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Identification of genomic rearrangements in Parkinson's disease genes by multiplex ligation-dependent probe amplification

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CONTRIBUTIONS

Study design	Prof. Nicholas Wood
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Day-to-day supervision	Dr. Emma Deas
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LIST OF ABBREVIATIONS

A	Adenine
ABCG2	ATP-Binding Cassette, sub-family G, member 2
AD	Autosomal dominant
Apo E	Apolipoprotein E
AR	Autosomal recessive
ATOH1	Atonal Homolog 1 gene
ATP13A2	ATPase 13A2 gene
bp	Base pair
c.	Complementary DNA
CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
CAV1	Caveolin 1 gene
CAV2	Caveolin 2 gene
Cytosine	Cytosine
ddH20	Double distilled water
DLB	Dementia with Lewy bodies
DMP1	Dentin Matrix Acidic Phosphoprotein gene
DNA	Deoxyribonucleic acid
DSPP	Dentin Sialophosphoprotein gene
EDTA	Ethylenediaminetetraacetic acid
EOPD	Early onset Parkinson's disease
FAM13A1	Family with Sequence Similarity 13, member A1 gene
FAM13A1OS	Family with Sequence Similarity 13, member A1 Opposite Strand
FISH	Fluorescent in situ hybridisation
G	Guanine
g.	Genomic DNA
GCH1	GTP Cyclohydrolase 1 gene
GIGYF2	Grb10-interacting GYF Protein gene
GPRIN3	GPRIN Family Member 3
GRID2	Glutamate Receptor, Ionotropic, Delta 2
GW	Genome-wide
HERC3	Hect Domain and RLD 3 gene
HERC5	Hect Domain and RLD 5 gene
HERC6	Hect Domain and RLD 6 gene
HSP90AB3P	Heat Shock Protein 90kDa alpha, class B member 3 gene
HtrA2	HtrA2 Serine Peptidase 2 gene
IBSP	Integrin-Binding Sialoprotein gene
kb	Kilobase
LOC	Locus
LPA	Lipoprotein gene Lp(a) gene
LRRK2	Leucine-rich repeat kinase 2 gene
Mb	Megabase
MEPE	Matrix, Extracellular Phosphoglycoprotein with ASARM Motif gene
MIM	Mendelian inheritance in man

MLPA	Multiplex ligation-dependent probe amplification
MMRN1	Multimerin 1
NAP1L5	Nucleosome Assembly Protein 1-like 5 gene
NUDT9	Nudix (Nucleoside Diphosphate Linked Moiety X)-type motif 9 gene
p.	Protein
PCR	Polymerase chain reaction
PD	Parkinson's disease
PD-D	Parkinson's disease-dementia
PIGY	Phosphatidylinositol Glycan Anchor Y gene
PINK-1	PTEN Induced Putative Kinase 1 gene
PKD2	Polycystic Kidney Disease 2 gene
PPM1K	Protein Phosphatase 1 K gene
qPCR	Quantitative PCR
SB	Southern blot
SN	Substantia nigra
SNCA	α-Synuclein gene
SNP	Single nucleotide polymorphysm
SPARCL1	SPARC-Like 1 gene
SPP1	Secreted Phosphoprotein 1 gene
T	Thymine
TE	Tris/EDTA buffer
TIGD2	Tigger Transposable Element Derived 2 gene
TMSL3	Thymosin-Like 3 gene
TNFRSF9	Tumor Necrosis Factor Receptor Superfamily, member 9 gene
UCHL-1	Ubiquitin C-terminal Hydrolase L1 gene
YOPD	Young onset Parkinson's disease

ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder, characterised clinically by tremor, rigidity, bradykinesia, and postural instability. To date, 13 genetic loci have been directly associated with disease. Pathogenic mutations in PD genes associated include single nucleotide changes, small deletions and insertions, as well as large genomic rearrangements.

The aim of this study was to screen all familial PD samples held within the Institute of Neurology for genomic rearrangements in *SNCA, Parkin, LRRK2, PINK1 and DJ-1* using the MLPA technique.

The DNA samples from 83 patients with familial PD were included, as well as 39 additional DNA samples extracted from the brains of pathologically confirmed PD patients. MLPA analysis was performed using the P051 and P052 probe mixes.

We detected heterozygous genomic rearrangements in 9 familial PD patients. These consisted of a rare *SNCA* duplication, multiple *Parkin* rearrangements such as exon 2 duplication, exon 8 deletion (2 patients), exon 3 deletion and exons 3 and 4 deletion, *PINK1* exon 8 deletion (2 patients) and *DJ-1* exons 1 and 3 duplication. For the patients with *SNCA* and *DJ-1* genomic rearrangements, we also described clinical findings. The rare *SNCA* duplication was confirmed by a genome-wide single nucleotide polymorphysm assay and as a direct result of this project, the family have been contacted and offered genetic counselling.

1. INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease (deLau *et al.*, 2006). It affects more than 1.8% of the European population aged 65 years or over, with age at onset later than 50 years in most cases (de Rijk *et al.*, 2000). Young-onset (YOPD) or early-onset PD (EOPD) is variably defined in studies as PD starting at the age of less than 40 or 50 years (Quinn *et al.*, 1987; Schrag *et al.*, 2000; Butterfi *et al.*, 2006).

Classical clinical signs of PD are tremor, rigidity, bradykinesia, and postural instability. Pathologically, it is characterised by selective loss of dopaminergic neurones within the substantia nigra (SN) and the presence of intracellular inclusions known as Lewy bodies in surviving neurones (Hughes *et al.*, 1992). A high proportion of PD patients develop dementia several years after disease onset. In these cases, the term PD-dementia (PD-D) is used. PD-D and dementia with Lewy bodies (DLB) are clinically and pathologically similar. An arbitrary "1-year rule" is often used to distinguish between the two: if the onset of dementia is within 1 year of Parkinsonism the diagnosis of DLB is considered (Aarsland *et al.*, 2003; McKeith *et al.*, 2005).

Familial forms of Parkinsonism are rare compared with idiopathic disease. They are estimated to account for less than 10% of all PD cases (deLau *et al.*, 2006). Linkage analyses of families with monogenic forms of Parkinsonism have revealed 13 genetic loci directly associated with disease (Table 1) (Belin *and* Westerlund, 2008). For six of these loci (PARK 1/4, 2, 6, 7, 8, 9 and 13), the genes have been identified

and confirmed by several groups to cause familial Parkinsonism (Polymeropoulos *et al.*, 1997; Kitada *et al*, 1998; Singleton *et al.*, 2003; Bonifati *et al*, 2004; Paisan-Ruiz *et al*, 2004; Valente *et al.*, 2004a; Zimprich *et al.*, 2004; Ramirez *et al.*, 2006). A mutation in *ubiquitin C-terminal hydrolase L1 (UCHL-1)* has only been linked to familial Parkinsonism in one family (Leroy *et al.*, 1998) and Grb10-Interacting GYF Protein 2 gene *(GIGYF2)* has only recently been added to the list (Lautier *et al.*, 2008). Analysis of the proteins encoded by these genes has provided important insights into the molecular pathways that may be important in sporadic forms of PD (Tan *and* Skipper, 2007).

Table 1. ***Genetic causes of parkinsonism (adapted from Belin and Westerlund, 2008)***

Locus	Gene	Chromosome	Mode of inheritance
PARK 1/4	*SNCA* (Polymeropoulos *et al.*, 1997)	4q21	AD
PARK 2	*Parkin* (Kitada *et al.*, 1998)	6q25.2-q27	AR
PARK 3	unknown	2p13	AD
PARK 5	*UCHL1* (Leroy *et al.*, 1998)	4q21	AD
PARK 6	*PINK1* (Valente *et al.*, 2004a)	1p35-p36	AR
PARK 7	*DJ-1* (Bonifati *et al.*, 2003)	1p36	AR
PARK 8	*LRRK2* (Paisan-Ruiz *et al.*, 2004; Zimprich *et al.*, 2004)	12q12	AD
PARK 9	*ATP13A2* (Ramirez *et al*, 2006)	1p36	AR
PARK 10	unknown*	1p32	AR
PARK 11	*GIGYF2* (Lautier *et al.*, 2008)	2q36-q37	AD
PARK 12	unknown	X	NA
PARK 13	*Omi/HtrA2* (Strauss *et al.*, 2005)	2p13	NA

AD=autosomal dominant, AR=autosomal recessive, *SNCA=α-Synuclein, UCHL1= Ubiquitin C-terminal Hydrolase L1, PINK1=PTEN Induced Putative Kinase 1, LRRK2=Leucine-Rich Repeat Kinase 2, ATP13A2=ATPase 13A2, GIGYF2= Grb10-Interacting GYF Protein 2, Omi/HtrA2=HtrA2 Serine Peptidase 2*, NA=no data available, * association studies suggested candidate genes (Li *et al.*, 2007).

Pathogenic mutations in genes associated with PD include single nucleotide changes, small deletions and insertions, as well as large genomic rearrangements. The latter have been reported for all PD genes except *UCH-L1* and *leucine-rich repeat kinase 2 (LRRK2)*, and vary in size from single exons to entire genes (Scarciolla *et al.*, 2007).

The following section provides an introduction to the PD genes of interest in our study with an emphasis on genomic rearrangements.

1.1 α-synuclein gene (SNCA) mutations

SNCA maps to chromosome 4q21 (PARK1/4; MIM#s 163890 and 168601) and consists of six exons. The first pathogenic missense mutation (c.209G>A) was identified by Polymeropoulos *et al.* (1997) in the autosomal dominant Contursi kindred and in three Greek families. Kruger *et al.* (1998) and Zarranz *et al.* (2004) found two additional missense mutations in a German (c.88G>C) and Spanish (c.136G>A) family, respectively. Identification of *SNCA* was followed by the discovery that alpha synuclein represents a major component of Lewy bodies (Spillantini *et al.*, 1997).

