IDENTIFICATION OF PATHOGENIC MUTATIONSIN NEURODEGENERATIVE DISORDERS: BIOINFORMATIC ANALYSIS OF NEXT GENERATION SEQUENCING DATA

by

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To my beloved mother,

Gönül Kartal,

who gives me inspiration and hope.

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ABSTRACT

IDENTIFICATION OF PATHOGENIC MUTATIONS IN NEURODEGENERATIVE DISORDERS: BIOINFORMATIC ANALYSIS OF NEXT GENERATION SEQUENCING DATA

Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and ataxias belong to a large group of disorders, characterized by progressive neuronal cell death in specific parts of the brain. They exhibit Mendelian and also complex inheritance patterns, thus a deeper understanding of genetic factors underlying disease pathogenesis is crucial to improve new and more targeted therapy methods.

Next generation sequencing is a highly efficient and powerful method for the identification of disease-causing variants in complex disorders. In the framework of this study, one dominant and 12 recessive pedigrees were subjected to exome sequencing. Selection of families was performed by considering factors, such as consanguinity and the presence of more than one affected member in the family. Homozygosity mapping was performed for determination of homozygous stretches. Candidate variants were validated in family members and segregation of mutations in the families was shown.

The data and bioinformatic analyses methods described above were for the first time adapted, developed and applied to several families in our laboratory within this thesis work. The analysis of next generation sequencing data is expected to unravel the genetic factors that cause neurodegeneration in these complex syndromes.

ÖZET

NÖRODEJENERATIF HASTALIKLARDA PATOJENİK MUTASYONLARIN BELIRLENMESI:
YENI NESIL DIZILEME VERILERININ BIYOİNFORMATIK ANALIZI

Alzheimer, Parkinson, amiyotrofik lateral sklerozveataksilergibi nörodejeneratif hastalıklar, beyninözgünbölgelerindekisinirhücrelerininprogresifkaybıiletanımlanangenişbirhastalıkgrubunadahildirler. Bu hastalıklar hem Mendel türü, hem de komplekskalıtımgösterdikleriiçin, hastalıklarınaltındayatangenetikfaktörleriderinlemesineanlamak, yeniveözgüntedaviyöntemlerinigeliştirmekaçısındanyaşamsalönemesahiptir.

Yeninesildizilemeyöntemleri, hastalıknedenitanımlanmasındaoldukçaetkinveyüksekölçekliyaklaşımlardır. Bu çalışmakapsamında, birdominantve 12 resesifgeçişliaile, ekzomdizilemeanaliziileincelenmiştir. Ailelerinseçimibirdenfazlaetkilenmişbireyveakrabaevliliğigibifaktörlergözetilerekyapılmıştır.Ayrıcahomozigotbölgelerintanımlanmasıiçinhomozigotlukharitalanmasıuygulanmıştır.Bulunanadaymutasyonlar/değişimler, ailebireylerindedoğrulanmışvemutasyonunaile-içigeçişigösterilmiştir.

Tarifedilenbiyoinformatikanalizyöntemleri, laboratuvarımızda ilk defabutezkapsamındaadapteedilmiş, geliştirmişvebirçokaileyeuygulanmıştır.Yeninesildizilemeanalizinin, buhastalıklardanörodejenerasyonanedenolangenetikfaktörlerinanlaşılmasınakatkıdabulunmasıbeklenmektedir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

*	Asterisk
%	Percentage
^{o}C	Centigrade degree
g	Gram
kb	Kilobase
µg	Microgram
µl	Microliter
µM	Micromolar
mM	Milimolar
mg	Miligram
ml	Mililiter
M	Molar
Mb	Million bases
ng	Nanogram
#	Number
V	Volt

LIST OF ACRONYMS/ABBREVIATIONS

AD	Alzheimer's Disease
ABL	Abelson Murine Leukemia Viral Oncogene Homolog 1
ACAN	Agreecan
ADCK3	AarF Domain Containing Kinase 3
ALS	Amyotrophic Lateral Sclerosis
ALS2	Alsin
ALS-FTD	Amyotrophic Lateral Sclerosis-Frontotemporal Dementia
ALSPDC	ALS-Parkinsonism-Dementia Complex
ALSoD	Amyotrophic Lateral Sclerosis Online Genetics Database
ANG	Angiogenin
ANO10	Anoctamin 10
AO	Age of Onset
AOA	Autosomal Recessive Ataxia With Oculomotor Apraxia
APTX	Aprataxin
AR	Autosomal Recessive
ARCA	Autosomal Recessive Cerebellar Ataxias
ARHGEF28	Rho Guanine Nucleotide Exchange Factor (GEF) 2
ARSACS	Autosomal Recessive Spastic Ataxia Of Charlevoix Saguenay
ATLD	Ataxia Telangiectasia Like Disorder
A-T	Ataxia–Telangiectasia
ATM	Ataxia Telangiectasia Mutated
ATP13A2	ATPase Type 13A2
ATXN2	Ataxin 2

BMAA	Beta-*N*-Methylamino-L-Alanine
BWA	Burrows-Wheeler Aligner
C	Cysteine
C9orf72	Chromosome 9 Open Reading Frame 72
ChIP-seq	Chromatin Immunoprecipitation
CHMP2B	Charged Multivesicular Body Protein 2B
Chr	Chromosome
Ca^{2+}	Calcium Ion
ConDel	CONsensusDELeteriousness
CNV	Copy Number Variation
Cu	Copper
CYP27A1	Cytochrome P450, Family 27, Subfamily A, Polypeptide
DAO	D-amino-acid oxidase
DCTN1	Dynactin 1
DENN	Differentially Expressed in Normal and Neoplastic Cells
DNA	Deoxyribonucleic Acid
DJ1	Parkinson Protein 7
DGV	Database of Genomic Variants
dNTP	Deoxyribonucleotide Triphosphate
DSB	Double-strand Break
EAAT2	Excitatory Amino Acid Neurotransmitter 2
EDTA	Ethylenediaminetetraacetic Acid
EOAH	Early-onset Ataxia with Ocular Motor Apraxia and Hypoalbuminemia
EOPD	Early-onset PD
ER	Endoplasmic Reticulum
ESP	Exome Sequencing Project

EtBr	Ethidium Bromide
EtOH	Ethanol
fALS	Familial Amyotrophic Lateral Sclerosis
FBXO7	F-Box Only Protein 7
FIG4	FIG4 phosphoinositide 5-phosphatase
FRDA	Friedreich's ataxia
FTD	Frontotemporal Dementia
FUS	Fused in Sarcoma
FXN	Frataxin
G	Glycine
GATK	Genome Analysis Toolkit
GOF	Gain of Function
H	Histidine
HEXA	Hexosaminidase A (Alpha Polypeptide)
HGMD	Human Gene Mutation Database
HIT	Histidine-triad
H_2O	Water
H_2O_2	Hydrogen Peroxide
HomSI	Homozygous Stretch Identifier
Ig	Immunoglobulin
IGV	Integrative Genomics Viewer
INAREK	Ethics Committee on Research with Human Participants
KRS	Kufor-Rakeb syndrome
LMN	Lower Motor Neuron
LOF	Loss of Function
MAF	Minor allele Frequency
Methyl-seq	Methyl Sequencing

MIRAS	Polymerase (DNA Directed), Gamm
MND	Motor Neuron Disease
MRE11	Meiotic Recombination 11 Homolog A
MRN	Mre11-Rad50-Nbs1
mRNA	Messenger Ribonucleic Acid
mt	Mutant
NEFH	Neurofilament, Heavy Polypeptide
ND	Neurodegenerative diseases
NGS	Next Generation Sequencing
NPC1	Niemann-Pick Disease, Type C1
NPHP-RC	Nephronophthisis-Related Ciliopathies
O_2^-	Superoxide
O_2	Oxygen
OPTN	Optineurin
OH^-	Hydroxyl radicals
PARK9	Parkinson's Disease 9
PBS	Phosphate Buffered Saline
PD	Parkinson's disease
PDC	Parkinsonism-Dementia Complex
PCR	Polymerase Chain Reaction
PEX	Peroxisomal Biogenesis Factor
PFN1	Profilin 1
PHYH	Phytanoyl-CoA 2-Hydroxylase
PIK3R5	Phosphoinositide-3-Kinase, Regulatory Subunit 5
PLA2G6	Phospholipase A2, Group VI (Cytosolic, Calcium-Independent)
PolyPhen2	Polymorphism Phenotyping v2

POLG	polymerase (DNA directed), gamma
PRPH	Peripherin
Provean	Protein Variation Effect Analyzer
Q	Glutamine
R	Arginine
RNA	Ribonucleic Acid
RNA-seq	RNA Sequencing
ROS	Reactive Oxygen Species
SACS	Sacsin
sALS	Sporadic Amyotrophic Lateral Sclerosis
SCA	Spinocerebellar Ataxia
SETX	Senataxin
SIGMAR1	Sigma Non-Opioid Intracellular Receptor 1
SIFT	Sorts Intolerant from Tolerant
SLC6A19	solute carrier family 6 (neutral amino acid transporter), member 19
SMA	Spinal Muscular Atrophy
SNP	Single Nucleotide Polymorphism
SOD1	Superoxide Dismutase1
SPG11	Spatacsin
SQSTM1	Sequestosome 1
SYT14	SynaptotagminXIV
SYNE1	Spectrin Repeat Containing, Nuclear Envelope 1
TAF15	TATA-Binding Protein-Associated Factor 2N
TARDBP/ TDP-43	TAR DNA Binding Protein
TBE	Tris/Borate/EDTA
TDP1	Tyrosyl-DNA Phosphodiesterase 1

TM	Melting Temperature
TM3	Third Multiple Three
TRPM7	Transient Receptor Potential Melastatin 7
TTPA	Tocopherol (Alpha) Transfer Protein
UAS	Upstream Activation Sequence
UBQLN2	Ubiquilin 2
UMN	Upper Motor Neurons
UPR	Unfolded Protein Response
UV	Ultraviolet
VABP	Vesicle-associated Membrane Protein-associated Protein B
VCP	Valosin-Containing Protein
WB	Western Blot
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
Y	Tyrosine
Zn	Zinc

1. INTRODUCTION

1.1. Application of Next-Generation Sequencing Technologies

The development of massively parallel nucleic acid sequencing or next generation sequencing (NGS),also known as high-throughput sequencing, has drastically changed our understanding of human genome over the last decade. In 2003, the first draft of the human genome was obtained; afterwards the number of individuals, whose genome has been sequenced, has exploded. The NGS technology allows performing various applications, such as whole genome sequencing (WGS), whole exome sequencing (WES), whole transcriptome analysis (RNA-seq), genome-wide profiling of epigenetic marks (methyl-seq) and chromatin structure (ChIP-seq). The technology has been adopted in many laboratories and dominates today the genetic and genomic research all around the world.

1.1.1. Whole Exome Sequencing

1.1.1.1. Significance

The exome, which contains 180.000 exons, constitutes about 1% of the whole genome. However, mutations in these regions of the genome are much more likely to have severe consequences by comparison with the entire genome (Stenson *et al.*, 2009).

Whole exome sequencing, is an efficient and powerful tool, which covers the protein-coding regions of the entire human genome. WES, with its high-throughput capacity and low cost, represents a highly enriched subset of the genome, useful in medical research for disease-causing variants, because the majority of disease-causing variants are in the protein-coding region.

Exome sequencing shows a high potential to identify causative variants in Mendelian disorders. A successful model strategy for gene discovery in Mendelian disorders is the trio sequencing where parents and the proband are genotyped. Complex disorders have a genetically heterogeneous background, so a large number of genes are thought to be associated to small degrees with disease(Han *et al.*, 2014). This heterogeneity means that very large sample sizes are required for gene discovery, and exome sequencing has the potential to detect causative genes in complex disorders, which previously was not possible with traditional methods. The most significant impact of NGS is in molecular diagnosis. It helps to detect the causative variants/genes in previously undiagnosed or clinically complex conditions(Nemeth *et al.*, 2013). WES allows clinicians to diagnose affected patients with conditions that have eluded traditional diagnostic approaches and makes the identification of cases possible in whom, mutations indifferent genes contribute to the phenotype. Identification of disease gene mutations has major implications for clinical diagnosis and therapy. The pathological pathways can be understood with the revelation of the genetic basis of the diseases, which paves the way for novel therapeutic approaches.

1.1.1.2. Limitations

Despite its many advantages, WES has also some technical and biological drawbacks:

- Exome sequencing is only able to identify variants in the coding region of the genome. There are missing parts elsewhere in genome, and variants outside of the protein-coding regions may alter gene expression profiles and may have a contributions to disease phenotype.
- PolyQ diseases (glutamine codon expansions), copy number variations (CNVs), repetitive and satellite regions are also difficult to identify with WES.
- A major drawback of WES is the unbalanced enrichment or capture of exons due to technical problems of target-probe hybridization. Sequencing of GC-rich exons may fail.

- Depending on the NGS platform used, the sequencing error rate of per nucleotide shows alterations, however it is still high, approximately 5% (Matullo *et al.*, 2013). The coverage of WES and also WGS should increase to prevent the false-positive rate.
- Another challenge is different program algorithms, which are used for assembly and alignment (Bamshad *et al.*, 2011, Kahvejian *et al.*, 2008).
- Public databases, such as dbSNP, contain some disease-associated variants with high minor allele frequencies (MAF), which leads to the eliminationof causative variants during the analysis. The definition of a higher threshold is necessary to detect the common variants.
- Genes for recessive disorders are easier to identify than dominant ones, because the number of candidates is lower; also having more than one rare nonsynonymous homozygous variant in a gene is less likely.
- Some online tools predict damaging variants according to conservation of amino acids, protein structure and function. PolyPhen2, SIFT, MutationTaster, Condel are some of these prediction tools, however some disease-causing variants can be predicted as tolerated or benign by these tools (Foo *et al.*, 2012, Healy *et al.*, 2008).

1.1.1.3 Study Design for Exome Sequencing

The major issue for the proper design of WES is choosing the appropriate samples to sequence. Consanguineous families, inbred populations and families with multiple affected members are more suitable to study. There are several approaches to find new genes/variants. Sequencing of subjects from a population, sharing a homogenous environment and consanguinity in extended families, which increases the level of long chromosomal stretches and recessive alleles, is an approach that was thought to find rare variants with high medical relevance (Hou *et al.*, 2013).In extreme-trait design approach, participantswere chosen from small populations at the extreme ends of phenotypic trait distribution, which may allow the identification of rare and deleterious variants. Family-based approach needs more than one-affected individual in the same family(Ng *et al.*, 2010). Another approach, genotype imputations, includes the

extrapolation of genetic correlations from a panel (Howie *et al.*, 2011). Lastly, a study proposed a novel strategy to integrate different NGS data sets such as WGS, WES, ChIP-seq together(Chen *et al.*, 2012).

After determination of the strategy and the individuals, the sequencing protocol needs to be determined. The coverage level should allow identifying the vast majority of protein-coding variants with high specificity. The efficiency of enrichment requires an average depth of 60-80X of each individual. Also, choosing the perfect sequencing platform is crucial. All the platforms have their own limitations and length of reads, so the long-read platform should be chosen for de novo assembly, whereas a short-read platform should be taken for comparative assembly (Figure 1.1.).

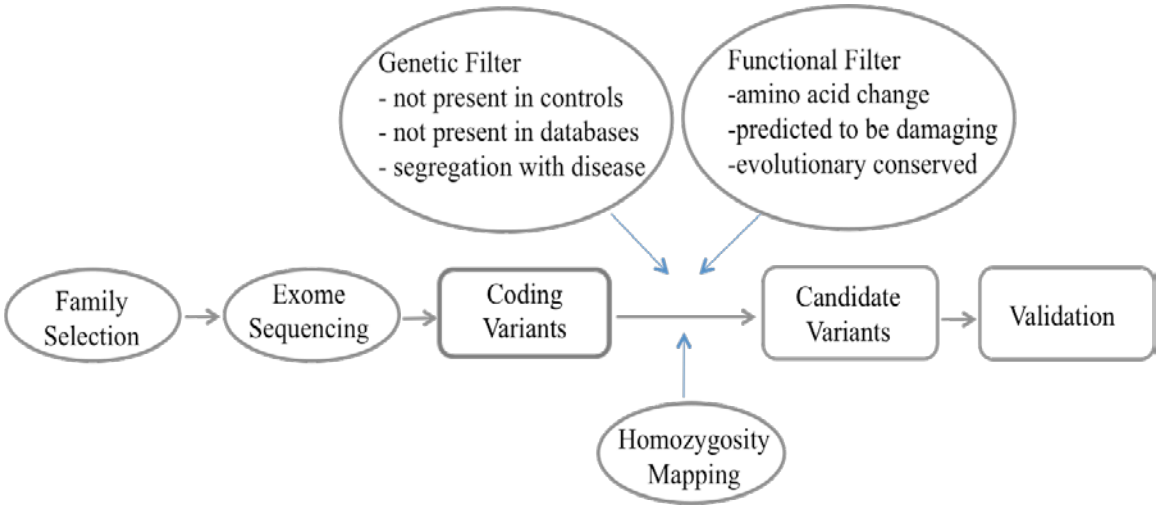

Figure1.1. The overview of the strategy used in identifying candidate variants. (Modified from (Kuhlenbaumer *et al.*, 2011).

1.1.2. Homozygosity Mapping

In consanguineous families or inbred populations, as a result of inheritance of the same genomic regions through both parents, the individuals have the homozygous stretches in the genome. Several recessive disease-causing genes have been discovered with the help of this technique in consanguineous families(Lander and Botstein, 1987).

Homozygosity mapping serves as an extremely powerful tool applicable to autosomal recessive disease research. SNP arrays are typically used to determine the homozygous regions.Recently, the advent of NGS enables the coexisting identification of homozygous stretches and the detection of mutations relevant for diagnosis, using data from a single sequencing experiment.

Homozygous Stretch Identifier (HomSI) is a novel tool that identifies homozygous regions using deep sequence data. HomSI identifies the majority of homozygous regions found by SNP genotype data, using .vcf file as an input (Gormez *et al.*, 2014). In this thesis, HomSI was used to perform homozygosity mapping.

1.2. Neurodegenerative Disorders

The term neurodegenerative disorder (ND), includes a large spectrum of diseases which primarily affect the neurons in the human brain and are characterized by progressive nervous system dysfunction, such as Alzheimer's Disease (AD) and other dementias, hydrocephalus, stroke, Parkinson's Disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), Huntington's Disease, prion diseases. Approximately 90% of patients are sporadic, however familial cases are also seen, enabling identification of disease causing genes. Although NDs share several features, the affected cell types such as distinct neuron types, glial cells and brain parts show differences (Ambegaokar *et al.*, 2010). For instance, in AD, the temporal lobe is affected and neurofibrillary tangles or plaques are accumulated, whereas loss of dopaminergic neurons in the nigrostriatal system is observed with the Lewy body formations in PD (Gama Sosa et al. 2012).

1.2.1. Motor Neuron Disease or Amyotrophic Lateral Sclerosis

Motor Neuron Disease (MND), also known as Amyotrophic Lateral Sclerosis or Lou Gehrig’s Disease, is an adult-onset devastating neurodegenerative disorder caused by the progressive death of the upper motor neurons (UMNs) in the cortex and the lower motor neurons (LMNs) in the brainstem and spinal cord(Pasinelli and Brown, 2006).

ALS is a complex disorder with a large heterogeneity on clinical, genetic and mechanistic levels. The clinical hallmark of ALS is progressive motor weakness without sensory disturbance. Neuronal degeneration leads to muscle atrophy, weakness and spasticity; in 60% of patients, the symptoms start with weakening of the limbs, and bulbar dysfunction leading to dysarthria is seen in ~30% of patients (Baumer *et al.*, 2014). Frontotemporal dementia (FTD) and respiratory weakness are also present in about 5% of patients. Cognitive impairment can occur in the early stage of the disease, usually characterized by a rapid progressive motor weakness leading to behavioral changes. Bulbar-onset patients, whose symptoms start with speech and swallowing difficulties and continue with limb features, have more rapid progression when compared with limb-onset patients.

ALS is the most common adult-onset motor neuron disease and evenly occurring throughout the world. In a population-based study of Europe, the incidence is found to be 2.6 per 100,000 population per year in women and 3.9 per 100,000 for men, resulting in a lifetime risk of 1 in 472 and 1 in 350, respectively (Alonso *et al.*, 2009). The prevalence, on the other hand, is similar to the incidence of the disease, being 4-6 in 100,000 individuals (Pasinelli and Brown, 2006).

The genetic contribution to ALS is thought to be significant for only 5-10% of cases as familial ALS (fALS) with multiple affected family members, whereas the remaining portion is considered as sporadic ALS (sALS) (Andersen and Al-Chalabi, 2011). The age of onset is lower in fALS, being around 47 as opposed to 57 in sALS though a distinguished phenotype is impossible between sALS and fALS(Kiernan *et al.*, 2011). With the similarities in pathology of fALS and sALS, the discrimination is partly applicable in genetic terms (Andersen and Al-Chalabi, 2011).There is no cure for ALS yet and the current Riluzoletreatment allows extending the life span for only two months (Bensimon *et al.*, 1994). Availability of new treatment approaches will only be possible with the unraveling of the pathogenesis of ALS.For two decades, first linkage analyses and more recently next generation sequencing techniquesin extended ALS families enabled the identification of genetic loci implicated in ALS and the discovery of several causal genes (Table 1.1.) (DeJesus-Hernandez *et al.*, 2011, Renton *et al.*, 2011, van

Blitterswijk *et al.*, 2012). The full list of ALS associated genes can be found in the ALSoD database (http://alsod.iop.kcl.ac.uk/als/). Mutations in genes identified so far are only able to clarify 60-65% of fALS cases (Baumer *et al.*, 2014). Some of these genes also contribute to sALS with a lower frequency. Familial ALS appears with different modes of inheritance, autosomal-dominant, autosomal-dominant with incomplete penetrance, autosomal-recessive and X-linked inheritance pattern(Andersen and Al-Chalabi, 2011, Deng *et al.*, 2011, Robberecht and Philips, 2013).

