Molecular Characterization of

Oculopharyngodistal Myopathy (OPDM) and Mimicking Disorders

by

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Koc University

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ABSTRACT

Ph.D. Thesis Title
Molecular Characterization of Oculopharyngodistal Myopathy (OPDM) and Mimicking Disorders

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Oculopharyngodistal myopathy (OPDM) is a rare, progressive inherited muscle disease involving the ocular, pharyngeal, and distal muscles. It is reported more frequently in Turkish, Japanese, and Chinese than in other communities. There were not any genes associated with OPDM since 2019. Although four different genes were identified between August 2019-November 2022 related to OPDM, none of the genes still cover majority of the patients. The aim of this thesis is to conduct molecular characterization of patients who admitted to Koç University Hospital Center for Muscle Diseases and were clinically diagnosed with OPDM.

Seven cases with a clinical diagnosis of OPDM were referred to the medical genetics department between March 2018-2020. Four cases had a definite molecular diagnosis of the diseases in differential diagnoses, whereas the remaining three patients' molecular diagnoses were not definite.

It has been shown that patients clinically followed up on OPDM should primarily be examined at the molecular basis for diseases in the differential diagnosis list. Four out of seven patients were diagnosed in a similar fashion. All studies including whole-genome sequencing were performed on one out of the three patients without a molecular diagnosis, and for this specific patient genetic etiopathogenesis still could not be clarified. Therefore, it is considered that genetic analysis should be studied more extensively in a genomic and functional manner with a wider gene pool through international teams.

OZETCE

Doktora Tez Başlığı
Okülofaringodisal miyopati (OPDM) ve taklit eden hastalıkların moleküler Karakterizasyonu

Hücresel ve Moleküler Tıp, Doktora
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Okülofaringodistal miyopati (OPDM) oküler, farinks ve distal kasları tutan, nadir, ilerleyici kalıtsal bir kas hastalığıdır. Türkiye, Japon ve Çin topluluklarında diğer ülkelerden daha sık rapor edilmiştir. 2019 yılına kadar OPDM ile herhangi bir gen ilişkilendirilmemişti. Ağustos 2019-Kasım 2022 arasında OPDM ile ilişkili dört farklı gen tanımlanmakla birlikte, bu genler hastaların tamamını kapsamamaktadır. Bu tez çalışması ile amacımız Koç Üniversitesi Hastanesi Kas Hastalıkları Merkezi'ne başvuran ve klinik olarak OPDM tanısı alan hastalarda moleküler karakterizasyonun tanımlanmasıdır.

Mart 2018-Mart 2020 arasında KUH Tıbbi Genetik Anabilim Dalı'na başvuran OPDM klinik tanılı yedi vakadan dördü ayırıcı tanıda yer alan hastalıklarla ilgili tanı almıştır. Üç hastada ise moleküler tanı kesinleşmemiştir.

Bu tez ile OPDM klinik tanısı alan hastalarda öncelikli olarak ayırıcı tanıda bulunan hastalıkların dışlanması gerektiği gösterilmiştir. Yedi hastadan dördü böylece tanı alabilmiştir. Tanı almayan üç hastadan birinde ise tüm genom analizi dahil tüm çalışmalar yapılmış olup, genetik etyopatogenez aydınlatılamamıştır. Bu nedenle daha fazla sayıda vaka ve uluslararası işbirlikleri ile genomik ve fonksiyonel çalışmaların yürütülmesinin uygun olacağı öngörülmektedir.

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ABBREVIATIONS

a.a.:	aminoacid
AD:	autosomal dominant
AR:	autosomal recessive
CK:	creatine kinase
CNV:	Copy Number Variations
COX:	Cytochrome C Oxidase
DM1:	Myotonic Dystrophy type 1
DMD:	Duchenne Muscular Dystrophy
DMPK:	Dystrophia Myotonica Protein Kinase
DNA:	Deoxyribonucleic Acid
ECG:	Electrocardiography
EMG:	Electromyography
ENT:	Ear, Nose, Throat
FXTAS:	Fragile X tremor/ataxia syndrome
GATK:	Genome Analysis ToolKit
GDC:	Genetic Diagnostic Center
KUH:	Koç University Hospital
LoH:	Loss of Heterozygosity
MLPA:	Multiplex ligation-dependent probe amplification
MRI:	Magnetic Resonance Imaging
mtDNA:	mitochondrial DNA
NGS:	Next-Generation Sequencing
NIID:	Neuronal Intranuclear Inclusion Disease
NMD:	Neuromuscular Diseases
OPDM:	OculoPharyngoDistal Myopathy
OPDM1:	OculoPharyngoDistal Myopathy 1
OPDM2:	OculoPharyngoDistal Myopathy 2
OPDM3:	OculoPharyngoDistal Myopathy 3
OPDM4:	OculoPharyngoDistal Myopathy 4
OPMD:	OculoPharyngeal Muscular Dystrophy
OPML:	Oculopharyngeal Myopathy with Leukoencephalopathy

PABPN1:	PolyAdenine Binding Protein Nuclear
PD:	Parkinson's Disease
PEG:	Percutaneous Endoscopic Gastrostomy
PEO:	Progressive External Ophthalmoplegia
PolyPhen-2:	Polymorphism Phenotyping v2
qPCR:	Quantitative PCR
RRF:	Ragged-red fibers
RT:	Room Temperature
SCA:	Spinocerebellar Ataxia
SDH:	Succinate Dehydrogenase
SIFT:	Sorting Intolerant From Tolerant
SSCP:	Single-strand Conformation Polymorphism
TMD:	Tibial Muscular Dystrophy
TP PCR:	Triplet Repeat Primed PCR
UTR:	Untranslated Region
VUS:	Variant of Unknown Significance
WES:	Whole Exome Sequencing
WGS:	Whole Genome Sequencing

Chapter 1

INTRODUCTION

Neuromuscular diseases (NMD) are genetically and phenotypically heterogeneous group of disorders that affect the peripheral nerves, neuromuscular junction, and muscle. Muscle diseases (myopathies) are a large group of NMD which cause muscle dysfunction and weakness. Although myopathies' main symptoms are muscle weakness and wasting, other symptoms such as cramps, myotonia, droopy eyelids, double vision, breathing and swallowing difficulties, and cardiovascular symptoms may accompany. However, the clinical picture's severity can vary from asymptomatic to life-threatening, and the onset ranges from *in utero* to late adulthood, even adulthood, and the elderly. Symptoms tend to be progressive [Morrison, 2016].

Etiopathogenesis of the vast majority of myopathies is genetic mutations. However, a small group of myopathies is caused by viral infections, autoimmune diseases, hormonal or metabolic disorders, and toxic-iatrogenic manner.

Many genes have been identified for myopathies in the past 30 years, the Duchenne muscular dystrophy (DMD) gene being the first one. The inheritance pattern can be autosomal dominant (AD), autosomal recessive (AR), X-linked, mitochondrial DNA (mtDNA) related, and/or somatic mosaic. The mutations vary greatly, including missense, nonsense, small insertions/deletions, large deletions/duplications, and repeat expansions/contractions. Still, the genetic etiopathogenesis of many myopathies is unknown.

Myopathies' diagnostic workup necessitates a multidisciplinary approach; neurologists, neuropathologists, electrophysiologists, and molecular geneticists have to be the primary essential partners. During the follow-up of the patient, cardiologists, pulmonologists, physical therapists, orthopedists, ophthalmologists, and ear-nose-throat (ENT) specialists should get involved in the team depending on the type of myopathy and/or patient’s symptoms.

Before the implementation of molecular genetics in the diagnostic workup of myopathy patients, the diagnosis was based on the clinical expertise of the neurologist, blood tests (creatine kinase (CK), lactate level,...), electromyography (EMG), and muscle biopsy.

With the advancement of molecular diagnostic technology, namely next-generation sequencing (NGS) techniques, genetic testing became widespread and less costly, allowing the diminished need for invasive diagnostic procedures. Molecular genetic testing is currently based on NGS and Sanger sequencing analysis primarily. Molecular testing is a rapid, non-invasive, and affordable procedure for the definite diagnosis of genetically inherited NMD's. Since now, 1079 different diseases and 608 different genes are associated with NMD. Sixty-six mapped loci are associated with NMD, and further investigations are needed for the new gene identification [http://www.musclegenetable.fr].

We know that one gene-one phenotype or vice versa is not a proper knowledge anymore. For example, the TTN gene is associated with six different phenotypes, while spinocerebellar ataxia (SCA) is associated with more than 40 genes [https://www.omim.org]. Having a definite diagnosis is an essential step for NMD patients to determine inheritance pattern, clinical course, prognosis, and potential therapeutic options. Genetic counseling will be possible only with a definite diagnosis, to screen the family members at risk and for reproductive options.

Oculopharyngodistal myopathy (OPDM) is an adult-onset slowly progressive myopathy, was first described by Satoyoshi and Kinoshita in 1977 [Satoyoshi and Kinoshita, 1977]. OPDM is inherited both in autosomal dominant (AD) and recessive (AR) forms and is characterized by ptosis, external ophthalmoplegia, facial/distal limb muscle weakness and atrophy, and laryngeal and pharyngeal involvement. Skeletal muscle biopsy shows myopathic changes with rimmed vacuoles. OPDM has been primarily reported from Turkey, China and Japan [Durmuş et al., 2011, Ishiura et al., 2019, Deng et al., 2020].

We aimed to determine the genetic etiopathogenesis of OPDM and mimicking disorders. If the responsible gene(s) for OPDM and mimicking diseases are identified, it will be beneficial for the patients and their families. Possible outcomes of the work-up are listed below.

- **Definite diagnosis** will be achieved without the need for interventional, invasive, or expensive investigations.
- **Genetic counseling** will impact the family planning of affected patients, and alternative reproductive options can be offered.
- **Presymptomatic screening**; if the targeted mutation testing is performed for the relatives who are at risk of developing the disease (over 18 years of age) before the onset of symptoms, they can plan their lives accordingly.
- It may be possible to determine the **phenotype-genotype correlation** with the families.
- It may be possible to initiate research projects on the **treatment of the disease**.

There was no gene associated with OPDM until August 2019. However, although four genes related to OPDM between August 2019 and November 2022, it is still not possible to genetically diagnose majority of OPDM patients.

To sum up, if the responsible gene is identified for all OPDM patients, as one of the three countries where the disease is the most common, Turkey's prestige in the scientific community will increase. Further research can be initiated for treatment options. The patients will also benefit from the knowledge of the inheritance pattern (which may affect family planning), the progress of the disease (planning of life), and possible therapeutic options.

Chapter 2

DIAGNOSIS OF OPDM AND MIMICKING DISORDERS

2.1 OPDM

OPDM was described for the first time in 1977 in six individuals from four unrelated families who showed common clinical findings. Satoyoshi and Kinoshita observed the weakness of the jaw, face, bulbar area, eye, and distal muscles of the patients. They stated that the disease was inherited in an autosomal dominant manner. No other systemic diseases were observed in the patients, and the initial finding of the disease was ptosis having an onset around 40 years of age. Muscle biopsy of the patients showed degeneration of fiber structure, vacuolar formation, increased internal nuclei/endomysial fat/connective tissue, and fiber diameter variation. Satoyoshi and Kinoshita listed oculopharyngeal muscular dystrophy (OPMD), myotonic dystrophy (DM1), and Kears-Shy syndromes in the differential diagnosis of OPDM. OPDM had been previously described as “distal involvement of the extremities in ocular myopathy.” After Satoyoshi and Kinoshita report a further six cases, it was renamed as “oculopharyngodistal myopathy” [Satoyoshi and Kinoshita, 1977].

The first AR form of OPDM was described by Uyama (et al., 1998) in two Japanese siblings who were born to second-degree cousins. The parents and four other siblings were healthy. The patients' first symptom was the weakness of the anterior tibialis muscles at the age of 35-40. Other symptoms such as bilateral ptosis, dysphagia, and distal dominant upper limb weakness, external ophthalmoplegia, developed over time. EMG revealed myogenic changes, CK levels were mildly elevated (1397 – 2500 units/l; normal values:32-166 unit/l), and muscle biopsy showed abnormal variation in fiber size and occasional rimmed vacuoles at atrophic fibers [Uyama et al., 1998]. Up till now, 363 OPDM cases have been reported in 16 various manuscripts. The first detailed progression of the disease in 43 patients from 9 families was described in Turkey [Durmuş et al., 2011]. The summary of manuscripts is shown in Table 2.1.

Table 2.1: Summary of all published manuscripts about OPDM

MS /date	No of affecteds	No of families	Inheritance	Biopsy	First symptom	Onset age	Country
Satoyoshi and Kinoshita, 1977	17	4	3 AD	myopathic pattern wo specific changes	ptosis, diplopia, distal muscle weakness	40-63	Japan
Amato et al., 1995	1	1	AD?	rimmed vacuole + fiber size variability	dysarthria, dysphagia	9	USA
Uyama et al., 1998	2	1	AR	rimmed vacuole + fiber size variability	Tibialis anterior weakness	35-40	Japan
Minami et al., 2001	4	4	4 AD	rimmed vacuole + fiber size variability	Ø	Ø	Japan
van der Sluijs et al., 2004	2	1	AR	rimmed vacuole + fiber size variability	walking on toes, ptosis	18-25	Netherlands
Witoonpanich et al., 2004	2	1	AR	rimmed vacuole	ptosis	33-35	Thailand
Lu et al., 2008	6	1	AR	rimmed vacuole + fiber size variability	distal muscle weakness	22	China
Thevathasan et al., 2010	2	1	AD	rimmed vacuole + fiber size variability	ptosis, diplopia	18-22	England
Durmuş et al., 2011	47	9	5AD 2AR 2?	rimmed vacuole + myopathic changes	ptosis, dysphagia	22	Turkey
Mignarri et al., 2012	1	1	AD	rimmed vacuole + fiber size variability	ptosis, dysphonia	32	Italy
Shimizu et al., 2013	1	1	AD?	rimmed vacuole + fiber size variability	ptosis	47	Japan
Zhao et al., 2015	14	10	1AD 2AR	rimmed vacuole + fiber size variability	ptosis, distal muscle weakness	14-36	China
Maeda et al., 2015	1	1	AD?	rimmed vacuole + muscular atrophy	ptosis	36	Brazil
Ishiura et al., 2019	34	34	AD	myopathic changes with rimmed vacuole	NA	NA	Japan
Deng et al., 2020	18	18	AD	myopathic changes with rimmed vacuole	NA	NA	China
Ogasawara et al., 2020	211	201	AD	intranuclear inclusions and rimmed vacuoles	NA	Adulthood	Japan
Yu et al., 2022	11	11	AD	rimmed vacuoles and filamentous intranuclear inclusions	ptosis and limited eye movements,	20-30	China

The most prominent features of OPDM are ocular (ptosis/extraocular) and pharyngeal (dysphagia, dysphonia, dysarthria) involvement. The first symptom of patients is usually ptosis or dysphagia; dysarthria occurs around five years later from the first symptom. The patients have respiratory insufficiency around ten years after the onset, and lose their ability to walk in twenty years after initial signs [Durmuş et al., 2010]. Intrafamilial and interfamilial clinical variability has been observed in both inheritance patterns. The disease's genetic cause is unknown for all OPDM patients, and it is hypothesized that the disease is genetically heterogeneous [Ishiura et al., 2019].

The diagnostic of OPDM is mainly based on clinical and histopathological findings.

Clinical findings include;

- Facial muscle atrophy/weakness (myopathic face),
- Pharyngeal/laryngeal weakness (dysphagia, dysphonia, dysarthria),
- Ptosis, external ophthalmoplegia,
- Distal/proximal muscle weakness, difficulty in walking,
- Respiratory insufficiency due to muscle weakness and recurrent pneumonia due to aspiration (later stages).

Laboratory and histopathological findings:

- Muscle biopsy showing myopathic changes with rimmed vacuoles (Figure 2.1),
- Serum CK level: normal or slightly increased (2-5 fold),
- EMG: myogenic changes (in some patients).

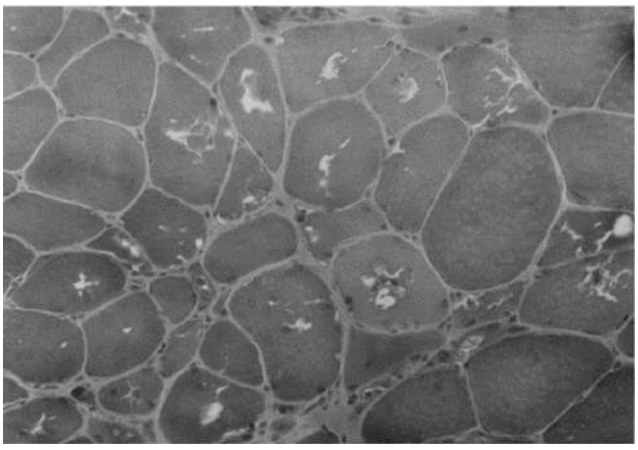


Figure 2.1: Histopathology of OPDM patient showing; abnormal variation in fiber sizes and abundant rimmed vacuoles [Uyama et al., 1997].

In all articles (Table 2.1), OPMD has been excluded by genetic testing, which is on the top of the differential diagnosis list for OPDM. In some manuscripts, in addition to OPMD, DM1, distal myopathy with rimmed vacuole (*GNE*-related), FSHD, PEO related genes (*POLG*) are also included in the differential diagnosis list. In one of the most recent articles published by Ishiura (et al., 2019), WGS analysis was performed in an OPDM patient, and CGG repeat expansion was detected in the *LRP12* gene 5'UTR region. *LRP12* was the first OPDM-related gene identified in 38% (OPDM1; 13/34) of the patients. Later, *GIPC1* gene 5'UTR GGC expansion (OPDM2; 12/24 patients; 50%), *NOTCH2NLC* noncoding region CGG repeat expansion (OPDM3; 7/211 patients; 0,03%), and *RILPL1* gene 5'UTR CGG expansion (OPDM4; 11/11 patients; 100%) is related with OPDM. These genes will be discussed in detail in the mimicking disorders part.

2.2 OPDM Mimicking Disorders

Differential diagnosis of OPDM is based on the clinical features of disorders mimicking OPDM. OPMD is at the top of the list due to the ocular and pharyngeal involvement and rimmed vacuoles on muscle biopsy. DM1 is in the list due to similar muscle involvement, and progressive external ophthalmoplegia (PEO) with mtDNA deletions are in the list due to extraocular muscle involvement, muscle weakness, and the age of onset of the disease. Although OPDM and OPDM1/2/3/4 are overlapping disorders, the genes identified in OPDM1/2/3/4 are not mutated in all OPDM patients. The following section briefly discusses the differential diagnosis list and its common/distinctive features.

2.2.1. Oculopharyngeal Muscular Dystrophy (OPMD)

OPMD was described in 1915 by Taylor (et al., 1915) as the combination of ptosis and pharyngeal palsy but was named 47 years later by Victor (et al., 1962). OPMD is an autosomal dominant progressive neuromuscular disorder characterized by ptosis, swallowing difficulties, and proximal muscle weakness at later stages of the disease. Other symptoms may include tongue weakness, wet voice due to pooling of saliva, mild limitation of extraocular muscles in upward gaze, and facial muscle weakness.

In OPMD patients, serum CK level may be normal or slightly elevated, EMG may be compatible with myopathy, and muscle biopsy shows rimmed vacuole and fiber size variation. The age of onset of the disease is the fourth-fifth decade of life [Trollet et al., 2001].

The prevalence of OPMD is 1/100,000 in Europe but higher in Bukhara Jews (1/700) and French Canadian (1/1,000) populations [De Braekeleer, 1991; Brais et al., 1998; Blumen et al., 2000].

Polyadenine binding protein nuclear (*PABPN1*) gene exon 1 pathological repeat expansions (GCG) are associated with OPMD [Brais et al., 1998]. *PABPN1* gene exon 1 contains (GCG)8-13 repeats at heterozygous state in patients, whereas the normal size is (GCG)6 repeat in the healthy population. Since now, only one pathogenic missense mutation was identified in the *PABPN1* gene. The mutation (c.35G>C) is located on exon 1 and causes glycine to alanine a substitution at 12th amino acid (p.Gly12Ala) [Robinson et al., 2006].

A study in the French population showed that there is a correlation between the size of the expansion and the severity/progression of the disease [Richard et al., 2017].

Genotype[a]	No.	Age at diagnosis, y, mean ± SD	CK, UI/L	Clinical symptoms		
				Ptosis	Dysphagia	Proximal weakness
Heterozygous						
10/11	6	72 ± 11	<100	±	+	Late
10/12	33	73 ± 10	<100	±	+	Late
10/13	176	64 ± 10	<100	+	+	Late
10/14	55	61 ± 8	<300	+	+	Late
10/15	56	60 ± 10	300-500	+/Diplopia	+/++	Yes
10/16	4	51 ± 6	500-1,000	+/Facial weakness	++	Yes/wheelchair
10/17	2	53 ± 4	700-1,000	++	+++	Yes
Compound heterozygous						
11/12	9	58 ± 11	300-600	+	+	Yes
Homozygous						
11/11	9	73 ± 7	<100-500	+	+	Yes
12/12	2	49 ± 1	300-500	++	++	Yes
13/13	2	37 ± 9	300-700	+++	++	Yes

Abbreviations: +/− = inconstant; + = mild; ++ = present; +++ = severe; CK = creatine kinase (normal value <100 UI/L).
[a]For clarity, genotype (GCN)x/(GCN)y is indicated as x/y.

Figure 2.2: Genotype-phenotype correlation of OPMD disease severity and *PABPN1* exon 1 (GCN) repeat expansion [from Richard et al., 2017].

Richard (et al., 2017) evaluated 354 unrelated OPMD patients and genotyped them for *PABPN1* gene (GCN) repeat expansion. They had previously described (GCG) repeat expansion in correlation with the disease. In some genetic diagnostic laboratories, the (GCN) repeat expansion is taken into consideration instead of (GCG) repeat, as Richard (et al., 2017) did. In the control population (GCG)6 or (GCN)10 repeats are observed. Figure 2.2 shows that as the number of repeat expansions increases, the onset age of the disease is getting earlier, and patients develop a more severe phenotype. The most severe phenotype was observed at (GCG)9/(GCG)9 or (GCN)13/(GCN)13 genotype as expected. The age of onset in the most severe phenotype is around 37±9, and all the patients have ptosis (severe), dysphagia, proximal muscle weakness, and increased serum CK level.

2.2.2. Myotonic Dystrophy 1 (DM1):

DM1; is an autosomal dominant disorder that affects multiple systems and causes myotonia, muscular dystrophy, cataracts, dysphagia, hypogonadism, frontal balding, intellectual disability, and electrocardiographic (ECG) changes. Skeletal and smooth muscles, eyes, heart, endocrine system, reproduction, and central nervous system may be affected in DM1 patients [Bird, 1999]. The prevalence of DM1 is estimated to be 1 in 20,000 worldwide [Theadom et al., 2014].