SNCA gene dosage changes

In several large studies of familial and sporadic cases of PD no *SNCA* dosage changes were found (Hope *et al.*, 2004; Johnson *et al.*, 2004; Gispert *et al.*, 2005; Deng *et al.*, 2006; Williams-Gray *et al.*, 2006). Other studies identified families with *SNCA* triplications or duplications, but the hit rate was very low (Chartier-Harlin *et al.*, 2004; Farrer *et al.*, 2004; Ibanez *et al.*, 2004; Nishioka *et al.*, 2006; Ahn *et al.*, 2008). Of note, Ahn *et al.* (2008) recently reported two apparently sporadic cases of

PD with *SNCA* duplication but in general, SNCA multiplications appear to be a rare event. The studies of *SNCA* dosage changes in PD are summarized in Table 1.1a.

Table 1.1a ***Overview of studies of gene dosage changes in PD.***

	No of patients	***SNCA* duplications**
Chartier-Harlin *et al.*, 2004	9 families	1 family with duplication
Farrer *et al.*, 2004	42 familial	1 triplication
Hope *et al.*, 2004	50 familial	0
Ibanez *et al.*, 2004	119 familial	2 patients
Johnson *et al.*, 2004	101 familial 325 sporadic 366 normal controls 65 DLB	0
Gispert *et al.*, 2005	156 familial 190 sporadic	0
Deng *et al.*, 2006	180 familial 106 sporadic 10 negative controls 1 positive control	0
Nishioka *et al.*, 2006	113 familial AD PD 200 sporadic	2 familial 0 sporadic
Williams-Gray *et al.*, 2006	538 sporadic 923 controls	0
Ahn *et al.*, 2008	28 familial 878 sporadic 200 MSA	1 familial 2 sporadic 0 MSA

MSA=multiple system atrophy, DLB=dementia with Lewy bodies.

Families with SNCA dosage changes

Ten families with Parkinsonism due to *SNCA* multiplication have been identified worldwide: with the majority harbouring *SNCA* duplications (Chartier-Harlin *et al.*, 2004; Ibanez *et al.*, 2004, Nishioka *et al.*, 2006; Fuchs *et al.*, 2007; Ahn *et al.*, 2008; Ikeuchi *et al.*, 2008). *SNCA* triplication segregated with the disease in the Iowa kindred and the Swedish-American family (Singleton *et al.*, 2003; Farrer *et al.*, 2004; Fuchs *et al.*, 2007). Notably, the Swedish and Swedish–American kindreds have been shown to be related as they both have ancestors from the Lister family complex (Fuchs *et al.*, 2007). Information on families with *SNCA* dosage changes are summarized in Table 1.1.b.

Table 1.1.b ***Families with SNCA gene dosage changes.***

	***SNCA* dosage change**	**The size of the multiplicated region**
Iowa family (Singleton *et al.*, 2003; Ross *et al.*, 2008)	Triplication	1.61-2.04 Mb (contains 17 genes)
Swedish-American family, Lister complex, branch I (Farrer *et al.*, 2004; Fuchs *et al.*, 2007; Ross *et al.*, 2008)	Triplication	0.9 Mb (0.7987 – 0.9359 Mb, 2 genes)
Swedish family, Lister complex, branch J (Fuchs *et al.*, 2007; Ross et al., 2008)	Duplication	0.9 Mb (0.7987 – 0.9359 Mb, 2 genes)
French family (Chartier-Harlin *et al.*, 2004; Ross *et al.*, 2008)	Duplication	4.93-4.97 Mb (31 transcripts)
French family (Ibanez *et al.*, 2004)	Duplication	0.5 Mb
Italian family (Ibanez *et al.*, 2004)	Duplication	0.5 Mb
Japanese family A (Nishioka *et al.*, 2006; Ross *et al.*, 2008)	Duplication	0.5 Mb
Japanese family B (Nishioka *et al.*, 2006; Ross *et al.*, 2008)	Duplication	0.4 Mb
Japanese family (Ikeuchi *et al.*, 2008)	Duplication in 3 patients, homozygous duplication in 1 patient	5 Mb
Korean family (Ahn *et al.*, 2008)	Duplication	NA

Mb=megabase, NA=no data available.

Clinical phenotype in SNCA dosage changes

Affected members in *SNCA* duplicated families usually present in their fifth or sixth decade with clinical features similar to idiopathic PD. In general, the disease progresses slowly and responds well to levodopa treatment. Cognitive decline late in the disease course and autonomic dysfunction have both been described, but neither seem to be a prominent feature (Chartier-Harlin *et al.*, 2004; Ibanez *et al.*, 2004; Nishioka *et al.*, 2006; Fuchs *et al.*, 2007).

In contrast, patients with *SNCA* triplication present earlier (on average in their mid-thirties) with rapidly progressive parkinsonism, autonomic dysfunction, prominent cognitive decline and visual hallucinations consistent with clinical diagnoses of DLB or PD-D (Muenter *et al.*, 1998; Gwinn-Hardy *et al*, 2000; Fuchs *et al.*, 2007). Furthermore, a Japanese patient with a homozygous *SNCA* duplication (equivalent *SNCA* dosage as triplication patients) was described to have a similar clinical phenotype (Ikeuchi *et al.*, 2008).

Several groups have suggested that the aggressive disease course and earlier occurrence of symptoms in *SNCA* triplication cases, compared to *SNCA* duplication cases, reflect a direct relation between *SNCA* gene dosage and clinical phenotype (Ibanez *et al.*, 2004; Fuchs *et al.*, 2007; Ross *et al.*, 2008).

1.2 Parkin gene mutations

The *Parkin* gene maps to chromosome 6q25.2–q27 (PARK2; MIM#s 600116 and 602544) and consists of 12 exons. The first *Parkin* mutations identified were

genomic rearrangements (deletion of exons 3 to 7 and deletion of exon 4) (Kitada *et al.*, 1998). Over 100 different mutations have now been described and affect all exons (Tan *and* Skipper, 2007; Hedrich *et al.*, 2004b).

According to studies by Lucking *et al.* (2000) and Periquet *et al.* (2003), *Parkin* mutations account for nearly 50% of familial EOPD patients with an autosomal recessive mode of inheritance and approximately 15% of sporadic EOPD cases.

Parkin genomic rearrangements

A comprehensive review of 379 *Parkin* mutation carriers revealed exon rearrangements in more than 50% of cases, the most common being deletions of exon 4, exon 3 or both (Hedrich *et al.*, 2004b).

Clinical phenotype in Parkin mutations

Parkin mutation carriers most commonly present with EOPD, although the age of onset varies considerably. Compared with idiopathic PD, the progression is slower with a good and sustained response to lower doses of levodopa. Common observations are dystonia at onset and symmetrical parkinsonism, especially in those with younger onset. A considerable proportion of reported cases are carriers of heterozygous *Parkin* mutations, and in these cases the symptoms tend to start later in life (Lucking *et al.*, 2000; Kann *et al.*, 2002; Lohmann *et al.*, 2003; Pramstaller *et al.*, 2005).

1.3 PINK1 gene mutations

The *PINK1* gene maps to chromosome 1p35 (the PARK6 locus; MIM #605909) and consists of 8 exons. A truncating nonsense and a missense mutation in *PINK1* were originally identified in two families (Italian and Spanish) with autosomal recessive familial PD (Valente *et al.*, 2004a), followed by a number of further missense and frameshift mutations (Hatano *et al.*, 2004; Rogaeva *et al.*, 2004; Valente *et al.*, 2004b; Klein *et al.*, 2005; Ibanez *et al.*, 2006; Tan *et al.*, 2006).

The frequency of *PINK1* mutations in patients with mainly sporadic EOPD was estimated at 5% to 7% (Klein *et al.* 2005; Valente *et al.* 2004b), but in two large cohorts from Ireland and North America much lower carrier rates were found (Healy *et al.*, 2004; Rogaeva *et al.*, 2004).

PINK1 genomic rearrangements

Whilst missense mutations, small deletions and duplications resulting in truncation mutants are common, larger gene rearrangements seem to be rare (Tan *and* Skipper, 2007). Only one case of an entire *PINK1* deletion has been described to date in an Italian sporadic EOPD patient. The mutation was in a compound heterozygous state with a 23 bp deletion across the junction between intron 6 and exon 7 (g.15445>15467del23) (Marongiu *et al.*, 2007). Homozygous deletion of exons 6 to 8 has been identified in Japanese EOPD patients by two authors (Li *et al.* 2005, Atsumi *et al.*, 2006).

Clinical phenotype in PINK1 mutations

Clinical findings in patients with PINK1 mutations resemble those in patients with Parkin mutations and show early age at onset, slow disease progression, good response to levodopa therapy and a common presence of dystonia as a presenting feature (Hatano *et al.*, 2004; Valente *et al.*, 2004b; Ibanez *et al.*, 2006; Tan *et al.*, 2006).

1.4 DJ-1 gene mutations

The *DJ-1* gene maps to chromosome 1p36 (the PARK7 locus; MIM # 602533) and consists of eight exons. It encodes a 189-amino-acid protein of approximately 20 kDa. The first *DJ-1* mutations were identified in two aurosomal recessive kindreds, a Dutch family with a deletion encompassing exons 1 to 5 and an Italian family harbouring a homozygous c.497T>C missense mutation (Bonifati *et al.*, 2003). Several other point mutations in *DJ-1* have been identified including homozygous, heterozygus and compound heterozygous cases (Abou-Sleiman *et al.* 2003; Hague *et al.* 2003; Hering *et al.*, 2004; Annesi *et al.*, 2005; Tang *et al.*, 2006).