In the framework of this thesis, the following rare genes were shown to cause ALS:

- *SQSTM1:*The gene encodes an ubiquitin-binding protein (p62) with an important role in protein degradation via the proteasome. Moreover, it is a major pathologic protein involved in neurodegeneration. The gene was associated both with sALS and fALS mechanisms (Fecto *et al.*, 2011).
- *SPG11* encodes for the 2,443-residue long protein, spatacsin and it consists of 40 exons. The physiological role of spatacsin is still unknown, however the protein contains four putative transmembrane domains, suggesting that it may be a receptor or a transporter, which is presumed to be involved in axonal transport (Salinas *et al.*, 2008). Mutations in the *SPG11* gene represent the most common form of autosomal recessive (AR) hereditary spastic paraplegia with thin corpus callosum (HSP-TCC) (Stevanin *et al.*, 2008, Stevanin *et al.*, 2007). The clinical spectrum of ALS becamebroader with the identification of *SPG11* mutations in autosomal recessive juvenile amyotrophic lateral sclerosis (ARJALS) patients (Orlacchio *et al.*, 2010).
- The *OPTN* gene encodes a 577-amino acid protein, which consists of three noncoding exons in the 5’-untranslated region (UTR) and 13 exons. *OPTN* regulates diverse cellular processes, including membrane trafficking, protein secretion, cell division and host defense against pathogens. Mutations in *OPTN* may give rise to a wide range of clinical phenotypes (Kachaner *et al.*, 2012).Dominant missense, recessive deletion and nonsense mutations of *OPTN* have been identified in patients both with fALS and sALS(Maruyama *et al.*, 2010).

Table 1.1. ALS-associated genes, adapted from (Ajroud-Driss and Siddique, 2015).

Gene	**Locus**	**Mode of inheritance**	**Mean AO**	**Putative protein function**
SOD1	21q22.11	AD, AR, de novo	47	Superoxide metabolism
ALS2	2q33.2	AR	1	Vesicle trafficking
SETX	9q34.13	AD	18	RNA metabolism
SPG11	15q21.1	AR	16	DNA damage repair
FUS	16p11.2	AD, AR, de novo	46	RNA metabolism
VAPB	20q13.33	AD	44	Vesicle trafficking
ANG	14q11.1	AD	55	Angiogenesis
TARDBP	1p36.22	AD, AR	55	RNA metabolism
FIG4	6q21	AD	55	Vesicle trafficking
OPTN	10p13	AD, AR	51	Vesicle trafficking
ATXN2	12q23-q24.1	AD	57	Endocytosis; RNA translation
VCP	9p13	AD	49	Proteasome; vesicle trafficking
UBQLN2	Xp11.21	SD	41	Proteasome
SIGMAR1	9p13	AR	1	-
PFN1	17p13.3	AD	45	Cytoskeletal dynamics
C9orf72	9p21.2	AD	57	DENN domain protein
CHMP2B	3p12.1	AD	-	Vesicle trafficking
NEFH	22q12.1-q13.1	AD	60	Axonal transport
DCTN1	2p13	-	55	Axonal transport
PRPH	12q12	-	-	Unknown
DAO	12q24	-	44	Unknown
SQSTM1	5q35	AD	-	Ubiquitination; autophagy

AD: autosomal dominant, AR: autosomal recessive

1.2.2. ALS-Parkinsonism-Dementia Complex

After identification of ALS with high-prevalence during World War II among the indigenous Chamorros of Guam, a related disorder, parkinsonism-dementia complex (PDC), was characterized in the same population (Hirano *et al.*, 1961, Kurland and Mulder, 1954). ALS-parkinsonism-dementia complex (ALSPDC) of Kii peninsula, which was defined first by Brait and colleagues, is an endemic disease with diverse phenotypic features of classical ALS, parkinsonism and dementia(Brait *et al.*, 1973). Extrapyramidal signs and symptoms due to nigrostriatal system dysfunction have been shown in patients. ALS, parkinsonism and dementia may develop together in patients with a frequent speech difficulty, cognitive decline, pyramidalism and amyotrophy (Gilbert *et al.*, 2010, Park *et al.*, 2011).

However, the clinical manifestation of ALSPDC has a wide spectrum. Hence, during the course of the disease, the prominent clinical finding can change in the index case or between distinct ALSPDC patients (Annesi *et al.*, 2005). Neuropathologic findings in ALS-PDC include neurofibrillary tangles containing tau proteins in the spinal cord and brain. This finding leads to the idea that tau pathology could be a central pathological event in ALSPDC.

Although the cause of PDC remains uncertain, epidemiological and animal studies have identified two candidate environmental triggers: toxins from a traditional food source, the cycad plant (Spencer *et al.*, 1987), and altered mineral content of the soil and drinking water (Garruto *et al.*, 1991). There are two neurotoxins, beta-*N*-methylamino-L-alanine (BMAA) and sterol-D-glucosides,that cause glutamate excitotoxicity. (Cox and Sacks, 2002, Khabazian *et al.*, 2002).Slowness of gait with postural instability, dominant axial rigidity and bradykinesia with poor response to levodopa therapy are the most striking features seen in ALSPDC patients. ALSPDC has been described to be associated with the mutations of *DJ1 (PARK7*), a gene causing

early-onset PD (EOPD).

In the framework of this thesis, two genes were shown to be responsible for ALSPDC:

- *DJ1,* is a gene with seven exons,encodes 189 amino acids. It is a member of peptidase C56 family of proteins. It acts as a positive regulator of androgen receptor-dependent transcription and may also function as a redox-sensitive chaperone, a sensor for oxidative stress. DJ1 protein protects neurons against oxidative stress and cell death. *DJ1* gene is responsible for autosomal recessive early onset Parkinson's disease and also ALSPDC phenotypes (Bonifati *et al.*, 2003b). Studies indicate that mutations in the *DJ1* gene could cause a wide range of disorders (Abou-Sleiman *et al.*, 2003, Bonifati *et al.*, 2003a, Steele, 2005).
- *TRPM7,*transient receptor potential melastatin 7, is a bifunctional protein containing both channel and kinase domains. It is involved in the homeostatic regulation of intercellular Ca^{2+}, Mg^{2+} ions and tracing metals and also in anoxic neuronal death. Despite contrary information, it has been observed that *TRPM7* contributes to the pathogenesis of ALSPDC.Furthermore;a study suggested that mutations of OPTN (optineurin) might lead to ALSPDC with incomplete penetrance(Kaji *et al.*, 2012). The inheritance pattern is not clear, since *DJ1* and *OPTN* present a recessive trait whereas *TRPM7* shows a dominant inheritance pattern.

1.2.3. Autosomal Recessive Ataxias with Oculomotor Apraxia

Ataxia is a condition that is characterized by difficulties in coordinating movements. Ataxias are a heterogeneous group of neurodegenerative disorders with notably clinical overlaps among the different forms (Table 1.2.). Autosomal recessive ataxia with oculomotor apraxia (AOA) is a subgroup of the hereditary autosomal recessive cerebellar ataxias (ARCAs), which are characterized by cerebellar ataxia and combination of different ophthalmological and neurological signs (Di Donato *et al.*, 2001, Fogel and Perlman, 2007). AOA includes ataxia oculomotor apraxia type 1

(AOA1), ataxia oculomotor apraxia type 2 (AOA2), ataxia telangiectasia (A-T) and ataxia telangiectasia like disorder (A-TLD) (Le Ber *et al.*, 2005).

Table 1.2. Ataxia classification, genes, and basic clinical phenotypes associated with autosomal-recessive cerebellar ataxia, adapted from(Anheim *et al.*, 2014).

Ataxia	**Gene**	**Key additional clinical findings**
Friedreich ataxia	*FXN*	Ataxia, neuropathy, diabetes, cardiac
MIRAS	*POLG*	Neuropathy, deafness
ARSACS	*SACS*	Early onset, spastic, neuropathy
Recessive ataxia	*KIAA0226*	Cognitive impairment, epilepsy
SCAR11	*SYT14*	Cognitive impairment
Pure cerebellar ataxias		
ARCA1	*SYNE1*	Ataxia and marked dysarthria
ARCA2	*ADCK3*	Childhood onset ataxia
SCAR10	*ANO10*	Ataxia, proximal and distal atrophy
DNA repair deficiency		
Ataxia telangiectasia	*ATM*	Telangiectasia, cancer, immunodeficiency
AT-like disorder	*MRE11*	Resembles AT, no telangiectasia
AOA1	*APTX*	Oculomotor apraxia, neuropathy
AOA2	*SETX*	Oculomotor apraxia, neuropathy
SCAN1	*TDP1*	Neuropathy
AOA3	*PIK3R5*	Oculomotor apraxia, neuropathy
Metabolic		
AVED	*TTPA*	Decreased vitamin E, resembles FRDA
Abetalipoproteinemia	*ABL*	Fat malabsorption, neuropathy, spasticity
Niemann-Pick type C	*NPC1*	Supranuclear palsy, dementia, seizures
Refsum syndrome	*Pex7*, *Pex10*, *PHYH*	Predominantly neuropathy, retinopathy
Hartnup disease	*SLC6A19*	Pellagra, aminoaciduria
Cerebrotendinousxanthomatosis	*CYP27A1*	Spasticity, neuropathy, xanthomas
Tay–Sachs disease	*HEXA*	Adult onset, mild cognitive problems

1.2.3.1. Autosomal Recessive Ataxias with Oculomotor Apraxia 1

In 1998, ataxia with oculomotor apraxia was defined as a new syndrome, which is different from A-T with the absence of multisystemic involvement (Aicardi *et al.*, 1988). Genetic studies subsequently revealed non-allelic heterogeneity in AOA, and two subgroups, named AOA1 (Date *et al.*, 2001, Moreira *et al.*, 2001) and AOA2 (Moreira *et al.*, 2004), were described. AOA1 (MIM #208920), also called early-onset ataxia with ocular motor apraxia and hypoalbuminemia (EOAH). It was first described in Japanese families presenting with early-onset cerebellar ataxia, oculomotor apraxia, mental retardation, hypercholesterolemia and hypoalbuminemia. The hallmark of the disease is oculomotor apraxia, occurring in approximately 80% of the cases. Cerebellar ataxia and chorea are usually the most prominent features, followed by neuropathy that dominates the phenotype. The mean disease duration is 11 years (5-20 years).

AOA1 is caused by mutations in *APTX* that encodes the aprataxin protein with seven exons(Moreira *et al.*, 2001). APTX is a member of the histidine triad protein superfamily, and is comprised of three domains. The *APTX* gene encodes aprataxin, a histidine-triad (HIT) protein the function of which is still unknown, however some evidences suggest that it could have a role in single-strand DNA break repair(Clements *et al.*, 2004). Most mutations are located in the nucleotide binding HIT domain.

1.2.3.2. Autosomal Recessive Ataxias with Oculomotor Apraxia 2

Autosomal Recessive Ataxias with Oculomotor Apraxia 2 (AOA2, Omim #606002) or autosomal recessive spinocerebellar ataxia-1 (SCAR1) is an autosomal recessive disorder, which is characterized by its age of onset between two and 30 years (Moreira *et al.*, 2004). Disease progression leads to severe disability. Its symptoms are cerebellar atrophy, axonal sensorimotor neuropathy, oculomotor apraxia, and elevated serum concentration of alpha-fetoprotein (AFP) (Moreira *et al.*, 2001). The elevated AFP

level enables to exclude A-T and AOA1 in AOA2 patients. AOA2 has a worldwide distribution, and its prevalence is estimated around 1 in 900,000 (Anheim *et al.*, 2010, Anheim *et al.*, 2012). The responsible gene for AOA2 is senataxin*(SETX)*, which encodes a 2677 amino acids long protein and contains 24 exons. Senataxin is suspected to be a DNA/RNA helicase (Chen *et al.*, 2006, Moreira *et al.*, 2004, Ursic *et al.*, 2004). It is considered to be involved in the defense against DNA damage and in processing non-coding RNAs (Moreira *et al.*, 2004, Suraweera *et al.*, 2007). *SETX* missense mutations are mostly located at either the N-terminal domain or the C-terminal helicase domain (HD), supporting the fact that they are both key functional domains (Bassuk *et al.*, 2007). The protein has a DNA/RNA helicase domain conserved orthologous Sen1p RNA helicase protein in yeast. The certain biological function of senataxin is still unknown, however SETX mutation carriers show an increased sensitivity to oxidative DNA damage induced by H_2O_2, camptothecin, mitomycin C, which lead to generation of cross-links, DNA single-strand breaks (SSB) or double-strand breaks (DSB). Furthermore, the yeast research supports the neurotoxic damage of SETX in neurons. To these respects, SETX may have a role in DNA damage response and possibly DSB repair system, however there is no evidence for a defect in SSB defect in AOA2 (Suraweera *et al.*, 2007). Functional analysis showed an exciting result that hypersensitivity to H_2O_2, camptothecin, mitomycin C does not depend on the loss of function of wild-type senataxin in AOA2 patients or in other neurodegenerative diseases associated with DNA repair defects. An explanation for this is the possibility of abnormal interactions between mutated *SETX* and other proteins being responsible in oxidative damage. Different mutations, in both homozygous and compound heterozygous status, were shown in AOA2 patients with or without oculomotor apraxia (Asaka *et al.*, 2006, Criscuolo *et al.*, 2006, Moreira *et al.*, 2004, Tazir *et al.*, 2009).

Dominant mutations in *SETX* are also associated with a rare autosomal dominant form of ALS (ALS4) characterized by juvenile onset, severe distal muscle weakness and atrophy, normal sensation and pyramidal signs associated with degeneration of motor neurons in the brain and spinal cord. ALS4 usually shows a slow progression, sparing of bulbar and respiratory muscles and a normal life span (Chance *et al.*, 1998, Chen *et al.*, 2004, De Jonghe *et al.*, 2002, Rabin *et al.*, 1999). Chen et al identified a family with a

combined phenotype of AOA2 and ALS4, which is widening the spectrum of *SETX* mutation phenotypes.

1.2.3.3. Ataxia–telangiectasia-Like Disorder 1

Ataxia–telangiectasia (A-T, MIM #208900) is a progressive autosomal recessive disease caused by mutations in the ataxia telangiectasia mutated (*ATM*) gene both in homozygous and compound heterozygous forms (Savitsky *et al.*, 1995). A-T can be diagnosed with presence of progressive cerebellar ataxia and telangiectasia (dilated blood vessels, usually in the eyes). The symptoms appear in young children at about the time they start to walk (Sedgwick and Collins, 1991).

Ataxia–telangiectasia-like disorder 1 (ATLD1) is a rare autosomal recessive disorder characterized clinically by progressive cerebellar degeneration resulting in ataxia and oculomotor apraxia. The clinical picture of ATLD1 is very similar with A-T (Table 2.1.); however, ATLD1 patients show no telangiectasia (Hernandez *et al.*, 1993, Klein *et al.*, 1996). ATLD1 has a later onset of the neurological features, slower progression, longer survival and normal levels of IgA, IgG and IgM(Stewart *et al.*, 1999). The presence of mutations in the meiotic recombination 11 homolog A (*MRE11*) gene leads to ATLD1. The *MRE11* gene encodes a protein, which interacts with Rad50 and Nbs1 proteins. It has nuclease and intrinsic DNA-binding activity. *MRE11* is a member of the Mre11-Rad50-Nbs1 (MRN) complex, involved in double-strand break (DSB) repair mechanism, DNA recombination and multiple cell-cycle checkpoints (van Gaalen *et al.*, 2011). Defects in *MRE11* can be also a cause of nephronophthisis-related ciliopathies (NPHP-RC), a group of recessive diseases that affect kidney, retina and the brain.

1.2.4. Autosomal Recessive Spastic Ataxia Of Charlevoix Saguenay

Autosomal Recessive Spastic Ataxia Of Charlevoix Saguenay (ARSACS, MIM #270550), is a distinct form of hereditary early-onset spastic ataxia mainly restricted to the Quebec region in Canada with a carrier prevalence estimated to 1/22(Bouchard *et al.*, 1978, De Braekeleer *et al.*, 1993). The incidence and prevalence worldwide remain

unknown but ARSACS is very rare in other countries with cases described from Japan, Netherlands, Italy, Belgium, France and Spain and Turkey in this thesis (Kamada *et al.*, 2008, Miyatake *et al.*, 2012, Ouyang *et al.*, 2006, Richter *et al.*, 2004, Synofzik *et al.*, 2013, Thiffault *et al.*, 2013, Vermeer *et al.*, 2008). Reported clinical features are early-onset spasticity, dysarthria, distal muscle wasting, foot deformities, truncal ataxia, and absence of sensory evoked potentials in the lower limbs, retinal striation reminiscent of early Leber's atrophy and the presence of mitral valve prolapse (Bouchard *et al.*, 1998). These symptoms generally appear between the ages of 12 to 24 months. The spasticity observation in infant period is specific to ARSACS disease(Fogel and Perlman, 2007).

ARSACS is caused by autosomal recessive mutations in the *SACS* gene (13q11), which encodes a large protein named sacsin (Engert *et al.*, 1999). Presence of two domains, DnaJ motif at the C-terminal and a putative ubiquitin-like (UbL), suggests that sacsin may integrate the ubiquitin proteasome system (UPS) and also chaperon activity. The pathological mechanism of sacsin protein is still unknown, however it thought to be interacting with Ataxin-1 and to act as a chaperon in protein folding (Parfitt *et al.*, 2009). Treatment aims to control the spasticity and should include physiotherapy, pharmacotherapy. Most patients become wheelchair-bound by the 5th decade of life. Death generally occurs during the sixth decade of life.

1.2.5. Kufor-Rakeb Syndrome

Parkinson's Disease is a neurodegenerative disorder caused by both genetic and environmental factors. Only 5-10% of affected individuals have a family history. Pedigree and cohort studies identified several genes and loci associated with the PD phenotype (Bonifati, 2014).Autosomal recessive mutations in *Parkin, Pink1, DJ-1, ATP13A2, PLA2G6,* and *FBXO7* genes lare known to ead to PD.

Kufor-Rakeb syndrome (KRS) or Parkinson's Disease 9 (PARK9) is a severe, early-onset autosomal recessive form of levodopa-responsive type of Parkinsonism, which was defined in a consanguineous family from Kufor-Rakeb, Jordan(Najim al-Din

et al., 1994). It is characterized by pyramidal degeneration, supranuclear palsy, cognitive impairment and dementia (Williams *et al.*, 2005). KRS is likely to be the consequence of homozygous and compound heterozygous mutations leading to loss of function of ATP13A2 protein. Wild-type ATP13A2 localizes to the lysosome, while mutated forms localize to the endoplasmic reticulum (ER) (Ramirez *et al.*, 2006).Several studies provide further evidence that *ATP13A2* is required for the preventing ∝-synuclein aggregation (Yang and Xu, 2014). Mutant protein causes increased ER stress and goes to proteasomal degradation via the ER-associated degradation pathway(Ugolino *et al.*, 2011).

Mutations in the *ATP13A2* gene are linked to Parkinson's disease, neuronal ceroidlipofuscinoses (NCLs) and also Kufor-Rakeb syndrome (Ramirez *et al.*, 2006). About 20 missense and nonsense mutations were identified in the entire *ATP13A2* gene (Yang and Xu, 2014). There is still no cure for KRS however; ATP13A2 is an appropriate target for both PD and KRS therapies.

2. PURPOSE

The rapidly aging world of today is facing a rise in the emergence of many late onset diseases, which primarily include an array of neurodegenerative disorders. Technological developments enable to gain a deeper understanding in neurodegeneration, especially next generation sequencing technologies change all habits in medical researchallowing us to analyze neurodegenerative disorders in great detail.

The purpose of this study is to identify the genes/variants responsible for different neurodegenerative diseases, which could not be solved by conventional PCR-based techniques. These include amyotrophic lateral sclerosis, related syndromes and recessive ataxias.

3. MATERIALS

3.1. Subjects

In the framework of this thesis, 13 families were analyzed. The initial clinical diagnoses of these 13 families were either motor neuron disease or dominant or recessive ataxias, egSCAs or FRDA. All affected individuals in those 13 families and their close family memberswere screened for the respective genes SOD1,C9Orf72, TDP-43, FUS, UBLQN2 in ALS and ATXN 1,2,3,6,7, TBP and FXN in ataxias. When they were negative for these common genes, family selection for exome sequencing analysis was performed in accordance with close consanguinity in parents and/or the presence of more than one affected member in the family.

Consent was obtained from all family members who participated in this study. The study protocol was approved by the Ethics Committee on Research with Human Participants (INAREK) at Boğaziçi University. Clinical evaluation of all index cases and family members was performed by expert neurologists from several hospitals throughout Turkey. Peripheral blood samples were collected into EDTA-containing tubes for analysis. Available samples for analysis indicated by asterisk (*) in all pedigrees.

3.1.1. Family 1

Two affected juvenile siblings in Family 1 showed limb onset disease with weakness in right hand fingers and fasciculations, tenor and hypo-tenor atrophy in the right arm and atrophy of interosseous muscles of right hand. The probands were tested for the five common ALS genes, they were found negative for all of them. Samples of the parents and unaffected sister were not available to us, to the best of our knowledge,the parents and sister were healthy at the time of diagnosis (Figure 3.1.).

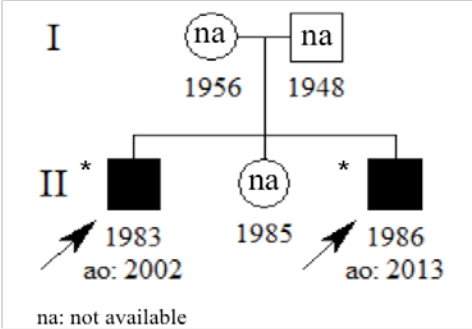

Figure 3.1. Pedigree of family 1.

3.1.2. Family 2

In Family 2, cognitive impairment, bilateral leg weakness and upper extremity weakness were present in the proband. No episodic loss of vision, loss of consciousness, frank tremor, incoordination and seizures were observed. Five frequent ALS genes were screened and any variant was detected in these regions. GAA expansion in the frataxin gene, which leads to FRDA, was also excluded. Sample of the affected father was not available (Figure 3.2.).

