2001).

VHL disease is classified into two types. Patients without pheochromocytomas are categorized as VHL Type 1, whereas, patients with pheochromocytomas are classified as VHL Type 2. Type 2 disease is also divided into three groups as Type 2A, Type 2B and Type 2C. Type 2A has retinal and central nervous system hemangioblastoma, but lacks renal cell carcinoma, whereas Type 2B has also renal cell carcinoma besides the retinal and central nervous system hemangioblastomas. Type 2C shows only pheochromocytomas, but lacks the other tumors seen in 2A and 2B (Shuin *et al.*, 2006).

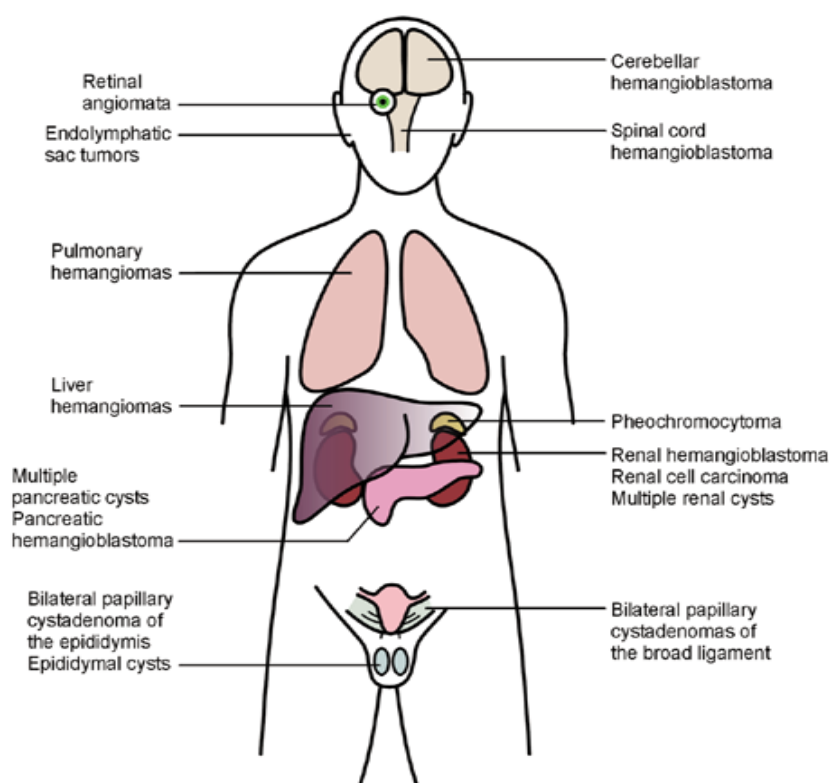


Figure 1.8. VHL Patient: Tumors seen at different locations (Friedrich, 2001).

The diagnosis of VHL is based on two different criteria:

- Having a family history of developing retinal angioma (RA), Central Nervous System (CNS) hemangioblastoma (HB), renal cell carcinoma (RCC) or pancreatic tumors or cysts and epididymal cystadenoma
- Having no family history, but developing HB or RA in combination with other tumors, like pheochromocytoma, epididymal cystadenoma, pancreatic tumors and RCC (Shuin *et al.*, 2006)

1.2.3. Molecular Genetics of VHL Disease

1.2.3.1. The VHL Gene Structure and Mutations. VHL is a tumor suppressor gene, with three exons and 639 nucleotides. It is widely expressed in fetal and adult tissues. During embryogenesis, the VHL protein (pVHL) is found in all connective tissues and mesenchymal cells, but the high level of expression is observed specifically in urogenital system, brain, spinal cord, sensory ganglia, eyes and bronchial epithelium (Richards, 1996). pVHL is conserved in organisms, ranging from *Caenorhabditis elegans* to humans. The first exon of the VHL gene carries two starting codons (codon 54) resulting in two different isoforms (Iliopoulos 1998; Shuin *et al.*, 2006; Kim and Kaelin, 2004).

The two-hit model of Knudson explains, how a tumor suppressor gene causes an autosomal dominant disease (Knudson, 1971). According to this model, in familial cases, the wild-type allele is hypermethylated or mutated somatically and loses its function. In sporadic cases, the first and the second hit occur somatically, rather than in the germline (Figure 1.9.). In different tissues, different inactivation types are observed, for instance 50 per cent of the sporadic RCC is mutated somatically whereas, hypermethylation is seen in only 10 per cent to 20 per cent of these cases. On the other hand, in sporadic hemangioblastomas, hypermethylation is never seen, somatically mutated alleles are observed in 30 per cent of HB. In pheochromocytomas, the situation is different; somatic mutations are observed very rarely. Pheochromocytomas result from haploinsufficiency of pVHL, when it is inactivated during a critical time in development; or a field defect, which is a defect, shared by all of the cells in the tissue, rather than an individual clone (Richards, 2001; Kaelin Jr, 2007a; Kim and Kaelin, 2004).

1.2.3.2. The VHL Protein. Resulting from two initiation sites, there are two different-sized VHL proteins, pVHL30 and pVHL19, having molecular weights of ~30 kDa and 19 kDa, respectively; pVHL30 contains 213 and pVHL19 160 amino acids. When one of the two isoforms are introduced into renal cells, with VHL disease, they are able to suppress the tumor growth. Most of the mutations seen in the disease, are at the C terminal of the protein up to residue 54, implying that, when a mutation occurs in the first 54 amino acids, the short form -pVHL19- can still be produced and used in the cell (Ohh and Kaelin Jr, 1999).

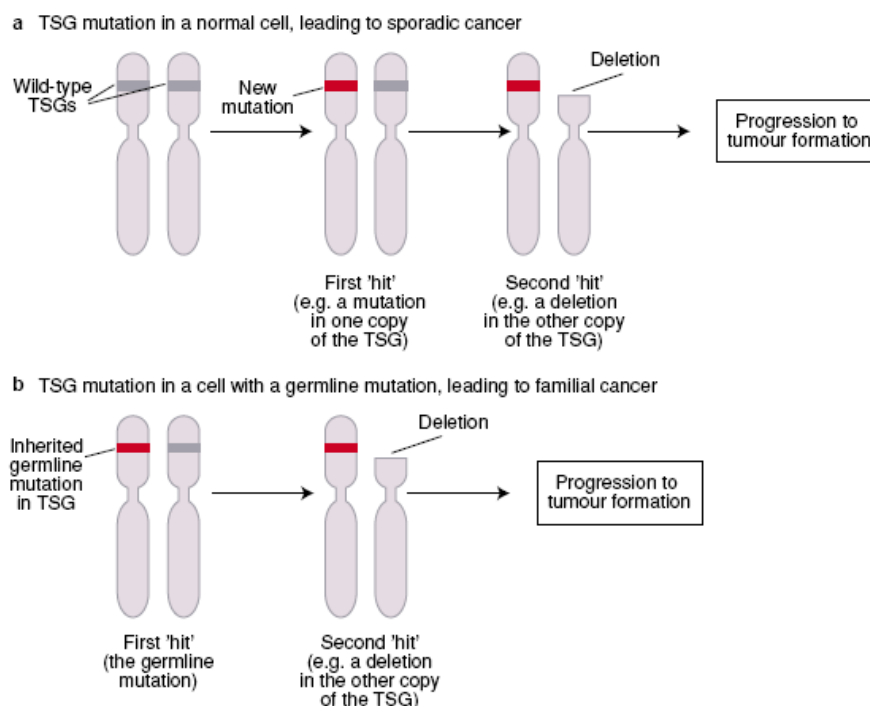


Figure 1.9. The general principle of Knudson's two-hit hypothesis, shows how a tumor suppressor gene (TSG) causes an autosomal dominant disease; a) in sporadic cases b) in familial cases (Richards, 2001)

The cellular location of pVHL30 was studied with immunohistochemistry and biochemical fractionation. pVHL30 is found to be usually in the cytoplasm and sometimes in the nucleus, even associated with the endoplasmic reticulum. (Iliopoulos *et al.* 1998, Ohh *et al.*, 1998; Pause *et al.*, 1997) The protein is thought to be shuttling back and forth between the nucleus and the cytoplasm to respond to particular signals. Recent findings suggest that exon 2 has a role in this shuttling. During its transport from the nucleus to the cytoplasm and back, pVHL30 also binds to its complex, carrying it along from the cytoplasm to the nucleus and back again (Bonicalzi *et al.*, 2001). Findings showed that, this movement of pVHL is mediated by Ras-related Nuclear Protein (Ran), and this reaction is ATP-dependent. pVHL19 does not have the N-terminal pentameric repeat, which is similar to a sequence of membrane protein, observed in *Trypanosoma brucei*. Similar with this finding, pVHL19 was not found to be associated with any membranes. There is no evidence, that pVHL19 also shuttles back and forth between the nucleus and the cytoplasm like its longer isoform (Ohh and Kaelin Jr., 1999; Groulx, et al., 2000).

Primary nucleotide or amino acid sequences of pVHL are not found to be in homology with any other proteins with known functions. Both of the isoforms of pVHL do not carry any domain with known enzymatic activity. To understand their activity, several labs studied the proteins that can bind to pVHL. pVHL seems more likely to be a multipurpose adaptor protein, which participates in several protein-protein interactions, having a role in regulating transcription in cellular processes (Leung and Ohh, 2002; Frew and Krek., 2008).

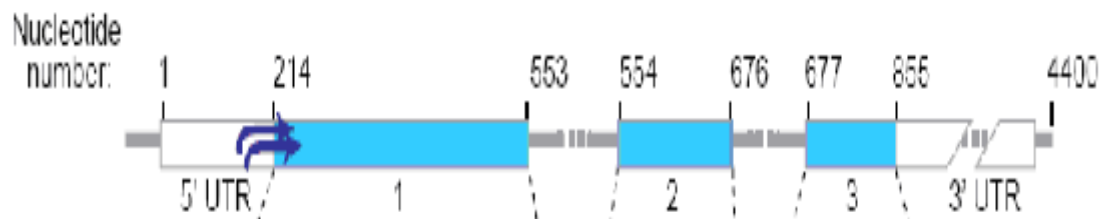
pVHL has two domains, called α and β , in the 3' or in the middle portion of the protein. The smaller α domain is translocated between amino acids 155 and 192, and has a role in binding proteins, such as Elongin C. It consists of three α -helices. Many missense mutations are seen in this region, especially in Arg 167. The amino acids, involved in forming the α -helices, have a role in stabilizing the α domain structure. The β -domain, having a role in binding target proteins, consists of a seven-stranded β -sandwich between amino acids 63 and 154 and an α -helix between the amino acids 193 and 204. (Figure 1.10.) (Stebbins *et al.*, 1999; Richards, 2001; Shuin *et al.*, 2006).

The promoter region of pVHL seems to contain GC-rich, but TATA and CCAAT-less regions. Sixty bp upstream of the first methionine is the putative Sp1-binding site, where transcription is initiated. (Kuzmin *et al.*, 1995) Several predicted binding sites for transcription factors are defined, but there is no information on how VHL expression is controlled. Part of the 3' UTR is found to be conserved in rodents, but only humans contain 11 Alu repeats (Renbaum, *et al.*, 1996; Richards, 2001).

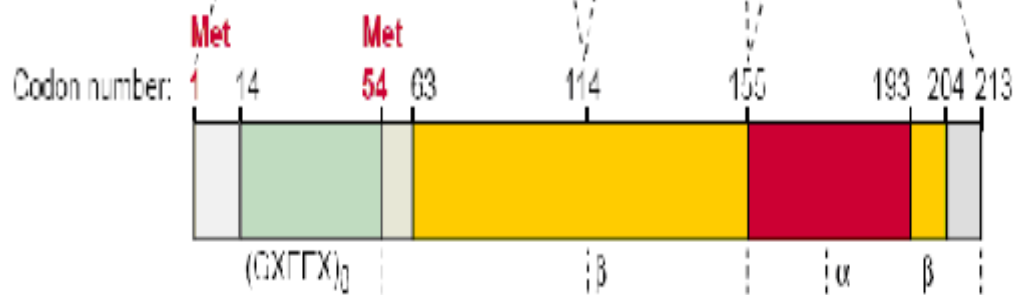
1.2.3.3. Function and Downtargets of the pVHL. Being a multipurpose adaptor protein, pVHL has several functions in the cell (Table 1.4.). However, the best known function of pVHL is being an adaptor protein of a multiprotein complex called VCB-Cul2 E3 ubiquitin ligase complex (also called VEC). Besides Cul-2 and Rbx1 (also known as ROC1 and Hrt1), this complex also contains Elongin C, which directly binds pVHL and Elongin B. Elongin B and C normally bind to each other and have a role in enhancing the rate of elongation by RNA polymerase II. Like Cul-1, Cul-2 is a member of the Cullin family, and they both have a similarity with the yeast protein Cdc53, which participates in

ubiquitination and degradation of proteins (Ohh and Kaelin Jr., 1999; Ivan and Kaelin Jr., 2001; Leung and Ohh, 2002;).

a VHL gene



b VHL protein



c Functional regions of VHL protein

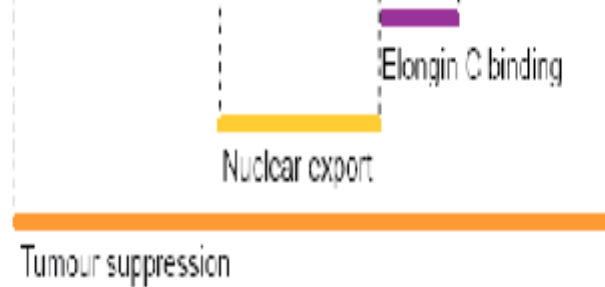


Figure 1.10. The VHL gene and its protein structure and function; $(GXEEEX)_8$ is the pentameric repeat region (Richards, 2001).

Table 1.4. Some of the known functions of the pVHL as an adaptor protein.

Complex formed by VHL	Target protein	Outcome
VCB-Cul2 E3 ubiquitin ligase	HIF (Hypoxia-inducible factor)	Control of hypoxia inducible genes like VEGF, GLUT-1 and erythropoietin
VCB-Cul2 E3 ubiquitin ligase	Rbp1 (Retinol binding protein 1)	Stress –induced transcription
Casein Kinase II (CKII) and caspase recruitment domain-containing-scaffold protein (CARD9)	Inhibitor of Bcl10-Malt1 protein.	Overexpression of Nuclear Factor KB (NF-KB) giving rise to increased survival
p53, p300, ATM	p53	Protection of p53 from degradation
Unknown	p400 (inhibitor of senescence)	Senescent cells observation
Atypical protein kinase C APKC	JunB	Inhibition of apoptosis
Collagen IV Or fibronectin	MMP-2, MMP-9 and MT1-MMP (metalloproteinases) Mechanism is not known	Tumor invasiveness and progression
Par-3-Par-6-aPKC complex	Unknown microtubule assembly proteins	Primary cilium loss
VCB-Cul2 E3 ubiquitin ligase	E-cadherin expression by HIF (Hypoxia-Inducible Factor) depending pathway	Tumor invasiveness
Unknown	Jade-1	Tumor suppressor protein

- The first target of the VEC protein complex is the Hypoxia-inducible factor (HIF), which is a regulator of oxygen-dependent gene expression. HIF is a heterodimeric transcriptional factor that consists of α and β structural domains. With the help of these domains, HIF is able to bind to DNA at particular sequences. When there is oxygen in the cell, the HIF- α subunit is hydroxylated by prolyl hydroxylases on two proline residues at positions 402 and 564. Hydroxylation makes the subunit suitable for pVHL to bind, and marks it for degradation. Under hypoxic condition, the hydroxylation of HIF- α subunit is inhibited. It runs away from degradation, translocates to the nucleus and binds with its other subunit. The β subunit is also called aryl hydrocarbon receptor nuclear translocator (HIF-1 β). This mechanism controls the transcription of many hypoxia-inducible genes, like vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT-1) and erythropoietin. Studies showed that, when cells lack pVHL, HIF- α cannot be degraded in the presence of oxygen and these genes are transcribed excessively (Masson et al, 2001, Leung and Ohh, 2002;).
- Another ubiquitin-ligase activity of the pVHL protein, using the same complex, is ubiquitination of Rbp1, which is the large subunit of the RNA polymerase II complex. Like the recognition of HIF- α , Rbp1 should also be hydroxylated from its proline residues by the prolyl-hydroxylase-domain-containing enzymes. Recent findings showed that Rbp1 ubiquitination is triggered by ultra-violet light (Kuznetsova et al., 2003). Oxidative stress is another factor that causes ubiquitination of Rbp1, however instead of being degraded, it is just recruited to chromatin (Mikhaylova *et al.*, 2008). The direct consequences of these ubiquitination reactions of Rbp1 are not known. However, a theory states, that the transcriptional responses under certain circumstances, may be regulated by pVHL, by inhibiting an important factor of basal transcriptional machinery (Frew and Krek, 2008).
- pVHL also acts on other transcription factors like nuclear factor κ B (NF- κ B). NF- κ B is an important transcription factor, that regulates several cellular processes, like inflammatory and immune responses, apoptosis and proliferation. For inhibiting this pathway, pVHL uses different proteins, other than its ubiquitin-ligase complex. Two proteins, caspase-recruitment-domain-containing-scaffold-protein (CARD9) and

casein kinase II (CKII), are linked to each other by binding pVHL (Yang *et al.*, 2007). CK II phosphorylates and inhibits the CARD9 activity. Normally CARD9 activates a complex (Bcl10-Malt1) to stimulate the inhibitor of NF- κ B kinase. In other words, when pVHL is absent, CARD9 cannot be inhibited and as a result NF- κ B cannot be inhibited, too. This explains why elevated NF- κ B activity is observed in the clear-cell renal cell carcinoma (ccRCC) and why the ccRCC is resistant to apoptosis-inducing therapies (Frew and Krek, 2008).