DJ-1 genomic rearrangements

It is estimated that *DJ-1* mutations account for 1% of cases with EOPD (Abou-Sleiman *et al.*, 2003). Several groups have screened large cohorts of PD patients for gene dosage changes in *DJ-1* gene, but most of these studies gave negative results and are summarised in Table 1.4 (Abou-Sleiman *et al*, 2003; Clark *et al.*, 2004; Hedrich *et al.*, 2004a; Lockhart *et al.*, 2004; Karamohamed *et al.*, 2005; Klein *et al.*, 2005; Pankratz *et al.*, 2006).

Table 1.4 ***Summary of DJ-1 gene dosage studies.***

Study	No of patients	*DJ-1* dosage changes
Abou-Sleiman *et al.*, 2003	190 pathologically proven cases with sporadic PD 185 YOPD cases - *Parkin* mutations excluded 96 controls 124 Ashkenazi Jewish controls 20 Afro-Caribbean controls	0 (tested only for exons 1-5 deletion – 14,082 bp)
Clark *et al.*, 2004	89 EOPD patients (onset <50 years)	0 (Tested only for exons 1-5 deletion – 14,082 bp)
Hedrich *et al.*, 2004a	100 YOPD patients (onset <40 years)	2 heterozygous carriers (1 deletion of exons 5-7, 1 small deletion of 11 bp (IVS5+2-12 del)
Lockhart *et al.*, 2004	41 EOPD patients (<50 years) – *Parkin* mutations excluded (39 sporadic, 2 familial)	0
Karamohamed *et al.*, 2005	292 familial PD cases from different families	0 (tested only for exons 1-5 deletion – 14,082 bp)
Klein *et al.*, 2005	65 EOPD patients (onset 25-51 years, 62 sporadic, 3 familial)	0
Pankratz *et al.*, 2006	93 PD patients from 64 families with linkage to the DJ-1 region (18-80 years onset)	0 (MLPA used)

Apart from the family with a homozygous exon 1 to 5 *DJ-1* deletion identified by Bonifati *et al.* (2003), no other homozygous *DJ-1* dosage changes or exon rearrangements have been reported. Hedrich *et al.* (2004a) found *DJ-1* deletions in a heterozygous state, including a deletion of exons 5 to 7 and a small 11-base pair deletion (IVS5>2-12del).

Clinical phenotype in DJ-1 gene mutations

The affected members with exon 1 to 5 *DJ-1* deletion from the Dutch family presented with EOPD with slow disease progression and good response to levodopa. Some of them had dystonic features (van Duijn *et al.*, 2001; Bonifati *et al.*, 2003). The carrier of heterozygous *DJ-1* exon 5-7 deletion described by Hedrich *et al.* (2004a) presented at age 42 with levodopa responsive parkinsoinsm. No psychiatric features were present. The patient with the 11-base pair deletion presented at age 17 with tremor predominant Parkinsonism. The response to levodopa was not tested. No cognitive impairment or psychiatric abnormalities were noted (Hedrich *et al.*, 2004a).

1.5 LRRK2 gene mutations

The *LRRK2* gene maps to chromosome 12p (the PARK8 locus; MIM# 609007) and consists of 51 exons. Several point mutations in *LRRK2* were first identified in large kindreds with autosomal dominant Parkinsonism (Paisan-Ruiz *et al.*, 2004; Zimprich *et al.*, 2004).

Paisan-Riuz *et al.* (2008) performed an analysis of the *LRRK2* locus for whole gene multiplications or deletions in 275 PD cases. However, no major genome rearrangements were found. Johnson *et al.* (2007) screened 79 North American patients with familial PD with the same outcome. To our knowledge, no larger rearrangements in *LRRK2* have been identified to date.

1.6 Current methods for detection of gene dosage changes

Routine mutation screening procedures readily detect point mutations, as well as small deletions and insertions. In contrast, detection of larger genomic rearrangements (exons, whole genes) is more challenging. The diagnostic usefulness of techniques such as quantitative real time polymerase chain reaction (qPCR), southern blot (SB) and fluorescent in situ hybridisation (FISH) are hindered by high costs, reduced sensitivity or low throughput (Scarciolla *et al.*, 2007).

Multiplex ligation-dependent probe hybridisation (MLPA)

Multiplex ligation-dependent probe amplification (MLPA) is a relatively new method for gene copy number assessment (Schouten *et al.*, 2002). Instead of amplifying and quantifying genomic DNA sequences, it is based on comparative quantification of specifically bound probes that are amplified by PCR with universal primers. Because of the universal primers, amplifying numerous targets in one reaction is much easier. Furthermore, only one fluorescent primer is required for the detection of products (Sellner *and* Taylor, 2004). In comparison to other gene dosage detection techniques, MLPA is rapid, cost effective, sensitive, and relatively easy to perform (Scarciolla *et al.*, 2007).

With MLPA it is possible to establish the copy number of up to 45 DNA sequences in a single reaction (Fihgre 1.6) (Schouten *et al.*, 2002).

During MLPA, genomic DNA is hybridised in solution to approximately 45 specific probes. MLPA probes consist of two halves: one short synthetic oligonucleotide and one long probe oligonucleotide. The short synthetic oligonucleotide contains a 20-30 nucleotide target-specific sequence at the 3' end and a common 19 nucleotide sequence at the 5' end. The 19 nucleotide sequence is complementary to a universal labelled PCR primer.The long oligonucleotide has a 25-43 nucleotide target-specific sequence at the 5' end, a stuffer sequence and a common 36 nucleotide sequence complementary to an unlabelled universal PCR primer at the 3' end. The stuffer sequence is of variable length (19-370 nucleotides) and is necessary for the generation of PCR products that differ in size and thus allow electrophoretic resolution (Sellner *and* Taylor, 2004; Schouten *et al.*, 2002).

The target specific sequences of both probe halves bind adjacently to the target DNA. Only probes that have hybridised to the target DNA are then joined by a ligase. This generates a contiguous probe flanked by universal 19 and 36 nucleotide binding sites for PCR primers which permit amplification of the probe by PCR. If the sequence complementary to probes in the target DNA is missing (due to a deletion, point mutation or a single nucleotide polymorphism (SNP)), the probes cannot bind and consequently there is no amplification. In contrast, a genomic multiplication in the target region will result in increased probe amplification. (Sellner *and* Taylor, 2004; Djarmati *et al.*, 2006).

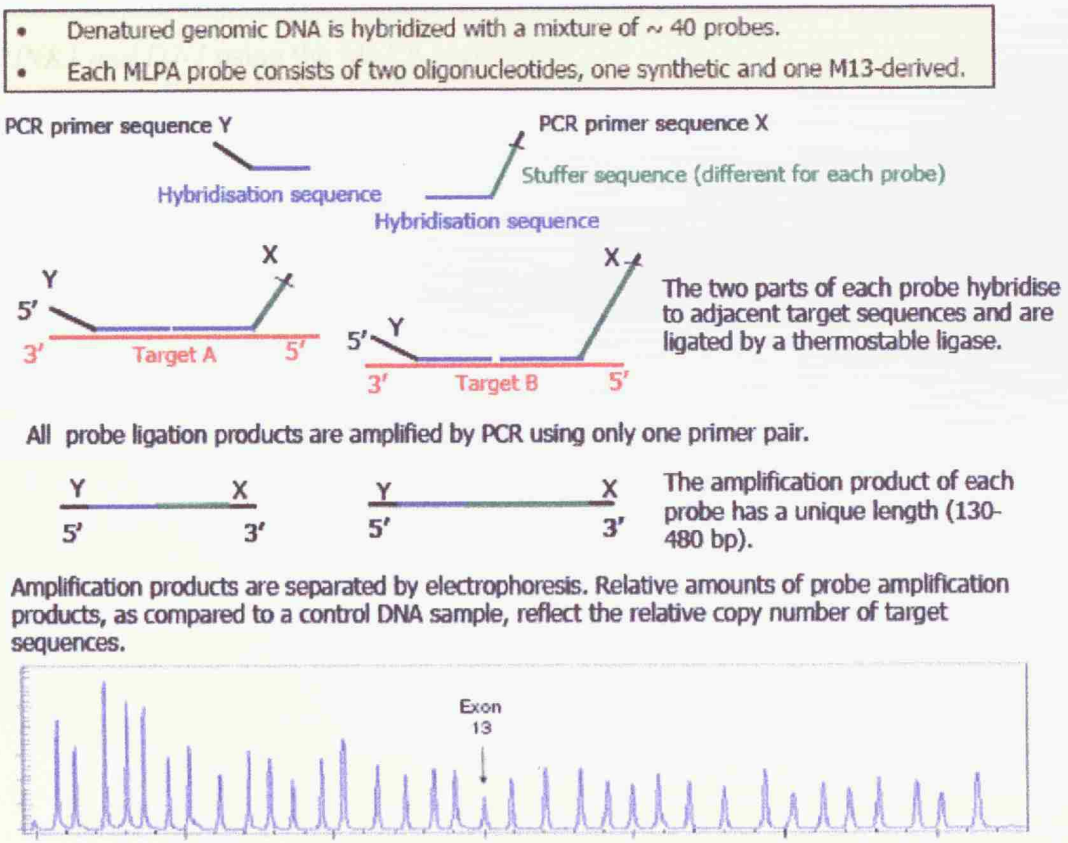
The amount of ligated probe produced is thus proportional to the target copy number. Amplified products are separated by sequence type electrophoresis. The peak heights are then compared with results from normal controls that are analysed

in the same experiment. Relative peak heights reflect the presence of deletions or duplications of target sequences (Sellner *and* Taylor, 2004).

Theoretically, a loss of one allele copy, an allele duplication or triplication should produce a relative peak ratio of 0.5, 1.5 and 2.0, respectively. In practice, allele copy losses usually produce relative peak values close to theoretical value of 0.5, while allele multiplications show lower than theoretical values (Scarciolla *et al.*, 2007).