DM1 is caused by CTG repeat expansions of the dystrophia myotonica protein kinase (*DMPK*) gene, at a noncoding, untranslated (UTR) region. In a healthy population, the *DMPK* gene has 5-34 CTG repeat expansions at 3'UTR. DM1 patients are categorized into three groups depending on the molecular and clinical findings: mild DM1 (50-150 repeats), classic DM1 (100-1,000 repeats), and congenital DM1 (>1,000 repeats). Other than triplet repeat expansion, one missense mutation, c.625G>T; p.D209Y, has been considered as a possible pathogenic variant causing DM1 [Tian et al., 2015; Wang et al.; 2016].

The clinical symptoms and other characteristic features of DM1 phenotypes are described in Table 2.2 [International Myotonic Dystrophy Consortium, 2000].

Table 2.2: DM1 phenotypes and repeat size correlation.

DM1	Age of onset	Repeat size	Clinical signs	Lifespan
Mild	20-70 yrs	50-150	• Cataract • Myotonia	Normal
Classic	10-30 yrs	100-1,000	In addition to Mild DM1; • Muscle weakness • Cardiac arrhythmia • Balding	Shortened (48-55 yrs)
Congenital	Birth to 10 yrs	>1,000	In addition to Classical DM1; • Infantile hypotonia • Respiratory deficits • Intellectual disability	Early death (<45 yrs), stillbirth

In DM1 families, clinical anticipation is observed. *DMPK* gene alleles that are greater than (CTG)34 are unstable and may expand in length during meiosis. The parents who carry >(CTG)34 are at risk of having offspring with expanded repeats. The onset age of the disease and the severity increases in every successive generation [Bird et al., 1999]. In mild–congenital DM1 patients, the expanded allele is generally inherited on maternal allele [Martorell et al., 2007]. Paternal transmission of *DMPK* expanded alleles has also been reported [Moxley & Meola 2008].

Muscle biopsy is not a critical diagnostic procedure by conventional stains in DM1 patients. However, the increased central nuclei, ring fibers, pyknotic nuclear clumps, and selective atrophy of type 1 fibers are considered strongly suggestive for the diagnosis of DM1. Fiber atrophy is more profound in DM1 than other dystrophies [Thornton, 2014].

2.2.3. Progressive external ophthalmoplegia (PEO) with mtDNA deletions:

PEO with mtDNA deletion syndromes is characterized by ptosis, ophthalmoplegia, impaired eye movements due to paralysis of the extraocular muscles, and additional multisystemic features such as cerebellar ataxia, intellectual disability, sensorineural hearing loss, dysphagia, exercise intolerance, muscle weakness, cardiac problems, and endocrinopathy [Goldstein and Falk, 2013]. Ten nuclear-encoded genes are associated with the phenotype and both AD and AR inheritance have been reported (see Table 2.3).

Molecular diagnosis of PEO with mtDNA deletion syndrome; is based on mtDNA deletion analysis of the affected tissue or mutation analysis of nuclear-coded genes in the blood sample. The mtDNA deletion occurs between 1.1 to 10 kb, and mostly a single deletion pattern is shown in the affected tissue. mtDNA deletion analysis is not preferred as a first-step analysis due to invasive procedure (muscle biopsy requirement) or the difficulty of mtDNA deletion analysis. Muscle biopsy of PEO with mtDNA deletion patients may show; ragged-red fibers (RRF), decreased or no cytochrome c oxidase (COX) activity, hyperactive fibers with succinate dehydrogenase (SDH) activity, and abnormal mitochondria accumulation (See Figure 2.3). Biochemical studies of affected tissue also show decreased activities of complexes containing mtDNA-encoded subunits (I, III, IV) [Goldstein and Falk, 2013].

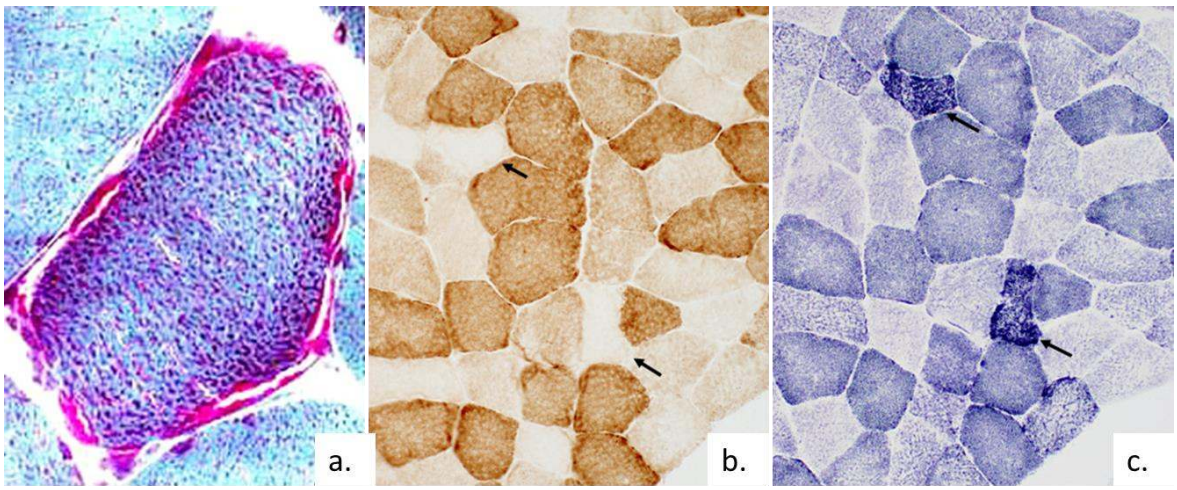


Figure 2.3: Muscle biopsy showing (a.) RRF, (b.) COX-negative fibers and (c.) excessive SDH stained fibers (https://neuromuscular.wustl.edu)

Table 2.3: PEO with mtDNA deletions syndrome-associated genes and other phenotypes

Gene	Abbr.	Onset	Inheritance	Other phenotypes
DGUOK	PEOB4	adult	AR	mtDNA depletion syndrome • 3 (hepatocerebral type) (AR)
DNA2	PEOA6	childhood - adulthood	AD	Seckel syndrome (AR)
POLG	PEOA1	adult	AD	mtDNA depletion syndrome • 4A (Alpers type) (AR)
POLG2	PEOA4	infancy to adulthood	AD	mtDNA depletion syndrome • 16 (hepatic type) (AR)
RNASEH1	PEOB2	adult	AR	
RRM2B	PEOA5	adult	AD	mtDNA depletion syndrome • 8B (MNGIE type) (AR) • 8A (encephalomyopathic type with renal tubulopathy) (AR)
SLC25A4	PEOA2	adult	AD	mtDNA depletion syndrome • 12A (cardiomyopathic type) (AD) • 12B (cardiomyopathic type) (AR)
TK2 (?)	PEOB3	adult	AR	mtDNA depletion syndrome • 2 (myopathic type) (AR)
TOP3A (?)	PEOB5	67 yrs (single case)	AR	Microcephaly, growth restriction, and increased sister chromatid exchange 2 (AR)
TWNK	PEOA3	adult	AD	Perrault syndrome 5 mtDNA depletion syndrome • 7 (hepatocerebral type) (AR)

In the literature, the *POLG* gene was considered as an overlapping disease due to similar clinical features of OPDM [Thevathasan et al., 2010, Durmuş et al., 2011, Zhao et al., 2015]. Since the *POLG* gene mutations are associated with PEO with mtDNA deletions, all PEO with mtDNA deletion-related genes are included in the differential diagnosis list.

PEOB4; is caused by biallelic mutations of the *DGUOK* gene. The disease's onset varies between 20-70 years of age, and clinical features of the disease are PEO, ptosis, muscle weakness (proximal), dysphonia, and dysphagia. Additional features (hearing loss, distal muscle weakness, mental retardation, rhabdomyolysis an others) may be observed in the patients. The severity of the disease varies between patients [Ronchi et al., 2012].

PEOA6; is a slowly progressing disorder characterized by PEO, ptosis, muscle weakness (lower limbs), and exercise intolerance. Myalgia, muscle cramps, obstructive sleep apnea may be seen in the patients. The age of onset ranges from childhood to adulthood. *DNA2* gene heterozygous mutations are responsible for PEOA6 [Ronchi et al., 2013].

PEOA1; is an AD, progressive, adult-onset disorder caused by heterozygous *POLG* mutations. The most common clinical features include PEO and muscle weakness. The less common symptoms are; dysphagia, cataracts, ataxia, parkinsonism, and hypogonadism [Hirano and DiMauro, 2001].

PEOA4; is a progressive disorder, and the severity of the disease varies. The onset of the disease ranges from infancy to adulthood. The disease's most common features are ptosis, PEO, and generalized muscle weakness. Developmental delay, seizures, cerebellar atrophy, and gastrointestinal symptoms may seen in the patients. *POLG2* mutations are responsible for PEOA4, and the inheritance pattern is AD [Young et al., 2011].

PEOB2; is caused by biallelic mutations of *RNASEH1,* and clinical features are ptosis, PEO, dysphagia, dysarthria, muscle weakness(proximal), and cerebellar atrophy. Respiratory insufficiency may be seen in the later stages of the disease. The onset varies from the early twenties to forties [Reyes et al., 2015]. Due to respiratory insufficiency and the onset of the disease, this is the most relevant overlapping gene with OPDM.

PEOA5; is characterized by ptosis, PEO, and proximal muscle weakness. Dysarthria, dysphagia, and anxiety may seen in patients. The age of onset is early twenties. *RRM2B* gene heterozygous mutations cause PEOA5 [Tyynismaa et al., 2009].

PEOA2; is caused by heterozygous mutations of *SLC25A4*(*ANT1*). PEO and ptosis are the most common features and the disease's onset is in adulthood. Hearing loss (sensorineural), exercise intolerance, and muscle weakness are the less common symptoms. [Kaukonen et al., 1999].

PEOB3; is suspected to be caused by biallelic mutations of *TK2*. Only one Finnish family has been reported so far. Two Finnish sisters had PEO, dysphagia, and proximal muscle weakness in their forties. One of them had in addition scapular winging and dysarthria [Tyynismaa et al., 2012].

PEOB5; is suspected to be associated with biallelic mutations of *TOP3A*. Up till now, one woman has been reported with PEOB5. She has slowly progressive ptosis, PEO, dysarthria, and proximal muscle weakness. She also had mild sensory neuropathy and cerebellar atrophy. Echocardiogram showed; impaired left ventricular function, ectopic beats, and tachycardia [Nicholls et al., 2018].

PEOA3; is an autosomal dominant, adult-onset myopathy caused by *TWNK* (*C10ORF2*) gene mutations. Clinical features of PEOA3 are PEO, ptosis, muscle weakness (proximal and distal), and exercise intolerance. The additional features are highly variable: psychomotor retardation, dysphagia, dysphonia, cardiovascular symptoms, and ataxia. One out of three PEO patients carries *TWNK* mutations [Fratter et al., 2010].

To sum up, in all PEO-related mitochondrial genes, *POLG, DNA2,* and especially *RNASEH1* have the most overlapping features with OPDM.

2.2.4. Oculopharyngodistal myopathy-1 (OPDM1; #164310):

OPDM1 (OPDM type 1) is an adult-onset disease characterized by ptosis, external ophthalmoplegia, facial muscle weakness, distal limb muscle weakness/atrophy, and pharyngeal involvement. OPDM1 is inherited in AD manner. Muscle biopsy of OPDM1 shows myopathic changes with rimmed vacuoles [Ishiura, 2019].

Ishiura (et al., 2019); performed whole-genome sequencing (WGS) to a familial OPDM patient and directly searched for CGG repeat expansions. They identified the *LRP12* gene 5'UTR region CGG repeat expansion (>150) in a group of OPDM patients (13/34; 38.2%) and named this group as OPDM1.

Ishiura (et al., 2019) screened clinically diagnosed OPDM patients who did not show rimmed vacuoles on their muscle biopsy for repeat expansion. None of the 19 patients carried the expanded repeat. When they screened their cohort (OPDM clinical phenotype without muscle biopsy specimen/result; 54 patients) for the CGG repeat expansion of the *LRP12* gene, they found nine additional cases (16.7%) who have expanded repeat expansion.

When the control group (1000 subjects) was checked for *LRP12* gene 5'UTR CGG repeat expansion, 998 subjects had a repeat of 13-45, while 2 subjects had expanded alleles. They stated in the article, as with the expansion of repeats observed in healthy controls of Fragile-X and *C9orf72*-related ALS at a low rate (Fragile-X; in men: 0.21%, United States and *C9orf72;* 0.4%, United Kingdom), could be the explanation of the repeat expansion in the *LRP12* gene in the healthy population (0.2%)

2.2.5. Oculopharyngodistal myopathy-2 (OPDM2; #618940):

Oculopharyngodistal myopathy-2 (OPDM2) is characterized by distal muscle weakness and/or PEO as an initial symptom, and the onset of the disease is 20-30s. The inheritance of the disorder is AD. Later in the patient's life, they develop facial muscle weakness and difficulty of walking or climbing stairs due to the disease's progressive nature. In most patients, they have lower limb involvement, but they noticed upper limb involvement and respiratory insufficiency in some cases. OPDM2 patients' muscle biopsy shows myopathic changes with abnormal cytoplasmic and intranuclear inclusions [Deng et al., 2020].

Deng (et al., 2020) screened eight familial and 16 sporadic OPDM cases. They excluded OPMD, DM1, OPDM1, and all known genes by NGS analysis. In one family, genome-wide linkage analysis was performed on 16 family members. Five cases and 100 healthy controls' WGS data were checked for short tandem repeat (STR) comparatively. *GIPC1* gene 5'UTR GGC repeat region was significantly had the highest score in the disease association. OPDM2 patients had more than 100 repeats, while healthy controls had 13-32 repeats.

12 out of 24 cases (50%) had increased GGC expansion at *GIPC1* gene 5'UTR. When they identified the gene in Chinese OPDM patients, they collaborated with Japanese groups and screened additional 194 patients. Seven out of 194 (3,61%) of OPDM patients had increased GGC expansion at *GIPC1* gene.

The analysis of *GIPC1* gene 5'UTR GGC expansions showed that the mRNA level of GIPC1 is elevated in patients suggesting that it may have a toxic effect on the cells while the protein level is not altered.

2.2.6. Oculopharyngodistal myopathy-3 (OPDM3; #619473):

OPDM3 is a rare progressive muscle disease affecting ocular, pharyngeal, and distal limb muscles. Rimmed vacuoles are seen in muscle histopathology. Dysphagia, dysarthria, and muscle weakness are observed in some patients with Neuronal intranuclear inclusion disease (NIID), but OPDM is not included in the differential diagnosis since the most characteristic features of OPDM "ptosis/PEO or rimmed vacuoles" are not seen in NIID. Ogasawara et al. (2020) screened patients diagnosed with OPDM for *NOTCH2NLC* gene 5'UTR CGG repeat expansions which is previously related to NIID [Ishiura et al., 2020].

Two hundred eleven clinically diagnosed OPDM patients from 201 families screened for CGG expansions in the *NOTCH2NLC* gene, and seven unrelated OPDM patients showed increased repeat size. These patients had some additional clinical features of NIID, such as leukoencephalopathy, retinal degeneration, and tremor/ataxia [Ogasawara et al., 2020]. *NOTCH2NLC* gene GGC repeat expansion was further checked in 1000 sporadic Parkinson disease (PD) patients and 1076 controls. 13 patients had expanded allele (greater than 40) while none of the controls have the variant [Ma et al., 2020]. Three different phenotypes up till then have been associated with *NOTCH2NLC* gene repeat expansions: NIID, OPDM, and Parkinson's disease.

2.2.7. Oculopharyngodistal myopathy-4 (OPDM4; #619790):

The phenotype of OPDM4 overlaps with other OPDM phenotypes, and CGG repeat expansion was detected in the 5'UTR region of the RILPL1 gene. The number of CGG repeats was observed between 139-197 in 11 Chinese patients, while the number of repeats was found between 9-16 in the control group. The increase in CGG in the 5'UTR region in all four OPDM phenotypes suggests that the disease mechanism overlaps with this mechanism.

2.3 Summary of OPDM and Mimicking Disorders

OPDM and OPMD are two similar disorders. Ptosis, dysphagia, and muscle weakness are observed in both diseases, but proximal muscle weakness is more evident in OPMD while distal muscle weakness in OPDM. Since muscle weakness is not usually observed in the early stages of the disease, it is highly likely to confuse these two phenotypes. “Rimmed vacuoles" are also observed in muscle biopsy in both disorders. One of the distinguishing features is the age of onset of the disease. Although OPMD symptoms are observed at a later age, at the 5th and 6th decades, as the number of repeats increases early age of onset and more severe phenotypes are encountered. Therefore, it is crucial to exclude OPMD whenever OPDM is considered in the diagnosis. In cases where the disease is more severe or the onset is earlier, both the increase in the number of repeat expansions and biallelic inheritance of OPMD should be considered. Biallelic inheritance should be taken into account in all populations where high consanguinity rates are observed.

The common features of OPDM and DM1 are ptosis, dysphagia, and distal muscle weakness, while DM1 patients also show myotonia. When performing the neurological examination the finding of myotonia should lead to DM1 diagnosis. Anticipation is also a highly specific observation in family trees. In DM1 cases, the age of onset gets earlier in successive generations and more severe phenotype. When muscle biopsy is performed in DM1, "atrophy of type 1 fiber" is observed. Muscle biopsy, myotonia, and anticipation are the distinctive features of DM1 from OPDM.

PEO with mtDNA deletions have been associated with ten different genes. Some have AD inheritance while others have AR forms. Although different phenotypes are observed in PEO with mtDNA deletions, findings of OPDM overlap especially in the PEOB2 (*RNASEH1*-related) phenotype. Ptosis, PEO, dysphagia, respiratory failure, and muscle weakness are observed in both disorders. Although other PEO types also show signs of ptosis and PEO, additional different findings may occur.

PEOB2 should be considered at the top of the differential diagnosis list when both findings, dysphagia and respiratory failure are observed. PEOB2 is inherited autosomal recessively and distinguishing features are muscle biopsy and cerebellar findings. Mitochondrial signs (RRF, COX-negative, SDH-positive) are observed in the muscle biopsy of PEOB2 patients and, elevated serum lactate levels may accompany. Cerebellar atrophy is an additional feature revealed by cranial MRI from the early stages of the disease, and it progresses over the years.

OPDM and OPDM1, OPDM2, OPDM3, OPDM4 are overlapping phenotypes. Although there is no phenotypic difference between them, OPDM1, OPDM2, OPDM3, and OPDM4 are inherited in only AD manner. Genetic etiopathogenesis have not been identified in all OPDM patients up till now.

The features of OPDM and all four mimicking disorders are summarized in Table 2.4.

Table 2.4: Features of OPDM and mimicking disorders

	OPDM	OPMD	DM1	PEO	OPDM1	OPDM2	OPDM3	OPDM4
Gene(s)	n.a.	PABPN1	DMPK	Several (see table 2.3)	LRP12	GIPC1	NOTCH2NLC	RILPL1
Mutation location	n.a.	exon 1	3'UTR	Exonic/splice	5'UTR	5'UTR	5'UTR	5'UTR
Mutation type	n.a.	triplet repeat	triplet repeat	SNV	triplet repeat	triplet repeat	triplet repeat	triplet repeat
Inheritance	AD/AR	AD	AD	AD/AR	AD	AD	AD	AD
Onset age	adult	late-onset	infancy to adulthood	infancy to adulthood	adult	adult	adult	adult
Ocular	+	+	+	+	+	+	+	+
Pharyngeal	+	+	+	+	+	+	+	+
limb	D>P	P>D	D		D>P	D>P	D>P	D>P
Other findings								
MRI	-	n.r.	White matter hyperintense lesions	+	n.r.	n.r.	Leukoencephalopathy	n.r.
histopathological	rimmed vacuole	rimmed vacuole	-	RRF, COX negative, SDH positive	myopathic changes with rimmed vacuole	myopathic changes with rimmed vacuole	intranuclear inclusions and rimmed vacuoles	rimmed vacuoles and filamentous intranuclear inclusions

n.a.:not applicable, **n.r.:**not reported, **D:** distal, **P:** proximal, **SNV:** single nucleotide variant

Chapter 3

MATERIAL and METHODS

Seven cases from seven different families were included who were referred from Koç University Hospital (KUH) Neurology Department Neuromuscular Diseases Division to the Medical Genetics Department with a preliminary clinical diagnosis of OPDM. The index patients and several other family members were consulted, sampled (blood and muscle biopsy) and investigations were performed between March 2018 – March 2020 after obtaining informed consent.

3.1 Patients

A physical examination of the index case and family members was performed, and detailed disease history was obtained at the first visit. The pedigrees were drawn according to the declaration of index cases. Pedigrees were updated at each visit. Patient examination forms (given as Appendix A) were filled by neurologists or medical geneticists who are experts in neuromuscular disorders.

Family 1 (A.A.Y. Family): The index case had his first symptom, ptosis, at the age of 60. At 62-63 years of age, he developed dysphagia and walking difficulty. Family history revealed several similarly affected members (See Figure 3.1). He had a healthy son and daughter, aged 29 and 30 respectively. Parents of A.A.Y. are not consanguineous.

Physical examination of A.A.Y. showed bilateral ptosis, restriction of upward gaze, weakness of the tongue, and distal extremity muscles. He was wheelchair-dependent, but he still could walk without support for a short distance. CK level was slightly increased (396 U/L, normal range: 22-198 U/L), EMG was myopathic accompanied by myotonia. Outer center muscle biopsy was consistent with "myopathic changes," and cranial MRI was reported as normal.

The pedigree and clinical information were consistent with the autosomal dominant form of OPDM.

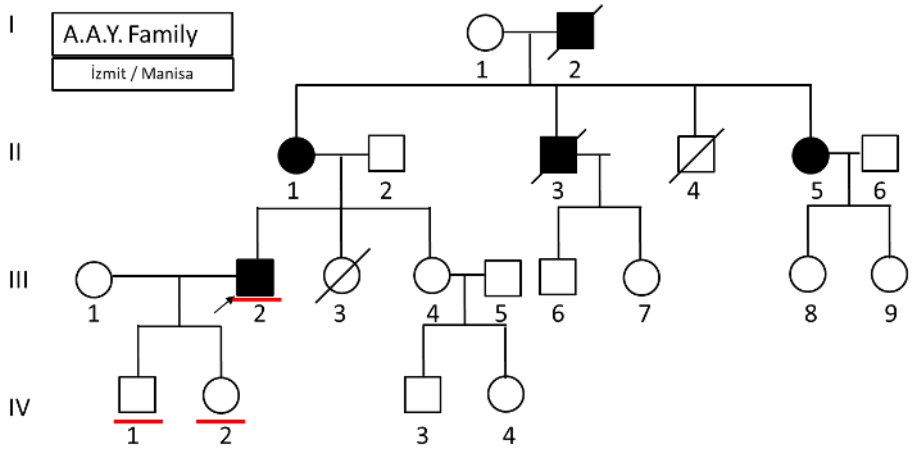


Figure 3.1: Pedigree of Family 1 (A.A.Y.)