- Another important study showed that p53, which is one of the best known and most common proteins to play a role in cancer, is affected by pVHL activity. p53 is a tumor suppressor protein and plays a role in cell cycle arrest and apoptosis. pVHL is found to be activating the transcription activity of p53 by linking it to some other p53-activating proteins, like ATM and p300. pVHL also suppresses degradation of p53 (Roe *et al.*, 2006; Frew and Krek, 2008).
- An interesting study by Young and coworkers (2008) showed, that in mouse embryo fibroblasts, deletion of VHL results in reduction of p400 protein, which acts like an inhibitor in senescence pathway. After VHL deletion, senescent cells were seen in mouse kidneys. Senescence is a barrier in tumor formation, therefore a new theory which arises with these finding implies, that secondary mutations should occur in VHL patients, to overcome this barrier for tumor formation and proliferation of pVHL deficient cells (Frew and Krek, 2008).
- The last role of pVHL on transcriptional regulatory factors, is on JunB. The exact pathway is unknown, but somehow, by binding and inhibiting atypical protein kinase C (aPKC), pVHL regulates JunB activity (Lee *et al.*, 2005; Okuda *et al.*, 2001; Frew and Krek, 2008).

pVHL has also roles, independent from transcription regulatory functions. It also functions in regulation of extracellular matrix (ECM), primary cilium formation and microtubule cytoskeleton.

- ECM assembly is impaired in all types of VHL disease. The exact mechanism is unknown, but reduced fibronectin staining is observed in ccRCC cases. Recent findings showed that pVHL binds directly to collagen IV $\alpha 2$ chain (especially on the N terminal tail that projects from ER to cytosol), and it also indirectly binds to fibronectin. These interactions are important for ECM formation. Also matrix metalloproteinases (MMPs), which are peptidases, that are able to degrade all kinds of ECM proteins, are shown to be regulated by pVHL. Especially, expressions of MMP-2, MMP-9 and MT1-MMP (membrane type 1-MMP) are up-regulated, when pVHL is absent. This would also result in tumor invasiveness and progression. Recent findings demonstrate that fibronectin deposition is also regulated by pVHL (Tang 2006, Nyhan et al., 2008).
- Cilia are the structures that have longitudinal strands of microtubules that project from the surface of polarized epithelial cells. In kidneys, primary cilia are the non-motile organelles that function in flow-sensing, as well as growth-factor-signal-transduction, having a negative role in proliferation. (Kuehn et al., 2007). In Type 1 and Type 2A VHL patients, loss of primary cilium is observed. The exact regulatory mechanisms between pVHL and the primary cilium are not known. On the other hand, studies imply that pVHL regulates primary cilium by having a role in microtubule assembly. A study showed that pVHL may do this by interacting with the Par-3-Par-6-aPKC complex, which is a component of intraflagellar transport complexes (Schermer et al., 2006). An important protein that regulates pVHL is glycogen synthase kinase 3 β (GSK3 β). GSK3 β is found to bind, phosphorylate and inhibit pVHL activity on regulation of microtubules. However, GSK3 β also regulates the microtubule assembly. Therefore, when both of them are active, GSK3 β inhibits pVHL, but when GSK3 β is inactive, it is pVHL, which regulates the microtubule and primary cilium maintenance. (Hergovich et al., 2006) As expected, GSK3 β is inactivated in cysts of the VHL disease patients (Nyhan et al., 2008; Kaelin Jr., 2007 b).
- Apart from its functions listed above and depicted in Figure 1.11, pVHL also functions in cell-cell junctions by acting on E-cadherin. Loss of E-cadherin is associated with loss of cell-cell adhesion capabilities. Another finding is that in RCC

cell lines, E-cadherin expression is decreased. pVHL is found to be regulating the expression of E-cadherin by HIF- α depending pathway. When pVHL expression is lost in the cell, HIF- α is stabilized and transactivates the repressors of E-cadherin, which acts on the E2 boxes on the E-cadherin promoter. In human RCC cells, when the E-cadherin is knocked down, the invasive abilities of these cells increase (Esteban *et al.*, 2006; Evans *et al.*, 2007; Nyhan *et al.*, 2008).

- Yeast-two-hybrid studies identified a novel protein, called Jade-1, which is thought to be a renal cancer tumor suppressor protein, involved in apoptosis. Jade-1 is found to be bound and stabilized by pVHL (Zhou *et al.*, 2002; Zhou *et al.*, 2004; Nyhan *et al.*, 2008)

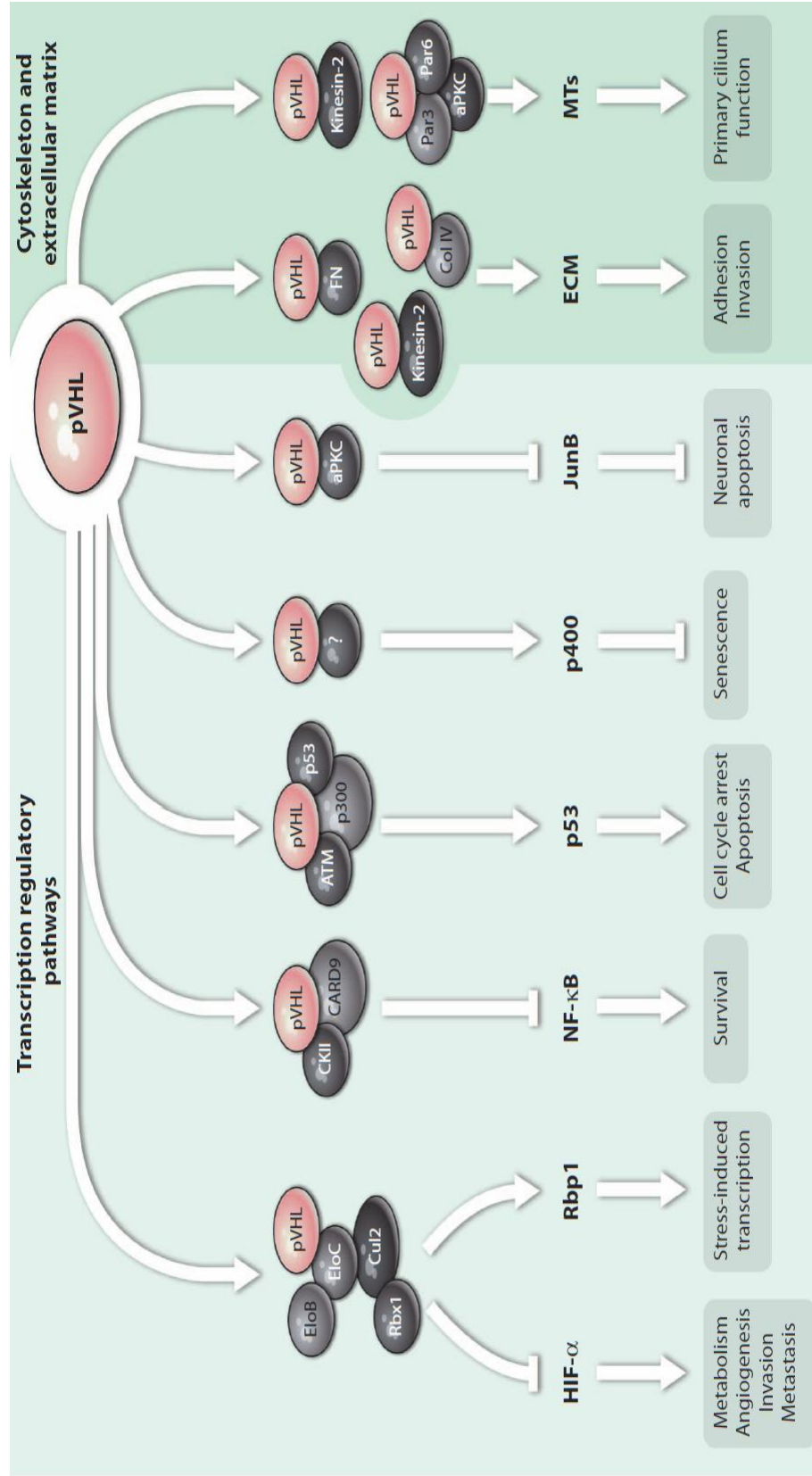


Figure 1.11. Summary of some of the known functions of the protein complexes formed by binding of pVHL; the left part shows the roles of pVHL by acting on transcription, the right part shows the roles in cytoskeleton and ECM maintenance (Frew and Krek, 2008)

1.2.4. Molecular Basis of VHL

In familial VHL disease, more than 300 mutations have been described so far. The estimated new mutation rate is between 3 per cent and 20 per cent. The mutation types, seen in different types of the disease, show correlations: VHL deletions and truncating mutations are usually found in Type 1 families, whereas missense mutations, resulting in amino acid substitutions, are more common in Type 2 patients (Zbar *et al.*, 1996; Sims, 2001; Kim and Kaelin, 2004).

Studies on affected families provide information about the spectrum of germline VHL mutations. Netherlands, China, North America and Japan conducted large-scale studies on many families to provide mutation status (Houwelingen *et al.*, 2005, Zbar *et al.*, 1996; Zhang *et al.*, 2007). The overall mutation frequencies in different exons are shown in Figure 1.12. Most mutations identified are on exon 3, primarily at the elongin binding site. In codons 1-54 and 201-213 no germline mutations are found.

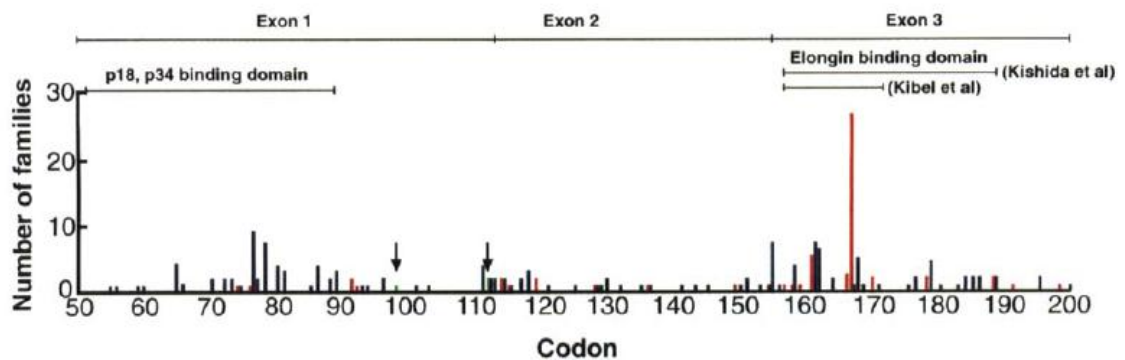


Figure 1.12. Germline mutations observed in North America, Japan and Europe: Black bars show Type 1 VHL germline mutations, whereas, red bars show Type 2 VHL germline mutations. Arrows indicate positions of Type 2A germline mutations (Zbar *et al.*, 2006).

1.2.5. VHL Disease in Turkey

There are only a few studies on VHL disease, conducted in Turkey. In 1991, Bilge and coworkers, in the first paper on VHL disease in Turkey, clinically examined a large family, but genetic testing was not applied. The first molecular study was performed in 2007 by Karabagli and coworkers. They applied PCR and sequence analysis on possible VHL patients, however, they could not report any mutations. In 2007, Akcaglar and coworkers, used karyotype analysis, and successfully detected a few deletions at the VHL gene region on chromosome 3. As this was unsatisfactory for direct diagnosis of the disease, the paper concluded, that molecular genetic testing should be introduced. This was the last study published on VHL disease in Turkey.

In the framework of this thesis, a large kindred from Erzincan, several members of whom are affected with VHL Disease, was investigated for mutations in the VHL gene.

2. PURPOSE

In the framework of this thesis, the molecular bases of two different autosomal dominant syndromes, Myotonic Dystrophy Type 1 and von Hippel-Lindau Disease were investigated. The molecular analysis of these two disorders were largely neglected in Turkey, mainly due to their complicated molecular analyses.

Myotonic dystrophies are classified into two types (DM1 and DM2), and the more common Type 1 is caused by a high CTG repeat expansion in the 3' untranslated region of the DMPK gene. As DM1 and DM2 show clinical similarities, the molecular analysis of DM carries great importance, not only for the differential diagnosis of DM1 and DM2, but also for distinguishing DM from other neurological disorders, which often have overlapping clinical features. Thus, there is a huge demand from medical schools all over Turkey for the molecular diagnosis of DM. DM1 starts to manifest its characteristics when the repeats expand over the threshold of 50 to 100 CTGs. Since this high CG content is hard to amplify with conventional PCR techniques, the molecular diagnosis of DM is usually carried out with Southern Blotting, which is time-consuming and expensive, thus not appropriate for routine molecular diagnosis.

Von Hippel-Lindau disease is an inherited cancer syndrome, caused by mutations in the VHL tumor suppressor gene. Large family studies reveal interesting results in gaining insights into the tumor diversity in patients. Many countries conducted studies on several families and constructed a mutation frequency database for VHL. Here we present a large Turkish kindred from Erzincan, several members of whom have a variety of benign and malignant tumors.

In the framework of this thesis we had two main goals:

- Establish the Triplet-Repeat-Primed-PCR technique in our laboratory and apply this technique to Turkish DM patients.
- Identify the disease-causing mutation in the large kindred with VHL disease.

3. MATERIALS

3.1. Subjects

3.1.1. Myotonic Dystrophy

Blood samples of DM patients were provided by the Neurology Departments of the following Medical Schools: İstanbul University, İstanbul and Cerrahpaşa Medical Schools, İstanbul; Çukurova University, Adana; Dokuz Eylül University, İzmir; Gaziosmanpaşa University, Tokat; Bakırköy Research Hospital for Psychiatry and Neurology, İstanbul. A total number of 117 DM patients, all of whom originated from Turkey, were investigated in the framework of this thesis. Informed consent was obtained from all individuals.

3.1.2. Von Hippel-Lindau Disease

Blood sample of the VHL family was provided by Assoc. Prof. Doğan Başak, MD, İstanbul. Twenty-six VHL patients from the family were genotyped. Informed consent was obtained.

3.2. Oligonucleotide Primers

3.2.1. Myotonic Dystrophy

Sequences of the primers, used in the amplification of the DMPK gene CTG repeat region, are given in Table 3.1.; they were purchased from Genova, İstanbul.

Table 3.1. Oligonucleotide primer pairs used in the PCR of the DMPK gene.

Primer Name	Primer Sequence
DM-P1 (5' Fam)	Forward: 5'-AGAAAGAAATGGTCTGTGATC-3'
TAILCTG	Reverse: 5'-TACGCATCCCAGTTTGAGACGTGCTGCTGCTGCTGCTGCTGC-3'
TAILR	Reverse: 5'-TACGCATCCCAGTTTGAGACG-3'

3.2.2. Von Hippel-Lindau Disease

Sequences of the primers, used in the amplification of the VHL gene are given in Table 3.2.; these primers were purchased from Iontek and Elips, Istanbul

Table 3.2. Oligonucleotide primer pairs used in the PCR of the VHL gene

Primer Name	Primer Sequence
VHLE1F1	Forward: 5'-GGTCTGGATCGCGGAGGGA-3'
VHLE1R1	Reverse: 5'-GCCCGGCCTCCATCTCCT-3'
VHLE1F2	Forward: 5'-AGTCGGGCGCCGAGGAG-3'
VHLE1R2	Reverse: 5'-CCGTCTGAAGTTGAGCCATAC-3'
VHLE1F3	Forward: 5'-CCCAGGTCATCTTCTGCAAT-3'
VHLE1R3	Reverse: 5'-CTGCTGGGTCGGGCCTAAG-3'
VHLE2F	Forward: 5'-GTGGCTCTTTAACAACCTTTGC-3'
VHLE2R	Reverse: 5'-CCTGTACTTACCACAACAACCTTATC-3'
VHLE3F1	Forward: 5'-CACTGAGGATTTGGTTTTTGC-3'
VHLE3R1	Reverse: 5'-TCCAGGTCTTTCTGCACATTT-3'
VHLE3F2	Forward: 5'-GACATCGTCAGGTCGCTCTA-3'
VHLE3R2	Reverse: 5'-TCAAAAGCTGAGATGAACAGTG-3'

3.3. Enzymes

Taq DNA Polymerase : 5U/ μ l, Fermentas, Lithuania

3.4. Chemicals

All chemicals used in this study, were purchased from Merck (Germany), Sigma (Germany) and Amresco (USA), unless stated otherwise in the text.