Some probes are also designed to specifically recognise the wild type or the mutant allele in selected mutations (e.g. G2019S mutation in *LRRK2*) (Scarciolla *et al.*, 2007).

Figure 1.6 ***MLPA technique outline (taken from: Schouten et al.., 2002).***



Genome-wide single nucleotide polymorphism (GW-SNP) assays

Due to advances in technology, high-throughput SNP genotyping has become more accessible for research and diagnostic purposes. It has proven to be a powerful tool for detection of known and novel genomic rearrangements (copy number variations) and has lead to recent findings of extensive DNA copy number variation in the population and identification of many previously unrecognized submicroscopic chromosomal aberrations (Beaudet *and* Belmont, 2008).

1.7 Aim

The aim of this study was to screen all familial PD samples held within the Institute of Neurology, and a subset of pathologically confirmed PD patients with proven alpha-synucleinopathies, for genomic rearrangements in *SNCA, Parkin, LRRK2, PINK1 and DJ-1* using the MLPA technique.

2. MATERIALS AND METHODS

2.1 Patient selection

The DNA samples from 83 patients with PD with a positive familial history of the disease collected from 1990 through to 2005 were included. All the patients had been previously screened for point mutations in *Parkin* and *PINK1*.

Additionally, 39 DNA samples extracted from the brains of PD patients with a pathological diagnosis of alpha-synucleinopathy were included.

In each experiment, normal control samples and positive control samples (for which gene dosage changes had been previously confirmed with other techniques) were run in parallel with the patient samples. Negative controls were also included and consisted of blank (double distilled water – ddH_2O) as well as blank samples containing no DNA.

All DNA samples were obtained according to the joint reasearch ethics committee of the National Hospital for Neurology and Neurosurgery and Institute of Neurology guidelines. Written consent was given by all donor patients.

2.2 DNA extraction

DNA was extracted from blood samples or brain tissue using standard procedures.

2.3 MLPA analysis

MLPA analysis was performed for all the samples using the SALSA MLPA kit (MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's protocol. P051 Parkinson-1 (lot 0107) probe mix was used for all samples. Where dosage changes were detected in either *SNCA* or *Parkin*, an additional independent analysis with probe mix P052 Parkinson-2 (lot 0907) which contains different probes for these two genes was performed for confirmation. Table 2.3 shows a list of specific probes supplied in both probe mixes.

Table 2.3 ***List of specific probes in the MLPA P051 and P052 kits (adapted from Djarmati et al., 2006).***

	SALSA MLPA KITS	
Gene	**P051**	**P052**
SNCA	Exons 1, 3, 4, 5, 6 + p.A30P wild type	Exon 2
Parkin	Exons 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	Exons 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 + intron 1
UCHL1	No probe	Exons 1, 4, 5, 9
PINK1	Exons 1, 2, 3, 4, 5, 6, 7, 8	No probe
DJ-1	Exons 1, 2, 3, 4, 5, 6, 7	No probe
LRRK2	p.G2019S specific	Exons 1, 2, 10, 15, 27, 41, 49 + p.G2019S specific
GCH1	No probe	Exons 1, 2, 3, 4, 5, 6
Probes for other genes	*LPA, TNFRSF9*	*CAV1, CAV2*
No. of control probes	6	9
Σ	41	41

GCH1= GTP cyclohydrolase 1 gene, *LPA*= lipoprotein gene, Lp(a), *TNFRSF9*= tumor necrosis factor receptor superfamily, member 9 gene, *CAV1*= caveolin 1 gene, *CAV2*= caveolin 2 gene.

DNA denaturation and hybridisation of the SALSA MLPA probes:

Two microliters of genomic DNA (at a concentration of 50 ng/μl) were used. DNA was diluted with TE to 5 μl and denatured for 5 minutes at 98°C and then cooled to 25°C. After adding 1.5 μl of SALSA probemix and 1.5 μl of MLPA buffer, the samples were incubated at 95 °C for 1 minute and hybridised at 60°C for 16 hours.

Ligation reaction:

After hybridisation, the temperature was reduced to 54 °C and 32 μl of Ligase-65 mix (3 μl of Ligase-65 buffer A, 3 μl of Ligase-65 buffer B, 25 μl of ddH_2O) were added to each reaction tube. Following incubation at 54 °C for 15 minutes, the temperature was increased to 98°C for 5 minutes to inactivate the ligase.

PCR reaction:

Four microliters of SALSA PCR buffer and 26 µl of ddH_2O was added to 10 µl of MLPA ligation reaction and the temperature was increased to 60°C. Ten microliters of polymerase mix (2 µl of SALSA PCR-primers, 2 µl of SALSA enzyme dilution buffer, 2 µl of PCR ddH_2O, 0.5 µl of SALSA polymerase) were added to each reaction in a Gene Amp PCR System 9700 thermal cycler. The PCR reaction was started immediately. PCR conditions were as follows: 35 cycles at 95 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 60 seconds. The cycles were followed by a 20 minute step at 72 °C.

Denaturation and electrophoresis:

0.3 µl of GeneScan 500 Liz size standard and 12 µl of HiDi Formamide (both Applied Biosystems - ABI) were added to 1.5 µl of PCR reaction. The samples were then denatured in a thermal cycler at 98°C for 3 min and subsequently cooled on ice for 3 min. Finally, samples were electrophoresed using an ABI 3730xl DNA analyser with ABI DNA Analyser Data Collection Software V3.0.

Analysis:

Files were exported to GeneMarker V1.70 software (SoftGenetics, State College, Pennsylvania, USA) where the peaks were first visually inspected. Automated normalisation and analysis comparing peak heights with average peak hights from normal control samples were then carried out. A ratio of less than 0.75 was regarded as a deletion and more than 1.25 as a duplication. Scatter plots generated by the software were analysed.

2.4 Confirmation of SNCA duplication

A GW-SNP assay was performed using the Human 610-Quad BeadChip that features 550,000 tag SNPs derived from HapMap data and 60,000 additional markers developed in collaboration with deCODE Genetics. It provides a high-density genomic coverage and is suitable for detection of both known and novel copy number variation regions (http://www.illumina.com/).

Genotyping was carried out according to the manufacturer's instructions (Infinium HD Super assay manual, Illumina Inc) by the SNP technology platform. Briefly, 1 μg of genomic DNA was amplified at 37°C overnight. After overnight incubation, the amplified DNA was enzymatically fragmented and precipitated with 100% 2-propanol after the addition of PM1 buffer. The dried precipitated pellet was then resuspended in RA1 buffer and hybridised to a beadchip along with RA1 and formamide. The arrays were then incubated overnight at 48°C, after which they underwent single-base extension on a Teflow chamber rack system (Tecan, Mannedorf, Switzerland) using XC1, XC2, and TEM buffers. After the single-base extension step, the beadchips were stained, dried for 1 h and then imaged using a BeadArray Reader System (http://www.illumina.com/).

The genotyping and scan data from each sample was analysed and normalised using the GenomeViewer tool within BeadStudio V3.2 Genotyping module (Illumina Inc., San Diego, CA). Normalised fluorescence signals were compared with the signal intensities of a set of reference genotypes from 120 normal samples, and the log2 ratios between the sample and the reference signals were calculated. Additionally,

the frequencies of B alleles for the samples were estimated based on reference genotype clusters. Two metrics which allow the visualization of copy number changes and homozygosity were assessed: log *R* ratio and B allele frequency. B allele frequency gives an estimate of the proportion of times an individual allele is called A or B; thus an individual homozygous for the B allele would have a score close to 1, an individual homozygous for the A allele a score close to 0, and a score of 0.5 would indicate a heterozygous genotype. The log *R* ratio gives an indirect measure of copy number of each SNP by plotting the ratio of observed to expected hybridization intensity. An *R* above 1 is indicative of an increase in copy number (duplication or triplication), and values below 1 suggest a deletion. A value equal to zero indicates a normal copy number (Gibbs *and* Singleton, 2006).

3. RESULTS

The DNA samples from 83 patients (49 males, 34 females) with familial PD were analysed. Additionally, 39 PD DNA samples extracted from the brains of patients with a pathological diagnosis of alpha-synucleinopathy (28 males, 11 females) were included. Average age at disease onset in this group was 64.8 years (42-78), 8 patients had positive familial history of PD. All together, 91 patients had familial PD.

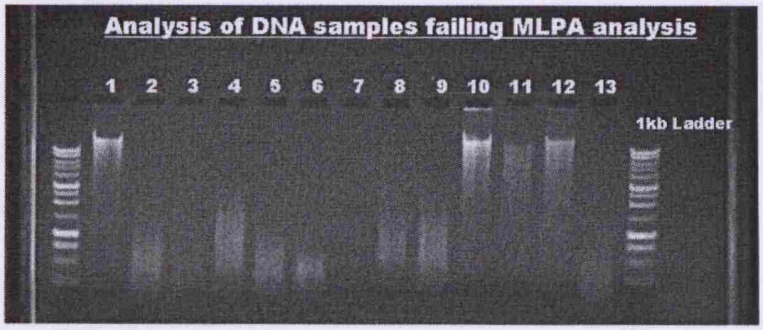
Genomic rearrangements were detected with MLPA using P051 probe mix in 9 out of 91 familial PD patients. All the mutations were in the heterozygous state (Table 3). No rearrangements were detected in the samples from patients without a family history of the disease. The samples with genomic rearrangements in *Parkin* were independently analysed with MLPA P052 probe mix that contains probes for *Parkin* exons that are specific for different target sequences. Similarly, we performed the analysis with MLPA P052 probe mix for the *SNCA* duplication sample. P052 contains a probe for SNCA exon 2 that is not provided in P051 probe mix and can thus serve as independent confirmation.