Family 2 (F.K.): F.K.'s parents are first-degree cousins, and according to F.K.'s declaration, she is the only one affected in the family. The first symptom was bilateral droopy eyelids at 44 yrs of age, and she was operated on. She developed dysphagia (45 yrs), dysphonia (45 yrs), walking difficulty (55 yrs), respiratory difficulty (55 yrs), and facial muscle weakness. No cranial MRI, CK level, or other testing was performed. The autosomal recessive form of OPDM was considered with the pedigree evaluation.

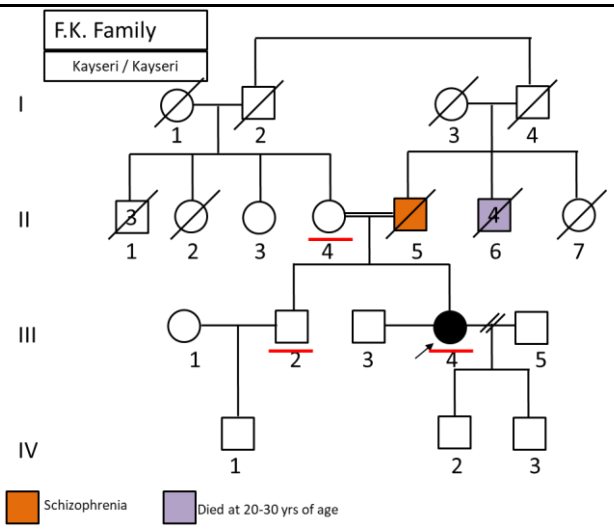


Figure 3.2: Pedigree of Family 2 (F.K.)

Family 3 (Y.İ.): Y.İ. was born to consanguineous parents. Y.İ. stated that his mother has reported muscle weakness of him since childhood. At 34 yrs of age, he developed bilateral ptosis and dysphagia. One year later, he experienced dysphonia and unsteady gait. He had many family members who are similarly affected. At physical examination, external ophthalmoplegia and cerebellar signs were also noticed. EMG revealed myogenic involvement, and outer center cranial MRI reported slightly widened 4th ventricles, arachnoid cyst, nonspecific gliotic foci at the cerebral subcortical white matter. CK level was in the normal range. The muscle biopsy was not performed. Clinical findings were suggestive for AR form of OPDM.

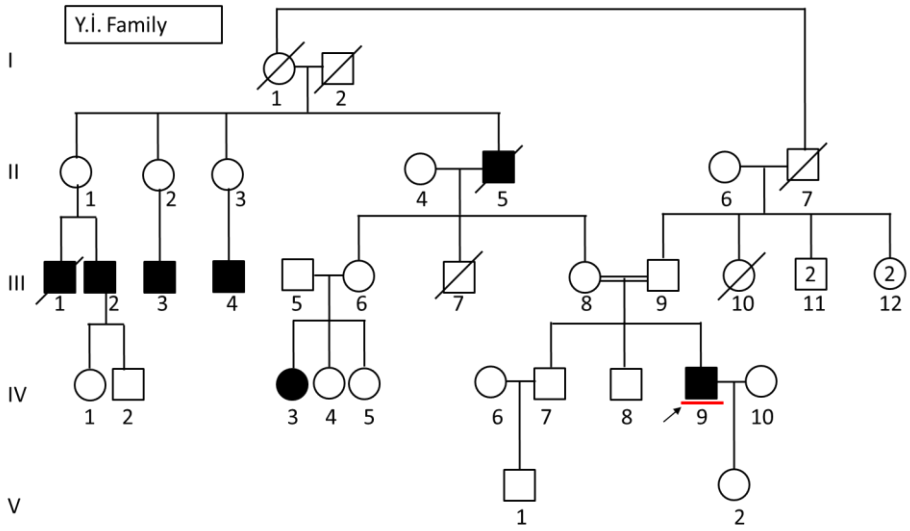


Figure 3.3: Pedigree of Family 3 (Y.İ.)

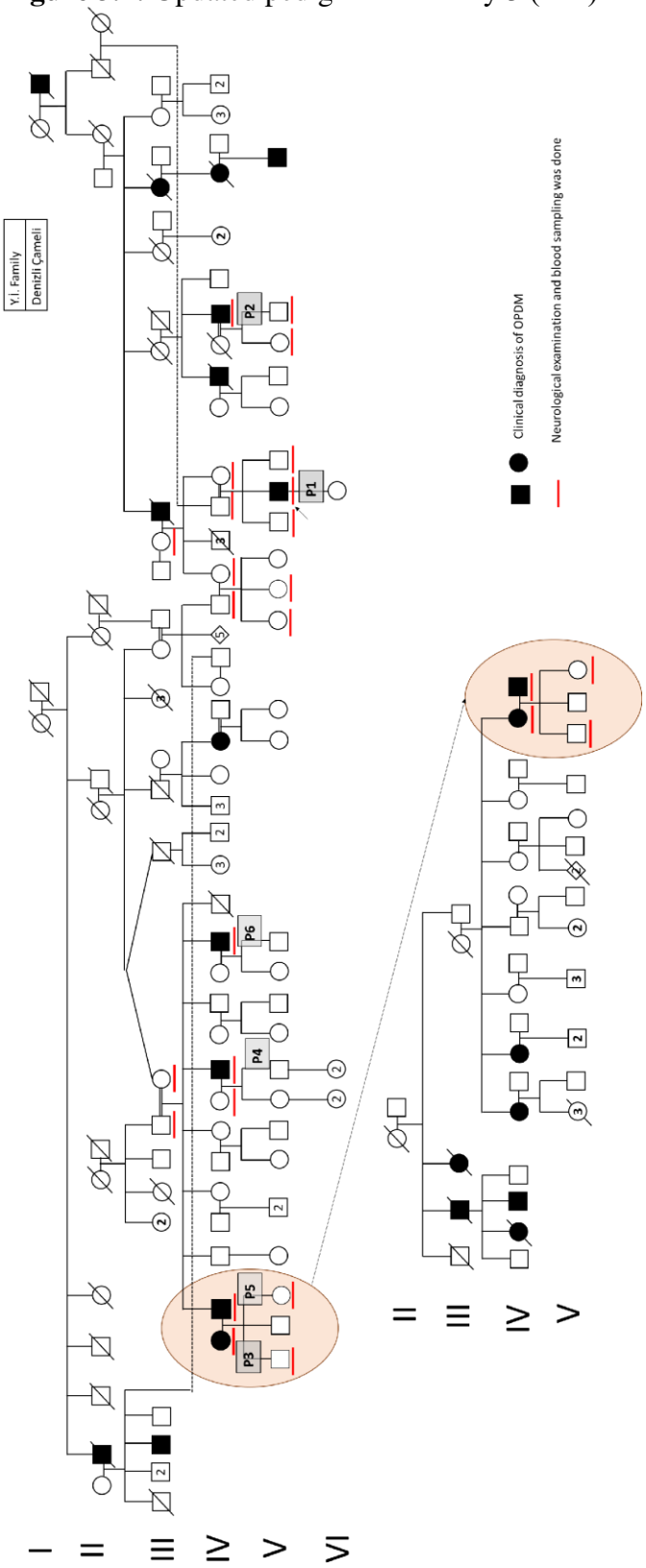
Home visits were planned for Y.İ.'s affected family members who live in Aydın, Denizli, and Antalya. The pedigree was updated at each home visit (see Figure 3.4). Six affected family members were examined, and blood samples were collected from all affected and unaffected relatives. Examined members are underlined with a red line on the updated pedigree (Figure 3.4), and the summary of the clinical examination is shown in the table (Table 3.1).

Table 3.1: Clinical finding of the affected patients in Family 3

	Y.İ.	**M.Ö.**	**A.O.**	**H.O.**	**A.İ.O.**	**B.O.**
date of birth (year)	1984	1971	1978	1966	1977	1971
age at examination	35	48	41	53	42	48
sex	M	M	F	M	M	M
consanguinity	+					
first symptom	ptosis	muscle weakness (D)	ptosis (left)	ptosis	ptosis	muscle weakness (D)
age of onset	30	30	30	24	30	37
ptosis	+ (30)	+ (32)	+ (30)	+ (24)	+ (30)	+ (39)
PEO	+	+	+	+	+	+
dysphagia	+ (32)	+ (35)	-	+ (43)	+ (32)	+ (41)
dysarthria	+ (34)	+ (35)	+ (30)	+ (48)	+ (32)	+ (39)
walking difficulty	-	+ (30)	+ (38)	+ (38)	+ (35)	+ (37)
wheelchair dependence	-	+ (40)	-	-		
facial involvement						
orbicularis oculi	+ (mild)	+ (mild)	+ (mild)	+ (mild)	+ (mild)	+
malar	+ (mild)	+	+ (mild)	+ (mild)	+ (mild)	+
zygomatic	+ (mild)	+	+ (mild)	+ (mild)	-	+
respiratory insufficiency	-	-	-	- (RLI)	+ / -	-
DTR	+	-	NA	NA	NA	NA
muscle strength scale						
neck flexion/extension	5 - 5 /5	4 - 4 / 5	3 - 5 /5	2 - 5 / 5	3 - 4 / 5	3 - 5 / 5
upper extremity D-P	5 - 5 /5	4 - 4 / 5	4 - 5 /5	4 - 5 /5	4 - 5 / 5	4 - 5 / 5
lower extremity D-P	5 - 5 /5	4 - 4 / 5	5 - 5 / 5	4 - 5 /5	4 - 5 / 5	5 - 5 / 5

D:distal; P:proximal; RLI: recurrent lung infection

Figure 3.4: Updated pedigree of Family 3 (Y.İ.)



Family 4 (E.G.): E.G., was born to second-degree cousin marriage (see Figure 3.5). He and his brother (N.G.) had symptoms of NMD since 18-22 years of age. E.G., had unilateral (left) ptosis at 18 years old. Ten years later, swallowing difficulty and dysphonia accompanied. He had lost 35 kg in the past five years. Neurological examination at 38 years of age showed external ophthalmoparesis and facial muscle weakness. DTR was absent. Neck and lower-proximal muscle strength were decreased. He had a muscle biopsy performed in Germany, but there was no access to the reports.

E.G.(IV-4)'s brother N.G.(IV-2)'s first symptom was nasal speech at 22 years of age. Ptosis and dysphagia developed at 28 yrs of age, ophthalmoparesis, and dysphagia at 30 years of age. Neurological examination at 39 yrs of age showed facial and lower distal muscle weakness. He could walk with a cane or support. N.G.'s upper muscle strength was much better than lower distal muscles. The findings and family history were considered as suggestive for the AR form of OPDM.

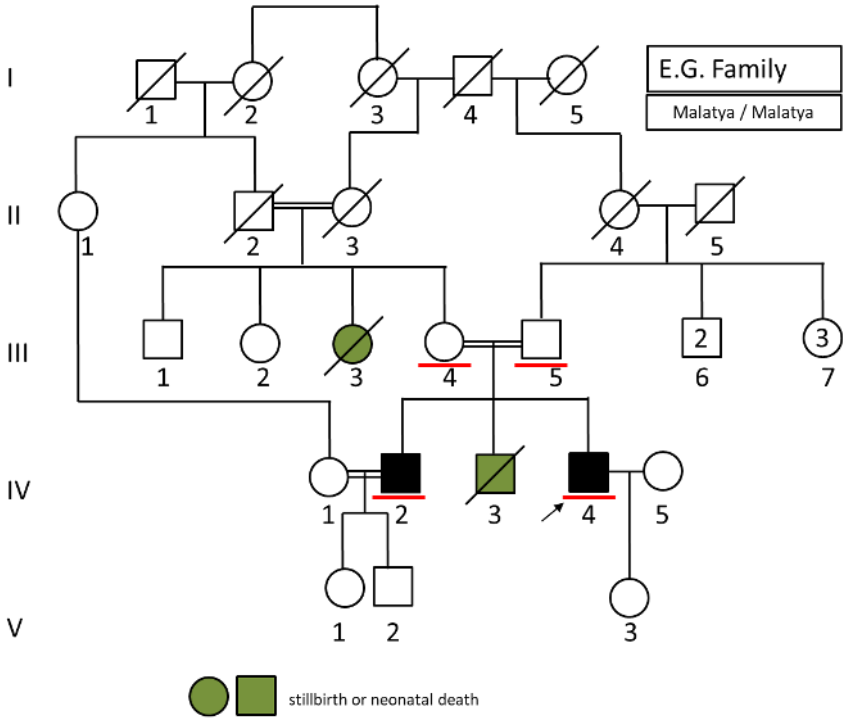


Figure 3.5: Pedigree of Family 4 (E.G.)

Family 5 (M.S.): M.S.'s first symptom was droopy eyelids at 30 yrs of age and was operated on at 36 yrs of age. EMG was suggestive of myopathic involvement. For the past five years, he had weakness of lower extremities with onset at 41 years of age. He could walk unsteadily and had difficulty in climbing stairs. He spoke with a slightly hoarse voice, had swallowing difficulty with solid foods.

Neurological examination at 46 years of age revealed external ophthalmoplegia. Muscle biopsy showed rimmed vacuoles at an outer center in 2016. His CK level was slightly increased (4-7 times, 795, 1463 U/L, normal range: 22-198 U/L). His family history revealed that his paternal uncle's two children, one girl and one boy, and two grandchildren of each affected with a neuromuscular disease which was more severe and fatal in the second and third decades. There were no medical reports related to affected patients in the family. The clinical features and family history were considered suggestive for AD or AR form of OPDM.

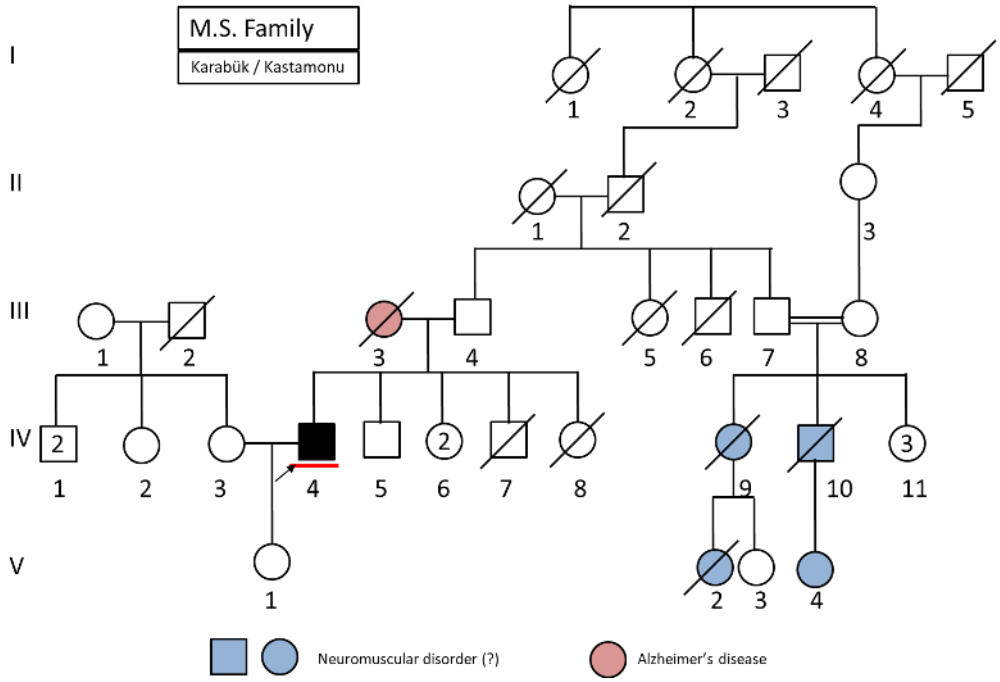


Figure 3.6: Pedigree of Family 5 (M.S.)

Family 6 (M.G.): M.G.'s first symptom was droopy eyelids at 20 yrs. His parents are not consanguineous but from the same small village. In his thirties, dysphonia became evident, and later dysphagia developed. Neurological examination at the age of 39 revealed external ophthalmoparesis. His father (Y.G.) and brother (F.G.) were similarly affected. His father had ptosis, dysphonia, dysphagia, and distal muscle weakness. He could walk with a cane. His symptoms had developed at the age of 25. Y.G.'s muscle biopsy was performed at an outer center which stated the presence of rimmed vacuoles, as being suggestive for OPMD/OPDM. Affected brother F.G.; had ptosis, dysphagia, and dysphonia at the age of 33. Family history and clinical findings were considered as suggestive for AD (AR?) form of OPDM.

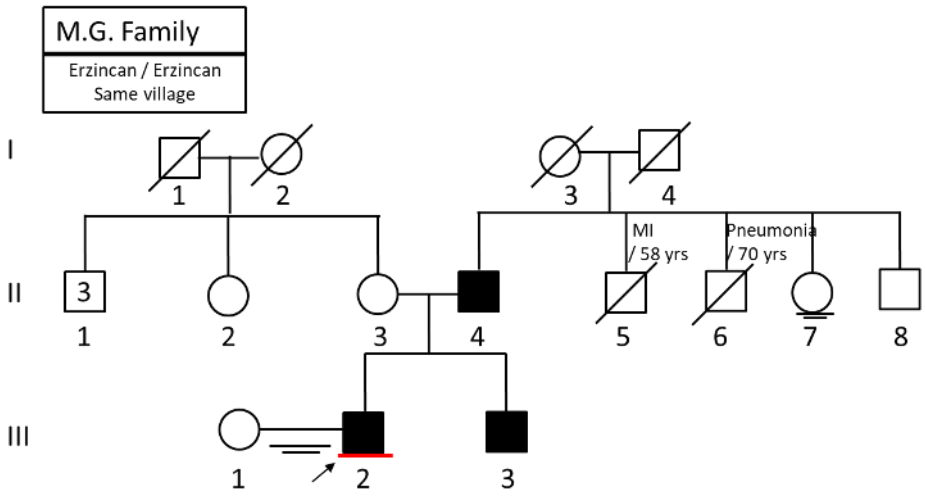


Figure 3.7: Pedigree of Family 6 (M.G.)

Family 7 (M.A.B.): M.A.B.'s first symptom was droopy eyelids at 62 yrs. Dysphonia and slurred speech became overt at 63 yrs of age. Neurological examination at the age of 64 revealed external ophthalmoparesis. Cranial MRI of the index case was reported as cerebral/cerebellar atrophy and millimetric ischemic gliotic lesions in frontoparietal subcortical white matter and periventricular white matter bilaterally and centrum semiovale. M.A.B.'s parents are not consanguineous but from the same village.

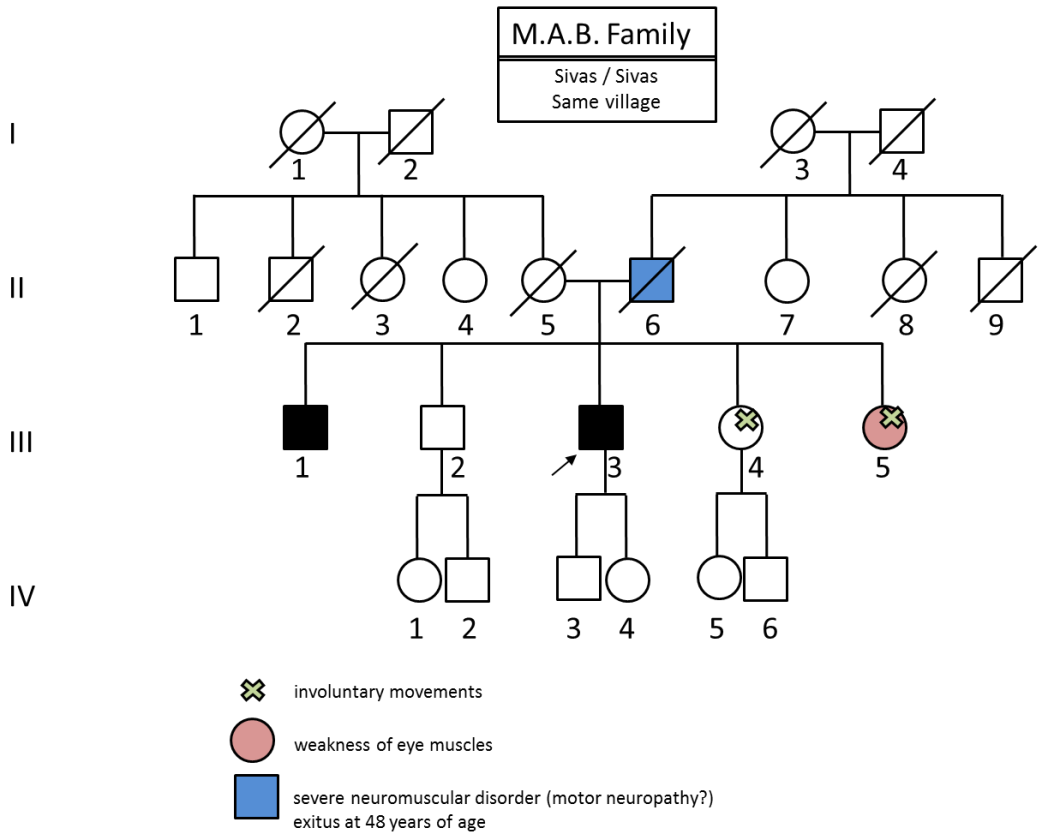


Figure 3.8: Pedigree of Family 7 (M.A.B.)

M.A.B.'s elder brother (III-1) had similar symptoms with muscle weakness of his legs. Younger sister (III-5) had involuntary movements like her elder sister (III-4) and also eye muscle weakness. The parents are not consanguineous but from the same village. Father (II-6) died at 48 years of age due to progressive neuromuscular disease. He has had dysphagia, walking difficulty, and drooling. The neurologist had a preliminary diagnosis of motor neuropathies, but there was no clinical/molecular diagnostic report.

All the index cases had ptosis, external ophthalmoplegia, facial muscle weakness, and dysphagia. dysarthria (except Family 5) and muscle weakness (except the index of Family 3) were observed in patients. Cerebellar signs with different severity were observed in Family 3. The age of onset varied between 18-62 years of age mean age being 37. Ptosis or dysphagia was generally the first symptom of the disease. All index cases were male beside patient 2. Four pedigree was compatible with AR inheritance, one with AD form, while two of them (Family 5 and 6) are not certain. Only four cases had muscle biopsies, and three biopsies showed rimmed vacuoles. The summary of all affected cases' clinical features is shown in Table 3.2.

