

**Genetic Susceptibility in  
Parkinson's Disease**

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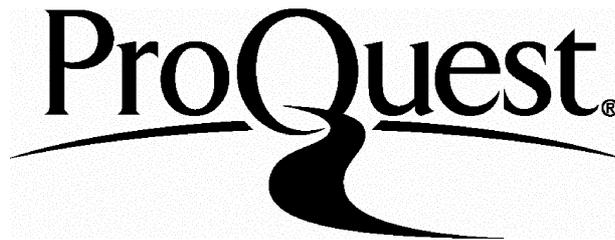
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## ABSTRACT

Until 4 years ago, the role of genetic factors in the aetiology of PD was controversial but subsequently, a number of families have been identified, in whom parkinsonism is inherited as an apparently monogenic mendelian trait with high penetrance and in several of these families, disease genes have been identified. However, most cases of PD appear to be sporadic, with no clear mendelian mode of inheritance.

The formation of the Genetic Susceptibility in Parkinson's disease (GSPD) enabled the clinical assessment, collection and analysis of DNA samples from a large number of affected sibling pairs (ASPs). Since 1996, three autosomal dominant PD loci have been described (PARK1, 3 AND 4), an average of 125 ASPs were therefore genotyped for evidence of linkage to these regions. For three markers D4S1647 (PARK1, alpha-synuclein, SNCA), D4S405 (Ubiquitin-C-terminal hydrolase, UCH-L1) and D2S1394 (PARK3, locus at 2p13), nominal p-values of 0.05 or lower were observed suggesting the presence of susceptibility regions. A PARK4 (locus 4p15) haplotype segregating with Parkinson's disease and postural tremor was detected in an Italian family.

A large intra-familial association study using ASPs obtained by GSPD found that the slow acetylator genotype for N-acetyltransferase 2 (NAT2) was over-represented in familial PD. GSPD collaborative analysis of the SNCA and UCH-L1 gene failed to detect mutations by direct sequencing of SNCA and UCH-L1 in index cases from a series of PD kindreds or screening of 230 index familial PD cases for the two known mutations in SNCA. Hence, although of great interest, mutations in these genes are a very rare cause of familial PD. In contrast, analysis of the Parkin gene in selected families, sporadic and juvenile-onset cases showed that coding mutations in Parkin are a common cause of autosomal recessive parkinsonism in Europe. The neuropathology of the first UK case with a mutation in the Parkin is described.

Two large familial PD kindreds in the UK were identified. No mutations were found in the SNCA gene and linkage studies to the other dominant familial PD loci were also negative. Further linkage and PET studies are ongoing to identify a novel familial PD locus in these individuals.

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## ABBREVIATIONS

AD	Alzheimer's Disease
ALS	Amyotrophic lateral sclerosis
APOE	Apolipoprotein E
APP	Amyloid precursor protein
APS	Ammonium persulphate
AR-JP	Autosomal recessive juvenile-onset parkinsonism
Asn	Asparagine
Asp	Aspartic acid
ASP	Affected sibling pair
BDNF	Brain-derived neurotrophic factor
bp	Base pair
C	Cytosine
CAT	Catalase
cDNA	Complementary DNA
CEPH	Centre d'Études du Polmorphismes Humaine
CHLC	Cooperative Human Linkage Centre
cM	CentiMorgan
CNS	Central nervous system
CT	Computerised tomography scan
CYP	Debrisoquine 4-hydroxylase
dNTP	Deoxynucleoside triphosphate
ddNTP	Dideoxynucleoside triphosphate
DNA	Deoxyribose nucleic acid
DOPA	Dihydroxyphenylalanine
DRD	Dopa-responsive dystonia
DZ	Dizygotic
EDTA	Ethylene diamine tetra-acetic acid
EEG	Electroencephalogram
EU	European Union
<sup>18</sup> F-DOPA	Positron-emitting fluoro-dopa
bFGF	Basic fibroblast growth factor
FISH	Fluorescent in situ hybridization
FPD	Familial Parkinson's Disease
FTDP	Frontotemporal dementia
G	Guanine
GCI	Glial cell inclusion
GDB	Genome database
GFAP	Glial fibrillary acidic protein
GIRK	G-protein inward rectifier potassium channel
GPX	Glutathione peroxidase
GSPD	Genetic Susceptibility to Parkinson's Disease consortium
GST	Glutathione S-transferase
HGMP	Human genome mapping project
5-HT	5-hydroxytryptamine
H&Y	Hoehn & Yahr score
IBD	Identity by descent
IBS	Identity by state
kb	Kilobase
LB	Lewy body