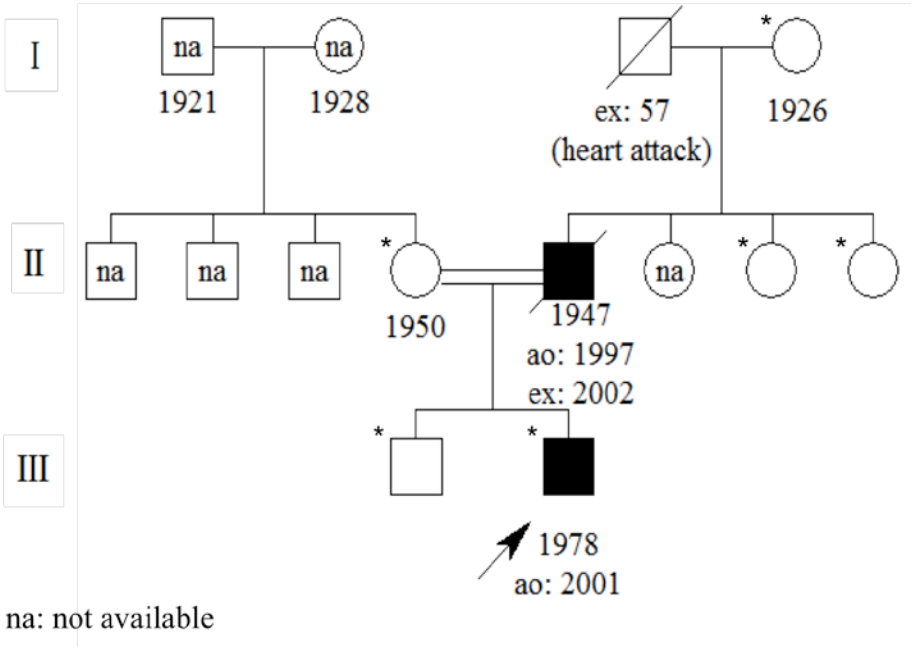

Figure 3.2. A consanguineous family with two affected members.

3.1.3. Family 3

In Family 3, two affected siblings had died with ALS. Older brother had a mixed (bulbar+limb) site of onset at 33 years, involvement of anterior horn was present,he passed away within eight years. Younger brother had limb onset disease followed by bulbar signs, he succumbed to disease within seven years.Both affected siblings were tested for ALS genes and analysis revealed no mutation (Figure 3.3.).

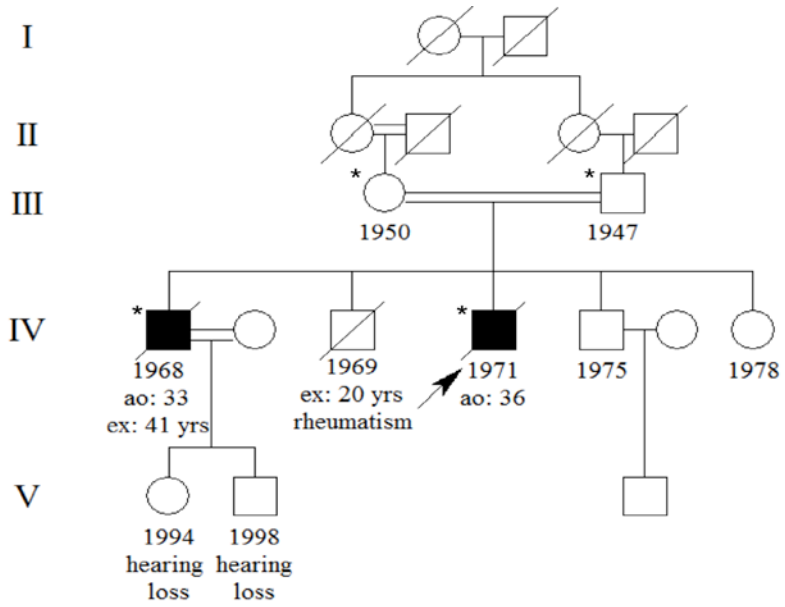

Figure 3.3. Two affected siblings in a consanguineous family.

3.1.4. Family 4

The initial diagnosis of the index case was ALS. She had muscle weakness in her limbs and survival was six years. Two affected older brothers had passed away in eight and two years. No mutation was found in any region of five common ALS genes. The parents of the index case were first cousin. The samples of the affected brothers were not available to us (Figure 3.4.).

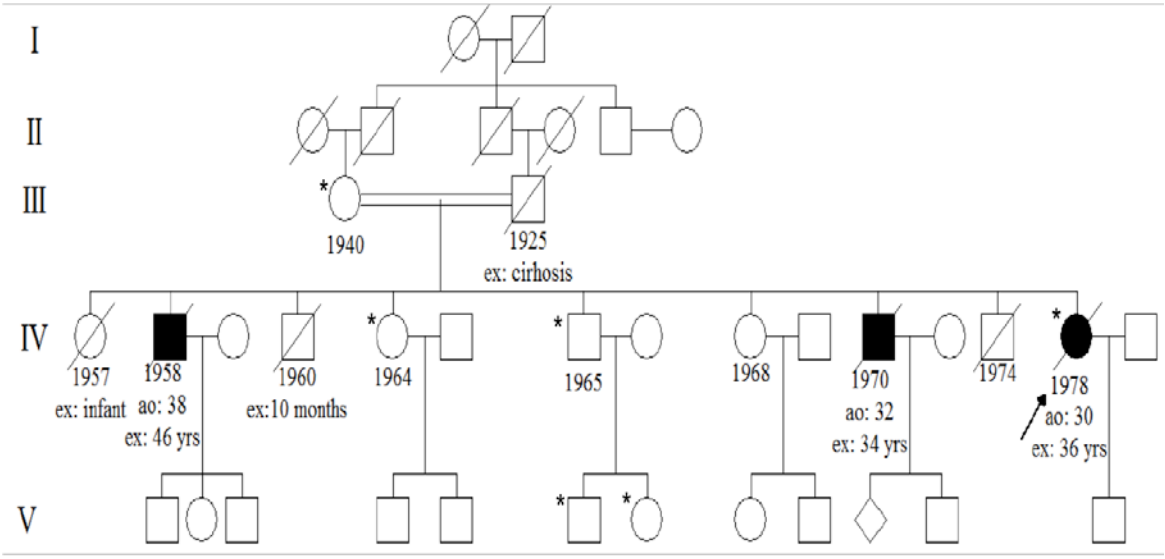

Figure 3.4. Family 4 with three affected siblings.

3.1.5. Family 5

The index case initially showed bradykinesia, rigidity, tremor and speech problems. Blepharospasm, psychosis and hallucination followed the initial symptoms. There are two affected siblings with similar clinical features in the family (Figure 3.5).

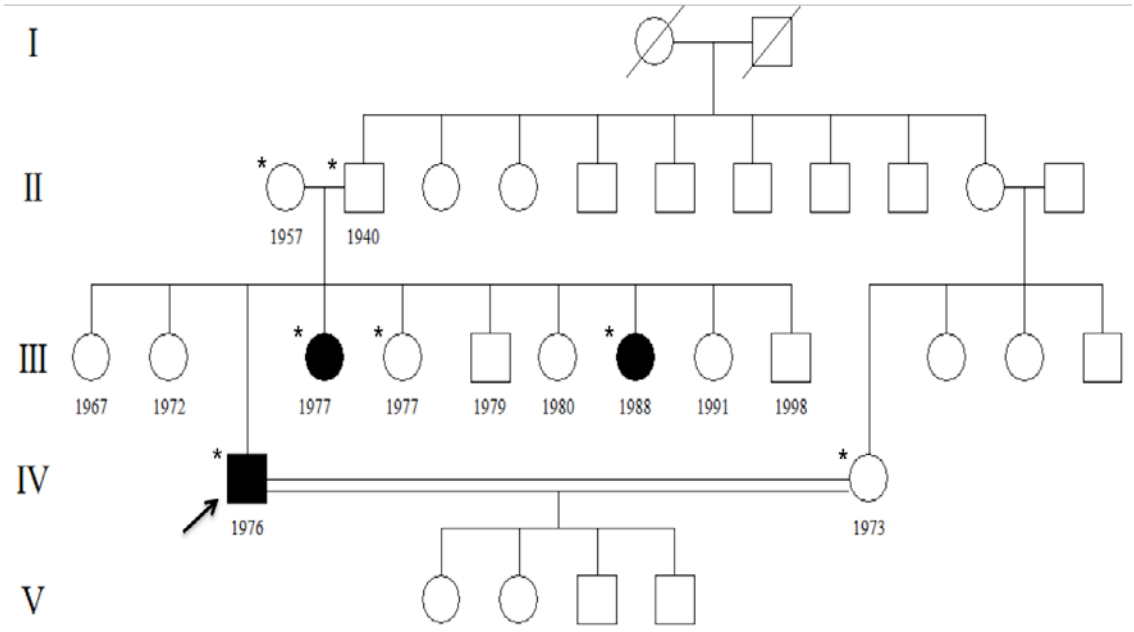

Figure 3.5. Family with three affected individuals.

3.1.6. Family 6

There are four affected members diagnosed with ataxia in this family. Balance disorder, walking difficulties, cerebellar atrophy and polyneuropathy are present in the index case with an age of onset at 11 years. She was diagnosed with SCA and anyCAG trinucleotide repeat expansion was not found in the *ATXN1* and *ATXN3* locus. FRDA also excluded with the screening of GAA expansion in the *FXN* gene. Similar phenotypes are observed in other affected members in the family (Figure 3.6.).

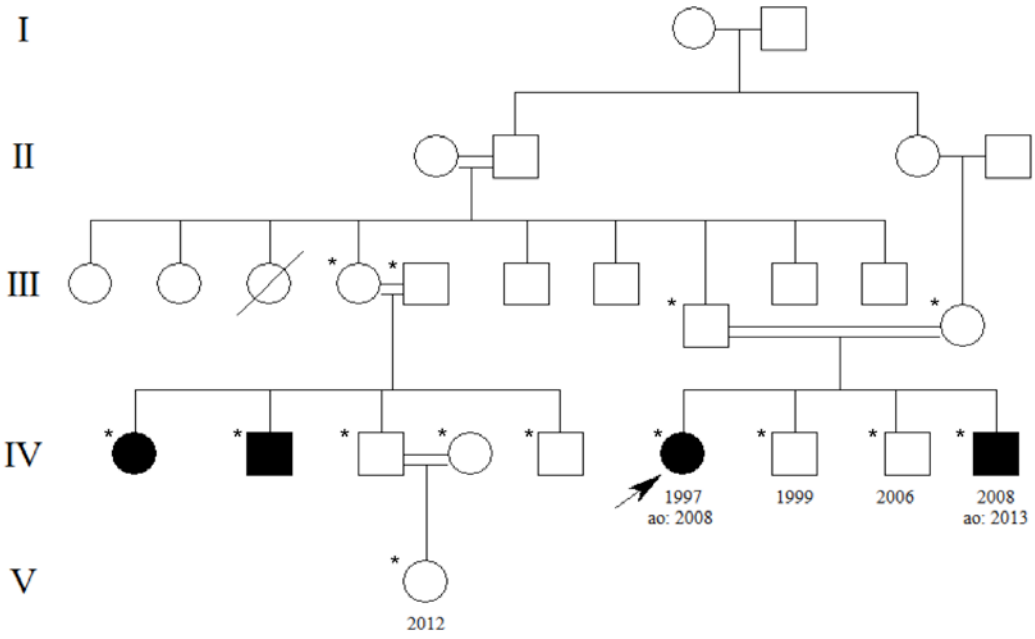

Figure 3.6. Family with four affected members.

3.1.7. Family 7

The older brother had mental retardation and walking difficulties at the age of 14 and the younger brother had walking difficulties without mental retardation at the age of 13. They were first diagnosed with SCAand found negative for SCA types 1,2,3,6,7,17. Then, they were tested for FRDA and there was no GAA expansion the *FXN* gene (Figure 3.7.).

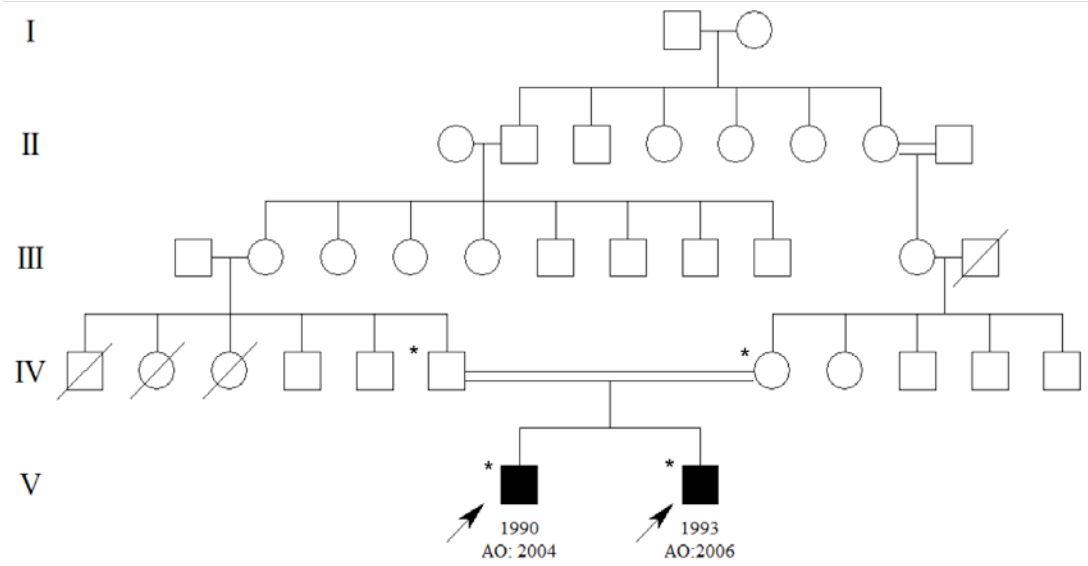

Figure 3.7. Family tree with two affected juvenile siblings.

3.1.8. Family 8

The proband,offspring of a first-cousin marriage, was first diagnosed with FRDA at age 15, presenting with speech problems, walking difficulties and cerebellar ataxia. All affected members have a juvenile onset disease (Figure 3.8.). No mutations were detected in the SCA 1,2,3,6,7,17 and FRDA locus of the proband.

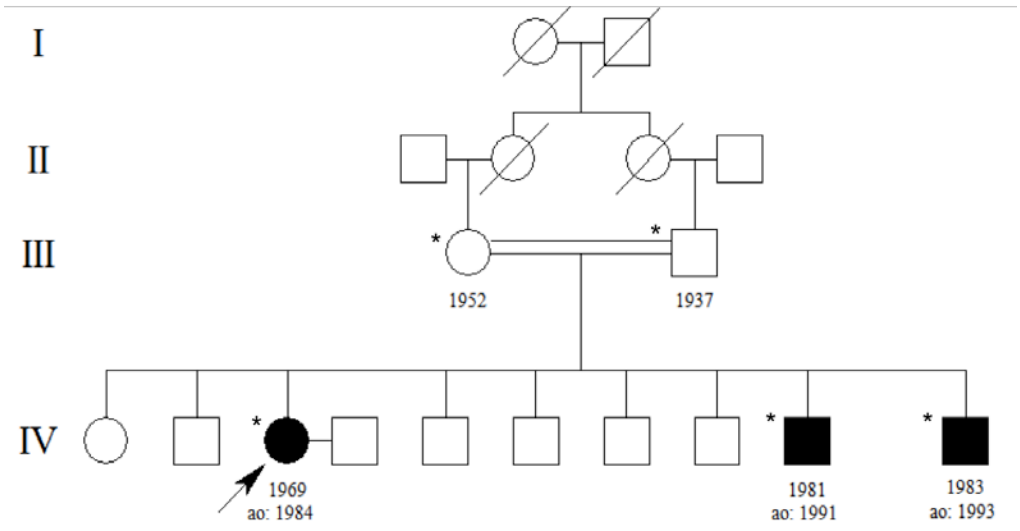

Figure 3.8. Family with three affected siblings.

3.1.9. Family 9

The affected sister had walking difficulties at the age of two and dysarthria was added at 12 years. Brother had dysarthria at the age of two; walking difficulties appeared at 13 years of age. In both siblings, spinocerebellar atrophy is present (Figure 3.9.). Both siblings were negative for the CAG and GAA expansion in SCA 1,2,3,6,7,17 and FRDA.

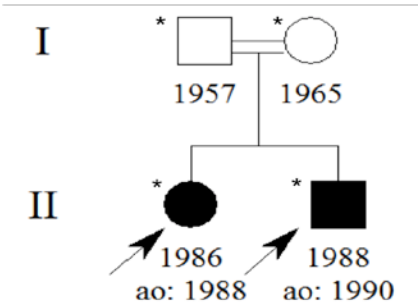

Figure 3.9. Two juvenile siblings in a consanguineous family.

3.1.10. Family 10

The index case, who was referred to us with FRDAdiagnosis and found negative, hadtenar, hypotenar, interosseal, tibialis anterior and intrinsic foot muscle atrophy,symmetrical weakness, dysarthria and spondylosis. Patient had pronounced ataxic gait and lower limbs were spastic. Similar complaints were present in her three sisters.(Figure 3.10.).

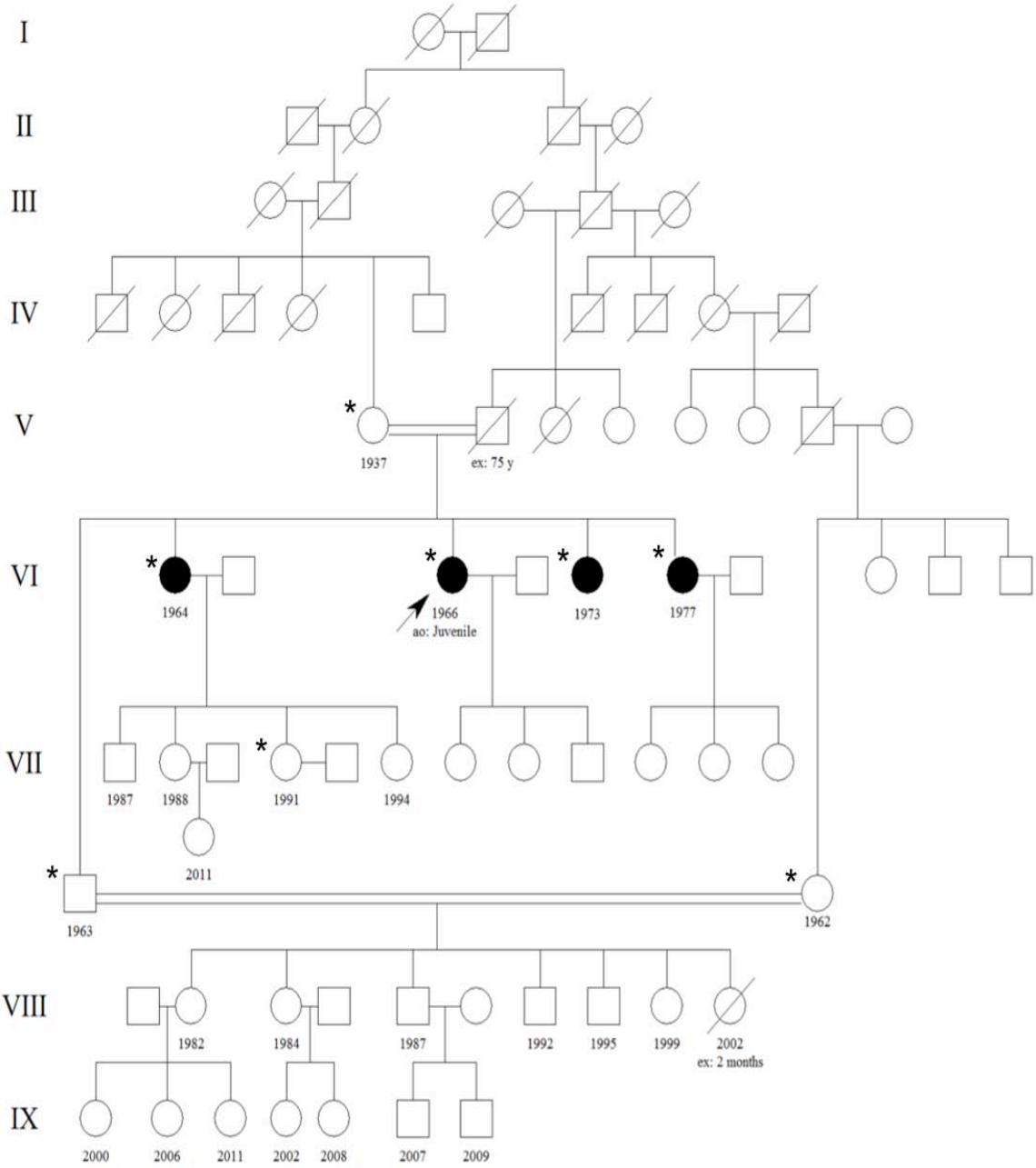

Figure 3.10. A consanguineous family with four affected patients.

3.1.11. Family 11

The family with four affected members was referred to our laboratory with the clinical diagnosis of SCA. Analysis revealed no expanded CAG repeats in the SCA 1,2,3,6,7,17 alleles. All the affected individuals shared the same phenotype.

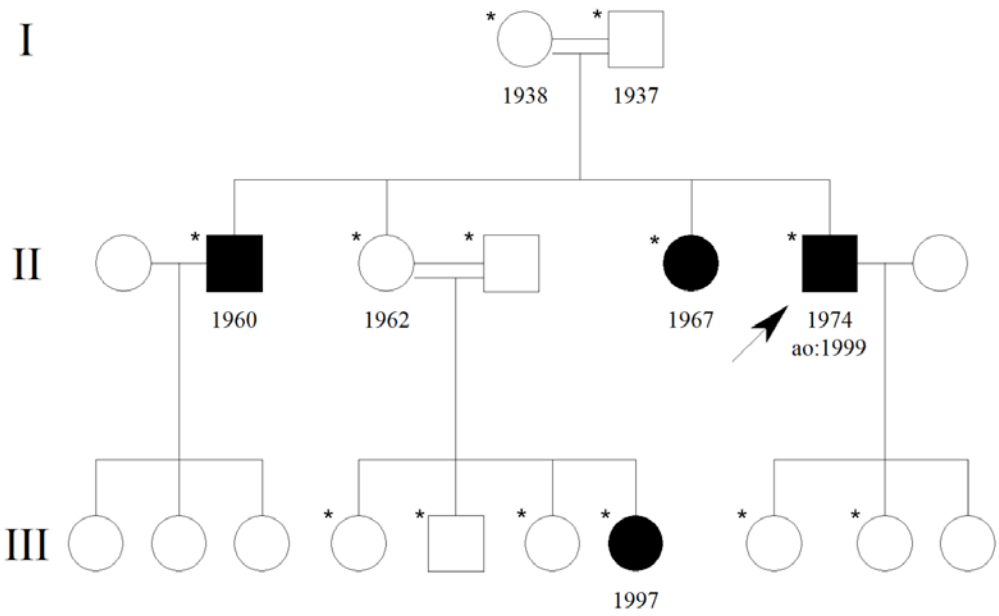

Figure 3.11. Four affected members of an inbred family.