Table 3. ***Genomic rearrangements in familial PD samples***

Case	Gene	Genomic rearrangement	Probemix used
1	PARK1 (*SNCA*)	Exons 1, 2, 3, 4, 5, 6 duplication Exon 2 duplication	P051 P052 (contains only one probe for *SNCA* – exon 2)
2	PARK2 (*Parkin*)	Exon 8 deletion	P051/P052
3	PARK2 (*Parkin*)	Exon 8 deletion	P051/P052
4	PARK2 (*Parkin*)	Exon 2 duplication	P051/P052
5	PARK2 (*Parkin*)	Exon 3 deletion	P051/P052
6	PARK2 (*Parkin*)	Exons 3 and 4 deletion	P051/P052
7	PARK6 (*PINK1*)	Exon 8 deletion	P051
8	PARK6 (*PINK1*)	Exon 8 deletion	P051
9	PARK7 (*DJ-1*)	Exons 1 and 3 duplication	P051

Unfortunately, MLPA analysis failed in 13 samples. However, gel electrophoresis of these samples revealed that the cause of failures was sheared DNA in 10 cases. In the remaining 3 samples, the cause were probabaly contaminants (Figure 3). We were not able to obtain alternative samples for 8 of these patients and consequently further analysis was not pursued.

Figure 3. ***Gel electrophoresis of DNA samples failing DNA analysis.***



Samples 1, 10 and 12 show a clearly visible band indicating that DNA is not fragmented. In samples 2-9, 11 and 13 the band is absent indicating sheared DNA.

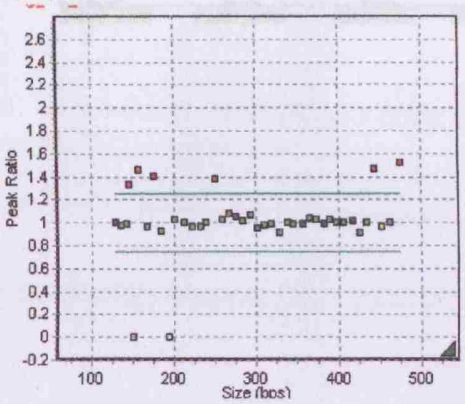
3.1 Case 1 – SNCA duplication patient

Genetic studies

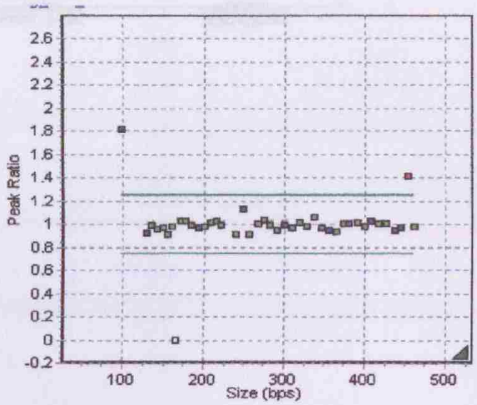
Strikingly, despite their rare occurrence, MLPA analysis showed duplication of all *SNCA* exons in one patient. Peak heights representing exons 1 to 6 of *SNCA* (Figure 3.1) were approximately 1.3-1.5 times larger than the average peak height of normal control samples.

Figure 3.1. ***Case 1 – SNCA duplication – MLPA figures.*** A and B) MLPA peak ratio plots for P051 and P052 probemixes, respectively. Red dots represent probes for *SNCA* exons 1 to 6 (P052 probemix only contains one *SNCA* probe – exon 2). Blue dots represent control probes supplied in P051 and P052 MLPA kits. Blue dot with an unusually high peak ratio in B represents control 1 and is probably an artifact. Green dots represent probes for other PARK genes. C-E) MLPA trace overlay. Comparison of intensities of the sample peaks (in blue) and average control peaks (in red).

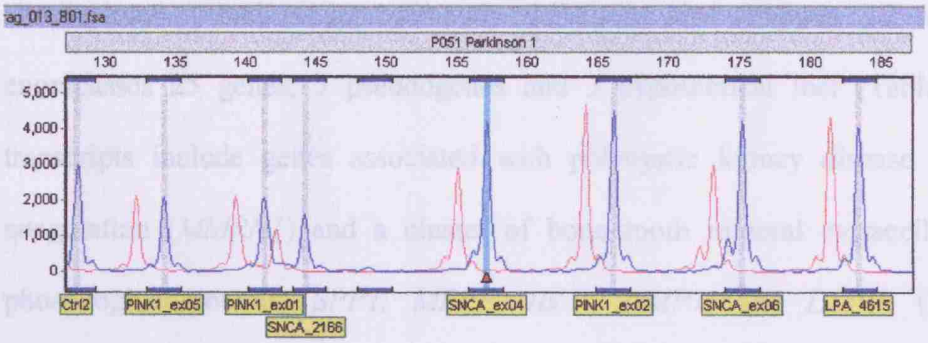
A P051



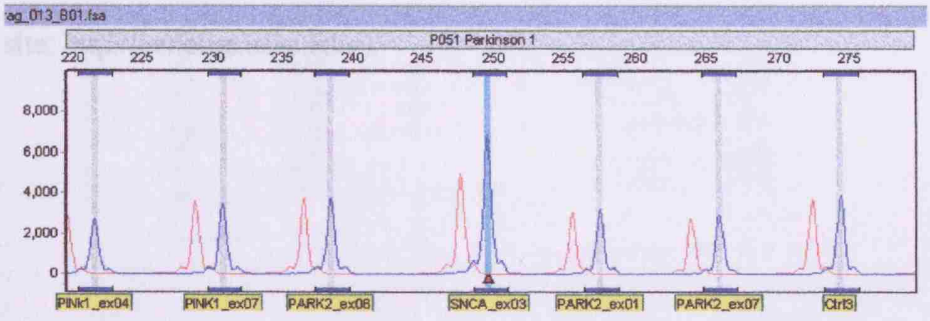
B P052



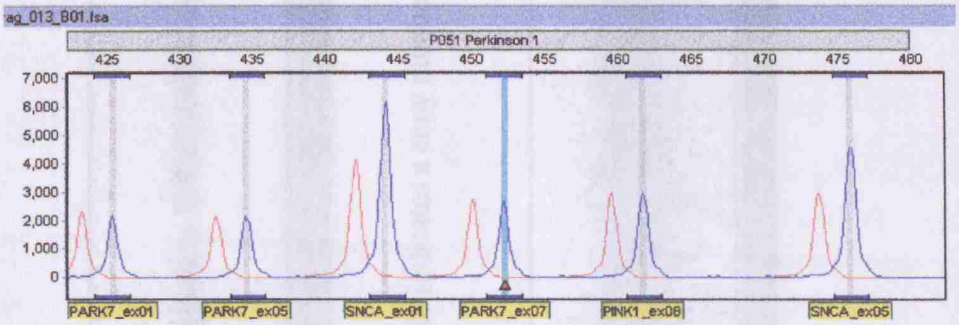
C



D



E



The duplication was confirmed using the genome-wide SNP assay. The duplicated region is estimated to extend from the position 88566392bp to 95168203bp on chromosome 4 and is approximately 6.6Mb in size (Figures 3.2 and 3.3). It enompasses 25 genes, 5 pseudogenes and 3 hypothetical loci (Table 3.2). The transcripts include genes associated with polycystic kidney disease II (*PKD2*), coagulation (*MMRN1*) and a cluster of bone–tooth mineral extracellular matrix phosphoglycoproteins (*SPP1*, *MEPE*, *IBSP*, *DMP1*, and *DSPP*) (http://www.ncbi.nlm.nih.gov; Rowe *et al.*, 2000). Four transcripts including *SNCA* are expressed at high levels in the brain (microarray expression data retrieved from UCSC Web site: http://genome.ucsc.edu/).

Figure 3.2. Visualisation of structural genomic variability across chromosome 4. B allele frequencies are depicted on the Y axis. Normal values cluster around 0 (homozygous for the A allele of a SNP), 1 (homozygous for the B allele) and 0.5 (heterozygous A/B genotype). The duplicated region is indicated by a deviation of values from these normal scores.

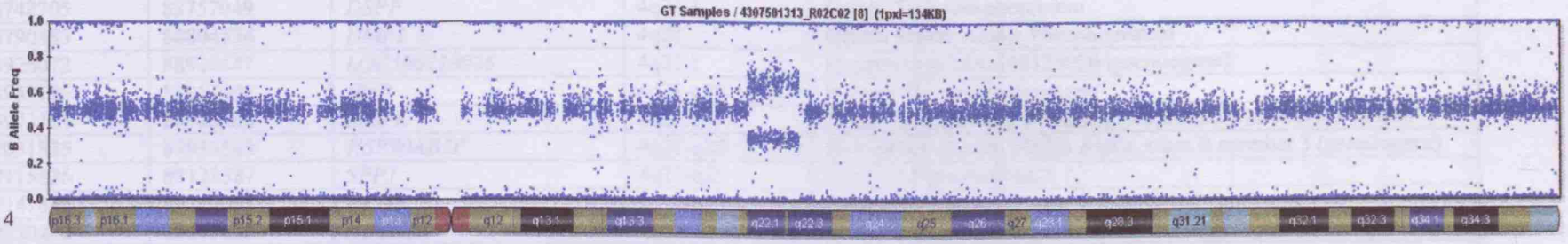


Figure 3.3. Visualisation of structural genomic variability across chromosome 4. An increase in Log R ratio indicates a copy number increase.