Table 3.2: Clinical findings of the probands in seven families

Family no	1	2	3	4	5	6	7
Family name	A.A.Y.	F.K.	Y.İ.	E.G.	M.S.	M.G.	M.A.B.
Pedigree index	III-2	III-4	IV-9	IV-4	IV-4	III-2	
Gender	M	F	M	M	M	M	M
Age of onset	55	44	32	18	30	20	62
First symptom	dysphagia	dysphagia	ptosis	ptosis	ptosis	ptosis	ptosis
Inheritance pattern	AD	AR	AR	AR	AD-AR	AD(AR?)	AR
Affected family members	Yes	Yes	Yes	Yes	No	Yes	Yes
Facial muscle weakness	+	+	+	+	+	+	+
Myopathic face	+	+	+	+	+	+	+
Ptosis	+	+	+	+	+	+	+
External ophtalmoplegia	+	+	+	+	+	+	+
Respiratory insufficiency	-	-	-	-	-	-	-
Dysarthria / nasal voice	+	+	+	+	-	+	+
Dysphagia	+	+	+	+	+	+	-
Proximal muscle weakness	-	-	-	+	+	+	-
Distal muscle weakness	+	+	-	+	+	+	-
Myotonia	+	-			-	-	-
EMG results	Myotonic	n.a.	Myogenic	n.a.	Myopathic	n.a.	
Muscle biopsy	Myopathic	RV	n.a.	n.a.	RV	RV	n.a.
Normal or slightly elevated CK level	+	n.a.	+	n.a.	+	n.a.	+

n.a.: not available; RV: rimmed vacuoles

3.2 Biological samples

Biological samples were obtained from all affected patients and family members with informed consent. The study was approved by the ethical committee of Koç University (approval no: 2019.051.IRB2.019; given as Appendix B). Blood, skin, and muscle biopsy samples from affected patients, non-affected family members, and healthy controls (age, and sex-matched) were obtained and stored for molecular testing and functional analysis.

Blood: Routine blood collection procedure was applied. Peripheral blood samples were collected to dry K_2EDTA tubes and kept at 4°C.

• Blood samples were collected both from affected and non-affected (all sampled individuals are shown with a red line on pedigrees).

Skin biopsy: Biopsies were obtained from the patients' and healthy controls' medial forearms. Biopsy was performed on the arm (usually the left arm) that patients did not use. Forearm medial side was sterilized with alcohol and shaved. The local anesthetic agent was applied intracutaneously. During the waiting period of local anesthesia, the area to be biopsied was sterilized with Bactrim (combination of two antibiotics: sulfamethoxazole and trimethoprim). After one minute of sterilization, a punch biopsy tool (3 mm in diameter) was applied, and the dermis, epidermis layer was cut. The biopsy sample was transferred to a medium with antibiotics directly. The biopsied area was covered with sterile gauze, and suggestion to change the dressing two days later.

• Skin biopsy samples were collected from Family 3 (index), Family 4 (index and affected brother). Healthy controls (H.C.-1, H.C.-2, and H.C.-3) were chosen from age-matched (one female and two males) subjects who do not have any clinical sign of neuromuscular disease or NMD affected in their families.

Muscle biopsy: The muscle (affected) biopsy was obtained under sterile conditions by a neuromuscular disease specialist. One specimen was snap-frozen for muscle histochemistry and enzyme-histochemistry. One other specimen was directly transferred to medium with antibiotics and was transferred to the cell culture lab for functional studies (and ¼ of the tissue is stored at -80 degrees for future studies).

• Muscle biopsy was obtained from the index (Y.İ.) of Family3.

Cell culture (fibroblast and muscle tissue culture): Biopsy samples (skin and biceps muscle) were cut into small pieces by a surgical blade. Collagenase IV was added to minced tissue (3-5 cc) and incubated at 37°C for 1 hour. After incubation, collagenase IV was discarded, and trypsin was added (3-5 cc) and incubated at 37°C for 1,5 hours. After incubation, cells were collected in a falcon tube and centrifuged at 1,200 rpm for 10 minutes. The supernatant was discarded, and cells were washed with PBS twice (5 ml PBS was added, cells were dissolved and centrifuge at 1,200 rpm for 10 min, PBS discarded). Cells were resuspended at 5 ml of DMEM (with high glucose + %10 FBS + antibiotic cocktail) and transferred to the T25 flask. When cells were reached >70 percent confluency, cells were passaged into one T75 flask (Passage 1 – P1). Passage 2 (P-2) cells were transferred into three T75 flasks after trypsinization.

3.3. DNA/ RNA/Protein isolation

DNA, RNA, and cDNA quantification was performed by spectrophotometric method at 260 (for DNA and cDNA) and 280 nm (for RNA) by Nanodrop 2000 (Thermo Fisher).

DNA isolation: QIAmp DNA mini kit (Qiagen, Germany) was used to isolate DNA from blood, cell culture, and tissues as the manufacturer's protocol. As a quality control, the DNA (>50 ng/µl), A260/A280 (between 1.8-2.0), and A260/A230 (1.8-2.0) ratios were checked. If needed, isolation procedures were reperformed.

RNA isolation and cDNA synthesis: Hybrid-R kit (GeneAll Biotechnology Co. Ltd, Korea) was used to isolate total RNA from blood and cell culture. The concentration of RNA (>50 ng/µl), A260/A280 (between 1.8-2.0), and A260/A230 (1.8-2.0) ratios were checked as quality control. First-strand cDNA was synthesized from total RNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany).

Protein isolation: Cultured cells were resuspended with RIPA buffer + 1X protease buffer (Roche) + 1X phosphatase buffer (Roche) and incubated on ice for 30 minutes. After centrifugation at 17.000rcf for 30 minutes at 4°C, the supernatant was transferred into a clean eppendorf tube and kept at -20°C till used. Protein levels were measured by Bradford assay BIO-RAD).

3.4. PCR: To exclude DM1 and OPDM1-2 by fragmentation analysis, triplet repeat primed PCR (TP-PCR) was applied to DNA. Warner developed the TP-PCR method (et al., 1996) to analyze CTG repeat expansions of the *DMPK* gene on DM1 patients. The primer list is mentioned on Table 3.3. TP-PCR; results in a mixture of different sizes of fragments that can be analyzed by fragment analysis as seen on Figure 3.9. The limitation of the TP-PCR method is that high-sized repeat expansions can obtain no reliable info due to the extinction of higher-sized region signals (Figure 3.10). DM1 and OPDM1-2 will be excluded by TP-PCR described previously [Kamsteeg et al., 2012; Ishiura et al., 2019].

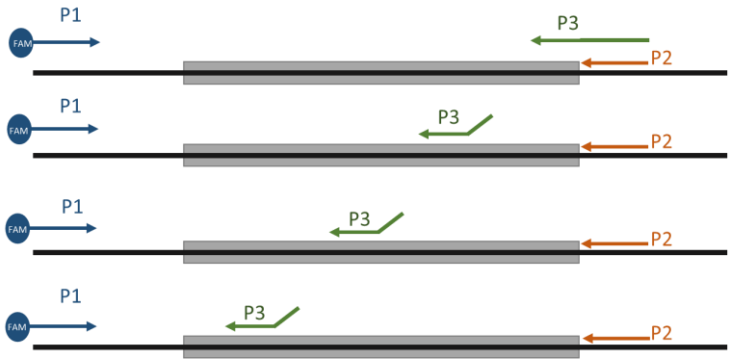


Figure 3.9: Schematic view of TP-PCR. The gray area represents the "CTG" repeat region. P1: Fluoresecently (FAM) labeled forward primer, P2: Reverse primer starting before CTG repeat, P3: Reverse primer starting with 5 "CTG" repeats lets to amplify the different size of PCR products.

Table 3.3: The primer list used to exclude DM1 and OPDM1-2-3

Genes	P1	P2	P3
DM1	gaagggtccttgtagccgggaa	tacgcatcccagtttgagacg	tacgcatcccagtttgagacg(cag)5
LRP12	ccgggagctgcatgtgtcagagg	ggagggaggagaagctggaggtagacg	ccgggagctgcatgtgtcagagg(cgg)5
GIPC1	cagacacatccttctcgcagaggccac	caggaaacagctatgacc	caggaaacagctatgaccggcggaggcagc(ggc)3
NOTCH2NLC	agcgcccacagcagagcggc	ccgggagctgcatgtgtcagagg	ccgggagctgcatgtgtcagagg(cgg)5

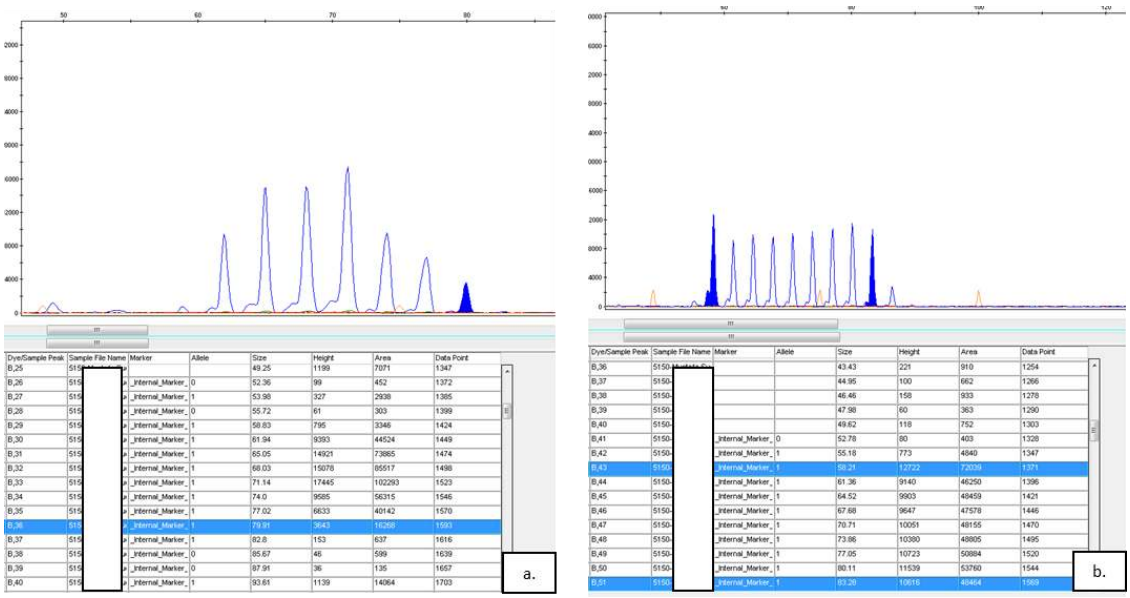


Figure 3.10: The normal range repeat expansions of (a.) *LRP12* and (b.) *DMPK* gene by TP-PCR.

3.5. Sanger sequencing: Sequence analysis of the genes listed below was performed using an ABI PRISM 3500xL Genetic Analyzer (Applied Biosystems®). For sequencing PCR; both forward and reverse primers are used in different tubes. Sequence analysis was done by using FinchTV (Geospiza) and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

***PABPN1* gene exon 1:** Short GCG repeat expansion was amplified on genomic DNA by PCR using forward (5'-CGCAGTGCCCCGCCTTAGA-3') and reverse (5'-ACAAGATGGCGCCGCCGCCCCGGC-3') primers as described by Brais et al. [1998].

PEO with mtDNA deletion syndrome-related genes: Sanger sequence is performed to PEO-related genes which have less than 9 exons (*SLC25A4, RNASEH1, TWBNK, MGME1*). WES analysis was performed in all the remaining cases with unidentified etiology.

Also, the genes localized at the LoH region, which are associated with muscle function, were sequenced as a possible candidate gene. *CELF6* was sequenced in Family 3.

3.6. SNParray: SNParray is a method mainly to analyse deletion/duplications. But it is also a method to determine the loss of heterozygosity (LoH) regions in the families in which the parents are consanguineous, and the pedigree is suggestive for autosomal recessive inheritance. Illumina 300K single-nucleotide polymorphism-based microarray chips are used to detect LoH regions, and chips are scanned at the iScan platform (Illumina, USA). The data is analyzed at Bluefuse multi-software program.
SNParray analysis was performed in Family 3 (Y.İ.), Family 4 (E.G.), and Family 6 (M.G.) since AR inheritance was considered possible due to the consanguinity of the parents or their origin of birth being from the same small villages. The possibility of the candidate gene(s) residing in the LOH region is a high possibility in AR inherited disorders.

3.7. WES/WGS: Whole Exome Sequencing (WES)/Whole Genome Sequencing (WGS) is a method for sequencing all protein-coding regions/complete DNA sequences, including non-coding regions of the genome. WES includes ~20,000 genes (~180,000 exons), constituting approximately 1% of the whole genome. It is estimated that 85% of the mutations causing the disease are in the exons [www.centogene.com]. For this reason, when the results cannot be obtained in the targeted gene sequence in patients, WES analysis was planned for five families (Family 3-4-5-6-7), and WGS for Family 4. For WES/WGS analysis, the DNA is sequenced after passing through a series of wet-lab stages, depending on the platform used. These stages consist of DNA fragmentation, amplification, indexing, PCR amplification of desired exons, purification, and quality control by Qubit. Then the processed DNA is loaded to next-generation sequencing instrument. After the raw data (fastq files) obtained passes certain stages (raw data quality assessment, pre-processing, alignment, post-processing, variant calling, annotation), it is finally converted to an excel file, and data analysis is initiated. The pipeline used for these steps is the Genome Analysis ToolKit (GATK), and annotation is done with the Annovar software before file conversion.

Bioinformatics's main task is to know which mutations to prioritize in 120,000-140,000 variants. The analysis method consists of mainly eight stages:

1. “Pathogenic” or “likely pathogenic” variants are filtered. If no related gene is identified, proceed to step 2,
2. “Variant of unknown significance-VUS” variants are added to the filter.
3. Variants with minor allele frequencies (MAF) below 1% according to databases such as ExAC, gnomAD, 1000genome, esp6500,… are filtered (http://www.1000genomes.org/; https://gnomad.broadinstitute.org/),
4. Heterozygous and homozygous changes are separated,
5. “Exonic,” “Exonic-splicing,” and “Splicing" parameters are filtered while “noncoding, UTR” regions are excluded,
6. Synonymous single nucleotide changes (SNV’s) are excluded,
7. Genes are sorted by name (to identify compound heterozygous changes for AR disorders),
8. *In silico* analysis software tools’ scores for pathogenic variants are sorted; such as mutation taster, Sorting Intolerant From Tolerant (SIFT), Polymorphism Phenotyping v2 (PolyPhen-2) [Schwarz et al., 2014; Ng & Henikoff, 2003; Adzhubei et al., 2010].

Note: If one or more LoH regions were obtained from SNParray analysis, it can be sorted as a third step. If no gene is selected as a candidate from the LoH region, the data can be analyzed with no prioritization.

WES wet-lab part was provided from an outer center (Centogene AG, Germany) as a service procurement due to lack of WES/WGS machine track. The raw data was downloaded from an iCloud system, and all analyses were performed at KUH-Genetic Diagnostic Center (GDC), as stated above.

3.8 Functional studies

In case of uncertain results of the genetic studies, functional studies were being planned to clarify the variants' pathogenicity. The functional studies were designed considering previous studies related to the gene and its function at cell/tissue. Initially, gene expression and protein levels were measured. Gene-specific studies will be explained in the next step.

3.8.1. Quantitative PCR (qPCR): qPCR is used to measure RNA level quantification. RNA was obtained from cultured fibroblasts and muscle cells by RNeasy mini kit (Qiagen). cDNA was synthesized by Transcriptor High Fidelity cDNA Synthesis Kit (Roche). SYBR Green PCR master mix (Thermo Fisher, Cat No:4309155) and Rotor-Gene Q (Qiagen) machines are used for qPCR according to manufacturer's instructions. The primers used for quantifications are listed in Table 3.4. Delta delta CT values are used for the comparison of RNA levels.

Table 3.4: The primer list used for qPCR

Gene name	F	R
GAPDH	AGGTCGGAGTCAACGGATTTGG	TGCCATGGGTGGAATCATATTGG
mtDNA_12S	CCACGGGAAACAGCAGTGATT	CTATTGACTTGGGTTAATCGTGTGA
RNASEH1	GTTCCTGGCCCACAGAGTC	TGCACTCATTCCAGGTCAGAA
ND1	GCACGAAACGGGATCAAACA	AGAGAAGTAAGCCGAGGGCG
COI	CACCTAGCAGGTGTCTCCTC	AGAGGGGCGTTTGGTATTGG
CYB	GCACGAAACGGGATCAAACA	AGAGAAGTAAGCCGAGGGCG

3.8.2. Western-blot: To compare the candidate gene(s) protein level and housekeeping gene (Vinculin), Western blot method is performed. 20 mg protein and 5µl marker are loaded to Mini Protean Gel (Bio-rad). The gel is run at 200 Volt for 30 minutes. And the semi-dry transfer is carried out to transfer proteins to the PVDF membrane (Bio-rad).

The PVDF membrane is incubated at 4°C overnight at blocking solution + primary antibody. The membrane is washed with TBS-T solution for five times (5 minutes each at room temperature - RT). And secondary antibody (in blocking solution) is incubated at RT for an hour. The membrane is washed with TBS-T solution for five times (5 minutes each at RT). ECL Western Blot Substrate Kit (Abcam) is used to screen the desired protein.
For RNASEH1, Abcam ab229078 (Anti-RNase H1/RNH1 antibody) is ordered, but due to the covid-19 pandemic, the antibody could not reach on time. But the optimization of Western blot protocol is done and ready to run.

3.8.3. Southern blot: Southern blot method is necessary for DM1 and other repeat disorders to detect the actual repeat size which cannot be detected by TP-PCR. DM1 is a triplet repeat expansion disorder, and the diagnosis is based on TP-PCR (for detection 150 repeat or less, not higher) and/or Southern blot (gives the approximate repeat size). Classical and congenital types cannot be distinguished by TP-PCR (without clinical info).

For Southern blot analysis, "DIG Labeling and Detection Kit (Roche)" is used. The probe is obtained from a confirmed congenital DM1 patient's long-range PCR products.
DM1-LR_F: GAACGGGGCTCGAAGGGTCCTTGTAG
DM1-LR_R: CTTCCCAGGCCTGCAGTTTGCCCATC
The PCR products are labeled by DIG by following the manufacturer's instructions. The optimized Southern blot protocol is given as a table (Table 3.5).

Table 3.5: Southern blot protocol for DM1

Note	Step	**DAY 1**	Date:
		LR-PCR of the patients DNA	
without EtBr	1.1.	%1 agarose gel is prepared in 1X TAE buffer	15x20 cm
	1.2.	PCR products are run in 1% agarose gel for 100 Volt, 5 hours	100V - 300 min
by ruler	1.3.	The membrane was cut at the same size with the agarose gel	15 x 20 cm
shake gently, at RT	1.4.	After running gel, the gel is transferred to 0,25 M HCl	15 min
	1.5.	The gel is rinsed with dH2O	2 times
shake gently, at RT	1.6.	The gel is incubated at 0,5M NaOH + 1,5M NaCl	30 min
		*2 pieces of Whatman paper and membrane soaked on 2X SSC	5 min
	1.7.	Prepare transfer setup (See Figure 4.2)	✓
	1.8.	Leave overnight at RT	✓ **(16:30)**
		DAY 2	Date:
		*Set hot plate to 80°C	
	2.1.	Transfer membrane to hot plate for 2 hours	✓ **(11:00)*** **
*** Keep at 4°C		*Set hybridization oven to 65°C	
		* Denaturate Salmon DNA at 99°C for 10 min	500µl
	2.2.	Add denaturated Salmon DNA to hybridization solution (HS)	20 ml + 500µl
No foam, bubble!!!	2.3.	Place membrane to hybridization tube and add the solution prepared at 2.2.	✓ **(17:00)**
	2.4.	Balance with another tube	
	2.5.	Hybridize at 65°C for 1 hour	
		* Denaturate probe at 95°C for 2 min	30 µl
	2.6.	Add denaturated probe to 3 ml HS	
	2.7.	Replace buffer with 2.6 and incubate at 65°C for 15 min	✓ **(18:00)**
	2.8.	Decrease the temperature to binding temperature and incubate overnight	✓ **(18:15) 45°C**
		DAY 3	Date:
	3.1.	Incubate membrane at low stringency buffer (LSB) for 5 min at RT	✓ **(10:00)**
HSB=1:4 LSB	3.2.	Incubate at 65°C with high stringency buffer (HSB) for 15 minutes	2 times
at RT	3.3.	Wash membrane with Wash-Buffer	15 min, 2 times
	3.4.	Prepare 2 ml Blocking solution + 18 ml Maleic acid	20 ml
		*Centrifuge vial8 at 13.000rpm for 5 min	
shake gently, at RT	3.5.	Add 4µl of vial 8 to the solution at 3.4, pour to the membrane	30 min
	3.6.	Wash membrane with Wash-Buffer	15 min, 2 times
	3.7.	Add 15 ml Detection Buffer + 75µl of vial 9, incubate at dark	
	3.8.	Stop the reaction with TE buffer when the bands appear	around 1 hour

1. Healthy control (5 CTG repeat; confirmed)
2. Mild DM1
3. Classical DM1
4. Healthy control (5 CTG repeat; confirmed)
5. Congenital DM1
6. Marker

Figure 3.11: The result of Southern blot for DM1

The diagnosis of DM1 by TP-PCR and Southern blot methods was optimized. In case of uncertain results, parents or other first/second-degree relatives may be included in the study for confirmation.

3.8.4. Silver staining: This method is used to visualize mtDNA deletions, which cannot be seen by routine agarose gel screening. To screen mtDNA deletions, mtDNA is amplified from genomic DNA (extracted from blood and fibroblast) by two primers, including 16,208 base pairs (bp) out of 16,569 bp. For LR-PCR, Roche Expand Long Template PCR System kit is used as manufacturer's instruction, and the primer sequences to amplify mtDNA are given below.

mtDNA_LR-PCR_F: accgcccgtcaccctcctcaagtatacttcaaagg
mtDNA_LR-PCR_R: accgccaggtcctttgagttttaagctgtggctcg

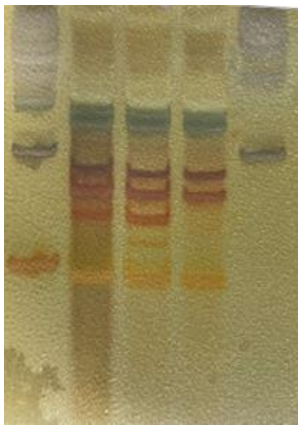


Figure 3.12: Silver staining protocol optimization

Both agarose gel (small mtDNA deletion) and polyacrylamide gel (for possible large mtDNA deletion) staining procedures were applied.

The protocol applied for silver staining is previously described by Prieto (et al., 1997). Briefly, after horizontal electrophoresis of agarose (0,8%), gels were submerged to 10% acetic acid at RT for 10 min. Then gels were rinsed with redistilled water. The gels were soaked 20 mM $AgNO_3$ for 20 min at 40°C (For polyacrylamide gels; 2mM $AgNO_3$). The gel is rinsed with redistilled water for 30 seconds (10 times). Gels were developed in 3% NaOH containing 37% formaldehyde at RT until bands became visible (brown). As an optimization, four PCR products were mixed and run in polyacrylamide gel (see Figure 3.11).

3.8.5. mtDNA depletion and recovery analysis

In PEO with mtDNA deletion patients, mtDNA depletion and decrease of mtDNA recovery rate can be observed by qPCR. qPCR method was optimized, and delta-delta Ct methods were used to analyze the mtDNA depletion and recovery analysis [https://toptipbio.com/delta-delta-ct-pcr/]. In summary, the housekeeping gene Ct average will be compared with the gene of interest's Ct. Fibroblast culture cells (all in passage 4) will be used for the analysis.

For mtDNA depletion analysis, the nuclear DNA gene (*GAPDH*) will be the housekeeping gene, *12S mtDNA* will be the gene of interest. It could be possible to visualize mtDNA deletion by qPCR analysis by comparing *12S mtDNA* with *COI, ND1,* or *CYB*.