3.5. Buffers and Solutions

3.5.1. DNA Extraction

Lysis Buffer	:	155 mM NH_4Cl 10 mM KHCO_3 1 mM Na_2EDTA (pH 7.4)
Nuclease Buffer	:	10 mM Tris-HCl (pH 8.0) 400 mM NaCl 2 mM Na_2EDTA (pH 7.4)
Sodiumdodecylsulfate (SDS)	:	10 per cent SDS (w/v) (pH 7.2)
Proteinase K	:	20 mg/ml in dH_2O , Promega, USA
Sodium Chloride (NaCl)	:	2.5 M saturated stock solution
Absolute Ethanol (EtOH)	:	Riedel-de Haën, Germany
TE Buffer	:	20 mM Tris-HCl (pH8.0) 1 mM Na_2EDTA (pH8.0)

3.5.2. Polymerase Chain Reaction (PCR)

3.5.2.1. Myotonic Dystrophy.

CM103 2X Custom PCR Master Mix :	1.25 units Taq DNA polymerase 67 mM Tris-HCl (pH 8.0 at 25° C) 16 mM (NH ₄) ₂ SO ₄ 3.7 mM MgCl ₂ 0.085 mg/ml BSA 6.7 µM EDTA 0.75 M of each of dATP, dCTP, dGTP and dTTP Thermo Scientific, USA
Betaine	5 M, Sigma, Germany

3.5.2.2. Von Hippel-Lindau Disease.

10X Taq Buffer	:	Proprietary formulation (MgCl ₂ free) Fermentas, Lithuania
Magnesium Chloride (MgCl ₂)	:	25 mM, Fermentas, Lithuania
Deoxyribonucleotides (dNTPs)	:	100 mM of each dNTP Fermentas, Lithuania
DMSO	:	Proprietary formulation Qiagen, Germany

3.5.3. Agarose Gel Electrophoresis

10X TBE Buffer	:	0.89 M Tris-Base 0.89 M Boric Acid 20 mM Na ₂ EDTA (pH 8.3)
Ethidium Bromide (EtBr)	:	10 mg/ml
Agarose	:	Agarose Basica Le, Prona, EU
6X Loading Dye	:	10 mM Tris-HCl (pH 7.6) 0.03 per cent Bromophenol Blue 0.03 per cent Xylene Cyanol FF 60 per cent glycerol 60 mM EDTA Fermentas, Lithuania
DNA Ladder	:	100 base pair (bp) Fermentas, Lithuania

3.6. Kits

PCR Purification Kit	:	Qiagen, Germany
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3.7. Equipments

Autoclaves	:	Model MAC-601, Eyela, Japan Model ASB260T, Astell, UK
Balances	:	440-47N, Kern, France

Centrifuges	:	5415, Eppendorf, Germany Universal 16R, Hettich, Germany Allegra X22-R, Beckman Coulter, USA
Deep Freezers	:	2021D (-20 ⁰ C), Arçelik, Turkey Sanyo (-70 ⁰ C), Sanyo, Japan
DNA Isolation Instrument	:	MagNA Pure Compact Instrument Version 1.0 Roche, Germany
Documentation System	:	GelDoc Documentation System BIO-RAD, USA
Electrophoretic Equipments	:	i-MyRun-N, CosmoBio., Ltd. Japan Minicell Primo E320, Thermo, USA Mini Sub Cell GT, BIO-RAD, USA Wide Sub Cell GT BIO-RAD, USA Sequi-Gen Cell, BIO-RAD, USA
Heat Block	:	Thermostat Heater 5320 Eppendorf, Germany
Magnetic Stirrers	:	Chiltern Hotplate Magnetic Stirrer HS31, UK
Ovens	:	MD 554, Microwave Oven Arçelik, Turkey EN 400 (37°C), Nuve, Turkey BD53 (56°C), Binder, Germany
Water bath	:	1083 Shaking Water Bath, GFL, Germany

Power Suppliers	:	EC 135-90 Thermo, USA Model 200, BRL, USA
Refrigerators	:	4250T, Arçelik, Turkey
Spectrophotometer	:	NanoDrop ND-1000, Thermo, USA
Thermocyclers	:	TC 312, Techne, UK Techgene, Progene, UK Techne, Progene, UK Touchgene Gradient, Progene, UK
Vortex	:	Fisons WhirliMixer, UK Reax Top, Heidolph, Germany
Water Purification	:	WaTech Water Technologies, Turkey

4. METHODS

4.1. DNA Extraction from White Blood Cells

4.1.1. Sodium Chloride Extraction

Ten milliliters of peripheral blood sample were collected from each individual into EDTA-containing tubes to prevent coagulation; the blood was stored at 4°C until extraction.

Blood samples were transferred into 50 ml sterile Falcon tubes, and 30 ml of ice-cold lysis buffer was added. Then they were incubated for 15 minutes at 4°C to lyse the cells. To precipitate the nuclei of the lysed cells, after the incubation, the samples were centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 10 ml of lysis buffer by vortexing. The samples were centrifuged for a second time at 5000 rpm for 10 minutes at 4°C. The supernatant was again discarded. The last suspension and centrifugation steps were repeated, if the precipitate was dark reddish, indicating contamination with red blood cell debris. Then, the supernatant was discarded. To lyse the nuclear envelope, three ml nuclei lysis buffer was added to the remaining nuclear pellet and vortexed. Then, 30 µl Proteinase K (20 mg/ml) and 50 µl 10 per cent SDS were added and vortexed to mix the contents. The samples were incubated either at 56°C for three hours, or at 37°C overnight, to degrade the proteins.

When the incubation was complete, 10 ml 2.5 M NaCl was added to the samples, the tubes were shaken and vortexed very well before centrifugation at 5000 rpm for 40 minutes at room temperature. The supernatant was transferred into a new 50 ml Falcon tube very carefully; to precipitate the DNA, two volumes of ice-cold absolute ethanol were added slowly to the supernatant. The tubes were inverted gently for several times until the DNA precipitate became visible. With the help of a micropipette tip, the DNA was transferred into an Eppendorf tube which was kept open for ethanol to evaporate. DNA was dissolved

in 100-150 μ l TE buffer and kept at room temperature for overnight. The final storage was at 4⁰C.

If there was no visible DNA precipitation after the addition of ethanol, the tubes were kept at -70⁰C for at least two hours. After the samples were completely thawed, they were centrifuged at 14000 rpm for 30 minutes at 18⁰C. After centrifugation, the supernatant was discarded and the ethanol evaporated. The DNA pellet was dissolved in 80-100 μ l TE buffer.

4.1.2. Extraction by MagNA Pure Compact Instrument

MagNA Pure Compact Instrument is a robotic nucleic acid extraction system that uses the specifically designed MagNA Pure Compact Nucleic Acid Isolation Kit. The extraction principle of this system is based on the affinity of magnetic beads to the DNA. After lysing the cells and the nuclear envelope, the DNA binds to the magnetic beads, and is then removed from the beads in the elution step. There are two different isolation kits, for small and large volumes, they use 500 μ l and 1 ml of blood, respectively. From the small volume kit, 100 μ l of DNA is eluted, and from the large volume kit 200 μ l DNA is obtained.

4.2. Analysis of the Extracted DNA Samples

4.2.1. Agarose Gel Electrophoresis

The quantity and quality of the DNA samples were evaluated first by running the samples on a one per cent agarose gel. One gram of agarose was dissolved in 100 ml of 0.5X TBE buffer and boiled to prepare the agarose gel. Ethidium bromide, intercalating between the stacked bases of DNA, enables to visualize the DNA under UV light. Ethidium bromide is used in a final concentration of 0.5 mg/ml and was added to the agarose after cooling it to 50⁰C. The solution was poured onto a gel plate and the combs were inserted. After the gel solidified, the combs were removed and the plate was put into an electrophoresis tank, containing 0.5X TBE buffer. Loading dye (6X) was mixed with

each DNA sample to a final concentration of 1X. The samples were loaded into the slots of the gel with a micropipette. The gel was run at 150 V for 10-15 minutes; DNA bands were visualized under UV light and recorded; the quality and quantity of the DNA samples were determined by comparing their intensity with known DNA samples on the gel.

4.2.2. Spectrophotometric Measurement of DNA

The exact amount and concentration of the DNA sample is measured by spectrophotometry using a NanoDrop Spectrophotometer. The light source of the spectrophotometer is provided by a pulsed xenon flash lamp. A PC-based software controls the instrument, and the data is logged in an archive file on the PC. The software calculates the concentration of the DNA and also its purity by the ratio of absorbance at 260 to 280 nm.

1 μ l of DNA was pipetted into the end of the receiving fiber optic cable of the NanoDrop Spectrophotometer, and the source fiber optic cable was then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. Optical density of the DNA was measured at 260 nm (OD₂₆₀).

4.3. Polymerase Chain Reaction

Fifty to hundred ng of genomic DNA was used as template in all PCR mixtures. After addition of DNA and all PCR components, the volume of each PCR mixture was completed to 25-27 μ l.

After the PCR amplification was terminated, the samples were run in a two per cent agarose gel, to check the quality and quantity of the amplified sample. For this purpose, one μ l of 6X loading dye was mixed with five μ l of each PCR product and run at 175 volt for 15-20 minutes. The 100 bp-ladder was used as the DNA weight marker on the gel. The gel was visualized and recorded under UV light as described in 4.3.1.

4.3.1. TP-PCR and F-PCR Protocol for DMPK Gene CTG Repeat Region

To amplify the CTG region of the DMPK gene, two different PCR protocols were used:

- DM-Triplet-Repeat-Primed-PCR (DMTP) and
- DM Flanking PCR (DMF).

4.3.1.1. DMTP. The following PCR conditions were applied with the PCR components shown in Table 4.1.:

Initial Denaturation	:	94 ⁰ C	4 minutes	
Denaturation	:	94 ⁰ C	1 minute	} 10 cycles
Annealing	:	66 ⁰ C	1 minute	
Extension	:	72 ⁰ C	6 minute	
Denaturation	:	94 ⁰ C	1 minute	} 20 cycles
Annealing	:	66 ⁰ C	1 minute	
Extension	:	72 ⁰ C	4 minute	
Final Extension	:	60 ⁰ C	1 hour 30 minutes	

Table 4.1. DMTP-PCR components

Components	Volume (μl)	[Stock]	[End]
CM103 custom PCR master mix	12.5	2X	1X
DMP1-Fam	2.5	10 μM	1 μM
Tail CTG	0.5	10 μM	0.2 μM
Tail R	2.5	10 μM	1 μM
Betaine	6.25	5M	1.25 M
Water	0.25		

4.3.1.2. DMF. For flanking PCR, the following PCR conditions were applied with the PCR components shown in Table 4.2.:

Initial Denaturation	:	94 ⁰ C	4 minutes		
Denaturation	:	94 ⁰ C	1 minute	}	5 cycles
Annealing	:	66 ⁰ C	1 minute		
Extension	:	72 ⁰ C	5 minute		
Denaturation	:	94 ⁰ C	1 minute	}	5 cycles
Annealing	:	64 ⁰ C	1 minute		
Extension	:	72 ⁰ C	5 minute		
Denaturation	:	94 ⁰ C	1 minute	}	5 cycles
Annealing	:	62 ⁰ C	1 minute		
Extension	:	72 ⁰ C	5 minute		
Denaturation	:	94 ⁰ C	1 minute	}	18 cycles
Annealing	:	60 ⁰ C	1 minute		
Extension	:	72 ⁰ C	5 minute		
Final Extension	:	72 ⁰ C	5 minutes		

Table 4.2. DMF-PCR components

Components	Volume (μl)	[Stock]	[End]
CM103 custom PCR master mix	12.5	2X	1X
DMP1-Fam	2	10 μM	0.8 μM
DMP2	2	10 μM	0.8 μM
Betaine	6.25	5M	1.25 M
Water	0.25		

4.3.2. PCR Protocols for the VHL Gene

To find the mutation of the family, three exons of the VHL gene were amplified prior to sequencing. As some exons are too large to be sequenced, they were analyzed separately. Exon 3 was amplified in two different reactions. Distinct annealing temperatures were used (Table 4.3). Touchdown PCR was applied, starting from five degree higher than the actual annealing temperature and lowering one degree for every five cycles.

Initial Denaturation	:	94 ⁰ C	5 minutes	
Denaturation	:	94 ⁰ C	40 seconds	} 6 X 5 cycles
Annealing	:		40 seconds	
Extension	:	72 ⁰ C	40 seconds	
Final Extension	:	72 ⁰ C	1.30 minute	

Table 4.3. Annealing temperatures of the VHL PCR assays

Exon parts	Annealing temperatures
Exon 2 (E2)	60
5' end Exon 3 part 1 (E3P1)	55
3' end Exon 3 part 2 (E3P2)	55

4.4. Determination of Fragment Sizes by GeneScan Analysis

To identify CTG repeat genotypes in the DMPK gene, all samples were subjected to GeneScan Analysis (ABI PRISM[®] 310 or ABI PRISM[®] 3130 XL Genetic Analyzer, Refgen Biyoteknoloji, Ankara). GeneScan system is a method of capillary electrophoresis, that is used to determine fragment sizes. The GeneScan software compares the length of PCR fragments with its size marker, draws a standard curve, and calculates the fragment sizes according to this standard curve.

Table 4.4. VHL PCR components for different reactions

		VOLUMES (μ l)			
Components	[Stock]	E2	E3P1	E3P2	[End]
MgCl₂	25 mM	1	1.5	1.5	1 – 2.5 mM
DNTP	10 mM	0.7	0.7	0.7	0.7 mM
Forward Primer	10 μ M	2.5	2.5	2.5	0.5 μ M
Reverse Primer	10 μ M	2.5	2.5	2.5	0.5 μ M
Buffer	10X	2.5	2.5	2.5	1X
DMSO	100 per cent	1	-	-	1.5 per cent-4 per cent
DNA Polymerase	5 units/ μ l	0.2	0.2	0.2	0.04 units/ μ l

To determine PCR fragment sizes by GeneScan Analysis, PCR products are labeled by a fluorescent dye. For this purpose, a 5' FAM-labeled forward primer was used to amplify the CTG repeat region in the DMPK gene. For every run, positive, negative samples and a sample without DNA were analyzed, to see if there were any differences in the standards of the Genescan analyzer.

Before running the samples on the ABI PRISM[®] 3130 XL Genetic Analyzer, the samples were treated with heat or formamide for denaturation. For one μ l of PCR product, 9.8 μ l of formamide were used. Also 0.5 μ l of size standard was added to this mixture. The samples were run at 1.5kV with 15 seconds injection time. The injection voltage is 1.6 kV. A 500 bp size standard is used in all analyses.

If the samples were run on the ABI PRISM[®] 310 Genetic Analyzer, the resulting data were analyzed by the Chromas. If the samples were run on ABI PRISM[®] 3130 XL Genetic Analyzer, the ABI PRISM peak scanner was used to analyze the results.

4.5. DNA Sequencing

4.5.1. Purification and Preparation of PCR Samples Prior to DNA Sequencing

Prior to DNA sequencing, the PCR products were purified from excess nucleotides, primers, polymerase and salts with the QIAquick PCR Purification Kit by following the instructions of the manufacturer. Then, the purified PCR samples were run in two per cent agarose gels to visualize the quality and quantity of the purification. The samples were then referred either to Iontek Ltd., Istanbul, or to Refgen Biyoteknoloji, Ankara for further analysis.

5. RESULTS

5.1. Myotonic Dystrophy

5.1.1. Distribution and Epidemiology

One hundred seventeen Turkish patients, with a probable clinical diagnosis of DM, were screened in the framework of this study. These included 21 DM1 families with 63 members and 54 isolated cases. Out of 117 patients, 67 were found to be DM1-positive and 50 patients were DM1-negative (Figure 5.1.). Of 67 DM1-positive samples, 27 were isolated cases (23 per cent) and 40 belonged to 15 families (34 per cent) (Figure 5.2. and Figure 5.3.).