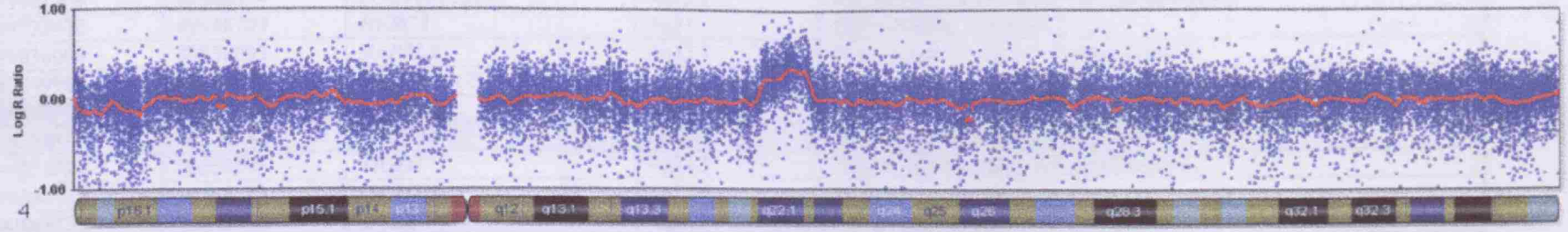


Table 3.2. ***The genes in the duplicated region (obtained from www.ncbi.nlm.nih.gov).***

From (position)	To (position)	Gene found in this region	Chromosome	Protein
88562759	88598523	*NUDT9*	4q22.1	Nudix (Nucleoside Diphosphate Linked Moiety X)-Type Motif 9
88613511	88669530	*SPARCL1*	4q22.1	SPARC-Like 1
88748705	88757049	*DSPP*	4q21.3	Dentin Sialophosphoprotein
88790483	88804534	*DMP1*	4q21	Dentin Matrix Acidic Phosphoprotein
88926072	88926547	*LOC100128026*	4q22.1	Hypothetical LOC100128026 (pseudogene)
88939726	88952098	*IBSP*	4q21-q25	Integrin-Binding Sialoprotein
88973164	88986968	*MEPE*	4q21.1	Matrix, Extracellular Phosphoglycoprotein with ASARM Motif
89031835	89034580	*HSP90AB3P*	4q21-q25	Heat Shock Protein 90kDa Alpha, class B member 3 (pseudogene)
89115826	89123587	*SPP1*	4q21-q25	Secreted Phosphoprotein 1
89147844	89217953	*PKD2*	4q21-q23	Polycystic Kidney Disease 2 (autosomal dominant)
89230440	89299035	*ABCG2*	4q22	ATP-Binding Cassette, sub-family G (WHITE), member 2
89402041	89424771	*PPM1K*	4q22.1	Protein Phosphatase 1K
89518915	89583272	*HERC6*	4q22.1	Hect Domain and RLD 6
89597291	89646337	*HERC5*	4q22.1	Hect Domain and RLD 5
89648765	89650577	*LOC728333*	4q22.1	Similar to Nuclear Receptor Coactivator 4 (pseudogene)
89661158	89663978	*PIGY*	4q22.1	Phosphatidylinositol Glycan Anchor Biosynthesis, class Y
89667334	89668344	*LOC100129137*	4q22.1	Hypothetical LOC100129137 (pseudogene)
89732670	89848709	*HERC3*	4q21	Hect Domain and RLD 3
89836090	89838003	*NAP1L5*	4q22.1	Nucleosome Assembly Protein 1-like 5
89849963	89870277	*FAM13A1OS*	4q22.1	Family with Sequence Similarity 13, member A1 Opposite Strand
89866129	90197346	*FAM13A1*	4q22.1	Family with Sequence Similarity 13, member A1
90250887	90252542	*LOC731282*	4q22.1	Hypothetical protein LOC731282
90252991	90255075	*TIGD2*	4q22.1	Tigger Transposable Element Derived 2
90384452	90448184	*GPRIN3*	4q22.1	GPRIN Family Member 3
90865728	90977156	*SNCA*	4q21	Synuclein, alpha
90976196	90980769	*LOC644248*	4q22.1	Hypothetical LOC644248
91035075	91094803	*MMRN1*	4q22	Multimerin 1
91375205	91922178	*MGC48628*	4q22.1	Similar to KIAA1680 protein
91978659	91979292	*TMSL3*	4q22.1	Thymosin-like 3
92459208	92465474	*LOC728394*	4q22.1	Hypothetical protein LOC728394
93322646	93324225	*LOC133083*	4q22.1	Similar to Peptidase (mitochondrial processing) Alpha (pseudogene)
93444573	94912672	*GRID2*	4q22	Glutamate Receptor, Ionotropic, Delta 2
94969101	94970165	*ATOH1*	4q22	Atonal Homolog 1 (Drosophila)

Clinical presentation of identified SNCA duplication patient

The patient presented aged 29 years with slight right hand weakness and tremor while writing. In addition to that, the speed of writing became slower and the letters smaller. He learned to overcome this by using his left hand. He also noticed a loss of swing in his right arm when walking and clumsiness of his right leg when playing football.

When examined one year later he was noted to have a slight loss of facial expression and a pill-rolling rest tremor of his right arm. There was right predominant rigidity and poverty of movement. The rest of the examination was normal apart from the consistent finding of extensor plantar responses for which no alternative cause was found. This finding persisted throughout the course of his disease. No postural instability was observed.

Head CT was unremarkable. Autonomic testing, EMG and sensory evoked potentials were all normal. Formal psychometry aged 30 years demonstrated a verbal IQ of 108 and a performance IQ of 118 without perceptual or frontal impairment.

He initiated levodopa aged 33 years with good benefit. This allowed him to continue playing football until the age of 37 years although he was using up to 1300 mg of levodopa per day as well as selegiline and bromocriptine. He developed motor fluctuations and peak-dose dyskinesia after 5 years of treatment. At the age of 38 years, he spent 30-50% of his day in a state of severe dyskinesia while most of the rest of the day was characterized by marked off-period akinesia and rest tremor.

Subcutaneous apomorphine and postero-ventral pallidotomy were considered but not used.

Aged 39 years, after ten years of symptoms, it was clear that the patient was overly self-medicating; he took 26 tablets of 2.5 mg bromocriptine per day in addition to a minimum of 2000 mg levodopa. He was hoarding and hiding tablets. Depression and cognitive problems became more evident manifesting as erratic behavior, unsafe and careless driving, multiple encounters with the police and relationship difficulties including psychosexual problems. Repeat psychometry demonstrated a fall in verbal IQ to 89 (performance IQ could not be measured because of dyskinesia), a decline in memory, word retrieval, visual perceptual and visual spatial function as well as widespread frontal and subcortical abnormalities.

At the age of 45 years, after 16 years of PD, behavioral and cognitive problems dominated medical management. His thoughts were frequently delusional such as accusing his nurses of trying to harm him. He was verbally, and occasionally physically, aggressive towards carers and family. These symptoms were partially controlled using quietapine which did not cause a demonstrable change in his parkinsonian symptoms. Hallucinations were not recorded at any stage during this illness. The last years of his illness were characterized by severe akinesia and abulia. Whilst no postmortem examination was conducted, he reportedly died from a heart attack aged 47 years.

Notably, this patient's mother developed an akinetic-rigid syndrome at the age of 46 years. It is reported that her symptoms started with typical rest tremor initially

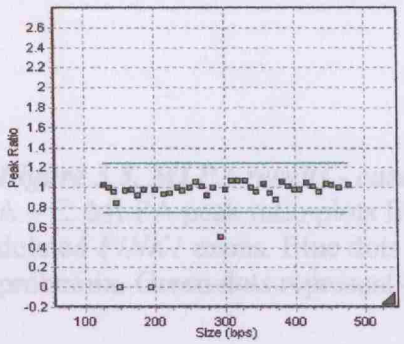
affecting the right arm. She responded well to levodopa, but developed motor fuctuations after 5 years of treatment. The later stages of her illness were characterized by progressive dementia and severe psychiatric co-morbidity. She died 10 years after disease onset following a fracture of the right leg and a pulmonary embolus.

3.2 Cases 2 through 6 – genomic rearrangements in Parkin

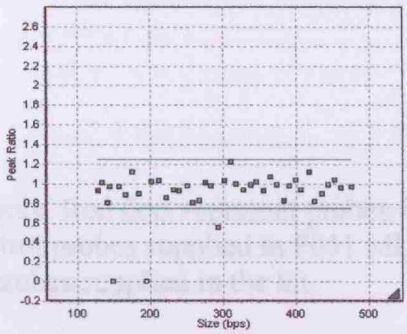
Deletions and duplications of *Parkin* exons were detected in 5 patients (Figure 3.4).

Figure 3.4. ***MLPA results - cases 2 through 6.***
A – E: MLPA peak ratio plots for P051 probemix (P052 data not shown). Red dots represent probes for deleted or duplicated *Parkin* exons. Blue dots represent control probes supplied in P051 MLPA probemix. Green dots represent other PARK probes supplied in the kit.

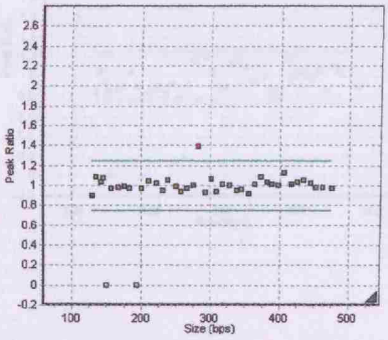
A Case 2 – *Parkin* exon 8 deletion.



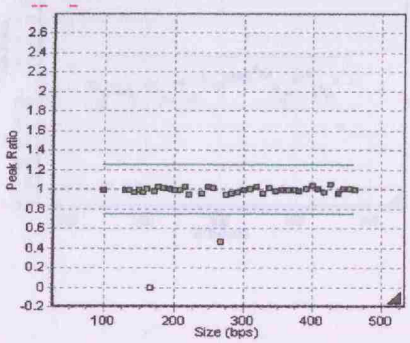
B Case 3 – *Parkin* exon 8 deletion.