For mtDNA recovery analysis: mtDNA recovery experiment was achieved by adding 15 ng/ml EtBr to the culture medium for 96 hrs, and recovery was followed up for 192 hours after the removal of the EtBr.

Day 0, 2, 4 (incubation with EtBr)

Day 6, 8, 10, 12 (removal of EtBr and with the regular medium)

Chapter 4

RESULTS

All index cases from seven families were subjected to testing for OPMD, DM1, OPDM1-2 (not OPDM3 and OPDM4 due to Covid-19 pandemic), and PEO with mtDNA deletions (*RNASEH1, SLC25A4, TWNK*). Families that were not diagnosed with OPMD, DM1, and OPDM1-2 were considered for further research (SNParray, WES, functional studies).

The individuals who were tested at molecular level will be indicated as below in pedigrees.

N	: homozygous wild type
black filled	: affected, ● ■
+/-	: carrier
red framed	: molecularly confirmed affected, ○ □
✦	: obligatory affected; not molecularly tested

4.1. Family 1 (A.A.Y.):

A.A.Y. (III-1) has (GCG)6/9 repeat at the *PABPN1* gene at exon 1 (Figure 4.1). The result is consistent with **OPMD**.

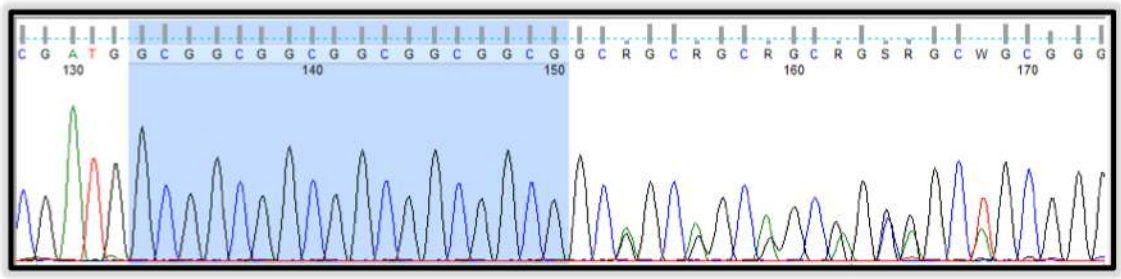


Figure 4.1: Sequence analysis of *PABPN1* gene exon 1 heterozygous expanded allele in Family 1(A.A.Y.)

The children of A.A.Y. were tested who have a 50% risk of inheriting expanded allele from their father. His daughter (IV-2) has 6/6, and his son (IV-1) has 6/9 repeats (Figure 4.2). At the genetic counseling session, the son (IV-1) was directed to neurology and psychiatry outpatient clinics for follow-up. Molecular testing was offered for the affected (II-1, II-5) and other family members who are at risk of inheriting the disease (III-4, III-6/7, III-8/9) (See Figure 4.2).

The index case was consulted to Neurology, Pulmonology, and Ear, Nose, Throat (ENT) departments. A.A.Y.'s chest BT revealed; centrilobular and paraseptal emphysema of both lung parenchyma and widespread degenerative changes of bony structures. Percutaneous endoscopic gastrostomy (PEG) was suggested by a neurologist, but the patient denied. Annual follow-up of the patient by the neurology department is recommended.

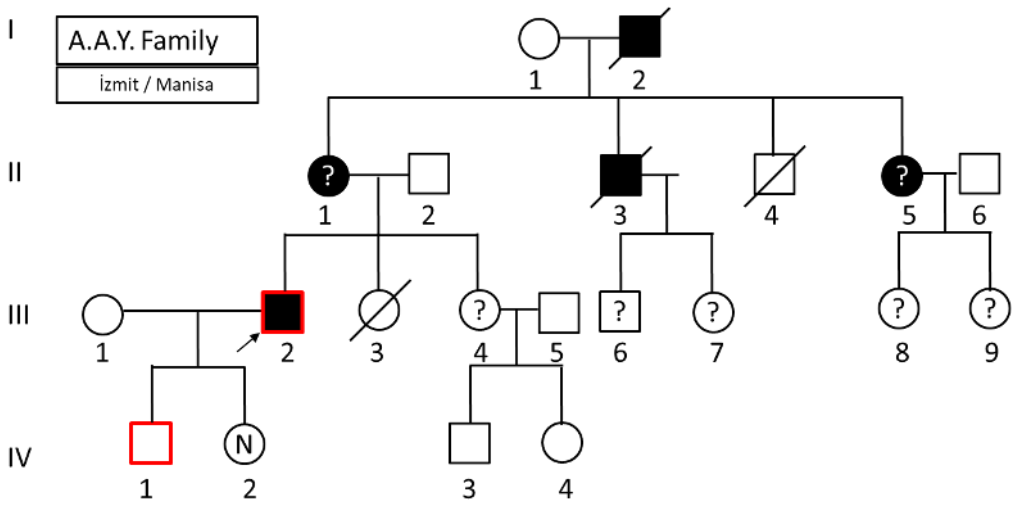


Figure 4.2: Updated pedigree of Family 1 (A.A.Y.)

4.2. Family 2 (F.K.):

F.K. (III-1) has (GCG)8/8 repeat at the *PABPN1* gene exon 1 (Figure 4.3). The result is consistent with the **homozygote state of autosomal dominant disorder, OPMD**. We further tested the healthy mother and brother for OPMD.

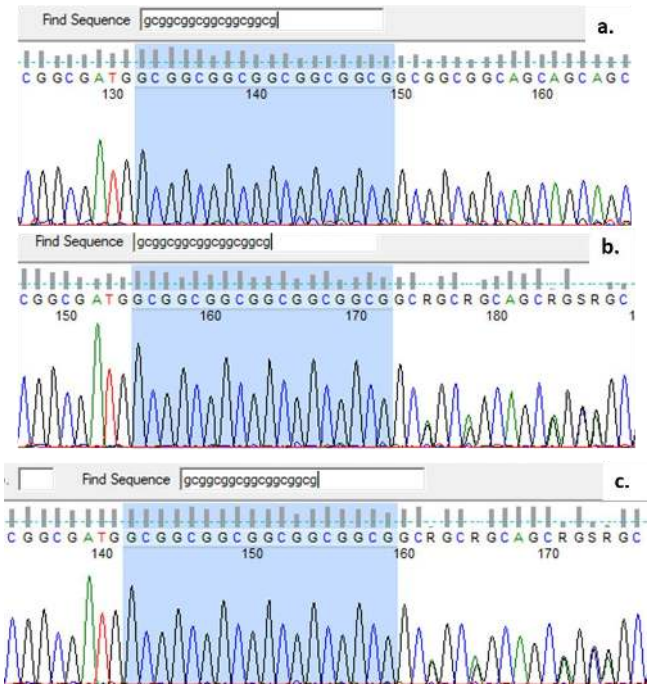


Figure 4.3: Sequence analysis of the *PABPN1* gene exon 1 in Family 2 (F.K.). (a.): index case; homozygous (GCG)8/8, (b, c): mother and brother; heterozygous (GCG)6/8 expanded allele

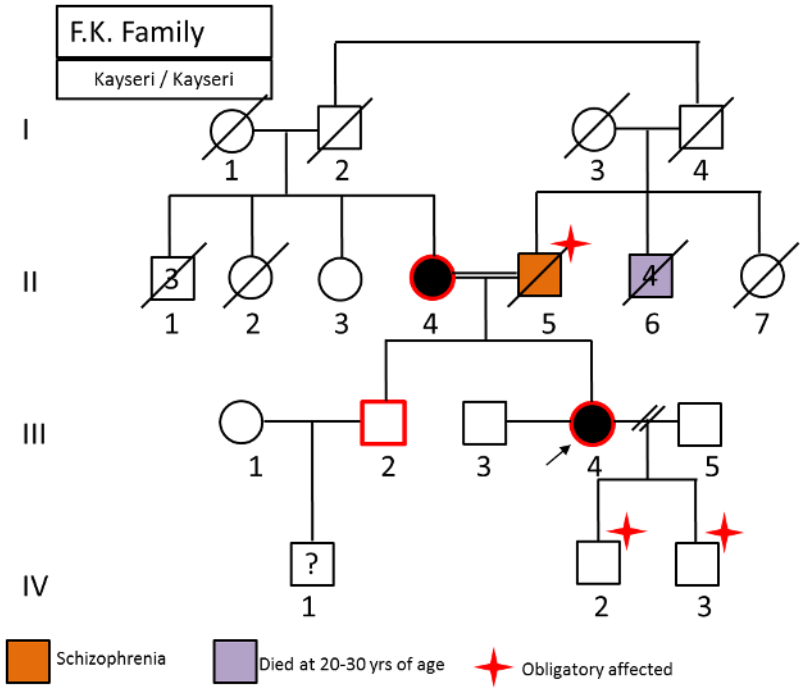


Figure 4.4: Updated pedigree of Family 2 (F.K.)

Physical examination of the mother (II-4) revealed that she has distal muscle weakness and could walk with a cane from 70 years of age on. She also has bilateral ptosis and dysphagia, although all the family members believed that these were due to advanced age. The father (II-5) could not be tested due to the unavailability of the biological sample since he died at the age of 48 when he was asymptomatic. There were no living family members on the father's side to be tested. The brother (III-2) carries the expanded allele at the heterozygous state, although he did not show any symptoms at the age of 66.

The children of the index patient (IV-2, IV-3) and the affected brother's children (IV-1) were not tested. IV-2 and IV-3 were considered as obligatory carriers for AD form OPMD since they should have inherited one of the mother's expanded alleles. For IV-1, the probability of being affected is 50%. The father was informed that after the age of 18, pre-symptomatic genetic testing could be performed according to the individual's will and consent.

4.3. Family 3 (Y.İ.):

Y.İ. was subjected to OPMD, DM1, and OPDM1 molecular testing, which were all noninformative.

The research algorithm was initiated to identify the etiopathogenesis of the OPDM-mimicking disorder. SNParray analysis was performed to determine the common LoH region in P1 and P2 (**Figure 4.5**) due to consanguineous marriages in the families. Common LoH regions and the genes residing in LOH regions are listed in Table 4.1.

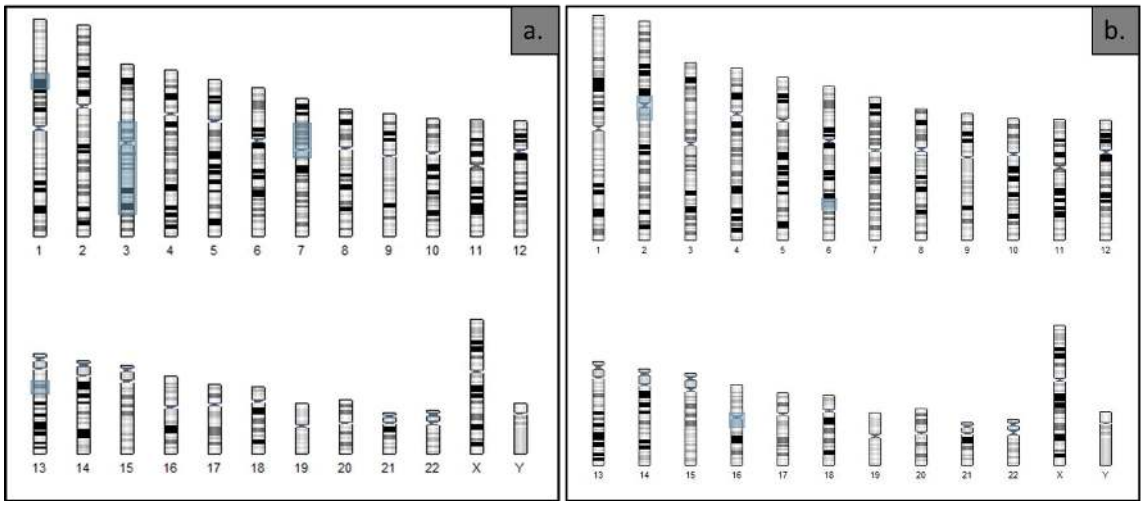


Figure 4.5: LoH regions and their localization on chromosomes of P1 (a.) and P2 (b.) in Family 3 (Y.İ.)

CELF6 gene residing in the LoH region of chromosome 15 was considered as a possible candidate gene due to its role in transcription. It is known that; *PABPN1* plays a role in controls of the poly(A) tail length on the RNA transcripts and therefore shows tissue-specific symptoms by accumulating the protein in the cell [Apponi et al., 2010]. *CELF6*'s role is to control muscle-specific splicing mechanisms [Ladd et al., 2004]. Since the *CELF6* gene was considered as a possible gene, Sanger sequencing was performed which revealed no disease-causing variants.

Table 4.1: Common LoH regions in Family 3 (Y.İ.) and genes list residing in LOH regions

Gene name	**Chromosome 2**		**Gene name**	**Chromosome 13**
TSSC1	2 : 3192696 - 3381653		*FRY-AS1*	13 : 32599451 - 32605776
TSSC1-IT1	2 : 3302112 - 3305236		*FRY*	13 : 32605437 - 32870794
TRAPPC12	2 : 3383446 - 3488865			**Chromosome 15**
TRAPPC12-AS1	2 : 3485013 - 3486180		*MYO9A*	15 : 72114632 - 72410918
ADI1	2 : 3501132 - 3523507		*RNA5SP399*	15 : 72150911 - 72151027
RNASEH1	2 : 3592383 - 3606206		*RNU2-65P*	15 : 72337524 - 72337708
RNASEH1-AS1	2 : 3606082 - 3609324		*SENP8*	15 : 72406599 - 72433311
RPS7	2 : 3622795 - 3628509		*GRAMD2*	15 : 72452148 - 72490126
COLEC11	2 : 3642426 - 3692048		*PKM*	15 : 72491370 - 72524164
TMSB4XP2	2 : 3665138 - 3665347		*PARP6*	15 : 72533522 - 72565340
ALLC	2 : 3705785 - 3750261		*CELF6*	15 : 72577068 - 72612470
GAPDHP48	2 : 3735611 - 3736571		*HEXA*	15 : 72635775 - 72668817
DCDC2C	2 : 3751453 - 3836122		*HEXA-AS1*	15 : 72668454 - 72671129
	Chromosome 3		*TMEM202*	15 : 72690614 - 72700370
RNU6-1094P	3 : 96327658 - 96327761		*ARIH1*	15 : 72766667 - 72879692
MTRNR2L12	3 : 96335981 - 96337000		*MIR630*	15 : 72879558 - 72879654
RPL18AP8	3 : 96338148 - 96338675		*RN7SL485P*	15 : 72904161 - 72904401
RCC2P5	3 : 96472029 - 96473515		*GOLGA6B*	15 : 72947079 - 72958735
CDV3P1	3 : 96495789 - 96496527		*RN7SL853P*	15 : 72956896 - 72957171
EPHA6	3 : 96533425 - 97471304		*HIGD2B*	15 : 72968124 - 72978490
ARL6	3 : 97483365 - 97519953		*BBS4*	15 : 72978527 - 73030817
CRYBG3	3 : 97595819 - 97663810		*ADPGK*	15 : 73043710 - 73078187
MINA	3 : 97660662 - 97691301		*ADPGK-AS1*	15 : 73075176 - 73090540
GABRR3	3 : 97705517 - 97754148		*NPM1P42*	15 : 73191740 - 73192601
	Chromosome 13		*NEO1*	15 : 73344051 - 73597547
RXFP2	13 : 32313674 - 32377009		*FKBP1AP2*	15 : 73435577 - 73435859
EEF1DP3	13 : 32420978 - 32527609		*NPM1P43*	15 : 73454120 - 73455269
LINC01073	13 : 32559822 - 32565134		*HCN4*	15 : 73612200 - 73661605

WES analysis revealed 110,695 variations at P1. After the 8 step analysis (with the prioritization of LoH region), *RNASEH1* (NM_002936) gene exon 4:c.G493C (p.G165R) variation was considered as a strong candidate. The *RNASEH1* gene is located on 2p25.3, and biallelic mutations of the gene are associated with “Progressive external ophthalmoplegia with mitochondrial DNA deletions, autosomal recessive 2 (PEOB2)”.

RNASEH1 gene has 8 exons and encodes a 286 amino acid (a.a.) endonuclease present in both the nucleus and mitochondria that specifically digests the RNA component of RNA-DNA double-stranded hybrids (Figure 4.6).

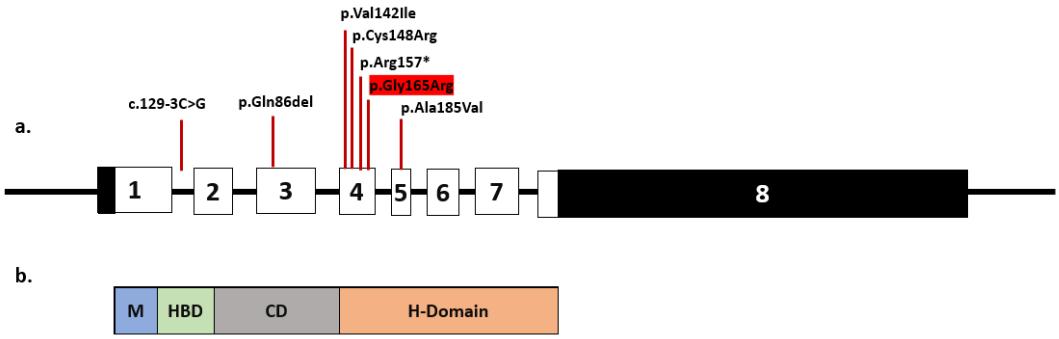


Figure 4.6: *RNASEH1* gene schematic view (a.) exons and the location of mutations described in PEOB2 patients (red-colored; KUH patient). (b.) domains of the protein;
M: mitochondrial Targeting Sequence (MTS) that directs the protein to mitochondria and is cleaved after import
HBD: hybrid binding domain (HBD) involved in recognition of DNA-RNA heteroduplexes
CD: catalytic domain responsible for the cleavage of the RNA component in the heteroduplex
Connection (H) Domain: links the last two (HBD and CD) domains.

Only six different mutations were described in eight unrelated PEOB2 families up till now. Most of the mutations are located in exons 4 and 5 (see Figure 4.6), which is the connection domain of the last two domains. Common features of PEOB2 patients were CPEO, dysphagia, dysphonia, muscle weakness and wasting, cerebellar involvement, and respiratory impairment at later stages. Muscle biopsies show COX-negative, SDH-positive fibers with RRF. Electron microscopy of muscle biopsy was consistent with elongated, hyper-fused mitochondria. In most cases, cerebellar atrophy was observed, especially in the later stages of the disease [Reyes et al.; 2015, Bugiardini et al.; 2017, Sachdev et al.; 2018, Carreno-Gago et al.; 2019].

After considering *RNASEH1* as a strong candidate, segregation analysis was performed of the family members aged over 35. The segregation was compatible with the pedigree for affected and non-affected cases (Figure 4.7). Further analysis was performed in the P1 to confirm the diagnosis.

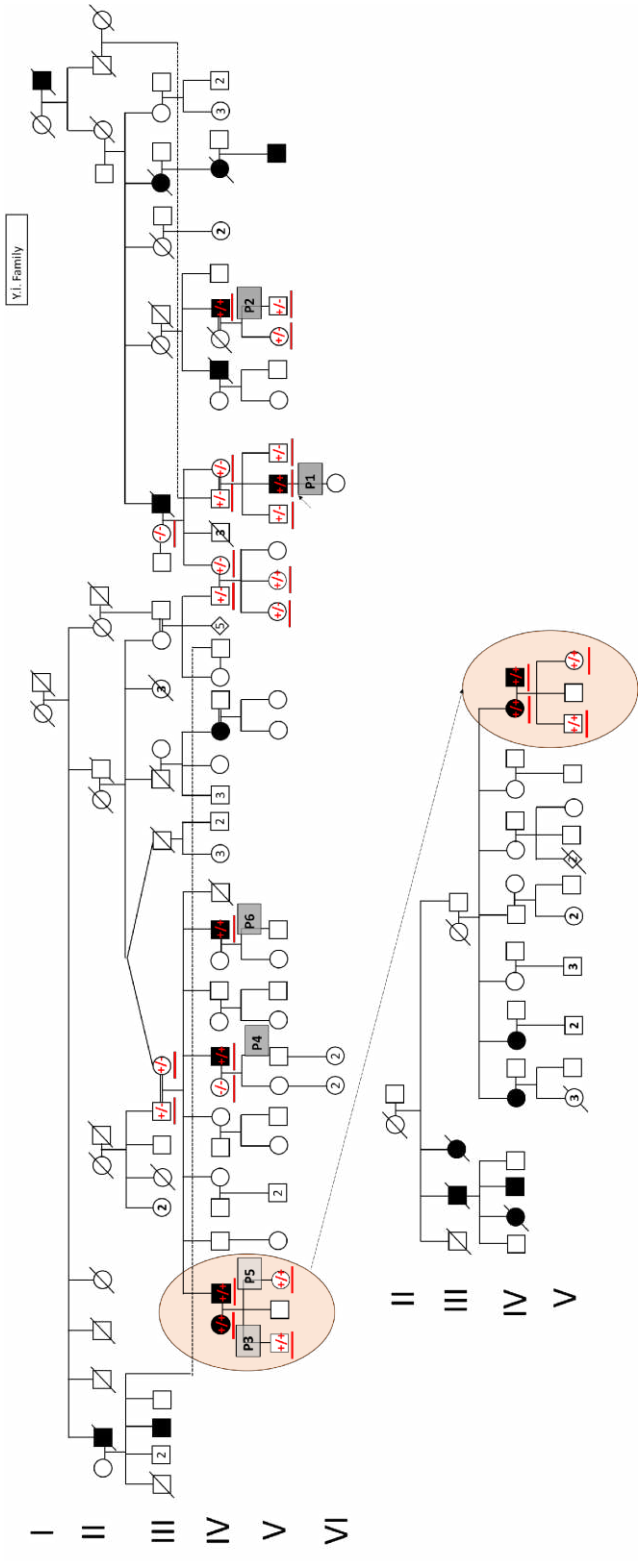


Figure 4.7: Segregation analysis of *RNASEH1* mutation in Family 3 (Y.İ.)

Muscle biopsy performed on the index case (Figure 4.8) showed; COX-negative, SDH-positive, and RRF, which was suggestive of mitochondrial diseases.

Cranial MRI of the index case (P1) and P2 showed cerebellar atrophy, which was more prominent in P2 (48 years of age) since he is at a more advanced stage of the disease (Figure 4.9). P2 had lost his ability to walk independently and also had respiratory insufficiency.

