

PARKINSON'S DISEASE IN TURKISH PATIENTS:
MOLECULAR DEFECTS IN FAMILIAL AND ISOLATED CASES

by

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*To my beloved parents
and
to my grandparents lost before time*

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ABSTRACT

PARKINSON'S DISEASE IN TURKISH PATIENTS: MOLECULAR DEFECTS IN FAMILIAL AND ISOLATED CASES

Parkinson's disease (PD) is worldwide the second most common neurodegenerative disorder after Alzheimer's disease. Recently, several genes have been shown to result in PD phenotype. Mutations in these genes may lead to autosomal dominant (*α -synuclein*, *LRRK2*), autosomal recessive (*Parkin*, *PINK1*, *DJ1*) or sporadic PD. In the framework of this study, the above five genes were investigated in Turkish patients. For the *Parkin* gene, we have performed dHPLC analysis prior to sequencing. The high percentage of abnormal dHPLC profiles observed in the patient population revealed only one mutation and 136 polymorphisms. Exon rearrangements were investigated by semi-quantitative multiplex PCR and by MLPA; 12 rearrangements were described in the *Parkin* gene. *PINK1* and *DJ1* exons were also sequenced; 75 variations were identified, two being heterozygous mutations. Point mutations in *α -synuclein* are a rare cause of PD and were not found in our patient population. In contrast, triplications and duplications of the whole gene are found more frequently in autosomal dominant forms of the disease. A heterozygous duplication of exons 3 and 4 was described in several patients of a large kindred, and the whole family was subjected to haplotype analysis for chromosome 4q. *LRRK2* consists of 51 exons and most mutations are seen in 15 of these. Sequencing of these exons revealed a novel S1508R and a G2019S mutation. G2019S frequency differs among populations and it is associated with three different haplotypes so far. The haplotype of our patient has been shown to have a great homology with the Japanese haplotype. Although an independent origin of the Turkish haplotype cannot be excluded at this point, the huge and centuries-long migration of the Turkic people in Central Asia rather supports a common origin.

ÖZET

TÜRK HASTALARDA PARKINSON HASTALIĞI: AİLESEL VE İZOLE VAKALARDA MOLEKÜLER BOZUKLUKLAR

Parkinson hastalığı (PH) dünyada Alzheimer hastalığından sonra en sıklıkla görülen nörodejeneratif hastalıktır. Bugün, PH'ya neden olduğu bilinen bir dizi gen tanımlanmıştır. Bu genlerdeki mutasyonlar otozomal dominant (α -sinüklein, *LRRK2*), otozomal resesif (*Parkin*, *PINK1*, *DJ1*) ya da sporadik PH'ya yol açarlar. Bu çalışma çerçevesinde yukarıdaki beş gen, Türk Parkinson hastalarında incelendi. *Parkin* geni için dizi analizi öncesi dHPLC uygulandı. dHPLC'de görülen yüksek sayıda anormal profiller, sadece bir mutasyon ve 136 polimorfizm tanımlanması ile sonuçlandı. Ekson düzen değişimleri semikantitatif multipleks PCR ve MLPA ile incelendi, ve *Parkin* geninde 12 farklılık bulundu. *PINK1* ve *DJ1* genlerinin eksonları dizi analizine tabi tutuldu, ve ikisi heterozigot mutasyon olmak üzere, toplam 75 farklılık tanımlandı. *Alfa-sinüklein* genindeki nokta mutasyonları nadirdir, bu tez çerçevesinde incelenen hasta popülasyonunda nokta mutasyonlarına rastlanmadı. Buna karşılık tüm genin triplikasyon ve düplikasyonları otozomal dominant geçişli hastalıkta daha sıktır. Ekson 3 ve 4'ün heterozigot düplikasyonu geniş bir ailenin hasta bireylerinde bulundu. Bunun üzerine tüm aile bireyleri kromozom 4 haplotip analizi ile incelendi. *LRRK2* geni 51 ekson'dan oluşur, mutasyonların çoğu bu eksonların 15'inde yoğunlaşmıştır. Bunların doğrudan dizi analizi, incelenen hasta popülasyonunda, hem literatürde ilk defa tanımlanan bir mutasyon bulmamızı sağladı (S1508R), hem de sık görülen G2019S mutasyonunu gösterdi. G2019S'nin sıklığı toplumdan topluma değişir ve bugüne kadar üç değişik haplotipte görülmüştür. Hastamızın haplotipi incelendiğinde, Japon haplotipiyle büyük benzerlik bulundu. Her ne kadar bu aşamada Türk haplotipinin bağımsız gelişmiş olma olasılığını göz ardı edemesek de, Türk toplumlarının Orta Asya'da yüzyıllar süren geçmişleri ve göçleri, Türk ve Japon haplotiplerinin aynı kökenden gelme olasılığını güçlendirmektedir.

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LIST OF ABBREVIATIONS

4q	Long arm of chromosome 4
A	Adenine
APS	Ammonium Persulfate
ATP	Adenosine Triphosphate
bp	Base pair
C	Cytosine
COR	C-terminal of ROC
dH ₂ O	Distilled water
dHPLC	Denaturing High-Performance Liquid Chromatography
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ds	Double stranded
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders-revision IV
DUB	Deubiquitinating enzyme
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin protein ligase
EDTA	Ethylenediaminetetraacetate
EtBr	Ethidium Bromide
EtOH	Ethanol
ExoI	Exonuclease I
Fam	Carboxyfluorescein
G	Guanine
GABA	Gamma-Amino Butyric Acid
GBA	Glucocerebrosidase
GIGYF2	Grb10-Interacting GYF Protein-2
GP _e	Globus Pallidus Externa
GP _i	Globus Pallidus Interna

H ₂ O ₂	Hydrogen peroxide
HCN	Hyperpolarization-activated and cyclic nucleotide gated cation channels
Hsp75	Heat Shock Protein 75
HTRA2	High Temperature Requirement protein A2
IBR	In Between RING
IVS	Intervening Sequence Variation
kb	Kilo base
kDa	Kilo Dalton
L-Dopa	Levodopa
LRRK2	Leucine-Rich Repeat Kinase 2
MAOB	Monoamine oxidase B
MAPKKK	Mitogen-Activated Protein Kinase Kinase Kinase
Mb	Mega base
MgCl ₂	Magnesium chloride
min	Minute
MLPA	Multiplex Ligation-Dependent Probe Amplification
MPP ⁺	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine
mRNA	Messenger RNA
NAC	Non-amyloid component
NaCl	Sodium chloride
NURR1	Nuclear Receptor Related protein 1
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PET	Positron Emission Tomography
PINK1	Phosphatase and Tensin Homologue-Induced Kinase 1
RE	Restriction Enzyme
RING	Really Interesting New Gene
RNA	Ribonucleic acid
ROC	Ras in complex protein

ROS	Reactive Oxygen Species
rpm	Revolutions per minute
rs	Reference sequence
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium Dodecyl Sulfate
sec	Second
SMMT	Standardized Mini-Mental Test
SNc	Substantia Nigra Pars Compacta
SNr	Substantia Nigra Pars Reticulata
SNP	Single Nucleotide Polymorphism
ss	SNP assay
STN	Subthalamic nucleus
T	Thymine
Taq	Thermus aquaticus
TBE	Tris-Boric acid-EDTA
TE	Tris-EDTA
TEMED	N',N',N',N'-Tetramethylethylenediamine
TRAP1	Tumor necrosis factor Receptor-Associated Protein 1
Ubl	Ubiquitin-like domain
UCHL1	Ubiquitin Carboxy-terminal Hydrolase L1
UPDRS	Unified Parkinson's Disease Rating Scale
UPS	Ubiquitin-Proteasome System
UTR	Untranslated Region
UV	Ultra Violet

1. INTRODUCTION

1.1. Clinical Features and Epidemiology of Parkinson's Disease (PD)

First described by James Parkinson in his seminal essay documenting six cases of “shaking palsy”, Parkinson’s disease (PD) was named by Jean Martin Charcot in 1862 and its clinical description was refined (Figure 1.1). Today, after Alzheimer’s disease, PD is the second most common neurodegenerative disorder in the Western world (OMIM #168600). The prevalence of the condition is age-dependent: PD affects 1-2 per cent of the population above the age of 60, but this rate is increased up to 4-5 per cent in 85-year-olds (Fahn, 2003).

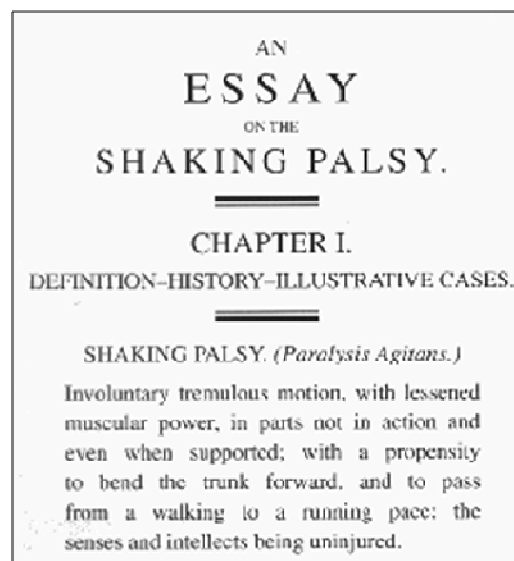


Figure 1.1. Cover page of Parkinson's classical essay on shaking palsy (1817)

The classical triad of signs of PD includes bradykinesia, rigidity and resting tremor. Bradykinesia refers to the generalized slowness of the movement; it is interchangeably used with akinesia (absence of movement) or hypokinesia (poverty of movement). Rigidity is defined as “an unvarying increased resistance within the range of passive movement about a joint”. Resting tremor can be cited as the most obvious manifestation of PD; it is

“an involuntary rhythmic oscillation of a body region that is produced by alternating contractions of reciprocally innervated muscles”. A fourth feature, postural instability, is often mentioned, although it tends to occur in the later stages of the disease (Ebadi and Pfeiffer, 2005). PD is also associated with a good response to levodopa (L-Dopa) treatment.

1.2. Anatomy

PD is characterized by a selective degeneration of the dopaminergic nigro-striatal pathway. Typically in PD, dopaminergic neuronal cell loss occurs in the *substantia nigra*, the changes being concentrated in the central part of the *zona compacta* (SNc). It has been estimated that often, diagnosis is made, when dopamine levels in the striatum are already reduced to 60-70 per cent of the normal level (Schapira, 2006) (Figure 1.2).

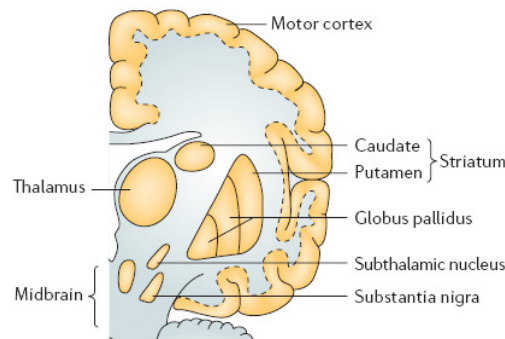


Figure 1.2. Brain regions involved in PD (Farrer, 2006)

In physiological conditions, the substantia nigra forms a part of the movement initiation chain. There are two different pathways from the sensory cortex to the premotor area (Figure 1.3); the direct and indirect pathways.

1.2.1. The Direct Pathway

It involves stimulation of striatal neurons, which results in disinhibition of the thalamus via the globus pallidus interna (GPI) and the substantia nigra pars reticulata

(SNr). The resulting activation of thalamocortical neurons is thought to facilitate movement by exciting the premotor area and supplementary areas thus stimulating the motor cortex, the brain stem and the spinal cord.

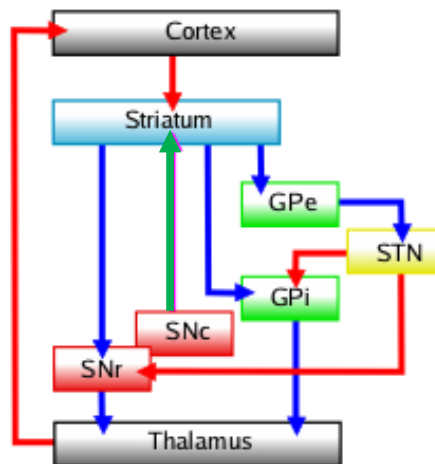


Figure 1.3. Major pathways of the basal ganglia (excitatory -glutamatergic- and inhibitory -GABAergic- pathways are indicated, respectively, by red and blue arrows; the dopaminergic pathway is shown in green) (Gillies, 2005)

1.2.2. The Indirect Pathway

Corticostriatal excitation results in stimulation of the subthalamic nuclei via the external segment of the globus pallidus (GPe) and the subthalamic nucleus (STN), which in turn inhibits the thalamus via GPi and SNr. The consequence of thalamic inhibition is decreased excitation of the premotor and supplementary areas.

Moreover, the dopaminergic neurons of the substantia nigra have a dual effect on the striatal neurons; they excite the direct pathway while they inhibit the indirect pathway, thus dopamine is a key modulator of both pathways. In PD, this effect is reversed, therefore leading to a pathological condition.

1.3. Neuropathology

The dramatic loss of neuromelanin containing dopaminergic neurons is accompanied by the presence of Lewy bodies in the remaining neurons. These are fibrillar cytoplasmic inclusions consisting of aggregates of abnormally accumulated proteins, the most prominent being α -synuclein, neurofilaments, ubiquitin and ubiquitinated proteins (McNaught and Olanow, 2006) (Figure 1.4).

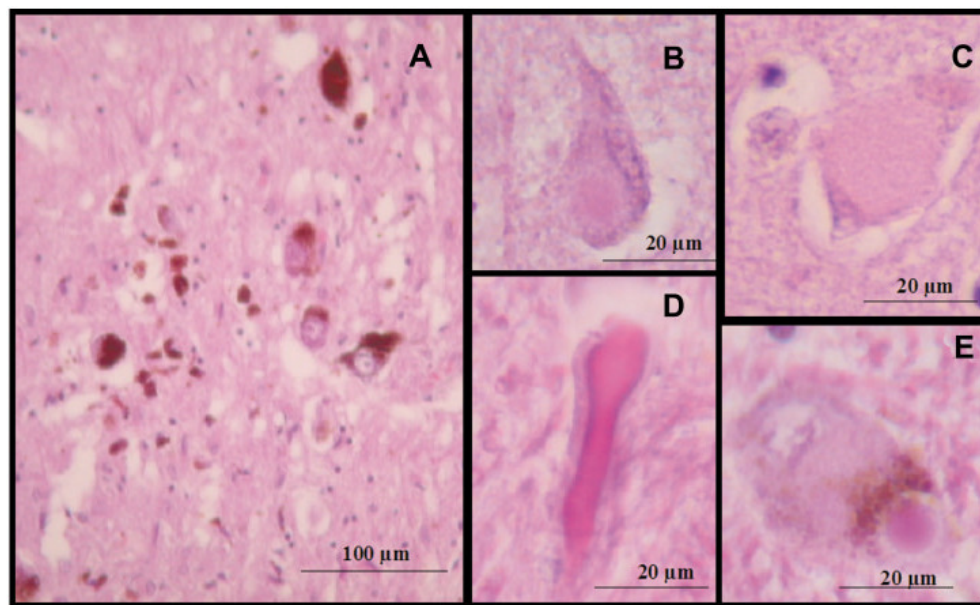


Figure 1.4. Pathological findings of the patient in whom the α -synuclein E46K mutation has been discovered. Hematoxylin and eosin have been used to stain different brain regions A. severe neuronal loss and secondary spongiosis in the substantia nigra; B-C. two Lewy bodies; one concentric and one non-concentric in the cingular cortex; D. an atypical elongated rod-like Lewy body in the pontine tegmentum; E. classic Lewy body in the locus coeruleus (Zarranz *et al.*, 2004)

Originally, the disease was considered exclusively sporadic and attributed to shared exposure to a common environment, despite 10 to 30 per cent of PD sufferers reporting a first-degree relative with Parkinsonism (Farrer, 2006). Recent epidemiological studies have revealed that less than 10 per cent of PD cases are of familial origin, with the majority of PD cases being sporadic (Thomas and Beal, 2007).

1.4. Some of the Hypothetical Mechanisms Leading to PD

1.4.1. The Ubiquitin-Proteasome Pathway

The ubiquitin-proteasome system is essential for the non-lysosomal degradation of proteins that are damaged, misfolded or mutant. This pathway is believed to be implicated in the pathogenesis of many different neurodegenerative diseases such as Parkinson's, Huntington's and Alzheimer's diseases. In all of these conditions, ubiquitinated protein aggregates are found in the brain regions where neurodegeneration occurs. In the case of PD, these ubiquitinated aggregates are Lewy bodies that are dispersed in the remaining dopaminergic neurons of the substantia nigra. However, the nature of these aggregates is not very clear: are they toxic, thus causing the disease? Or are they a way for neurons to detoxify their environment, becoming then neuroprotective? It is already known that often in Parkin-related cases, Lewy bodies are absent; as Parkin is an E3 ubiquitin-ligase, its dysfunction may lead to an inability to form these proteinaceous inclusions, explaining the earlier age of onset of Parkin-related PD cases compared with α -synuclein patients, in whom Lewy bodies are frequently observed. Therefore, we may think, that inclusions are protective and just a consequence of the presence of mutated proteins that will augment the load on the ubiquitin-proteasome pathway, indirectly causing apoptosis of the dopaminergic neurons (Ross and Pickart, 2004) (Figure 1.5).

1.4.2. Mitochondrial Dysfunction

The observation that exposures to 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), a herbicide, results in Parkinsonism, has been a convincing evidence of an environmental involvement in the etiology of PD. MPTP is metabolized to 1-methyl-4-phenylpyridinium (MPP^+) by the enzyme monoamine oxidase B (MAOB) in astrocytes, and is selectively imported in the dopaminergic neurons via the dopamine transporter. In neurons, MPP^+ inhibits the complex I of the mitochondrial electron transport chain, resulting in ATP depletion accompanied by oxidative stress, leading finally to neuronal death.

Other environmental toxins, such as paraquat and rotenone, have been proposed to induce PD. In particular, rotenone, which is also a mitochondrial respiratory chain complex I inhibitor, is used to obtain reliable animal models of PD. Rats develop a PD-like syndrome with a selective degeneration of the nigro-striatal pathway and inclusion bodies containing α -synuclein, reminiscent of Lewy bodies (Abou-Sleiman *et al.*, 2006a). Complex I deficiency has been estimated to be 30-40 per cent in the substantia nigra of PD patients (Shapira *et al.*, 1990).

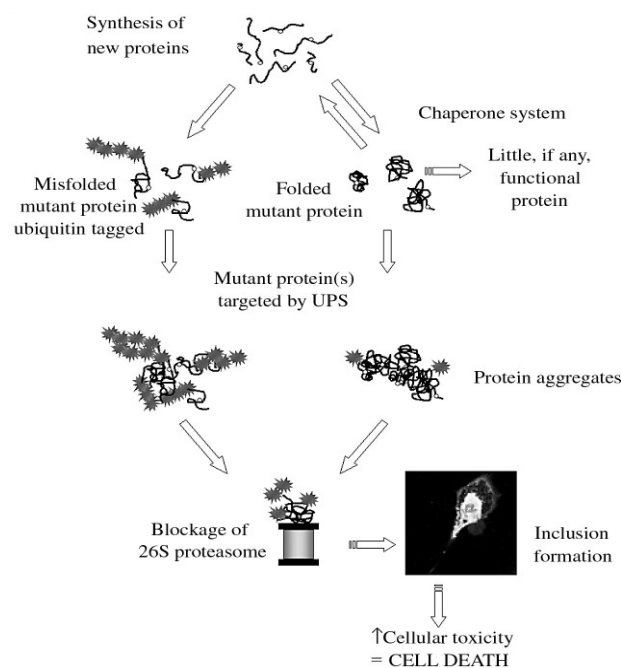


Figure 1.5. Formation of inclusion bodies (Ardley and Robinson, 2004) (UPS: Ubiquitin-Proteasome System)

1.4.3. Oxidative Stress

The neuronal metabolism of dopamine is responsible for the selective vulnerability of dopaminergic neurons to oxidative stress; the auto-oxidation of dopamine itself and its catabolism by MAOB is known to generate free radicals.

Besides, epidemiological studies prove that the greatest risk factor for the development of sporadic PD is ageing; and oxidative stress is the main cellular pathway

leading to ageing. Oxidative stress unifies many pathological pathways leading to PD (Figure 1.6):

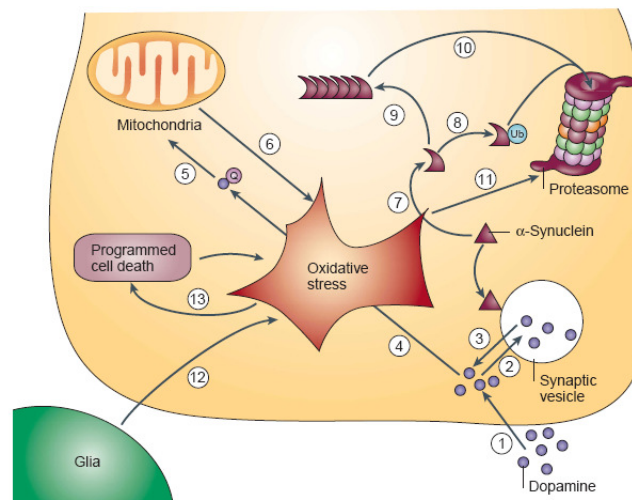


Figure 1.6. Oxidative stress pathways in a dopaminergic neuron (Andersen 2004)

Dopamine (purple circles) is uptaken into the dopaminergic neuron (1) then to the synaptic vesicle (2) and is released by α -synuclein (red triangle) (3). Dopamine is oxidized to dopamine quinone (4), which subsequently produces potential mitochondrial inhibitors (5), resulting in mitochondrial production of oxidative stress (6). Alpha-synuclein is oxidized (7), ubiquitinated, and finally degraded by the proteasome (8). Alpha-synuclein may also form oligomers (9), which will interact with the proteasome (10). Other toxic oxidative by-products will interact with the proteasome (11). Moreover inflammation in glial cells will also increase the oxidative stress burden (12) leading to the induction of programmed cell death (13).

1.4.4. PD Etiology is Complex

The complex I deficiency enhances free radical generation, which impairs the function of the mitochondrial respiratory chain. Mitochondrial dysfunction and the increased substrate load of oxidized protein decrease the activity of the ubiquitin-proteasome system. Abnormalities in protein phosphorylation might have an effect on the

ubiquitin-proteasome system and on the mitochondrial activity. Inflammation is known to increase free-radical production, which enhanced by excitotoxicity, will damage the mitochondria (Figure 1.7).

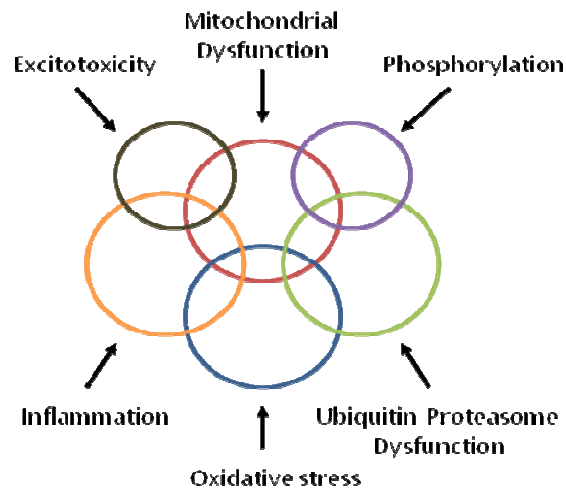


Figure 1.7. Factors that interact in the pathogenesis of PD (adapted from Schapira, 2008)

1.5. Monogenic Forms of PD

Since 1997, seven genes have been implicated in the disease pathogenesis in Mendelian forms of PD; the mutation spectrum underlines the importance of the mutated proteins and the pathways in which they are implicated. These provide new clues in understanding PD pathogenesis.

To date three genes are known to cause autosomal dominant PD: *α-synuclein*, *Leucine-Rich Repeat Kinase 2 (LRRK2)*, and *Grb10-Interacting GYF Protein-2 (GIGYF2)*. In addition four genes cause autosomal recessive PD: *Parkin*, *Phosphatase and Tensin Homologue (PTEN)-Induced Kinase 1 (PINK1)*, *DJ1* and *ATP13A2* (Table 1.1).

Table 1.1. Genes implicated in PD

Locus	Map position	Gene	Age of Onset	Inheritance
PARK1/4	4q21	<i>α-synuclein</i>	variable	AD
PARK2	6q25-q27	<i>Parkin</i>	<40	AR
PARK6	1p35-p37	<i>PINK1</i>	<40	AR
PARK7	1p36	<i>DJ1</i>	30-40	AR
PARK8	12p11-q13	<i>LRRK2</i>	variable	AD
PARK9	1p36	<i>ATP13A2</i>	<20	AR
PARK11*	2q36	<i>GIGYF2</i>	late	AD

*This gene has been proposed as being causative of PD; further confirmation is needed

(AD: autosomal dominant; AR: autosomal recessive)

1.5.1. Genes Responsible for Dominantly Inherited PD

1.5.1.1. Alpha-synuclein. In 1997, the identification of *α -synuclein* as the first gene implicated in PD resulted from linkage analysis in the “Contursi” kindred, with the point mutation A53T being associated with the disease in this family (Polymeropoulos *et al.*, 1997). Since then, two other point mutations, A30P (Kruger *et al.*, 1998) and E46K (Zarranz *et al.*, 2004), as well as duplications and triplications of the *α -synuclein* gene, have been identified among PD patients (Singleton *et al.*, 2003; Farrer *et al.*, 2004).

The point mutations and gene rearrangements result in rare autosomal dominant, middle to late onset PD. *Alpha-synuclein* point mutations produce an early onset, severe PD phenotype with cognitive impairment. Additionally, duplications of the whole gene result in typical PD, whereas triplications cause an earlier onset and a more aggressive disease, with features ranging from typical PD to dementia with Lewy bodies (Ibanez *et al.*, 2004 and 2009; Fuchs *et al.*, 2007).

The loss of dopaminergic neurons and the presence of Lewy bodies, especially in the substantia nigra, but also in the locus coeruleus, have been observed in *α -synuclein*-linked PD (Polymeropoulos *et al.*, 1997) (Figure 1.8). Alpha-synuclein is a major component of

Lewy bodies; with abnormal deposition being observed in other neurodegenerative disorders with Parkinsonian features (Spillantini *et al.*, 1997).

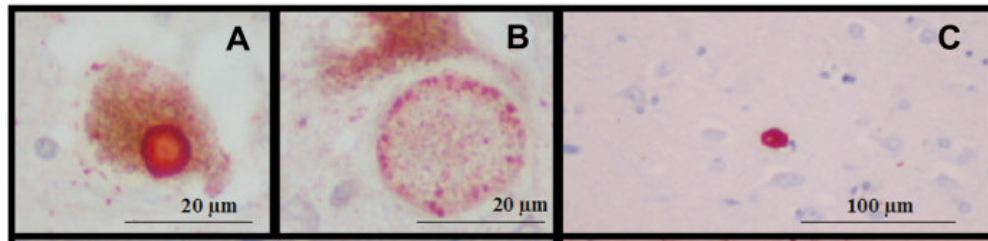


Figure 1.8. Immunocytochemical findings in the index case in whom the E46K mutation has been discovered A. a typical Lewy body inclusion in the substantia nigra; B. an axonal spheroid in the substantia nigra; C. some isolated Lewy bodies in the frontal neocortex (Zarranz *et al.*, 2004)

The α -synuclein gene has 6 exons, encoding an abundant 140 amino-acid cytosolic protein, which is found at presynaptic terminals and thought to be involved in synaptic function, modulating the dopamine neurotransmission (Abeliovich *et al.*, 2000).

The α -synuclein protein is organized in three distinct domains: an N-terminal amphipathic region with conserved repeats (KTKEGV), a central hydrophobic NAC domain (non-amyloid component) and an acidic C-terminal region. The three point mutations reported, are located in the amphipathic region and are believed to exacerbate the toxic protofibril and fibril formation. Indeed, monomeric mutant α -synuclein has the propensity to form stable β -pleated sheets; the ensuing fibrillogenesis (formation of oligomers, protofibrils and finally fibrils) resulting in the generation of pathological inclusions known as Lewy bodies (Conway *et al.*, 2000; Goldberg and Lansbury, 2000) (Figure 1.9). Moreover, dopamine has been shown to modulate α -synuclein aggregation, explaining the selective vulnerability of the substantia nigra in PD (Conway *et al.*, 2001). Studies in drosophila indicate that Lewy bodies may be a way of detoxifying the cell from damaged α -synuclein (Chen and Feany, 2005).

The enhanced toxicity of mutant α -synuclein may also be explained by the assembly of oligomeric α -synuclein into annular structures (Lashuel *et al.*, 2002), integrating cellular

membranes and forming pores, thereby affecting membranes permeability (Kostka *et al.*, 2008).