L-dopa	Levodopa
Leu	Leucine
LOD	Logarithm of odds ratio
Lys	Lysine
MAO	Monoamine oxidase
Mb	Megabase
MMSE	Mini mental status examination score
MPTP	N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRS	Magnetic resonance spectroscopy
MSA	Multiple system atrophy
MZ	Monozygotic
NAC	Non-amyloid beta component (of $\alpha$ -synuclein)
NAT	N-acetyl transferase
OD	Optical density
OMIM	On-line Mendelian Inheritance in Man database
OR	Odds ratio
PAGE	Polyacrylamide gel electrophoresis
Parkin	Parkin gene
PCR	Polymerase chain reaction
PD	Parkinson's Disease
PET	Positron emission tomography
PIC	Polymorphism information content
Pro	Proline
PSP	Progressive supranuclear palsy
RFLP	Restriction fragment length polymorphism
RNA	Ribose nucleic acid
RR	Relative risk
SD	Standard deviation
SE	Standard error
SNCA	$\alpha$ -synuclein gene
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SSCP	Single stranded conformational polymorphism
SSTR	Simple sequence tandem repeat
T	Thymine
TBE	Tris-borate EDTA solution
TE	Tris EDTA solution
TEMED	N, N, N', N' - tetramethylethylenediamine
TH	Tyrosine hydroxylase
UCH-L1	Ubiquitin C-terminal hydroxylase L1
UPDRS	Universal Parkinson's Disease Rating System
WT	Wild type
Z	LOD score

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## **CHAPTER 1: GENERAL INTRODUCTION**

## 1.1 OUTLINE OF CHAPTER

Parkinson's disease (PD), a progressive neurodegenerative disorder, is a common cause of disability. Although there are symptomatic treatments, none modify disease progression. Up until 1996 the role of genetic factors in PD remained controversial, as although epidemiological studies had suggested a familial risk, lack of concordance in twin studies had been used to argue against this. However, genetic markers on chromosome 4q21-q23 were found to be linked to the PD phenotype in a large Italian-American family (the Contursi kindred) in 1996. The subsequent finding of a mutation in the  $\alpha$ -synuclein gene (SNCA) in this family in 1997 led to intensive global research into the genetics of PD. The EU consortium, Genetic Susceptibility in Parkinson's Disease (GSPD), of which I was one of the UK partners, has played a central role in this research since 1996 and my own work within this consortium is described in this thesis.

Two novel genes, SNCA (Polymeropoulos *et al.*, 1997) and Parkin (Kitada *et al.*, 1998a) as well as three further genetic loci (Farrer *et al.*, 1999b; Gasser *et al.*, 1998; Valente *et al.*, 2001) have now been implicated in the pathogenesis of familial PD. These represent significant progress in our understanding of the disease, considering the rarity of large families, low heritability and genetic heterogeneity. Mutations in a further gene, UCH-L1, (Leroy *et al.*, 1998), have also been described in familial PD and are discussed in later chapters. Characterisation of the gene products encoded by these PD genes in the future should help to elucidate the molecular mechanisms of neurodegeneration in this disorder and perhaps also in other neurodegenerative diseases where defects in particular metabolic pathways or in the intracellular handling of proteins may be a common feature. Nigral degeneration with Lewy body formation and the resulting clinical picture of PD may represent a final common pathway of a multifactorial disease process in which both environmental and genetic factors have a role.

In order to set my own work in context, this introduction outlines the importance of multifactorial disease in neurology and the relevant genetic principles upon which much of the experimental work performed as part of this thesis was based. The major

advances in the field of PD genetics are described, illustrating how the existence of genetic factors in PD has now become firmly established.