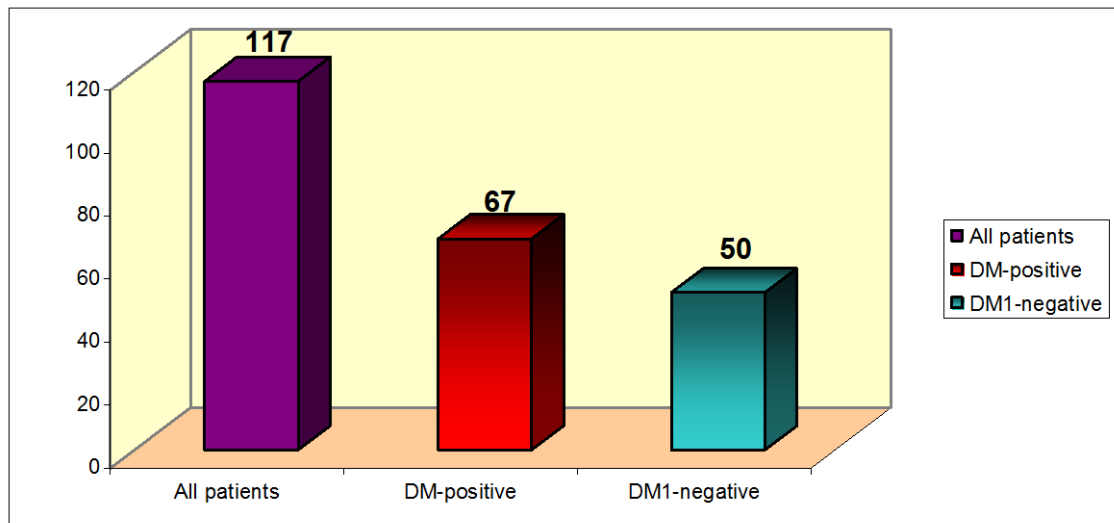


Figure 5.1. Total number of affected and DM1-negative individuals screened in this study

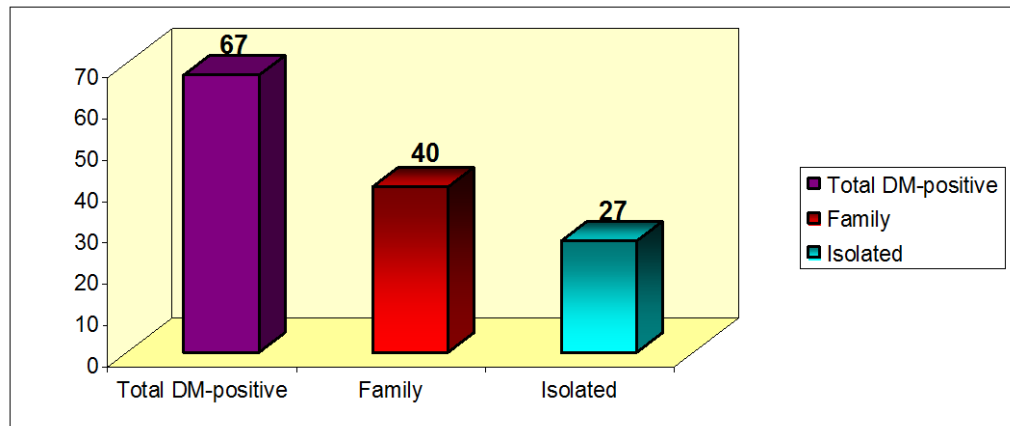


Figure: 5.2. Total number of families and isolated DM1 patients in this study.

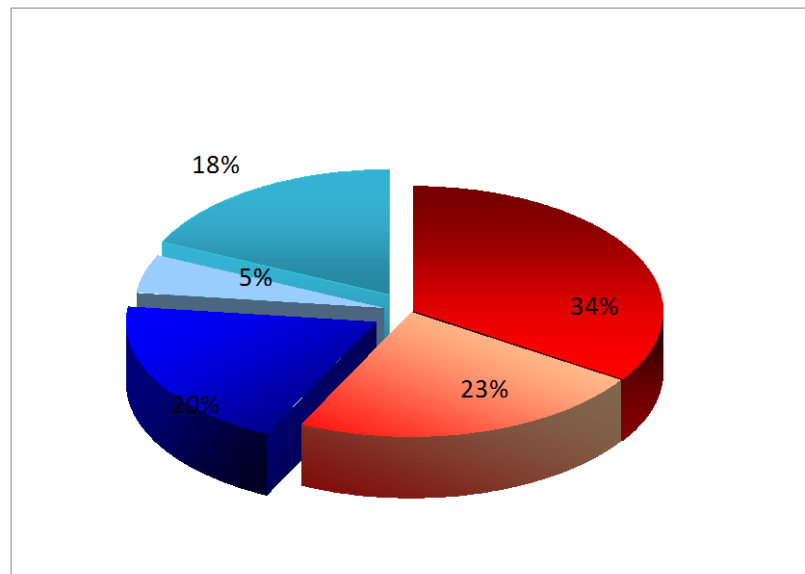


Figure 5.3. Percentage of the DM1-affected and DM1-negative patients.

Red: DM1-positive, blue: DM1-negative, dark red: DM1-positive family members, light red: DM1-positive isolated patients, light blue: healthy members of DM1-affected families, middle blue: isolated cases DM1-negative, dark blue: DM1-negative families

Of 67 DM1-positive patients, 39 were males and 28 were females, the male to female ratio was 1.39:1 (Figure 5.4.).

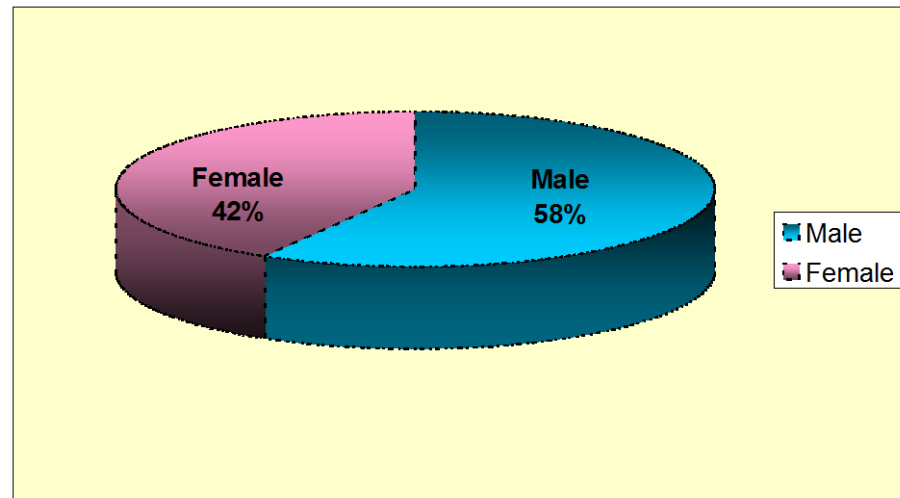


Figure 5.4. Male/female ratio in 67 DM1-affected patients.

Figure 5.5. classifies the DM1-affected patients according to their origins and Figure 5.6. shows the distribution of these cities to geographical regions of Turkey.

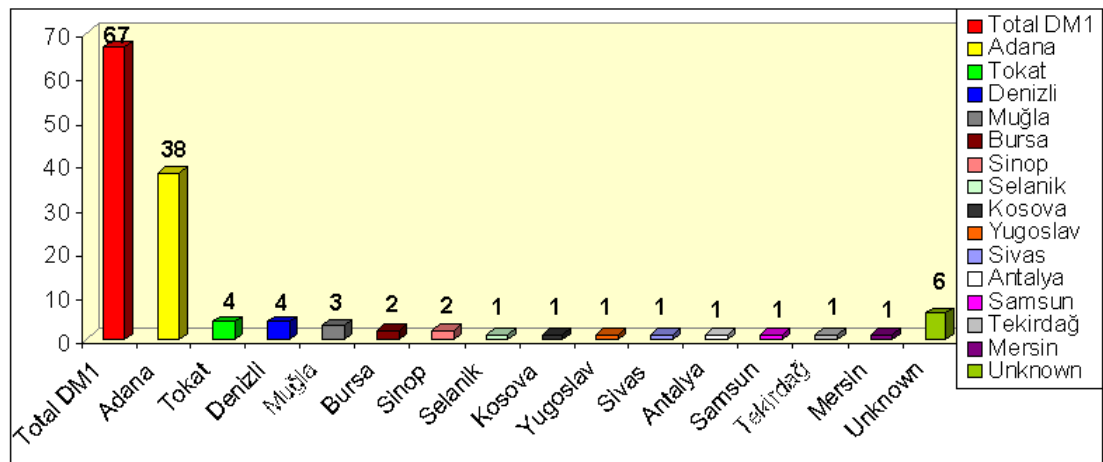


Figure 5.5. The origins of the DM1-affected patients.

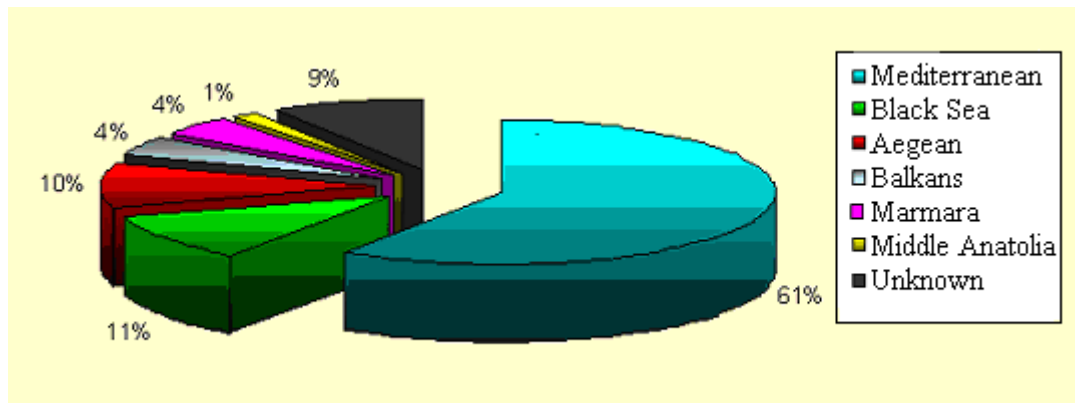


Figure 5.6. The geographical distribution of DM1 patients.

5.1.2. Analysis of the DMPK Gene

The CTG region of the DMPK gene was amplified by Triple-Repeat-Primed- and flanking PCR (Figure 5.7 and Figure 5.8). The samples with expanded CTG repeats, differing by three nucleotides, resulted in a smear pattern in the TP-PCR gel (indicated by an arrow in Figure 5.7.). All samples were further analyzed with ABI PRISM genetic analyzer. Figures 5.9 to 5.12 show typical fragment analysis results of positive and negative controls.

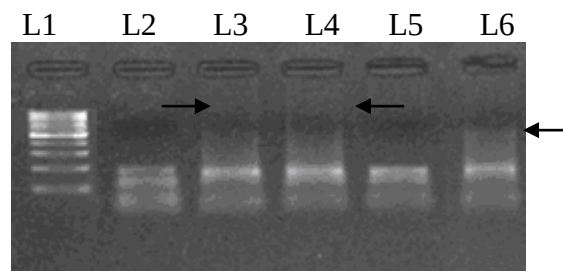


Figure 5.7. TP-PCR results of the samples. L1: ladder, L2: negative control, L3: positive control, L4: DM1-positive L5: DM1-negative, L6: DM1 patient with a low CTG number.

Arrows indicate the smear appearance.

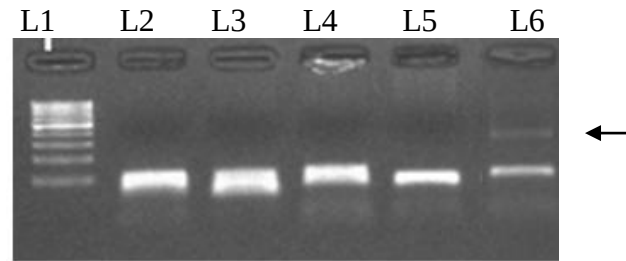


Figure 5.8. Flanking PCR results of the samples from Figure 5.7. L1: ladder, L2: negative control, L3: positive control, L4: DM1-affected, L5: DM1-negative, L6: DM1 patient with a low CTG repeat number: the low-CTG-containing mutated allele could be amplified in this patient (arrow).

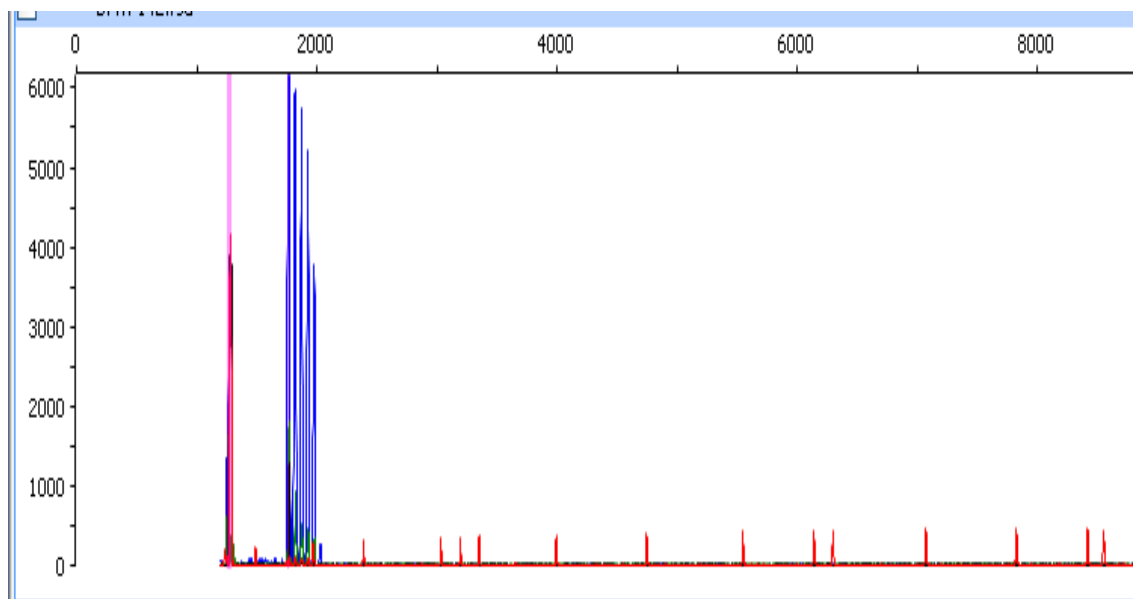


Figure 5.9. TP-PCR fragment analysis of a negative control with ABI Prism peak scanner: peaks abruptly end behind the large allele.

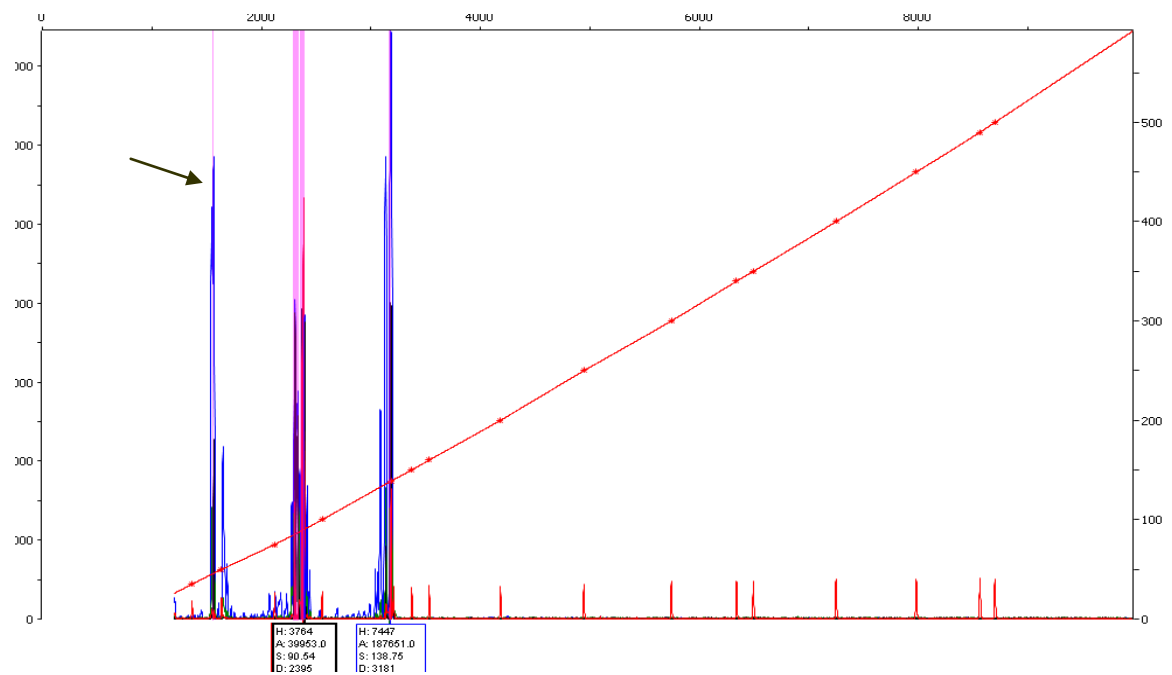


Figure 5.10. F-PCR fragment analysis of a DM1-negative control. Two peaks can be observed, the first small peak about 50 bp is a primer dimer (arrow).