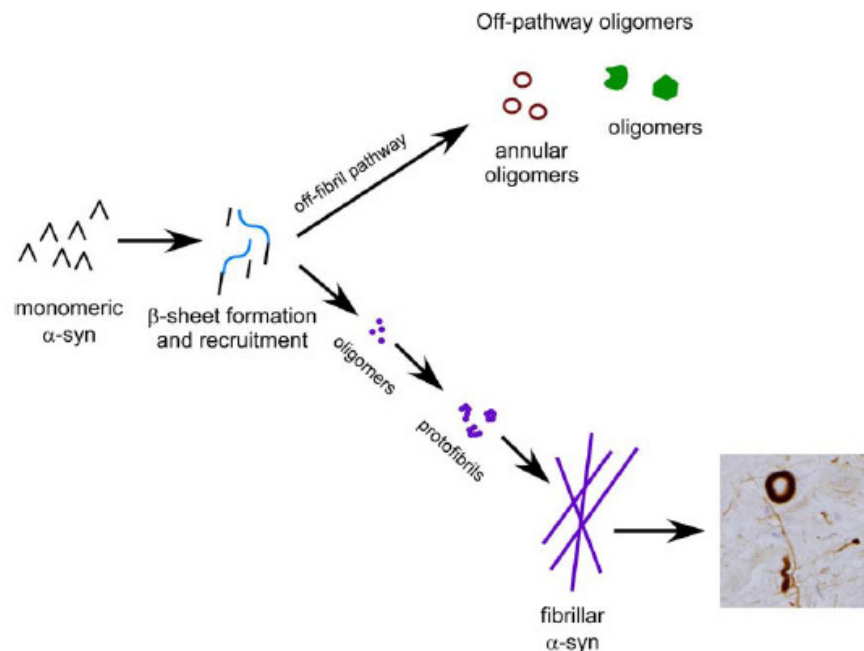


Figure 1.9. Formation of α -synuclein oligomers and fibrils (Waxman and Giasson, 2008)

Of note, the overexpression of the wild-type protein is sufficient to cause PD, as duplications or triplications of the wild-type α -synuclein produce the disease in patients. Furthermore, the overexpression of human α -synuclein in rats is enough to produce degeneration of the nigrostriatal system (Kirik *et al.*, 2002).

The pathological mechanism induced by mutant α -synuclein, either point mutations or gene rearrangements, point toward a dominant gain of function mechanism, compatible with the dominant inheritance pattern.

1.5.1.2. Leucine-Rich Repeat Kinase 2 (LRRK2). Today, the *LRRK2* gene is recognized as the most common cause of familial autosomal dominant, as well as sporadic forms of PD. *LRRK2* is a large gene consisting of 51 exons encoding a 2,527 amino-acid protein, named dardarin from the basque word *dardara*, meaning tremor. Sequence homology has assigned dardarin to the Ras / GTPase superfamily; dardarin has multiple domains

including an N-terminal leucine-rich repeat, a GTPase ROC (Ras of complex proteins) / COR (C-terminal of Roc) domain, a mitogen-activated protein kinase kinase kinase (MAPKKK) and C-terminal β -propeller (WD40 repeats) (Paisan-Ruiz *et al.*, 2004; Zimprich *et al.*, 2004) (Figure 1.10).

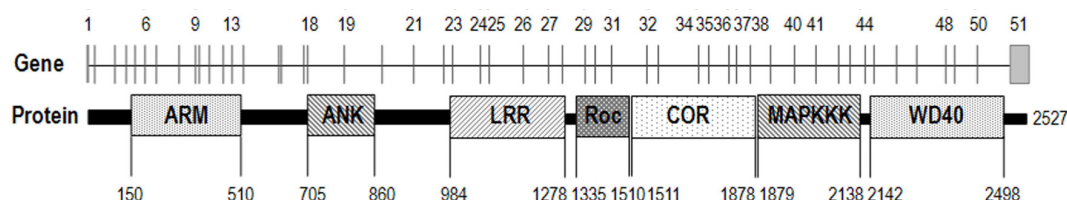


Figure 1.10. Schematic representation of LRRK2 with its several conserved domains:

ARM (Armadillo), ANK (Ankyrin repeat), LRR (leucine rich repeat), Roc (Ras of complex proteins: GTPase), COR (C-terminal of Roc), MAPKKK (mitogen-activated kinase kinase kinase) and WD-40 domain (Lesage *et al.*, 2008)

Out of at least 40 different *LRRK2* sequence variants in the *LRRK2* gene, described so far, only seven are proven to be pathogenic since they co-segregate with PD in the families they were reported in (Table 1.2) (Mata *et al.*, 2006; Lesage *et al.*, 2008).

LRRK2-associated PD is generally late-onset, with patients displaying the cardinal signs of the disease and having a good response to L-Dopa. Neuropathological findings vary significantly from α -synuclein-positive Lewy bodies in the brainstem, to Lewy bodies in the brainstem and cerebral cortex, to tau pathology, or no aggregation at all (Wszolek *et al.*, 2004).

The G2019S substitution (c.6055 G>A: Gly2019Ser) in exon 41 shows age-dependent, reduced penetrance (Healy *et al.*, 2008; Hulihan *et al.*, 2008). The G2019S mutation is present in several populations with a highly variable frequency according to the geographic / ethnic origin (Nichols *et al.*, 2007). Although only found in 0.1 per cent of PD cases in Asia (Tomiya *et al.*, 2006), this mutation is much more frequent in Europeans with 2-6 per cent of familial cases and 1-2 per cent of sporadic cases (Brice, 2005). Interestingly, studies involving North African Berber Arabs and Ashkenazi Jews revealed

that this mutation is much more common in those populations, representing 20-40 per cent of familial and sporadic cases (Lesage *et al.*, 2006; Ozelius *et al.*, 2006, Lesage *et al.*, 2008).

Table 1.2. Potentially pathogenic vs. proven pathogenic amino-acid substitutions found in the *LRRK2* gene (Lesage *et al.*, 2008)

LRRK2 exon	Potentially pathogenic mutations	Recurrent proven pathogenic mutations
Exon 9	E334K	
Exon 24	Q1111H	
Exon 25	I1122V	
Exon 26	I1192V	
Exon 27	S1228T	
Exon 29		I1371V
		R1441H
Exon 31	A1442P	R1441C
		R1441G *
Exon 35		Y1699C
Exon 37	L1795F	
Exon 41		I2020T **
		G2019S
Exon 48	T2356I	

* frequent in Basques (Simon-Sanchez *et al.*, 2006); ** frequent in Japanese (Tomiya *et al.*, 2006)

Three haplotypes associated with the G2019S mutation have been described in different populations. Haplotype 1 is the most frequent, predominant in European Americans, North African Arabs and Ashkenazi Jews, resulting from a common founder (Kachergus *et al.*, 2005), initially dated 725 years back by Lesage and colleagues (2005) and reevaluated as 2,250 years old by Zabetian and colleagues (2006a). Haplotype 2, being

rarer, is shared by few cases among Western Europeans, and haplotype 3 originally being found in the Japanese population (Zabetian *et al.*, 2006b).

Regarding the pathological outcomes of *LRRK2* mutations, the two *LRRK2* mutants lying in the kinase domain (G2019S and I2020T) and one mutation on the ROC / COR GTPase domain (R1441C) have been reported to result in an increased autophosphorylation activity (West *et al.*, 2005; Gloeckner *et al.*, 2006). It has been further demonstrated that an increased kinase activity of the mutant *LRRK2* may mediate neuronal toxicity in mouse primary cortical neurons (Smith *et al.*, 2006), due to mitochondria-dependent apoptosis (Iaccarino *et al.*, 2007). The GTPase Roc domain has been proposed to regulate the kinase activity. Although no substrates of dardarin are known, it has been shown to interact with Parkin (Smith *et al.*, 2006). The precise function of dardarin awaits future research.

1.5.1.3. Grb10-Interacting GYF Protein-2 (*GIGYF2*). *Grb10-Interacting GYF Protein-2 (*GIGYF2*)* was very recently identified as a candidate gene for the PARK11 locus on chromosome 2q36-37 (Lautier *et al.*, 2008). In this study, all coding exons of the shortest isoform of the *GIGYF2* gene were sequenced in Italian and French PD patients and origin-matched controls. Ten different missense mutations were identified in the *GIGYF2* gene in index PD patients, and no mutations were reported in controls (Table 1.3).

The segregation of the disease with *GIGYF2* mutations was confirmed in family members available. The *GIGYF2* mutations matched highly conserved regions, and they were found in heterozygous state, indicating an autosomal dominant inheritance pattern. Moreover, one patient, with earlier onset and severe clinical course, was found to carry the *GIGYF2* mutation Ile278Val and the previously described *LRRK2* Ile1371Val mutation simultaneously. *GIGYF2* was primarily identified through its binding to the Grb10 adapter protein, thought to function by linking proteins to tyrosine kinases; thus underlying a possible interaction with *LRRK2*.

Table 1.3. *GIGYF2* mutations identified in Italian-French PD patients (Lautier *et al.*, 2008)

GIGYF2 exon	Mutation
Exon 2	c.167 A>G: Asn56Ser ¹
Exon 4	c.334 A>G: Thr112Ala
Exon 8	c.832 A>G: Ile278Val
Exon 9	c.1003 T>A: Ser335Thr
Exon 11	c.1370 A>C: Asn457Thr ²
Exon 14	c.1818 C>G: Asp606Glu
Exon 25	c.3625_3648del124: Del LPQQQQQQ 1209-1216 ³
Exon 25	c.3648_3649insCAACAG: Ins QQ 1217
Exon 25	c.3661_3672del12: Del PPQQ 1221-1224
Exon 26	c.3724 G>A: Val1242Ile

¹ found in 4 unrelated PD patients ² found in 3 unrelated PD patients ³ found in 2 unrelated PD patients

(Variants considered as pathogenic in the publication are shown in bold)

However, the causative link between *GIGYF2* and PD failed to replicate in another study, involving Portuguese and North American cohorts (Bras *et al.*, 2009). Indeed, the Asn457Thr and His1171Arg mutations were also found in controls, suggesting a minor role for *GIGYF2* in PD development.

Grb and *GIGYF* proteins are thought to be involved in insulin-like growth factor and insulin signaling; functional studies are needed to determine whether this protein may have a role in PD pathogenesis.

1.5.2. Genes Responsible for Recessively Inherited PD

1.5.2.1 Parkin. A year after the discovery of the *α -synuclein* gene, *Parkin* has been associated with PD (Kitada *et al.*, 1998). In contrast to *α -synuclein*, mutations in the *Parkin* gene turned out to be recurrent, accounting for nearly half of autosomal recessive juvenile Parkinsonism and also ~20 per cent of isolated early-onset PD cases (Lücking *et al.*, 2000). *Parkin* is one of the largest genes in the human genome, mapping 1.38 Mb and comprising

12 exons (Kitada *et al.*, 1998). The *Parkin* gene encodes a ubiquitous 465 amino-acid protein divided into three parts: an N-terminal ubiquitin like domain (Ubl), a C-terminal RING (Really Interesting New Gene) box, and a PDZ linker region. The RING box can be further divided into three domains: RING1, RING2, and IBR (In Between Ring) (Shimura *et al.*, 2000).

Various point mutations and numerous combinations of exon deletions and multiplications have been found on the *Parkin* gene (Hedrich *et al.*, 2004b). Clinically, *Parkin*-related PD cases have a slow progression of disease, presence of dystonia at onset accompanying the triad of signs and a very good response to L-Dopa, despite early and severe development of L-Dopa-induced motor fluctuations and dyskinesias (Lohmann *et al.*, 2003).

Parkin is most likely a cytosolic protein, although it may localize to the mitochondrial outer membrane (Darios *et al.*, 2003), or to the endoplasmic reticulum (Takahashi *et al.*, 2003) under stress conditions. Parkin is an E3 ubiquitin-ligase which forms a complex with an E2 protein (ubiquitin-conjugating enzyme), via its RING-IBR-RING domain, for the ubiquitination of cellular proteins, thus directing them to the 26S proteasome for degradation (Shimura *et al.*, 2000) (Figure 1.11).

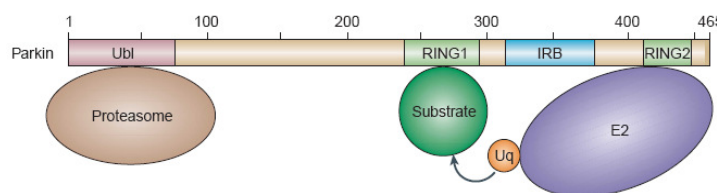


Figure 1.11. Schematic representation of Parkin interactions: the Ubl domain binds to the proteasome, substrate binding may be mediated by the RING1 domain and E2 enzyme binding by the IBR and RING2 domains (Bossy-Wetzel *et al.*, 2004)

Overexpression of Parkin has been suggested to protect against mutant α -synuclein-induced toxicity, through Lewy body formation (Lo Bianco *et al.*, 2004). In the vast majority of PD cases, point mutations or exon rearrangements affecting *Parkin*, lead to an

inability to form aggregates; thus explaining the absence of Lewy bodies (Mori *et al.*, 1998). It seems that mutations in the *Parkin* gene prevent interactions of the protein with E2 enzymes or their substrates; hence its E3 ligase activity is reduced or abolished, resulting in an abnormal accumulation of non-ubiquitinated intracellular proteins, primary to the loss of dopaminergic neurons. Parkin is therefore neuroprotective, through its role in the ubiquitin-proteasome system (Figure 1.12).

Although not fully explored, Parkin has also a proteasome-independent function and many Parkin substrates have been proposed: CDCREL1, PAELR, and aminoacyl-tRNA synthetase cofactor p38 (Corti *et al.*, 2003; von Coelln *et al.*, 2004; Ko *et al.*, 2005). In addition to the classical polyubiquitination known to be achieved through the lysine 48 (K48) residue, evidence for proteasome-independent K63-linked monoubiquitination has been recently presented (Lim *et al.*, 2005; Hampe *et al.*, 2006) (Figure 1.12).

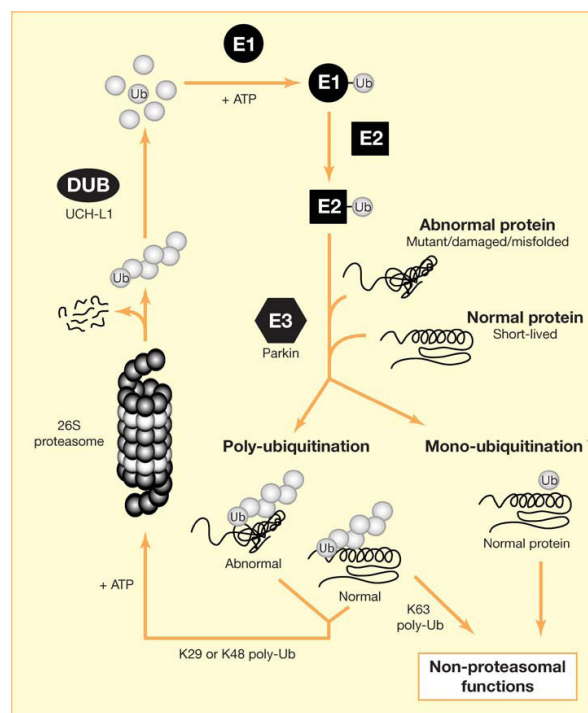


Figure 1.12. The ubiquitin-proteasome system (E1: ubiquitin-activating enzyme; E2: ubiquitin-conjugating enzyme; E3: ubiquitin protein ligase; DUB: deubiquitinating enzyme) (Moore *et al.*, 2005)

Analyses of *Parkin* knock-out mice have revealed no dopaminergic degeneration and no accumulation of putative substrates, although adeno-virus driven p38 overexpression in the substantia nigra leads to loss of dopaminergic neurons in mice (Ko *et al.*, 2005). *Parkin* knock-out drosophila display mitochondrial dysfunction and increased oxidative stress. The drosophila studies have stressed the importance of the mitochondrial dysfunction in *Parkin*-related PD (Greene *et al.*, 2005), confirming that proteasome dysfunction may not be the unique cause of the disease (see details in *PINK1* section). Indeed the identification of mutations in *DJI*, a possible redox sensor, in 2002, has brought additional proofs for the involvement of oxidative stress in the pathogenesis of PD. This hypothesis was further asserted by identification of mutations in a mitochondrial kinase, *PINK1* in 2004.

1.5.2.2. DJ1. Homozygous mutations in the *DJI* gene were first described in two European families: a large deletion of 14kb, comprising exons 1 to 5, in a Dutch family; and a missense mutation (L166P) in an Italian family (Bonifati *et al.*, 2002; Bonifati *et al.*, 2003). Further missense, splice-site and exonic deletion mutations have been identified (Table 1.4) but these studies have shown that *DJI* rarely associates with PD (<1 per cent of early-onset cases). Phenotypically, *DJI* patients are indistinguishable from *Parkin* and *PINK1*-linked PD cases (Klein *et al.*, 2007a).

DJI has 8 exons, the first two being non-coding and alternatively spliced, exons 2-7 encode for a 189 amino-acid protein being very conserved and ubiquitously expressed. It was initially described as an oncogene, and then involved in human male fertility (Nagakubo *et al.*, 1997; Klinefelter *et al.*, 1997; Wagenfeld *et al.*, 1998). The distribution of wild-type DJ1 is either nuclear or cytoplasmic, whereas mutated DJ1 shows reduced nuclear localization and translocation to mitochondria (Zhang *et al.*, 2005). DJ1 has been shown to act as a redox-sensitive molecular chaperone preventing the aggregation of α -synuclein and the neurofilament subunit NFL (Shendelman *et al.*, 2004).

Table 1.4. Mutations identified in the *DJ1* gene

DJ1 mutations	State	Reference
Deletion exons 1-5	homozygous	Bonifati <i>et al.</i> , 2003
L166P	homozygous	Bonifati <i>et al.</i> , 2003
M26I	homozygous	Abou-Sleiman <i>et al.</i> , 2003
D149A	heterozygous	Abou-Sleiman <i>et al.</i> , 2003
A104T	heterozygous	Hague <i>et al.</i> , 2003
c.56delC and c.57G>A	compound heterozygous	Hague <i>et al.</i> , 2003
IVS6-1G>C	compound heterozygous	Hague <i>et al.</i> , 2003
IVS5+2-12del	heterozygous	Hedrich <i>et al.</i> , 2004a
c.253-322 del	heterozygous	Djarmati <i>et al.</i> , 2004
E64D	homozygous	Hering <i>et al.</i> , 2004

The DJ1 protein exists as a homodimer with the active site of the protein being at the junction of the subunits. The L166P mutant protein has proven to be highly unstable due to its ability to disrupt the C-terminal helical domain, leading to an impaired self-dimerization of the DJ1 protein. Unstable monomers are then rapidly degraded by the 20S/26S proteasome (Macedo *et al.*, 2003; Miller *et al.*, 2003; Moore *et al.*, 2003). The L166P mutation, therefore, underlies a loss of function mechanism. Moreover, cellular studies have proven that DJ1 knock-out cells are sensitized to H₂O₂-driven oxidative stress, being rescued by overexpression of wild-type DJ1 but not L166P mutated protein (Yokota *et al.*, 2003). Under oxidative stress conditions, DJ1 undergoes an acidic shift in isoelectric point value (from 6.2 to 5.8) oxidizing its cysteine 106 residue and thus quenching reactive oxygen species (ROS). DJ1 is then translocated to the outer membrane of mitochondria where the cell is protected from apoptosis that may be induced by this organelle (Mitsumoto and Nakagawa, 2001). DJ1 seems to act as a redox sensor, exerting protective effects under oxidative stress conditions.

1.5.2.3. PTEN-Induced Kinase 1 (PINK1). After the identification of the PARK6 locus in 2001, two point mutations have been reported in the *PINK1* gene: G309D in a Spanish family and W437X in two Italian families (Valente *et al.*, 2001; Valente *et al.*, 2004a).

Several studies have then described various point mutations and less frequently exon deletions (Hatano *et al.*, 2004a; Healy *et al.*, 2004; Rogaeva *et al.*, 2004; Valente *et al.*, 2004b; Bonifati *et al.*, 2005; Klein *et al.*, 2005; Li *et al.*, 2005; Ibanez *et al.*, 2006). *PINK1* has an intermediate frequency between *Parkin* and *DJ1*, estimated as 1-9 per cent with a great variability being present among different ethnicities (Thomas *et al.*, 2007). Clinically, *PINK1*-linked PD cases have a slow progression, a good and sustained response to L-Dopa. Additionally, there is a similar phenotype with *Parkin*-related cases (Ibanez *et al.*, 2006).

The *PINK1* gene encodes a 581 amino-acid ubiquitous protein consisting of an N-terminal 34 amino-acid mitochondrial targeting motif, a conserved serine-threonine kinase domain (156-509) and a C-terminal autoregulatory domain. The majority of the identified mutations are in the kinase domain, indicating the importance of PINK1 enzymatic activity in PD pathogenesis (Valente *et al.*, 2004a).

PINK1 or Parkin-deficient drosophila have been shown to be phenotypically very similar. They have flight muscle degeneration and morphological abnormalities of mitochondria in muscle and gonadal cells, indicating that Parkin and PINK1 may be on the same mitochondrial signaling pathway (Poole *et al.*, 2008). Indeed, it has been reported that the mutant phenotype of PINK1 deficient drosophila can be rescued by Parkin overexpression but not vice versa, demonstrating clearly that Parkin acts downstream of PINK1 (Clark *et al.*, 2006; Park *et al.*, 2006; Yang *et al.*, 2006) (Figure 1.13).

A substrate of PINK1 has been recently identified as TNF receptor-associated protein 1 (TRAP1). TRAP1 is a mitochondrial molecular chaperone, known also as Hsp75 (Heat shock protein 75). Wild-type PINK1 has been shown to phosphorylate TRAP1, this phosphorylation being significantly increased in response to oxidative stress induced by H₂O₂. Interestingly, the G309D or L347P, PD-linked PINK1 mutants abolish this kinase activity, although not affecting either the PINK1-TRAP1 interaction or their mitochondrial colocalization. Over-expression of wild-type PINK1 further revealed to reduce cytochrome c release from mitochondria under oxidative stress conditions. TRAP1 is therefore proposed as being a down-stream effector of PINK1: when phosphorylated by PINK1,

TRAP1 may indirectly suppress cytochrome c release from mitochondria, protecting against oxidative stress-induced cell death (Pridgeon *et al.*, 2007) (Figure 1.10).

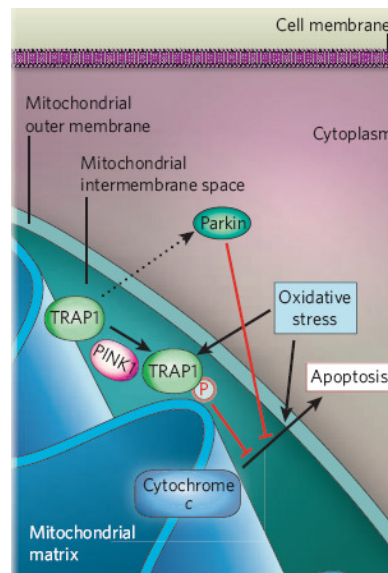


Figure 1.13. Parkinsonism and signaling within mitochondria: Parkin, PINK1 and TRAP1 are interacting in the same signaling pathway (Abeliovich, 2007)

It is therefore plausible that Parkin, PINK1 and TRAP1 are interacting in the same signaling pathway, the defects of which may lead to PD.

1.5.2.4. ATP13A2. In 2006, the *ATP13A2* gene was associated with Kufor-Rakeb syndrome, an atypical form of recessively inherited Parkinsonism. Kufor-Rakeb is described as an L-Dopa-responsive juvenile Parkinsonism with akinesia, rigidity, dyskinesia, visual hallucinations, pyramidal signs, supra nuclear gaze paresis, cognitive deterioration, and diffuse moderate atrophy (Al Din *et al.*, 1994). Truncating mutations have been described in the original Jordanian kindred, from Kufor-Rakeb, as well as in a family from Chile. The PD patients from Chile are compound heterozygous for the 1306+5G>A in exon 13 and the 3057delC mutations in exon 26, and the Jordanian are homozygous for a 22 bp duplication in exon 16 (Ramirez *et al.*, 2006). More recently, a novel homozygous missense mutation (Gly504Arg) was described in one sporadic case

from Brazil with juvenile Parkinsonism, and two heterozygous missense mutations (Thr12Met, Gly533Arg) in two Italian cases with young onset PD (Di Fonzo *et al.*, 2007).

ATP13A2 encodes a lysosomal type5 P-type ATPase of 1180 amino-acids, highly expressed in brain, especially in the substantia nigra (Ramirez *et al.*, 2006). This novel gene may indicate the importance of lysosomes in the pathogenesis of PD. Indeed, α -synuclein is known to be degraded by lysosomes and a lysosomal enzyme, glucocerebrosidase, is linked to Lewy body disorders (Lwin *et al.*, 2004; Aharon-Peretz *et al.*, 2004; Cuervo *et al.*, 2004; Lee *et al.*, 2004; Goker-Alpan *et al.*, 2006). Autophagy may, therefore, be directly related to PD.

In the last decade, since the breakthrough discovery of α -synuclein mutations, as the first recognized cause of familial PD, many key developments have lead to the identification of genes for the Mendelian forms of PD. These findings have given a different perspective to the pathogenesis of PD. Identifying PD patients, carrying mutations in those genes, are expected to give clues to the function of the encoded proteins, thereby contributing to the elucidation of the mechanisms leading to PD.

2. PURPOSE

The rapid expansion of disease-causing genes in PD is not only important in paving the way to novel molecular mechanisms, which will ultimately lead to new treatment options, but it is also of major importance in neurological practice, in which the differential diagnosis of neurodegenerative disorders present with great difficulties.

- The aim of this study is to investigate the molecular basis of the familial and isolated forms of PD associated with mutations in *Parkin*, *PINK1*, *DJ1*, *α-synuclein* and *LRRK2*.
- Investigation of these rare monogenic forms of PD is expected:
 - to simplify the differential diagnosis of PD,
 - to shed light to disease pathogenesis, and
 - to hopefully give insights into the complex mechanisms of not only the genetic, but also the more common idiopathic form of PD.

In spite of great progress in PD genetics, the molecular and clinical data are still very limited in the literature, for both recessive and dominant PD cases; and to the best of our knowledge, the molecular basis of PD is completely unexplored in the Turkish population. Thus, within the framework of the above purpose, our objectives were:

- to describe the distribution of the above five genes in Turkish PD patients,
- to determine the frequencies and types of mutations in those genes,
- to define the age-dependence of PD mutations in the Turkish PD families.

The answers to the above issues are expected to assist in understanding the complex genotype-phenotype correlation of the disease in PD patients, and will hopefully help clinicians in identifying clinical signs solely associated with one gene, or one mutation; or conversely these particular genetic / clinical signs may give further insights into the mechanisms leading to PD.

3. MATERIALS

3.1. Subjects

Peripheral blood samples from PD patients and their family members used in this study are provided by Prof. Sibel Ertan, Prof. Sibel Özekmekçi, Prof. Hülya Apaydın and Prof. Güneş Kızıltan at Cerrahpaşa School of Medicine; by Assoc. Prof. Haşmet Hanağası at İstanbul School of Medicine; by Assoc. Prof. Dilek Ince Günal and Dr. Mesrur Köseoğlu at Marmara School of Medicine; by Assoc. Prof. Hülya Tireli at Haydarpaşa Numune Hospital; by Dr. Serpil Moralı at Okmeydanı Training and Research Hospital; by Dr. Ali Zırh at VKV American Hospital; by Dr. İlknur Aydın at Göztepe Training and Research Hospital; by Dr. Sema Targıt at Şişli Etfal Training and Research Hospital; and by Dr. Murat Alemdar, at Kocaeli University School of Medicine.

In this study, 78 Turkish PD patients and 65 of their relatives were investigated; 47 were males and 31 were females; 52 had a positive family history of PD (32 compatible with dominant and 20 with recessive inheritance); 26 were isolated cases; 12 index cases had consanguineous parents [mean age at onset 39 ± 14.13 years (12-69); mean disease duration 10 ± 5.84 years (1-27)].

All patients met the diagnostic criteria of clinically definite PD: at least two of the triad of signs (akinesia, rigidity, resting tremor), >30 per cent improvement with levodopa at least at onset, and absence of exclusion criteria. Dominant inheritance was defined by the presence of at least two affected cases in two successive or two different generations; and families with at least two affected siblings in only one generation were compatible with a recessive pattern of inheritance. The ethics committee of each participating institution approved this study, and all subjects gave an informed consent (Appendix A).

3.2. Oligonucleotide Primers

Sequences of the primers, used in the amplification of the *Parkin*, *PINK1*, *DJ1*, α -*synuclein* and *LRRK2* genes are given in Tables 3.1, 3.3, 3.4, 3.5 and 3.7 respectively. Sequences of the primers, used for exon dosage of the *Parkin* and α -*synuclein* genes are given in Tables 3.2 and 3.6 respectively. These primers are purchased from Iontek, Turkey and Operon, Germany.