## **1.2 MULTIFACTORIAL GENETIC DISEASE**

### **1.2.1 The importance of multifactorial genetic diseases in neurology**

Of the 7000 human diseases inherited in a mendelian fashion, most are rare conditions, such as the autosomal dominant spinocerebellar ataxias (SCA 1-3, 6-8), Huntington's disease and myotonic dystrophy, occurring in less than 0.01% of the population. To date, more than 1000 genes associated with monogenic disorders have at least been localized to a chromosomal region by linkage analysis. By comparison, relatively few genes have been similarly mapped for the common diseases. Determining the genetic component of a disease may not be a simple task, as the occurrence of a rare disease in multiple biological relatives is likely to have arisen from a single cause, whereas familial aggregation of a common disease could have multiple causes which have occurred by chance. The more common neurological disorders are probably not caused by single genes but result from the interaction of multiple genes acting in concert with various environmental factors.

To date, disease-causing genes have been described in certain forms of PD, epilepsy, motor neuron disease and Alzheimer's disease as a result of parametric and non-parametric linkage analysis as well as positional candidate and positional cloning approaches (Bell and Lathrop, 1996; Gunel *et al.*, 1996; Wood, 1997). These are invariably the relatively uncommon familial variants of these diseases but molecular analysis of these rare families may help to clarify the pathogenesis of the far more common sporadic forms of these diseases and how a particular genotype correlates with phenotype.

Cloning of disease genes has provided clinicians with the means for both diagnostic testing and predictive testing of at-risk individuals, including prenatal diagnosis (for example, for Huntington's disease). These advances have improved genetic counselling and increased its accuracy, thus directly benefiting patients. Classification of neurological diseases in particular has been aided by their definition at the molecular as well as clinical levels, as seen in the autosomal dominant spinocerebellar ataxias. This is also exemplified by genotype-phenotype studies of PD, as a result of which it is now clear that several genes may result in clinically indistinguishable phenotypes and that a

single locus may cause more than one phenotypic manifestation (Farrer *et al.*, 1999b). Classification of many similar neurodegenerative disorders will no doubt in future be based on genetic profile as well as phenotype. Despite being considered an archetypal 'non-genetic' neurological disorder, genetic analysis of PD has revealed new insights into this disease. The identification of mutations in SNCA in the Contursi kindred (Polymeropoulos *et al.*, 1997) and the subsequent identification of  $\alpha$ -synuclein protein in pathological deposits in other 'multifactorial' disorders has led to the suggestion that PD may share pathogenic mechanisms with multiple system atrophy and Alzheimer's disease (Farrer *et al.*, 1999a).

### **1.3 THE HUMAN GENOME AND STRATEGIES FOR GENE IDENTIFICATION**

The human genome contains approximately  $3 \times 10^9$  base pairs of DNA. The whole of the human genome has been recently sequenced and comprises 25,000-35,000 genes (Lander *et al.*, 2001). Four general strategies exist for identification of disease genes: functional cloning, positional cloning, positional candidate approaches and position-independent candidate approaches. The suitability of each approach depends on the degree of understanding of the molecular pathology of the disease, the availability for study of patients and families with the disease, and the completeness of the available gene map. Much of the work carried out as part of this thesis made use of linkage techniques outlined below. Other work involved mutation analysis of cloned genes. Positional and functional cloning work was not carried out by myself and therefore the theoretical background to these techniques will only be described in brief.

### **1.4 CLONING OF DISEASE GENES**

Functional cloning makes use of prior information about the presumed biochemical defect of a disease gene. This technique does not rely on knowledge of the chromosomal map position. In most cases this approach relies on demonstration of an abnormal or deficient protein and subsequent purification of the protein. Functional cloning was the first method of gene identification to be developed. The genes for phenylketonuria (Robson *et al.*, 1982) and factor VIII (Gitschier *et al.*, 1984) were

identified using this method. Functional cloning as a technique is limited, as it depends on a high level of understanding of the molecular pathology of a disease to predict the abnormal protein involved, yet the biochemical defect involved in most single gene disorders is not known.

The term positional cloning refers to the isolation of a gene solely on the basis of its chromosomal map position. The principle upon which positional cloning is based is the initial localisation of the gene (by linkage techniques) to a specific subchromosomal region, followed by successive narrowing of this candidate interval, which eventually results in the location of the gene itself. Alternatively, the detection of chromosomal deletions or translocations (cytogenetic abnormalities) have been used to accurately identify disease genes. Positional cloning is now considered a standard means of gene identification, although the positional candidate approach (see below) is currently considered the most efficient way of identifying disease genes.