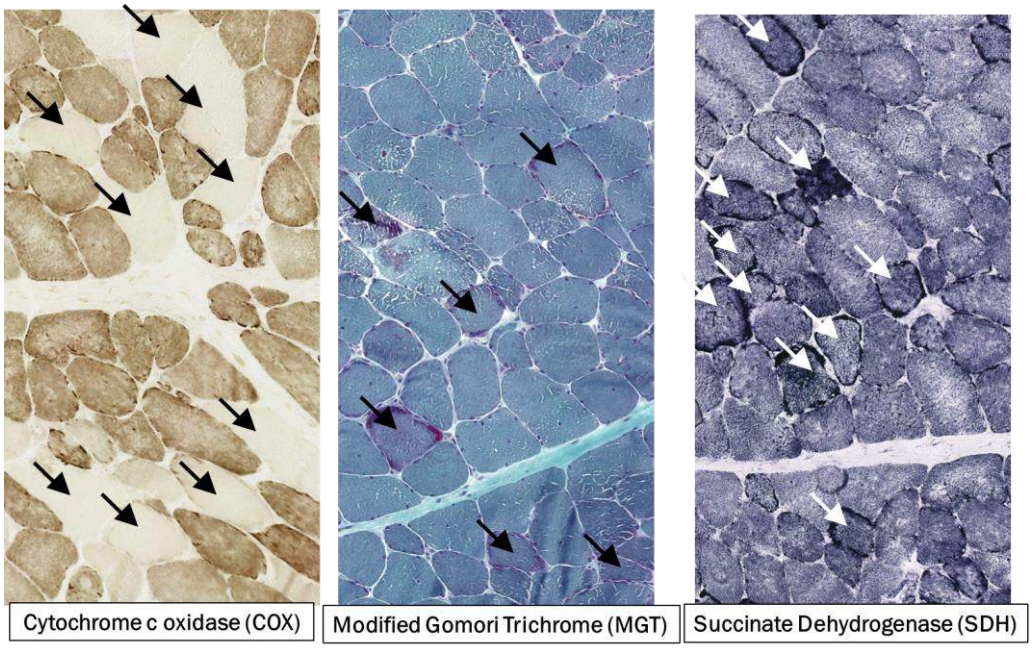


Figure 4.8: Muscle biopsy of the index case in Family 3 (Y.İ.). COX-negative fibers, RRF, SDH-positive fibers (20 X)

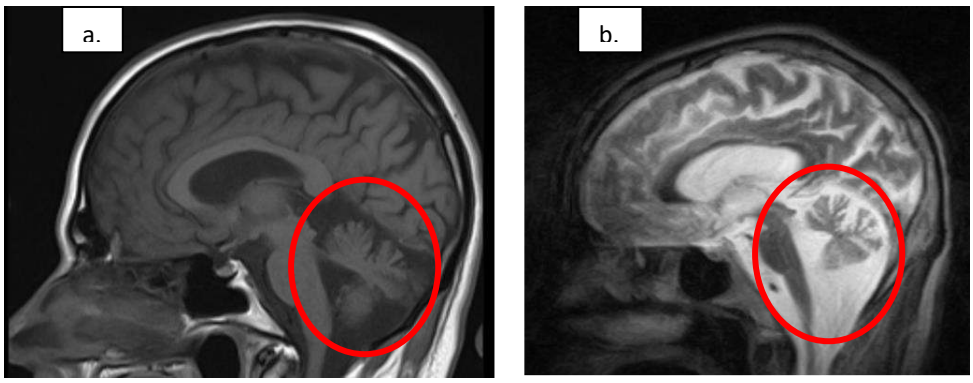


Figure 4.9: Cranial MRI sections showing cerebellar atrophy. a. P1 at the age of 35 b. P2 at the age of 48 in Family 3. Note that b. shows a more advanced stage of cerebellar atrophy.

Segregation analysis of the family members, muscle biopsy, and cranial MRI findings of P1, P2 was considered compatible with the PEOB2 diagnosis.

To investigate the mitochondrial function of *RNASEH1* mutation, fibroblast samples of P1 along with two age-matched healthy controls, and functional analysis were planned.

Fibroblast culture (P1; control 1, control 2) mitochondrial transcription levels were measured on the first day. According to the results obtained, 12S DNA was expressed more in the individual carrying the *RNASEH1* mutation, showing no mtDNA depletion (Figure 4.10). It was also observed that there was an increase in the expression of mitochondrial DNA in the affected patient's cell (1,2-3 fold), *RNASEH1* transcript level was stable, but mitochondria were less active in the affected patient's fibroblast culture when compared to healthy controls. This experiment needs to be confirmed repeatedly, including *RNASEH1* mutation carrier individuals and also in muscle tissues.

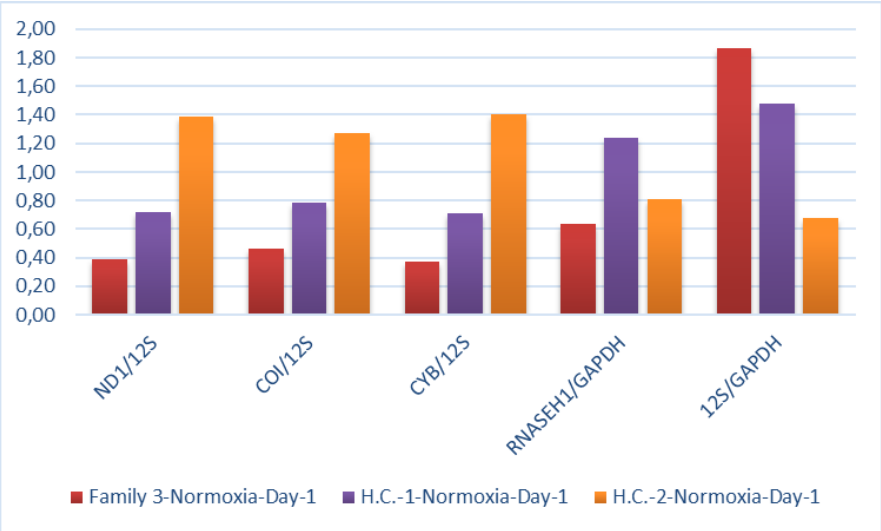


Figure 4.10: Relative mtDNA copy number was assessed in cells grown in glucose

For mitochondria recovery analysis, a certain amount of EtBr was added to the cell culture for four days, and then EtBr was removed, and the cells were fed with a regular medium for a week. All results were demonstrated in Figure 4.11.

Figure 4.11 shows that recovery of mtDNA count (GAPDH/12S) to the baseline level is completed in about four days in healthy individuals (Day 8), while the patient did not return to its baseline level in 7 days (Day 11). The data suggests that the mtDNA recovery rate may be decreased in patients compared to healthy individuals.

As shown in the table, an unbalanced expression was observed after EtBr was added in the transcription of CYB, COI, and ND1 genes synthesized from mtDNA. To explain the expression level change, it is necessary to repeat the experiment in more affected/carrier individuals and comparing the expression levels in different tissue samples.

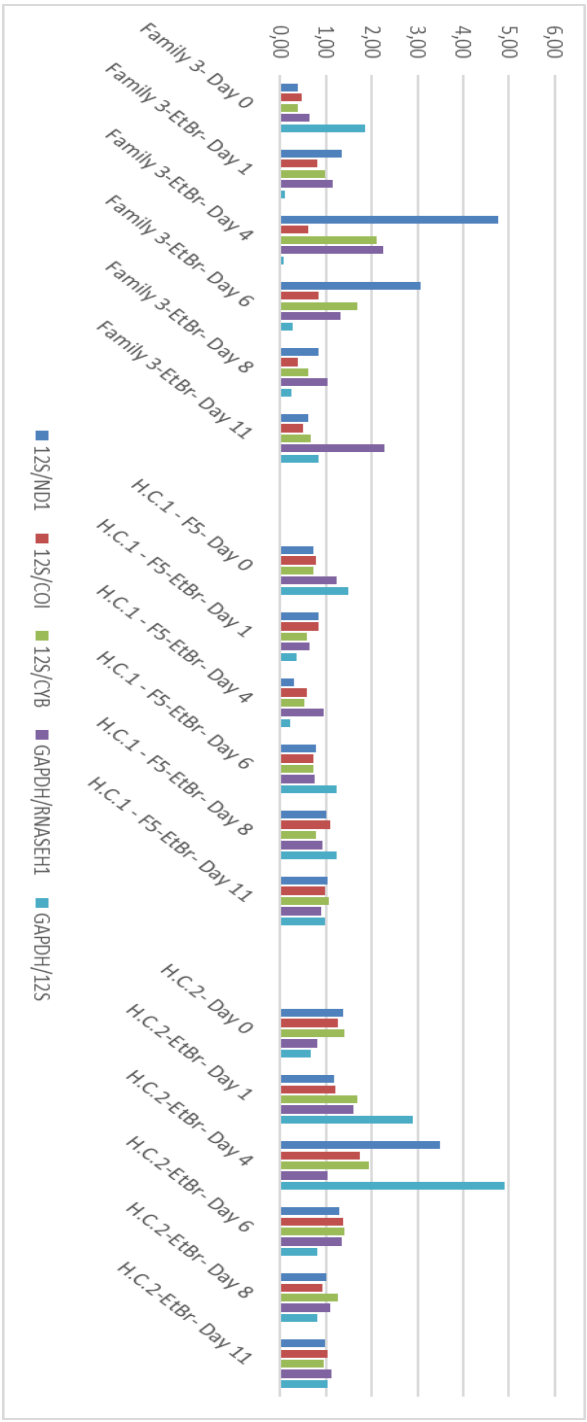


Figure 4.11: Q-PCR analysis of Family 3 (Y.İ.) index case and two healthy controls' fibroblasts. Day 1-4: EtBr medium; Day 6-8-11: Removing EtBr, adding regular medium

4.4. Family 4 (E.G.): OPMD, DM1, and OPDM1/2 were excluded in the index case. Quatro-WES analysis had been performed in Germany, but the only result they had was that the index case has *DNA2* gene p.Met259Val variant with uncertain significance (VUS) as a possible disease-causing variant (neither a report nor images were available). The *DNA2* gene mutations are associated with "Progressive external ophthalmoplegia with mitochondrial DNA deletions, autosomal dominant 6 (PEOA6)". Segregation analysis was planned for the index, affected brother, and healthy parents. Two affected brothers and unaffected father were heterozygous for the variant. The segregation result excluded the diagnosis of PEOA6. The parents of the index were second-degree cousins once removed, further LoH analysis was planned for the affected brothers and the parents.

Table 4.2: LoH regions of the index case in Family 4 (E.G.)

Region	Start Cyto	End Cyto	Type	LOH Ratio	Chromosome	Start	End	Size (bp)
1	2q22.3	2q31.1	LOH	3033.00	2	145,208,193	174,436,869	29,228,677
2	3p26.3	3p26.1	LOH	847.00	3	66,894	5,806,750	5,739,857
3	3q21.1	3q22.2	LOH	968.00	3	122,325,467	134,636,796	12,311,330
4	5q35.2	5q35.3	LOH	895.00	5	174,278,912	180,705,539	6,426,628
5	6p21.2	6p12.1	LOH	2245.00	6	37,524,986	53,896,155	16,371,170
6	6q16.1	6q21	LOH	554.50	6	95,215,623	106,303,274	11,087,652
7	7q22.1	7q22.1	LOH	214.00	7	98,685,224	100,254,213	1,568,990
8	8p21.2	8q11.23	LOH	1872.00	8	24,632,351	53,927,134	29,294,784
9	9p21.1	9p13.1	LOH	620.00	9	28,542,803	38,709,952	10,167,150
10	14q11.2	14q11.2	LOH	90.00	14	21,534,908	23,857,351	2,322,444
11	14q23.3	14q24.1	LOH	335.00	14	66,290,126	69,493,375	3,203,250
12	16p13.12	16p13.11	LOH	410.00	16	12,885,808	16,090,801	3,204,994
13	19p12	19q11	LOH	55.20	19	23,832,026	27,977,423	4,145,398

Table 4.3: LoH regions of the affected brother in Family 4 (N.G.)

Region	Start Cyto	End Cyto	Type	LOH Ratio	Chromosome	Start	End	Size (bp)
1	3q13.31	3q21.1	LOH	382.00	3	116,968,649	122,288,210	5,319,562
2	4p14	4q12	LOH	926.50	4	37,470,428	58,012,732	20,542,305
3	4q13.1	4q13.3	LOH	567.00	4	62,130,085	74,455,381	12,325,297
4	4q23	4q25	LOH	657.00	4	99,317,251	109,315,729	9,998,479
5	5p15.2	5p14.3	LOH	405.00	5	14,372,691	20,621,364	6,248,674
6	5q35.2	5q35.3	LOH	905.00	5	174,278,912	180,705,539	6,426,628
7	6p21.1	6p12.1	LOH	1803.00	6	41,619,050	53,896,155	12,277,106
8	7p13	7p11.2	LOH	1378.00	7	43,561,814	54,709,978	11,148,165
9	7q22.3	7q36.1	LOH	1239.00	7	105,587,441	149,142,729	43,555,289
10	9p21.2	9p13.1	LOH	722.00	9	27,619,290	38,709,952	11,090,663
11	9q31.2	9q33.1	LOH	1088.00	9	110,642,150	121,979,181	11,337,032
12	17q21.2	17q21.31	LOH	103.50	17	40,052,321	41,800,656	1,748,336
13	19p12	19q11	LOH	221.00	19	23,774,942	28,497,091	4,722,150
14	20q11.23	20q13.12	LOH	474.00	20	36,920,753	42,114,419	5,193,667

Table 4.4: LoH regions of the father in Family 4

Region	Start Cyto	End Cyto	Type	LOH Ratio	Chromosome	Start	End	Size (bp)
1	3q13.31	3q13.33	LOH	215.00	3	116,968,649	119,503,005	2,534,357
2	4q13.1	4q13.3	LOH	566.00	4	62,130,085	74,455,381	12,325,297
3	6p11.2	6q11.1	LOH	126.50	6	57,165,948	62,752,460	5,586,513
4	7q22.1	7q22.1	LOH	217.00	7	98,685,224	100,254,213	1,568,990
5	8q11.21	8q11.21	LOH	41.80	8	48,936,111	50,253,358	1,317,248
6	11p11.2	11p11.12	LOH	68.20	11	47,385,923	50,429,467	3,043,545
7	13q34	13q34	LOH	425.00	13	111,424,409	114,179,631	2,755,223
8	19p12	19q11	LOH	46.33	19	23,832,026	27,977,423	4,145,398

Table 4.5: LoH regions of the mother in Family 4

Region	Start Cyto	End Cyto	Type	LOH Ratio	Chromosome	Start	End	Size (bp)
1	1p34.3	1p34.2	LOH	32.67	1	39,430,262	40,796,468	1,366,207
2	1p34.2	1p31.1	LOH	4464.00	1	42,499,365	84,761,174	42,261,810
3	1p22.2	1p21.1	LOH	897.00	1	90,327,555	106,221,029	15,893,475
4	1p13.2	1q21.3	LOH	1450.00	1	114,906,992	152,546,889	37,639,898
5	1q23.2	1q23.3	LOH	352.00	1	160,452,218	164,720,061	4,267,844
6	1q42.13	1q42.2	LOH	316.00	1	229,018,916	232,804,107	3,785,192
7	1q43	1q43	LOH	576.00	1	237,385,440	241,258,422	3,872,983
8	1q44	1q44	LOH	322.00	1	245,075,384	247,513,049	2,437,666
9	2p25.2	2p25.1	LOH	327.50	2	6,692,014	11,118,836	4,426,823
10	2p22.3	2p14	LOH	2017.00	2	34,059,750	65,804,266	31,744,517
11	2q11.2	2q12.2	LOH	300.00	2	102,248,081	106,091,003	3,842,923
12	3q24	3q25.2	LOH	586.00	3	147,774,712	154,819,781	7,045,070
13	3q26.31	3q26.33	LOH	1022.00	3	171,559,827	182,426,534	10,866,708
14	4q24	4q28.3	LOH	2525.00	4	101,128,211	136,681,841	35,553,631
15	5q23.2	5q31.3	LOH	905.00	5	126,982,737	142,353,097	15,370,361
16	5q34	5q34	LOH	460.00	5	162,736,772	167,653,364	4,916,593
17	6p22.1	6p21.2	LOH	271.00	6	29,545,284	37,524,986	7,979,703
18	6p11.2	6q11.1	LOH	121.00	6	57,205,920	62,717,871	5,511,952
19	6q16.1	6q21	LOH	1106.00	6	95,342,961	106,299,822	10,956,862
20	7p22.3	7p21.3	LOH	284.00	7	46,239	8,560,944	8,514,706
21	7p21.2	7p14.3	LOH	1733.00	7	14,091,524	29,498,356	15,406,833
22	8p23.2	8p22	LOH	2236.00	8	2,763,578	17,226,856	14,463,279
23	9p24.2	9p23	LOH	1034.00	9	3,603,238	10,603,845	7,000,608
24	10p15.3	10p13	LOH	2268.00	10	135,708	16,712,610	16,576,903
25	10q26.11	10q26.13	LOH	462.00	10	119,846,522	123,150,301	3,303,780
26	11p15.5	11p15.4	LOH	289.00	11	2,044,150	4,450,821	2,406,672
27	12q24.13	12q24.23	LOH	622.00	12	114,182,892	118,234,080	4,051,189
28	12q24.23	12q24.32	LOH	1394.00	12	118,316,834	128,304,265	9,987,432
29	12q24.32	12q24.33	LOH	348.00	12	128,910,398	133,770,975	4,860,578
30	13q34	13q34	LOH	522.00	13	111,429,684	115,106,996	3,677,313
31	15q21.1	15q22.2	LOH	1229.00	15	47,472,264	60,966,891	13,494,628
32	15q23	15q25.1	LOH	1355.00	15	71,129,781	81,218,306	10,088,526
33	17q23.3	17q24.2	LOH	303.00	17	62,060,718	66,950,453	4,889,736
34	18q21.31	18q22.2	LOH	944.00	18	55,828,612	67,399,208	11,570,597
35	18q22.3	18q23	LOH	895.00	18	71,467,050	78,014,582	6,547,533
36	19p13.3	19p13.3	LOH	501.00	19	267,039	3,697,775	3,430,737
37	19p12	19q11	LOH	218.50	19	23,774,942	28,497,091	4,722,150
38	20p13	20p13	LOH	98.50	20	63,244	1,278,770	1,215,527
39	22q11.23	22q12.1	LOH	224.00	22	24,622,470	26,448,197	1,825,728
40	Xp11.22	Xp11.21	LOH	261.00	X	54,781,603	57,329,089	2,547,487
41	Xq28	Xq28	LOH	620.00	X	151,421,554	155,235,833	3,814,280

Table 4.6: Common LoH regions in affecteds in Family 4 (E.G.)

Start Cyto	End Cyto	Type	LOH Ratio	Chromosome	Start	End	Size (bp)
5q35.2	5q35.3	LOH	905.00	5	174,278,912	180,705,539	6,426,628
6p21.1	6p12.1	LOH	1803.00	6	41,619,050	53,896,155	12,277,106
9p21.1	9p13.1	LOH	620.00	9	28,542,803	38,709,952	10,167,150

These three LoH regions include 214 genes. And only 42 genes out of 214 are associated with the disease although none of them were associated with NMDs, and the remaining 172 genes have not been associated with any known human diseases.

Cranial MRI imaging was performed to exclude the possibility of PEO with mtDNA deletion syndromes. Cranial MRI showed; hyperintense, perpendicular to the ventricle lesions in T2AG in periclosal, periventricular, and white matter, which were suspicious for demyelinating diseases and relative atrophy of cerebral and cerebellar regions (Figure 4.12).

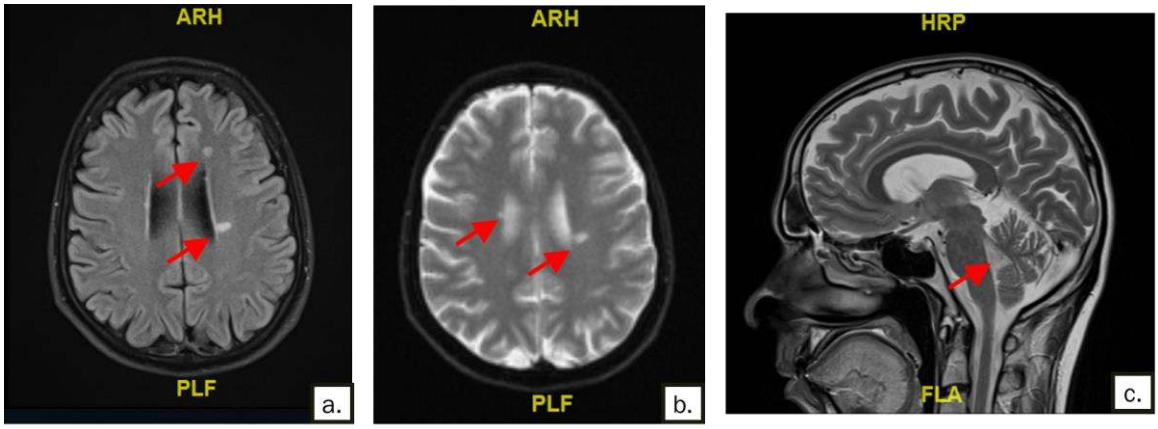


Figure 4.12: Cranial MRI sections showing hyperintense lesions (a and b) and relative atrophy of cerebral and cerebellar regions (c) of the index case of Family 4 (E.G.)

WES analysis was performed since there was no access to the patient's previous WES data. As an initial step, changes with a frequency below 0.01 were listed (13,054 variants). The genes in the LoH region (63 genes) were listed, and non-synonymous changes in the exonic regions of 9 genes were evaluated as candidates.

5q35.2 – 5q35.3 region: 1 gene; no exonic variant
6p21.1 – 6p12.1 region : 31 gene; 2 exonic variants (*AARS2*, *MUT*)
9p21.1 – 9p13.1 region : 31 gene; 7 exonic gene variants (*AQP7, FAM205A, FAM214B, HRCT1, PIGO, PRSS3, RPP25L*)

Three out of 8 genes have already been associated with a human phenotype. These are*; AARS2* associated with "Leukoencephalopathy, progressive with ovarian failure," *MUT* associated with "Methylmalonic aciduria due to methylmalonyl-CoA mutase deficiency," and *PIGO* associated with "Hyperphosphatasia with mental retardation syndrome 2". These three genes were discarded since the phenotypes did not overlap with the patients.

AQP7, FAM205A, FAM214B, PRSS3 variants were also eliminated since the most recent eight patients evaluated by WES (adult patients with no neuromuscular involvement) also carried the same variants. The variant on the *HRCT1* gene has not been reported before related to a human disease so the gene did not have an OMIM number.

HRCT1 gene (NM_001039792) exon1;
c.317_318insCCACCACCACCCCCACCACACCCCTCACCACCTCCACCACCACC ACCACCACCA; (p.P106delinsPHHHPHHTPHHLHHHHHHH) variation is reported as homozygous in Family 4, and in two more families' (Family 5 and 6) affected were heterozygous for the same variant and also carried a different variation in a different position of the same gene (*-cis; -trans* status is not known).

Segregation analysis was planned on affected brothers, unaffected parents in the family, and in 10 healthy subjects to determine whether the variation is related to the disease or not. The segregation analysis revealed; it is compatible with the pedigree in the family, but two out of ten healthy subjects also carried the repeat expansion at homozygous state. So, the gene is eliminated from candidacy.