Table 3.1. Oligonucleotide primers for *Parkin* sequencing (5'→3') (Kitada *et al.*, 1998)

Parkinprom258f	GCATTTGTTTAAGCTCAGGGTCTCT
Parkin_Ex1_For	GCGCGGCTGGCGCCGCTGCGCGCA
Parkin_Ex1_Rev	GCGGCGCAGAGAGAGGCTGTAC
Parkin_Ex2_For	ATGTTGCTATCACCATTTAAGGG
Parkin_Ex2_Rev	AGATTGGCAGCGCAGGCGGCATG
Parkin_Ex3_For	ACATGTCACTTTTGCTTCCCT
Parkin_Ex3_Rev	AGGCCATGCTCCATGCAGACTGC
Parkin_Ex4_For	ACAAGCTTTTAAAGAGTTTCTTGT
Parkin_Ex4_Rev	AGGCAATGTGTTAGTACACA
Parkin_Ex5_For	ACATGTCTTAAGGAGTACATTT
Parkin_Ex5_Rev	TCTCTAATTTTCTGGCAAACAGT
Parkin_Ex6_For	AGAGATTGTTTACTGTGGAACA
Parkin_Ex6_Rev	GAGTGATGCTATTTTATAGATCCT
Parkin_Ex7_For	TGCCTTTCCACACTGACAGGTACT
Parkin_Ex7_Rev	TCTGTTCTTCATTAGCATTAGAGA
Parkin_Ex8_For2	CGTCTGTGTCCTCCCAAAG
Parkin_Ex8_Rev2	GCCCAAACGTCTCATTAGC
Parkin_Ex9_For	GGGTGAAATTTGCAGTCAGT
Parkin_Ex9_Rev	AATATAATCCCAGCCCATGTGCA
Parkin_Ex10_For2	GACCCATCATCTCTGTAGCCCTG

Table 3.1. Oligonucleotide primers for *Parkin* sequencing (5'→3') (Kitada *et al.*, 1998)

(continued)

Parkin_Ex10_Rev2	AATGCTCACATGCCTTGTCCT
Parkin_Ex11_For	ACAGGGAACATAAACTCTGATCC
Parkin_Ex11_Rev	CAACACACCAGGCACCTTCAGA
Parkin_Ex12_For2	TTGTATTGCTTGTGATTATTCG
Parkin_Ex12_Rev2	TAGAAAATGAAGGTAGACACTG

Table 3.2. Oligonucleotide primers for *Parkin* exon dosage (5'→3') (Lücking and Brice, 2003)

TTRfam_For	Fam-ACGTTCTCTGATAATGGGATC
TTRdos_Rev	CCTCTCTCTACCAAGTGAGG
TTRinner_dos_Rev	AGGTGTCATCAGCAGCCT
Promfam_For	Fam-GCATTGTGTTTAAGCTCAGGGTC
Promdos_Rev	GCCGTGTTGACCAGTCGCTT
1suzfam_For	Fam-GGAGGCCTGGAGGATTTAAC
1atgdos_Rev	TTTGGCCCCGTCATTGACAGTT
2fam_For	Fam-ATGTTGCTATCACCATTTAAGGG
2dos_Rev	AGATTGGCAGCGCAGGCGGCATG
3ifam_For	Fam-AATTGTGACCTGGATCAGC
3idos_Rev	CTGGACTTCCAGCTGGTGGTGAG
4fam_For	Fam-ACAAGCTTTTAAAGAGTTTCTTGT
4dos_Rev	AGGCAATGTGTTAGTACACA
5fam_For	Fam-ACATGTCTTAAGGAGTACATTT
5dos_Rev	TCTCTAATTCCTGGCAAACAGTG
6fam_For	Fam-AGAGATTGTTTACTGTGGAAACA
6dos_Rev	GAGTGATGCTATTTTTAGATCCT
7fam_For	Fam-TGCCTTTCCACACTGACAGGTACT
7dos_Rev	TCTGTTCTTCATTAGCATTAGAGA
8fam_For	Fam-TGATAGTCATAACTGTGTGTAAG

Table 3.2. Oligonucleotide primers for *Parkin* exon dosage (5'→3') (Lücking and Brice, 2003) (continued)

8dos_Rev	ACTGTCTCATTAGCGTCTATCTT
9fam_For	Fam-GGGTGAAATTTGCAGTCAGT
9dos_Rev	AATATAATCCCAGCCCATGTGCA
10fam_For	Fam-ATTGCCAAATGCAACCTMTGTC
10dos_Rev	TTGGAGGAATGAGTAGGGCATT
11fam_For	Fam-ACAGGGAACATAAACTCTGATCC
11dos_Rev	CAACACACCAGGCACCTTCAGA
12fam_For	Fam-GTTTGGGAATGCGTGTTTT
12dos_Rev	AGAATTAGAAAATGAAGGTAGACA

Table 3.3. Oligonucleotide primers for *PINK1* sequencing (5'→3') (Valente *et al.*, 2004a)

Pink1_Ex1a_For	TCACTGCTAGAGGCGCCAGT
Pink1_Ex1a_Rev	GCACCACGAACTGCCGCTG
Pink1_Ex1b_For	TCGGGCTCGGGCTCCCTAA
Pink1_Ex1b_Rev	CGGCCCTCGATCTGCTCAG
Pink1_Ex2_For	ATTGATCTGGTCGACGTGGAC
Pink1_Ex2_Rev	CCTTTCCTGTGGATAATCTGTC
Pink1_Ex3_For	CTCGAAGGTCAGAGCCAATTC
Pink1_Ex3_Rev	CTGTCATATCAGACACTGTACC
Pink1_Ex4_For	GAATGTCAGTGCCAGTGTTGG
Pink1_Ex4_Rev	AGATATGTTCCCTTTGCATGGC
Pink1_Ex5_For	CGTATTGGGAGTCGTTCGATGT
Pink1_Ex5_Rev	GACCTGAAGAGTCAGTCCTAAA
Pink1_Ex6_For	GTCAGCTATGTCTTGCTGGTG
Pink1_Ex6_Rev	ATCACAAGGCATCGAGTCTCC
Pink1_Ex7_For	TGGATCAGGTGATGTGCAGGA

Table 3.3. Oligonucleotide primers for *PINK1* sequencing (5'→3') (Valente *et al.*, 2004a)

(continued)

Pink1_Ex7_Rev	AGGATCTGTCACTGTGGCTCT
Pink1_Ex8_For	GAGAAGGGAAGACCCTCACTA
Pink1_Ex8_Rev	CAGACTGAACTCTCACTCAAGT

Table 3.4. Oligonucleotide primers for *DJ1* sequencing (5'→3') (Bonifati *et al.*, 2003)

Dj1_Ex1A/B_For	GAGGTAGACTCGGCCGGAC
Dj1_Ex1A/B_Rev	TTCTGGACGCTTCAGCGTTG
Dj1_Ex2_For	TAGGAAGTACTTACTCTGCTTG
Dj1_Ex2_Rev	TATTTATTCTTATGTCATCTCTG
Dj1_Ex3_For	CAGCTGTGTAAACGTTACTC
Dj1_Ex3_Rev	ATTCTGTATCAAGCAATTGCC
Dj1_Ex4_For	CTATCTCCTGTACTTCCCAC
Dj1_Ex4_Rev	ACAGAACATAAGCAGATGCTG
Dj1_Ex5_For	TGAGAAATGCCTTGCTTGGG
Dj1_Ex5_Rev	GCTATTTGGAATCAAACCATCG
Dj1_Ex6_For	TTTGCCAGATGTGCTCAGCAAATCG
Dj1_Ex6_Rev	ACTGCACTCCAGCCTGGGCGATGG
Dj1_Ex7_For	CACATAGCCCATTAGGATGTC
Dj1_Ex7_Rev	AGCTGCAAATGAAGGTGATAC

Table 3.5. Oligonucleotide primers for α -synuclein sequencing (5'→3') (Polymeropoulos *et al.*, 1997; Krüger *et al.*, 1998; Zarranz *et al.*, 2004)

Syn_Ex3_For	AAGTGTATTTTATGTTTTCC
Syn_Ex3_Rev	AACTGACATTTGGGGTTTACC
Syn_Ex4_For	GCTAATCAGCAATTTAAGGCTAG
Syn_Ex4_Rev	GATATGTTCTTAGATGCTCAG

Table 3.6. Oligonucleotide primers for *α -synuclein* exon dosage (5'→3') (Ibanez *et al.*, 2004)

Syn_Dosage_Ex3afam_For	Fam-AAGTGTATTTTATGTTTTCC
Syn_Dosage_Ex3ados_Rev	AACTGACATTTGGGGTTTACC
Syn_Dosage_Ex4afam_For	Fam-GCTAATCAGCAATTTAAGGCTAG
Syn_Dosage_Ex4ados_Rev	GATATGTTCTTAGATGCTCAG

Table 3.7. Oligonucleotide primers for *LRRK2* sequencing (5'→3') (Zimprich *et al.*, 2004; Di Fonzo *et al.*, 2006)

LRRK2_Ex19_For	GAAGTTTGATTTGCCAGTCTCC
LRRK2_Ex19_Rev	TCAAACCTGGCATGAATAACCAC
LRRK2_Ex21_For	AAGTGAAAAACCAACATGGC
LRRK2_Ex21_Rev	ACATCAGGGAAATCCCTACC
LRRK2_Ex23_For	AGCCTGATTGCTAGGAGGTG
LRRK2_Ex23_Rev	GGGGGACTTATCACCCAGTG
LRRK2_Ex25_For	TCCTCTTTGATGCTGTTCTTTG
LRRK2_Ex25_Rev	TGCCACTTTTAAATCCACAAC
LRRK2_Ex27_For	GGTGGTTCAACTTCAGGCTC
LRRK2_Ex27_Rev	AATGGAAATTAAATTAAGTGACACAT
LRRK2_Ex29_For	TTTACCAAACATTATCAACTACCC
LRRK2_Ex29_Rev	TCCCTGTTCCAAACAAATGG
LRRK2_Ex31_For	GAATGTGAGCAGGCCAGT
LRRK2_Ex31_Rev	AGAAAACCCACAATTTTAAGTG
LRRK2_Ex32_For	CTGAATTTGCCAACCATTG
LRRK2_Ex32_Rev	GAACCGTATGGATATTCTCTCAAC
LRRK2_Ex33_For	AAAATGAGGAAGTTGGACTAG
LRRK2_Ex33_Rev	TGCTCTGGGCGCCATCTG
LRRK2_Ex35_For	AGGTTGGGTGTTTTGTGAGG
LRRK2_Ex35_Rev	ATGCCATCTCCCTAATTCTC

Table 3.7. Oligonucleotide primers for *LRRK2* sequencing (5'→3') (Zimprich *et al.*, 2004; Di Fonzo *et al.*, 2006) (continued)

LRRK2_Ex38_For	TAGAGAAATATAGGATTGGTTAG
LRRK2_Ex38_Rev	ATTATAACAAATATATGAAGACAG
LRRK2_Ex39_For	CAATGAAACAAGTAGGTCAGG
LRRK2_Ex39_Rev	GTATGCAAAATAAGAGTTCCAG
LRRK2_Ex41_For	TTTTGATGCTTGACATAGTGGAC
LRRK2_Ex41_Rev	CACATCTGAGGTCAGTGGTTATC
LRRK2_Ex47_For	AAGATCTTCCTTATGAATTATCAACAG
LRRK2_Ex47_Rev	CTGTTGATAATTCATAAGGAAGATCTT
LRRK2_Ex48_For	TATAAGGTTGTATTACACGTAG
LRRK2_Ex48_Rev	AAAATTCTCTATTTCAGAGGCAG

3.3. Enzymes

AccuPrime Taq DNA Polymerase	:	5U/μl, Invitrogen, USA
BioTaq DNA Polymerase	:	5U/μl, Bioline, UK
GoTaq DNA Polymerase	:	5U/μl, Promega, USA
Qiagen Taq DNA Polymerase	:	5U/μl, Qiagen, Germany
Exonuclease I	:	20U/μl, Fermentas, Lithuania
Phosphatase, Alkaline from shrimp	:	1U/μl, Sigma, Germany
MvaI	:	10U/μl, Fermentas, Lithuania
StyI	:	10U/μl, Fermentas, Lithuania

Tsp45I : 10U/μl, Fermentas, Lithuania

3.4. Chemicals

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

3.5. Buffers and Solutions

3.5.1. DNA Extraction

Lysis Buffer	:	155 mM NH ₄ Cl 10 mM KHCO ₃ 1 mM Na ₂ EDTA (pH 7.4)
Nuclease Buffer	:	10 mM Tris-HCl (pH 8.0) 400 mM NaCl 2 mM Na ₂ EDTA (pH 7.4)
Sodiumdodecylsulfate (SDS)	:	10 per cent SDS (w/v) (pH 7.2)
Proteinase K	:	20 mg/ml in dH ₂ O, Promega, USA
Sodium Chloride (NaCl)	:	2.5 M saturated stock solution
Absolute Ethanol (EtOH)	:	Riedel-de Haën, Germany
TE Buffer	:	20 mM Tris-HCl (pH 8.0) 1 mM Na ₂ EDTA (pH 8.0)

3.5.2. Polymerase Chain Reaction (PCR)

10X Bioline Buffer	:	160 mM (NH ₄) ₂ SO ₄ 670 mM Tris-HCl (pH 8.8) 0.1 per cent Tween-20 (MgCl ₂ free) Bioline, UK
5X GoTaq Flexi Buffer	:	Proprietary formulation (MgCl ₂ free) Promega, USA
10X Invitrogen Buffer	:	200 mM Tris (pH 8.4) 500 mM KCl (MgCl ₂ free) Invitrogen, USA
10X Qiagen Buffer	:	Proprietary formulation contains 15 mM MgCl ₂ Qiagen, Germany
Magnesium Chloride (MgCl ₂)	:	50 mM, Bioline, UK 50 mM, Invitrogen, USA 25 mM, Promega, USA 25 mM, Qiagen, Germany
Deoxyribonucleotides (dNTPs)	:	100 mM of each dNTP Promega, USA
Q Solution (5X)	:	Proprietary formulation Qiagen, Germany

Betaine Solution : 5 M, Sigma, Germany

Dimethylsulphoxide (DMSO) : Sigma, Germany

3.5.3. Restriction Enzyme Analysis

Buffer R (10X) : 10 mM Tris-HCl (pH 8.5)
100 mM KCl
10 M MgCl₂
0.1 mg/ml BSA
Fermentas, Lithuania

Buffer O (10X) : 50 mM Tris-HCl (pH 7.5)
100 mM NaCl
10 mM MgCl₂
0.1 mg/ml BSA
Fermentas, Lithuania

3.5.4. Agarose Gel Electrophoresis

10X TBE Buffer : 0.89 M Tris-Base
0.89 M Boric Acid
20 mM Na₂EDTA (pH 8.3)

Ethidium Bromide (EtBr) : 10 mg/ml, Sigma, Germany

1 or 2 per cent agarose gel : 1 or 2 per cent agarose (w/v) in 0.5X
TBE Buffer, with 0.5 µg/ml EtBr

10X Loading Dye : 2.5 mg/ml Bromophenol Blue
1 per cent SDS in glycerol

DNA Ladder : 100 base pair (bp)
Fermentas, Lithuania

3.5.5. Polyacrylamide Gel Electrophoresis

40 per cent Acrylamide-Bisacrylamide : 38 per cent Acrylamide
Stock (19:1) 2 per cent N, N'-Methylene
Bisacrylamide

8 per cent Denaturing : 8 per cent Acrylamide-Bisacrylamide
Instagel Solution Solution (19/1)
8.3 M Urea
1X TBE buffer

Ammonium Persulfate (APS) : 10 per cent APS (w/v) in dH₂O

TEMED : N,N,N,N-tetramethylethylenediamine

10X Sample Buffer : 95 per cent formamide
20 mM EDTA
0.05 per cent xylene cyanol
0.05 per cent bromophenol blue

3.5.6. Silver Staining

Staining Buffer : 0.1 per cent AgNO₃ (w/v) in dH₂O

Developing Buffer : 1.5 per cent NaOH
0.01 per cent NaBH₄
0.015 per cent formaldehyde in dH₂O

3.6. Kits

BigDye Terminator v3.1	:	Sequencing Buffer (5X)
Cycle Sequencing Kit		Ready reaction premix (2.5X)
		Applied Biosystems, USA
MagNA Pure	:	Roche, Germany
Nucleic Acid Isolation Kit		
Multiplex Ligation-dependent	:	P051 and P052, MRC, Holland
Probe Amplification (MLPA)		
Qiaquick PCR Purification Kit	:	Qiagen, Germany

3.7. Equipment

Autoclaves	:	Model ASB260T, Astell, UK
		Model Midas 55, Prior Clave, UK
Balances	:	440-47N, Kern, France
Centrifuges	:	5415C, Eppendorf, Germany
		Spectrafuge, Labnet, USA
		Allegra X22-R, Beckman Coulter,
		Germany
		Universal 16R, Hettich, Germany
Deep Freezers	:	2021D (-20°C), Arçelik, Turkey
		Sanyo (-70°C), Sanyo, Japan

Denaturing High-Performance Liquid Chromatography (dHPLC)	:	WAVE 3500 Transgenomic, USA
DNA Analyzer	:	ABI 3730, Applied Biosystems, USA
DNA Isolation	:	MagNA Pure Compact Instrument Version 1.0 Roche, Germany
Documentation System	:	GelDoc Documentation System BIO-RAD, USA
Electrophoretic Equipments	:	i-MyRun-N, CosmoBio Co, Japan Minicell Primo E320, Thermo, USA Mini Sub Cell GT, BIO-RAD, USA Wide Sub Cell GT BIO-RAD, USA Sequi-Gen GT Cell, BIO-RAD, USA
Magnetic Stirrer	:	Heidolph MR3001, Germany
Ovens	:	MD554, Microwave Oven Arçelik, Turkey EN400 (37°C), Nuve, Turkey ED115 (56°C), Binder, Germany
pH meter	:	pH720, InoLab, Germany
Power Suppliers	:	EC250-90 Thermo, USA Power Pac Basic, BIO-RAD, USA Power Pac HV, BIO-RAD, USA

Refrigerators	:	3011EC, Arçelik, Turkey
Spectrophotometer	:	NanoDrop ND-1000, Thermo, USA
Thermocyclers	:	TC312, Techne, UK Techgene, Progene, UK Techne, Progene, UK Touchgene Gradient, Progene, UK
Vortex	:	Fisons WhirliMixer, UK Reax Top, Heidolph, Germany
Water Purification	:	WaTech Water Technologies, Turkey

4. METHODS

4.1. DNA Extraction from White Blood Cells

4.1.1. Sodium Chloride Extraction

Ten ml of peripheral blood samples from PD patients and their relatives were collected into sterile vacutainer tubes containing K₂EDTA to prevent coagulation, and stored at 4°C until extraction.

Ten ml of blood was added to 30 ml ice-cold red blood cell lysis buffer in a sterile Falcon tube, inverted and put into cold room (4°C) for 15 min to allow the cells to lyse. Tubes were then centrifuged at 5000 rpm for 10 min (4°C) to precipitate nuclei of the lysed cells, and supernatants were discarded. Pellets were then resuspended in 10 ml lysis buffer by vortexing. Tubes were centrifuged again at 5000 rpm for 10 min (4°C) and supernatants were discarded. If pellets were not white and contaminated by remains from red blood cells, the last resuspension and centrifugation steps had to be repeated. Nuclear pellets were then resuspended in three ml ice-cold nuclease buffer by vortexing, until clumps were dissolved. The samples were then incubated in 400 µl dH₂O, 30 µl proteinase K (20 mg/ml), 50 µl SDS (10 per cent) either over night at 37°C, or for three hours at 56°C.

After incubation, 10 ml 2.5 M NaCl was added to the samples which were shaken thoroughly. Tubes were then centrifuged at 5000 rpm for 30 min at room temperature. Supernatants were then transferred into new sterile Falcon tubes. Two volumes of ice-cold absolute EtOH were added, and samples were shaken by slowly inverting motion, until DNA threads became visible. DNA was then fished out, transferred into Eppendorf tubes and kept at room temperature until EtOH evaporated. DNA was then dissolved in 100-1000 µl TE buffer, left at room temperature overnight, and then stored at +4°C.

If there was no visible DNA precipitation after the addition of EtOH, the tubes were kept at -70°C overnight. Then, they were centrifuged at 14000 rpm for 30 min at 18°C. After centrifugation, supernatants were discarded, and DNA pellets were dissolved in 100-200 µl TE buffer.

4.1.2. Extraction by MagNA Pure Compact Instrument

MagNA Pure Compact Instrument is a robotic nucleic acid extraction system, which uses the specifically designed MagNA Pure compact nucleic acid isolation kit. The extraction principle of this system is based on the affinity of magnetic beads for DNA, which first binds to the magnetic beads, and is then removed from the beads in the elution step. Of one ml fresh blood, 200 µl DNA in buffer solution is obtained.

4.2. Analysis of the Extracted DNA Samples

4.2.1. Agarose Gel Electrophoresis

The quality and quantity of the extracted DNA was tested on a one per cent agarose gel. To prepare 100 ml of a one per cent agarose solution, one g agarose was measured into a flask and 100 ml 0.5X TBE was added. The mixture was stirred on a hot plate until agarose was dissolved and the solution was clear. The solution was allowed to cool before pouring, and EtBr, which enables to visualize the DNA under UV light, was added at this point to a concentration of 0.5 µg/ml. Combs were placed to the gel tray and the solution was poured. The gel solidifies in 15 min at room temperature. To run the gel, combs were gently removed, and the tray was placed in the electrophoresis chamber, and covered (just until wells were submerged) with 0.5X TBE electrophoresis buffer. To prepare samples for electrophoresis, two µl of 5X gel loading dye was added for every one µl of DNA solution. Samples were loaded, and electrophoresis was performed at 150 V for 10 min. Fragments were then detected under UV light. The quality and quantity of the DNA sample was determined by comparing its intensity with known DNA samples.

4.2.2. Spectrophotometric Measurement of DNA

The exact amount and concentration of the DNA sample was determined by spectrophotometric measurement using a NanoDrop Spectrophotometer. In the NanoDrop Spectrophotometer, a pulsed xenon flash lamp provides the light source and the spectrometer is used to analyze the light after passing through the sample. One μl of DNA was pipetted into the end of the receiving fiber optic cable of the NanoDrop Spectrophotometer, and the source fiber optic cable was then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends.

Optical density of the DNA was measured at 260 nm (OD_{260}). Since 50 μg of double stranded DNA has an absorbance of 1.0 at 260 nm, the concentration of DNA in the sample was calculated by using the formula:

$$[50 \mu\text{g/ml} \times \text{Optical Density } (\text{OD}_{260}) \times \text{Dilution Factor} = \text{Concentration in } \mu\text{g/ml}].$$

4.3. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) amplifies the DNA region of interest. Fifty to hundred ng of genomic DNA was used as template in all PCR mixtures. After addition of DNA and all PCR components, the volume of each PCR mixture was completed to 25 μl with distilled water, and amplification was initiated in a thermal cycler. After the PCR amplification was terminated, the samples were run on a two per cent agarose gel to check the quality and quantity of amplified samples. For this purpose, five μl of each PCR product was mixed with two μl of 5X loading dye and run at 175 V for 15-20 min. The 100 bp ladder was used as the DNA size marker on the gel. The gel was visualized under UV light and recorded, as described in 4.2.1.

4.3.1. Parkin PCR Protocol

To amplify the promoter-exon 1 region of the *Parkin* gene, the following touchdown PCR conditions were applied, with the PCR components shown in Table 4.1:

Initial Denaturation	:	94°C	5 min		
Denaturation	:	94°C	30 sec	}	11 cycles
Annealing	:	60°C→50°C	30 sec		
Extension	:	72°C	40 sec		
Denaturation	:	94°C	30 sec	}	24 cycles
Annealing	:	54°C	30 sec		
Extension	:	72°C	30 sec		
Final Extension	:	72°C	7 min		

To amplify exons 2 to 12 of the *Parkin* gene, the following PCR conditions were applied with the PCR components shown in Table 4.1:

Initial Denaturation	:	94°C	5 min		
Denaturation	:	94°C	30 sec	}	35 cycles
Annealing	:	A°C*	30 sec		
Extension	:	72°C	30 sec		
Final Extension	:	72°C	7 min		

*The annealing temperature (A°C) is:

- 55°C for exons 2, 4, 8, 9 and 12;
- 57°C for exons 7 and 11;
- 58°C for exon 10; and
- 59°C for exons 3, 5, 6.

Table 4.1. *Parkin* PCR components

Components	Volume (μl)	[Stock]	[Final]
Buffer	5	5X	1X
MgCl ₂	1.5	25 mM	1.5 mM
dNTP	0.2	25 mM	0.2 mM
Forward primer	0.5	20 μM	0.4 μM
Reverse primer	0.5	20 μM	0.4 μM
Q Solution (for promoter-exon 1 only)	5	5X	1X
GoTaq DNA Polymerase*	0.1	5U/μl	0.02U/μl

* 0.2 μl of GoTaq DNA Polymerase is used for exon 3 (final concentration 0.04U/μl)

4.3.2. PINK1 and DJ1 PCR Protocol

To amplify exons 1A of the *PINK1* gene and exon 1 of the *DJ1* gene, the following PCR conditions were applied with the PCR components shown in Table 4.2:

Initial Denaturation	:	94°C	5 min	
Denaturation	:	94°C	30 sec	} 35 cycles
Annealing	:	59°C	30 sec	
Extension	:	72°C	30 sec	
Final Extension	:	72°C	7 min	

To amplify exons 1B to 8 of the *PINK1* gene and exons 2 to 7 of the *DJ1* gene, the following touchdown PCR conditions were applied with the PCR components shown in Table 4.2:

Initial Denaturation	:	94°C	5 min	
Denaturation	:	94°C	30 sec	} 11 cycles
Annealing	:	64°C→54°C	30 sec	
Extension	:	72°C	40 sec	
Denaturation	:	94°C	30 sec	} 24 cycles
Annealing	:	54°C	30 sec	
Extension	:	72°C	30 sec	
Final Extension	:	72°C	7 min	

Table 4.2. *PINK1* and *DJ1* PCR components

Components	Volume (μl)	[Stock]	[Final]
Buffer	5	5X	1X
MgCl ₂	1.5	25 mM	1.5 mM
dNTP*	0.2	25 mM	0.2 mM
Forward primer	0.5	20 μM	0.4 μM
Reverse primer	0.5	20 μM	0.4 μM
DMSO**	1.25	100%	5%
GoTaq DNA Polymerase	0.1	5U/μl	0.02U/μl

* 0.3 μl dNTP is added for *PINK1* exon 1A and *DJ1* exon 1 (final concentration 0.3 mM)

** DMSO was added for exons 1A, 1B, 2 and 8 of the *PINK1* gene; and exons 1 and 3 of the *DJ1* gene only

4.3.3. Alpha-synuclein PCR Protocol

To amplify exon 3 of the *α-synuclein* gene, the following PCR conditions were applied with the PCR components shown in Table 4.3:

Initial Denaturation	:	94°C	4 min	
Denaturation	:	94°C	1 min	} 30 cycles
Annealing	:	50°C	1 min	
Extension	:	72°C	1 min	
Final Extension	:	72°C	4 min	

To amplify exon 4 of the *α-synuclein* gene, the following PCR conditions were applied with the PCR components shown in Table 4.3:

Initial Denaturation	:	94°C	5 min	
Denaturation	:	94°C	1 min	} 35 cycles
Annealing	:	52°C	1 min 30 sec	
Extension	:	72°C	1 min	
Final Extension	:	72°C	10 min	

Table 4.3. *Alpha-synuclein* PCR components

Components	Volume (μl)	[Stock]	[Final]
Buffer	5	5X	1X
MgCl ₂	1.5	25 mM	1.5 mM
dNTP	0.2	25 mM	0.2 mM
Forward primer	0.5	20 μM	0.4 μM
Reverse primer	0.5	20 μM	0.4 μM
GoTaq DNA Polymerase	0.2	5U/μl	0.04U/μl

Ten microsatellite markers were used to construct the PD-linked haplotype in a large PD family: D4S2409, D4S2622, D4S1542, D4S2371, D4S2461, D4S2304, D4S1544, D4S414, D4S2404, D4S2364 (sequences are available at the Human Genome Database, <http://www.gdb.org>). The following PCR conditions were applied with the PCR components shown in Table 4.4:

Initial Denaturation	:	94°C	5 min	
Denaturation	:	94°C	30 sec	} 35 cycles
Annealing	:	57°C	30 sec	
Extension	:	72°C	30 sec	
Final Extension	:	72°C	7 min	

Table 4.4. *Alpha-synuclein* region microsatellites PCR components

Components	Volume (μl)	[Stock]	[Final]
Buffer	5	5X	1X
MgCl ₂	1.5	25 mM	1.5 mM
dNTP	0.2	25 mM	0.2 mM
Forward primer	0.5	20 μM	0.4 μM
Reverse primer	0.5	20 μM	0.4 μM
GoTaq DNA Polymerase	0.1	5U/μl	0.02U/μl

4.3.4. LRRK2 PCR Protocol

To amplify exons 19, 21, 23, 25, 27, 29, 31, 32, 33, 35, 38, 39, 41, 47 and 48 of the *LRRK2* gene, the following PCR conditions were applied with the PCR components shown in Table 4.5:

Initial Denaturation	:	94°C	5 min		
Denaturation	:	94°C	30 sec	}	11 cycles
Annealing	:	64°C→54°C	30 sec		
Extension	:	72°C	40 sec		
Denaturation	:	94°C	30 sec	}	24 cycles
Annealing	:	54°C	30 sec		
Extension	:	72°C	30 sec		
Final Extension	:	72°C	7 min		

Table 4.5. *LRRK2* PCR components

Components	Volume (μl)	[Stock]	[Final]
Buffer	5	5X	1X
MgCl ₂	1.5	25 mM	1.5 mM
dNTP	0.2	25 mM	0.2 mM
Forward primer	0.5	20 μM	0.4 μM
Reverse primer	0.5	20 μM	0.4 μM
GoTaq DNA Polymerase	0.2	5U/μl	0.04U/μl

To amplify the 21 microsatellite markers used to construct the G2019S-linked haplotype, the following PCR conditions were applied, with the PCR components shown in Table 4.6:

Initial Denaturation	:	95°C	15 min	
Denaturation	:	96°C	30 sec	} 35 cycles
Annealing	:	55°C	1 min 30sec	
Extension	:	72°C	1 min 30sec	
Final Extension	:	72°C	10 min	

4.4. Restriction Enzyme Analysis

The *α-synuclein* gene was investigated by restriction enzyme (RE) analysis for the three known mutations: A53T, A30P and E46K (Polymeropoulos *et al.*, 1997; Krüger *et al.*, 1998; Zarranz *et al.*, 2004). In RE analysis, the PCR products were incubated at 37°C for at least four hours, with the restriction enzymes and reaction mixture components given in Table 4.7.

Table 4.6. G2019S haplotype microsatellites PCR components

a. Multiplex I b. Multiplex II

a.

G2019S-haplotype Multiplex I	Volume (μl)
Bioline Buffer 10X	2.5
MgCl ₂ (50 mM)	0.75
dNTP (25 mM)	0.2
Q Solution (5X)	5
D12S1701 For (20 μM)	1
D12S1701 Rev (20 μM)	1
D12S87 For (20 μM)	0.08
D12S87 Rev (20 μM)	0.08
D12S2515 For (20 μM)	0.75
D12S2515 Rev (20 μM)	0.75
D12S2516 For (20 μM)	0.15
D12S2516 Rev (20 μM)	0.15
D12S2514 For (20 μM)	0.1
D12S2514 Rev (20 μM)	0.1
D12S1301 For (20 μM)	0.25
D12S1301 Rev (20 μM)	0.25
D12S2517 For (20 μM)	0.75
D12S2517 Rev (20 μM)	0.75
D12S2520 For (20 μM)	1
D12S2520 Rev (20 μM)	1
D12S2522 For (20 μM)	1.1
D12S2522 Rev (20 μM)	1.1
dH ₂ O	1.09
Bioline Taq (5U/μl)	0.1

b.