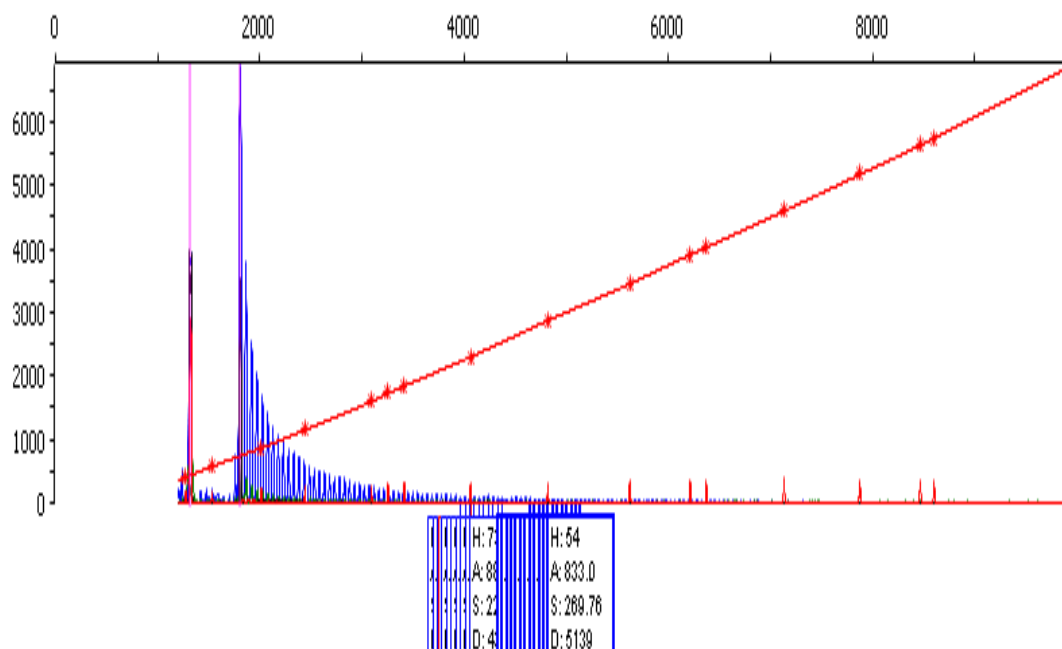


Figure 5.11. TP-PCR fragment analysis of a DM1-positive control. Peaks, observed in every three bases, decrease in size to gradually diminish.

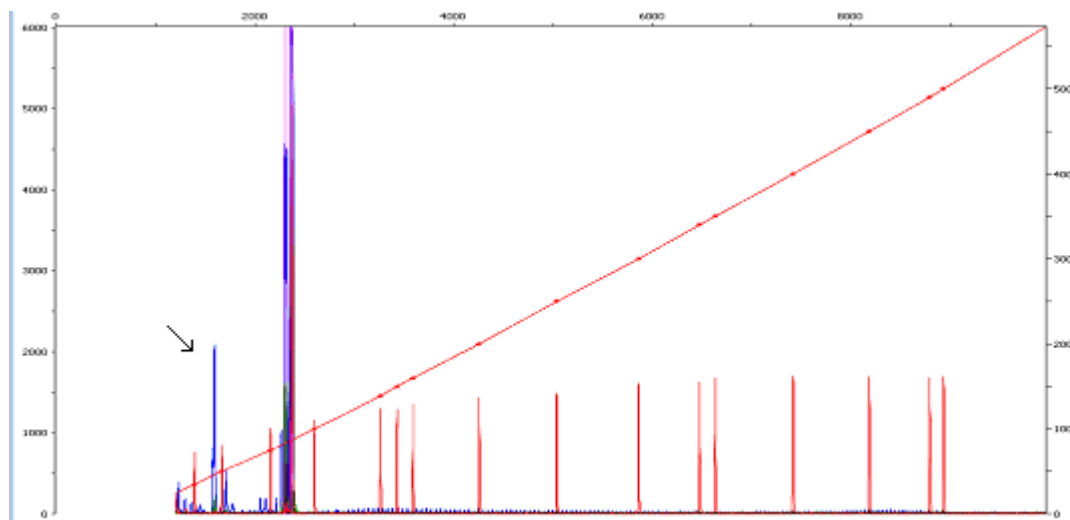


Figure 5.12. F-PCR fragment analysis of a DM1-positive control. One peak, showing only one allele, can be amplified with the conventional PCR. The small peak before the large allele is a primer dimer (arrow).

In the next section are examples of some representative families, who were diagnosed by TP-PCR.

5.1.3. Turkish DM Families

5.1.3.1. Family 1: A Large DM Family. A large family with myotonic dystrophy from Adana was investigated in the framework of this thesis. Seven family members had similar symptoms and were clinically diagnosed with DM Type 1. TP- and F-PCR were applied to five family members and all of them were found to carry expanded CTG repeats (Figure 5.3. ID numbers: 3, 5, 6, 13, 14).

The index case (ID#14) was a 22-year-old male patient with clinical signs of DM. He had difficulty in relaxing his hand muscles after contraction. According to the patient, this symptom existed from early childhood; his problem increased especially in cold weather. The patient had frontal baldness and atrophy in jaw muscles, clinical examination showed also myotonia.

In his 45-years old uncle (ID#5), the symptoms started as pain in the waist region. For the last three years, the patient had difficulty in walking. Clinical examination showed myotonic discharges.

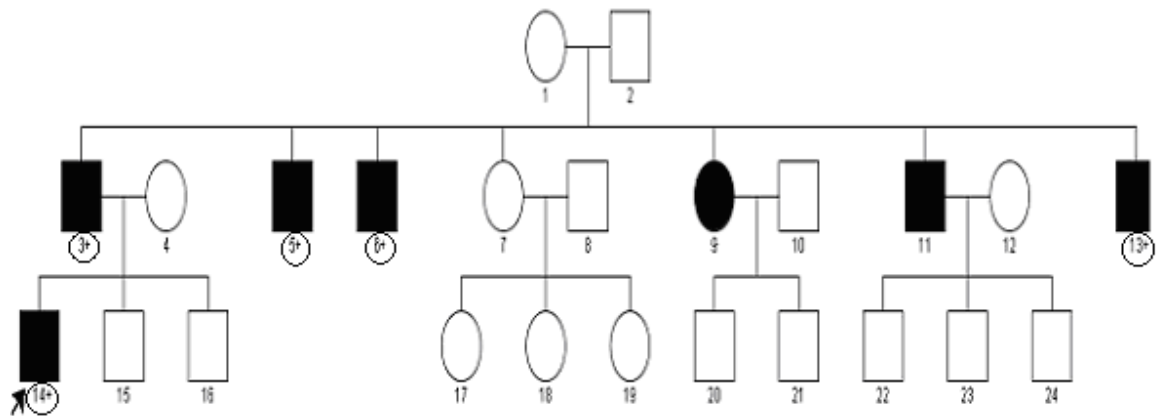


Figure 5.13. Pedigree of Family 1. Samples shown in circles were available for DNA analysis. The index case is indicated by an arrow.

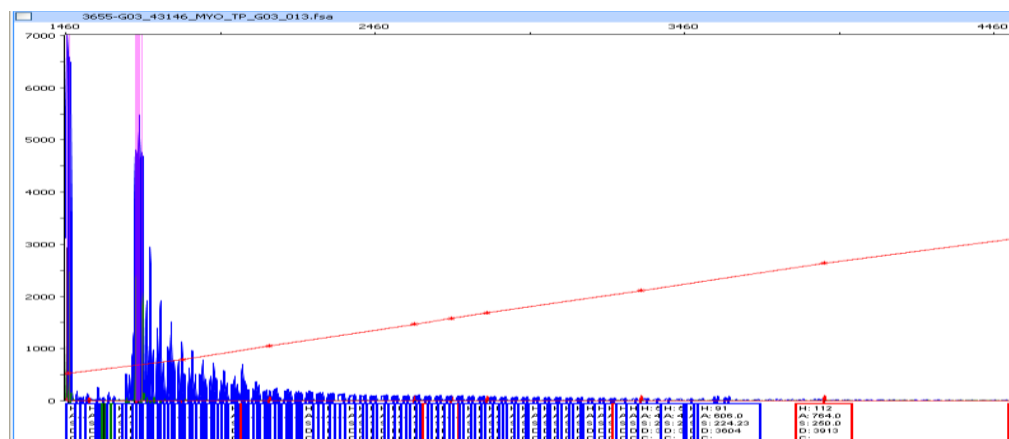


Figure 5.14. Family 1: TP-PCR fragment analysis of the index case (ID#14).

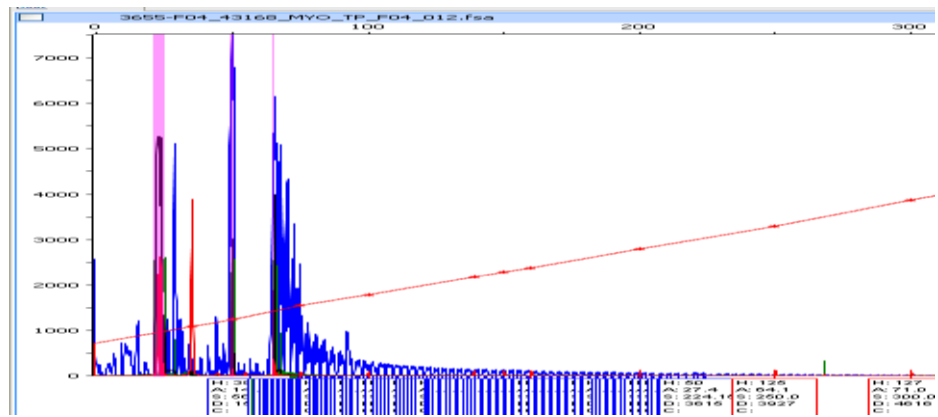


Figure 5.15. Family 1: TP-PCR fragment analysis of index patient's affected father (ID#3).

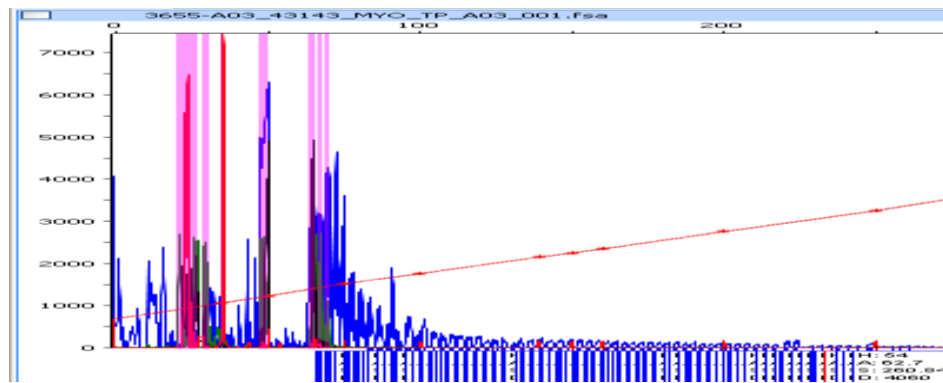


Figure 5.16. Family 1: TP-PCR fragment analysis of one of index patient's affected uncles (ID#5).

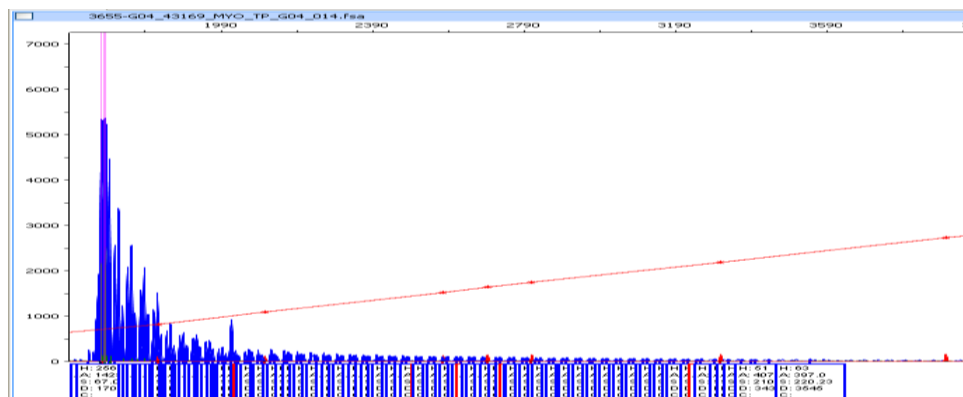


Figure 5.17. Family 1: TP-PCR fragment analysis of index patient's second affected uncle (ID#6).

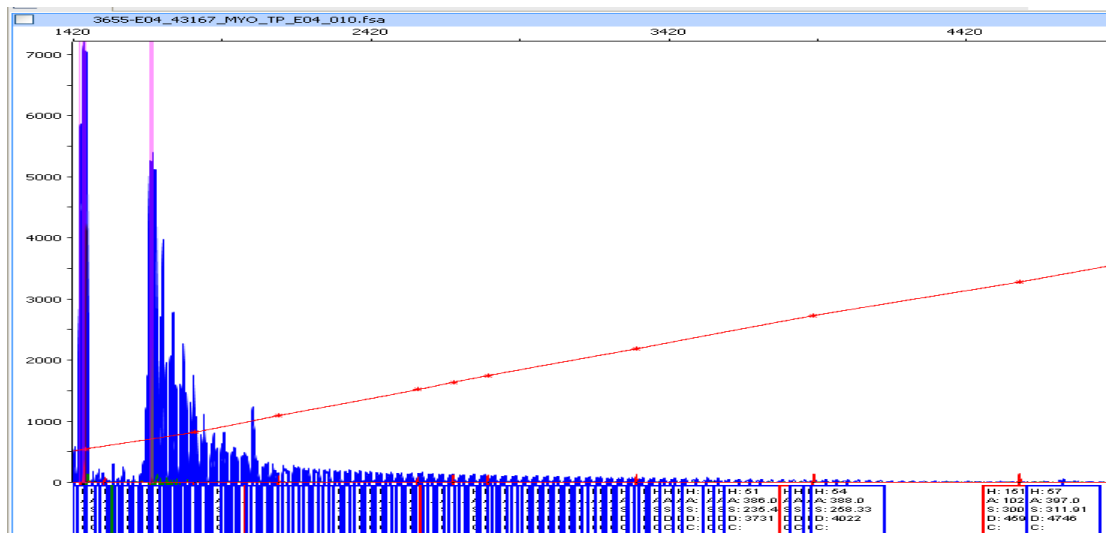


Figure 5.18. Family 1: TP-PCR fragment analysis of index patient's third affected uncle (ID#13).

5.1.3.2. Family 2: Sizeable Expansion. The index case was referred to our laboratory for molecular diagnosis of DM1. Blood samples were also collected from her parents, although neither of them had symptoms yet (Figure 5.19.). Interestingly, the mother was found to be affected with a sizeable expansion, which presents as a ladder in fragment analysis (Fig. 5.20). The father was normal, regarding his DM loci (Fig. 5.21), and the index case had a full expansion (Fig. 5.22).

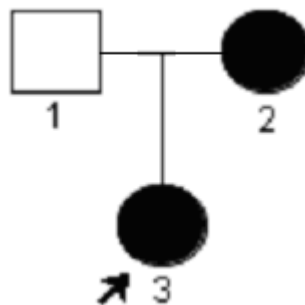


Figure 5.19. Pedigree of Family 2.