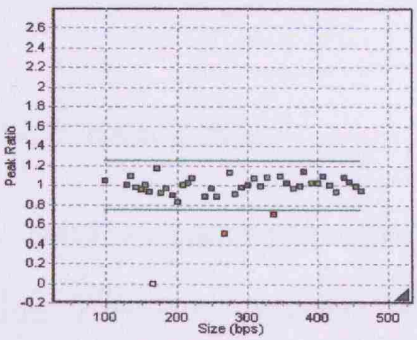
C Case 4 – *Parkin* exon 2 duplication.



D Case 5 – *Parkin* exon 3 deletion.



E Case 5 – *Parkin* exons 3 and 4 deletion.

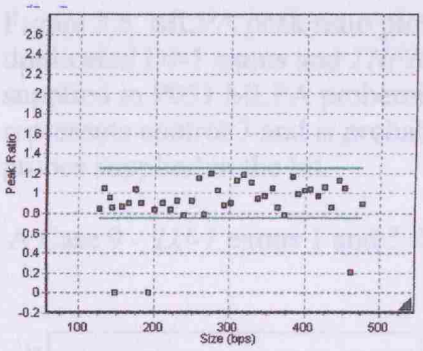


3.3 Cases 7 and 8 – Genomic rearrangements in PINK1

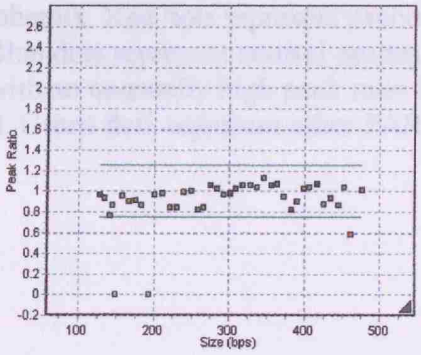
PINK1 exon 8 deletion was detected twice (Figure 3.5).

Figure 3.5. MLPA results - cases 7 and 8
A – E: MLPA peak ratio plots for P051 probemix. Red dots represent probes for deleted *PINK1* exons. Blue dots represent control probes supplied in P051 MLPA probemix. Green dots represent other PARK probes supplied in the kit.

A Case 7 – *PINK1* exon 8 deletion



B Case 8 – *PINK1* exon 8 deletion

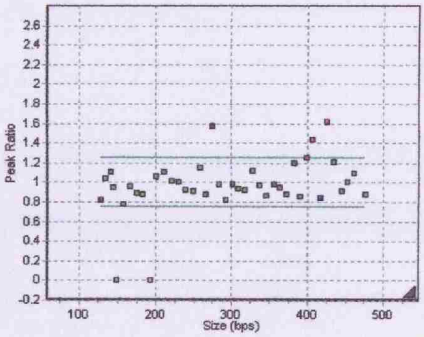


3.4 Case 9 – genomic rearrangement in DJ-1

Duplication of *DJ-1* exons 1 and 3 was detected in one patient (Figure 3.6). MLPA P051 probemix does not contain a probe for *DJ-1* exon 2 and P052 has no probes for the *DJ-1* gene. Consequently, we were not able to test for a dosage change in exon 2. However, along with changes in *DJ-1* exons 1 and 3, a duplication of *TNFRSF9* gene was detected. Since *TNFRSF9* is located 23 kb p-telomeric of *DJ-1* gene, the most likely explanation is that this whole segment is duplicated.

Figure 3.6. MLPA peak ratio plot for P051 probemix. Red dots represent probes for duplicated DJ-1 exons and *TNFRSF9* probe. Blue dots represent control probes supplied in P051 MLPA probemix. Blue dot with an unusually high peak ratio in B represents control 3 and is probably an artifact. Green dots represent other PARK probes supplied in the kit.

A Case 9 – *DJ-1* exons 1 and 3 duplication.



Clinical presentation

Limited information was available on this South African male patient. He presented aged 48 years with impaired dexterity and stiffness of his left hand. A year later he was noticed to limp with his left leg and stumble when walking. His past medical

history includes lumbar spine laminectomy. On neurological examination two years after disease onset he had a slight loss of facial expresion, mild intermittent pill-rolling rest tremor of the left hand, bradikinesia and fatigue of the left limbs. Apart from absent reflexes in his lower limbs, the rest of the neurological examination was unremarkable. He was treated with levodopa and ropinirole. However, the response was questionable as the patient noted little improvement. Of note, his father was diagnosed with PD aged 60 and he died at the age of 75.

4. DISCUSSION

We identified 9 carriers of genomic rearrangements among 91 patients with familial PD. Since our samples were selected from two different sources, it is not representative of the population of familail PD patients. Therefore, we cannot make any conclusions about the prevalence of detected genomic rearrangements.

4.1 Genomic rearrangements in Parkin

Parkin mutations are a relatively frequent finding in patients with familial PD, with deletions and duplications of one or more exons representing more than 50% of the reported cases (Hedrich *et al.*, 2004). The high frequency of *Parkin* exon rearrangements can be explained by its localisation within the common fragile site *FRA6E* (Denison *et al.*, 2003).

It is therefore not surprising that *Parkin* exon rearrangements were detected in 6 out of 9 mutation carriers in our study. All the mutations were independently confirmed using the MLPA P052 probe mix. According to the literature, the most common rearrangements are deletions of exon 4, exon 3 or both (Hedrich *et al.*, 2004). Indeed, we identified a deletion of exon 3 as well as a deletion of exons 3 and 4. A rarer exon 8 deletion was present in two patients. Careful search of the patient database revealed that these two patients were related (although the rest of our samples were obtained from unrelated subjects).

Because of the recessive nature of Parkin associated PD, the pathogenicity of heterozygous mutations can be questioned. Our patients had been previously screened for point mutations in *Parkin*. Although both cases with *Parkin* exon 8 deletion had been identified, no other Parkin mutations were found in our samples, so we can exclude compound heterozygotes. However, it is still possible that our patients had a second mutation in a different gene. It is also worth mentioning that several studies identified a large proportion of cases with only a single heterozygous *Parkin* mutation even when extensive mutational screening was performed. Most of the authors hypothesise that the presence of heterozygous *Parkin* mutations increases susceptibility for PD (Kann *et al.*, 2002; Lucking *et al.*, 2000; Periquet *et al.*, 2003; Clark *et al.*, 2006; Lohmann *et al.*, 2003).

4.2 Genomic rearrangements in PINK1

We also detected two cases with *PINK1* exon 8 deletion. To our knowledge, an isolated exon 8 deletion has not been identified so far. However, cases with deletions of exons 6 through 8 have been described in the literature (Li *et al.*, 2005; Atsumi *et al.*, 2006). Although the MLPA probe for *PINK1* exon 8 is designed complementary to a sequence where no SNPs have been identified (www.ensembl.org), a rare variant that would prevent the probe from binding and thus produce a result consistent with deletion cannot be excluded. A point mutation or a small deletion or insertion are also possible. However, these samples have previously been sequenced for *PINK1* mutations and none were initially found, although a small exonic deletion could have been missed. The time we had available for our study did not permit us to

re.amplify the genomic region contatinig exon 8. However, we are going to perform it in the near future.

4.3 Genomic rearrangements in DJ-1

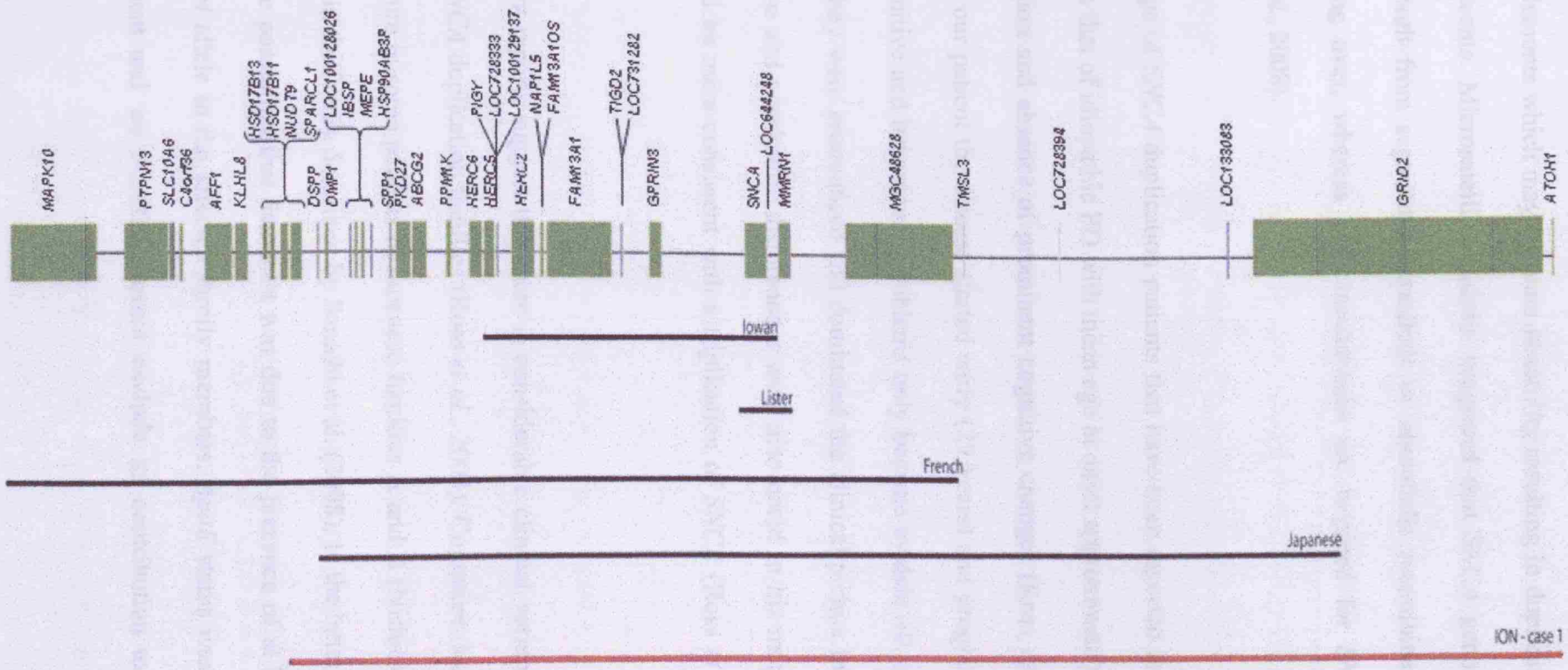
Genomic rearrangements in *DJ-1* are rare (Belin *and* Westerlund, 200) and we are not aware of any cases of *DJ-1* exon duplications in the literature.