G2019S-haplotype Multiplex II	Volume (μl)
Bioline Buffer 10X	2.5
MgCl ₂ (50 mM)	0.75
dNTP (25 mM)	0.2
Q Solution (5X)	5
D12S2519 For (20 μM)	0.15
D12S2519 Rev (20 μM)	0.15
D12S2080 For (20 μM)	0.15
D12S2080 Rev (20 μM)	0.15
D12S2194 For (20 μM)	0.2
D12S2194 Rev (20 μM)	0.2
D12S2521 For (20 μM)	0.25
D12S2521 Rev (20 μM)	0.25
D12S1648 For (20 μM)	0.15
D12S1648 Rev (20 μM)	0.15
D12S2518 For (20 μM)	0.15
D12S2518 Rev (20 μM)	0.15
D12S1048 For (20 μM)	0.2
D12S1048 Rev (20 μM)	0.2
D12S2523 For (20 μM)	0.2
D12S2523 Rev (20 μM)	0.2
dH ₂ O	8.55
Bioline Taq (5U/μl)	0.1

Table 4.7. Components used in restriction enzyme analysis

α-synuclein mutations	PCR product	RE (10U/μl$\rightarrow$5U/μl)		Buffer (10X$\rightarrow$1X)		dH₂O
A53T	5 μ l	Tsp45I	0.5 μ l	Buffer R	1.5 μ l	8 μ l
A30P	5 μ l	MvaI	0.5 μ l	Buffer R	1.5 μ l	8 μ l
E46K	5 μ l	StyI	0.5 μ l	Buffer O	1.5 μ l	8 μ l

After incubation, the digested PCR products were directly mixed with four μ l of 5X loading dye and electrophoresed on a two per cent agarose gel together with a 100 bp size marker at 175 V for 15-20 min. The gel was then visualized under UV light and the bands were recorded (Table 4.8).

Table 4.8. Restriction enzymes used, their recognition sites and the expected results of the enzyme digestion ($\downarrow$: cutting site sense strand)

α-synuclein mutations	α-synuclein exons	RE	Recognition Site	Position on the Gene	Result
A53T	Exon 4	Tsp45I	5'- $\downarrow$ GT(G/C)AC-3'	GTGTGGCAA	undigested
				GT $\downarrow$ GTG <u>A</u> CAA	digested
A30P	Exon 3	MvaI	5'-CC $\downarrow$ (A/T)GG-3'	GAAGCAGCA	undigested
				GAAC <u>C</u> $\downarrow$ AGCA	digested
E46K	Exon 3	StyI	5'-C $\downarrow$ CAAGG-3'	CCAAG <u>A</u> AGG	undigested
				C $\downarrow$ CAAGGAGG	digested

(Mutations are shown in bold and underlined characters)

4.5. Denaturing High-Performance Liquid Chromatography (dHPLC)

The dHPLC analysis is based on heteroduplex formation through hybridization after heating and cooling of two PCR-amplified double-stranded DNAs, which differ in one nucleotide. The separation of heteroduplexes from homoduplexes is accomplished under partially denaturing conditions. DNA variations are detected as a pattern of peaks that discriminate variant double stranded DNA from wild-type DNA.

The principle of fragment separation lies in the ability of the separation cartridge to bind double-stranded DNA and release it, as the helix of the molecule is unwound. The DNA is eluted from the column, as an increasing concentration of acetonitrile flows across the matrix. Heteroduplexes formed in heterozygous samples will elute before homoduplexes, due to their instability, appearing ideally as two or more peaks in chromatograms (Figure 4.1).

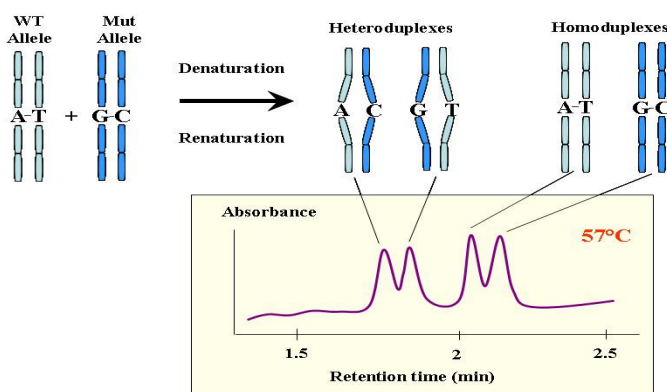


Figure 4.1. The principle of dHPLC and a chromatogram example. Heteroduplexes are released from the column earlier than homoduplexes (WT: wild-type; Mut: mutated)

Two different wild-type and three independent positive controls were used for the dHPLC analysis of each exon of the *Parkin* gene. For each sample, either test or control, 10 µl PCR product of the corresponding *Parkin* exon of the wild-type individual was mixed with 10 µl PCR product of the same *Parkin* exon of the patient tested. All PCR products were first denatured at 96°C for five min and then cooled gradually for another 20 min. The mixed PCR products were eluted using a linear acetonitrile gradient in a 0.1M triethylamine acetate buffer at a constant flow rate of 0.9 ml/min. The injection volume was fixed to five µl for each sample. The temperatures and timeshifts used, are indicated in Table 4.9.

Table 4.9. dHPLC conditions for analysis of the *Parkin* gene

Parkin Exons*	dHPLC Temperatures (°C)	Corresponding Timeshifts**
Exon 2	59	0
	62	0.4
	63.4	0.5
Exon 3	59	0.5
	65	1.2
Exon 4	57.8	0.3
	59.8	0.5
	63	0.9
Exon 6	56.5	0.3
	59.5	0.6
Exon 7	58.4	0.1
	60.4	0.2
	61.4	0.3
Exon 9	61.4	0.6
	64	1
	65.7	1.2
Exon 10	55	0.2
	60	0.8
	61	0.9
Exon 11	58.4	0.2
	60.4	0.5
	61.5	0.7
	66	0.9
Exon 12	61.5	0.46
	65	0.7

* Exons 1, 5 and 8 of the *Parkin* gene give inconclusive results with dHPLC, and were directly sequenced

** A timeshift assured the separation of the injection peak and the homo/heteroduplex from the sample

4.6. DNA Sequencing

The initial sequencing procedures were performed on an ABI 3730 automated sequencer in the INSERM U679 laboratory in Paris, later they were sent to commercial core facilities (Iontek, Istanbul; Refgen, Ankara).

4.6.1. Purification and Preparation of PCR Samples for DNA Sequencing

Prior to DNA sequencing, the PCR products were purified from excess nucleotides, primers and salts with the QIAquick PCR Purification Kit, following the instructions of the manufacturer. Then, the purified PCR samples were run on a two per cent agarose gel to visualize the quality and quantity of the purified products.

In the sequencing procedures performed personally, two enzymes were used to purify the products: ExonucleaseI (ExoI) and Shrimp Alkaline Phosphatase (SAP). ExoI digests the single-stranded DNA and SAP dephosphorylates the remaining primers. A mix was prepared with: [2 μ l SAP (1U/ μ l) and 0.5 μ l ExoI (20U/ μ l) x number of PCR]. To each 10 μ l PCR product, 2.5 μ l of this ExoI-SAP mix was added. To activate the enzymes, PCR samples were incubated at 37°C for 30 min, and then, they were placed at 80°C for another 30 min, to inactivate the enzymes.

4.6.2. Sequencing Reaction

The following sequencing conditions were applied to the ExoI-SAP purified PCR products, with the components shown in Table 4.10:

Initial Denaturation	:	96°C	5 min	} 28 cycles
Denaturation	:	96°C	10 sec	
Annealing	:	A°C*	5 sec	
Extension	:	60°C	4 min	
Final Extension	:	60°C	10 min	

*The annealing temperature (A°C) was calculated for each primer used.

Table 4.10. Sequencing reaction components

Components	Volume (µl)	[Stock]	[Final]
BigDye Mix (v3.1)	0.5	2.5X	0.125X
Buffer	2	5X	1X
Primer	0.5	20 µM	1 µM
ExoI-SAP purified PCR product	3	-	-
dH ₂ O	4	-	-

4.6.3. G50 PCR Purification

The purification of the sequencing reactions was performed with a G50 Sephadex resin, which was prepared by dissolving 2.5 g of G50 in 50 ml dH₂O. Each well of a Millipore 96-well plate, was filled with 250 µl of the G50 mixture and the plate was centrifuged for two min at 2500 rpm. A Greiner plate was placed under the Millipore plate to retrieve the excess water. This step was repeated, in order to completely dehydrate the Sephadex resin. The ExoI-SAP purified PCR products (10 µl) were mixed with 20 µl dH₂O and added to the center of each well of the G50-Millipore plate. A sterile 96-well PCR plate was positioned underneath to receive the samples and centrifuged for five min at 2500 rpm. The 96-well PCR plate was directly inserted in the ABI 3730 apparatus tray for the sequencing analysis.

4.7. Exon Dosage

4.7.1. Exon Dosage for the Parkin Gene

Screening for exon rearrangements in the *Parkin* gene was performed using a semi-quantitative multiplex PCR assay. The exons were amplified in four different

combinations. For each combination, the corresponding PCR conditions were applied, with the PCR components shown in Table 4.11:

- Combination I and III:

Combination I includes exons 4, 7, 8, 11 and combination III includes exons 2, 3, 9, 12.

Initial Denaturation	:	94°C	5 min		
Denaturation	:	94°C	30 sec	}	23 cycles
Annealing	:	53°C	45 sec		
Extension	:	72°C	2 min 30 sec		
Final Extension	:	72°C	10 min		

- Combination II (includes exons 5, 6, 8 and 10):

Initial Denaturation	:	94°C	5 min		
Denaturation	:	94°C	30 sec	}	23 cycles
Annealing	:	53°C	45 sec		
Extension	:	68°C	2 min 30 sec		
Final Extension	:	68°C	10 min		

- Combination IV (includes the promoter region, exons 1 and 9):

Initial Denaturation	:	94°C	5 min		
Denaturation	:	94°C	30 sec	}	22 cycles
Annealing	:	53°C	30 sec		
Extension	:	68°C	45 sec		
Final Extension	:	68°C	10 min		

Table 4.11. *Parkin* semi-quantitative multiplex PCR assay components
(a. Combination I ; b. Combination II ; c. Combination III ; d. Combination IV)

a.

Combination I exons 4/7/8/11	Volume (µl)
Invitrogen Buffer 10X	2.5
MgCl ₂ (50 mM)	1.5
dNTP (25 mM)	0.2
4 Fam For (20 µM)	1.6
4 Dos Rev (20 µM)	1.6
7 Fam For (20 µM)	1.3
7 Dos Rev (20 µM)	1.3
8 Fam For (20 µM)	1.25
8 Dos Rev (20 µM)	1.25
11 Fam For (20 µM)	0.8
11 Dos Rev (20 µM)	0.8
dH ₂ O	8.7
Invitrogen Taq (5U/µl)	0.2

b.

Combination II exons 5/6/8/10	Volume (µl)
Qiagen Buffer (10X)	2.5
MgCl ₂ (25 mM)	1.5
dNTP (25 mM)	0.2
5 Fam For (20 µM)	1.2
5 Dos Rev (20 µM)	1.2
6 Fam For (20 µM)	1.4
6 Dos Rev (20 µM)	1.4
8 Fam For (20 µM)	1.3
8 Dos Rev (20 µM)	1.3
10 Fam For (20 µM)	0.8
10 Dos Rev (20 µM)	0.8
dH ₂ O	9.2
Qiagen Taq (5U/µl)	0.2

For combinations I, II and III, two µl (20 ng/µl) DNA was used, whereas for combination IV, four µl (20 ng/µl) was taken. PCR products were diluted by a ratio of 1:20 (5 µl PCR product in 95 µl dH₂O). A denaturing mix was prepared with 12.9 µl formamide and 0.1 µl rox500, per well. Two µl of the diluted PCR products, merged with 13 µl formamide-rox500 mix, were run on an ABI 3730. Two samples carrying a heterozygous exon 8 and 9 deletion, and two healthy samples were used as positive and negative internal controls, respectively. For each individual, each peak intensity corresponding to an exon, was compared to the other exons of the same individual, amplified in the same reaction. The resulting ratios were then normalized with the ratios of the two normal samples.

Table 4.11. *Parkin* semi-quantitative multiplex PCR assay components (a. Combination I ; b. Combination II ; c. Combination III ; d. Combination IV) (continued)

c.

Combination III exons 2/3i/9/12/TTR	Volume (μl)
Invitrogen Buffer 10X	2.5
MgCl ₂ (50 mM)	1.5
dNTP (25 mM)	0.2
2 Fam For (20 μM)	1
2 Dos Rev (20 μM)	1
3i Fam For (20 μM)	0.5
3i Dos Rev (20 μM)	0.5
9 Fam For (20 μM)	1.3
9 Dos Rev (20 μM)	1.3
12 Fam For (20 μM)	1.8
12 Dos Rev (20 μM)	1.8
*TTR Fam For (20 μM)	1.2
*TTR Dos Rev (20 μM)	1.2
dH ₂ O	7
Invitrogen Taq (5U/μl)	0.2

* TTR: transthyretin gene (reference)

d.

Combination IV promoter/exon 1/exon 9	Volume (μl)
Qiagen Buffer (10X)	2.5
dNTP (25 mM)	0.2
Q Solution	5
Ex1suz Fam For (20 μM)	1.2
Ex1atg Dos Rev (20 μM)	1.2
Prom Fam For (20 μM)	1.2
Prom Dos Rev (20 μM)	1.2
9 Fam For (20 μM)	1.2
9 Dos Rev (20 μM)	1.2
dH ₂ O	5.9
Qiagen Taq (5U/μl)	0.2

4.7.2. Exon Dosage for the Alpha-synuclein Gene

Screening for exon rearrangements in the *α-synuclein* gene was performed using a semi-quantitative multiplex PCR assay, amplifying exons 3 and 4. The following PCR conditions were applied, with the PCR components shown in Table 4.12:

Initial Denaturation	:	94°C	5 min	
Denaturation	:	94°C	30 sec	} 23 cycles
Annealing	:	51°C	45 sec	
Extension	:	72°C	2 min 30 sec	
Final Extension	:	72°C	10 min	

Table 4.12. *Alpha-synuclein* semi-quantitative multiplex PCR assay components

α-synuclein dosage	Volume (μ l)
Qiagen Buffer (10X)	2.5
MgCl ₂ (50 mM)	1.5
dNTP (25 mM)	0.2
3a Fam For (20 μ M)	1.2
3a Dos Rev (20 μ M)	1.2
4a Fam For (20 μ M)	1.2
4a Dos Rev (20 μ M)	1.2
4parkin Fam For (20 μ M)	1.2
4parkin Dos Rev (20 μ M)	1.2
*TTR Fam For (20 μ M)	0.3
*TTR inner Dos Rev (20 μ M)	0.3
Q Solution (5X)	5
dH ₂ O	2.8
Qiagen Taq (5U/ μ l)	0.2

* TTR: transthyretin gene (reference)

For semi-quantitative multiplex PCR assay of the *α -synuclein* gene, five μ l (10 ng/ μ l) DNA was used. One sample, carrying a heterozygous duplication of *α -synuclein* exon 3 and 4, and one sample, carrying a heterozygous deletion of *Parkin* exon 4, were used as positive controls. The formamide-rox500 mix and the subsequent PCR dilutions, as well as the genescan analysis were performed as described in 4.7.1.

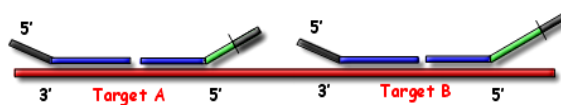
4.8. Multiplex Ligation-Dependent Probe Amplification (MLPA) Analysis

This method was first described by Schouten *et al.* in 2002. Each MLPA probe consists of two oligonucleotides, one synthetic and one M13-derived single-stranded DNA fragment. For each probe, there is a target-specific sequence that can be ligated, when correctly hybridized to its target. All probes have the same PCR primer sequences at their ends, enabling the amplification of up to 45 different hybridization probes in one PCR-based reaction. The non-hybridizing stuffer sequence of each probe has a different length and sequence, allowing separation by electrophoresis (Figure 4.2.a).

The PD-specific MLPA probe mix was added and allowed to hybridize to its respective target overnight. Probes were then ligated by a thermostable ligase. A universal primer pair was used to amplify all ligated probes (Figure 4.2.b).

This PCR reaction is very robust since only one pair of PCR primers is used for the amplification of all fragments. Amplification products ranged in size from 130 - 490bp and were analyzed by sequence type electrophoresis. The PD-specific P051 (lot 0107) and P052 (lot 1206) MLPA probe mix kits were used according to the manufacturer's recommendations (Appendix B and C).

a.



b.

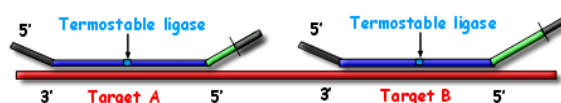


Figure 4.2. MLPA concept a. Hybridization probes, in blue, are specific to target sequences. Stuffer sequences are shown in green and PCR primer sequences are depicted in black. b. A thermostable enzyme ligates hybridization probes

4.9. Haplotype Analyses

4.9.1. Haplotyping the Alpha-synuclein Region in a Large PD Family

To identify the *α-synuclein* gene region haplotypes of a large PD family, the samples were run on an eight per cent polyacrylamide gel.

4.9.1.1. Preparation of Denaturing Polyacrylamide Gel. The 38x50 cm sequencing apparatus was assembled using 0.75 mm spacers. Hundred ml of eight per cent instagel was mixed with 625 µl of ten per cent APS and 62.5 µl TEMED, and immediately poured between two glass plates. The sharks-tooth comb was inserted in inverted position. The gel was allowed to polymerize for at least one hour before the electrophoretic run.

4.9.1.2. Denaturing Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out in 1X TBE buffer pre-heated for 15 min in a microwave oven to 40°C. The gel was pre-run in the hot buffer for 20 min at a constant power of 60-70 W to allow the gel temperature to rise to 50-55°C. The DNA samples were mixed with PAGE loading dye (15 µl PAGE loading dye for 20 µl PCR product), denatured in a thermocycler for five minutes at 96°C, and instantly disposed on ice. The comb was reoriented into the correct position to form wells, and the upper part of the gel was cleaned from urea and gel particles by rinsing the slots with electrophoresis buffer. Two µl of each sample was loaded into wells, and the gel was run at a constant power of 65 W. The electrophoresis running time was adjusted according to allele sizes and the distances between alleles.

4.9.1.3. Silver Staining. After electrophoresis, the glass plates were separated. The gel usually remained attached to one of them, and was removed with the help of a filter paper. Then it was carefully transferred to a container; after addition of staining buffer, it was shaken cautiously for five minutes. Then, the gel was washed twice with dH₂O to remove excess silver. It was then incubated in the developing buffer until bands appeared. If the bands were weakly stained, and alleles hard to discriminate, the staining procedure was

repeated. Finally, the gel was washed with dH₂O twice and was sealed in a transparent folder.

4.9.1.4. Haplotype Construction. Ten extragenic 4q microsatellite markers were used to construct the PD-linked haplotype in 12 members of the large PD family: D4S2409, D4S2622, D4S1542, D4S2371, D4S2461, D4S2304, D4S1544, D4S414, D4S2404, D4S2364 (sequences are available at the Human Genome Database, <http://www.gdb.org>).

In order to identify alleles shared among affected and unaffected members of the family, haplotypes of the 12 members, with and without *α-synuclein* gene multiplication, were constructed with Simwalk 2.91, allowing a minimum number of recombinations.

4.9.2. LRRK2 Haplotype in a Patient with G2019S

Using 20 microsatellite markers and 54 Single Nucleotide Polymorphisms (SNPs) on chromosome 12q, an extensive haplotype was established for the index patient with the G2019S mutation, her daughter and her husband, as well as for two Japanese PD patients carrying the G2019S mutation. This region spans 16 Mb, includes the PARK8 region and is delimited by the D12S2194 and D12S2520 short tandem repeat (STR) markers.

The STR markers were genotyped by multiplexing appropriate fluorescently-labeled primers. The fluorescent PCR products were then pooled for analysis on an ABI 3730 automated sequencer with Genescan 3.7 software (Applied Biosystems). Two DNA samples from CEPH reference families 1331-01 and 1331-02 were used as external standards, to control the consistency between runs.

The 54 *LRRK2* SNPs were genotyped by sequencing with the Big Dye Terminator Cycle Sequencing Ready Reaction kit and analysis with DNA Sequencing Analysis (version 5.1) and Seqscape (version 2.1.1) softwares (Applied Biosystems). Haplotypes were constructed using the Merlin program (Center for Statistical Genetics). Marker positions were described elsewhere (Kachergus *et al.*, 2005; Lesage *et al.*, 2009 in preparation).

4.10. Overall Strategy of Mutational Analysis of Five PD Genes

In the framework of this thesis, the strategy described in Table 4.13 was followed.

Table 4.13. Overall strategy of mutational analysis of five PD Genes

Dominant PD Patients		Recessive PD Patients		Sporadic PD Patients
DNA Extraction from Blood Leucocytes by “MagNA Pure® Compact Versatile Nucleic Acid Purification System” (Roche)				
PCR Amplification of the Following Genes and Exons:				
ALPHA SYNUCLEIN (exons 3 and 4)		LRRK2 (exons 19, 21, 23, 25, 27, 29, 31, 32, 33, 35, 38, 39, 41, 47 and 48)	PARKIN (12 exons + promoter)	LRRK2 (exon 41)
RE analysis: ▪ MvaI for A30P ▪ StyI for E46K ▪ Tsp45I for A53T	Analysis of Exon Rearrangements by Semi-quantitative PCR		Analysis of Exon Rearrangements (ER) by Semi-quantitative PCR	dHPLC Analysis
			2 ER	0/1 ER Checking the Number of ER
	Sequencing Analysis	Sequencing Analysis		Sequencing Analysis
Investigation of the G2019S Mutation				
Verification / Confirmation of Exon Amplifications and deletions in α-synuclein, Parkin, PINK1 and DJ1 with “Multiplex Ligation-dependent Probe Amplification” (MLPA)				
			Sequencing of PINK1 and DJ1	

4.11. Breakdown of the Turkish PD Cohort Investigated for Recessive PD Genes

In the framework of this thesis, 64 early-onset Turkish PD patients were investigated for mutations and rearrangements in *Parkin* and / or *PINK1* and / or *DJ1*. The first cohort consisted of 48, and the second cohort of 16 early-onset PD patients. The second cohort, chronologically more recent, is composed of patients who were comprehensively selected according to their clinical data. For the first cohort, a pre-screen was performed with dHLPC and only patients with aberrant profiles in the corresponding *Parkin* exons were sequenced. For this group, *Parkin* exon dosage was achieved by semi-quantitative PCR, whereas in the second group, patients were subjected to MLPA, and directly sequenced for *Parkin*. Besides, a third cohort emerged from a meticulous screen conducted among the 64 early-onset PD patients, who could not be linked to *Parkin*. Thirty-two Turkish PD patients with middle age of onset and / or compatible with recessive inheritance (positive family history) were selected for *PINK1* / *DJ1* sequencing (Figure 4.3).

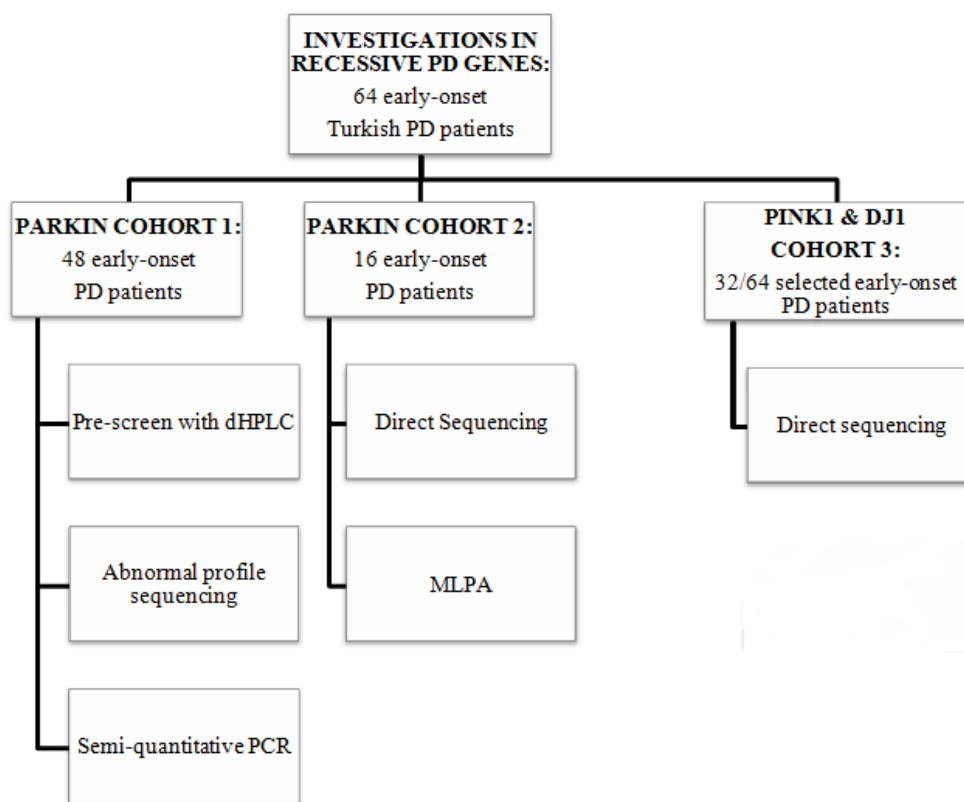


Figure 4.3. Breakdown of the Turkish PD cohort investigated for recessive PD genes

4.12. Breakdown of the Turkish PD Cohort Investigated for Dominant PD Genes

Thirty-two Turkish PD patients, compatible with a dominant inheritance pattern, were investigated for mutations and / or rearrangements in *α-synuclein* and *LRRK2* as described below (Figure 4.4).

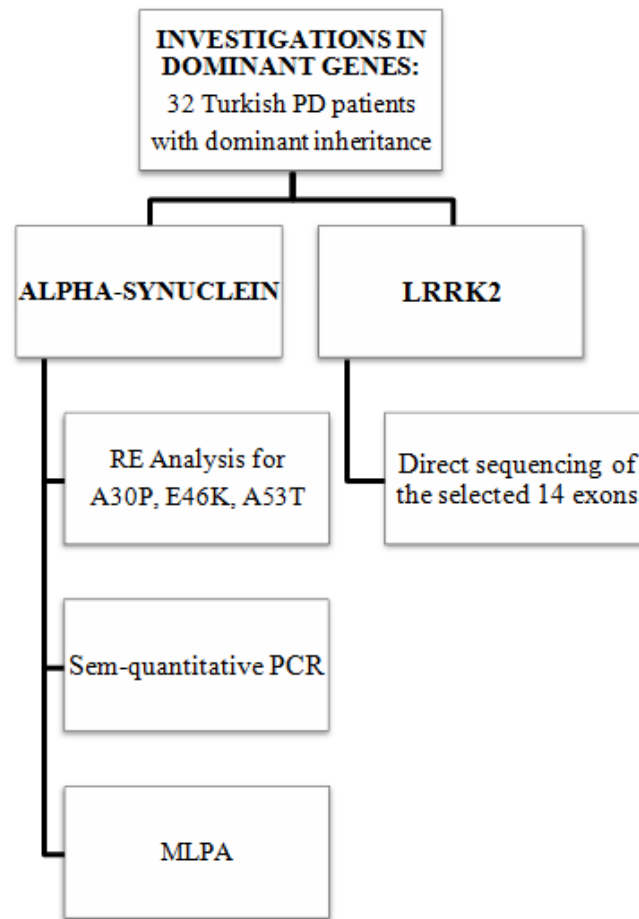


Figure 4.4. Breakdown of the Turkish PD cohort investigated for dominant PD genes

5. RESULTS

5.1. Exploring the Genetics of Recessive PD

5.1.1. dHPLC Analysis of the Parkin Gene

A cohort of 48 Turkish PD patients with early-onset PD and their 29 relatives were analyzed for exons 2, 3, 4, 6, 7, 9, 10, 11 and 12 of the *Parkin* gene using dHPLC. The promoter region and exons 1, 5 and 8, not giving conclusive results in dHPLC, were directly sequenced.

SNPs and mutations were identified by the presence of an elution profile different from the wild-type. The normalization of elution profiles was performed with eight samples at a time in order to better evaluate the tested samples and to level out the differences in DNA concentrations for each temperature applied and exon investigated. Seventy-seven samples were screened on the dHPLC with the pre-designed methods, for nine exons of the *Parkin* gene. Some of the results are compiled in Figures 5.1 and 5.2.