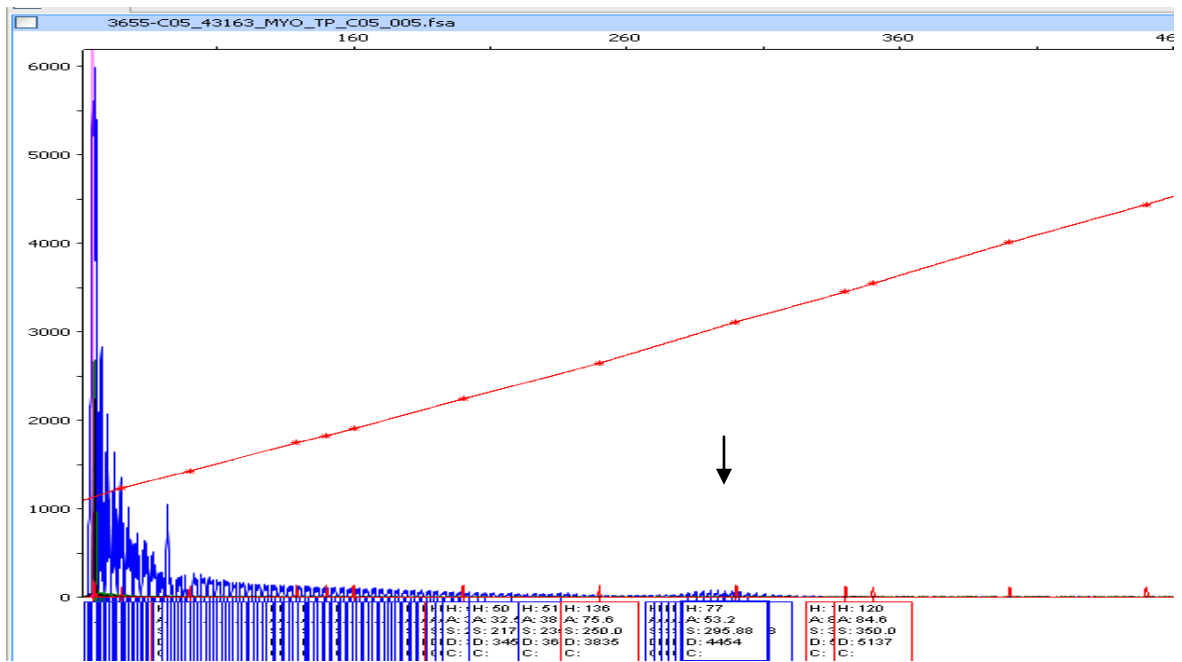


Figure 5.20. Family 2: TP-PCR fragment analysis of the mother: Arrow indicates the ladder-like peaks, which start to increase again, showing the expanded allele, having a size about 81 CTG repeats

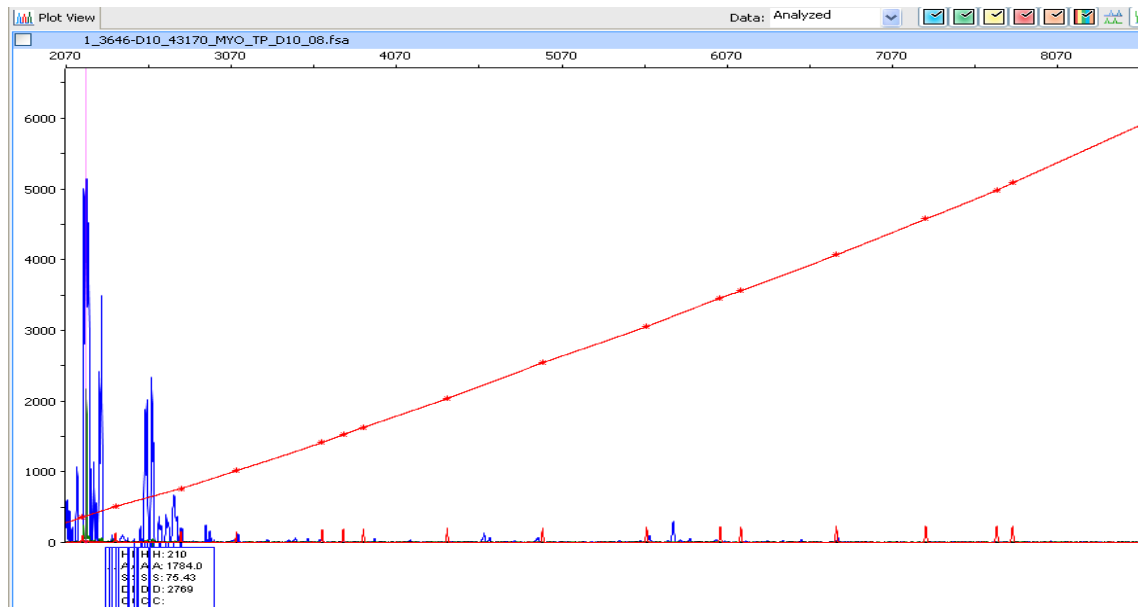


Figure 5.21. Family 2: TP-PCR fragment analysis of the father: the peaks finish after the large allele, sized 75 nucleotides, which correspond to about 8 CTG repeats.

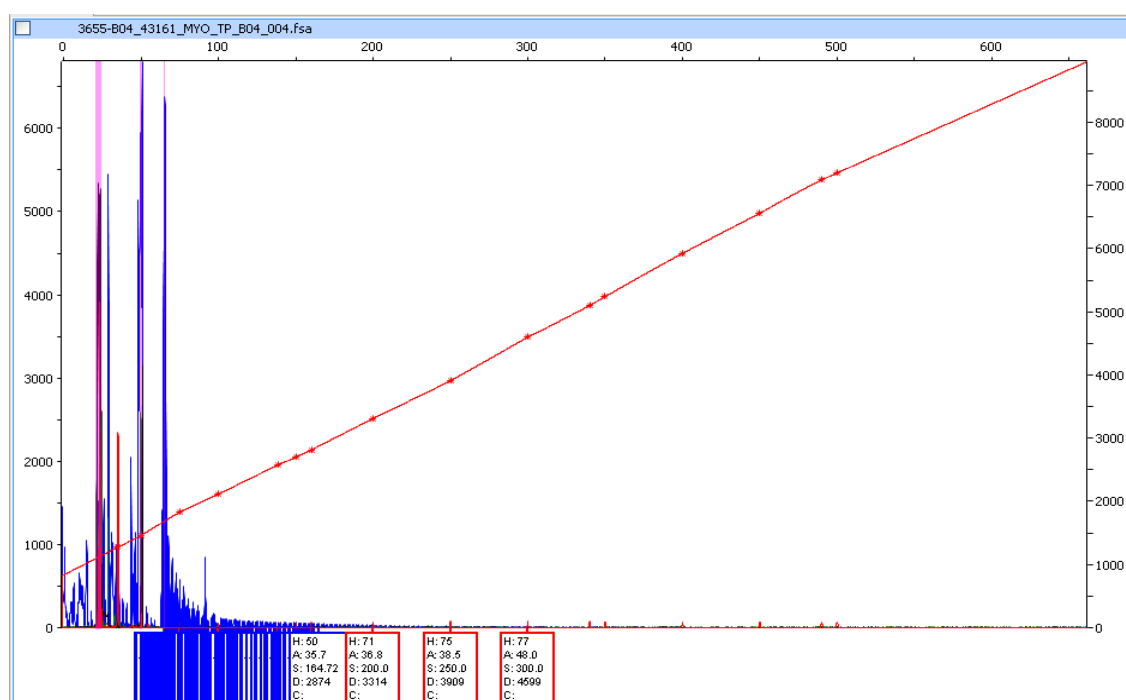


Figure 5.22. Family 2: TP-PCR fragment analysis of the index case: full expansion is observed. The expanded allele is larger than 100 CTG, thus cannot be sized.

5.1.3.3. Family 3: A Patient with Co-existing DM and CMT. Blood samples of a mother with her two sons were referred to our laboratory for DM1 analysis (Fig. 5.23). The mother, shown with an arrow in the pedigree, was 46 years old and complained from numbness in her feet and hands for 10 years (Figure 5.23). Eight years ago, she felt increasing weakness in her feet, spreading to her hands with time. The clinical examination revealed a bilateral ptosis. Atrophic muscles were present in her feet and myotonic discharges were observed in the EMG. Nerve conduction studies were consistent with demyelinating sensory-motor neuropathy, thus the patient was also clinically a candidate for possible Charcot-Marie-Tooth disease (CMT Type 1). Genetic analyses of both the CMT1 and DM1 loci revealed that she was both CMT1- and DM1-positive (Fig. 5.24.).

Her 28-years-old son (#3 in the pedigree), complained from weakness in his hands, especially increasing in cold weather. He had frontal baldness and dysarthria, myotonic discharges were also present. Molecular analysis revealed that the patient was affected with DM1 (figure 5.25), but not with CMT1. The blood sample of the daughter (21 years

old), who was asymptomatic, was not available. The younger son was not subjected to DM analysis, since he was only 12 years old and asymptomatic.

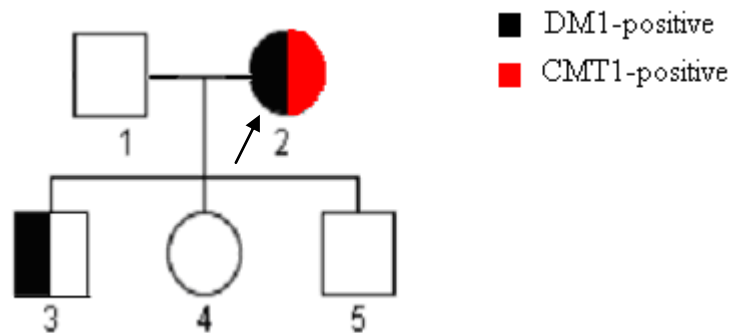


Figure 5.23. Pedigree of Family 3. The arrow indicates the mother, who carries two autosomal dominant diseases. Her affected elder son carries only DM1.

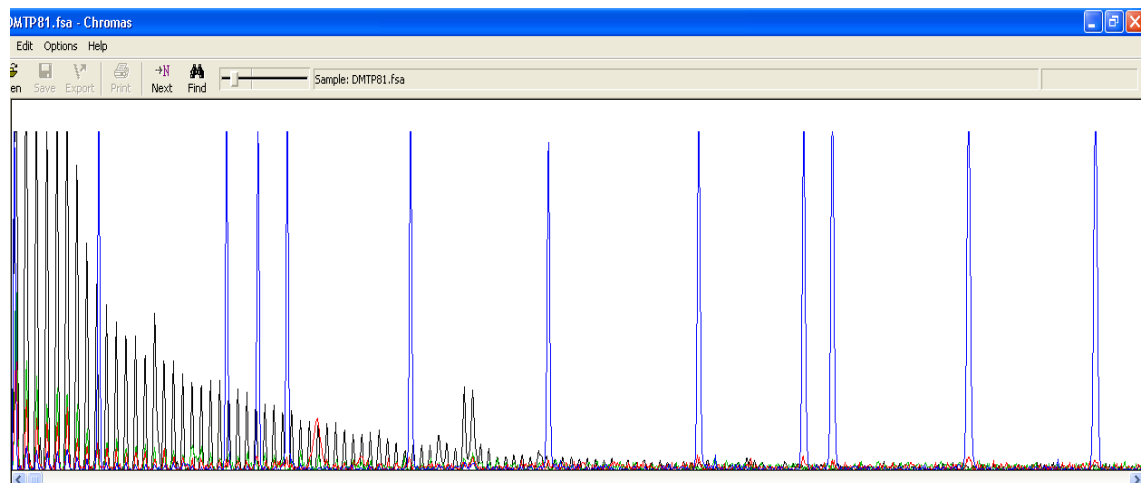


Figure 5.24. Family 3: TP-PCR fragment analysis of the index case. Full expansion is observed confirming the DM clinical diagnosis. The blue peaks show the size markers.

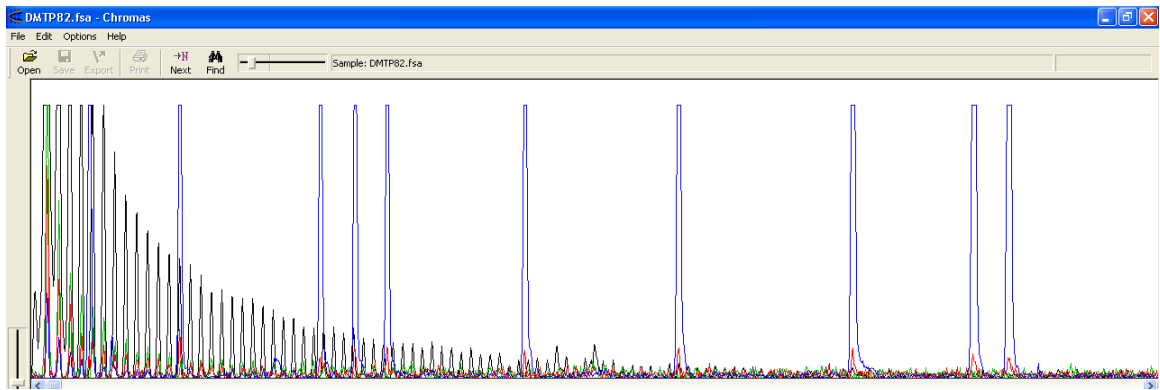


Figure 5.25. Family 3: TP-PCR fragment analysis result of the son: Expanded CTG confirms the DM diagnosis.

5.2. Von Hippel-Lindau Disease

A large Turkish kindred, many individuals of whom were affected with VHL disease, was also investigated in the framework of this thesis. The pedigree of the family consisting of five generations is shown in Figure 5.26.

The family originates from Erzincan, which is a medium-sized city in eastern Turkey. All available symptomatic patients and asymptomatic individuals at 50 per cent risk were examined. All patients were consulted by the physician in charge, and gave written informed consents.

There were three consanguineous marriages in the family. A consanguineous marriage between two VHL-affected individuals (33 and 65) was also present. Several types of cancer formation, including brain, renal, pancreas, spinal cord and cerebellum tumors were seen in this family, Figure 5.27 shows the distribution of tumor types. Renal carcinomas were the most predominant carcinomas in the family (Figure 5.28).

Since the index case (#36 in the pedigree) declared to have low blood indices, upon request he and his son were analyzed in our laboratory and shown to heterozygously carry the IVS-I-1 (G → A) Mediterranean mutation.

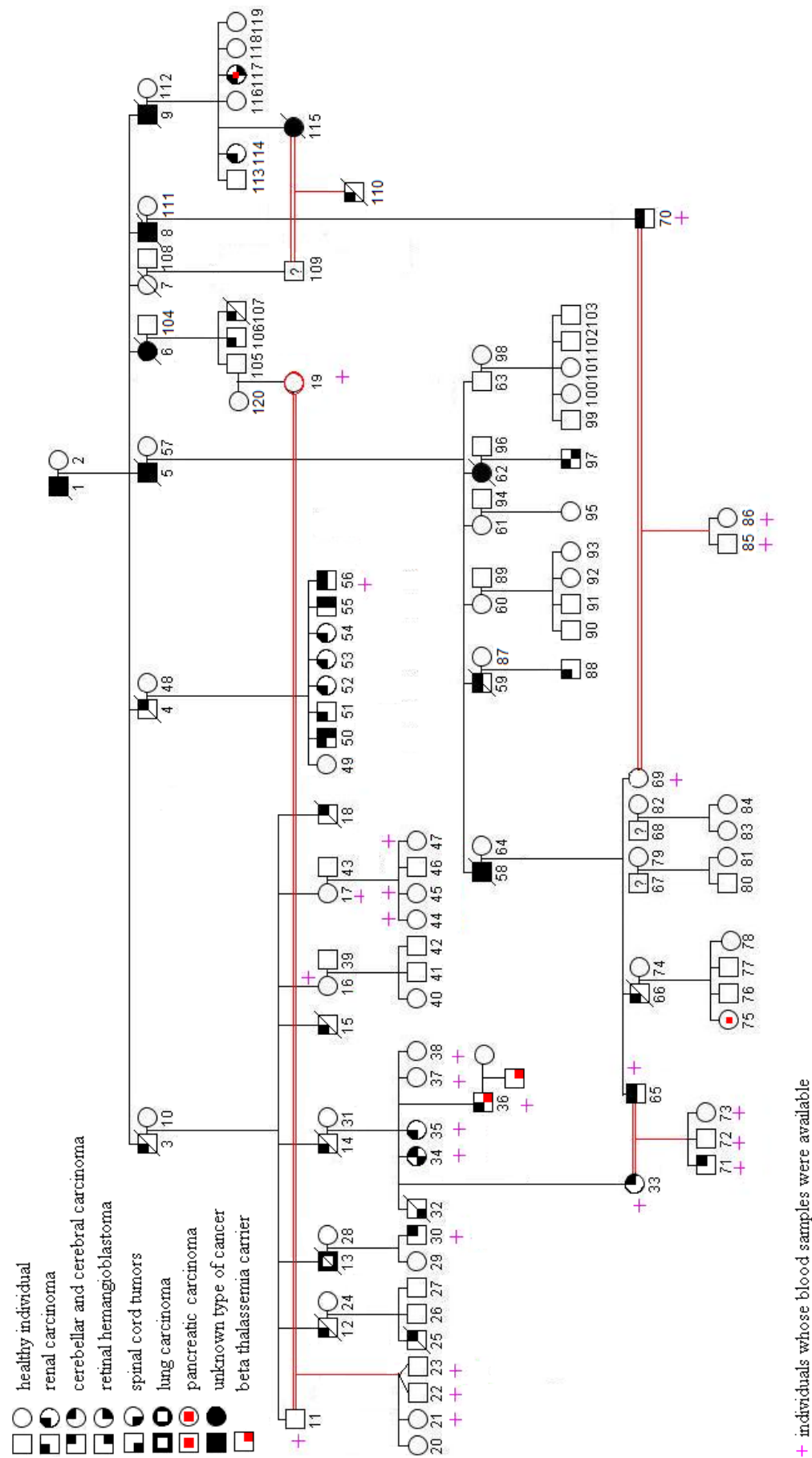


Fig. 5.26. Pedigree and distribution of tumors in the family.