3.1.12. Family 12

In Family 12, there are two affected juvenile siblings diagnosed with ataxia, both presenting with cerebellar dysfunction, nystagmus and speech problems. (Figure 3.12.).Both affected siblings were tested for FRDA and SCA 1,2,3,6,7,17 diseases. The older brother was negative for all of them, while youngest brother is a carrier of GAA expansion inherited from unaffected father, which leads to FRDA in homozygous state.

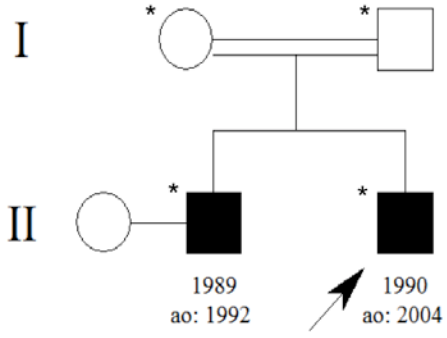

Figure 3.12. Family 12 with two juvenile cases.

3.1.13. Family 13

Both affected siblings have a slowly progressive cerebellar ataxia and dementia. Speech, balance and walking problems are also present.The parents are first cousin. The index case was referred to our laboratory with the clinical diagnosis of SCA. CAG expansion screening analysis showed that there was no expanded CAG allele in the SCA 1,2,3,6,7,17 locus of him.

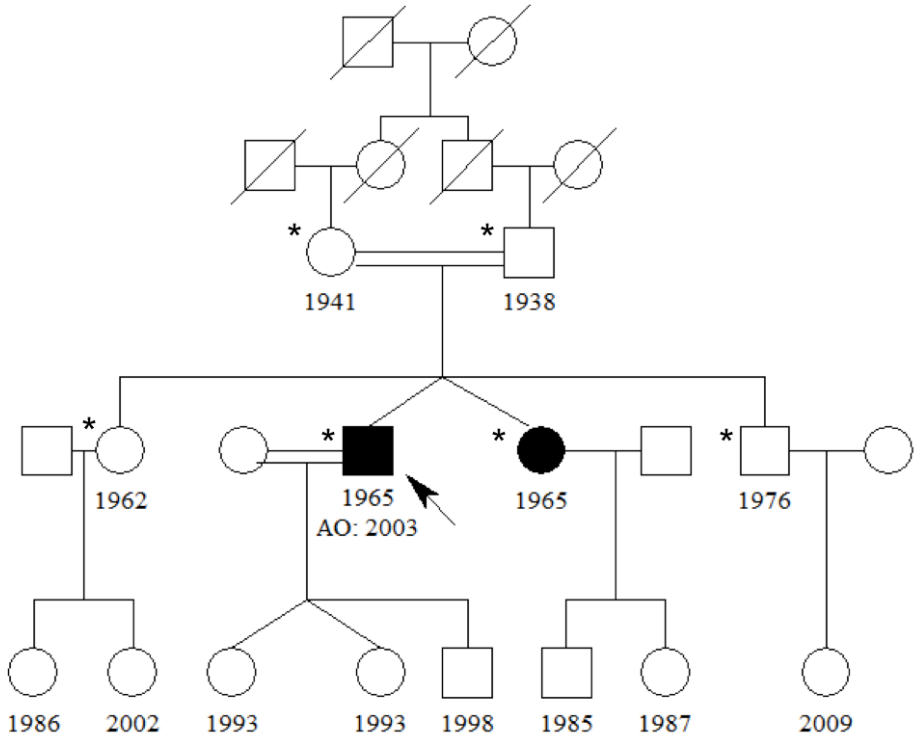

Figure 3.13. Family 13 with affected twins.

3.2. Buffers and Solutions

Buffers and solutions used for gel electrophoresis and validation PCR are listed in Table 3.1. and 3.2.

3.2.1. Agarose Gel Electrophoresis

All the necessary chemicals for agarose gel electrophoresis are listed in Table 3.1.

Table 3.1. Chemicals used in gel electrophoresis.

0.5X TBE buffer (pH 8.3)	0.89 M Tris-Base
	0.89 M Boric Acid
	20 mM Na_2 EDTA
6X DNA Loading Dye	100 mMTris-HCl (pH 7.6) 0.03 per cent Bromophenol Blue 0.03 per cent Xylene Cyanol FF 60 per cent glycerol 60mM EDTA Fermentas, Lithuania
Agarose	Prona, Poland
DNA ladder, 1kb	Fermentas, Lithuania
Ethidium Bromide (EtBr)	10 mg/ml (MP Biomedicals, France)

3.2.2. PCR for Validation of NGS Results

Table 3.2. Reagents for PCR.

5 mMdNTP	12.5mM each of dATP, dCTP, dGTP, dTTP in dH2O, Roche, Germany / Fermentas, Lithuania
$MgCl_2$	25mM MgClRoche, Germany
5X Reaction Buffer	5X Colorless GoTaq® Reaction Buffer with $MgCl_2$, Promega, USA
GoTaq DNA Polymerase	Promega, USA

3.3. Kits

Kits used in this thesis for

DNA purification:

- QIAquick Gel Extraction Kit, Qiagen, USA
- QIAquick PCR Purification Kit, Qiagen, USA

Whole exome sequencing:

- IlluminaTruSeq Sample Preparation Kit, Illumina, USA
- KAPA High Throughput Library Preparation Kit, Kapabiosystems, USA
- Roche NimbleGen 2.1M Exome Array, 454 Life Sciences, USA
- llluminaTruSeq Exome Enrichment Kit, Illumina, USA
- Roche NimbleGenSeqCap EZ Human Exome Library, 454 Life Sciences, USA
- IlluminaTruSeq PE Cluster Kit v3-cBot-HS, Illumina, USA
- IlluminaTruSeq Rapid PE Cluster Kit – HS, Illumina, USA

3.4. Primers

Primers used in this thesis for validation of exome sequencing analysis results are complied in Tables 3.3.

Table 3.3. Sequences of primers.

Primer Name	Tm (°C)	Sequence
APTX_E6F	56.9	5'- TGGGAATTAAGTGACTTAGTG -3'
APTX_E6R	56.9	5'- GGGTCTCAGTGCAATATGTG -3'
ARHGEF28_26F1	63.6	5'- TGCGTTTTGTGTTTCGAGGT -3'
ARHGEF28_E26R	63.6	5'- GTTAGTGTCCTAGGAGCCCC -3'
ATP13A2_E	66	5'- CCCAGCTGTCATCATATTCTGCC -3'
ATP13A2_E	66	5'- CCCACGTCATCTATTCTGGGACC -3'
DJ1_E3F	59	5'- TTAAAGACAGTGTTACTCTGAATT -3'
DJ1_E3R	59	5'- CATCCAGCCACCCACTTAC -3'
MRE11_E6F	59.2	5'- TGCTTTTAACAGGTGATACGA -3'
MRE11_E6R	59.2	5'- TCTCCAAATTTCTCAATTGTTT -3'
OPTN_E9F	56.6	5'- TGTGTTAAATCCCTTGCATTTC -3'
OPTN_E9R	56.6	5'- AACATTTGACCTCCGGTGAC -3'
OPTN_E10F	59	5'- ACCTTCCCTAGGAAGCATGG -3'
OPTN_E10R	59	5'- GACAGTGAGTGCTGTTTGGG -3'
OPTN_E11F	59	5'- AAACCCTACAGCCCTAAAATTC -3'
OPTN_E11R	59	5'- TGCTAGGACTCCTTCAGATAAGTG -3'

Table 3.3. Sequences of primers (continued).

Primer Name	Tm (°C)	Sequence
SACS_E9F	56.5	5'- AGCTTGAGCCATAAGAAATTG -3'
SACS_E9R	56.5	5'- AGCTTGAGCCATAAGAAATTG -3'
SACS_E10.1F	56.5	5'- TTTGTGAGAATAGAAAGCTGTTG -3'
SACS_E10.1R	56.5	5'- ATCTGCAAAACAGCACTTGG -3'
SACS_E10.14R	65.2	5'- CGAATAATCAGTGAAGGAATATGG-3'
SACS_E10.14R	65.2	5'- CAAGATTCTGAATTCCTCTAACATC-3'
SACS_E10.16F	61.7	5'- TTGCACAATGTTCAGATTTCC -3'
SACS_E10.16R	61.7	5'- AAATGGCAGCCCAGTCTC -3'
SETX_E10.9F	66	5'- GTACTTGCCAACAGTAACAG -3'
SETX_E10.9R	66	5'- GGTTTAGATGCAGGAGGAG -3'
SPG11_E33F	65	5'- CAATAGGCCAAGGGTTTCAA -3'
SPG11_E33R	65	5'- TATAACTCCTGCTGGAGGGC -3'
SPG11_E40F	65	5'- AATTAGCCAGGGTGGTGACA -3'
SPG11_E40R	65	5'- CCCACAAAGGACTGATATGG -3'
SQSTM1_E6F	60	5'- TCTGTAGTCTCCACAGGCCA -3'
SQSTM1_E6R	60	5'- CTGCAGAGGTGCTGAGGATG -3'
TRPM7_E28F	62	5'- ACTTAGAGTAATGGGTTGAAATTTAG -3'
TRPM7_E28R	62	5'- TGTAAATGAGTATGTTTTCTGACAC -3'

3.5. Equipment

The equipment used in this study is listed in Table 3.4.

Table 3.4. Equipment list.

Equipment	**Model/ Company**
Autoclave	ASB260T, Astell, UK
Balance	TE612, Sartorius, Germany
Centrifuge	Allegra X22-R, Beckman Coulter, USA Centrifuge 2-16K, Sigma, USA
Computers	• HP Z820 Workstation (64GB RAM, 12 core, SSD harddisk, nvidiaquadrogpuquadro 4000) Apple Macbook Pro (2.5 GHz Intel Core i5, 4 GB 1600 MHz DDR3, 500GB)
Deep Freezers	2021D (-20 °C), Arçelik, Turkey HT5786-A (-86 °C), Hettich, Germany
DNA extraction	MagNA Pure, Roche, Germany
Documentation System	GelDoc Documentation System, BIO-RAD, USA Image-Lab v5.0, BIO-RAD, USA
Electrophoretic Equipment	EC250-90 Compact Power Supply, Thermo Scientific, USA
Falcon Tubes	EasyOpen 50-ml Centrifuge Tubes, JETBIOFIL, USA
Hood	IP44/I, Wesemann, Germany
Microwave	Arçelik, Turkey

Table 3.4. Equipment list (continued).

Equipment	Model/ Company
Micropipettes	Rainin 0,5-2 µl, 2-20 µl, 20-200 µl, 200-1000 µl, USA
Nanodrop	ND-2000c, Thermo Scientific, USA
pH Meter	PB-11, Sartorious, Germany
Tips	10 µl, 100 µl, 200 µl, 1000 µl, Universal Fit Filter Tips, Axygen, USA
Vortex	FisonsWhirliMixer, UK
Water Bath	Gemo DT104, TEST LaboratuvarCihazları, TR
Water Purification System	Arium® 611UV Ultrapure Water System, Sartorius, Germany

3.6. Sequencing Platforms

Whole exome sequencing was performed in collaboration with Yale University and University of Massachusetts Medical School (UMASS) or outsourced from two different companies using IlluminaHiSeq 2000, IlluminaHiSeq 2500 IlluminaMiSeq and IonTorent Life Technologies sequencing platforms.

3.7. Electronic Databases and Bioinformatics Tools

The open-source bioinformatics software, bioinformatics tools and electronic databases used in whole exome sequencing analysis are given in Table 3.5.

Table 3.5. Open-source bioinformatics software, bioinformatics tools and electronic databases.

Name & website	Description
Basic Sources	
Ensembl Genome Browser http://www.ensembl.org	Reference genome database for vertebrates and other eukaryotic species
GeneCards http://www.genecards.org	A searchable database which includes gene-related data, including genomic, transcriptomic, proteomic, genetic, clinical, and functional information
NCBI UniGene http://www.ncbi.nlm.nih.gov/unigene/	Expression analysis by tissue, age, and health status; and related proteins reports (protEST) and clone resources
UniProt http://www.uniprot.org	An open-source protein sequence and functional information database
Online Mendelian Inheritance in Man http://www.omim.org	An online catalog of human genes and associated disorders
Databases used for annotation	
1000 Genomes http://www.1000genomes.org	Human genetic variation catalog
dbSNP http://www.ncbi.nlm.nih.gov/SNP/	A catalog of short human genome variations
NHLBI GO Exome Sequencing Project http://evs.gs.washington.edu/EVS/)	A database of 6500 human exome results for NHLBI Exome Sequencing Project (ESP)

Table 3.5. Open-source bioinformatics software, bioinformatics tools and electronic databases (continued).

Name & website	Description
Database of Genomic Variants (DGV) http://dgv.tcag.ca/dgv/app/home	A human genomic structural variation database
Human Gene Mutation Database http://www.hgmd.cf.ac.uk/ac/index.php	A collection of known variations responsible for human disease
Human Genome Variation Society http://www.hgvs.org	A database of genomic variants with associated clinical information
Amino acid substitution prediction	
Polymorphism Phenotyping v2 (PolyPhen2) http://genetics.bwh.harvard.edu/pph2/	Straightforward physical and comparative considerations prediction
SIFT http://sift.jcvi.org	Based on the degree of conservation of amino acid residues
Analysis of exome sequencing data	
Protein Variation Effect Analyzer (Provean) http://provean.jcvi.org/index.php	Biological function of a protein
Mutation Taster http://www.mutationtaster.org	Mutation prediction for the deep-sequencing variants
Annovar http://www.openbioinformatics.org/annovar/	Functional annotation of genetic variations
BamView http://bamview.sourceforge.net	Free interactive display of read alignments in BAM data file

Table 3.5. Open-source bioinformatics software, bioinformatics tools and electronic databases (continued).

Name & website	Description
Integrative Genomics Viewer (IGV) http://www.broadinstitute.org/igv/	Visualization tool for interactive exploration of large, integrated genomic datasets
BedTools http://bedtools.readthedocs.org/en/latest/	A fast, flexible toolset for genome arithmetic
Burrows-Wheeler Aligner (BWA) http://bio-bwa.sourceforge.net	Software package for mapping low-divergent sequences against a large reference genome, such as the human genome.
Genome Analysis Toolkit (GATK) http://gatkforums.broadinstitute.org	Focus on variant discovery and genotyping as well as strong emphasis on data quality assurance
PICARD http://broadinstitute.github.io/picard/	Picard comprises Java-based command-line utilities that manipulate SAM text and binary files (BAM) for creating new programs that read and write SAM files.
SamTools http://samtools.sourceforge.net	Manipulating alignments in the SAM format, including sorting, merging, indexing and generating alignments
Varsifter http://research.nhgri.nih.gov/software/VarSifter/	A Java program designed to display, sort, filter, and generally sift variation data from high throughput data

4. METHODS

4.1. Whole Exome Sequencing

4.1.1. DNA isolation and Quality Control

DNA isolation from peripheral blood samples was performed according to the instructions of MagNA Pure Compact Instrument (Roche), a system for extracting nucleic acids from tissue or blood samples. Genomic DNA quality was tested by running the sample on a 1% agarose gel. DNA concentration was measured using a NanoDrop spectrophotometer at 260 nm optical density (OD 260). 260/230 nm and 260/280 nm ratios were also evaluated and documented.

4.1.2. Exome Enrichment and Sequencing

Whole exome sequencing was performed on DNA sample of the index cases, affected family members or/and parents in each family either in collaboration with Yale University and UMASS or outsourced to two different companies, The Scientific and Technological Research Council Of Turkey (Tübitak) and BGI.

Preparation of an exon-enriched gDNA library for massively parallel sequencing requires three steps: (i) creation of a genomic DNA library, (ii) enrichment of the library for exon targets, and (iii) verification of library quality and enrichment. An overview of exome sequencing is shown in Figure 4.1. The genomic DNA is sheared into several fragments and single strand overhangs are converted to blunt ends to obtain double stranded DNA. Fragments between 300-400 bp in size are selected from gel and adapters are ligated to the blunt ends. Fragments are amplified via PCR using adaptor-specific primers. Enriched DNA library is mixed with biotinylated RNA-based capture probes, which target protein coding sequences and flanking sites of the genome. Streptavidin

magnetic beads are used to separate genomic DNA-bait hybrids. A second round of PCR is used to amplify the library to sufficient levels for sequence. The enriched library is controlled by real-time PCR to determine whether the exon enrichment was successful.

The enriched DNA library is hybridized to the flow cell, a solid support including forward and reverse primers. Template fragments are amplified in clusters by the bridge amplification and sequenced via the cyclic reversible termination technique. Fluorescently labeled nucleotides (each one labeled with a different fluorescent dye) are incorporated on the primed templates in this method. DNA synthesis is terminated after addition of a single nucleotide. After washing the unincorporated nucleotides, imaging is performed. With the separation of a hindering fluorophore a new cycle begins.

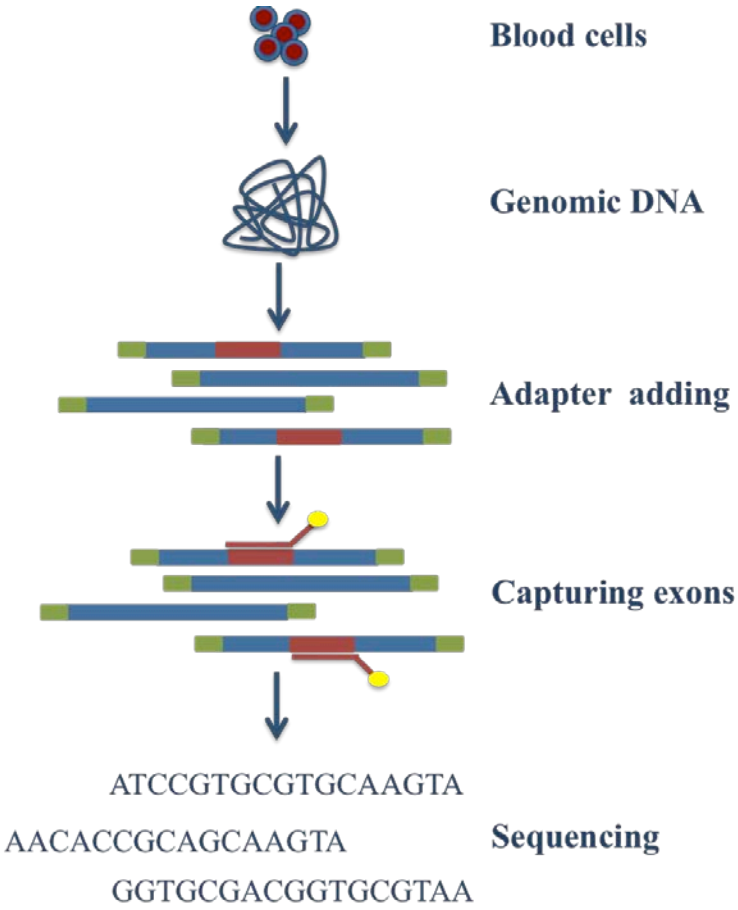

Figure 4.1. Representation of Exome Sequencing Workflow

4.2. Exome Sequencing Data Analysis

4.2.1. Base Calling, Alignment and Variant Calling

An overview of exome sequencing data analysis is represented in Figure 4.2.Short paired-end reads are generated using Illumina's Real Time Analysis Software (CASAVA version 1.13) with default parameters. The paired-end reads (*.fastq) are mapped to the reference human genome hg19 using Burrows Wheeler Aligner (BWA) which allows approximately 2% mismatches. The SAM (Sequence Alignment/ Map) file, which is output of BWA, is converted to a compressed binary version, BAM. SAMTools was used for sorting and indexing the BAM files and removing PCR duplicates. Realignment is applied with the Genome Analysis Toolkit (GATK). BEDTools was used to compute the coverage of target regions. Variant calling (SNV and indels) is performed by SAMTools and GATK. Single nucleotide variations and indels are detected by comparing the samples data to the reference genome.

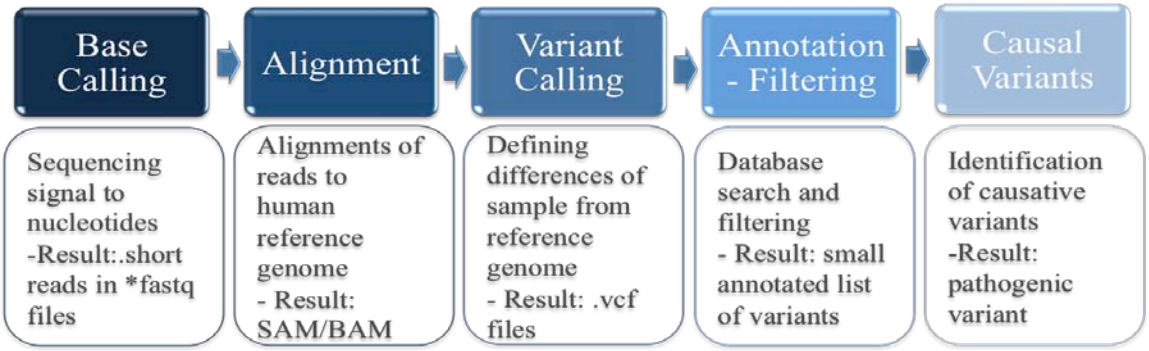

Figure 4.2. Bioinformatics workflow for next generation sequencing.

4.2.2. Variant Annotation

The remaining bioinformatics analyses including variant annotation, prioritization and validation were performed in our laboratory. All bioinformatics analyses in our laboratory were executed on Ubuntu 14.04 operating systems. The commands used in this analysis are listed in Table 4.1.

Table 4.1. Command lines used in annotation.

Commands	**Function**
convert2annovar.pl file_GATK.vcf –format vcf4 –outfilefile.anno	Converting GATK output to compatible ANNOVAR format
perl summarize_annovar.pl -out file.annotated -buildver hg19 -verdbsnp 138 -ver1000g 1000g2012apr -veresp 6500 -remove -alltranscriptfile.annohumandb	Annotation
file.annotated.genome_summary.csv > file.annotated.genome_summary.txt	Tab-limited format
python wannotate_pinar.py file_GATK.vcf file.annotated.genome_summary.txt	Adding omimdatabe information

Functional annotation of the variants is obtained with the ANNOVAR annotation tool by using several databases and online tools. Chromosomal location, base change, region (exonic, intronic, intergenic, splicing, UTR, non-coding RNA), read depth, dbSNP ID (if available), type of change (synonymous, nonsynonymous, stopgain, stoploss, frameshift), the MAF in 1000 Genome and Exome Variant Server (EVS) databases are added with ANNOVAR. OMIM database information, which presents the association of genes with linked disorders, is inserted. Additionally, online prediction tool scores are used to predict whether an exonic substitution affects protein function. Aligned exome data is visualized by Integrative Genome Viewer (IGV).