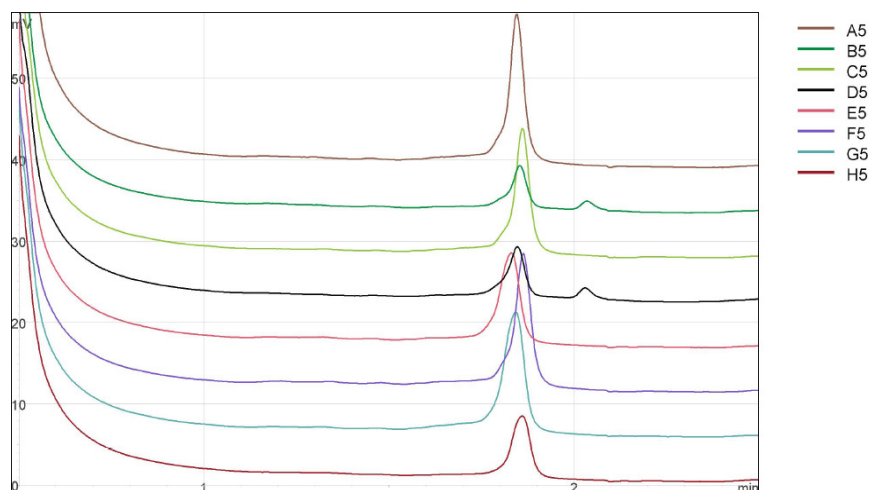


Figure 5.1. Chromatograms of eight Turkish PD samples for *Parkin* exon 2 at 62°C; individuals B5, D5, E5, G5 and H5 show a clear abnormal elution profile

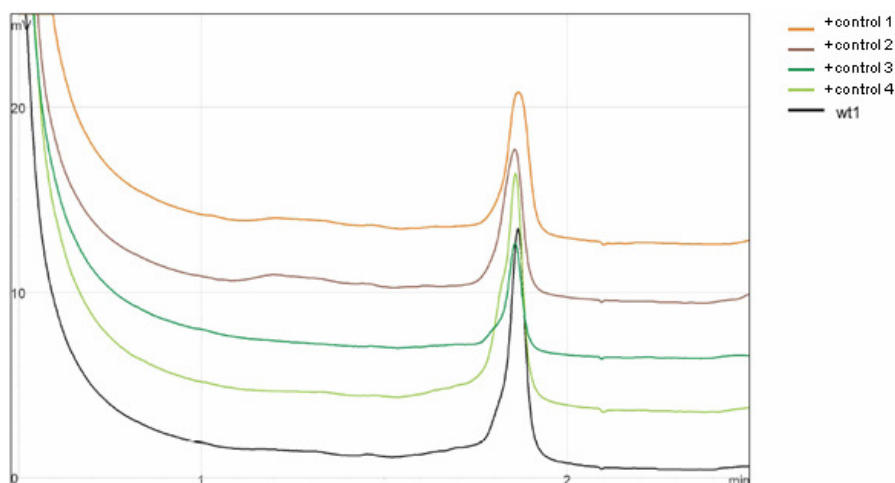


Figure 5.2. Chromatograms of the positive controls (+ controls) and of the wild-type (wt1) individual for *Parkin* exon 2 at 62°C; a sharp peak is expected for the tested individuals, as for the wt1

We were able to detect elution profiles, different from their respective wild-types, in all *Parkin* exons investigated; furthermore a great majority of our population under study revealed an unexpected profile for several exons of the *Parkin* gene (Table 5.1).

Table 5.1. Results of dHPLC screening for the *Parkin* gene in a total of 77 individuals

<i>Parkin</i> Exon	Number of patients with abnormal profiles	% of abnormal profiles
Exon 2	55	71.5%
Exon 3	29	37.7%
Exon 4	38	49.4%
Exon 6	16	20.8%
Exon 7	28	36.4%
Exon 9	32	41.6%
Exon 10	73	95%
Exon 11	45	58.5%
Exon 12	77	100%

5.1.2. Exon Dosage Analysis

5.1.2.1. Semi-quantitative Multiplex PCR of the Parkin Gene. The semi-quantitative technique detects exon rearrangements and small deletions or insertions. The intensities of the peaks obtained from GeneScan analysis were compared within the group, i.e. in relation to the other exons amplified in the same reaction. The resulting ratios were compared to the ratios of the peaks of known normal subjects, always run in parallel. An exon rearrangement was confirmed only, if all of the ratios concerning the exon were abnormal in four independent experiments. The comparison yielded the following rules:

- values of 0.6 or less were interpreted as a heterozygous deletion of an exon,
- values of 0.8 to 1.2 were interpreted as normal,
- values of 1.3 to 1.7 were interpreted as a heterozygous duplication, and
- values of 1.8 to 2.2 were interpreted as a homozygous duplication.

The same cohort of 48 Turkish PD patients with early-onset PD and their 29 relatives who were subjected to dHPLC analysis, were investigated in parallel, for exon deletion or multiplication of the *Parkin* gene; these results were further confirmed by MLPA analysis:

- a heterozygous deletion of exons 3 and 4 in two siblings,
- a heterozygous deletion of exon 2 in two siblings, and
- a heterozygous duplication of exons 7, 8 and 9 have been identified (Figure 5.3).

5.1.2.2. Multiplex Ligation-dependent Probe Amplification (MLPA). MLPA analysis was performed with the Parkinson-P051 and -P052 kits independently, to investigate genetic variations of 16 other samples with early-onset PD. MLPA has the advantage of being sensitive and robust and was therefore applied only once. The data analysis was achieved as for the semi-quantitative PCR. With this method, we were able to describe four different rearrangements on the *Parkin* gene (Figure 5.4):

- a heterozygous deletion of *Parkin* exons 2, 3, and 4;
- a homozygous deletion of *Parkin* exons 3 and 4;
- a homozygous deletion of *Parkin* exons 5, 6, and 7;
- a homozygous deletion of *Parkin* exon 5 in two siblings; and
- a heterozygous deletion of *Parkin* exon 2.

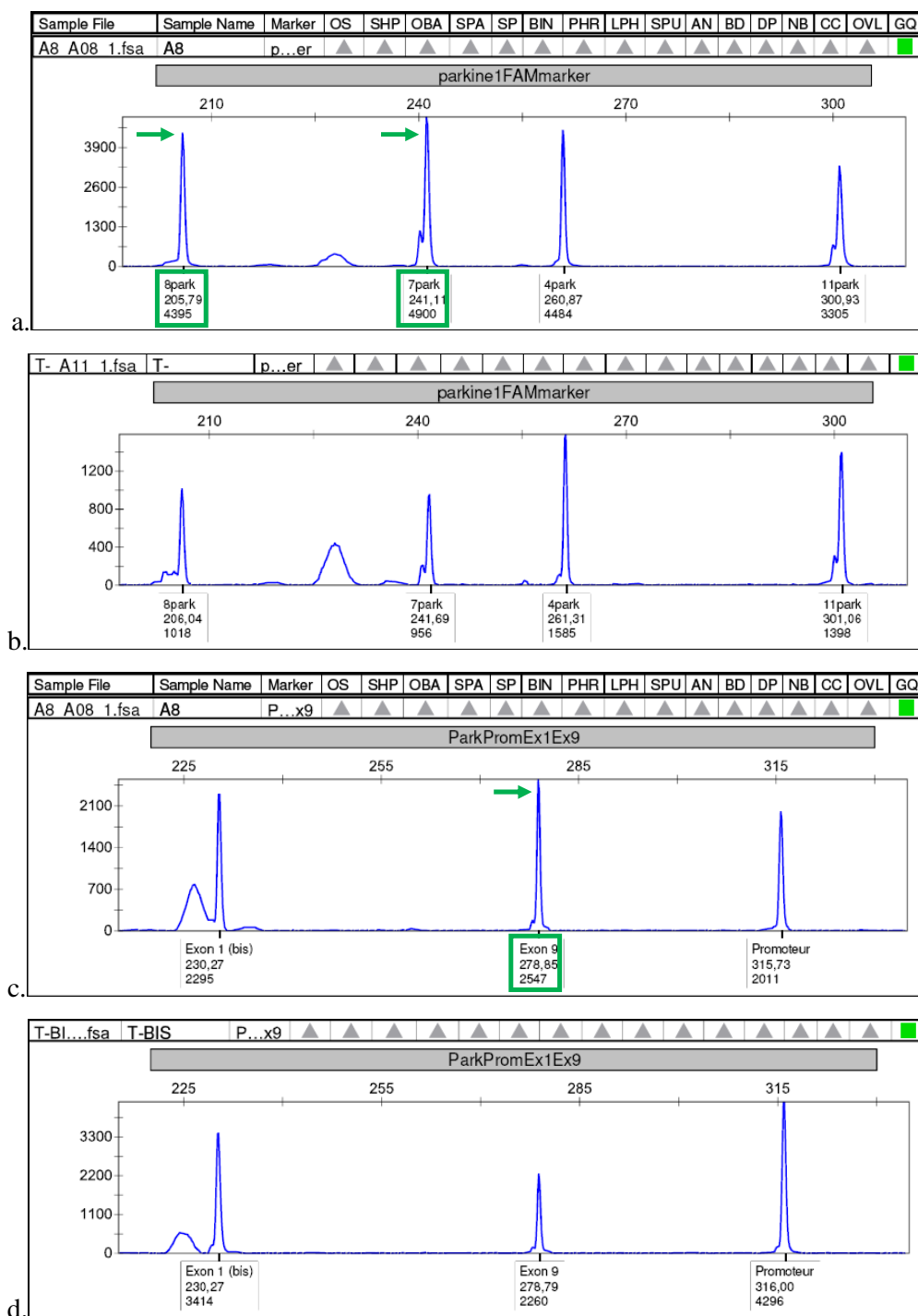


Figure 5.3. Four panels analyzed with GeneMapper 3.5 software; a and c: a Turkish PD patient is shown to have a heterozygous duplication of *Parkin* exons 7, 8 and 9, when compared to the negative controls (b and d)

No rearrangements were found for the two other recessive PD-linked genes, *PINK1* and *DJ1*, with the MLPA technique.

5.1.3. Sequencing Analyses

5.1.3.1. *Parkin*. After the pre-screen performed with dHPLC, samples with unexpected elution profiles were sequenced for the detection of genetic variants in the *Parkin* gene. The promoter region, exons 1, 5 and 8, inappropriate for dHPLC analysis, were directly sequenced for the 48 early-onset PD samples and their 29 relatives. Moreover, another 16 early-onset PD samples were included to the study population and subjected to sequencing for all exons of the *Parkin* gene and the promoter region (Table 5.2).

The variations found in the sequences analyzed were primarily intervening sequence variations (IVS). The IVS2-18 was found to be a single base insertion (+A), while the other eight variations detected were single nucleotide substitutions. Out of 137 variants identified, only four were exonic:

- Three of them were silent substitutions, having a minor effect on amino-acid class:
 - Ser167Asn: c.601G>A (exon 4) [rs1801474],
 - Val380Leu: c.1239G>C (exon 10) [rs1801582],
 - Asp394Asn: c.1281G>A (exon 11) [rs1801334].
- Another sample had a pathogenic mutation in exon 2: Val56Glu:c.268T>A (Hoenicka *et al.*, 2002). This pathogenic variant was described in heterozygous state in a female PD patient and her asymptomatic mother (Figure 5.5).

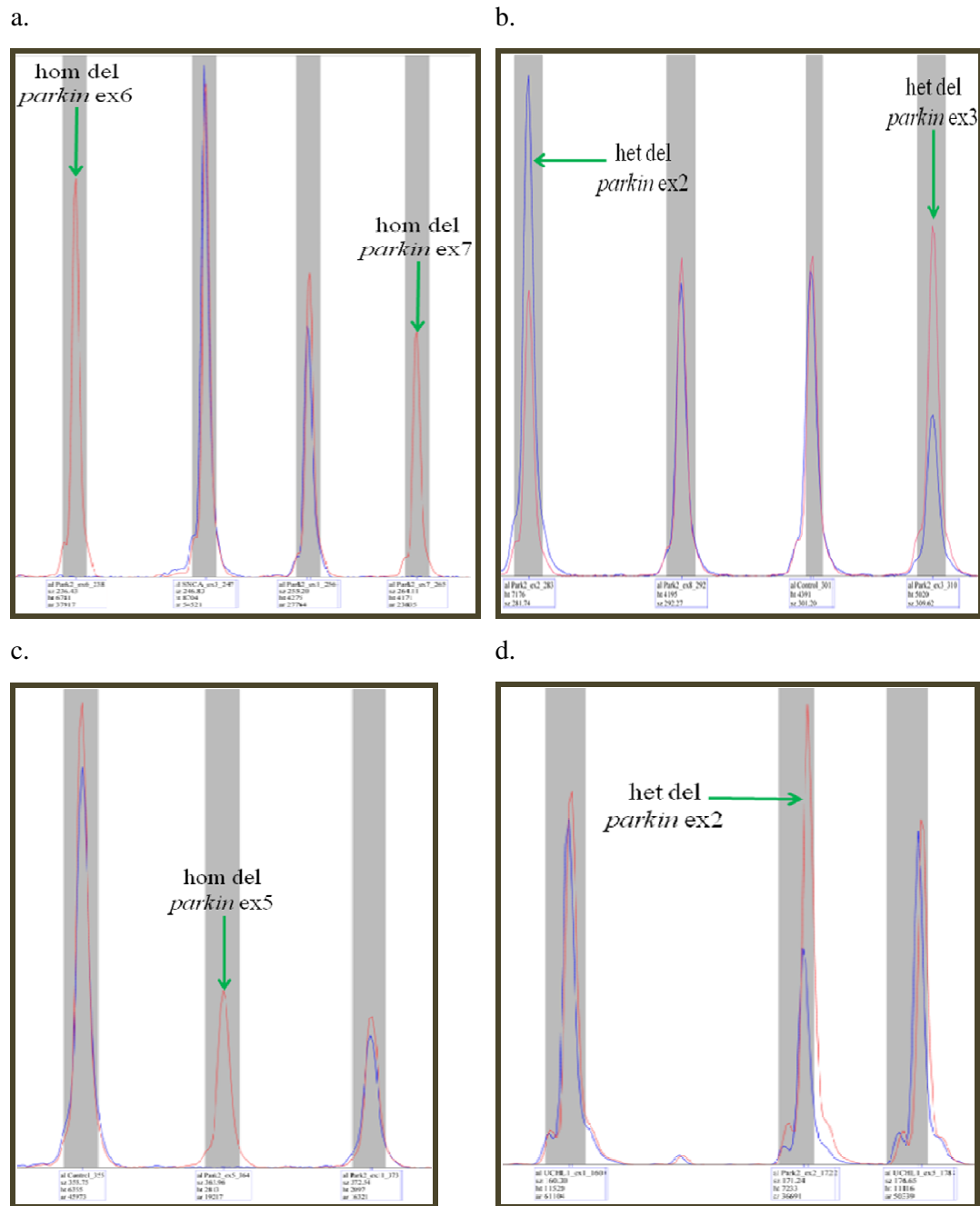


Figure 5.4. Four panels analyzed with GeneMapper 4.0 software; a, b, c are results obtained with MLPA P051 and d is obtained with MLPA P052. When compared to healthy controls (red line), four independent Turkish PD patients (blue line), are shown to have:

- a. homozygous deletion of *Parkin* exons 6 and 7 (data not shown for exon 5);
- b. heterozygous deletion of *Parkin* exons 2 and 3 (data not shown for exon 4);
- c. homozygous deletion of *Parkin* exon 5; d. heterozygous deletion of *Parkin* exon 2

(het: heterozygous, hom: homozygous, del: deletion)

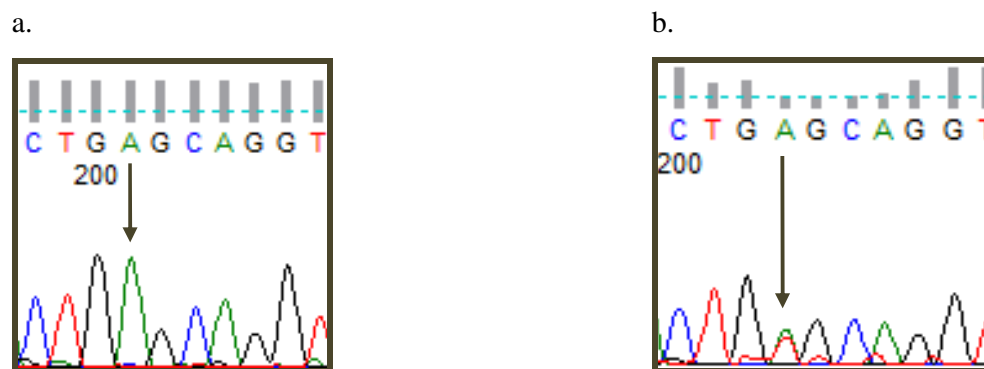


Figure 5.5. *Parkin* exon 2 sequencing chromatograms showing the Val56Glu mutation of the PD patient (a) having also a heterozygous deletion of *Parkin* exon 2 (compound heterozygous) and of her asymptomatic mother (b); the T>A substitution is pointed by an arrow

All sequencing results for the *Parkin* gene are summarized in Table 5.2. Clinical data of the Turkish PD patients with rearrangements and / or mutations in the *Parkin* gene are listed in Table 5.3.

Table 5.2. Thirteen variations either in heterozygous or homozygous condition were found in the *Parkin* gene; no sequence changes were detected for exons 1, 5, 7, 9 and 12

<i>Parkin</i> Exons	Number of <u>Heterozygous</u> Variations	Number of <u>Homozygous</u> Variations	Variation
Promoter	8	2	-227A>G
	20	4	-258T>G
Exon 2	<i>1</i>	-	<i>Val56Glu:c.268T>A*</i>
	15	-	IVS2+25T>C
Exon 3	1	-	IVS2-18insA
Exon 4	4	-	Ser167Asn:c.601G>A
Exon 6	1	-	IVS6+26C>T

Table 5.2. Thirteen variations either in heterozygous or homozygous condition were found in the *Parkin* gene; no sequence changes were detected for exons 1, 5, 7, 9 and 12

(continued)

<i>Parkin</i> Exons	Number of <u>Heterozygous</u> Variations	Number of <u>Homozygous</u> Variations	Variation
Exon 8	17	4	IVS7-35A>G
	8	-	IVS7-68G>C
	21	6	IVS8+48C>T
Exon 10	12	2	Val380Leu:c.1239G>C
	7	-	IVS10+159C>G
Exon 11	4	-	Asp394Asn:c.1281G>A

* This is the only pathogenic *Parkin* mutation found in the Turkish PD cohort

5.1.3.2. *PINK1*. Out of 64 early-onset PD patients, who could not be linked to *Parkin*, 32 with middle age of onset and / or compatible with recessive inheritance (positive family history) were selected for sequencing of the eight exons of the *PINK1* gene. Out of 42 variants described, only one was a pathogenic mutation. The Ala339Thr conversion was identified in two independent Turkish PD patients (Figure 5.6). The sequencing results are compiled in Table 5.4. Clinical data of the two *PINK1*-linked PD patients are compiled in Table 5.5.

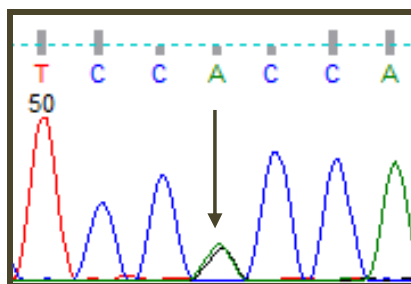


Figure 5.6. A *PINK1* exon 5 sequencing chromatogram showing the heterozygous Ala339Thr mutation; the G>A substitution is pointed by an arrow

Table 5.3. Overview of the clinical descriptions of the Turkish PD patients linked to *Parkin* (UPDRS on: Unified Parkinson's Disease Rating Scale on medication; het: heterozygous; hom: homozygous; del: deletion; NA: not available)

	PD117	PD141	PD167	PD193	PD206	PD211	PD212
Age at onset (years)	14	36	24	22	25	21	12
First degree relative affected	+	+	+	+	+	-	+
Consanguinity	-	-	-	+	+	+	-
Sex	M	M	M	M	M	F	F
Disease duration (years)	10	16	21	14	27	3	28
Signs at onset	rigidity	tremor	tremor	tremor	bradykinesia	dystonia	dystonia
Clinical signs at examination							
Rigidity	+	+	+	+	+	+	+
Bradykinesia	+	+	+	+	+	+	+
Resting tremor	+	+	+	+	-	+	+
Treatment							
*L-Dopa treatment duration (months)	43	188	36	132	123	24	135
*Improvement with L-Dopa ($\geq 30\%$)	+	+	+	+	+	+	+
*Dyskinesia	-	+	+	+	+	-	-
UPDRS on	11	NA	13	20	17	3	22
<i>Parkin</i> rearrangement	het del exons 3-4	het del exon 2	het dup exons 7-8-9	hom del exons 5-6-7	hom del exon 5	het del exon 2	het del exons 2-3-4
<i>Parkin</i> sequencing						Val56Glu	

Table 5.4. Five variations in heterozygous or homozygous condition were found in the *PINK1* gene; no sequence changes were found in exons 3, 4, 6, 7, and 8

<i>PINK1</i> Exons	Number of <u>Heterozygous</u> Variations	Number of <u>Homozygous</u> Variations	Variation
Exon 1	3	-	Arg4Arg:c.10C>A
	9	1	Leu63Leu:c.189C>T
Exon 2	12	14	IVS1-7A>G
Exon 5	1	-	IVS4-5G>A
	2	-	<i>Ala339Thr:c.1018G>A*</i>

* This is the only pathogenic *PINK1* mutation found in the Turkish PD cohort

Table 5.5. Clinical descriptions of the Turkish PD patients with *PINK1* mutations (UPDRS on: Unified Parkinson's Disease Rating Scale on medication; ND: not determined)

	PD205	PD227
Age at onset (years)	38	33
First degree relative affected	-	-
Consanguinity	-	+
Sex	M	M
Disease duration (years)	5	4
Signs at onset	tremor	bradykinesia
Clinical signs at examination		
Rigidity	+	+
Bradykinesia	+	+
Resting tremor	+	+
Treatment		
L-Dopa treatment duration (months)	60	24
Improvement with L-Dopa ($\geq 30\%$)	+	+
Dyskinesia	-	+
UPDRS on	11	ND
<i>PINK1</i> sequencing	Ala339Thr (het)	Ala339Thr (het)

5.1.3.3. *DJI*: The same 32 early-onset PD patients with middle age of onset and / or compatible with recessive inheritance (positive family history), who were selected for *PINK1* gene sequencing, were also analyzed for mutations and polymorphisms in the seven exons of the *DJI* gene. The results are summarized in Table 5.6.

Table 5.6. Four variations in heterozygous or homozygous condition were found in exons 3, 4 and 6 of the *DJI* gene; no sequence changes were detected in the other exons

<i>DJI</i> Exons	Number of <u>Heterozygous</u> Variations	Number of <u>Homozygous</u> Variations	Variation
Exon 3	4	-	IVS3-110C>T
Exon 4	5	-	IVS5+30T>G
Exon 6	19	4	IVS6-216G>A
	1	-	IVS6-251C>A

5.2. Exploring the Genetics of Dominant PD

5.2.1. Investigating the Alpha-synuclein Gene

5.2.1.1. RE Analysis. The *α -synuclein* gene was investigated for the three rare point mutations A53T, A30P and E46K by making use of their restriction enzyme recognition sites (Polymeropoulos *et al.*, 1997; Krüger *et al.*, 1998; Zarranz *et al.*, 2004). For this purpose, 32 Turkish PD patients, compatible with a dominant inheritance pattern, were selected and subjected to RE analysis with Tsp45I (A53T), MvaI (A30P) and StyI (E46K). No variations were observed. An example of restriction profiles is shown in Figure 5.7.

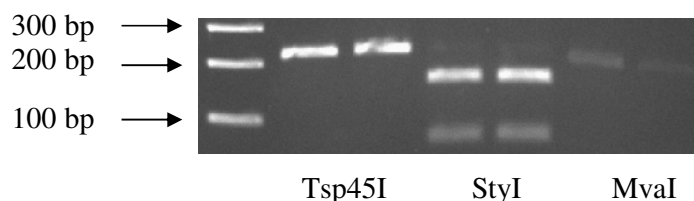


Figure 5.7. Digestion profiles of Tsp45I, StyI and MvaI, for two different samples. Tsp45I and MvaI did not digest the fragments, indicating the absence of A53T and A30P mutations, respectively; StyI restriction yielded two fragments, showing also an absence of the E46K mutation

5.2.1.2. Exon Dosage Analyses with Semi-quantitative Multiplex PCR and MLPA. The same 32 Turkish PD patients, with a dominant inheritance pattern, who were previously subjected to RE analysis, were analyzed for *α-synuclein* duplications and triplications with semi-quantitative multiplex PCR and with the MLPA method (P051 kit). A heterozygous duplication of *α-synuclein* was identified in a sibpair from Trabzon, in the Black Sea region of Turkey (Table 5.7); with the methods mentioned above (Figure 5.8 and Table 5.8). This duplication, definitely covering exons 3 and 4, will be referred in this thesis, as *α-synuclein* exons 3 and 4 duplication, as its boundaries have to be further investigated by haplotype analysis.

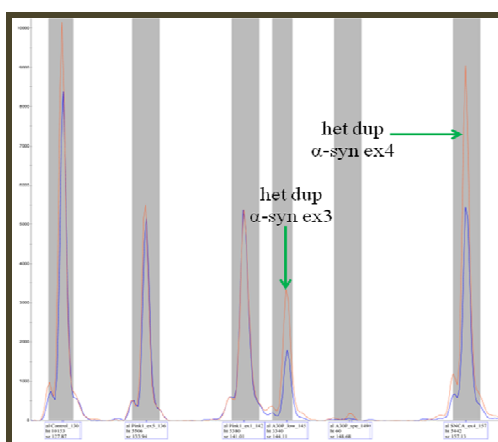


Figure 5.8. Result from MLPA P051 analysis with GeneMapper 4.0 software; when compared to the healthy control (blue line), patient PD169 (red line) was found to have a heterozygous duplication of *α-synuclein* exons 3 and 4

Table 5.7. Clinical description of the Turkish PD patient with the heterozygous duplication of *α-synuclein* exons 3 and 4 (UPDRS on: Unified Parkinson's Disease Rating Scale on medication)

	PD171
Age at onset (years)	51
First degree relative affected	+
Consanguinity	-
Sex	F
Disease duration (years)	7
Signs at onset	bradykinesia, tremor
Clinical signs at examination	
Rigidity	+
Bradykinesia	+
Resting tremor	+
Treatment	
L-Dopa treatment duration (months)	60
Improvement with L-Dopa ($\geq 30\%$)	+
Dyskinesia	+
UPDRS on	7
Rearrangement	Heterozygous duplication of <i>α-synuclein</i> exons 3-4

Table 5.8. Quantification and normalization of *α-synuclein* exons 3 and 4 for PD169 and PD171, using GeneMapper 3.5 software. Exons 3 and 4 of this gene were amplified with exon 4 of the *Parkin* and *Transthyretin* genes (TTR). The number of copies of *α-synuclein* exons was determined from the ratios of mutant to control alleles, compared to ratios obtained on two control subjects (T-, T-bis). A patient with a known heterozygous deletion of *Parkin* exon 4 and a patient with a known heterozygous duplication of *α-synuclein* exons 3 and 4 were used. Values below 0.6 were considered to indicate an individual with a heterozygous deletion of an exon, between 0.8 and 1.2, a normal individual, between 1.3 and 1.7, a heterozygous duplication

	Peak heights				Ratios							
	3a	4a	TTR	4Park	TTR /3a	TTR /4a	TTR /4Park	3a /4a	3a /4Park	4a /4Park	4Park /3a	4Park /4a
T-	6709	13518	8316	10390	1.28	0.61	0.81	0.48	0.63	1.32	1.59	0.76
T-bis	7022	13795	8341	10051	1.26	0.61	0.82	0.48	0.66	1.36	1.53	0.74
4Park het del	6870	12915	7628	4786	1.10	1.02	0.51	0.93	0.47	0.50	2.15	2.00
3a4a het dup	9929	19792	8491	10779	1.47	1.39	1.04	0.95	0.71	0.75	1.42	1.34
PD169	14666	25106	11279	13008	1.65	1.35	0.94	0.82	0.57	0.69	1.76	1.44
PD171	2119	3782	1611	1862	1.67	1.43	0.94	0.86	0.56	0.66	1.78	1.52

5.2.1.3. Haplotyping a Large Family from Trabzon. DNA samples of 20 family members of the sibpair from Trabzon, in whom a heterozygous duplication of *α-synuclein* exons 3 and 4 was identified, were collected (Figure 5.9). Informed written consent for participation was obtained from each individual and genetic counseling had been given by the medical school.

When semi-quantitative PCR and MLPA analyses were performed, as previously described, six asymptomatic family members were found to carry the heterozygous duplication of *α-synuclein* exons 3 and 4. In order to determine the PD-associated haplotype in this large Turkish family, 17 microsatellite markers distributed on a 11 Mb region in the 4q21.3 - 4q22.3 *α-synuclein* locus were selected (Figure 5.10). Twelve family members, either duplication carriers or controls, were included in the haplotyping study. An example of PAGE analysis is shown in Figure 5.11.