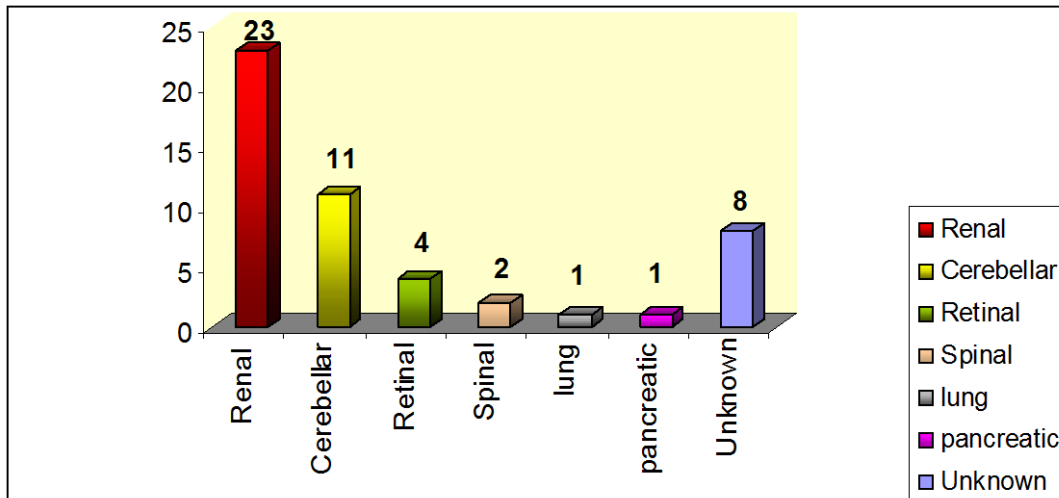


Figure 5.27. Types and numbers of the tumors seen in the family in several generations.

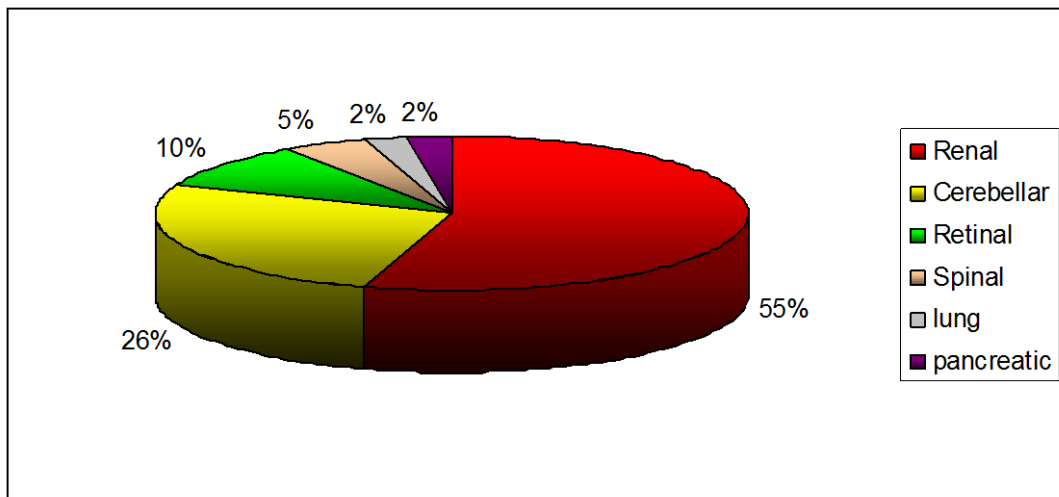


Figure 5.28. Per cent distribution of the tumors observed in the family.

5.2.1. PCR Amplification and DNA Sequencing

The search for a mutation in the VHL gene started with investigating Exon 2 and 3, which are known to be mutational hotspots. The product sizes of the amplified regions are shown in Table 5.1. and Figures 5.29.–5.31.

Table 5.1. Fragment lengths of the PCR products

Exon	Fragment length (bp)
Exon 2	194
Exon 3, 5' end	162
Exon 3, 3' end	150

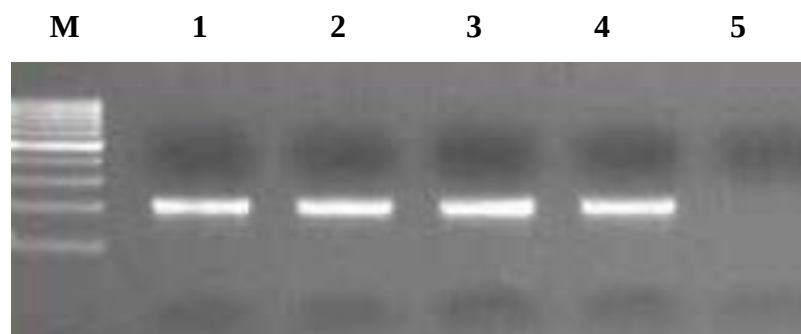


Figure 5.29. PCR amplification of the VHL gene, Exon 2.

M: 100 bp ladder, lanes 1, 2, 3, 4: Exon 2 PCR products of several VHL patients;
lane 5: no DNA.

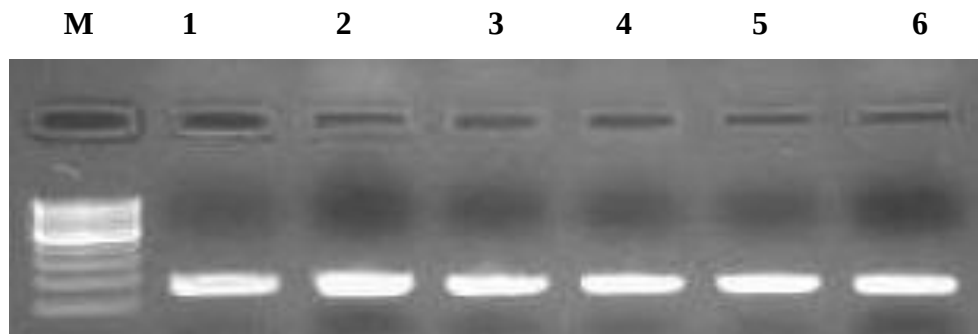


Figure 5.30. PCR amplification of the VHL gene Exon 3/ 5' end.

M: 100 bp ladder, lanes 1, 2, 3, 4, 5, 6: PCR products of
VHL patients.

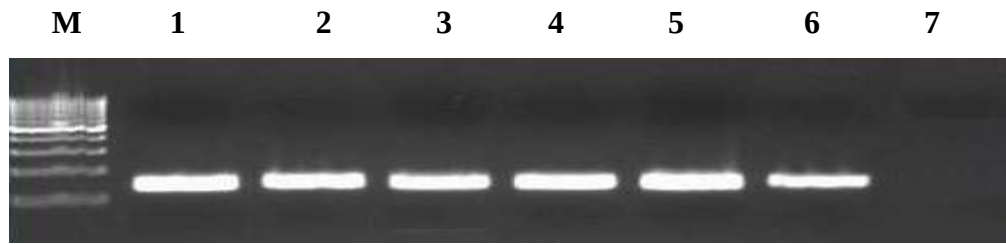


Figure 5.31. PCR amplification of the VHL gene Exon 3/3' end.

M: 100 bp ladder, lane 1, 2, 3, 4, 5, 6: second part of Exon 3 PCR products of several VHL patients, lane 5: no DNA.

Sequencing of all three regions in the index patient revealed the presence of a C to T substitution at position 694 of Exon 3 (Figure 5.32 and Figure 5.33).

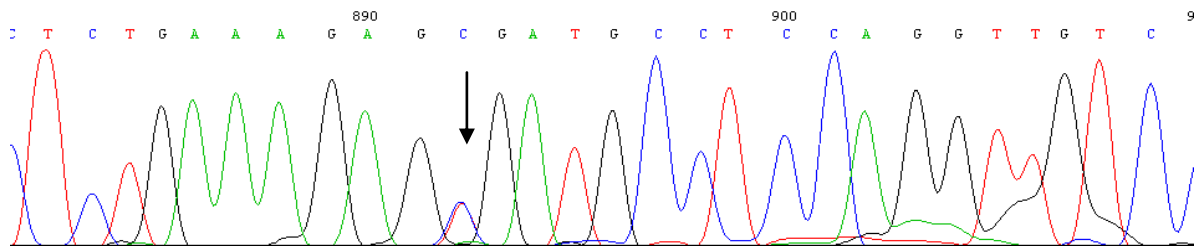


Figure 5.32. DNA sequencing of Exon 3 with reverse primer; the heterozygous presence of C and T is indicated by the arrow.

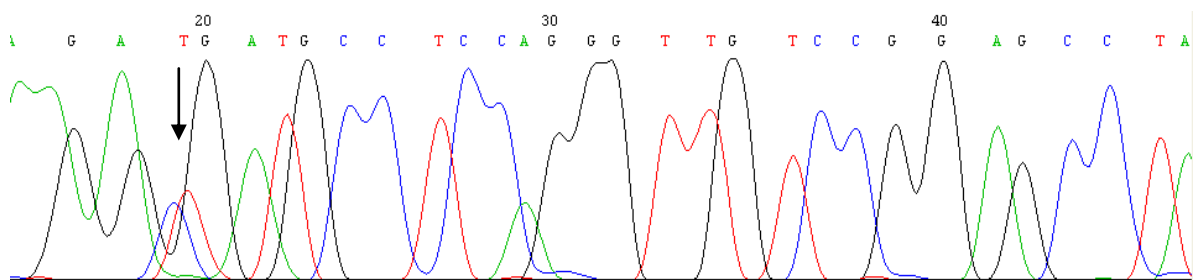


Figure 5.33. DNA sequencing of Exon 3 with forward primer, confirming the mutation.

In the protein the change of CGA, which codes for arginine, leads to a premature stop (TGA) at aminoacid position 161 (Zbar *et al.* 1996). All the other affected members of the family were subsequently shown to carry the same transition of C to T at position 694 of Exon 3.

6. DISCUSSION

6.1. Myotonic Dystrophy

Myotonic Dystrophy (DM) is a neuromuscular disorder with multi-systemic effects. Skeletal and cardiac muscles, endocrine, gastrointestinal and central nervous systems are the organ systems that are affected in DM1. Like in many other triplet repeat disorders, in DM, as the age of onset decreases, the severity of the disease increases. DM is the first disease to show autosomal dominant inheritance and a gain-of-function type of mechanism that is caused by a triplet repeat expansion within a non-coding region. Myotonic Dystrophy is classified into two types as DM1 and DM2. Although, there are some clinical differences between these two types, such as the muscle groups affected (DM1 usually affects the distal, and DM2 the proximal muscles) or the absence of congenital form in DM2, the remaining clinical features are remarkably similar. Even though, different mutation loci on different chromosomes are responsible for DM1 and DM2, similar molecular mechanisms are acting on disease pathology (Ranum and Cooper, 2006). The clinical similarities observed in these two types of DM, along with other neurological complaints, which may sometimes mimic DM, create the need for molecular differential diagnosis of the disease.

6.1.1. Establishment of the TP-PCR Technique

Molecular diagnosis of DM is usually carried out by Southern Blot Analysis. Although this method is very appropriate for determining the CTG repeat sizes on both alleles, it is not convenient to be used for routine analysis and for clinical diagnostic purposes. Conventional PCR techniques, on the other hand, face technical difficulties in high number repeat expansions: the expanded CTG repeats tend to form very strong hairpin loops, which are difficult to amplify. Also random enzymes cannot amplify long repeats. Another important problem is that PCR and agarose gel electrophoresis cannot distinguish healthy homozygous individuals from affected patients: Both of these individuals will show one allele in the gel; although the intensity of bands may differ, it is not appropriate to diagnose patients using this method.

The solution to the above drawbacks came from Warner and his coworkers (1996), who developed a new technique, the Triplet-Repeat-Primed-PCR (TP-PCR). Although this technique does not resolve the exact numbers of the expanded CTG repeats above 100, it can distinguish homozygous healthy from affected patients. High betaine concentration and very long annealing cycles are used to overcome the hairpin loop formation and a sequence analyzer is used for observation of PCR fragments.

Although TP-PCR lacks the CTG repeat expansion sizes of long alleles, this does not create a problem in clinical use, for two practical reasons. According to the recommendation of the International Myotonic Dystrophy Consortium, patients should only be provided with a simple positive or negative result and not with any further details. The reason for this recommendation is that there is a large somatic instability of CTG repeat numbers with increasing age. An affected person's overall average-repeat-length increases as the patient ages. Therefore, the repeat numbers can be variable according to the age of the person taking the test. The second important reason, not providing the CTG numbers is, the CTG repeat number in the blood cells are different than those in other tissues of the human body. It is interesting to note that the CTG repeat is much higher in muscle tissues (up to 1000 CTG higher) than in blood cells (Harper, 2008).

In this study, a custom-made ready mastermix was used for two reasons. First, this mastermix is much more appropriate for standardization and enables quick preparation. The second and more important reason is, as the ingredients except the primers are ready, the solution is much less prone to contamination, which is an important factor for diagnostic tests and routine laboratory settings.

6.1.2. Distribution of Patients

In Turkey, there are only very few internationally cited publications on Myotonic Dystrophy. In these reports, if there is any molecular analysis, it is carried out with Southern Blotting. The number of patients subjected to molecular analysis is also very limited. In this study, for the first time in Turkey, 117 DM1-suspected cases were

investigated and 67 were confirmed using a novel molecular technique that was established within the framework of this thesis.

Out of 117 suspected patients, 67 were found to be affected with DM1: the other 50 were free of any CTG expansion in the DM1 locus and as no point mutations have been reported so far in DM1, these patients are definitely DM1-negative. Since there are clinical similarities between DM1 and DM2, the next option should be the investigation for DM2.

Most of the patients analyzed within the framework of this study, were either referred to us from İstanbul Medical School, or from Çukurova Medical School (Adana). This explains the uneven distribution of our patients, the majority of whom originates from Adana and its surroundings.

We observed that there were more affected males in our patient cohort than females, the male to female ratio being 1.39 to 1. Surveys and studies showed that in 670 Japanese patients, the male to female ratio was found to be 1.6 to 1 (Matsuoka, 1988). Another study in Belgrade showed that the male to female ratio of DM1 patients was 1.1 to 1 (Mladenovic, 2006). In Iceland, the ratio was 1.2 to 1 (Leifsdottir *et al.*, 2005). In all populations investigated so far the male/female ratios were in favor for men and our data are in accordance with these results.

6.1.3. Families with Interesting Outcomes

The Turkish population and the Turkish family structure provide interesting results for genetic studies. Large families with many offspring can provide data on age of onset differences among family members. In one of our DM1 families from Adana, four out of seven siblings were affected and the index patient was a boy in the younger generation. Anticipation was very obvious in this family; however, since the CTG numbers were above 100, in all individuals investigated, a comparison between generations was not possible.

In another family genetic instability could be shown between generations. The mother, who did not have any clinical symptoms, had a repeat number of 81 CTGs. In the affected daughter, who was the index patient, the CTG repeat was found to be well over

100. Sizable expansions are important in TP-PCR, because our future aim is to further optimize the TP-PCR technique to amplify the triplets beyond 100 CTGs, thus small expansions are important as controls.

In third family, the mother carried two autosomal dominant neuromuscular diseases that have different effects on nerves. DM1 affects the brain and cognitive functions, whereas CMT1 affects peripheral nerves, causing delayed nerve conduction. A combination of these two disorders in the same patient is very rare in the literature and to the best of our knowledge, this is the first case reported in Turkey (Kurt *et al.*, unpublished data).