4.2.3. Candidate Gene Prioritization

A stepwise manner for filtering exome sequencing results is used to determine the candidate variants in the family:

- The result is sorted by a java-based software, Varsifter, according to the segregation of variations in the family.
- Variants, which are out of exons and splicing sites, are eliminated.
- Variants with high segmental duplication scores (≥ 0.9) are deleted.
- Variants with low MAF (< 0.1) are chosen. Normally, this score is 0.01, however we use 0.1 to prevent the elimination of candidates (we are looking for MAF in homozygous state, but it is not always in that state).
- Variants found in home database with high frequency are sifted.
- Among the remaining variants, those associated with disorders in the Omim database or/and genes with relevant function are selected.
- All candidate variations are validated by Sanger sequencing to prevent technical contamination and assemble problems. The segregation of the candidate mutation with the disease has to be shown.

4.2.4. Homozygosity Mapping

Determination of homozygous stretches by using massively parallel sequencing data enables us to narrow down the candidate region in the genome. With using HomSI or other homozygosity mapping programs, the candidate regions are defined and the variations coming from variant calling are evaluated. This method is useful for consanguineous families to detect the candidates.

4.3. Validation PCR

4.3.1. Validation PCR Conditions

Primers used for validation were either collected from literature or a new primer was designed with Primer3 software. The latter was checked via in-silico PCR tool on The Unified Soil Classification System (USCS) Genome Bioinformatics site (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start). Primers were purchased from

Sentromer, Turkey or MacrogenInc, Korea. Lyophilized primers were dissolved in 1 ml dH_2O and 10 µM dilutions were prepared.

PCR primers used for validation were designed to locate the desired mutation into the center of the PCR products with sizes around 200-600bp. All PCR reagents and PCR conditions used are listed in Appendix A. PCR products were analyzed on a 2% agarose gel. The intercalating dyeEtBr and/or GelRedfluorescent DNA stain were used for the visualization of the PCR products under UV light.

The total PCR product of 3 µl was mixed with 6X loading dye to a final concentration of 1X. The polymerized gel was placed into an electrophoresis chamber containing 0.5X TBE. After loading the samples, the gel was run at 120 V for around an hour, visualized under UV light and documented. The products were purified with Qiagen PCR Purification Kit for silica-membrane-based purification of PCR products.

Sequencing data was analyzed using the FinchTV v.1.4.0 and CLC Main Workbench v6.7 software.

5. RESULTS

All quality, depth, conservation scores, dbSNP and NHLBI GO Exome Sequencing Project (ESP) frequencies for all variations are complied in Table 5.14. and online prediction tool scores in Table 5.15. The inheritance patterns, initial-final diagnoses, variation status, association of genes with the disease are listed in Table 5.16.

5.1. Amyotrophic Lateral Sclerosis

5.1.1. ALS-Family 1

The DNA samples of both patients were subjected to WES. Novel and rare (MAF<0.01) exonic/splicing site variations were selected. After confirming the non-consanguinity in the parents, we focused on the heterozygous variants that are common for both patients. Among 4610 heterozygous variants, 157 were novel. One known heterozygous variant, T1482I on exon 28 of the *TRPM7* gene and one known heterozygous variant, E274D on exon 6 of the *SQSTM1* gene were found to be shared by two affected brothers (Figure 5.1. and Table 5.1.)(Ozoguz *et al.*, 2015).

The T1582I variation in the *TRPM7* gene is associated with the altered sensitivity to magnesium, which leads to Guamanian amyotrophic lateral sclerosis (ALS-G) and parkinsonism dementia (PD-G)(Hara *et al.*, 2010, Hermosura *et al.*, 2005).The mutation is in a conserved region of the protein and in silico analysis of the mutation predicts that the variant will have a negative effect on protein function. Heterozygous *SQSTM1* and *TRPM7* mutations are good candidates for the disease phenotype in the family. Since the parents known to be free of disease at the time of diagnosis were not available to us, results could not be confirmed with them (Figure 5.2.).

Table 5.1. Candidate variations inALS-Family 1.

Chr	Position	Gene name	dbSNP ID	Ref/Alt	Mutation
chr5	179260099	*SQSTM1*	rs55793208	G/C	c.G822C:p.E274D
chr15	50878630	*TRPM7*	rs8042919	G/A	c.C4445T:p.T1482I

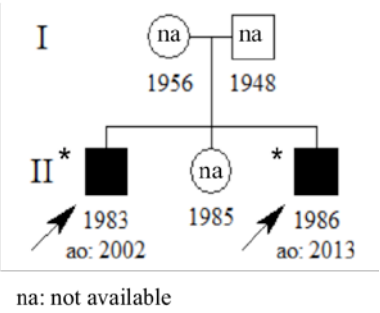

Figure 5.1. Both*TRPM7* and *SQSTM1* mutations in the affected siblings.

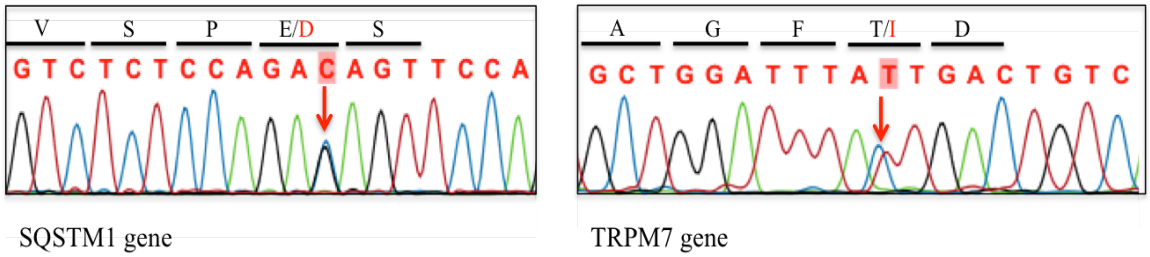

Figure 5.2. Heterozygous statuses of *SQSTM1*(E274D) and T*RPM7*(T1482I) mutations.

5.1.2. ALS-Family 2

The index case and his healthy mother and brother were subjected to WES. Since, the pedigree indicates a recessive inheritance pattern, exome sequencing results were evaluated accordingly. Out of 1196 exonic (except the synonymous ones) and splicing site variants in the homozygous state, two independent homozygous variants in the *SPG11* gene were detected(Ozoguz *et al.*, 2015). The Y2272X mutation in exon 38 is novel, and leads to a stop codon, whereas the asparagine to serine change at position 1962 (N1962S) in exon 33 is already reported (Table 5.2.).

The affected father died with classical ALS, his sample was not available to us.The segregation of both mutations in the family members was shown by Sanger sequencing (Figure 5.3., Figure 5.4. and Figure 5.5.). The mother and the paternal grandmother were carriers for both mutations. The unaffected older brother was only carrier for the N1962S change. The additional heterozygous P1235S variation with low MAF in the *ARHGEF28* gene was detected in the unaffected mother and in the older brother and was considered to be a benign polymorphism.

Table 5.2. Candidate variations in ALS-Family 2.

Chr	Position	Gene name	dbSNP ID	Ref/Alt	Mutation
chr15	44855496	*SPG11*	-	A/C	c.T6816G:p.Y2272X
chr15	44865000	*SPG11*	rs140824939	T/C	c.A5885G:p.N1962S
chr5	73205717	*ARHGEF28*	rs17634865	C/T	c.C3703T:p.P1235S

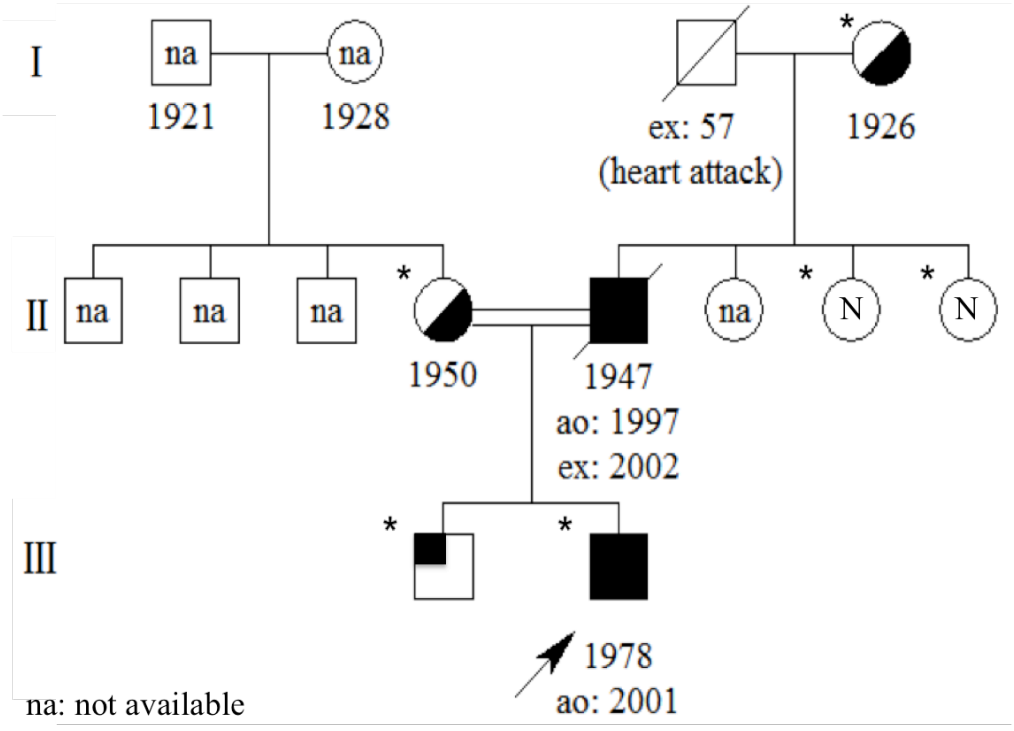

Figure 5.3. Tree of ALS-Family 2.

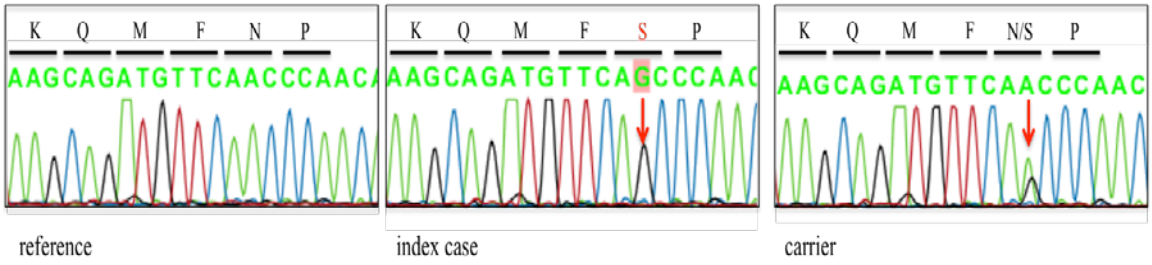

Figure 5.4. *SPG11*p.N1982S mutation in homozygous/heterozygous status.

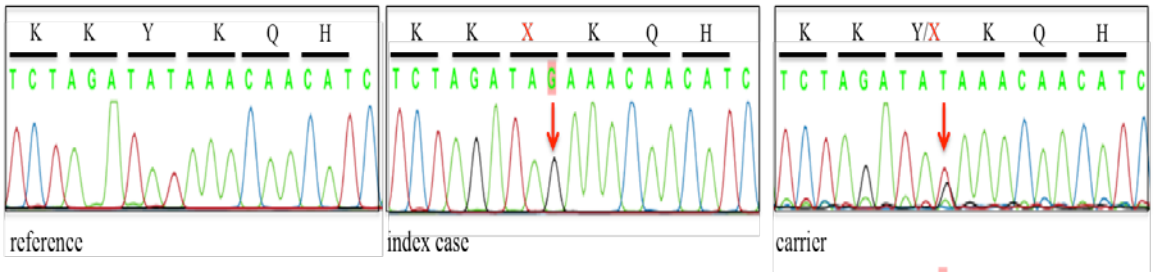

Figure 5.5. *SPG11*p.Y2272Xmutation in homozygous/heterozygous status.

5.1.3. ALS-Family 3

DNA samples of both affected members were subjected to WES. Two independent homozygous mutations in the *OPTN* gene were found to be shared by both (Table 5.3.). One of them is a known M98K missense mutation,which has a higher MAF in 1000 genomes and dbSNP138. The second variation is a novel G291fsX6 frameshift mutation, which results in a stop codon after five amino acids (Figure 5.6., Figure 5.7. and Figure 5.8.)(Ozoguz *et al.*, 2015). Validation results showed that the affected brothers were homozygous for both mutations and the unaffected parents carriers, as expected.

Table 5.3. Candidate variations inALS-Family 3.

Chr	Position	Gene name	dbSNP ID	Ref/Alt	Mutation
chr10	13152400	*OPTN*	rs11258194	T/A	c.T293A:p.M98K
chr10	13164477	*OPTN*	-	G/GC	c.873dupC:p.G291fsX6

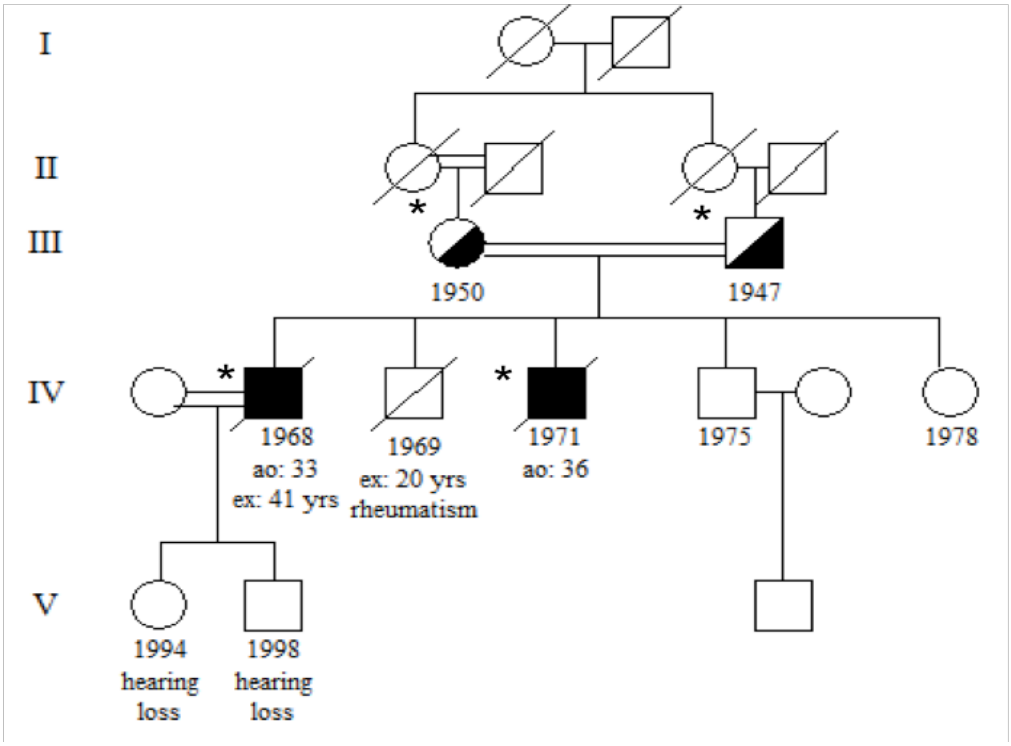

Figure 5.6. Tree of ALS-Family 3.

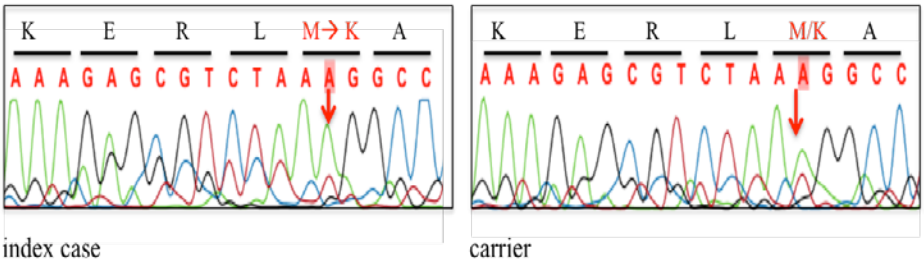

Figure 5.7. Homozygous/heterozygous*OPTN*p.M98K mutation.

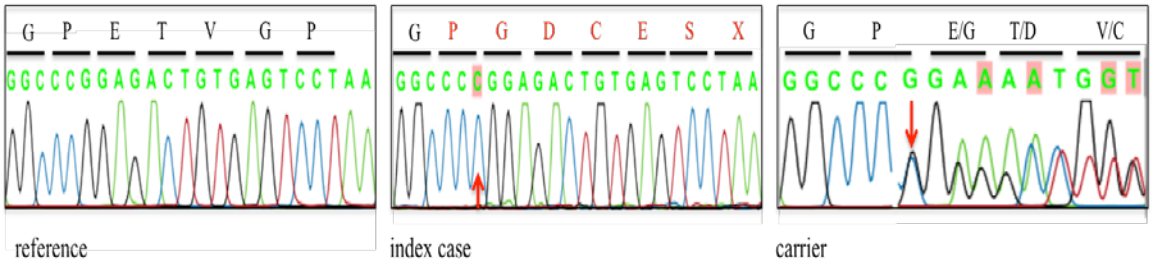

Figure 5.8. Homozygous/heterozygous*OPTN*p.G291fsX6 mutation.

5.1.4. ALS-Family 4

The youngest member of the family was referred to our laboratory with ALS diagnosis. Two affected members had died with a slow progressive form of ALS.Exome sequencing revealed a novel AA deletion in the *OPTN* gene, which leads to a frameshift at codon 359, resulting in a truncated protein (Table 5.4., Figure 5.9. and Figure 5.10.)(Ozoguz *et al.*, 2015).

Table 5.4. Candidate variation inALS-Family 4.

Chr	Position	Gene name	dbSNP ID	Ref/Alt	Mutation
chr10	13167492	*OPTN*	-	CAA/A	p.359_359del

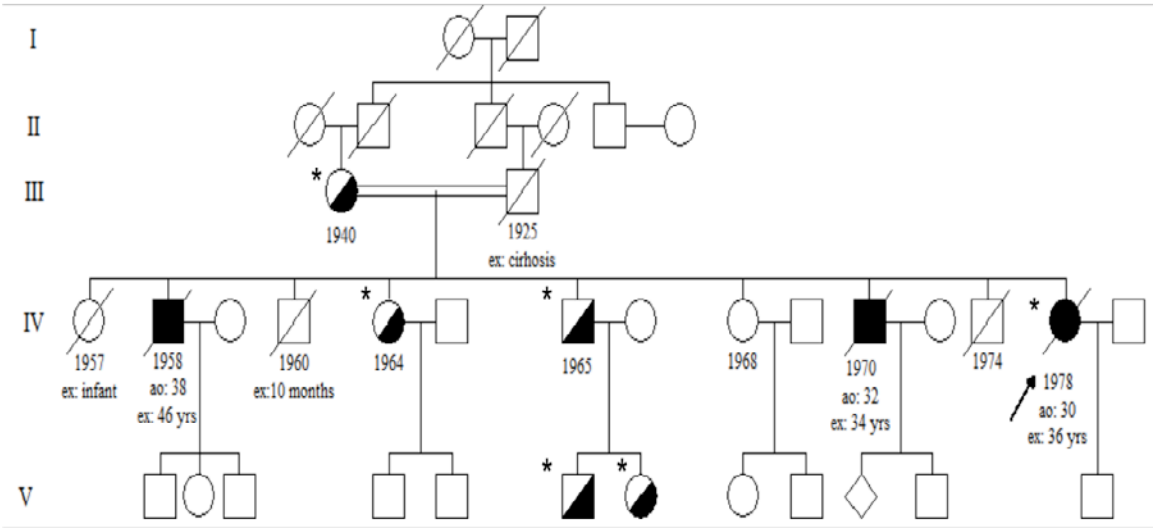

Figure 5.9. Family 4 with *OPTN* deletion.

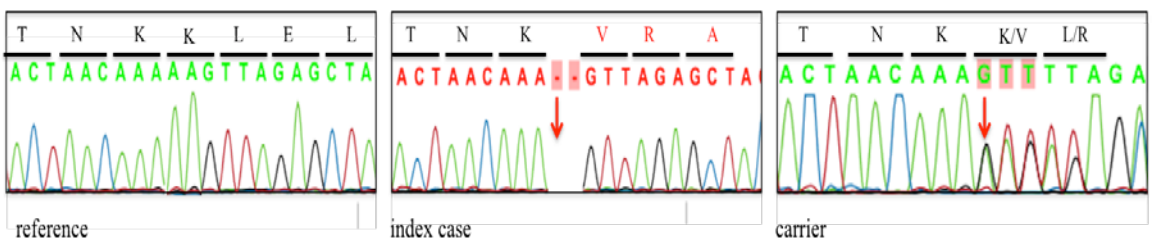

Figure 5.10. Homozygous/heterozygous *OPTN* p.359_359del mutation.

5.2. Family with ALS and Parkinsonism

In a consanguineous family, three out of ten individuals were reported with clinical features of motor neuron disease andparkinsonismwithout dementia. The index case, his parents and unaffected sister were subjected to exome sequencing analysis. The variants were filtered against the criteria of being homozygous in the index case and heterozygous in the parents. The only such SNV was a nonsense change in the *DJ1* gene in a homozygous stretch (Table 5.5. and Figure 5.11.). The mutation is a novel homozygous missense C to T change in exon 3 of the *DJ1* gene, resulting in the substitution of a highly conserved glutamine at position 45 of DJ1 protein by a stop codon. The mutation was not present in dbSNP138, the 1000 genomes, EVS and our in-house database including 110 exomes. The Q45X mutation in the available members of the family was confirmed by Sanger sequencing (Figure 5.12. and Figure 5.13.). All the affected individuals were shown to carry the Q45X mutation in homozygous state, whereas parents and the unaffected sister were carriers.

Table 5.5. Candidate variation in*DJ1* Family.