Bioinformatic analysis continued with various prioritization steps. Since the autosomal recessive inheritance model is considered in Family 4 and the parents are second-degree cousins once removed, homozygous changes were evaluated first. It was also assumed that the patients might have an X-linked recessive inheritance both being male. All genes with homozygous changes (<0.01 MAF) detected in Family 4 were scanned in other eight non-OPDM patients. The same method was applied to Family 5 and Family 6. In Family 4, only one gene remained after filtering: *ALYREF*. In contrast, the homozygous variant was not found in Family 4 in the *ALYREF* gene. WES analysis did not reveal the pathogenic variant responsible for the phenotype and further testing is suggested.

4.5. Family 5 (M.S.): OPMD, DM1, and OPDM1/2 were excluded in the index case. Since the parents were originating from different cities, LoH analysis was not performed.

Cranial MRI imaging was performed to exclude mtDNA deletion syndromes related to PEO. Cranial MRI revealed; chronic ischemic gliotic focis of millimeter-sized in the periventricular white matter, being more prominent in the periatrial areas. The depth and width of the sulci and fissures have increased mildly on both cerebral hemispheres. Cerebellar folios were mildly prominent (See Figure 4.13).

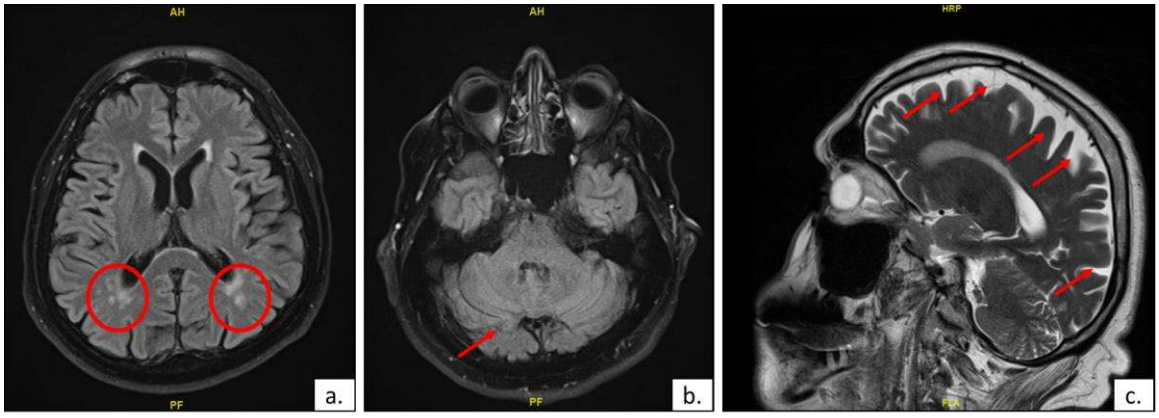


Figure 4.13: Cranial MRI sections showing ischemic gliotic focis in the periventricular white matter (a), cerebellar folios mildly prominent (b), and the depth and width of the sulci and fissures have increased (c) of the index case of Family 5 (M.S.)

For the bioinformatic analysis, WES changes with a frequency below 0.01 were listed (13,299 variants) as the initial step. There were 1,662 exonic or exonic splicing variants, and 1,116 of them were missense, frameshift deletion/insertion, nonframeshift deletion/insertion, stop gain, stop loss variants.

In Family 4 (E.G.), a candidate gene had variation in a homozygous state. In Family 5, WES analysis revealed the same mutation, *HRCT1* gene (NM_001039792) exon 1, p.P106delinsPHHHPHHTPHHLHHHHHHH in heterozygous state but also one more variant at heterozygous state exon1:c.299_300insCCACCACCACCA (p.L100delinsLHHHH). These variants have never been reported. The segregation analysis revealed; it is not compatible with the pedigree in the family, so the gene is eliminated from candidacy.

WES analysis revealed *TTN* gene (NM_001267550) exon 306, c.C63742T (p.L21248F) variant (rs771468174) at heterozygous state. This variant has a frequency of 0.000004 (1/246786, GnomAD_exome), 0.000009 (1/115194, ExAC) in databases [https://www.ncbi.nlm.nih.gov/snp]. *TTN* gene heterozygous mutations are associated with three different phenotypes. Tibial muscular dystrophy (TMD), tardive (MIM, #600334) is the possible overlapping phenotype with the patient's symptoms. TMD patients have; weakness of the lower leg muscles, steppage gait, and muscle biopsies reveal “rimmed vacuole.” TMD is an adult-onset, becoming symptomatic after 35 yrs, and slowly progressive disorder. The mutations related to TMD are located on the last exon of the gene. The *TTN* gene has 363 exons, and the variant detected is localized on exon 306. Segregation analysis for *TTN* variant is planned to clarify the inheritance pattern as (*de novo* or familial). The segregation analysis revealed; it is not compatible with the pedigree in the family, so the gene is eliminated from candidacy.

4.6. Family 6 (M.G.): OPMD, DM1, and OPDM1/2 were excluded for the index case. Since the parents of the index case originated from the same village, LoH analysis was planned for both affected brothers and the parents; unfortunately, no common LoH region was identified in affected family members.

The evaluation of WES analysis revealed 13,447 variants with a frequency below 0.01. There were 1,736 exonic or exonic splicing variants, and 1,181 of them were missense, frameshift deletion/insertion, nonframeshift deletion/insertion, stop gain, stop loss variations.

In Family 4 (E.G.), there was a candidate gene (*HRCT1*)with a variant at homozygous state. In Family 5 (M.S.), the same gene has the same variation (p.P106delinsPHHHPHHTPHHLHHHHHHH) in a heterozygous state with another heterozygous nonframeshit insertion (p.L100delinsLHHHH).

In Family 6, *HRCT1* gene (p.P106delinsPHHHPHHTPHHLHHHHHHH) variant was at heterozygous state. Another variant was also at heterozygous state with the same amino acid position of Family 5, with a longer insertion (c.299_300insCCACCACCACCACCACCCCCACCACCACCCCCGCCACACCCCT CACCACCTCCACCACCACCA; p.L100delinsLHHHHHPHHHPRHTPHHLHHHH). This variation had no reported frequencies in databases.

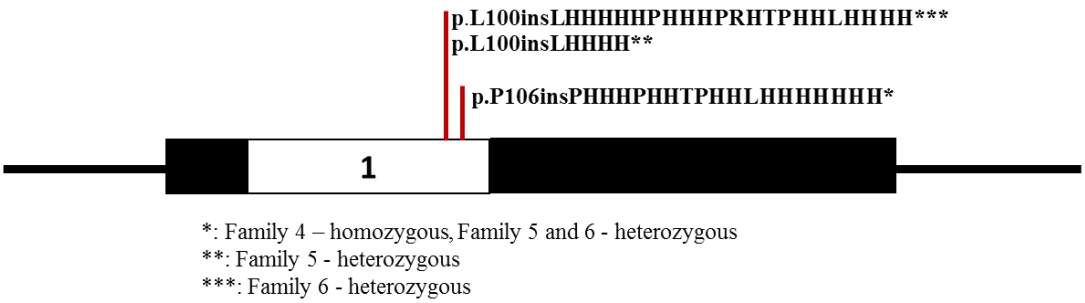


Figure 4.14: Schematic view of *HRCT1* gene variations and their localization

The variations were identified in two healthy controls at a homozygous state, and the gene is eliminated from candidacy.

WGS + mtDNA sequencing at an outer center (Centogene / Germany) also revealed no disease-associated variant, including OPMD1-2-3-4 related repeat expansions.

Family 7 (M.A.B.): M.A.B. was subjected to OPMD, DM1, and OPDM1/2 molecular testing, which were all noninformative.

To identify the OPDM-mimicking disorder's etiopathogenesis, WES+mtDNA sequence analysis is planned due to cranial MRI findings and suspicion of mitochondrial disease. Bioinformatic analysis revealed *RRM2B* gene (NM_001172477) p.Arg182His variant at homozygous state. mtDNA sequence analysis revealed two variants with uncertain significance. These are; mtDNA (NC_012920) *ND4* gene m.11622A>G (p.Tyr288Cys) in 9.8% heteroplasmy and *CO3* gene m.9331T>C (p.Leu42Pro) 19.8% heteroplasmy. To evaluate the pathogenicity of the variant in the *RRM2B* gene, segregation analysis was planned from the affected brother and healthy siblings, but family members refused to be tested. But the variant pathogenicity is updated to "likely pathogenic" and the molecular diagnosis was considered definite.

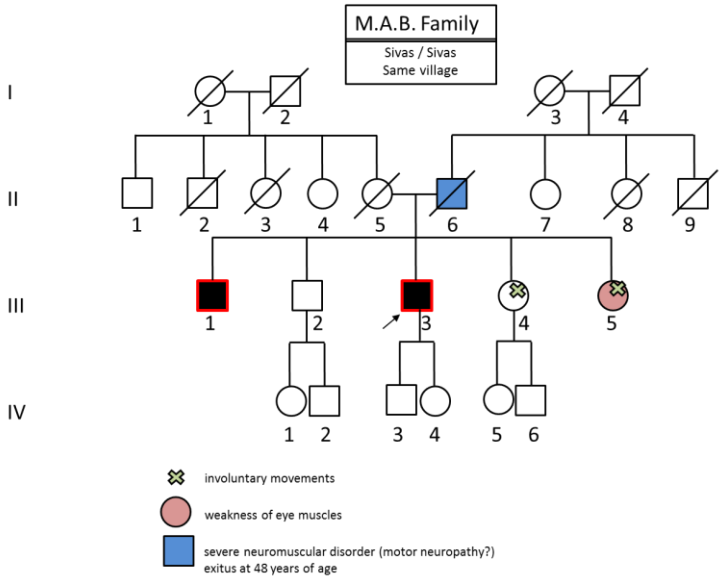


Figure 4.15: Updated pedigree of Family 7 (M.A.B.)

Chapter 5

DISCUSSION

OPDM is a progressive neuromuscular disorder that affects the extraocular, pharyngeal, laryngeal, and distal extremity muscles. The histopathological finding of OPDM is myopathic changes with rimmed vacuole is the most distinguishing feature of the disease. Although OPDM and OPMD are overlapping phenotypes, OPMD differs from OPDM with late age of onset and proximal muscle involvement. OPMD should be molecularly excluded in OPDM patients since muscle involvement usually occurs at later stages of the disease. Also, a more severe form of OPMD with onset at an earlier age may be due to the biallelic inheritance.

Repeat expansions play a role in the etiopathogenesis of several diseases that are on the differential diagnosis of OPDM. OPMD, being at the top of the DD list, is caused by GCG repeat expansions at the *PABPN1* gene. DM1 is caused by CTG repeat expansions at the *DMPK* gene 3' UTR. Considering repeat expansions as the possible cause of several neurological disorders, Ishiura (et al., 2019) analyzed CGG repeat expansion in WGS data of a group of NMD patients. These disorders were neuronal intranuclear inclusion disease (NIID), Oculopharyngeal myopathy with leukoencephalopathy (OPML), and Oculopharyngodistal Myopathy (OPDM). 5'UTR of *LRP12* gene CGG repeat expansion is associated with 38,2% of the OPDM patients, called OPDM1. The author stated, since OPDM is a heterogeneous disease, advanced genetic studies should be planned in patients whose etiopathogenesis could not be determined. Recently *GIPC1, NOTCH2NLC* and *RILPL1* genes have also been associated with OPDM. An increase in the number of CGG repeats was observed in all genes. Although four genes related to OPDM phenotype have been identified, still the majority of OPDM cases are unsolved at genetic basis.

As stated by Durmuş et al. in 2010, the OPDM diagnosis is still based on clinical and histopathological findings. In the past three years, 2019 on, repeat expansions in *LRP12, GIPC, NOTCH2NLC,* and *RILPL1* genes have been identified as the cause of OPDM, which leads us to repeat expansion testing of those four genes as the first-tier molecular testing of OPDM. However, as stated in the article, the negative outcome does not exclude the disease since it is determined only in 38,2% of the Japanese patients and 50% of the Chinese patients. None of the cases included in this study had expanded repeats in the *LRP12* or *GIPC1* gene. *NOTCH2NLC* and *RILPL1* genes repeat expansions could not be included due to covid-19 pandemia. Only one patient (Family 6; WGS data) is analyzed for the OPDM-related gene repeat expansions, which revealed a negative result.

Since the majority of OPDM cases are diagnosed clinically and histopathologically, a neurologist experienced in neuromuscular disorders is required when evaluating the patients. Although it is easier to diagnose the disease at advanced stages, genetic testing, muscle biopsy, cranial MRI, and other tests at early stages may guide to a definite diagnosis.

OPMD differs from OPDM due to proximal muscle involvement and later age onset. However, it is one of the diseases that should be excluded initially since the affected case presents similar findings like ptosis, dysphagia, muscle weakness, and rimmed vacuoles on muscle biopsy. It should be taken into consideration that the molecular findings may show an increased number of repeat expansions or biallelic inheritance when the patient has an earlier onset or severe phenotype.

To exclude DM1, which is the second disease in the differential diagnosis list, genetic testing or EMG showing myotonic discharges is compulsory. In cases who show no myotonia and when genetic testing is not possible, muscle biopsy or detailed family history for anticipation may guide the diagnosis of DM1. Muscle biopsies should always be evaluated by experienced neuropathologists. The pedigrees should also be evaluated for anticipation and parental transmission of the disease, maternal or paternal side.

PEO with mtDNA deletions are a group of disorders in which the clinical features of ptosis, PEO, and muscle involvement are observed. There are ten genes associated with PEO-related mtDNA deletions. Additional findings, cranial MRI and/or biochemical, could pinpoint the gene involved. Specific changes in muscle biopsies suggestive of mitochondrial disorders are revealed in all PEO cases with mtDNA deletions. If muscle biopsy and/or cranial MRI are performed in all patients with a preliminary clinical diagnosis of OPDM, this group of disorders can be eliminated.

OPDM1, OPDM2, OPDM3, and OPDM4 are clinically overlapping phenotypes of OPDM and there is no phenotypic finding that can differentiate one from the other.

We suggest the genetic diagnostic flow for diagnosis of OPDM as in Figure 5.1:

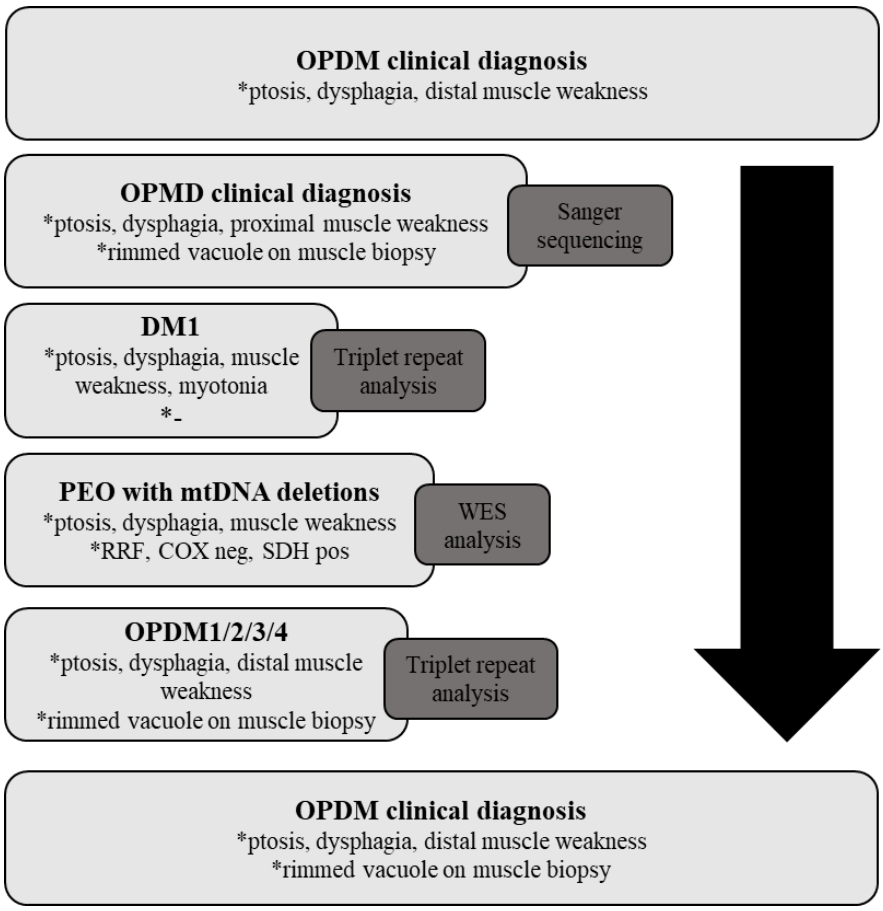


Figure 5.1: Flow diagram of clinically diagnosed OPDM patients

Seven patients who applied to Koç University Hospital (KUH) Neurology Department Neuromuscular Diseases Division between March 2018 and October 2019 and received a preliminary diagnosis of OPDM were included in the cohort. The diagnosis was confirmed in four patients: OPMD, PEOB2, and PEOA5. In three cases genetic etiopathogenesis could not be identified.

In Family 1 (A.A.Y), the genetic diagnosis of OPMD is confirmed with the phenotype of (GCG)6/9 repeat at the *PABPN1* gene. The initial referral diagnosis was DM1 from an outer center. Since the DM1 testing was noninformative, molecular testing was extended with the patient's permission. Physical examination led to clinical diagnosis of OPDM/OPMD. As the initial step, the *PABPN1* gene analysis was performed, and a definite diagnosis was achieved. Muscle biopsy of the patient was compatible with "myopathic changes" but did not show "rimmed vacuoles." Re-evaluation of muscle biopsy or performing muscle biopsy could be reconsidered when the diagnosis is not definite. The patient's clinical findings and molecular genetic testing results were compatible with OPMD.

In Family 2, the neurologists suspected of OPDM due to the early onset (44 years of age) of the disease. When the patient's pedigree was questioned, there was no similarly affected individual in the family. To exclude OPMD, molecular genetic testing was planned, and the molecular diagnosis of OPMD with (GCG)8/8 genotype is confirmed. With the patient's genotype result, her mother (around 80 years old) and brother (father died at 40 yrs due to schizophrenia(?)) were invited for clinical evaluation. Distal muscle weakness, ptosis, and dysphagia were noticed at the mother's neurological examination. Family members stated that they had thought of these symptoms as a sign of advancing age. Richard et al., (2017) state that individuals with a (GCG)6/8 genotype are expected to have a finding in the age range of 73±10. Genetic testing of the family members confirmed the heterozygous state of the mother but also the heterozygous state of the asymptomatic brother at his 60s. Genetic counseling session was planned for the asymptomatic brother.

In the literature, two more families carry the same allele ((GCG)8/8), like in Family 2 (F.K.) and they also originate from consanguineous families. The first patient had a family history of ptosis on both parental sides, maternal and paternal. On clinical examination at 60 years of age, he presented ptosis, dysphagia, and proximal muscle weakness. He had walking difficulty starting from his ‘50s. The second patient had bilateral ptosis and dysphagia around the age of his ’40s. His younger brother was also homozygous for the mutant allele and showed bilateral ptosis, dysphagia, ophthalmoparesis, and proximal and axial muscle weakness at 45 years of age [Richard et al., 2017].

The presence of the common LoH region identified in two distantly related, affected individuals from Family 3 (Y.İ.) led us to a definite diagnosis. The only “likely pathogenic” change in the LoH region was in the *RNASEH1* gene. Although the *RNASEH1* gene was not specific for the differential diagnosis in previous OPDM publications, we included PEO with mtDNA deletion in the differential diagnosis since one of them, the *POLG* gene, has been reported before [Thevathasan et al., 2011].

The identification of the *RNASEH1* gene with PEO disease was determined in 2015 as PEOB2. Most of the PEOB2 symptoms, dysphagia, ptosis, PEO, muscle weakness, respiratory impairment, and age of onset overlap with OPDM. While muscle biopsy can reveal suspicion of mitochondrial disease by RRF, decreased COX, and increased SDH activity [Reyes et al., 2015]. If the muscle biopsy had been performed in the index before molecular testing, the patient would not have a clinical OPDM diagnosis. However, since muscle biopsy is interventional, invasive, and costly and does not always lead to a definite diagnosis, except for specific diseases diagnosed by immunohistochemistry, recently it is postponed after the uninformative results of molecular testing [Domínguez-González et al., 2019].

In Family 3, there are several affected family members of different ages that have similar symptoms as the index case. Although family members are familiar with the disease, they did not consider the disease in family planning since genetic etiopathogenesis was not identified. The definite molecular diagnosis led to carrier testing and prenatal diagnosis in all individuals who are at risk.

In Family 7 (M.A.B.); homozygous mutation in the *RRM2B* gene leads to a diagnosis of RRM2B-related mitochondrial disease. The *RRM2B* gene heterozygous variants are associated with PEOA5 while biallelic mutations cause "Mitochondrial DNA depletion syndrome 8A (encephalomyopathic type with renal tubulopathy)-MTDPS8A" or "Mitochondrial DNA depletion syndrome 8B (MNGIE type)-MTDPS8B". PEOA5 is at the differential diagnosis of OPDM but not MTDPS8A or MTDPS8B. MTDPS8A/B are severe disorders characterized by neonatal hypotonia, lactic acidosis, and neurologic findings in the early stage of life. Since our patient did not have any of these symptoms (MTDPS8A/B), it is thought that the mutation is hypomorphic, leading to mild symptoms.

Family 4 (E.G.), Family 5 (M.S), and Family 6 (M.G) are unsolved cases of the cohort. The heterozygous variation in the *DNA2* gene reported from an outer center in Family 4 (E.G.) was excluded by segregation analysis since the healthy father carries the same variant. A nonframeshit insertion variation in the *HRCT1* gene was identified after the evaluation of WES data with prioritization of the LoH region. The variant which was in homozygous state in Family 4 (E.G.), was at compound heterozygous state in Family 5 (M.S) and Family 6 (M.G). The presence of another variant in a different region of the same gene in both individuals, strengthened the probability of the gene as a candidate one although segregation analysis and healthy control data eliminated the gene as a candidate.

The identification of OPDM related genes in the past three years has once again demonstrated the importance of triple repeat expansions in NMD's. In studies conducted, an increase in CGG repeats in the non-coding regions of *LRP12, GIPC1, NOTCH2NLC,* and *RILPL1* genes were associated with OPDM etiopathogenesis. WES analysis not covering non-coding regions of the genome could not detect repeat expansions. Repeat expansions in the non-coding regions of the genome could be identified only by WGS which should be considered as a first-tier research testing for unsolved NMDs.

Since all OPDM patients are not solved at molecular level further testing strategies such as RNAseq, tissue-specific testing to reveal possible mosaic states should be planned with international collaborations for the novel gene identification related to OPDM phenotype.