6.1.4. Potential Applications of TP-PCR and Future Directions

In several laboratories TP-PCR has been successfully applied not only to DM1, but also to FRDA. If TP-PCR could be further optimized to size larger expansions of CTG, Southern Blotting could be completely excluded from DM1 diagnosis for determining the allele sizes. For this purpose, new enzyme mixtures should be tried, as well as large size markers such as LIZ 1200, should be used for fragment analysis. TP-PCR can also be applied to other repeat disorders, DM2 being the first and most potential candidate.

6.2. Von Hippel-Lindau Disease

Von Hippel-Lindau Disease is a cancer syndrome, that is inherited in an autosomal dominant fashion. It is a rare disorder, seen in one in 36.000 individuals (Chan *et al.*, 2007). The major cysts described in VHL disease are hemangioblastomas in the retina and nervous system, pheochromocytoma, renal carcinoma and pancreatic cysts and tumors. VHL Disease is caused by mutations in the VHL tumor suppressor gene (Shuin *et al.*, 2006). As tumor suppressors take role in suppression of growth factors and other oncogenic factors, to form a tumor, two copies of the gene should be disabled. However, in VHL, a single mutation is sufficient to form a tumor. This phenomenon is explained by Knudson's two hit hypothesis, in which the first hit (or mutation) of one allele is inherited, and the second hit occurs somatically (Knudson, 1971). If two mutations on two alleles

were necessary to form a tumor, VHL disease would be much more rarely seen and only families with consanguineous marriages would be under high risk.

VHL disease is clinically grouped into two types, as VHL Type 1 and Type 2 Disease. In Type 1, pheochromocytoma is not observed at all, whereas in Type 2 pheochromocytomas can be seen. Type 2 is further divided into Types 2A, 2B and 2C, and each of these subtypes tend to show particular tumor formations. Recent findings indicate that there is a correlation between the clinical classification and the mutation types seen in the gene. For instance, Type 1 has usually microdeletions/insertions or nonsense mutations, whereas Type 2 has missense point mutations (Zbar *et al.*, 1996).

6.2.1. Clinical Features of the Turkish VHL Family Under Investigation

The family is from Erzincan, which is a medium-sized city in East Turkey. In the family, there was no pheochromocytoma case, reported by the clinician; this implied the presence of Type 1 VHL disease. DNA analysis confirmed a nonsense mutation in the VHL gene that changes arginine to a stop codon in the protein. Our results were consistent with the clinical categorization of the family and confirmed the affected patients in this family as having Type 1 VHL Disease.

In the literature, several studies agree that the most common cause of death in VHL disease are renal carcinomas and cerebellar hemangioblastomas; however, they report different prevalence of VHL manifestations. According to Leung and coworkers (2008), the most common manifestation of the disease are pancreatic cysts with a prevalence between 50 to 91 per cent, followed by cerebellar hemangioblastomas with 44 to 72 per cent and renal cysts with 59 to 63 per cent. The most common type of cancer seen in our patients were renal carcinomas with a prevalence of 55 per cent, followed by cerebellar hemangioblastomas with 26 per cent and retinal hemangioblastomas with 10 per cent. Pancreatic lesions were the least observed tumor types in the family under investigation. Although data in literature are not in full accordance with our results, it should be kept in mind that here we are dealing with one family and a single mutation. In the literature, however, these manifestations are the result of both VHL types with many different mutations.

There was a consanguineous marriage between two affected individuals, in this family and therefore a 25 per cent risk of a homozygous mutation in the affected child: However, this was not the case, and the child was heterozygously affected.

The index case was at the same time a β -thalassemia carrier with the IVS-I-1 mutation. The same β -thalassemia mutation was also found in his son. To the best of our knowledge, the co-presence of β -thalassemia and VHL disease has not been reported before. Thus, there is no information about how the combination of these two diseases acts on tumor formation and invasiveness. New insights may be gained upon further follow-up of the index case.

6.2.2. Establishment of the Protocol

VHL-PCR was straightforward to establish and the PCR products were rich in yield. This PCR was able to amplify very low concentrations of DNA, hence it was very prone to contamination. Therefore, extra attention had to be paid when conducting the VHL experiments.

To the best of our knowledge, this study is the first molecular investigation of a large Turkish family with VHL disease. Although there were earlier attempts in Turkey to define VHL mutations by techniques like FISH, these studies were not successful in defining the exact position of the mutations.

6.2.3. Mutation Site and Importance in Signaling

pVHL has two isoforms, differing by the first 54 amino acids at the N-terminal domain of the protein. The large pVHL consists of 213 amino acids and has two functional domains, α and β . The β domain is located at the N terminus of the protein. It has a seven-stranded β -sandwich and an α -helix, constituting the bigger part of the protein (Ohh and Kaelin Jr, 1999). This domain plays a role in HIF-1 α binding. The other smaller α -domain consists of three α -helices and functions in Elongin binding. (Figure 6.1.)

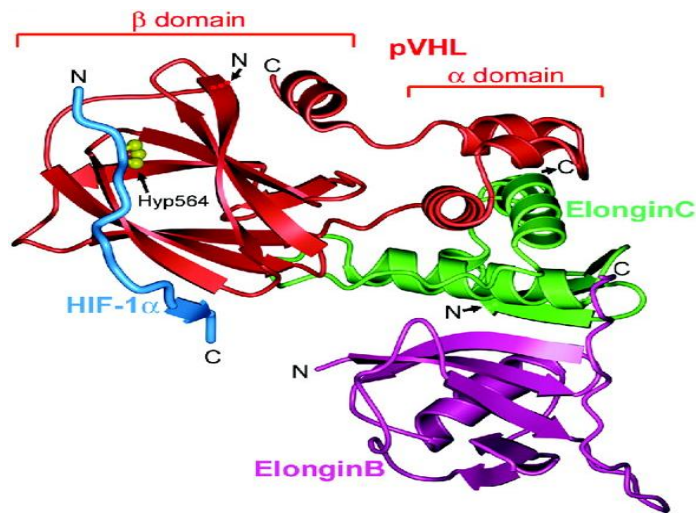


Figure 6.1. Domains and binding sites of pVHL (Min *et al.*, 2002)

The mutation that was detected in the Turkish VHL family resides in the Elongin binding domain of the protein, and changes arginine to a stop codon, causing a truncated protein. The Elongin binding site is important, because without Elongin C, pVHL cannot form the VCB-Cul2 E3 ubiquitin ligase complex. This complex acts on retinol binding protein that functions in stress-induced transcription. The most important target of pVHL is Hypoxia-Inducible Factor (HIF), which controls transcription of many genes, including E-Cadherin (Ohh and Kaelin Jr, 1999).

In our patients, early termination of the VHL protein at Elongin binding site and somatic inactivation of the remaining healthy allele in particular tissues, possibly disables the interaction between these proteins. Without formation of VCB-Cul2 E3 ubiquitin ligase complex, α -subunit of HIF cannot be inhibited, so the expression of the genes cannot be turned off. Excessive transcription of these genes, such as VEGF or erythropoietin, causes the tumor formation seen in the VHL patients (Leung and Ohh 2002).

6.3. Concluding Remarks

In the framework of this thesis, we have successfully established a new TP-PCR method and applied this novel technique to 117 DM-suspected cases. TP-PCR is proven to be a reliable and quick method and has already replaced other methods used in routine diagnostic laboratories. An efficient collaboration between clinicians and scientist would enable a better definition of genotype-phenotype correlation in DM1 patients.

In the framework of this thesis we have also conducted a molecular study on a large VHL family and identified the disease causing mutation. To the best of our knowledge, this thesis presents the first detailed molecular diagnosis of VHL disease in Turkey.

APPENDIX A: INFORMED WRITTEN CONSENTS

MİYOTONİK DİSTROFİ KATILIMCILARI İÇİN BİLGİ ve OLUR FORMU

Proje yürütücüsü: Prof. Dr. A. Nazlı Başak
Boğaziçi Üniversitesi Moleküler Biyoloji ve Genetik Bölümü,
34342, Bebek İstanbul.

Telefon (iş): (212) 359 66 79

Proje konusu: Miyotonik Distrofi Tip 1 (DM1) otozomal dominant kalıtım gösteren nöromusküler bir hastalıktır. Bireyde kaslarını gevşetmemek, katarakt, gündüz uykusu, gibi farklı organ sistemlerini etkileyen rahatsızlıklar gözlenebilir. Miyotonik Distrofi nesilden nesile aktarılırken, hastalık başlangıç yaşı azalır ve belirtiler ciddileşir.

Miyotonik Distrofiye neden olan mutasyon 19. kromozom'un uzun kolu üzerinde bulunmaktadır ve hasta bireylerde bu genin kodlanmayan kısmındaki CTG nükleotit tekrarlarında olağan dışı bir artış görülmektedir.

Öngörülen proje çerçevesinde DM1 şüphesi bulunan bireylerde DNA analizi yapılacaktır. Hastalık, otozomal dominant olarak kalıtıldığı için, hasta olan bir bireyin çocuğunun DM hastası olma riski %50'dir.

Çalışmaya katılmanız tamamen isteğinize bağlıdır. Araştırmaya katılmayı kabul ettiğiniz takdirde sizden on mililitre kan örneği alınacaktır. Yapacağımız araştırmanın size bir risk getirmesi beklenmemektedir. Kan aldırmanın genelde hiçbir zararı olmamasına karşın, nadiren çok az kanama ve morarmaya yol açabilir. Araştırmanın sonucunda aranan bilgi elde edildiği takdirde, ailenizin bireylerine genetik danışma hizmeti verilebilecektir. Çalışmaya kan verilerek gösterilecek gerekli toplumsal duyarlılık çalışma açısından büyük önem taşımaktadır.

Bu formu imzalamadan önce, çalışma ile ilgili sorularınız varsa lütfen sorun. Daha sonra sorunuz olursa Prof. Dr. A. Nazlı Başak'a (Telefon: 212 359 66 79 / 359 72 98) sorabilirsiniz. Araştırma ile ilgili haklarınız konusunda Boğaziçi Üniversitesi İnsan Araştırmaları Etik Kurulu'na danışabilirsiniz.

Adres ve telefon numaranız değişirse, bize haber vermenizi rica ederiz.

Bana anlatılanları ve yukarıda yazılanları anladım. Bu formun bir kopyasını aldım. Çalışmaya katılmayı kabul ediyorum.

Katılımcı veya vasisinin ismi ve imzası:

Tarih:

Hastaya yakınlığı:

Klinisyen ismi:

Adresi:

Telefonu:

İmzası:

VHL HASTALIĞI KATILIMCILARI İÇİN BİLGİ ve OLUR FORMU

Proje yürütücüsü: Prof. Dr. A. Nazlı Başak
Boğaziçi Üniversitesi Moleküler Biyoloji ve Genetik Bölümü,
34342, Bebek İstanbul.

Telefon (iş): (212) 359 66 79

Proje konusu: von Hippel Lindau (VHL) hastalığı otozomal dominant kalıtım gösteren bir kanser sendromudur. Bireyin çeşitli bölgelerinde (beyin, beyincik, böbrek, pankreas, retina, akciğer, omurilik, vb.) tümörler gözlemlenir. İki alt tipi vardır. Feokromasitoma denilen adrenal bezlerde tümörler gözlemlendiği zaman hastalar Tip 2 diye adlandırılır. Bu tip tümörler gözlemlenmediği zaman ise Tip 1 diye adlandırılır. Tip 2, 2A, 2B, 2C olmak üzere 3 alt tipe ayrılır. Hastalığın tiplerine göre gözlenen mutasyonlar değişiklik göstermektedir.

VHL geni 3. kromozom'un kısa kolu üzerinde bulunur. Bu gende meydana gelen mutasyonlar proteinin görevini yitirmesine ve daha bir çok genin aktive olup hücre bölünmesini denetleyememesine neden olur.

Öngörülen proje çerçevesinde VHL hastalığı görülen ailelere DNA analizi yapılacaktır. Hastalık, otozomal dominant olarak kalıtıldığı için, hasta olan bir bireyin çocuğunun VHL hastası olma riski %50'dir.

Çalışmaya katılmanız tamamen isteğinize bağlıdır. Araştırmaya katılmayı kabul ettiğiniz takdirde sizden on mililitre kan örneği alınacaktır. Yapacağımız araştırmanın size bir risk getirmesi beklenmemektedir. Kan aldırmanın genelde hiçbir zararı olmamasına karşın, nadiren çok az kanama ve morarmaya yol açabilir. Araştırmanın sonucunda aranan bilgi elde edildiği takdirde, ailenizin bireylerine genetik danışma hizmeti verilebilecektir. Önceden moleküler tanı yaptırmak pozitif tanı çıktığı takdirde oluşabilecek tümörlere erken müdahale açısından önem taşımaktadır. Pozitif çıkan bireyler düzenli kontrol sayesinde tümörler küçük ve iyi huylu iken yakalayabilirler. Çalışmaya kan verilerek gösterilecek gerekli toplumsal duyarlılık çalışma açısından büyük önem taşımaktadır.

Bu formu imzalamadan önce, çalışma ile ilgili sorularınız varsa lütfen sorun. Daha sonra sorunuz olursa Prof. Dr. A. Nazlı Başak'a (Telefon: 212 359 66 79 / 359 72 98) sorabilirsiniz. Araştırma ile ilgili haklarınız konusunda Boğaziçi Üniversitesi İnsan Araştırmaları Etik Kurulu'na danışabilirsiniz.

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Katılımcı veya vasisinin ismi ve imzası:

Tarih:

Hastaya yakınlığı:

Klinisyen ismi:

Adresi:

Telefonu:

İmzası:

APPENDIX B: PRODUCT OF THIS THESIS

Derleme

Miyotonik Distrofi: Kural Tanımayan Bir Üçlü-Tekrar Hastalığının Moleküler Biyolojisi ve TP-PCR Yöntemi ile DNA Tanısı

Myotonic Dystrophy: Molecular Biology and DNA Analysis of a Triplet-Repeat Disorder, that does not Obey Rules

Didem ERUSLU, A. Nazlı BAŞAK

Parkinson Hast. Hareket Boz. Der., 2009;12(1): 38-51

ÖZET

Miyotonik Distrofi (DM), otozomal dominant geçiş gösteren, multisistemik etkili nöromusküler bir hastalıktır. DM, hastalık patolojisi açısından diğer üçlü tekrar hastalıklardan farklıdır ve “toksik RNA” kavramını genetiğe kazandıran sendromdur. DM, Tip 1 ve Tip 2 olmak üzere ikiye ayrılır. Tip 1, hastalığın daha sık gözlenen formudur ve bu derleme Tip 1 üzerinde durmaktadır. DM’in iki tipi, farklı genlerdeki mutasyonlardan kaynaklansalar da, klinik bir takım benzerlikler gösterirler. Bu açıdan DM ‘in moleküler analizi büyük önem taşımaktadır. Yakın zamana kadar DM 1 genini tanımlamakta kullanılan en yaygın yöntem Southern Blot analizi idi. Bu yöntemin rutin laboratuvarında kullanım zorluğu ve otomasyona uygun olmaması, bilim adamlarını yeni yöntemler geliştirmeye yönlendirmiştir. Triple-primed-PCR (TP-PCR), polimeraz zincirleme reaksiyonuna dayalı yeni bir yöntemdir; DM tanısı günümüzde bu yöntem ile Southern Blot’dan çok daha hızlı ve güvenilir bir şekilde yapılabilmektedir. Bu makale çerçevesinde DM 1’in moleküler biyolojisi derlenecek ve TP-PCR yöntemi açıklanacaktır.

Anahtar Kelimeler: *Myotonik Distrofi, moleküler biyolojisi ve DNA tanısı, TP-PCR yöntemi*

SUMMARY

Myotonic Dystrophy (DM) is an autosomal dominant neuromuscular disease with multisystemic effects. DM is diverse from other triplet repeat disorders regarding its molecular pathology, being the first disease, which introduced the concept of “RNA toxicity” into genetics. DM is categorized into two distinct groups as Type 1 and Type 2. Type 1 is the more common observed form of the disease, and this review focuses to DM Type1. The two types of DM, caused by different mutations on different chromosomes, show several clinical similarities, thus, the molecular diagnosis of DM carries great importance in distinguishing DM 1 and DM 2. Until recently, Southern Blotting was the most common method used in the molecular analysis of DM 1. Because this method is tedious in a clinical setting and not appropriate for automation, scientists tried to devise new PCR-based techniques. Triple-primed-PCR (TP-PCR) is a novel technique, which is faster than and as reliable as Southern Blotting and has thus largely replaced the latter. In the framework of this review, the molecular biology of DM 1 will be revisited and the novel TP-PCR approach will be introduced.

Key Words: *Myotonik Distrofi, its molecular biology, and DNA diagnosis, TP-PCR technique*

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