Chr	Position	Gene name	dbSNP ID	Ref/Alt	Mutation
chr1	8025425	*DJ1*	-	C/T	c.C133T:p.Q45X

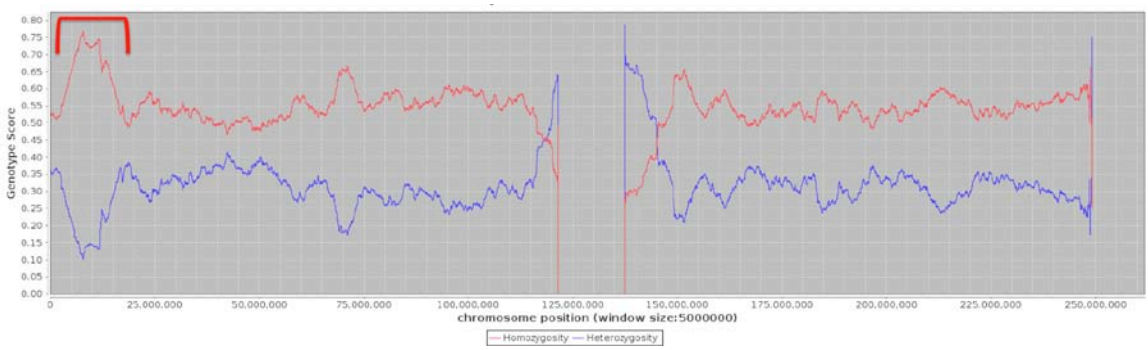

Figure 5.11. Homozygous stretch on chromosome 1.

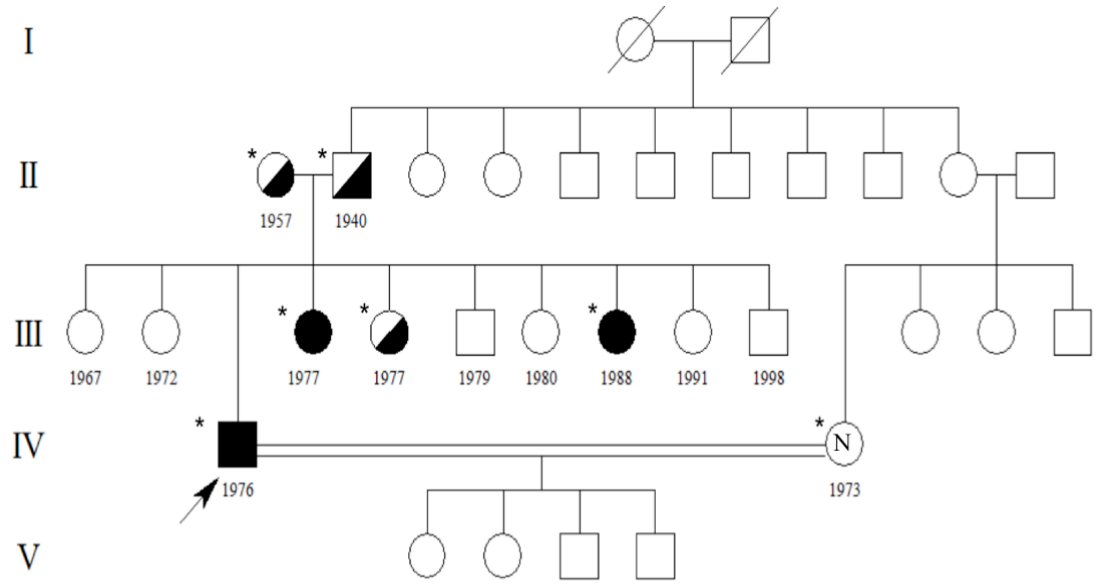

Figure 5.12. DJ1 mutation status in the family.

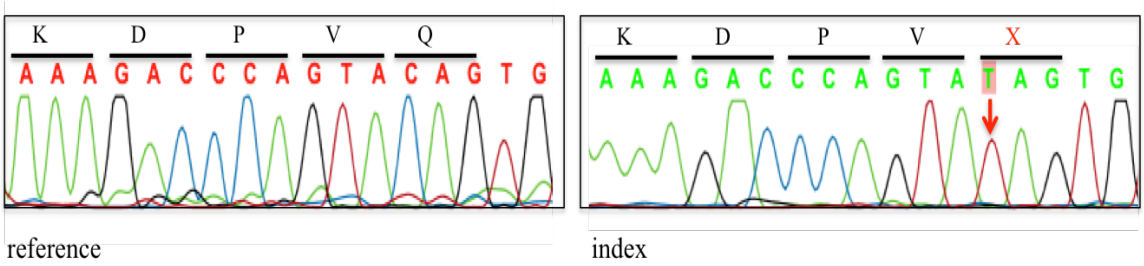

Figure 5.13. *DJ1*p.Q45X mutation.

5.3. Autosomal Recessive Ataxias with Oculomotor Apraxia 1

Two novel missense mutations were defined in two distinct families with ataxia phenotype. Both mutations were not present in dbSNP, the 1000 genomes, EVS and our in-home database.

5.3.1 AOA1-Family 1

In this family, the index case, her affected younger brother and her consanguineous parents were subjected to WES. Exome sequencing data was

investigated at the homozygous regions shared at least by the proband and her affected brother. Exome sequencing analysis revealeda homozygous A198V mutation was found in a highly conservedsequence of *APTX* gene. All prediction tools predicted the mutation as deleterious.Sanger sequencing revealed the expected segregation pattern in the family members (Table 5.6., Figure 5.14. and Figure 5.15.).

Table 5.6. Candidate variation in AOA1-Family 1.

Chr	Position	Gene name	dbSNP ID	Ref/Alt	Mutation
chr9	32984806	*APTX*	-	G/A	c.C473T:p.A158V

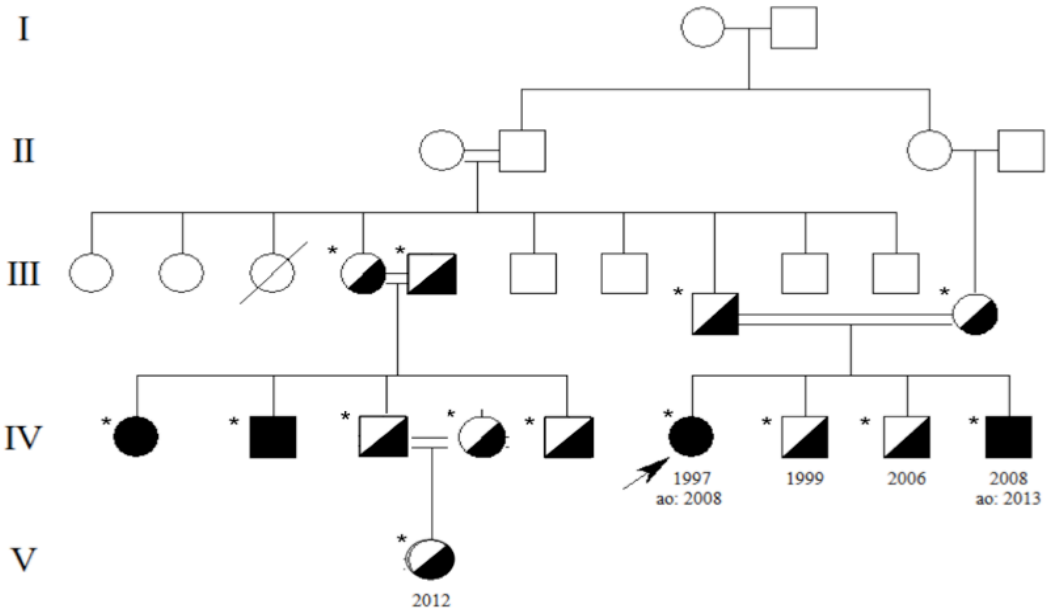

Figure 5.14. The *APTX* A158V mutation homozygotes and heterozygotes.

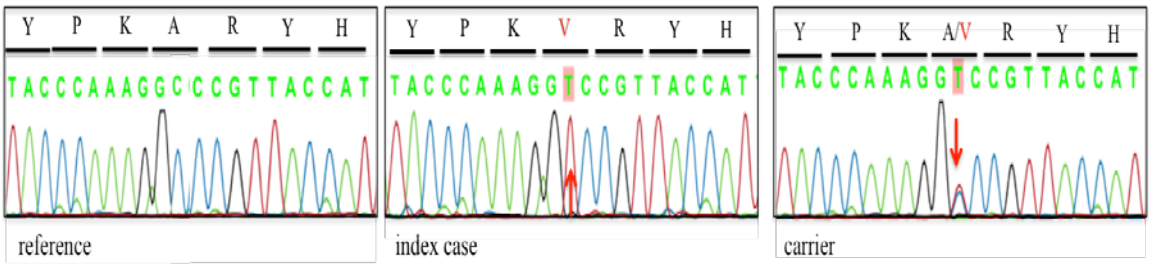

Figure 5.15. Sequencing result of *APTX*p.A198V mutation.

5.3.2 AOA1-Family 2

In a consanguineous family with two affected brothers, the olderpatients sample was subjected to WES. A missense mutationat position 190 of the*APTX* gene was found in both siblings,which replaced the conserved valine by glycine (Table 5.7.). The prediction tools majorly support the deleterious nature of the V190G mutation and it was validated by Sanger sequencing (Figure 5.16. and Figure 5.17.). As expected, the brothers were homozygous and the unaffected parents werecarriers for the V190G mutation.

Table 5.7. Candidate variation in AOA1-Family 2.

Chr	Position	Gene name	dbSNP ID	Ref/Alt	Mutation
chr9	32984710	*APTX*	-	A/C	c.T569G:p.V190G

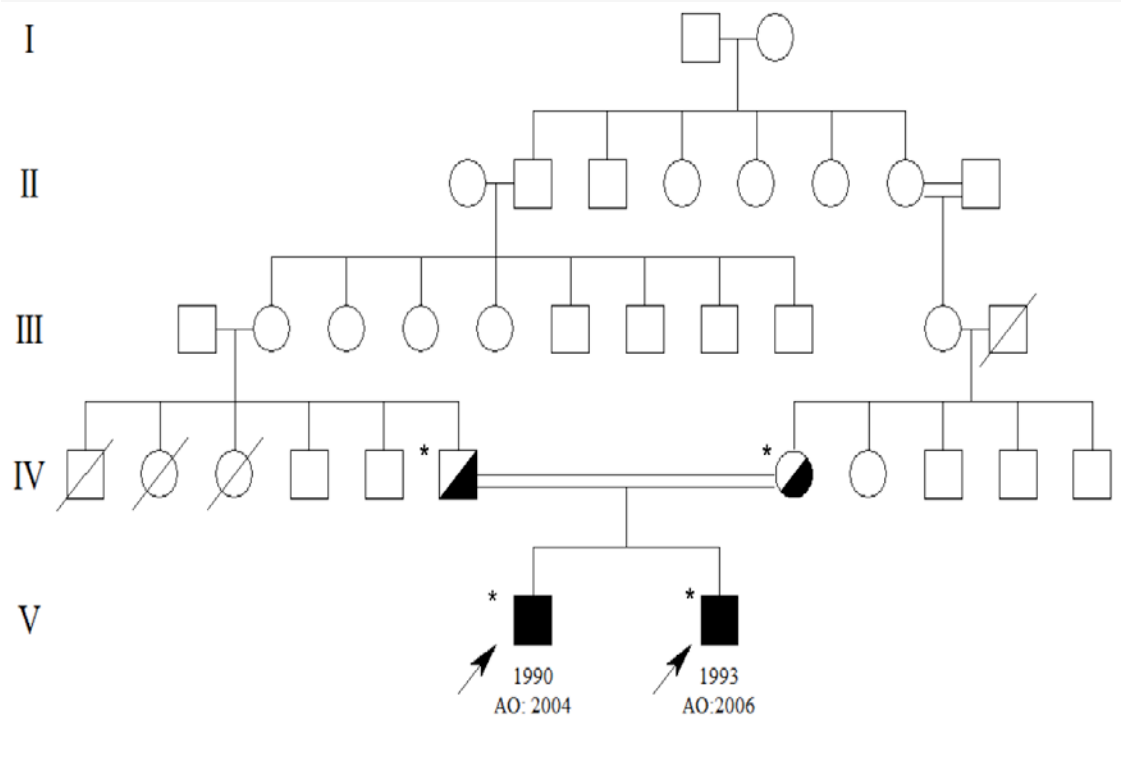

Figure 5.16. Status of *APTX* mutation in AOA1-Family 2.

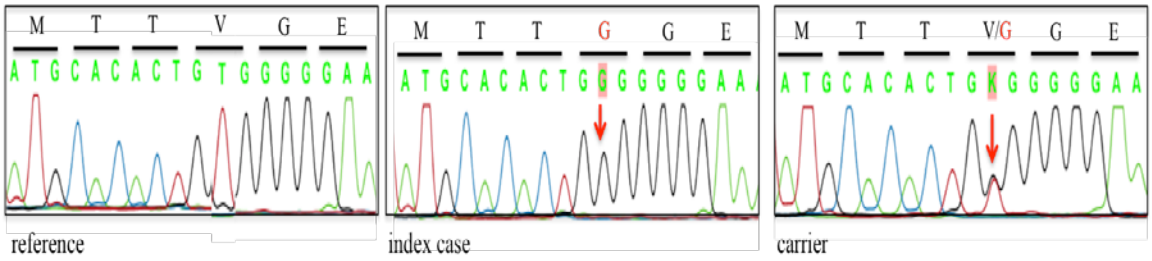

Figure 5.17. *APTX*p.V190G mutation.

5.4. Autosomal Recessive Ataxias with Oculomotor Apraxia 2

Three affected siblings, offsprings from a consanguineous marriage, were referred to our laboratory with the diagnosis of recessive ataxia. The whole family, including the parents, was subjected to exome sequencing. Variants, which were homozygous in the affected siblings and heterozygous in the parents, were chosen. A frameshift mutation,p.A1432fsX2, in the *SETX* gene, which leads to a stop codon at position 1434, was detected (Table 5.8. and Figure 5.18.). The mutation, which segregates with the disease in the family, was not found in all open-source databases and in-house database (Figure 5.19. and Figure 5.20.). The unaffected parents wereheterozygous and the three affected siblings were homozygous for the mutation.

Table 5.8. Candidate variation in the*SETX* family.

Chr	Position	Gene name	dbSNP ID	Ref/Alt	Mutation
chr9	135202689	*SETX*	-	T/TG	c.4296_4297insC:p.A1432fs

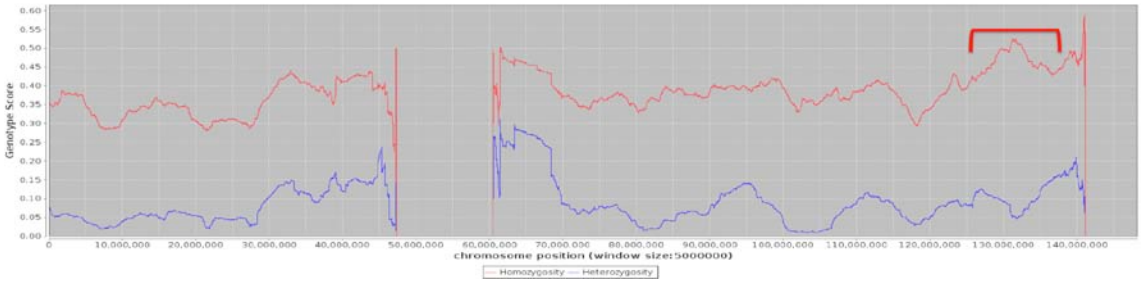

Figure 5.18. Homozygous stretch on chromosome 9.

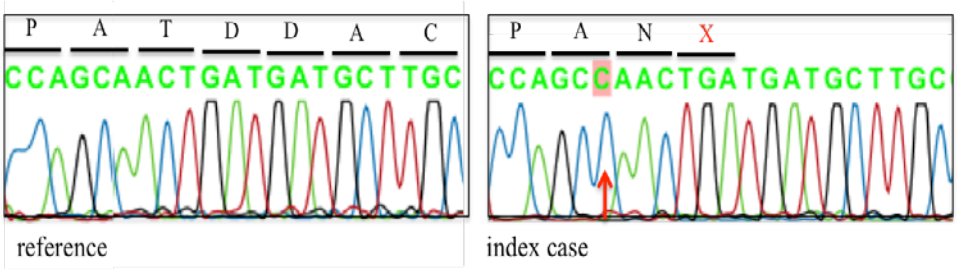

Figure 5.19. *SETX* p.A1432fsX2 mutation.

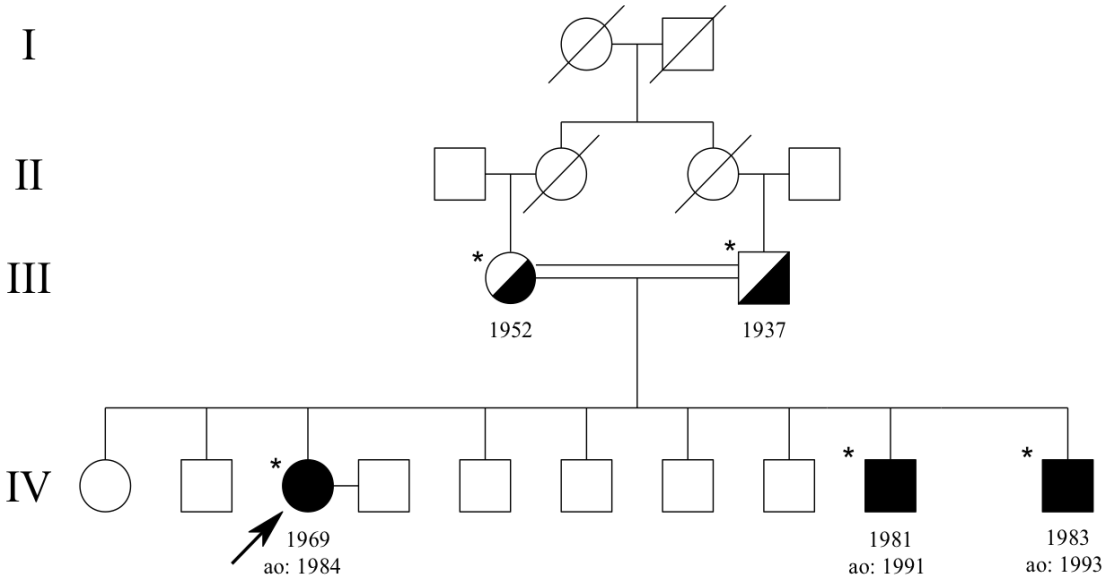

Figure 5.20. Segregation of the novel *SETX* mutation in the family.

5.5. Ataxia–telangiectasia-Like Disorder 1 (ATLD1)

DNA samples of two affected siblings with consanguineous parents were subjected to WES. The affected siblings had early childhood onset of progressive cerebellar degeneration, but no telangiectasia. According to exome results, a novel homozygous missense R155P mutation in the *MRE11* gene was present in both siblings (Table 5.9.). The conserved location of arginine and the findings of prediction tools support the deleterious nature of this variant. Parents and siblings were tested for the identified variant *MRE11*p.R155P by Sanger sequencing,which confirmed the exome result (Figure 5.21., Figure 5.22.).

Table 5.9. Candidate variation inthe ALTD1-Family.

Chr	Position	Gene name	dbSNP ID	Ref/Alt	Mutation
chr11	94211981	*MRE11*	-	C/G	c.G464C:p.R155P

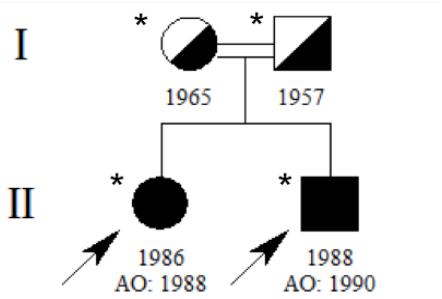

Figure 5.21. Pedigree with *MRE11* gene mutation.

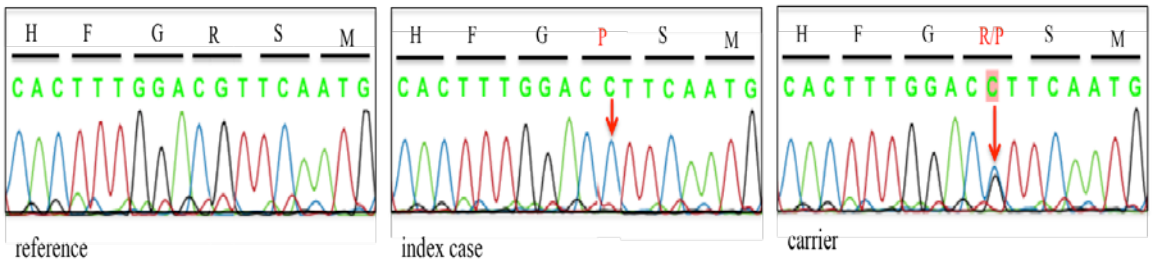

Figure 5.22. Chromatogram, showing the sequencing profile of the p.R155Pmutation.

5.6. Autosomal Recessive Spastic Ataxia of Charlevoix Saguenay

5.6.1. ARSACS-Family 1

A consanguineous couple was referred to our laboratory with four affected siblings in juvenile agewith ataxia andadditional skeletal deformities. The index case was subjected to exome sequencing. Exonic and splicing site variants were selected and a homozygous exonic *SACS* mutation **(**p.G2772A) anda splice site mutation (c.2093+1G>A)were identified (Table 5.10. and Figure 5.23.).The segregation and validation of the mutations were confirmed by Sanger sequencing. The splice site

variation c.2093+1G>A in the *SACS* gene was found to be false positive. The G2772A mutation was in a homozygous state in the affected members of the family and unaffected mother was carrier for this mutation (Figure 5.24. and Figure 5.25.). Additionally, two homozygous novel mutations (T1212I and A1213V) were present in the 5gene that is associated with a form of spondyloepiphyseal dysplasia (SED), which is characterized by skeletal deformities, they could explain the bone deformities present in the patients(Tompson *et al.*, 2009).

Table 5.10. Candidate variant list in ARSACS-Family 1.