BIBLIOGRAPHY

[International Myotonic Dystrophy Consortium, 2000] New nomenclature and DNA testing guidelines for myotonic dystrophy type 1 (DM1). *The International Myotonic Dystrophy Consortium (IDMC).* (2000). Neurology, 54(6), 1218-1221. doi:10.1212/wnl.54.6.1218

[Adzhubei et al., 2010] Adzhubei, I. A., Schmidt, S., Peshkin, L., Ramensky, V. E., Gerasimova, A., Bork, P., . . . Sunyaev, S. R. (2010). A method and server for predicting damaging missense mutations. Nat Methods, 7(4), 248-249. doi:10.1038/nmeth0410-248

[Amato et al., 1995] Amato, A. A., Jackson, C. E., Ridings, L. W., & Barohn, R. J. (1995). Childhood-onset oculopharyngodistal myopathy with chronic intestinal pseudo-obstruction. Muscle Nerve, 18(8), 842-847. doi:10.1002/mus.880180807

[Apponi et al, 2010] Apponi, L. H., Leung, S. W., Williams, K. R., Valentini, S. R., Corbett, A. H., & Pavlath, G. K. (2010). Loss of nuclear poly(A)-binding protein 1 causes defects in myogenesis and mRNA biogenesis. Hum Mol Genet, 19(6), 1058-1065. doi:10.1093/hmg/ddp569

[Bird, 1999] Bird TD.(2019) *Myotonic Dystrophy Type 1*. 1999 Sep 17 [Updated 2019 Oct 3]. In: Adam MP, Ardinger HH, Pagon RA, et al., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2020. Available from: https://www.ncbi.nlm.nih.gov/books/NBK1165/

[Blumen et al., 2000] Blumen, S. C., Korczyn, A. D., Lavoie, H., Medynski, S., Chapman, J., Asherov, A., . . . Brais, B. (2000). *Oculopharyngeal MD among Bukhara Jews is due to a founder (GCG)9 mutation in the PABP2 gene*. Neurology, 55(9), 1267-1270. doi:10.1212/wnl.55.9.1267

[Brais et al., 1998] Brais, B., Bouchard, J. P., Xie, Y. G., Rochefort, D. L., Chretien, N., Tome, F. M., . . . Rouleau, G. A. (1998). *Short GCG expansions in the PABP2 gene cause oculopharyngeal muscular dystrophy.* Nat Genet, 18(2), 164-167. doi:10.1038/ng0298-164

[Bugiardini et al., 2017] Bugiardini, E., Poole, O. V., Manole, A., Pittman, A. M., Horga, A., Hargreaves, I., . . . Pitceathly, R. D. S. (2017). *Clinicopathologic and molecular spectrum of RNASEH1-related mitochondrial disease.* Neurol Genet, 3(3), e149. doi:10.1212/nxg.0000000000000149

[Carreno-Gago et al., 2019] Carreno-Gago, L., Blazquez-Bermejo, C., Diaz-Manera, J., Camara, Y., Gallardo, E., Marti, R., . . . Garcia-Arumi, E. (2019). *Identification and Characterization of New RNASEH1 Mutations Associated With PEO Syndrome and Multiple Mitochondrial DNA Deletions.* Front Genet, 10, 576. doi:10.3389/fgene.2019.00576

[De Braekeleer, 1991] De Braekeleer, M. (1991). *Hereditary disorders in Saguenay-Lac-St-Jean* (Quebec, Canada). Hum Hered, 41(3), 141-146. doi:10.1159/000153992

[Domínguez-González et al., 2019] Domínguez-González, C., Hernández-Laín, A., Rivas, E., Hernández-Voth, A., Sayas Catalán, J., Fernández-Torrón, R., . . . Paradas, C. (2019). Late-onset thymidine kinase 2 deficiency: a review of 18 cases. Orphanet J Rare Dis, 14(1), 100. doi:10.1186/s13023-019-1071-z

[Durmuş et al., 2011] Durmus, H., Laval, S. H., Deymeer, F., Parman, Y., Kiyan, E., Gokyigiti, M., . . . Serdaroglu-Oflazer, P. (2011). *Oculopharyngodistal myopathy is a distinct entity: clinical and genetic features of 47 patients*. Neurology, 76(3), 227-235. doi:10.1212/WNL.0b013e318207b043

[Fratter et al, 2010] Fratter, C., Gorman, G. S., Stewart, J. D., Buddles, M., Smith, C., Evans, J., . . . Taylor, R. W. (2010). The clinical, histochemical, and molecular spectrum of PEO1 (Twinkle)-linked adPEO. Neurology, 74(20), 1619-1626. doi:10.1212/WNL.0b013e3181df099f

[Goldstein and Falk, 2013] Goldstein, A., & Falk, M. J. (1993). *Mitochondrial DNA Deletion Syndromes. I*n M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, L. J. H. Bean, K. Stephens, & A. Amemiya (Eds.), GeneReviews((R)). Seattle (WA): University of Washington, Seattle

[Hirano et al., 2001] Hirano, M., & DiMauro, S. (2001). ANT1, Twinkle, POLG, and TP: new genes open our eyes to ophthalmoplegia. Neurology, 57(12), 2163-2165. doi:10.1212/wnl.57.12.2163

[Ishiura et al., 2019] Ishiura, H., Shibata, S., Yoshimura, J., Suzuki, Y., Qu, W., Doi, K., . . . Tsuji, S. (2019). *Noncoding CGG repeat expansions in neuronal intranuclear inclusion disease, oculopharyngodistal myopathy and an overlapping disease*. Nat Genet, 51(8), 1222-1232. doi:10.1038/s41588-019-0458-z

[Kamsteeg et al., 2012] Kamsteeg, E. J., Kress, W., Catalli, C., Hertz, J. M., Witsch-Baumgartner, M., Buckley, M. F., . . . Scheffer, H. (2012). *Best practice guidelines and recommendations on the molecular diagnosis of myotonic dystrophy types 1 and 2.* Eur J Hum Genet, 20(12), 1203-1208. doi:10.1038/ejhg.2012.108

[Karczewski et al., 2020] Karczewski, K. J., Francioli, L. C., Tiao, G., Cummings, B. B., Alföldi, J., Wang, Q., . . . MacArthur, D. G. (2020). *The mutational constraint spectrum quantified from variation in 141,456 humans.* 531210. doi:10.1101/531210 %J bioRxiv

[Kaukonen et al, 1999] Kaukonen, J., Zeviani, M., Comi, G. P., Piscaglia, M. G., Peltonen, L., & Suomalainen, A. (1999). *A third locus predisposing to multiple deletions of mtDNA in autosomal dominant progressive external ophthalmoplegia.* Am J Hum Genet, 65(1), 256-261. doi:10.1086/302445

[Ladd et al., 2004]Ladd, A. N., Nguyen, N. H., Malhotra, K., & Cooper, T. A. (2004). *CELF6, a member of the CELF family of RNA-binding proteins, regulates muscle-specific splicing enhancer-dependent alternative splicing.* J Biol Chem, 279(17), 17756-17764. doi:10.1074/jbc.M310687200

[Lu et al., 2008] Lu, H., Luan, X., Yuan, Y., Dong, M., Sun, W., & Yan, C. (2008*). The clinical and myopathological features of oculopharyngodistal myopathy in a Chinese family.* Neuropathology, 28(6), 599-603. doi:10.1111/j.1440-1789.2008.00924.x

[Ma et al., 2020] Ma D., Tan YJ., Ng ASL., Ong HL., Sim W., Lim WK., …, Tan EK. *Association of NOTCH2NLC Repeat Expansions With Parkinson Disease.* JAMA Neurol. 2020 Aug 24;77(12):1–5. doi: 10.1001/jamaneurol.2020.3023. PMID: 32852534; PMCID: PMC7445625.

[Maeda et al., 2017] Maeda, M. Y., Hashimoto, T. Y., Oliveira Neto, I. C., & Neves, L. R. (2017). *Oculopharyngeal muscular dystrophy or oculopharyngeal distal myopathy: case report.* Braz J Otorhinolaryngol, 83(2), 235-238. doi:10.1016/j.bjorl.2015.07.019

[Martorell et al., 2007] Martorell, L., Cobo, A. M., Baiget, M., Naudo, M., Poza, J. J., & Parra, J. (2007). *Prenatal diagnosis in myotonic dystrophy type 1. Thirteen years of experience: implications for reproductive counselling in DM1 families.* Prenat Diagn, 27(1), 68-72. doi:10.1002/pd.1627

[Mignarri et al., 2012] Mignarri, A., Carluccio, M. A., Malandrini, A., Sicurelli, F., Galli, L., Mazzei, M. A., . . . Dotti, M. T. (2012*). The first Italian patient with oculopharyngodistal myopathy: case report and considerations on differential diagnosis.* Neuromuscul Disord, 22(8), 759-762. doi:10.1016/j.nmd.2012.03.010

[Minami et al., 2001] Minami, N., Ikezoe, K., Kuroda, H., Nakabayashi, H., Satoyoshi, E., & Nonaka, I. (2001). *Oculopharyngodistal myopathy is genetically heterogeneous and most cases are distinct from oculopharyngeal muscular dystrophy.* Neuromuscul Disord, 11(8), 699-702. doi:10.1016/s0960-8966(01)00227-9

[Morrison, 2016] Morrison, B. M. (2016). *Neuromuscular Diseases.* Semin Neurol, 36(5), 409-418. doi:10.1055/s-0036-1586263

[Moxley & Meola 2008] Moxley RT, Meola G. *The myotonic dystrophies. In: The Molecular and Genetic Basis of Neurologic and Psychiatric Disease*. Rosenberg RN, DiMauro S, Paulson HL, Ptacek L, Nestler EJ, eds. Philadelphia, PA: Wolters Kluwer; 2008:532-41

[Ng & Henikoff, 2003] Ng, P. C., & Henikoff, S. (2003*). SIFT: Predicting amino acid changes that affect protein function.* Nucleic Acids Res, 31(13), 3812-3814. doi:10.1093/nar/gkg509

[Nicholls et al, 2018] Nicholls, T. J., Nadalutti, C. A., Motori, E., Sommerville, E. W., Gorman, G. S., Basu, S., . . . Gustafsson, C. M. (2018). *Topoisomerase 3alpha Is Required for Decatenation and Segregation of Human mtDNA.* Mol Cell, 69(1), 9-23.e26. doi:10.1016/j.molcel.2017.11.033

[Prieto et al., 1997] Prieto, C. C., Leonardelli, R. I., & Zalazar, F. E. (1997). *A procedure of silver staining for nucleic acids in agarose gels without pretreatment or drying steps*. Anal Biochem, 252(1), 15-18. doi:10.1006/abio.1997.2288

[Reyes et al., 2015] Reyes, A., Melchionda, L., Nasca, A., Carrara, F., Lamantea, E., Zanolini, A., . . . Zeviani, M. (2015). *RNASEH1 Mutations Impair mtDNA Replication and Cause Adult-Onset Mitochondrial Encephalomyopathy*. Am J Hum Genet, 97(1), 186-193. doi:10.1016/j.ajhg.2015.05.013

[Richard et al., 2017] Richard, P., Trollet, C., Stojkovic, T., de Becdelievre, A., Perie, S., Pouget, J., & Eymard, B. (2017). *Correlation between PABPN1 genotype and disease severity in oculopharyngeal muscular dystrophy*. Neurology, 88(4), 359-365. doi:10.1212/wnl.0000000000003554

[Robinson et al., 2006] Robinson, D. O., Wills, A. J., Hammans, S. R., Read, S. P., & Sillibourne, J. (2006). *Oculopharyngeal muscular dystrophy: a point mutation which mimics the effect of the PABPN1 gene triplet repeat expansion mutation.* J Med Genet, 43(5), e23. doi:10.1136/jmg.2005.037598

[Ronchi et al., 2013] Ronchi, D., Di Fonzo, A., Lin, W., Bordoni, A., Liu, C., Fassone, E., . . . Comi, G. P. (2013). *Mutations in DNA2 link progressive myopathy to mitochondrial DNA instability.* Am J Hum Genet, 92(2), 293-300. doi:10.1016/j.ajhg.2012.12.014

[Ronchi et al., 2012] Ronchi, D., Garone, C., Bordoni, A., Gutierrez Rios, P., Calvo, S. E., Ripolone, M., . . . Sciacco, M. (2012). *Next-generation sequencing reveals DGUOK mutations in adult patients with mitochondrial DNA multiple deletions.* Brain, 135(Pt 11), 3404-3415. doi:10.1093/brain/aws258

[Sachdev et al., 2018] Sachdev, A., Fratter, C., & McMullan, T. F. W. (2018). *Novel mutation in the RNASEH1 gene in a chronic progressive external ophthalmoplegia patient.* Can J Ophthalmol, 53(5), e203-e205. doi:10.1016/j.jcjo.2018.01.005

[Satoyoshi and Kinoshita, 1977] Satoyoshi, E., & Kinoshita, M. (1977). *Oculopharyngodistal myopathy.* Arch Neurol, 34(2), 89-92. doi:10.1001/archneur.1977.00500140043007

[Schwarz et al, 2014] Schwarz, J. M., Cooper, D. N., Schuelke, M., & Seelow, D. (2014). MutationTaster2: mutation prediction for the deep-sequencing age. Nat Methods, 11(4), 361-362. doi:10.1038/nmeth.2890

[Shimizu et al., 2013] Shimizu, Y., Suzuki, S., Mori-Yoshimura, M., Nagasao, T., Toriumi, M., Oji, T., . . . Kishi, K. (2013). Surgical treatment of severe blepharoptosis and facial palsy caused by oculopharyngodistal myopathy. J Plast Reconstr Aesthet Surg, 66(10), e277-280. doi:10.1016/j.bjps.2013.03.034

[Taylor et al., 1915] Taylor, E. W. Progressive vagus-glossopharyngeal paralysis with ptosis: contribution to group of family diseases. J. Nerv. Ment. Dis. 42: 129-139, 1915

[Theadom et al., 2014] Theadom, A., Rodrigues, M., Roxburgh, R., Balalla, S., Higgins, C., Bhattacharjee, R., . . . Feigin, V. (2014*). Prevalence of muscular dystrophies: a systematic literature review.* Neuroepidemiology, 43(3-4), 259-268. doi:10.1159/000369343

[Thevathasan et al., 2011] Thevathasan, W., Squier, W., MacIver, D. H., Hilton, D. A., Fathers, E., & Hilton-Jones, D. (2011). Oculopharyngodistal myopathy--a possible association with cardiomyopathy. Neuromuscul Disord, 21(2), 121-125. doi:10.1016/j.nmd.2010.10.002

[Thornton et al., 2014] Thornton, C. A. (2014). *Myotonic dystrophy.* Neurol Clin, 32(3), 705-719, viii. doi:10.1016/j.ncl.2014.04.011

[Tian et al., 2015] Tian, X., Liang, W. C., Feng, Y., Wang, J., Zhang, V. W., Chou, C. H., . . . Jong, Y. J. (2015). *Expanding genotype/phenotype of neuromuscular diseases by comprehensive target capture/NGS*. Neurol Genet, 1(2), e14. doi:10.1212/nxg.0000000000000015

[Trollet et al., 2001] Trollet, C., Gidaro, T., Klein, P., Perie, S., Butler-Browne, G., & Lacau St Guily, J. (1993). *Oculopharyngeal Muscular Dystrophy*. In M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, L. J. H. Bean, K. Stephens, & A. Amemiya (Eds.), GeneReviews((R)). Seattle (WA): University of Washington, Seattle

[Tyynismaa et al., 2012] Tyynismaa, H., Sun, R., Ahola-Erkkila, S., Almusa, H., Poyhonen, R., Korpela, M., . . . Suomalainen, A. (2012). Thymidine kinase 2 mutations in autosomal recessive progressive external ophthalmoplegia with multiple mitochondrial DNA deletions. Hum Mol Genet, 21(1), 66-75. doi:10.1093/hmg/ddr438

[Tyynismaa et al., 2009] Tyynismaa, H., Ylikallio, E., Patel, M., Molnar, M. J., Haller, R. G., & Suomalainen, A. (2009). A heterozygous truncating mutation in RRM2B causes autosomal-dominant progressive external ophthalmoplegia with multiple mtDNA deletions. Am J Hum Genet, 85(2), 290-295. doi:10.1016/j.ajhg.2009.07.009

[Uyama et al., 1998] Uyama, E., Uchino, M., Chateau, D., & Tome, F. M. (1998*). Autosomal recessive oculopharyngodistal myopathy in light of distal myopathy with rimmed vacuoles and oculopharyngeal muscular dystrophy.* Neuromuscul Disord, 8(2), 119-125. doi:10.1016/s0960-8966(98)00002-9

[van der Sluijs et al., 2004] van der Sluijs, B. M., ter Laak, H. J., Scheffer, H., van der Maarel, S. M., & van Engelen, B. G. (2004). Autosomal recessive oculopharyngodistal myopathy: a distinct phenotypical, histological, and genetic entity. J Neurol Neurosurg Psychiatry, 75(10), 1499-1501. doi:10.1136/jnnp.2003.025072

[Victor et al., 1962] Victor, M., Hayes, R., & Adams, R. D. (1962). *Oculopharyngeal muscular dystrophy. A familial disease of late life characterized by dysphagia and progressive ptosis of the eyelids.* N Engl J Med, 267, 1267-1272. doi:10.1056/nejm196212202672501

[Wang et al.; 2016] Wang, Y., Peng, W., Guo, H. Y., Li, H., Tian, J., Shi, Y. J., . . . Feng, Z. C. (2016). *Next-generation sequencing-based molecular diagnosis of neonatal hypotonia in Chinese Population.* Sci Rep, 6, 29088. doi:10.1038/srep29088

[Warner et al., 1996] Warner, J. P., Barron, L. H., Goudie, D., Kelly, K., Dow, D., Fitzpatrick, D. R., & Brock, D. J. (1996). *A general method for the detection of large CAG repeat expansions by fluorescent PCR*. J Med Genet, 33(12), 1022-1026. doi:10.1136/jmg.33.12.1022

[Witoonpanich et al., 2004] Witoonpanich, R., Phankhian, S., Sura, T., Lertrit, P., & Phudhichareonrat, S. (2004). Oculopharyngodistal myopathy in a Thai family. J Med Assoc Thai, 87(12), 1518-1521.

[Young et al., 2011] Young, M. J., Longley, M. J., Li, F. Y., Kasiviswanathan, R., Wong, L. J., & Copeland, W. C. (2011). Biochemical analysis of human POLG2 variants associated with mitochondrial disease. Hum Mol Genet, 20(15), 3052-3066. doi:10.1093/hmg/ddr209

[Yu et al., 2022] Yu, J., Shan, J., Yu, M., Di, L., Xie, Z., Zhang, W., Lv, H., Meng, L., Zheng, Y., Zhao, Y., Gang, Q., Guo, X., and 9 others. The CGG repeat expansion in RILPL1 is associated with oculopharyngodistal myopathy type 4. Am. J. Hum. Genet. 109: 533-541, 2022.

[Zhao et al., 2015] Zhao, J., Liu, J., Xiao, J., Du, J., Que, C., Shi, X., . . . Wang, Z. (2015). Clinical and muscle imaging findings in 14 mainland chinese patients with oculopharyngodistal myopathy. PLoS One, 10(6), e0128629. doi:10.1371/journal.pone.0128629

Websites:

https://annovar.openbioinformatics.org/en/latest/

https://blast.ncbi.nlm.nih.gov/Blast.cgi

https://gatk.broadinstitute.org/hc/en-us

https://neuromuscular.wustl.edu/pathol/mitochondrial.htm

https://omim.org/

https://toptipbio.com/delta-delta-ct-pcr

http://www.1000genomes.org/

https://www.ncbi.nlm.nih.gov/snp

OPDM DEĞERLENDİRME FORMU

KOÇ ÜNİVERSİTESİ HASTANESİ

Ad - Soyad		Muayene tarihi	
Doğum tarihi		GTM No	
Cinsiyeti		Olgu No	
Telefon numarası			
Anne-baba akrabalık	Var / Yok	Boy	
Başlangıç yaşı		Kilo	
İlk bulgu		Baş çevresi	
		Kilo kaybı	

	Var / Yok	Başlangıç yaşı	Açıklama
Pitoz			
Oftalmoparezi			
Yutma güçlüğü (disfaji)			
Dizartri / Nazone konuşma /Ses kısıklığı			
Yürüme güçlüğü			
Yüz kasları tutulumu Orbikülaris okuli Orbikularis oris Malar Zigomatik			göz sıkma: sırıtma: ıslık çalma:
Solunum güçlüğü			yatarken tek nefeste kaça kadar saydığı: otururken tek nefeste kaça kadar saydığı:
Plantar refleks / DTR			
Kas gücü muayenesi	Boyun fleksör _____/5 ekstensor _____/5 Üst ekstremite distal _____/5 proksimal _____/5 Alt ekstremite distal _____/5 proksimal _____/5		

KOÇ ÜNİVERSİTESİ HASTANESİ

OPDM DEĞERLENDİRME FORMU

Ad - Soyad			Muayene tarihi	
Doğum tarihi			GTM No	
Cinsiyeti			Olgu No	

Antenatal ve erken çocukluk dönemi:

Çocukluk çağı hastalıkları :

Hastane yatışı / operasyon öyküsü :

Bulguları ile başvurduğu sağlık kuruluşları ve tetkikler:

Solunum muayenesi:

Kardiyovasküler muayene:

KBB muayenesi (Özellikle vokal kordlar):

İşitmenin nasıl olduğu:

Ek bulgular:

NOT:
1. Onam formu (araştırma ve fotoğraf/video)
2. Fotoğraf, video çekilmesi
3. Biyokimya tetkikleri
4. Kas biyopsisi/ EMG raporları

Rumelifeneri Yolu Sarıyer 34450 İstanbul T: 0212 338 10 00 F: 0212 338 12 05 www.ku.edu.tr

KOÇ
ÜNİVERSİTESİ

ETİK KURUL KARARI

Toplantı Tarihi:	13.02.2019
Karar No:	2019.051.IRB2.019
Sorumlu Araştırıcı:	Şahin Avcı
Araştırma Başlığı:	Otozomal Çekinik Kalıtımlı Okülofaringodistal Miyopatinin Moleküler Karakterizasyonu
Başlangıç tarihi:	18.02.2019
Etik Kurul izninin süresi:	1 yıl (Uzatma hakkı mevcut olarak)

Koç Üniversitesi Etik Kurulu'na değerlendirilmek üzere başvuruda bulunduğunuz yukarıda künyesi yazılı projenizin başvuru dosyası ve ilgili belgeleri, Üniversitemiz **"Biyomedikal Araştırmalar Etik Kurulu"** tarafından araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiştir.

Yapılan inceleme sonucunda çalışmanın gerçekleştirilmesinde etik ve bilimsel sakınca bulunmadığına karar verilmiştir.

Notlar:

- Araştırma başlangıç tarihinin 6 aydan daha fazla gecikmesi durumunda Etik Kurul'a başvurularak tarihlerin değiştirilmesi gereklidir.
- Etik Kurul incelemesi ve onayı olmadan bu araştırmada kullanılan prosedürler, formlar ya da protokollerde herhangi bir değişiklik yapılamaz.
- Etik bakımdan sorun çıkması ya da şüpheli bir olay/beklenmeyen etki görülmesi durumunda derhal etik kurul bilgilendirilmelidir.
- Araştırmanın gerçekleştirileceği birimlerin yöneticilerinden de ayrıca izin alınması gerekli olabilir.

Saygılarımla,

Hakan S. Orer
Başkan