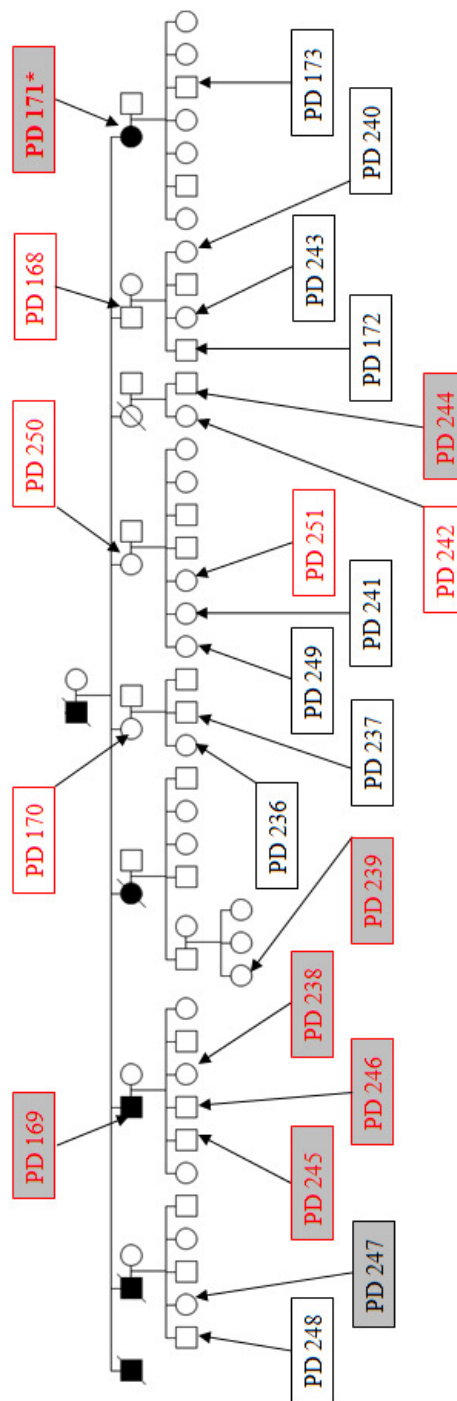


Figure 5.9. Pedigree of the large Turkish family from Trabzon. The specified 22 samples available for the semi-quantitative multiplex PCR and MLPA analyses are indicated in boxes; all individuals investigated for the α -synuclein haplotype are shown in red boxes. The individuals carrying the heterozygous duplication of α -synuclein exons 3 and 4 are further highlighted in grey. PD 171* is the index case

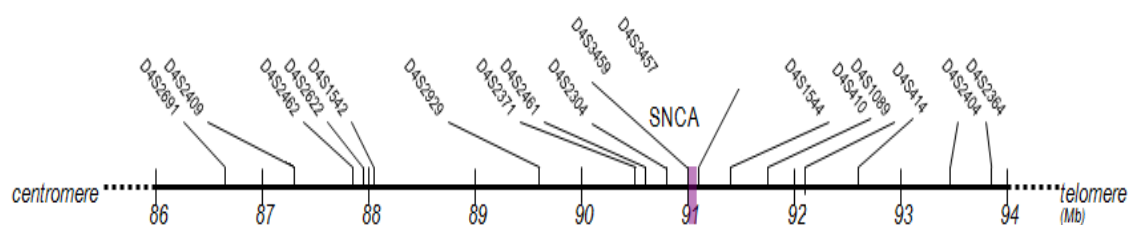


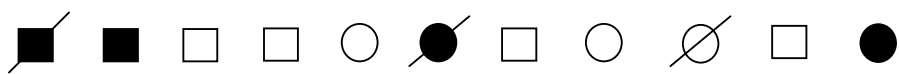
Figure 5.10. Chromosome 4 centromeric and telomeric microsatellite markers used for haplotyping the α -synuclein (SNCA) gene region; of the 17 microsatellite markers, chosen for this study, only two (D4S3457 and D4S3459) were intragenic

Out of the 17 markers used in the α -synuclein haplotype study, seven markers were found to be non-informative; therefore the haplotype was constructed with the results of ten microsatellite markers. Five individuals, carrying the exons 3 and 4 duplication, were shown to have the same haplotype on a 11 Mb region in the α -synuclein locus (Figure 5.12 and 5.13). The haplotyping results are compiled in Table 5.9.



Sample	Allele
PD168	1 / 3
PD169	2 / 4
PD170	1 / 3
PD171	2 / 4
PD238	2 / 2
PD239	1 / 2
PD242	1 / 4
PD244	1 / 2
PD245	2 / 2
PD246	2 / 2
PD250	1 / 3
PD251	1 / 3

Figure 5.11. PAGE analysis of the D4S2404 microsatellite marker; bands correspond to the alleles and samples designated below



Haplotype ID	8	12	27	28	29	13	33	39	16	77	18
PD #		<u>169</u>	<u>245</u>	<u>246</u>	<u>238</u>			<u>239</u>		<u>244</u>	<u>171</u>
D4S2409	2 <i>I</i>	<u>2</u> <i>1</i>	<u>1</u> <u>2</u>	<u>1</u> <u>2</u>	<u>1</u> <u>2</u>	<i>I</i> <i>2</i>	<i>I</i> <i>I</i>	<u>1</u> <u>2</u>	<i>I</i> <i>2</i>	<u>2</u> <i>1</i>	<u>1</u> <u>2</u>
D4S2622	3 <i>I</i>	<u>1</u> <u>1</u>	<u>1</u> <u>2</u>	<u>1</u> <u>2</u>	<u>1</u> <u>2</u>	<i>I</i> <i>I</i>	<i>I</i> <i>I</i>	<u>1</u> <u>1</u>	<i>I</i> <i>2</i>	<u>1</u> <i>1</i>	<u>1</u> <u>1</u>
D4S1542	<i>I</i> <i>I</i>	<u>1</u> <u>1</u>	<u>1</u> <u>1</u>	<u>1</u> <u>1</u>	<u>1</u> <u>1</u>	<i>I</i> <i>I</i>	<i>I</i> <i>I</i>	<u>1</u> <u>1</u>	<i>I</i> <i>I</i>	<u>1</u> <i>1</i>	<u>1</u> <u>1</u>
D4S2371	2 <i>I</i>	<u>2</u> <i>1</i>	<u>1</u> <u>2</u>	<u>1</u> <u>1</u>	<u>1</u> <u>2</u>	<i>I</i> <i>I</i>	<i>2</i> <i>I</i>	<u>2</u> <u>2</u>	<i>I</i> <i>2</i>	<u>2</u> <i>1</i>	<u>1</u> <u>2</u>
D4S2461	2 <i>I</i>	<u>2</u> <i>1</i>	<u>1</u> <u>2</u>	<u>1</u> <u>2</u>	<u>1</u> <u>2</u>	<i>I</i> <i>2</i>	<i>I</i> <i>I</i>	<u>1</u> <u>2</u>	<i>I</i> <i>2</i>	<u>2</u> <i>1</i>	<u>1</u> <u>2</u>
D4S2304	2 <i>2</i>	<u>3</u> <u>2</u>	<u>2</u> <u>3</u>	<u>2</u> <u>3</u>	<u>2</u> <u>3</u>	<i>2</i> <i>I</i>	<i>I</i> <i>2</i>	<u>1</u> <u>2</u>	<i>2</i> <i>3</i>	<u>1</u> <u>2</u>	<u>2</u> <u>3</u>
D4S1544	4 <i>3</i>	<u>1</u> <u>3</u>	<u>3</u> <u>1</u>	<u>3</u> <u>1</u>	<u>3</u> <u>1</u>	<i>3</i> <i>2</i>	<i>3</i> <i>3</i>	<u>3</u> <u>4</u>	<i>3</i> <i>I</i>	<u>1</u> <u>3</u>	<u>3</u> <u>1</u>
D4S414	3 <i>I</i>	<u>3</u> <u>1</u>	<u>1</u> <u>4</u>	<u>1</u> <u>4</u>	<u>1</u> <u>4</u>	<i>I</i> <i>3</i>	<i>I</i> <i>I</i>	<u>1</u> <u>2</u>	<i>I</i> <i>3</i>	<u>3</u> <i>1</i>	<u>1</u> <u>3</u>
D4S2404	3 <i>2</i>	<u>4</u> <u>2</u>	<u>2</u> <u>2</u>	<u>2</u> <u>2</u>	<u>2</u> <u>2</u>	<i>2</i> <i>I</i>	<i>I</i> <i>2</i>	<u>1</u> <u>2</u>	<i>2</i> <i>4</i>	<u>1</u> <u>2</u>	<u>2</u> <u>4</u>
D4S2364	2 <i>I</i>	<u>2</u> <i>1</i>	<u>1</u> <u>2</u>	<u>1</u> <u>1</u>	<u>1</u> <u>2</u>	<i>I</i> <i>I</i>	<i>2</i> <i>I</i>	<u>2</u> <u>2</u>	<i>I</i> <i>2</i>	<u>2</u> <i>1</i>	<u>2</u> <u>2</u>

Figure 5.12. Haplotype data of the affected members in the large family from Trabzon (with PD # underlined); the program infers the other haplotypes shown in italic. The disease-associated haplotype is shown in red

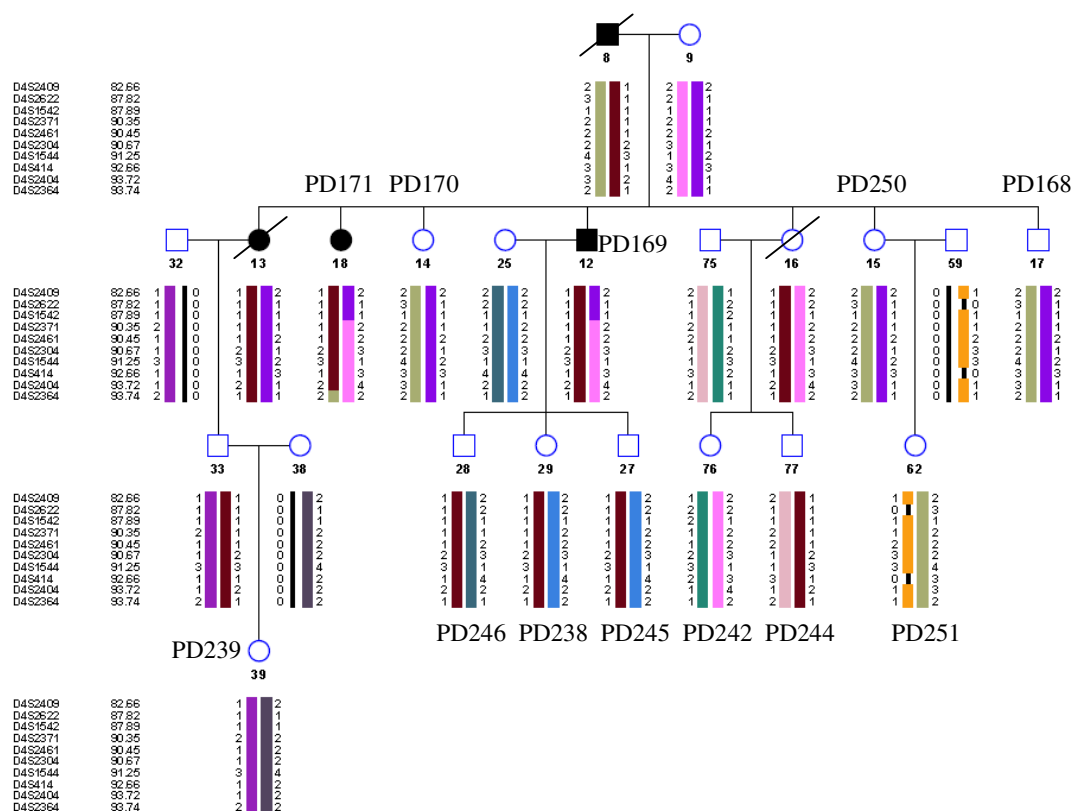


Figure 5.13. Simplified pedigree and haplotypes of the large family from Trabzon for chromosome 4q. The disease-associated haplotype is shown in red

5.2.2. Investigating the LRRK2 Gene

5.2.2.1. Sequencing Analysis. The same cohort of 32 Turkish PD patients, with a dominant inheritance pattern, was sequenced for *LRRK2* exons 19, 21, 23, 25, 27, 29, 31, 32, 33, 35, 38, 39, 47 and 48. As *LRRK2* consists of 51 exons, only those harboring putatively pathogenic amino-acid substitutions or amino-acid substitutions segregating with the disease were analyzed. One novel heterozygous mutation (Ser1508Arg) was identified in exon 31 of the *LRRK2* gene, in a female PD patient with age of onset 44 (Figure 5.14 and Table 5.10). The Ser1508Arg mutation was not found in neither of the 46 healthy Turkish samples (mean age: 42.5 ± 13.1). Moreover, 48 polymorphisms were described on the *LRRK2* gene, two being exonic; the results are summarized in Table 5.11.

Table 5.9. Summary of the data obtained by semi-quantitative PCR, MLPA and haplotype analyses on the large family from Trabzon (het dup syn ex3-4: heterozygous duplication of α -synuclein exons 3 and 4)

PD Samples	Year of Birth	Generation	Risk	Semi-Quantitative PCR & MLPA Analyses	Haplotype Analysis
PD168	1948	I st	50%	<i>healthy</i>	<i>confirmed</i>
PD169	1933			het dup syn ex3-4	<i>confirmed</i>
PD170	1936			<i>healthy</i>	<i>confirmed</i>
PD171	1950			het dup syn ex3-4	<i>confirmed</i>
PD250	1940			<i>healthy</i>	<i>confirmed</i>
PD248	1954	II nd	50%	<i>healthy</i>	-
PD247	1955			het dup syn ex3-4	-
PD245	1961	II nd	50%	het dup syn ex3-4	<i>confirmed</i>
PD246	1964			het dup syn ex3-4	<i>confirmed</i>
PD238	1967			het dup syn ex3-4	<i>confirmed</i>
PD236	1965	II nd	none	<i>healthy</i>	-
PD237	1966				-
PD249	1960	II nd	none	<i>healthy</i>	-
PD241	1962				-
PD251	1964				<i>confirmed</i>
PD242	?	II nd	25%	<i>healthy</i>	<i>confirmed</i>
PD244	?			het dup syn ex3-4	<i>confirmed</i>
PD172	1969	II nd	none	<i>healthy</i>	-
PD243	1970				-
PD240	1983				-
PD173	1980	II nd	50%	<i>healthy</i>	-
PD239	1982	III rd	25%	het dup syn ex3-4	<i>healthy?</i>

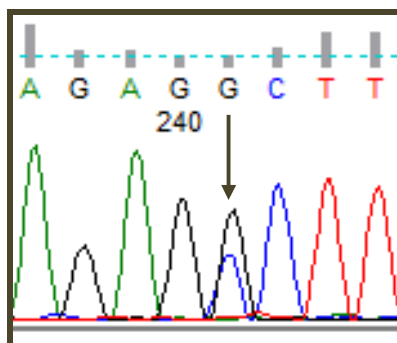


Figure 5.14. Sequencing chromatogram showing the heterozygous Ser1508Arg mutation; the C>G substitution is pointed by an arrow

Table 5.10. Clinical description of the Turkish PD patient with the *LRRK2* Ser1508Arg mutation (UPDRS on: Unified Parkinson's Disease Rating Scale on medication)

	PD124
Age at onset (years)	44
First degree relative affected	+
Consanguinity	-
Sex	F
Disease duration (years)	8
Signs at onset	bradykinesia tremor
Clinical signs at examination	
Rigidity	+
Bradykinesia	+
Resting tremor	+
Treatment	
L-Dopa treatment duration (months)	100
Improvement with L-Dopa ($\geq 30\%$)	-
Dyskinesia	-
UPDRS on	9
<i>LRRK2</i> sequencing	Heterozygous Ser1508Arg

Table 5.11. Eleven variations described in the *LRRK2* gene exons 19, 29, 31, 35, 38, 48; no sequence changes were found in the other exons

<i>LRRK2</i> Exons	Number of <u>Heterozygous</u> Variations	Number of <u>Homozygous</u> Variations	Variation
Exon 19	1	-	IVS19-25C>T
Exon 29	2	-	IVS29-105C>T
	1	-	IVS29-108C>T
	-	2	IVS29-170A>G
	1	-	Leu1325Leu:c.3976T>C
Exon 31	1	-	Ser1508Arg:c.4524C>G
Exon 35	14	7	IVS35+23T>A
Exon 38	3	-	IVS38+33G>A
Exon 48	1	-	IVS47-43A>G
	-	12	IVS47-9delT
	4	-	Gly2385Gly:c.7155A>G

* This is the novel pathogenic *LRRK2* mutation found in the Turkish PD cohort

5.2.2.2. Sequencing *LRRK2* Exon 41. A large cohort of Turkish PD patients was screened for the G2019S mutation. Direct sequencing of exon 41 in 78 PD patients (52 familial forms, including 32 with autosomal dominant inheritance and 20 with autosomal recessive inheritance, plus 26 isolated cases) and their 63 relatives, revealed a G2019S mutation in one female patient from Kastamonu, in North Western Turkey (Figure 5.14). Further genetic analyses in this family revealed the presence of the G2019S mutation in her 39-years-old clinically unaffected daughter, but surprisingly, not in the two female cousins of the index case, who were already bed-ridden with severe PD (Figure 5.15). Genetic counseling had been offered and written informed consent was obtained.

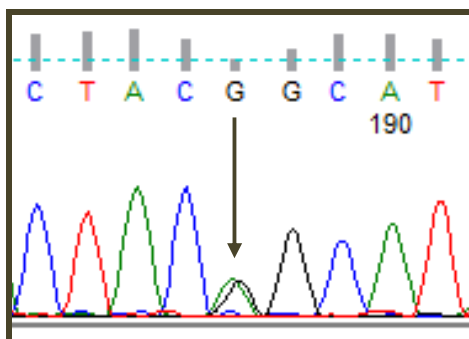


Figure 5.15. Sequencing chromatogram showing the heterozygous G2019S mutation; the G>A substitution is pointed by an arrow

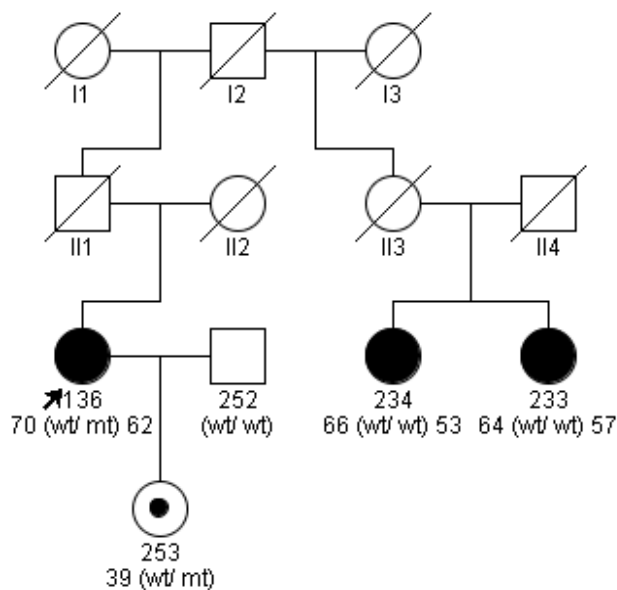


Figure 5.16. Simplified pedigree of the Turkish index patient (PD136) with the G2019S mutation; age at examination, genotype and age at onset are indicated under each individual investigated (wt: wild-type; mt: mutant)

5.2.2.3. Clinical Data of the Index Patient PD136 with the G2019S Mutation. The 70-year-old index case experienced left-sided resting leg tremor at the age of 60. PD diagnosis was made according to UK brain bank criteria. In ten years, she developed an asymmetric bilateral moderate bradykinesia, resting tremor, and rigidity more prominent on her left side. Her UPDRS score is 35, her symptoms still respond to the dopaminergic treatment, but she developed a “wearing off” phenomenon (predictable “off”) and has non-disturbing mild and inconsistent dyskinesias. She has no autonomic involvement, and her cognition is intact. She has minor depression according to DSM-IV. Her SMMT score is 28. She is

prescribed L-Dopa / carbidopa / entecapone (500mg / 125mg / 1000mg) /day and pridedil 150 mg/day.

5.2.2.4. Haplotyping the Patient with G2019S. Using 74 markers (20 microsatellites and 54 SNPs) within the PARK8 locus, the haplotype associated with the G2019S mutation was reconstructed in the index patient (PD136), her clinically unaffected daughter (PD253) and two Japanese PD patients (1107 and 1108). The investigation of the haplotype of our patient revealed an unexpected similarity to the Japanese patients, rather than to European, Jewish, or North African cases.

As shown in Table 5.12, the Turkish haplotype is identical to that found in two Japanese patients with G2019S on a large region of 8.3 Mb (from rs2128274 to rs10748075). To the best of our knowledge, it is the first time that the G2019S-associated Japanese haplotype has been reported in a different population than the Japanese (Pirkevi *et al.*, 2009; Lesage *et al.*, 2009, in preparation).

Table 5.12. G2019S-associated haplotypes in the Turkish family (136 / 253) compared with haplotypes 1, 2 and the haplotype found in Japanese cases (1107 / 1108). The homologous region between the Turkish and Japanese haplotypes is shown in bold and gray font. Both alleles are shown when the phase could not be unambiguously determined

Marker	Physical Map	Turkey 136 /253	Japan 1107 /1108	Haplotype 1	Haplotype 2
rs12823555	30750798	C	C	C	T
rs17458299	30751046	T	T	T	T
rs10082797	30751064	A	G	G	A
rs17442304	30751096	T	T	T	T
rs7302006	30779263	C	G	G	G
rs2896905	30779511	G	G	G	G
rs1482286	30779686	A	G	G	A
rs2128274	30808350	C	C	G	G
rs2128273	30808351	A	A	G	G
rs2046933	30808429	T	T	C	C

Table 5.12. G2019S-associated haplotypes in the Turkish family (136 / 253) compared with haplotypes 1, 2 and the haplotype found in Japanese cases (1107 / 1108). The homologous region between the Turkish and Japanese haplotypes is shown in bold and gray font. Both alleles are shown when the phase could not be unambiguously determined
(continued)

Marker	Physical Map	Turkey 136/ 253	Japan 1107/ 1108	Haplotype 1	Haplotype 2
rs716231	30838297	C	C	G	G
rs2638258	30838351	A	A	G	G
rs2708464	30838359	C	C	T	T
rs1873613	30838512	T	T	C	C
rs1873612	30838546	C	C	G	G
rs1873611	30838559	C	C	G	G
rs726624	30847959	A	C	C	C
rs728623	30848108	G	G	A	A
D12S5CAA	38745498	239	243	243	243
D12S9AG	38863278	178	178	178	178
rs715402	39017481	A	A	A	A
rs6581667	39017773	G	G	C	G
G>A	39017873	G	G	A	G
G2019S	39020469	A	A	A	A
rs10506155	39022206	A	A	G	A
A>G	39022277	G	G	G	G
T>A	39022310	A	A	A	A
rs919714	39022516	C	C	C	C
rs10784522	39026632	G	G	T	G
rs10784522	39026632	G	G	T	G
rs10878405	39028521	G	G	A	G
D12S2518	39034922	154	154	154	154
ss52051244	39043597	A	A	A	G
D12S8TA	39056001	152	152	154	154 / 152
rs3952596	39069322	G	G	C	G

Table 5.12. G2019S-associated haplotypes in the Turkish family (136 / 253) compared with haplotypes 1, 2 and the haplotype found in Japanese cases (1107 / 1108). The homologous region between the Turkish and Japanese haplotypes is shown in bold and gray font. Both alleles are shown when the phase could not be unambiguously determined (continued)

Marker	Physical Map	Turkey 136/ 253	Japan 1107/ 1108	Haplotype 1	Haplotype 2
rs3906483	39069415	A	A	G	G
rs11176402	39069641	C	C	C	T
rs4768250	39082622	G	G	G	T
rs7137809	39083044	C	C	G	C
rs7308560	39083119	A	A	T	A
rs10878551	39098194	C	C	T	C
rs10878552	39098301	C	C	A	C
rs7958987	39107938	G	G	A	G
rs7960062	39108083	C	C	T	C
rs7959343	39108167	G	G	A	G
rs2162470	39113550	C	C	C	T
D12S2519	39116885	140	140	132	138
rs10878584	39118388	C	C	T	C
rs11176635	39118423	T	T	C	T
rs11176638	39118798	G	G	C	G
rs10878586	39118835	C	C	T	C
rs10748075	39118853	C	C	T	T
D12S2520	39120098	254	257		
D12S2521	39128754	319	319		
D12S2522	39132223	283	285		
D12S2523	39147728	317	314		
D12S2517	39282976	190 /194	206		
D12S1048	39312730	229 /226	217		
D12S1301	42348911	108 /100	100		
D12S1701	46208478	99/93	93		

6. DISCUSSION

Twelve years ago, PD was not considered as a genetic disorder at all. Since 1997, fifteen loci have been linked to PD, six genes being implicated in PD with conclusive evidence from genome-wide linkage screens (*α -synuclein*, *LRRK2*, *Parkin*, *PINK1*, *DJ1*, *ATP13A2*), and five genes being proposed to be implicated in PD etiology in studies adopting candidate-gene approach (*UCHL1*, *GIGYF2*, *HTRA2*, *GBA*, *NURR1*, *synphilin-1*).

In the framework of this thesis, the role of mutations in five genes (*Parkin*, *PINK1*, *DJ1*, *α -synuclein* and *LRRK2*), linked to PD, have been evaluated in a series of 78 Turkish PD patients. Investigations have included not only sequencing analysis, but also search for exon rearrangements and haplotype analysis to increase the sensitivity of the study (Appendix D).

6.1. Recessive PD in Turkey

6.1.1. Limitations of the Techniques Used

6.1.1.1. dHPLC Analysis. The dHPLC analysis was performed on 48 Turkish PD patients and their 29 relatives, prior to *Parkin* sequencing. Although this pre-selection method decreased the number of our samples to be further sequenced, to 57 per cent, only 10 per cent of these revealed to be actual variations.

The main disadvantage associated with dHPLC is that, as melting temperatures of DNA double strands are dependent on the DNA sequence, the correct column temperature needs to be optimized for each different DNA fragment. In order to eliminate temperature errors, each *Parkin* exon studied, was analyzed at two to three different temperatures. Despite of these optimizations, the elution profiles obtained were misleading as demonstrated in the panel below (Figure 6.1).

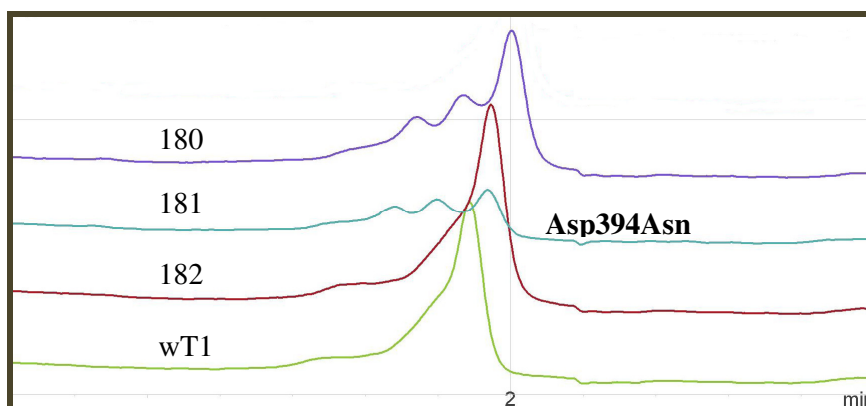


Figure 6.1. Elution profiles obtained for exon 11 of the *Parkin* gene at 58.4°C; the wild-type (wT1) individual is shown with three Turkish PD patients

Patients 180 and 181 reveal a clear abnormal profile, when compared to the wild-type individual. In spite of this, after sequencing of both samples, only the PD patient 181 turned out to carry the heterozygous Asp394Asn polymorphism. These false-positive results may be explained by inadequate column temperatures or by the purity of the PCR products. Some DNA samples were extracted with the common NaCl procedure and the dHPLC column may have been adversely affected by the excess salt. One alternative option would be to make use of the lightcycler technique and of its commercially available PCR kits, which are cost-effective, rapid, sensitive and reproducible (Gencer, 2006).

6.1.1.2. MLPA Analysis. Mutation screening protocols are mainly focused on detecting point mutations or small deletions / insertions by conventional sequencing techniques; genomic rearrangements (large deletions and multiplications) are however as common in PD as point mutations. The MLPA technique recently introduced into our laboratory, in the framework of this thesis, has proven to be rapid, cost-effective, sensitive and easy to perform. Thus, this novel platform and especially its optimized future versions are expected to greatly improve the analysis of comprehensive genetic testing in PD. The simultaneous detection of gene rearrangements in several distinct genes largely overcomes the diagnostic complexity, related to the wide genetic and allelic heterogeneity of PD.

As is the case for the semi-quantitative PCR approach, one of the limitations of the MLPA kit resides in the fact, that it cannot discriminate between heterozygous exon triplications and homozygous exon duplications or detect a combination of deletions and

duplications on the same exon. Similarly, in the case of rearrangements, involving adjacent exons, MLPA analysis is not able to discriminate whether deleted exons are on the same allele (heterozygous) or on two different alleles (compound heterozygous). In this case, further analysis of the parents of the patient, when available, is the simplest option. Given that in PD, point mutations and genomic rearrangements can present in homozygous or compound heterozygous states, in any possible combination, MLPA analysis should be accompanied by sequencing of the 12 exons and of the promoter region of *Parkin*, in order to completely rule out the presence of any mutations on this gene.

6.1.2. Discussion of the Parkin Gene Findings

6.1.2.1. Frequency of Parkin Mutations in the Turkish PD Cohort. Genetic investigation of familial PD has shown that not only point mutations, but also rearrangements (amplification or deletion of one or of a few exons) of *Parkin*, *PINK1* and *DJ1* are frequent. We tested, by semi-quantitative PCR and MLPA, a total of 64 Turkish PD patients with early-onset disease. One heterozygous duplication, four homozygous and seven heterozygous deletions were identified (Figure 6.2) and subsequent sequencing analyses were performed for all PD patients with and without heterozygous rearrangements.

- For one patient with a heterozygous deletion of exon 2, a second mutation (Val56Glu) was found on the trans exon 2 allele.
- No other point mutations could be detected in the sample population.
- For two siblings, a heterozygous exons 3 and 4 deletion could be identified. The samples of the parents were crucial for the establishment of the compound heterozygosity of their affected children, as the father had a heterozygous exon 3 deletion and the mother a heterozygous exon 4 deletion. As expected, both parents were asymptomatic carriers.
- In two additional patients, with heterozygous rearrangements of multiple exons, in the absence of the parents sample, it could not be deduced whether they were compound heterozygotes (e.g. exon 2 deletion on one allele and exons 3-4 deletion on the other; exons 7-8 duplication on one allele and exon 9 on the other) or heterozygote for a duplication / deletion, affecting only one of the two *Parkin* alleles, which would not explain disease manifestation.

- Regarding the three siblings with a heterozygous deletion of exon 2, sequencing did not uncover a second mutation. Therefore, their phenotype could not be fully explained; having a single mutation, the patients were considered to have a *Parkin*-related disease with the assumption, that the second mutation had either escaped detection on the analyzed genes with the methods used in this study or resides in an unknown location in the genome.