Chr	Position	Gene name	dbSNP ID	Ref/Alt	Mutation
chr13	23909699	*SACS*	-	C/G	c.G8315C:p.G2772A
chr13	23928656	*SACS*	-	C/T	c.2093+1G>A
chr15	89399451	*ACAN*	-	C/T	c.C3635T:p.T1212I
chr15	89399454	*ACAN*	-	C/T	c.C3638T:p.A1213V

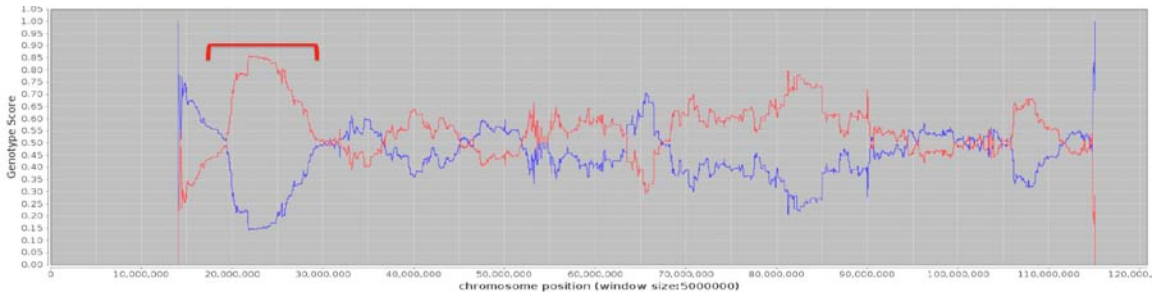

Figure 5.23. Homozygosity mapping result of chromosome 13.

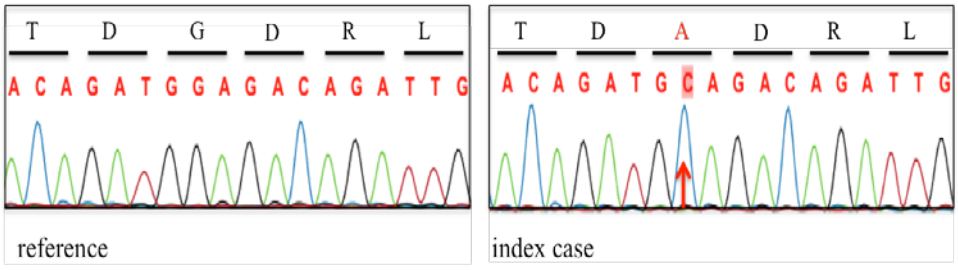

Figure 5.24. Chromatograms, showing the sequencing profile of ARSACS-Family 1.

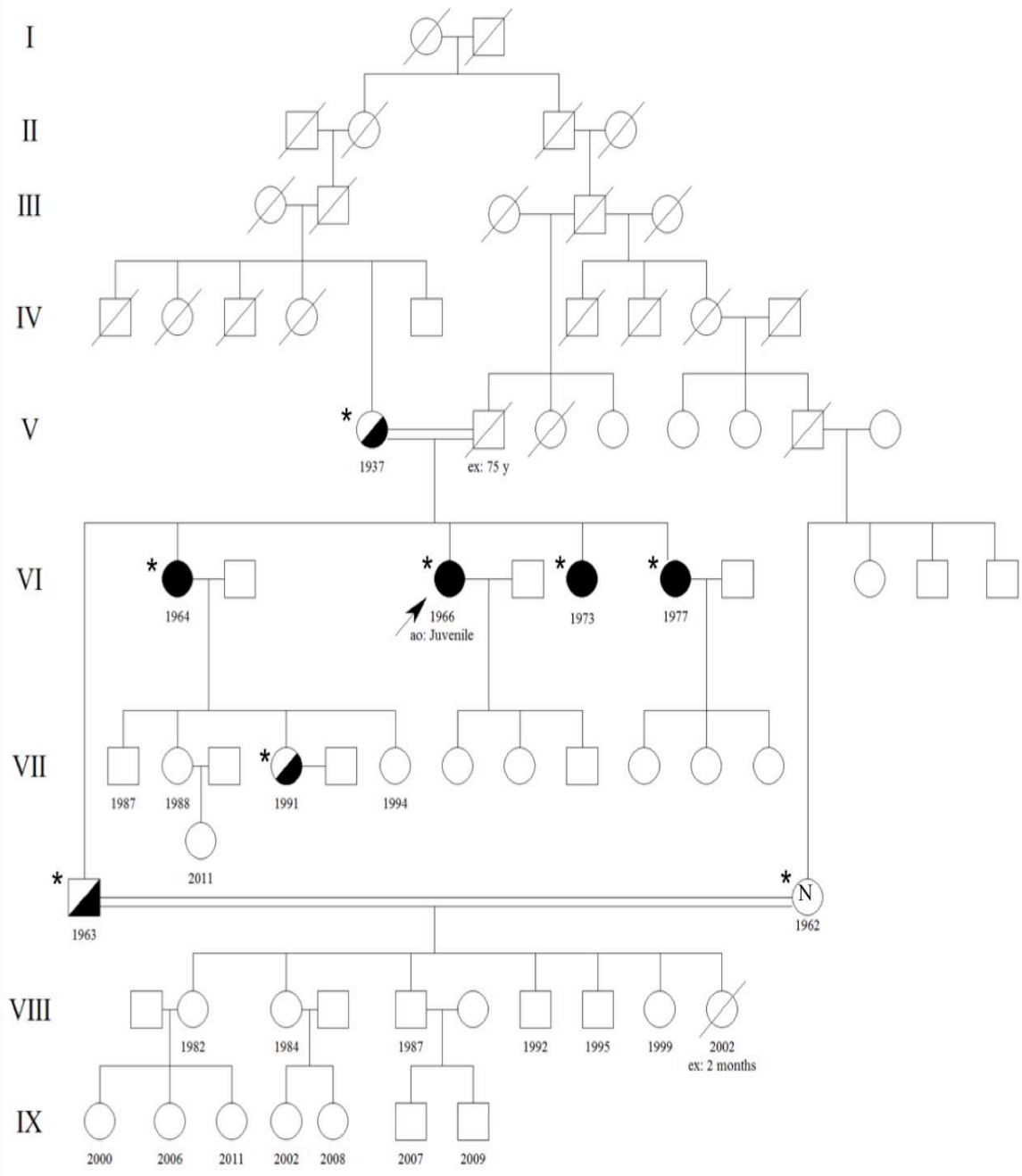

Figure 5.25. Pedigree of Family 1 with *SACS* mutation.

5.6.2. ARSACS-Family 2

In the ARSACS-Family 2 with four affected members, the index case was subjected to exome sequencing. Novel/rare exonic and splicing site variants were selected. A novel homozygousp.R2574fsX4 mutation was found in the *SACS* gene (Table

5.11.).The novel R2574fs mutation resulted in the substitution of the conserved amino acid arginine by lysine in the protein and a stop codon after three amino acids.The frameshift mutation was expected to have an adverse effect on the protein, and online prediction toolalso supported the damaging effect of the mutation, whichwas verified by Sanger sequencing in all available family members (Figure 5.26. and Figure 5.27).

Table 5.11. Variation list in ARSACS-Family 2.

Chr	Position	Gene name	dbSNP ID	Ref/Alt	Mutation
chr13	23910294	*SACS*	-	C/CT	c.7720dupA:p.R2574fs

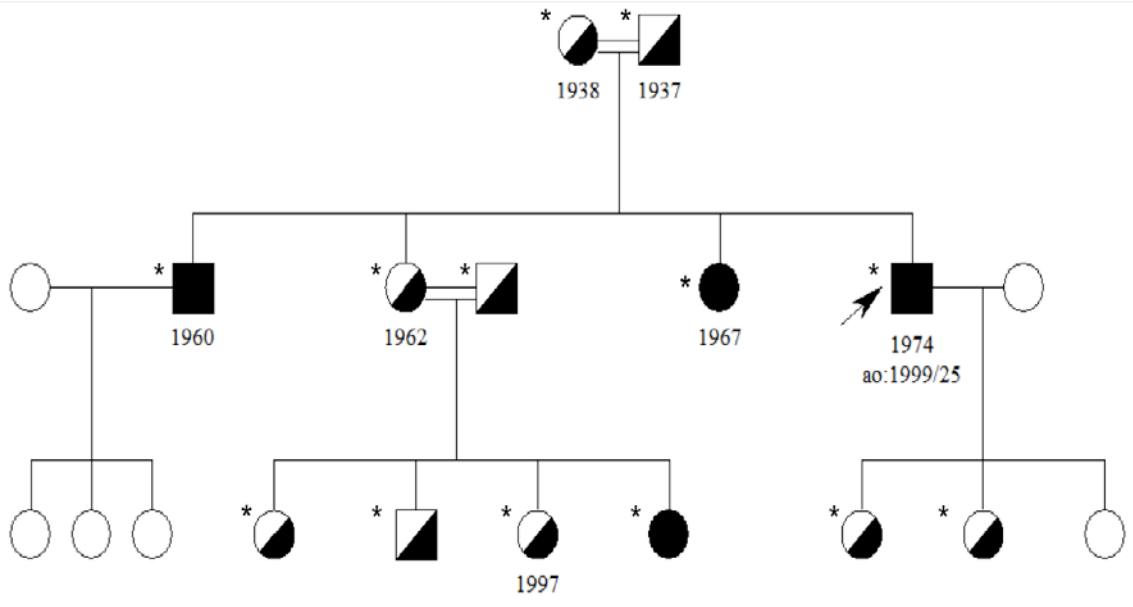

Figure 5.26. Genotypes of ARSACS-Family 2 with homozygous *SACS* mutation.

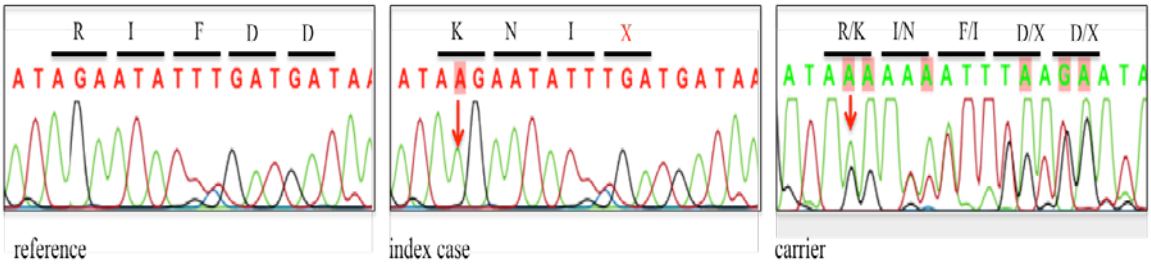

Figure 5.27. Chromatograms, showing the sequencing profile of ARSACS-Family 2.

5.6.3. ARSACS-Family 3

The WES analysis of the index case,with consanguineous parentsindicated that the *SACS* gene had aninsertion at position 2355_2356 (Table 5.12). The novel A insertion in the *SACS* gene leads to a frame-shift at codon 785, resulting in a truncated protein. The insertion was validated by Sanger sequencing in all family members(Figure 5.28. and Figure 5.29.).

Table 5.12. Candidate variation in ARSACS-Family 3.

Chr	Position	Gene name	dbSNP ID	Ref/Alt	Mutation
chr13	23915660	*SACS*	-	A/AT	c.2355_2356insA:p.N785fsX12

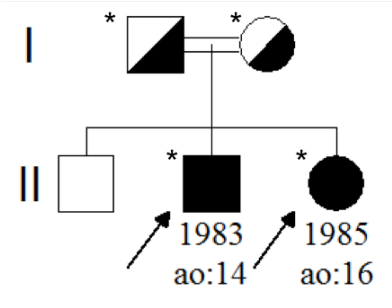

Figure 5.28. Genotypes of the index patients in ARSACS-Family 3 with a homozygous *SACS* mutation.

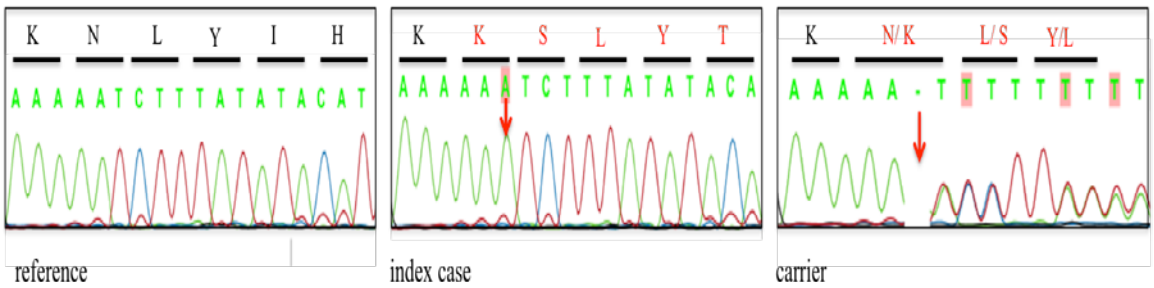

Figure 5.29. Chromatograms showing ARSACS N781fsX12 mutation.

5.7. Kufor-Rakep Syndrome

The index case, diagnosed with ataxia was subjected to WES along with his twin.A splicing site mutation, c.2747+2T>A in the ATP13A2 gene was identified via exome sequencing (Table 5.13.). The variationwas confirmed in both affected sibs, and as expected, unaffected parents and siblings werecarriers (Figure 5.30. and Figure 5.31.).

Table 5.13. Variant list in the family.

Chr	Position	Gene name	dbSNP ID	Ref/Alt	Mutation
chr 1	17314815	*ATP13A2*	-	A/T	c.2747+2T>A

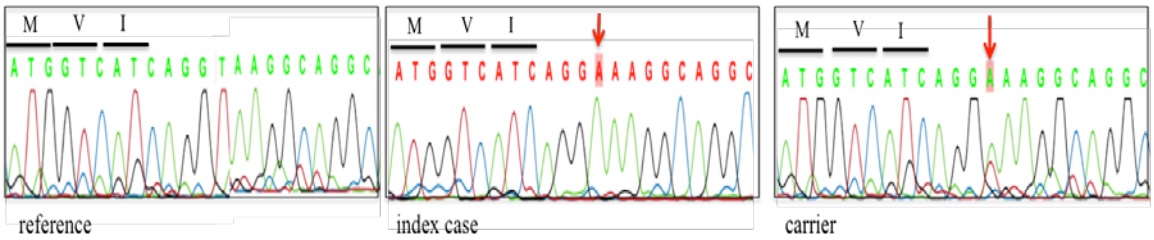

Figure 5.30. Chromatograms, showing the sequencing profiles of family members.

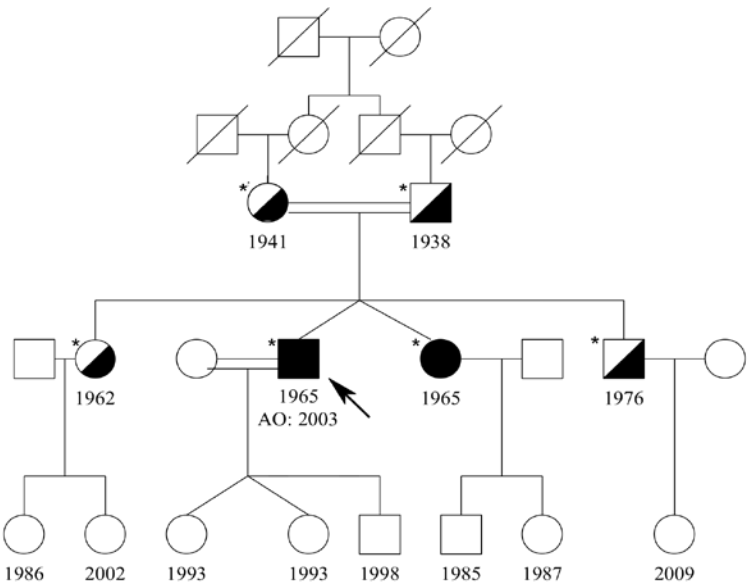

Figure 5.31. Family tree shows the segregation of the mutation in the family.

Table 5.14. The quality scores and minor allele frequencies of the variations.

Chr	Position	Gene name	Mutation	dbID	Quality	Approximate read depth	Conserved LOD	ESP6500 MAF	1000 Genome MAF
5	179260099	SQSTM1	p.E274D	rs55793208	1277.81	1815	0	0.018684	0.01
15	50878630	TRPM7	p.T1482I	rs8042919	10323.28	5068	399	0.07967	0.07
15	44855496	SPG11	p.N1962S*	-	3747.54	3072	422	-	-
15	44865000	SPG11	p.Y2272X	rs140824939	6509.54	4386	0	0.003618	0.0027
10	13152400	OPTN	p.G291fsX	rs11258194	9152.23	4134	0	0.061126	0.07
10	13164477	OPTN	p.M98K	-	5410.52	3594	0	-	-
10	13167493	OPTN	p.359del AA*	-	3544.74	3127	358	-	-
1	8025426	PARK7	p.Q45X*	-	7244.56	2104	521	-	-
9	32984710	APTX	p.A158V	-	2871.07	2835	278	-	-
9	32984806	APTX	p.V190G	-	986.22	1.474	545	-	-
9	135202689	SETX	p.A1482fs*	-	4263.84	895	0	-	-
11	94211981	MRE11	p.R155P	-	54.15	1756	658	-	-
13	23909700	SACS	p.G2772A*	TDB	1567.99	1486	475	-	-
15	89399451	ACAN	p.T1212I*	TDB	27.96	1491	0	-	-
15	89399454	ACAN	p.A1213V*	TDB	27.92	1521	0	-	-
13	23910294	SACS	p.R2574fs*	-	1298.02	1123	547	-	-
13	23915660	SACS	p.N785fs*	-	3215.35	1118	585	-	-
1	17314815	ATP13A2	c.2747+2T>A*	-	1491.79	1154	381	-	-

MAF: minor allele frequency, TDB: Tübitak Database, * novel variation

Table 5.15. Online prediction tool scores of the variations.

Chr	Position	Gene name	AA Change	PhyloP	SIFT	PolyPhen2	LRT	Mutation Taster	GERP ++
5	179260099	SQSTM1	c.G822C:p.E274D	0.031913	0.38	0.002	0.332125	0	-9.14
15	50878630	TRPM7	c.C4445T:p.T1482I	0.886245	0.96	0.003	0.523336	0.285706	2.28
15	44855496	SPG11	c.T6816G:p.Y2272X	0.936909	0.8999	0.712697	0.929139	1	2.77
15	44865000	SPG11	c.A5885G:p.N1962S*	0.02606	0.93	0	0.867116	0.266126	-6.54
10	13152400	OPTN	c.T293A:p.M98K	0.913907	0.06	0.011	0.959387	0.002869	3.33
10	13164477	OPTN	c.873dupC:p.G291fs	-	-	-	-	-	-
10	13167493	OPTN	c.1075_1076del:p.359_359del*	-	-	-	-	-	-
1	8025426	PARK7	c.C133T:p.Q45X*	0.98172	0.9024	0.732553	0.999999	1	4.39
9	32984710	APTX	c.T569G:p.V190G	0.998703	1	0.963	1	0.999988	5.24
9	32984806	APTX	c.C473T:p.A158V	0.993849	1	0.998	1	0.999949	4.38
9	135202689	SETX	c.4296_4297insC:p.A1432fs*	0	0	0	0	0	0
11	94211981	MRE11	c.G464C:p.R155P	0.9985	0.99	0.997	1	0.981086	4.99
13	23909700	SACS	c.G8315C:p.G2772A*	0.998617	0.5	0.889	1	0.98074	5.06
15	89399451	ACAN	c.C3635T:p.T1212I*	-	-	-	-	-	-
15	89399454	ACAN	c.C3638T:p.A1213V*	-	-	-	-	-	-
13	23910294	SACS	c.7720dupA:p.R2574fs*	-	-	-	-	-	-
13	23915660	SACS	c.2355_2356insA:p.N785fs*	-	-	-	-	-	-
1	17314815	ATP13A2	c.2747+2T>A*	-	-	-	-	-	-

* novel variant

Table 5.16.The overall results of the study.

Family	Inheritance	Initial diagnosis	Mutated gene	Mutation	Status	Final Diagnosis	Association of gene with disease (OMIM)
1	dominant	ALS	SQSTM1	p.E274D	het	ALS	Paget disease of bone
			TRPM7	p.T1482I	het		ALS-parkinsonism/dementia complex
2	recessive		SPG11	p.N1962S*	hom		Spastic paraplegia 11, autosomal recessive
				p.Y2272X	hom		
3	recessive		OPTN	p.G291fsX	hom		Glaucoma 1 open angle E
				p.M98K	hom		Amyotrophic lateral sclerosis 12
4	recessive		OPTN	p.359del AA*	hom		Paget disease of bone
5	recessive	ALD-PD	DJ1	p.Q45X	hom	ALS-parkinsonism	Parkinson disease 7 ar early-onset
6	recessive	SCA & FRDA	APTX	p.A158V	hom	AOA1	Ataxia ocular apraxia and hypoalbuminemia
7	recessive			p.V190G	hom		
8	recessive		SETX	p.A1482fsX*	hom	AOA2	Ataxia-ocular apraxia-2
9	recessive		MRE11	p.R155P	hom	A-TLD	Ataxia-telangiectasia-like disorder
10	recessive	FRDA	SACS	p.G2772A*	hom	ARSACS	Spastic ataxia, Charlevoix-Saguenay type
			ACAN	p.T1212I*	hom	SED	Spondyloepiphyseal dysplasia, Kimberley type
				p.A1213V*	hom		
11	recessive	SCA	SACS	p.R2574fsX*	hom	ARSACS	Spastic ataxia, Charlevoix-Saguenay type
12	recessive	SCA & FRDA	SACS	p.N785fsX12*	hom		
13	recessive	SCA	ATP13A2	c.2747+2T>A*	hom	Kufor-Rakeb Syndrome	Kufor-Rakeb Syndrome

* novel variation

6. DISCUSSION

6.1. Neurodegenerative Disorders

In the framework of this study, 13 Turkish families were investigated. The initial diagnoses, the final diagnoses, the mutated genes, the variations, their inheritance patterns and associated diseases are compiled in Table 5.16. Exome sequencing unraveled several known and novel mutations in *TRPM7, SQSTM1*, *SPG11* and *OPTN* genes in ALS families(Ozoguz *et al.*, 2015). Additionally a novel *DJ1* mutation was identified in a family with parkinsonism and ALS phenotype. Moreover, missense and nonsense mutations in *APTX, SETX, MRE11*, *SACS* and *ATP13A2* genes were identified in recessive ataxia families. All quality, depth and conservation scores for these variations are listed in Table 5.14. and online prediction tool scores in Table 5.15.