Nevertheless, a duplication of *DJ-1* exons 1 and 3 was detected in one patient, along with a duplication of the *TNFRSF9* gene which is located 23 kb p-telomeric of *DJ-1*. Although we were unable to assess the copy number of DJ-1 exon 2, due to the lack of specific probes in both MLPA mixes, the most likely explanation for this result is that the whole segment containing *TNFRSF9* and the first 3 exons of *DJ-1* is duplicated. Involvement of several contigous probes and reliability of MLPA assay in detecting other duplications (*SNCA*, *Parkin* exon 6 duplication in our control sample) increases our confidence in this result. Nevertheless, we are planning to confirm our finding and determine the size of the duplicated region with the Illumina GW-SNP assay.

4.4 SNCA duplication

The duplicated region including *SNCA* in our patient appears to be the largest reported so far (approximately 6.6 Mb, Figure 4.4), followed by a recently described 5 Mb duplication in a Japanese family (Ikeuchi *et al.*, 2008). The region in the French duplication family was 4.93–4.97 Mb in size (Chartier-Harlin *et al.*, 2004).

Figure 4.4 ***Genes in the duplicated region on chromosome 4.*** The approximate sizes of multiplicated regions in different affected families are shown below.



SNCA is located within the 4q21 chromosomal region harbouring a variety of transposable repeat elements which may promote instability resulting in duplication and recombination events. Microsatellite analysis suggested that *SNCA* genomic duplication events result from segmental intraallelic or interallelic recombination with unequal crossing over, whereas both mechanisms are required for *SNCA* triplication (Ross *et al.*, 2008).

The clinical phenotype of *SNCA* duplication patients that have been reported in the literature is similar to that of idiopathic PD with mean age at onset approximately 50 years, slow progression and absence of prominent cognitive changes (Ross *et al.*, 2008). Strikingly, in our patient the disease started early (29 years) and progressed rapidly. Though cognitive and behavioural problems only became evident 10 years after disease onset, they were pronounced and dominated the clinical picture in the later stages. Cognitive and psychiatric disturbances were also noted in his mother. These features would be more consistent with a triplication of *SNCA* (Ross *et al.*, 2008).

However, more recent reports suggest that there is considerable clinical variability within and among *SNCA* duplication families (Ross *et al.*, 2008). Cognitive decline was a prominent feature in some patients in Japanese families A and B (Nishioka *et al.*, 2006), as well as in the family described by Ikeuchi et al. (2008). In the latter, the authors suggested the possibility that dementia was due to the presence of at least one copy of *ApoE ε4* allele in the affected family members. *ApoE* status was not assessed in our patient and we therefore cannot exclude its contribution to the phenotype.

An additional unusual feature was extensor plantar responses for which no explanation was found. The patient manifested symptoms of dopamine dysregulation syndrome with overt self-medication, hoarding and hiding of tablets. Dopamine dysregulation syndrome has been recognized in a small sub-group of PD patients. These patients take excessive doses of dopamine replacement drugs that are much larger than those needed to relieve the motor symptoms. Consequently, they suffer from related motor and behavioural disturbances. Dopamine dysregulation syndrome is more common in males with YOPD (Evans *and* Lees, 2004), but it has so far not been reported in *SNCA* multiplication patients.

Since the duplicated region in this patient was larger than in any of the cases described before, any of the other duplicated genes may have contributed to the phenotype. Unfortunately, only limited expression and functional data are available on other genes within this region (Ross *et al.*, 2008).

One of the genes in the duplicated region that is worth mentioning is *MMRN1*. This gene encodes Multimerin 1, a protein that binds coagulation factor V in platelets. Multimerin 1 deficiency is associated with a bleeding disorder (Hayward *et al.*, 1996), but the effects of increased dosage have not been described, so we can only speculate whether overexpression would result in hypercoagulability. Interestingly, the patient's mother died because of pulmonary embolism, though this could be explained by increased risk for thrombotic events due to the fracture and her age. Furthermore, another patient with *SNCA* and *MMRN1* duplication from the Swedish family was described with deep vein thrombosis and pulmonary embolism. A

question of possible genetic susceptibility to thrombosis due to increased dosage of *MMRN1* was raised by the authors, but further investigations wer not pursued (Fuchs *et al.*, 2007).

The cause of the patient's sudden cardiac death remained unexplained. Two likely possibilities are an ischaemic event or a dysrhythmia. Cardiac dennervation has been described in PD, but we are not aware of the occurrence of life-threathening arrhythmias (Post *et al.*, 2008).

The unusual rash on extensor surfaces of the patient's hands might have been a side effect of treatment with Sinemet (Chou *and* Stacy, 2007).

As a direct result of confirming the SNCA duplication, the patient's family have been contacted regarding genetic counseling. If a sibling or offspring consents to genetic testing, the laboratory may be presented with the opportunity to obtain a fibroblast tissue sample with the aim of immortalizing these cells and utilising them to help unravel additional elements of the molecular basis of PD.

4.5 Genomic rearrangements in neurological disorders

Detection of a significant number of genomic rearrangements in our patient samples emphasises the importance of such events in PD. Genomic rearrangements are a known cause of several other neurological disorders, for example Charcot-Marie-Tooth disease type 1A. The relationship between subtle dosage changes and

phenotypes in several complex neurological traits is increasingly being recognised (Lee *and* Lupski, 2006).

4.6 MLPA technique

In our study, MLPA proved to be a reliable technique for the detection of genomic rearrangements. In parallel with our test samples, we repeatedly analysed samples with known mutations that had previously been confirmed with other methods. Samples with *Parkin* 2 exon 3 deletion, *Parkin* 2 exon 6 duplication and the *LRRK2* G2019S point mutation were analysed 10-, 7- and 6-times, respectively. MLPA gave accurate and reproducible results for all these samples.

Two other studies tested the effectiveness of MLPA P051 and P052 kits in detecting rearrangements in PD genes by using samples with known mutational status. Djarmati *et al.* (2006) tested 15 samples with various *Parkin* exon rearangements and a sample with *LRRK2* G2019S point mutation. Scarciolla *et al.* (2007) tested 12 samples with dosage changes in *SNCA, Parkin, PINK1 and DJ-1*, as well as one sample with *LRRK2* G2019S point mutation. In both studies, MLPA was able to precisely detect all present changes and in one case also detected a dinucleotide deletion in *Parkin* exon 9 of *Parkin* gene. The second study additionally tested 31 healthy control samples which had been previously confirmed negative for exon rearrangements in *Parkin, DJ-1, and PINK1*. MLPA detected all these cases as normal.

The disadvantages of MLPA

An obvious disadvantage of MLPA is that that any alteration in the target genomic sequence (exon deletion, point mutation, SNP) will prevent probe binding. Consequently, all these changes will appear as exon deletions (Djarmati *et al.*, 2006). Although MLPA probes are designed to bind to target sequences where no SNPs or point mutations have been described, there is always a slight possibility of a rare polymorphism. Exon deletions should thus ideally be analysed with a different probe or sequenced to exclude the possibility of a small sequence change (Djarmati *et al.*, 2006).

4.7 Limitations of our study

One of the main limitations of our study was that we did not have sufficient time to confirm all the mutations with other methods. However, all the *Parkin* mutations were confirmed in an independent reaction with the P052 probemix and sequencing had previously excluded point mutations. The *DJ-1* sample is scheduled for further investigation in August.

During this project, one additional problem was encountered: the use of DNA samples that had been stored for several years and repeatedly freezed and thawed. Consequently, MLPA reactions failed in 10 samples where DNA was degraded. We did not observe MLPA failures in freshly extracted DNA samples. As already

observed by other researchers, DNA quality is of crucial importance for MLPA performance (Kozlowski *et al.*, 2007).

MLPA P051 and P052 probe mixes lack probes for exons 2 and 4 of the *DJ-1* gene and several *LRRK2* exons. Although rearrangements in these exons have so far not been described, we cannot exclude the slight chance that these mutatations were present in our patients.

4.8 Conclusion

In this study we identified a total of 9 PD gene rearrangements in 122 patient samples including a rare *SNCA* duplication, *PINK1*, *DJ-1* exon rearrangements that had previously not been described and multiple *Parkin* rearrangements. Of note, previous screening for PD gene mutations did not identify these alterations. Furthermore, these findings may indicate that the number of genomic rearrangements present in PD patients may be underestimated. It will therefore be essential in future for diagnostic testing to include comprehensive genomic screening to determine the presence of genomic rearrangements. In our opinion, MLPA is a sensitive and reliable test that can be used in conjunction with gene sequencing to assess the genetic cause of PD in familial patients. Whilst, at the present time, MLPA is unable to assess all identified PARK genes, additional probes are consistently being generated and we hope in future that a comprehensive probe set will be avaliable.

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