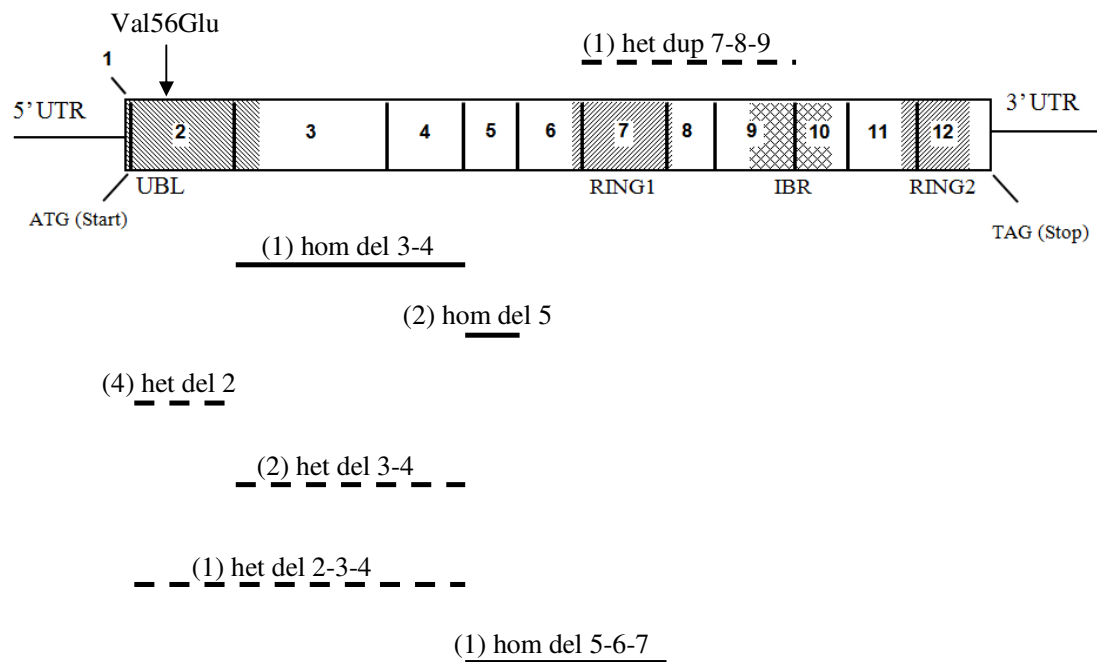


Figure 6.2. Schematic representation of the mutations found in the *Parkin* gene.

Frequencies are displayed in brackets. Heterozygous mutations are shown with dashed lines (UBL: Ubiquitin-like domain; RING finger domain; IBR: in-between-RING domain; hom: homozygous; het: heterozygous; dup: duplication; del: deletion)

Parkin mutations were detected in 11 out of 64 Turkish patients with early-onset PD (17 per cent) (Figure 6.3). When considering only the recessive cases (n=20), the frequency is increased to 55 per cent. Only one isolated early-onset PD patient was found to have a *Parkin*-related disease with a point mutation on one exon 2 allele, and a deletion on the other. As a group, the patients with *Parkin* mutations had a mean age at onset of 26.2 ± 8.5 years, compared to 39.8 ± 13.2 years for the patients without mutations in the *Parkin* gene; this difference is in accordance with previous publications. The deletion of exon 2 was the most common exon rearrangement in the *Parkin* gene (n=5). Overall, exon

rearrangements were largely predominant; only one point mutation was found in combination with a heterozygous exon rearrangement. Although genetic data on Turkish PD patients is scarce, it should be mentioned that two Turkish families with autosomal recessive juvenile Parkinsonism were mapped to the 6q25.2-q27 region, in the initial linkage study which lead to the identification of the *Parkin* gene (Jones *et al.*, 1998). Moreover, the first *Parkin* gene point mutations (Thr240Arg and Gln311Stop) were identified in two Turkish PD patients (Hattori *et al.*, 1998).

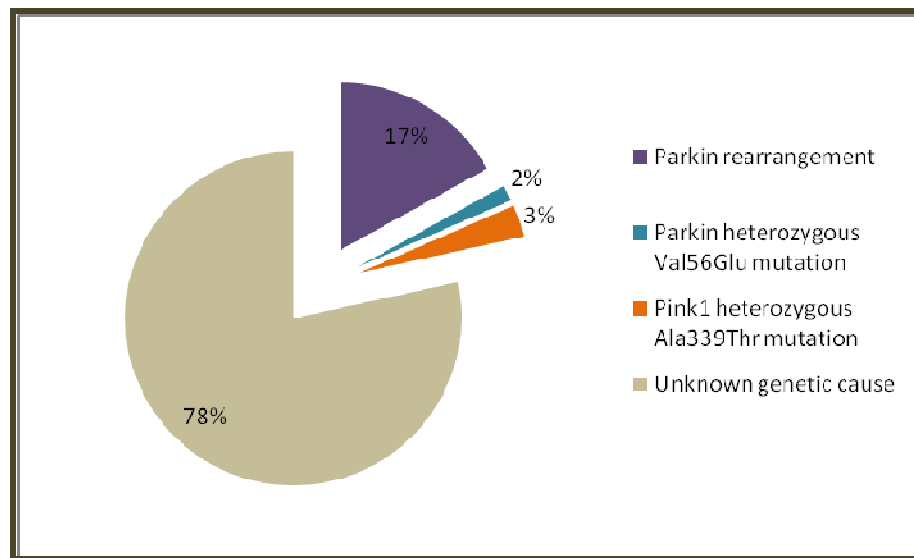


Figure 6.3. Distribution of the 64 early-onset Turkish PD patients screened in this study

6.1.2.2. Significance of the *Parkin* Gene Variations Found in the Turkish PD Cohort. The single pathogenic mutation found in the Turkish PD cohort is a change of the non-polar valine to a polar glutamic acid residue. The position is conserved among phylogenetically distant organisms like rat, mouse, drosophila and tetrahymena. This Val56Glu amino-acid change is located in the Ubl domain of Parkin, required for proteasome binding. Parkin harboring the Val56Glu mutation is probably unable to bind its target protein, which might result in pathological accumulation of the target and negative effects on the central nervous system (Hoenicka *et al.*, 2002).

The three additional amino-acid changes, detected in patients investigated for mutations in the *Parkin* gene were previously described polymorphisms: Ser167Asn in exon 4 [rs1801474], Val380Leu in exon 10 [rs1801582] and Asp394Asn in exon 11

[rs1801334]. These variations did not alter the class of the amino-acid: hydrophilic neutral for Ser167Asn and hydrophobic neutral for Val380Leu. However, Asp394Asn did change the class of the amino-acid from polar, hydrophobic and charged, to polar hydrophilic and neutral. All of these variations were observed in previous studies at a similar frequency, in cases and controls and thus were not considered to be associated with PD. The frequencies shown in 166 controls were 16 per cent for Val380Leu, 7 per cent for Asp394Asn and 1 per cent for Ser167Asn (Abbas *et al.*, 1999). In our Turkish PD cohort, the ratios were calculated as 22 per cent for Val380Leu and 6.25 per cent each for Asp394Asn and Ser167Asn.

The mutation spectrum of *Parkin* is very wide, complicating molecular diagnosis. Point mutations appear to be clustered into hot-spots throughout the functional domains of *Parkin*; Ubl and RING-IBR-RING. The various exon rearrangements however, are spread over the entire *Parkin* gene, although the occurrence is higher in exons 2-9. *Parkin* accounts for the largest number of familial PD cases, and although its function needs to be understood in detail, the protein appears to be a major player in the degenerative process.

6.1.3. Frequency and Significance of *PINK1* Mutations in the Turkish PD Cohort

In the first report involving PARK6 in autosomal recessive early-onset Parkinsonism, one Turkish family was linked to this locus (Hatano *et al.*, 2004b). Furthermore, recently a Turkish PD patient with familial PD was found to carry the homozygous c. 1309 T>C: p. W437R mutation in exon 7 of the *PINK1* gene (Kumazawa *et al.*, 2008). In our study, out of 32 early-onset cases investigated, one mutation, in heterozygous state was identified in two isolated Turkish PD patients (Ala339Thr); the mean age at onset is 35. The frequency of *PINK1* gene mutations is therefore assumed to be lower (6 per cent) than that of *Parkin* (19 per cent); this result is in accordance with previous studies, carried out on different ethnicities (Figure 6.3).

The mutated residue is highly conserved among vertebrates (Figure 6.4) and is located in the serine / threonine protein kinase domain. Besides, alanine is non-polar and threonine is polar, thus the Ala339Thr missense mutation is expected to affect the function of the protein.

However, in one recent report, this variation was predicted to be benign by the PolyPhen software, although found only in patients so far, and not in controls (Marongiu *et al.*, 2008). Nevertheless, the accuracy of the assumption can be questioned in the absence of a crystal structure of the protein, and a poor characterization of its functional domains. Furthermore, functional evidence is required to conclude on the pathogenicity of the variation. As an example, the E476K missense variant was shown to severely impair mitochondrial membrane potential after cellular stress induced by proteosomal inhibition, although E476K was poorly conserved even within mammals, present in heterozygous state both in patients and controls, and predicted to be benign (Abou-Sleiman *et al.*, 2006b). Thus, in the absence of functional assays, predictions on the pathogenic role of missense variants should be taken with caution.

PINK1: A339T	
Human (Homo sapiens)	PSPRLAAMLL
Chimpanzee (Pan troglodytes)	PSPRLAAMLL
Macaque (Macaca mulatta)	PSPRLATMML
Rat (Rattus norvegicus)	PSSRLATMML
Mouse (Mus musculus)	PSSRLATMML
Zebrafish (Danio rerio)	PKRTQASLMFL
Anopheles gambiae	VDMRTRILLFA
Drosophila melanogaster	LSTRNRILLLA
Caenorhabditis elegans	RNYWTGRVIA

Figure 6.4. Conservation across species (shaded in grey)
of the alanine residue affected in the Ala339Thr missense mutation

6.1.4. Are Single Heterozygous Mutations Risk Factors for PD?

It is widely accepted that monogenic forms of PD represent only 5-10 per cent of all PD cases, with mutations in *Parkin* occurring in 10-20 per cent of early-onset and *PINK1* mutations in 1-8 per cent of mostly young-onset PD patients. Accumulating evidence suggests that a considerable number of PD patients carry only one heterozygous mutation in *Parkin* or *PINK1* genes, known to be recessively inherited. In a recent report, compiling results from previous studies, the number of heterozygous mutation carriers in *Parkin* and *PINK1* genes, was shown to be significantly higher than that of homozygous or compound heterozygous cases (Klein *et al.*, 2007b) (Figure 6.5).

The pathogenicity of single heterozygous recessive mutations may be explained by at least three different mechanisms:

- Haploinsufficiency, (the single functional copy of the gene does not produce enough of the encoded protein leading to disease condition). In the case of *Parkin*, functioning as an E3-ubiquitin ligase, a 50% reduction in Parkin-dependent removal of substrates could sensitize neurons to toxicity.
- For mutations lying in an essential domain of the protein, a dominant-negative effect of the single recessive mutations, by which the mutant variant reduces the function of the wild-type protein, may also be proposed, although not yet supported by PD mouse models.
- A gain-of-function mechanism may also be postulated for the mutant mRNA or protein.

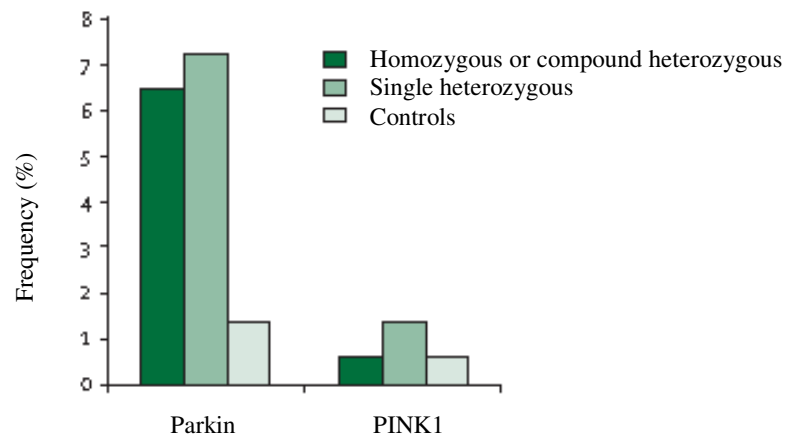


Figure 6.5. Mutation frequencies of *Parkin* and *PINK1* in PD patients, compared to healthy controls (adapted from Klein *et al.*, 2007b)

In the Turkish PD cohort investigated, a sibpair was found to carry a heterozygous exon 2 deletion in the *Parkin* gene; their asymptomatic sister, having the same genotype, would be interesting to follow up, to state on the pathogenicity of the heterozygous *Parkin* exon 2 deletion. Certainly, we cannot exclude yet the possibility of another genetic factor, either a still unknown mutated gene or a polymorphism acting as a risk factor, segregating both with the disease. We may speculate however, that as exon 2 is lying in the Ubl domain of Parkin, the “threshold” for PD development is achieved. On the other hand, in the absence of the parents’ data for the two patients carrying a multi-exon deletion or

duplication, the heterozygosity of the mutation is still debatable and needs further investigation.

Furthermore, two Turkish PD patients were found to carry a heterozygous Ala339Thr mutation in the *PINK1* gene. Even if the mutated residue lies in the serine-threonine kinase domain, the pathogenicity of the mutation is still controversial (see 6.1.3). It was recently proposed that heterozygous *PINK1* mutants have a dominant negative effect on the normal *PINK1* allele's function, by competing with endogenous wild-type PINK1 for the binding of its substrate TRAP1 (Pridgeon *et al.*, 2007). We may also suggest that this specific mutation acts as a risk factor in a toxic environment, as one of the Ala339Thr carrying PD patient is working in a dye factory.

6.2. Dominant PD in Turkey

6.2.1. The Alpha-synuclein Gene

Alpha-synuclein gene mutations were investigated in 32 PD patients compatible with a dominant inheritance pattern. Exons 3 and 4 of the *α-synuclein* gene were amplified and subjected to RE analysis for the presence of the three known mutations (A30P, E46K and A53T), revealing that none of them carried these. Although *α-synuclein* point mutations were the first to be reported in PD pathogenesis, their frequency is known to be very low; this is in accordance with the results obtained in the Turkish population (Figure 6.6).

The same 32 PD patients were analyzed for the presence of rearrangements in the *α-synuclein* gene by semi-quantitative PCR and by MLPA; a heterozygous duplication of *α-synuclein* (for at least exons 3 and 4) was identified in a sibpair from Trabzon.

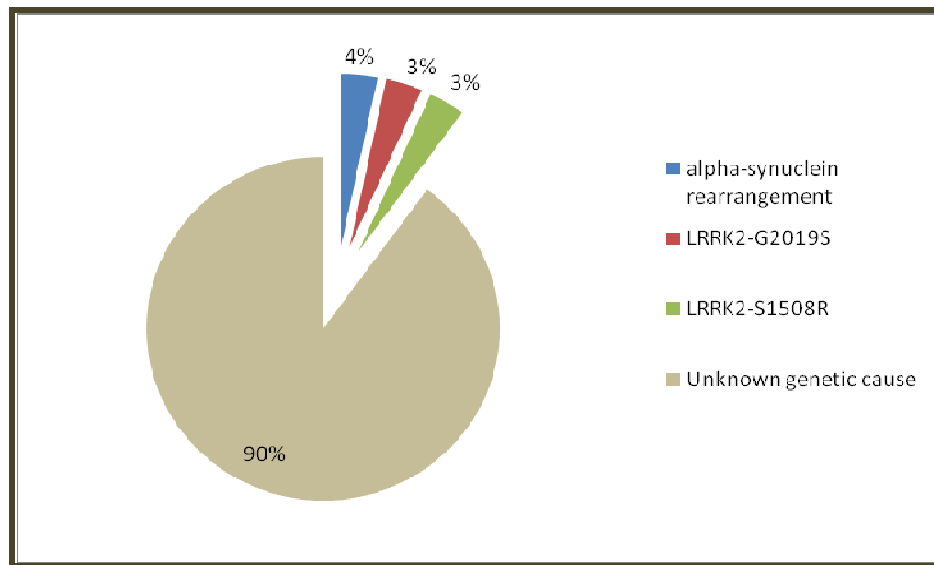


Figure 6.6. Distribution of the 32 dominant Turkish PD patients screened in this study

To get further insight in disease segregation, semi-quantitative PCR and MLPA analyses were performed to another 20 members of this large kindred: six asymptomatic members of the family were shown to be carriers of the same exons 3 and 4 heterozygous duplication, found initially in the sibpair.

In order to explore the common disease-haplotype, shared in this large PD family with no consanguinity, and to draw up the boundaries of this duplication, a haplotype analysis was conducted with a set of 17 microsatellite markers, spanning an 11 Mb region covering the *α-synuclein* locus. Ten markers were found to be informative and were used to construct the haplotype. This established, that five asymptomatic family members, as well as the index sibpair, shared the PD-linked haplotype thus indicating that, the duplication spans a larger region than simply exons 3 and 4.

Furthermore, an individual from the third generation (PD239), who was initially shown to have the heterozygous duplication of *α-synuclein* exons 3 and 4 by semi-quantitative PCR and MLPA, revealed unexpectedly to carry a “healthy” haplotype. This discrepancy may be explained by the fact, that the haplotype analysis was not performed either for her parents or for her grandparents. As her haplotype is inferred only from her aunts and uncles, the Simwalk 2.91 software used, may be misguided, rendering a false negative result.

To the best of our knowledge, this is the first family with a rearrangement in the α -synuclein gene identified in Turkey (personal communication, B. Elibol). Although, the disease-haplotype, shared by this large Turkish family, seems to span an 11 Mb region, the centromeric and telomeric breakpoints of the replicated region could not be firmly established by the method used. No recombinations have occurred in the α -synuclein locus over three generations, although the patient PD171 does not share the same allele as the affected members of the family, for the last microsatellite marker. In a previous study, the maximum length of the duplicated region was shown to be 4.5 Mb (Ibanez *et al.*, 2009), therefore our result needs further confirmation, either by quantification of microsatellite markers, or by dosage analysis, using an Affymetrix SNP microarray.

6.2.2. LRRK2 Mutations in the Turkish PD Cohort

6.2.2.1. Rare Mutational Hot-spots and their Frequencies. Sequencing of *LRRK2* mutational hot-spot exons 19, 21, 23, 25, 27, 29, 31, 32, 33, 35, 38, 39, 47 and 48 in a cohort of 32 Turkish PD patients, with a dominant inheritance pattern, yielded a novel heterozygous Ser1508Arg mutation in exon 31 (c.4524 C>G) in a female patient. This C>G nucleotide change is predicted not to be tolerated by the dardarin protein (J. Craig Venter Institute, www.sift.jcvi.org), as serine is a neutral amino-acid, whereas arginine is charged. Besides, the mutated serine residue is located in the Roc domain of the LRRK2 protein, believed to act as a GTPase, to regulate the protein kinase activity of the MAPKKK domain; this position is highly conserved among mammals (Figure 6.7).

LRRK2: S1508R	
Human (Homo Sapiens):	LRKTIINES S LNFKIRDQ
Chimpanzee (Pan troglodytes):	LRKTIINES S LNFKIRDQ
Macaque (Macaca mulatta):	LRKTIINES S LNFKIRDQ
Rat (Rattus norvegicus):	LRKTIINES S LNFKIRDQ
Mouse (Mus Musculus):	LRKTIINES S LNFKIRDQ
Chicken (Gallus gallus):	LRKII I KE S LHFKIRDQ
Tetraodon nigroviridis:	LRKAIARE V ANFKIQGQ

Figure 6.7. Conservation across species (shaded in grey) of the serine residue affected in the Ser1508Arg missense mutation

In an extensive study performed by Lesage and colleagues (2008), a total of 226 patients from families with autosomal dominant PD, mostly from France and North Africa, were screened for all the 51 coding exons of the *LRRK2* gene. Only eight potentially or proven pathogenic mutations were identified in 22 patients (9.7 per cent), in the above study, including the common G2019S in exon 41, the R1441H mutation in exon 31 and the I1371V mutation in exon 29. As our cohort is not as large, only a single mutation in the selected exons of the *LRRK2* gene in our sample population (3.2 per cent) is acceptable.

6.2.2.2. Frequency of the G2019S Mutation in Turkey. When the common *LRRK2* G2019S mutation in exon 41 (c.6055 G>A) was investigated in a series of 78 PD patients, both familial and sporadic, only one heterozygous G2019S mutation was identified. This reveals that the frequency of the *LRRK2* G2019S mutation is not high in Turkey (1.3 per cent). This number is comparable with that of European populations (2.8-6.6 per cent of familial cases, 0.7-1.6 per cent of sporadic cases) (Zabetian *et al.*, 2006a), but higher than in Far East Asia (0.1 per cent) (Tomiyama *et al.*, 2006).

Further genetic analyses of the Turkish G2019S family were necessary for the success of the subsequent haplotyping and were done with the consent of the family members. These analyses revealed the presence of the G2019S mutation in the 39-years-old clinically unaffected daughter of the patient, but surprisingly, not in the two female cousins of the index case, who are bed-ridden with severe PD. Given that the G2019S mutation is not fully penetrant, the asymptomatic daughter will either not suffer from PD at all, or show clinical signs at an advanced age like her mother (age at onset: 60). Considering that no cure for PD is currently available, the consequences of giving a positive test result for a low-penetrance mutation should be meticulously evaluated. Accordingly, offering a G2019S genetic test to asymptomatic, possible mutation-carriers should be avoided.

6.2.3. The G2019S Turkish-Japanese Haplotype

The investigation of the haplotype of our index patient revealed an unexpected similarity to the Japanese population, rather than to European, Jewish, or North African haplotypes. The Turkish haplotype is identical to that found in two Japanese PD patients

with G2019S, for all markers tested. To the best of our knowledge, it is the first time that the G2019S-associated Japanese haplotype has been reported in a different population. Although the possibility of an independent origin of this haplotype in Turkey cannot be excluded at this point, the results of our haplotype analysis, spanning a homologous region of 8.3 Mb, (from rs2128274 to rs10748075), rather supports the common origin hypothesis.

The Turkish and Japanese haplotypes may well be the result of a common ancestry, which can be plausibly explained by the huge and centuries-long migration of the Turkic people across Central Asia. This expansion has begun with the Huns in the 6th century, ending with the Seljuk Turks in the 11th century with the invasion of Anatolia. Further research is warranted to investigate the Turkish population, known to be ethnically and genetically very heterogeneous, by increasing the number of the Turkish PD cohort, and by grouping the patients according to their geographical origin. This should aid in revealing important clues about the nature and origin of this interesting haplotype.

7. CONCLUSION

7.1. Concerns about the Genetic Diagnosis of PD

The recent genetic discoveries have generated great interest and debate among patients, scientists and physicians, raising important questions concerning the role that the genetic testing might have in the near future. The high number of PD genes offers the exciting option of routine genetic testing in PD with an increasing demand for mutational analysis; however, the sensitivity and specificity of the available diagnostic tests, as well as the experience and expertise of the clinical settings are still very limited. Moreover, incomplete penetrance (70 per cent of heterozygous *LRRK2* variants do not lead to disease) and the observation that 50 per cent of PD patients have only one affected allele in the *Parkin*, *PINK1* and *DJ1* genes makes the PD diagnosis even more complicated (Tan and Jankovic 2006; Bonifati 2007; Klein *et al.*, 2007b). Combined with the unexpectedly large clinical and genetic heterogeneity of PD, and the still growing number of the currently known PD genes, the above limitations create a great deal of controversy. International guidelines for genetic counseling have to be introduced, and a firm collaboration between patients, scientists and clinicians urgently encouraged.

7.2. New Genes and New Perspectives

In the Turkish PD cohort investigated, *Parkin* mutations appear to be the most recurrent, with a frequency of 11 per cent. The ratio of heterozygous mutations in *PINK1* (3 per cent) is lower than in *Parkin*, but higher than the two dominant PD-linked, α -synuclein and *LRRK2* mutations (2 per cent each) (Figure 7.1). No mutations were found in the *DJ1* gene in our patient cohort. The vast majority (82 per cent) of the Turkish PD patients remain free from mutations, providing further evidence for the existence of additional unknown genes, linked to familial PD, or of unexplored risk factors interacting with precise environmental conditions.

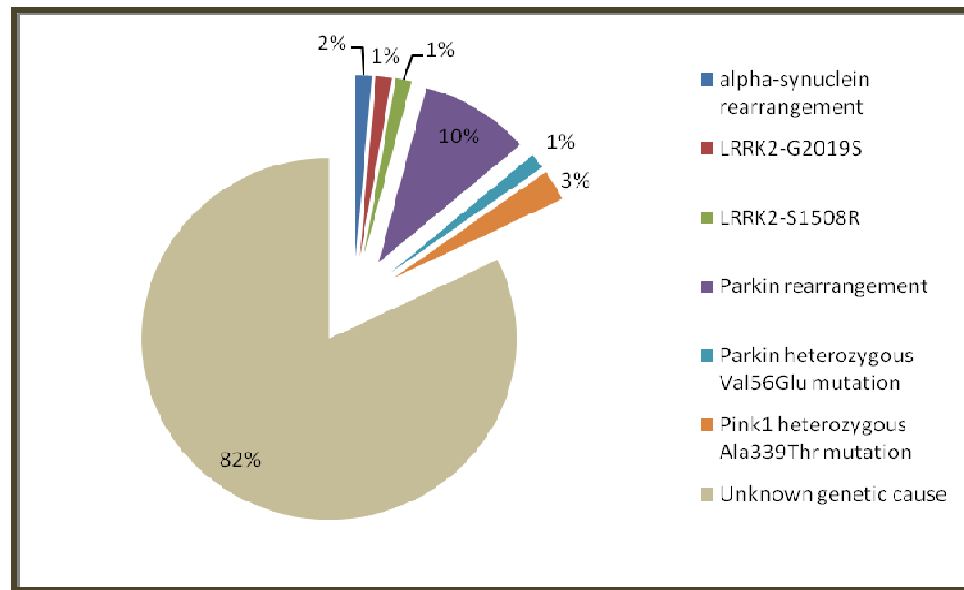


Figure 7.1. Distribution of the 78 Turkish PD patients screened in this study

Although PD-causing genes at several loci have not been identified yet, the mutational spectrum of the disease has been enlarged with the recent identification of two novel genes causing atypical PD:

- *PLA2G6* (encoding a calcium-independent group VI phospholipase A2), and
- *FBXO7* (a member of the F-box family, acting in the protein degradation involving the ubiquitin-proteasome system) (Paisan-Ruiz *et al.*, 2008; Shojaaee *et al.*, 2008).

Besides, in a recent paper published in 2009, the ubiquitin C-terminal hydrolase-L1 (UCHL1) was shown to associate with the endoplasmic reticulum when farnesylated. Intriguingly, when the farnesylation of UCHL1 was inhibited, α -synuclein levels were reduced, along with its subsequent toxicity, improving viability in cellular models. Thus, in the near future, farnesyl transferase inhibitors may be of therapeutical benefit in synucleinopathies, including PD (Liu *et al.*, 2009).

This exciting finding was immediately followed by the description of a new interaction, involving α -synuclein and ATP13A2. The elegant study proved, that α -synuclein toxicity is suppressed by ATP13A2 in yeast, in *C. elegans* and in primary neuron cultures. Moreover, ATP13A2 was shown to protect against manganese toxicity, linking gene products and environmental conditions (Gitler *et al.*, 2009).

However, none of these genes mentioned could account for the selective vulnerability of the substantia nigra pars compacta (SNc) dopaminergic neurons, precisely affected in PD. In an attempt to explain this ambiguity, Chan and colleagues (2007) have proposed that the SNc dopaminergic neurons express an unusual L-type calcium channel ($\text{Ca}_v1.3 \text{ Ca}^{2+}$) for their pacemaking activity. Surprisingly, the blockade of these channels with dihydropyridines, currently used against hypertension, induced a reversion to the juvenile form of pacemaking, implicating sodium and HCN (hyperpolarization-activated and cyclic nucleotide gated cation) channels, which in turn, protect SNc neurons both in *in vitro* and *in vivo* PD models. Therefore, identification of $\text{Ca}_v1.3 \text{ Ca}^{2+}$ inhibitors will bring new therapeutic promises for early stage PD patients.

7.3. Possible Pathways to Parkinsonism

In the last ten years, since the breakthrough discovery of *α -synuclein* mutations, as the first recognized cause of familial PD, many key developments have led to the identification of genes for the Mendelian forms of PD. As these genetic findings do not elucidate all familial PD cases, we may reasonably think of PD as a combination of environmental and genetic factors, underlining the importance of genetic findings in understanding how the much more frequent sporadic disease develops.

Moreover, a unifying molecular mechanism leading to PD has not been clarified yet (Figure 7.2). The situation may be more complicated, involving not a unique pathway, but a cross-talk from other pathways; parallel pathways may lead to the same disease, or multiple pathways may converge to a common phenotype. These challenging issues are still a matter of debate.

Analyzing the pathways, how these genes fit into and enhance our understanding of the molecular pathology of PD, will help to delineate the complex etiopathogenesis of the disease and the neurodegenerative processes involved. In the last decade, tremendous worldwide efforts have achieved great progress in PD research, thus, there is every reason to expect, that more effective therapeutical strategies will be developed in the near future to prevent or stop the nigral cell degeneration leading to PD. Our next goal is therefore a

genome-wide scan with Turkish patients, which has been already launched, in the hope of finding another piece to fit in Parkinson's Disease puzzle.