ALS is a complex disorder with a large spectrum of genetic background. Here, we represent four ALS families with several mutations. *SQSTM1* gene encodes a ubiquitin-binding protein with an important role in protein degradation via the proteasome. The gene has been associated both with sALS and fALS mechanisms (Fecto *et al.*, 2011). The identified E274D variation in the *SQSTM1* gene has a low MAF in 1000 genomes. The second variant in this family is *TRPM7*-T1582I. T1582I mutation in the *TRPM7* gene has been shown to be associated with the Guamanian amyotrophic lateral sclerosis (ALS-G) and parkinsonism dementia (PD-G), however the findings are contradictory (Hara *et al.*, 2010, Hermosura *et al.*, 2005). The conserved position supports the negative effect of the mutation on protein function. In our patients, with an ALS-PD complex type of disease manifestation, either only one of the above gene mutations may be sufficient for disease development, or the common action of both *SQSTM1* and *TRPM7* in mutated form, may have contributed disease. Since unfortunately the parents and the at the time of diagnosis unaffected sister were not available for further study, this question remains unsolved.

Mutations in the *SPG11* gene represent the most common form of autosomal recessive hereditary spastic paraplegia with thin corpus callosum (HSP-TCC) (Stevanin *et al.*, 2008, Stevanin *et al.*, 2007). The clinical spectrum of *SPG11*-based disease became wider with the identification of *SPG11* mutations in autosomal recessive juvenile amyotrophic lateral sclerosis (ARJALS) patients (Orlacchio *et al.*, 2010). In our family, two independent variations in the *SPG11* gene were homozygously present. The novel *SPG11*-Y2272X leads to a frameshift and results in a premature stop codon. The second mutation is an asparagine to serine change at position 1962.The affected father had died with classical ALS prior to this study, thus his sample was not available. The proband, offspring of a first-cousin consanguineous mating, has inherited both *SPG11* mutations above in homozygous format, and was diagnosed with juvenile onset ALS at the age of 23 with a slower disease progression as compared to his father. The father on the other hand, developed the disease at the much later age of 50 and may have carried only one of the above homozygous *SPG11* mutations. In the absence of his sample, it is very difficult to evaluate his fast progression.

Defects in *OPTN* are known to give rise to a wide range of clinical phenotypes (Kachaner *et al.*, 2012). Dominant missense, nonsense mutations and recessive deletions of *OPTN* have been identified in patients both with fALS and sALS(Maruyama *et al.*, 2010). *OPTN* mutations are very rare or absent in European populations and highly common in Japan. In our study, two families out of 13 have OPTN mutations and they have also been described earlier in the Turkish population (Ozoguz *et al.*, 2015). Two distinct homozygous mutations in the *OPTN* gene were found in both affected members of a family diagnosed with ALS. The first variation M98K has a higher MAF score in 1000 genomes and dbSNP databases. The second mutation, G291fs, is a novel C duplication, which leads to a stop codon after six amino acids. Both mutations segregate with the disease, again it is difficult to know how these mutations contribute to disease, eg if both of them are necessary for disease development stays so far elusive. In another ALS family with three affected members and consanguineous parents, exome sequencing revealed an AA deletion again in the *OPTN* gene. The two nucleotide deletion leads to a truncated *OPTN* protein after nine amino acids, which may explain the clinical phenotypes of all affected siblings with early onset ALS.

DJ1 gene is responsible for autosomal recessive early onset Parkinson's disease and also for ALSPDC phenotypes (Abou-Sleiman *et al.*, 2003, Bonifati *et al.*, 2003a, Bonifati *et al.*, 2003b, Steele, 2005) Our family shows parkinsonism and ALS with no dementia features so far, although it cannot be excluded in the later stages of disease. The novel Q45X mutation was not present in dbSNP, the 1000 genomes, EVS and our in-house database including more than 100 exome sequences. The novelty of the mutation supports the deleterious nature excluding the possibility of being a polymorphism. Only one family was reported with parkinsonism, dementia and ALS phenotype with two novel homozygous mutations in exon 7 (E163K) and in the promoter region of the *DJ1* gene (g.168_185dup) (Annesi *et al.*, 2005). To the best of our knowledge, our case is the second family in the literature with parkinsonism and ALS findings based on *DJ1* mutations.

Mutations in the *APTX* gene lead to a neurological disease ataxia-oculomotor apraxia 1. In the framework of this study two families with a mutated *APTX* gene were identified. The missense A158V mutation in the first family is in a highly conserved region of *APTX* and all tools predict the mutation as deleterious. The conserved valine replaced by glycine at position 190 (V190G), also novel, detected in the second family, was predicted as damaging, too by most prediction tools.

SETX gene is associated with a rare, juvenile onset, autosomal dominant ALS and autosomal dominant proximal spinal muscular atrophy. Its missense mutations are mostly located at either the N-terminal or the C-terminal helicase domain (HD), supporting the fact that they are both key functional domains (Bassuk *et al.*, 2007, Chance *et al.*, 1998, Chen *et al.*, 2006, Rudnik-Schoneborn *et al.*, 2012). In our family, the novel C insertion at position 1432 leads to a truncated protein at position 1434. The homozygous A1482fs mutation was not present in all variant databases including our in-house database. The mutation segregates with the disease and explains the phenotype in the family.

Defects in *MRE11* result in ATLD. Two affected siblings had early childhood onset of progressive cerebellar degeneration but no telangiectasia. The novel missense R155P mutation was found in homozygous state in both affected siblings; the

consanguineous parents were carriers, as expected. The arginine amino acid is well conserved and the substitution is predicted to be highly damaging.

Several mutations in *SACS* gene have been found to cause autosomal recessive spastic ataxia of Charlevoix-Saguenay. The 6594delT and C5254T mutations are very common in patients from in Quebec (Engert *et al.*, 2000). *SACS* mutations in three consanguineous families were defined in our study. Two independent homozygous mutations were detected in the exome analysis of a recessive ataxia family with three affected sisters. Only the exonic mutation proved to be real and the splice site mutation was shown to be false positive. The novel homozygous G2772A mutation in the *SACS* gene of all three patients explains the ataxic features, firmly excluding other causes of ataxia. The additional two homozygous novel mutations (T1212I and A1213V) in the *ACAN* gene associated with a form of spondyloepiphyseal dysplasia (SED) (Tompson *et al.*, 2009) are thought to explain the skeletal deformities of the index case and her affected sisters.

In another family with recessive ataxia, exome sequencing pointed to *SACS* as the strongest candidate gene. The novel *SACS*-R2574fsX mutation resulted in the substitution of the conserved amino acid arginine by lysine in the protein and a stop codon after three amino acids. The frameshift mutation (R2574fsX) is expected to have an adverse effect on the protein. The online prediction supports the negative effect of the mutation. We thus conclude that the mutation underlies the ataxia phenotype in the family.

Another ataxia family with consanguinity had two affected siblings, in whom a novel homozygous mutation in the *SACS* gene was identified. The N785fsX12 is a frame shift mutation, which leads to a truncated protein after 11 non-native amino acids. The mutation segregates with the disease, thus we conclude that it is associated with the disease phenotype.

Mutations in the *ATP13A2* gene are linked to Parkinson's disease, neuronal ceroidlipofuscinoses (NCLs) and also Kufor-Rakeb syndrome (Ramirez *et al.,* 2006). So far about 20 missense and nonsense mutations have been described in the *ATP13A2* gene (Yang and Xu, 2014). Defects in the *ATP13A2* gene lead to overlapping neurodegenerative disorders, which support a shared disease pathway. Exome sequencing of the consanguineous family with an affected twin couple led to the identification of a splicing site mutation, c.2747+2T>A in the gene.The mutation, possibly affecting the localization or the activity of the protein, may be the cause of the slowly progressive cerebellar and pyramidal signs in the affected twins.

The differential diagnosis in clinics is a challenge in neurodegenerative disorders due to the overlapping features and unspecified symptoms, since the pathways leading to cell death are poorly understood. Clinicians mostly need imaging, neuropathobiological, and biochemical tests to diagnose heterogeneous patients. Thus, the major assistant in complicated differential diagnosis is molecular screening(Han *et al.*, 2014). In our families mostly diagnosed with more frequent types of ataxias such as SCAs and FRDA, exome sequencing was able to distinguish between different types of ataxias via evaluating whole exome. Each genetic discovery broadens the phenotype associated with the traditional clinical definitions of neurodegenerative diseases. Mutations in the same gene result in a large spectrum of different phenotypes, for instance *DJ1* gene is frequently associated with autosomal recessive PD, however in our case, the novel mutation in the *DJ1* gene resulted in an ALS-parkinsonism complex without dementia.

Recent findings indicate that sporadic and familial cases may have a complex and even oligogenic inheritance pattern,this carries research to a new level of complexity. More genes in a combined form are required to cause the disease. In this thesis, we have seen oligogenic inheritance in three ALS families and in one ARSACS family. Several genes and/or mutations are implicated in these families and the responsible overlapping pathways are still unclear. The enigma is, how and where the distinct pathways involved in the disease cross and converge to cause the phenotype.

Neurodegenerative diseases show several inheritance patterns; dominant inheritance with complete or incomplete penetrance and recessive inheritance are most common. However, incomplete penetrance complicates the identification of genetic causes and lead to elimination of true-positives, because symptoms are not always present in individuals who have the mutation.

In neurodegenerative diseases, with the exception of Huntington disease and FRDA, approximately, 5% of disease cases are familial and the rest is sporadic in Caucasian population. Turkey is a large country with a high ethnic heterogeneity compared with Europe. This heterogeneity is despite the high numbers of the consanguineous and especially first-cousin marriages throughout Turkey between 20-60%, showing alterations between urban and rural areas respectively(Tuncbilek and Koc, 1994). This inbred population increases the recessive cases from 5% to 25%. Extended families with several living generations enable to see the intra- and inter-familial heterogeneity. In our study, 12 out of 13 families represent consanguineous mating with recessive inheritance patternsand several affected individuals in the same family.

This complexity makes neurodegenerative disease research challenging in Turkey, rendering genetic counseling crucial, but opens also many novel opportunities, which can be translated into more targeted treatments. This study illustrates an overview of the application of exome-focused NGS technologies in human neurodegenerative diseases.

6.2. Next Generation Sequencing

Next generation sequencing is an innovative development that overcomes the limitations in genomic research. NGS enables to sequence every genome, zoom into the targeted region, evaluate RNA and gene expression profiles, discover genome-wide methylation or DNA-protein interactions and analyze microbial diversity. Nowadays, NGS technology mainly focuses on whole genome and whole exome sequencing to determine the associations between variations and diseases.

Exome sequencing covers protein-coding regions, which accounts for 1-2% of the whole genome. However 85% of known disease-causing variants are located in the coding regions, which makes whole-exome sequencing a cost-effective alternative to whole-genome sequencing. The number of research articles published so far, demonstrates the power of exome sequencing approach in both Mendelian and complex diseases with significant success rates. It enables complete variant discovery and allows the analysis of phenotype-genotype correlations more accurately.WES is also efficient to do a rapid diagnosis in challenging patient cohorts (Nemeth *et al.*, 2013). Small sample sizes can be tolerated while working on Mendelian diseases, however, in complex disorders, investigation of large cohorts is crucial to detect the low-frequency variations with large effect sizes. Also, cases with recessive inheritance are more favorable to study when compared with sporadic and dominant cases. The high number of variants in sporadic and dominant samples may decrease the possibility of finding the disease-causing variant (Mitsui and Tsuji, 2014). Association studies of protein and disease are required when searching for the causative variant in hundreds of heterozygous variants.

Although next generation sequencing technology is rapidly developing, there are several technical and biological issues to be solved. It is still difficult to detect long indels, repeat regions, copy number variations, structural variations and chromosomal rearrangements with short reads generated by sequencing platforms. Exclusion of repeat expansions in ataxia cases is a big challenge. Additionally, there are some exonic regions with capturing problems, which may lead to miss some variations. Also, some pathogenic variations may be missed via WES and WGS due to low coverage and false-negatives.

WES and also WGS reveal high-quality and huge amount of data. The data interpretation and updating to new versions need high efforts with storage problems and require advanced computer platforms. There are several drawbacks at the computationalanalysis step. Variants in open-source databases do not represent the population frequencies; this may lead to eliminate causative variations, which have a false-positive allele frequency. Therefore, variations with a MAF above 0.1 could prevent missing. Furthermore, rare variants are more prone to be detected as false-negatives because of the geographical specificity. Online databases mostly contain the allele

frequency of Caucasian populations and there is no datarepresentsthe frequency of the Turkish population. Generating an in-house database, which contains the data of the Turkish population and collaboration with Tübitakwere the solution for evaluating the Turkish specific variations.

Exome sequencing methodology is still feasible for human diseases despite having several drawbacks. WES has a potential to be a routine analysis for molecular diagnosis laboratories if its processing cost and time are reduced. Despite the reduced cost of WES, there are still question marks about using WES as a routine tool in medicine. Medical services need to educate people who can understand and interpret the results of WES and transmit the information to the patients properly. For instance, several side results can be identified in addition to the variants of interest. At this point, a strong genetic counseling will be required.

Exome sequencing technology provides an opportunity to look and evaluate the human genome in an extensive manner. This pilot study in Turkish population tries to fill a big gap in understanding of several neurodegenerative disorders using next generation sequencing technologies.

CONCLUSION

APPENDIX A: PCR REAGENTS AND CONDITIONS

Table A.1. Validation PCR reagents for exon six of *APTX* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	3	5X	1X
dNTP	1.8	5 mM	600 µM
$MgCl_2$	1.5	25 mM	0.25 mM
Forward Primer	1	10 µM	0.66 µM
Reverse Primer	1	10 µM	0.66 µM
Go-Taq	0.2	5U/µl	1U

Table A.2. Validation PCR conditions for exon six of *APTX* gene.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	94	5 minutes	1
Denaturation	94	30 seconds	35
Annealing	56.9	45 seconds	
Extension	72	45 seconds	
Final extension	72	10 minutes	1

Table A.3. Validation PCR reagents for exon 26 of *ARHGEF28* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	5	5X	1X
dNTP	0.2	10 mM	0.2 mM
$MgCl_2$	2.5	25 mM	0.25 mM
Forward Primer	0.5	10 µM	0.2 µM
Reverse Primer	0.5	10 µM	0.2 µM
Go-Taq	0.2	5U/µl	1U

Table A.4. Validation PCR conditions for exon 26 of *ARHGEF28* gene.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	95	5 minutes	1
Denaturation	95	30 seconds	35
Annealing	63	30 seconds	
Extension	72	45 seconds	
Final extension	72	10 minutes	1

Table A.5. Validation PCR reagents for *ATP13A2* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	3	5X	1X
dNTP	1.8	5 mM	2.5 mM
$MgCl_2$	1.5	25 mM	0.6 mM
Forward Primer	1	10 µM	0.6 µM
Reverse Primer	1	10 µM	0.6 µM
Go-Taq	0.2	5U/µl	1U

Table A.6. Validation PCR conditions for *ATP13A2* gene.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	94	5 minutes	1
Denaturation	94	30 seconds	35
Annealing	66	30 seconds	
Extension	72	45 seconds	
Final extension	72	10 minutes	1

Table A.7. Validation PCR reagents for exon three of *DJ1*.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	3	5X	1X
dNTP	0.36	10 mM	0.2 mM
$MgCl_2$	1.5	25 mM	0.2 mM
Forward Primer	1	10 µM	0.66 µM
Reverse Primer	1	10 µM	0.66 µM
Go-Taq	0.2	5U/µl	1U

Table A.8. Validation PCR conditions for exon three of *DJ1*.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	94	5 minutes	1
Denaturation	94	30 seconds	35
Annealing	59	30 seconds	
Extension	72	45 seconds	
Final extension	72	10 minutes	1

Table A.9. Validation PCR reagents for exon six of *MRE11* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	3	5X	1X
dNTP	1.8	5 mM	0.6 mM
$MgCl_2$	1.5	25 mM	0.2 mM
Forward Primer	1	10 µM	0.66 µM
Reverse Primer	1	10 µM	0.66 µM
Go-Taq	0.2	5U/µl	1U

Table A.10. Validation PCR conditions.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	94	5 minutes	1
Denaturation	94	30 seconds	35
Annealing	59.2	30 seconds	
Extension	72	45 seconds	
Final extension	72	10 minutes	1

Table A.11. Validation PCR reagents for exon nine of *OPTN* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	5	5X	1X
dNTP	0.2	25 mM	0.2 mM
$MgCl_2$	2	25 mM	2 mM
Forward Primer	0.25	10 µM	0.1 µM
Reverse Primer	0.25	10 µM	0.1 µM
Go-Taq	0.2	5U/µl	1U

Table A.12. Validation PCR conditions for exon nine of *OPTN* gene.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	95	5 minutes	1
Denaturation	95	30 seconds	35
Annealing	56.6	30 seconds	
Extension	72	30 seconds	
Final extension	72	8 minutes	1

Table A.13. Validation PCR reagents for exon 10 and exon 11 of *OPTN* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	5	5X	1X
dNTP	0.2	25 mM	0.02 mM
$MgCl_2$	2	25 mM	0.2 mM
Forward Primer	0.4	10 µM	0.16 µM
Reverse Primer	0.4	10 µM	0.16 µM
Go-Taq	0.2	5U/µl	1U

Table A.14. Validation PCR conditions for exon 10 and 11 of *OPTN* gene.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	95	5 minutes	1
Denaturation	95	30 seconds	35
Annealing	59	30 seconds	
Extension	72	30 seconds	
Final extension	72	8 minutes	1

Table A.17. Validation PCR reagents for exon nine of *SACS* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	10	5X	1X
dNTP	1.2	25 mM	0.6 mM
$MgCl_2$	5	25 mM	2.5 mM
Forward Primer	2	10 µM	0.83 µM
Reverse Primer	2	10 µM	0.83 µM
Go-Taq	0.2	5U/µl	1U

Table A.18. Validation PCR conditions for exon nine of *SACS* gene.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	94	5 minutes	1
Denaturation	94	30 seconds	30
Annealing	56.5	30 seconds	
Extension	72	45 seconds	
Final extension	72	8 minutes	1

Table A.19. Validation PCR reagents for exon 10.1 of *SACS* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	3	5X	1X
dNTP	1.8	25 mM	0.6 mM
$MgCl_2$	1.5	25 mM	2.5 mM
Forward Primer	1	10 µM	0.8 µM
Reverse Primer	1	10 µM	0.8 µM
Go-Taq	0.2	5U/µl	1U

Table A.20. Validation PCR conditions for exon 10.1 of *SACS* gene.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	95	5 minutes	1
Denaturation	95	30 seconds	35
Annealing	56.5	30 seconds	
Extension	72	45 seconds	
Final extension	72	8 minutes	1

Table A.21. Validation PCR reagents for exon 10.14 of *SACS* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	3	5X	1X
dNTP	1.8	25 mM	0.6 mM
$MgCl_2$	1.5	25 mM	0.5 mM
Forward Primer	1	10 µM	0.6 µM
Reverse Primer	1	10 µM	0.6 µM
Go-Taq	0.2	5U/µl	1U

Table A.22. Validation PCR conditions for exon 10.14 of *SACS* gene.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	94	5 minutes	1
Denaturation	94	30 seconds	35
Annealing	65.2	30 seconds	
Extension	72	30 seconds	
Final extension	72	8 minutes	1

Table A.23. Validation PCR reagents for exon 10.16 of *SACS* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	10	5X	1X
dNTP	1.2	25 mM	0.6 mM
$MgCl_2$	5	25 mM	2.5 mM
Forward Primer	1	10 µM	0.83 µM
Reverse Primer	1	10 µM	0.83 µM
Go-Taq	0.2	5U/µl	1U

Table A.24. Validation PCR conditions for exon 10.16 of *SACS* gene.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	95	5 minutes	1
Denaturation	95	30 seconds	30
Annealing	61.7	30 seconds	
Extension	72	45 seconds	
Final extension	72	8 minutes	1

Table A.25. Validation PCR reagents for exon 10.9 of *SETX* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	3	5X	1X
dNTP	1.8	5 mM	0.6 mM
$MgCl_2$	1.5	25 mM	2.5 mM
Forward Primer	1	10 µM	0.6 µM
Reverse Primer	1	10 µM	0.6 µM
Go-Taq	0.2	5U/µl	1U

Table A.26. Validation PCR conditions for exon 10.9 of *SETX* gene.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	94	5 minutes	1
Denaturation	94	30 seconds	35
Annealing	66	30 seconds	
Extension	72	45 seconds	
Final extension	72	8 minutes	1

Table A.27. Validation PCR reagents for exon 33 and 40 of *SPG11* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	5	5X	1X
dNTP	0.2	10 mM	0.2 mM
$MgCl_2$	2.5	25 mM	0.25 mM
Forward Primer	0.5	10 µM	0.2 µM
Reverse Primer	0.5	10 µM	0.2 µM
Go-Taq	0.2	5U/µl	1U

Table A.28. Validation PCR conditions for exon 33 and 40 of *SPG11* gene.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	95	5 minutes	1
Denaturation	95	30 seconds	35
Annealing	65	30 seconds	
Extension	72	45 seconds	
Final extension	72	10 minutes	1

Table A.29. Validation PCR reagents for exon six of *SQSTM1* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	3	5X	1X
dNTP	0.36	10 mM	0.2 mM
$MgCl_2$	1.5	25 mM	0.2 mM
Forward Primer	1	10 µM	0.66 µM
Reverse Primer	1	10 µM	0.66 µM
Go-Taq	0.2	5U/µl	1U

Table A.30. Validation PCR conditions for exon six of *SQSTM1* gene.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	94	5 minutes	1
Denaturation	94	30 seconds	35
Annealing	60	30 seconds	
Extension	72	45 seconds	
Final extension	72	10 minutes	1

Table A.31. Validation PCR reagents for exon 28 of *TRPM7* gene.

Reagent	**Volume (µl)**	**[Stock]**	**[Final]**
Buffer	5	5X	1X
dNTP	0.2	10 mM	0.2 mM
$MgCl_2$	2.5	25 mM	0.25 mM
Forward Primer	0.5	10 µM	0.2 µM
Reverse Primer	0.5	10 µM	0.2 µM
Go-Taq	0.2	5U/µl	1U

Table A.32. Validation PCR conditions for exon 28 of *TRPM7* gene.

Process	**Temperature (°C)**	**Duration**	**# of cycles**
Initial denaturation	95	5 minutes	1
Denaturation	95	30 seconds	35
Annealing	62	30 seconds	
Extension	72	45 seconds	
Final extension	72	10 minutes	1

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