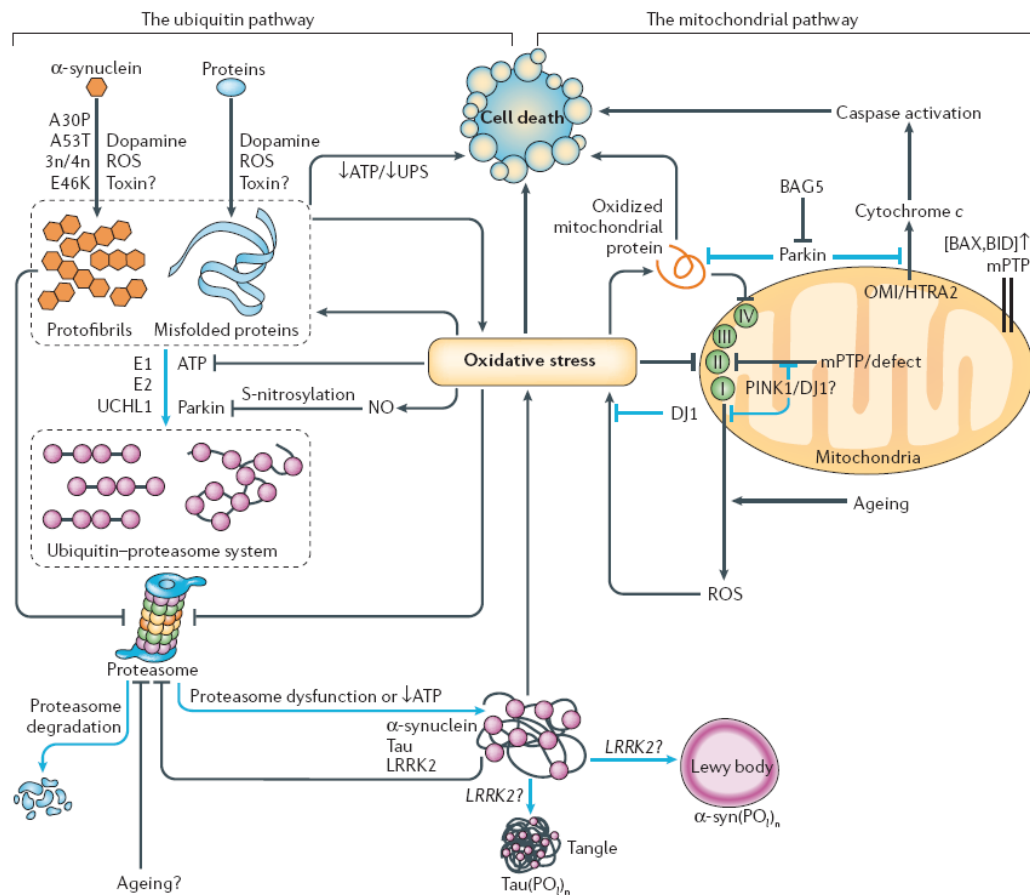


Figure 7.2. Possible pathways to Parkinsonism (Abou-Sleiman *et al.*, 2006a)

APPENDIX A: INFORMED WRITTEN CONSENT**KATILIMCILAR İÇİN BİLGİ ve OLUR FORMU**

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

Telefon (iş): (212) 359 66 79

Proje başlığı: Resesif Geçişli Parkinson Hastalığının Moleküler Patogenezi

Proje konusu:

Parkinson hastalığı, dünyada 4.000.000'dan fazla kişiyi etkileyen, sık rastlanan bir nörolojik hastalıktır. Kadın ve erkeklerde görülme oranı aynıdır; 65 yaş üzerindeki her 100 kişiden biri Parkinson hastasıdır. Parkinson yavaş ilerleyen bir hastalıktır; hayatı etkilediği halde, yaşamı tehdit etmez; beyinde dopamin adlı bir maddenin sentezlendiği sinir hücrelerinin kaybı sonucu ortaya çıkar. Parkinson'un ilk belirtileri beyindeki dopamin düzeyi normalin %20 altına düştüğü zaman görülür. Parkinson sadece yaşlıların hastalığı değildir. Genellikle ortalama 60 yaşında tanı konulduğu halde, Parkinson'lu her yirmi kişiden birinde ilk bulgular 40 yaşından önce görülmektedir. 21-40 yaş arasındaki hastalar, "Erken Başlangıçlı Parkinson Hastalığı" tanısı alırlar.

Günümüzde Parkinson ve benzeri beyin hastalıkları ile ilgili çalışmalar, özellikle de genetik çalışmalar büyük hız kazanmıştır. Genetik çalışmaların başında, hastalığa neden olan aday genlerde görülen genetik değişikliklerin (mutasyonların) test edilmeleri gelir. Parkinson hastalığına neden olan genlerin bulunması ve özelliklerinin anlaşılması, bu hastalığa yol açan mekanizmaların tanınması açısından büyük önem taşımaktadır.

Bu çalışmada, Erken Başlangıçlı Parkinson Hastalığı incelenecektir. Bu tip Parkinson'a neden olduğu bilinen üç gen vardır. Bunlar *Parkin*, *DJ1*, ve *PINK1* genleridir. Nörolog hekimin yaptığı klinik muayene sonucu bu gruba dahil edilen bireylerin hastalığının bu genlerden kaynaklanıp kaynaklanmadığı araştırılacaktır. Erken başlangıçlı Parkinson hastalığı resesif geçiş gösterir; hasta bireyin anne ve babası taşıyıcıdır, taşıyıcılar genelde belirti göstermezler, yani klinik açıdan sağlıklıdır. Bu yüzden hastanın ailesinden de kan alınması çok büyük önem taşımaktadır. Bu araştırmanın ileri aşamasında erken başlangıçlı Parkinson hastalığına yol açan yeni genlerin bulunması da hedeflenmektedir.

Çalışmaya katılmanız tamamen isteğinize bağlıdır. Sizden ücret talep edilmeyecektir ve size herhangi bir ödeme yapılmayacaktır. Araştırmaya katılmayı kabul ettiğiniz takdirde sizden on mililitre kan örneği alınacaktır. Yapacağımız araştırmanın size bir risk getirmesi beklenmemektedir. Kan aldırmanın genelde hiçbir zararı olmamasına karşın, nadiren çok az kanama ve morarmaya yol açabilir.

İstedığınız zaman çalışmaya katılmaktan vazgeçebilirsiniz. Bu durumda sizden almış olduğumuz örnek, eğer arzu ederseniz imha edilecektir; yoksa isminiz her zaman gizli tutulmak kaydı ile başka araştırmalar için de kullanılabilir. İlişikteki nörolog hekim tarafından doldurulan bilgi formundaki bilgiler araştırma ekibi tarafından görülecektir. Bu bilgilerin laboratuardaki araştırmacılarla paylaşılmasını kabul etmeniz gerekmektedir. Ayrıca, bu çalışmanın bazı aşamaları Paris INSERM U679'da Dr. A. Brice'in laboratuvarında gerçekleştirilecektir. Örneklerin deneysel çalışmanın bazı kısımları için yukarıdaki laboratuara gitmesi için onay vermeniz önemlidir.

Araştırmanın sonucunda, aranan bilgi elde edildiği takdirde, ailenizin bireylerine genetik danışma hizmeti verilebilecektir. Toplumumuzda genetik altyapının belirlenmesi, hastalığın moleküler temelini aydınlatılmasına katkıda bulunacaktır. Bunun sizin ailenize yararlı olup olmayacağını şimdiden söylemek mümkün değildir ve size bu konuda söz veremeyiz. Araştırmanın ileride başka bireylere yarar sağlaması olasılığı yüksektir. Çalışmaya kan verilerek gösterilecek gerekli toplumsal duyarlılık çalışma açısından büyük önem taşımaktadır.

Bu formu imzalamadan önce, çalışmayla ilgili sorularınız varsa lütfen sorun. Daha sonra sorunuz olursa Prof. Dr. A. Nazlı Başak'a (Telefon: 212- 359 66 79 / 359 72 98) ve ilgili nörolog hekiminize (adı ve telefon numarası formda belirtilmiştir) sorabilirsiniz. Araştırmayla ilgili haklarınız konusunda Boğaziçi Üniversitesi İnsan Araştırmaları Etik Kurulu'na danışabilirsiniz (Telefon: 212- 359 64 72, Prof. Dr. Aslı Tolun, Başkan). Adres ve telefon numaranız değişirse, bize haber vermenizi rica ederiz.

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

Katılımcı veya vasisinin ismi ve imzası:

Tarih:

Hastaya yakınlığı:

Şahit ismi ve imzası:

Klinisyen ismi:

Adresi:

Telefonu:

İmzası:

APPENDIX B: CONTENTS OF THE MLPA-P051 PROBE MIX

SALSA MLPA P051-B1 Parkinson-1 probemix

Length (nt)	SALSA MLPA probe	Chromosomal position		
		reference	other	PARK2
64-70-76-82	Q-fragments: DNA quantity; only visible with less than 100 ng sample DNA			
88-92-96	D-fragments: Low signal of 88 or 96 nt fragment indicates incomplete denaturation			
100	X-fragment: Specific for the X chromosome			
105	Y-fragment: Specific for the Y chromosome			
130	<i>Reference probe 0797-L00463</i>	5q31		
136	PINK1, probe 3695-L03590, exon 5		1p36.1	
142	PINK1, probe 3691-L03106, exon 1		1p36.1	
149 ⁵	SNCA, probe 2166-L01662, A30P mutation-specific		4q22	
157	SNCA, probe 2168-L01665, exon 4		4q22	
166	PINK1, probe 3692-L03107, exon 2		1p36.1	
172 *	ATP13A2 probe 11715-L12486, exon 9		1p36	
178 †	SNCA, probe 2169-L13410, exon 6		4q22	
184	LPA, probe 4615-L03891		6q26	
193 ⁵	LRRK2 probe 4575-L04006 G2019S mutation-specific		12q12	
200 *	PINK1, probe 12067-L12939, exon 3		1p36.1	
208 *	ATP13A2 probe 11716-L12487, exon 2		1p36	
220	PINK1, probe 3694-L03109, exon 4		1p36.1	
229	PINK1, probe 3698-L03154, exon 7		1p36.1	
238	PARK2 , probe 2178-L01676			Exon 6
247	SNCA probe 4616-L03892, exon 3		4q22	
256	PARK2 , probe 3203-L02564			Exon 1
265	PARK2 , probe 2179-L01677			Exon 7
274	<i>Reference probe 1665-L01244</i>	11q13		
283 †	PARK2 , probe 2174-L05093			Exon 2
292	PARK2 , probe 2180-L05094			Exon 8
301	Reference probe 0895-L00483		3p22	
310	PARK2 , probe 3205-L02566			Exon 3
319	PARK2 , probe 2181-L01679			Exon 9
328 ±	PINK1 probe 5653-L05107, exon 6		1p36.1	
337	PARK2 , probe 3363-L02747			Exon 4
346	PARK2 , probe 2182-L03127			Exon 10
355 *	<i>Reference probe 5865-L05117</i>	8q13		
364	PARK2 , probe 2177-L01675			Exon 5
373	PARK2 , probe 2183-L01681			Exon 11
382 *	<i>Reference probe 6594-L06152</i>	8q24		
391	PARK2 , probe 2184-L01682			Exon 12
400 †	TNFRSF9, probe 2185-L01683		1p36.2	
409	PARK7, probe 2187-L03592, exon 3		1p36.2	
418	<i>Reference probe 1617-L01199</i>	13q12		
427	PARK7, probe 3690-L03105, exon 1A		1p36.2	
436	PARK7, probe 2188-L01686, exon 5		1p36.2	
445	SNCA, probe 4096-L03102, exon 1		4q22	
454	PARK7, probe 2189-L02365, exon 7		1p36.2	
463	PINK1, probe 3697-L03153, exon 8		1p36.1	
478	SNCA, probe 3689-L03104, exon 5		4q22	

* New in version B1 (from lot 0608 onwards)

† Small change in length or amplification characteristics from lot 0608 onwards. No change in sequence detected.

⁵ The 149 nt fragment specific for the presence of the SNCA A30P mutation and the 193 nt fragment specific for the LRRK2 (PARK8) G2019S mutation will not generate an amplification product in the great majority of samples. At MRC-Holland we have no A30P mutation containing samples yet and test these probes on a synthetic plasmid that contains the mutant sequences.

± The sequence detected by this probe contains a SNP. This probe may be replaced in a future lot.

APPENDIX C: CONTENTS OF THE MLPA-P052 PROBE MIX

SALSA MLPA P052-C1 Parkinson-2 probemix

Length (nt)	SALSA MLPA probe	Chromosomal position		
		reference	other	PARK2
64-70-76-82	Q-fragments: DNA quantity; only visible with less than 100 ng sample DNA			
88-92-96	D-fragments: Low signal of 88 or 96 nt fragment indicates incomplete denaturation			
100	X-fragment: Specific for the X chromosome			
105	Y-fragment: Specific for the Y chromosome			
130	<i>Reference probe 0797-L00463</i>	5q31		
136	PACRG probe 3204-L02565		6q26	
142	CAV2, probe 3357-L02741, exon 3		7q31	
148	UCHL1, probe 3680-L03095, exon 4		4p14	
154	PARK2 , probe 3366-L02750			Exon 7
160	UCHL1, probe 3679-L03094, exon 1		4p14	
166 §	LRRK2 probe 4574-L04006. G2019S mutation-specific		12q12	
172	PARK2 , probe 3361-L03999			Exon 2
178	UCHL1, probe 3681-L03096, exon 5		4p14	
184	LRRK2 probe 6134-L05587, exon 2		12q12	
193	<i>Reference probe 1567-L01139</i>	22q12		
202	UCHL1, probe 2937-L05095, exon 9		4p14	
208	GCH1, probe 4405-L03099, exon 3		14q22	
214	PARK2 , probe 6135-L07959			Exon 12
220	LRRK2 probe 4282-L03686, exon 41		12q12	
225 *	ATP13A2 probe 11717-L12488, exon 14		1p36	
231 *	ATP13A2 probe 12581-L13662, exon 28		1p36	
238	PARK2 , probe 3365-L02749			Exon 6
247	<i>Reference probe 1164-L00720</i>	11q13		
256	GCH1, probe 4618-L03894, exon 1		14q22	
265	PARK2 , probe 5654-L05108			Exon 3
274	LRRK2 probe 4281-L03685, exon 27		12q12	
283	GCH1, probe 4404-L02610, exon 6		14q22	
292	PARK2 , probe 3367-L02751		6q26	Exon 8
301	<i>Reference probe 3255-L02692</i>	11p13		
310	GCH1, probe 3683-L03098, exon 2		14q22	
319	GCH1, probe 3685-L03100, exon 5		14q22	
328	LRRK2 probe 4283-L03687, exon 49		12q12	
337	PARK2 , probe 2176-L01674			Exon 4
346	PARK2 , probe 3369-L02753			Exon 10
355	<i>Reference probe 4014-L03437</i>	2q33		
364	PARK2 , probe 3364-L02748			Exon 5
373	LRRK2 probe 4278-L03682, exon 1		12q12	
382	<i>Reference probe 1136-L00694</i>	4q35		
391	PARK2 probe 4614-L03890		18q11	Exon 11
400	CAV1, probe 3359-L02743		7q31	
409	<i>Reference probe 0963-L09340</i>	2p14		
418	PARK2 , probe 3368-L02752			Exon 9
427	LRRK2 probe 4279-L03683, exon 10		12q12	
436	<i>Reference probe 2967-L02399</i>	7q31		
445	<i>Reference probe 1799-L01362</i>	13q14		
454	SNCA, probe 4092-L03103, exon 2		4q22	
463	LRRK2 probe 4280-L03684, exon 15		12q12	

* New in version C1 (from lot 1108 onwards)

§ The 166 nt fragment specific for the LRRK2 (PARK8) G2019S mutation will only generate an amplification product in samples containing this mutation.

APPENDIX D: OVERALL RESULTS OF THE TURKISH PD PATIENTS INVESTIGATED

Intronic variations are highlighted in green, exonic variations in orange and mutations in red. The blue background indicates that samples have been analyzed at the corresponding regions.

Table A.1. Thirty-two PD patients sequenced for exons 1 to 7 of *DJI*

	ex1	ex2	ex3	ex4	ex5	ex6	ex7
PD-124						IVS6-216 G>A hom	
PD-127						IVS6-216 G>A het	
PD-129						IVS6-216 G>A het	
PD-130						IVS6-216 G>A het	
PD-131						IVS6-216 G>A het	
PD-137							
PD-141							
PD-142						IVS6-216 G>A het	
PD-143				IVS5+30T>G het			
PD-156						IVS6-216 G>A het	
PD-158						IVS6-216 G>A het	
PD-174							
PD-178						IVS6-216 G>A het	
PD-181						IVS6-216 G>A hom	
PD-182			IVS3-110 C>T het	IVS5+30T>G het			
PD-187						IVS6-216 G>A het	
PD-189						IVS6-216 G>A het	
PD-193						IVS6-216 G>A het	
PD-203						IVS6-216 G>A het	
PD-204						IVS6-216 G>A het; IVS6-251 C>A het	
PD-205						IVS6-216 G>A het	
PD-206						IVS6-216 G>A hom	
PD-207						IVS6-216 G>A het	
PD-210							
PD-211							
PD-212						IVS6-216 G>A hom	
PD-217			IVS3-110 C>T het	IVS5+30T>G het		IVS6-216 G>A het	
PD-224							
PD-227						IVS6-216 G>A het	
PD-229							
PD-231			IVS3-110 C>T het	IVS5+30T>G het		IVS6-216 G>A het	
PD-232			IVS3-110 C>T het	IVS5+30T>G het		IVS6-216 G>A het	

Table A.2. Thirty-two PD patients sequenced for exons 1 to 8 of *PINK1*

	ex1	ex2	ex3	ex4	ex5	ex6	ex7	ex8
PD-124								
PD-127		IVS1-7 A>G het						
PD-129		IVS1-7 A>G hom						
PD-130	c.189 C>T het: L63L	IVS1-7 A>G hom						
PD-131		IVS1-7 A>G hom						
PD-137	c.189 C>T het: L63L	IVS1-7 A>G het						
PD-141	c.189 C>T het: L63L							
PD-142	c.189 C>T het: L63L	IVS1-7 A>G het						
PD-143		IVS1-7 A>G het						
PD-156	c.10 C>A het: R4R	IVS1-7 A>G hom						
PD-158		IVS1-7 A>G het						
PD-174		IVS1-7 A>G hom						
PD-178								
PD-181	c.189 C>T het: L63L	IVS1-7 A>G hom						
PD-182	c.10 C>A het: R4R	IVS1-7 A>G het						
PD-187	c.10 C>A het: R4R	IVS1-7 A>G het						
PD-189	c.189 C>T hom: L63L	IVS1-7 A>G hom						
PD-193								
PD-203		IVS1-7 A>G het						
PD-204		IVS1-7 A>G hom						
PD-205		IVS1-7 A>G het			c.1018 G>A het: A339T; IVS 4-5 G>A het			
PD-206		IVS1-7 A>G hom						
PD-207		IVS1-7 A>G hom						
PD-210	c.189 C>T het: L63L	IVS1-7 A>G hom						
PD-211		IVS1-7 A>G het						
PD-212								
PD-217		IVS1-7 A>G hom						
PD-224	c.189 C>T het: L63L	IVS1-7 A>G het						
PD-227		IVS1-7 A>G hom			c.1018 G>A het: A339T			
PD-229	c.189 C>T het: L63L	IVS1-7 A>G hom						
PD-231	c.189 C>T het: L63L	IVS1-7 A>G het						
PD-232								

Table A.3. Sixty-four PD patients and their 29 relatives sequenced for the promoter and
exons 1 to 12 of *Parkin*

	Prom-ex1	ex2	ex3	ex4	ex5	ex6	ex7	ex8	ex9	ex10	ex11	ex12
PD-111	-258 T>G het							IVS7-35 A>G het				PD-111
PD-112								IVS7-35 A>G het; IVS8-48 C>T het				PD-112
PD-113								IVS7-35 A>G het; IVS8-48 C>T het				PD-113
PD-114								IVS7-35 A>G het; IVS8-48 C>T het				PD-114
PD-115								IVS7-35 A>G het; IVS8-48 C>T het				PD-115
PD-116								IVS7-35 A>G het; IVS8-48 C>T het			Asp394Asn c.1281G>A het	PD-116
PD-117												PD-117
PD-118								IVS7-35 A>G hom				PD-118
PD-119	-258 T>G het							IVS7-35 A>G hom				PD-119
PD-120								IVS8-48 C>T hom		Val380Leu c.1239G>C hom; IVS10-159 C>G het		PD-120
PD-121										Val380Leu c.1239G>C hom		PD-121
PD-122												PD-122
PD-123												PD-123
PD-124												PD-124
PD-125	-258 T>G het							IVS7-35 A>G het; IVS8-48 C>T het				PD-125
PD-126	-227 A>G het							IVS8-48 C>T hom				PD-126
PD-127	-227 A>G het							IVS7-35 A>G hom				PD-127
PD-128	-258 T>G het							IVS7-35 A>G hom				PD-128
PD-129								IVS7-35 A>G het				PD-129
PD-130	-258 T>G het											PD-130
PD-131	-227 A>G het											PD-131
PD-132								IVS7-35 A>G hom				PD-132
PD-133	-258 T>G het							IVS8-48 C>T het				PD-133
PD-134	-227 A>G het							IVS8-48 C>T het				PD-134
PD-135												PD-135
PD-136												PD-136
PD-137								IVS7-35 A>G het; IVS8-48 C>T het				PD-137
PD-138								IVS7-468 G>C het; IVS7-35 A>G het				PD-138
PD-139										Val380Leu c.1239G>C het; IVS10-159 C>G het		PD-139
PD-140												PD-140
PD-141								IVS7-35 A>G het				PD-141
PD-142	-258 T>G hom							IVS8-48 C>T het				PD-142
PD-143	-227 A>G het							IVS8-48 C>T het				PD-143
PD-144								IVS8-48 C>T het				PD-144
PD-145												PD-145
PD-146												PD-146
PD-147												PD-147
PD-148												PD-148
PD-149												PD-149
PD-150	-258 T>G het	IVS2+25 T>C het						IVS8-48 C>T het		Val380Leu c.1239G>C het		PD-150
PD-151		IVS2+25 T>C het										PD-151
PD-152		IVS2+25 T>C het										PD-152
PD-153												PD-153
PD-154												PD-154
PD-155												PD-155
PD-156	-258 T>G het							IVS7-35 A>G het				PD-156
PD-157								IVS7-35 A>G het; IVS8-48 C>T het				PD-157

Table A.3. Sixty-four PD patients and their 29 relatives sequenced for the promoter and exons 1 to 12 of *Parkin* (continued)

	Prom-ex1	ex2	ex3	ex4	ex5	ex6	ex7	ex8	ex9	ex10	ex11	ex12
PD-158	-227 A>G hom							IVS8+48 C>T hom				PD-158
PD-159												PD-159
PD-160												PD-160
PD-161	-238 T>G het	IVS2+25 T>C het						IVS7-35 A>G het				PD-161
PD-162	-238 T>G het	IVS2+25 T>C het						IVS8+48 C>T het		Val380L eur. c.1239G>C het; IVS10+139 C>G het		PD-162
PD-163	-238 T>G het	IVS2+25 T>C het						IVS7-35 A>G hom				PD-163
PD-164	-227 A>G hom	IVS2+25 T>C het										PD-164
PD-165								IVS8+48 C>T hom				PD-165
PD-166	-238 T>G hom	IVS2+25 T>C het								Val380L eur. c.1239G>C het	Asp394Asn. c.1281G>A het	PD-166
PD-167												PD-167
PD-168												PD-168
PD-169	-238 T>G het	IVS2+25 T>C het		Ser167Asn. c.601G>A het				IVS7-35 A>G het; IVS8+48 C>T het		Val380L eur. c.1239G>C het; IVS10+139 C>G het		PD-169
PD-170												PD-170
PD-171	-238 T>G het	IVS2+25 T>C het						IVS7-35 A>G het; IVS8+48 C>T het				PD-171
PD-172												PD-172
PD-173												PD-173
PD-174	-227 A>G het	IVS2+25 T>C het						IVS7-35 A>G het; IVS8+48 C>T het				PD-174
PD-175												PD-175
PD-176												PD-176
PD-177	-238 T>G het	IVS2+25 T>C het										PD-177
PD-178								IVS7-35 A>G het; IVS8+48 C>T het		Val380L eur. c.1239G>C het; IVS10+139 C>G het		PD-178
PD-179												PD-179
PD-180												PD-180
PD-181	-238 T>G het							IVS8+48 C>T hom			Asp394Asn. c.1281G>A het	PD-181
PD-182	-238 T>G hom							IVS7-35 A>G het; IVS8+48 C>T het				PD-182
PD-183								IVS7-35 A>G het; IVS8+48 C>T het		Val380L eur. c.1239G>C het		PD-183
PD-184												PD-184
PD-185												PD-185
PD-186												PD-186
PD-187	-238 T>G het							IVS7-35 A>G het				PD-187
PD-189								IVS7-48 G>C het; IVS8+48 C>T het			Asp394Asn. c.1281G>A het	PD-189
PD-193	-238 T>G het											PD-193
PD-203	-238 T>G het							IVS8+48 C>T het				PD-203
PD-204	-238 T>G het							IVS7-68 G>C het				PD-204
PD-205	-238 T>G het			Ser167Asn. c.601G>A het				IVS7-68 G>C het				PD-205
PD-206												PD-206
PD-207												PD-207
PD-210	-227 A>G het	Val56Glu. c.268T>A het						IVS7-68 G>C het				PD-210
PD-211		Val56Glu. c.268T>A hom						IVS7-68 G>C het				PD-211
PD-212												PD-212
PD-217								IVS8+48 C>T het		Val380L eur. c.1239G>C het		PD-217
PD-224								IVS8+48 C>T het		Val380L eur. c.1239G>C het		PD-224
PD-227	-238 T>G hom											PD-227
PD-229								IVS8+48 C>T hom				PD-229
PD-231	-227 A>G het	IVS2+25 T>C het						IVS7-48 G>C het; IVS8+48 C>T het				PD-231
PD-232		IVS2+25 T>C het						IVS7-68 G>C het		Val380L eur. c.1239G>C het		PD-232

[illegible]

Table A.5. *Parkin*, *LRRK2* and *α -synuclein* investigations of all Turkish PD patients

	Parkin dHPLC	Parkin dosage	MLPA	LRRK2 ex41	α -synuclein ex3&4
PD-111	done	done		done	
PD-112	done	done		done	
PD-113	done	done		done	
PD-114	done	done		done	
PD-115	done	done		done	
PD-116	done	done		done	
PD-117	done	del het ex 3-4		done	
PD-118	done	del het ex 3-4		done	
PD-119	done	done		done	
PD-120	done	done		done	
PD-121	done	done		done	
PD-122	done	del het ex3		done	
PD-123	done	del het ex4		done	
PD-124	done	done	done	done	done
PD-125	done	done		done	
PD-126	done	done		done	
PD-127	done	done	done	done	done
PD-128	done	done		done	
PD-129	done	done	done	done	done
PD-130	done	done	done	done	done
PD-131	done	done	done	done	done
PD-132	done	done		done	
PD-133	done	done		done	
PD-134	done	done		done	
PD-135	done	done		done	
PD-136	done	done		c.6055G>A: G2019S het	
PD-137	done	done	done	done	done
PD-138	done	done		done	
PD-139	done	done		done	
PD-140	done	done		done	
PD-141	done	del het ex2	het del ex2 parkin	done	
PD-142	done	del het ex2	het del ex2 parkin	done	
PD-143	done	done	done	done	done
PD-144	done	done		done	
PD-145	done	done		done	
PD-146	done	done		done	
PD-147	done	del het ex2		done	
PD-148	done	done		done	
PD-149	done	done		done	
PD-150	done	done		done	
PD-151	done	done		done	
PD-152	done	done		done	
PD-153	done	done		done	
PD-154	done	done		done	
PD-155	done	done		done	
PD-156	done	done	done	done	done
PD-157	done	done		done	
PD-158	done	done	done	done	done
PD-159	done	done		done	
PD-160	done	done		done	
PD-161	done	done		done	
PD-162	done	done		done	
PD-163	done	done		done	
PD-164	done	done		done	
PD-165	done	done		done	
PD-166	done	done		done	
PD-167	done	dup het ex7-8-9		done	
PD-168	done	done	done	done	
PD-169	done	done	dup het syn ex3-4	done	
PD-170	done	done	done	done	
PD-171	done	done	dup het syn ex3-4	done	
PD-172	done	done		done	
PD-173	done	done		done	
PD-174	done	done	done	done	done
PD-175	done	done		done	
PD-176	done	done		done	
PD-177	done	done		done	
PD-178	done	done	done	done	done
PD-179	done	done		done	
PD-180	done	done		done	
PD-181	done	done	done	done	done
PD-182	done	done	done	done	done
PD-183	done	done		done	
PD-184	done	done		done	
PD-185	done	done		done	
PD-186	done	done		done	
PD-187	done	done	done	done	done

Table A.5. *Parkin*, *LRRK2* and *α-synuclein* investigations of all Turkish PD patients
(continued)

	Parkin dHPLC	Parkin dosage	MLPA	LRRK2 ex41	α-synuclein ex3&4
PD-188				done	
PD-189			done	done	done
PD-190				done	
PD-191				done	
PD-192				done	
PD-193			hom del ex5-6-7 parkin	done	
PD-194				done	
PD-195				done	
PD-196			done	done	done
PD-197				done	
PD-198				done	
PD-199				done	
PD-200				done	
PD-201			done	done	done
PD-202				done	
PD-203			done	done	done
PD-204			done	done	done
PD-205			done	done	
PD-206			hom del ex5 parkin	done	
PD-207			hom del ex5 parkin	done	
PD-208				done	
PD-209				done	
PD-210			done	done	
PD-211			het del ex2 parkin	done	
PD-212			het del ex2-3-4 parkin	done	
PD-213				done	
PD-214				done	
PD-215				done	
PD-216			done	done	done
PD-217			done	done	
PD-218				done	
PD-219				done	
PD-220				done	
PD-221				done	
PD-222				done	
PD-223				done	
PD-224			done	done	done
PD-225				done	
PD-226				done	
PD-227			done	done	done
PD-228				done	
PD-229			done	done	done
PD-230				done	
PD-231			done	done	
PD-232			done	done	done
PD-233			done	done	done
PD-234			done	done	done
PD-235				done	
PD-236				done	
PD-237				done	
PD-238			dup het syn ex3-4	done	
PD-239			dup het syn ex3-4	done	
PD-240				done	
PD-241				done	
PD-242			done	done	
PD-243				done	
PD-244			dup het syn ex3-4	done	
PD-245			dup het syn ex3-4	done	
PD-246			dup het syn ex3-4	done	
PD-247				done	
PD-248				done	
PD-249				done	
PD-250			done	done	
PD-251			done	done	
PD-252				done	
PD-253				c.6055G>A: G2019S het	

APPENDIX E: PRODUCTS OF THIS THESIS

Neurogenetics
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SHORT COMMUNICATION

A LRRK2 G2019S mutation carrier from Turkey shares the Japanese haplotype

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