## **1.5 FROM DISEASE TO GENE**

During the work of this thesis, a large number of affected sibling pairs with PD were collected for the original purpose of mapping and identifying genes which confer genetic susceptibility to PD as part of a genome-wide screen. From this pool of original families, two larger kindreds were identified (UK 402, UK 403). Exclusion studies of the known PD loci were performed on these two individual families and a genome-wide screen is now in progress in order to identify the disease locus and then the mutation in the gene responsible for the PD phenotype in these families. This work was not completed by myself and is currently being finished. The principles behind this strategy are as follows: the families in which the disease segregates are recruited and studied using genetic markers, with the aim of identifying a marker linked to the disease gene. Fine genetic mapping follows, to assign the gene to as small a genetic region as possible between defined markers. The resolution achieved by genetic mapping depends upon the number of informative meioses available in the pedigree material. In practice, it is unusual to be able to localise a gene to a smaller genetic interval than one centimorgan (cM), defined as the genetic distance over which two loci will be separated by recombination in 1% of meioses. Often, however, the candidate region containing the gene is larger. The minimum genetic localisation required for success in a purely

positional cloning strategy is considered to be approximately 2-3 cM. One cM corresponds to approximately 1 million base pairs (Mb) of DNA. A region, even of 2-3 cM may contain a substantial number of genes. As the techniques of physical mapping are both expensive and time-consuming a given candidate region should be as narrow as possible at the start of a cloning project.

## **1.6 GENETIC LINKAGE ANALYSIS**

Genetic linkage analysis is the first step in the positional cloning and positional candidate approaches to gene identification; the success of both are dependent to a large degree on the exactness of initial genetic mapping. Gene mapping by linkage analysis forms the basis of much of the work described in this thesis.

### **1.6.1 Principles of linkage analysis**

Genetic linkage analysis makes use of the exception to Mendel's Law of Independent Assortment which states that alleles at different genetic loci assort at random during meiosis. This applies to loci on different chromosomes which segregate independently of one another. During meiosis homologous chromosomes cross over and exchange genetic material, a process called recombination. As a result, widely separated loci on the same chromosome may be separated during meiosis. Where the probability of recombination between two syntenic loci during meiosis is 50%, these loci will also segregate independently. Observation of chiasmata shows that there are an average of 53 crossovers during a male meiosis; there is a minimum of one crossover per chromosome and there are on average 1.5 crossovers per (sex-averaged) chromosome (Ott, 1991). Regardless of the number of crossovers between two widely separated loci, the net result is that 50% of chromosomes will be recombinant, and 50% non-recombinant for these loci. Loci which are in close physical proximity on the same chromosome, however, are less likely than distant loci to be separated by recombination if chiasmata occur at random along the chromosome. Thus such loci do not segregate independently but tend to be inherited together more often than not. Two such loci are said to be linked. The degree to which two loci tend to be inherited together is therefore a measure of their physical proximity. Recombination will rarely separate loci that lie

very close together on a chromosome, because only a crossover located precisely in the small space between the 2 loci will create recombinants. Therefore, sets of alleles on the same small chromosomal segment tend to be transmitted as a block through a pedigree. Such a block of alleles is known as a haplotype. Haplotypes mark recognizable chromosomal segments which can be tracked through pedigrees and through populations. When not broken up by recombination, haplotypes can be treated for mapping purposes as alleles at a single highly polymorphic locus. This may be measured in practice by observation of the segregation of alleles in offspring.

### **1.6.2 Recombination Fraction**

The proportion of offspring in which two parental alleles are separated by recombination is the recombination fraction ( $\theta$ ). Quite simply,  $\theta$  is the probability that a parent will produce a recombinant offspring. The recombination fraction varies from 0 (for adjacent loci) to 0.5 (for distant loci) and may serve as a measure of the distance between the loci (Ott, 1991).

For closely linked loci (where  $\theta < 0.05 - 0.1$ ), it is reasonable to assume that the probability of more than one recombination occurring between the loci is small. In these circumstances the recombination fraction is equal to the genetic map distance between the loci, thus two loci showing recombination in 1% of meioses ( $\theta = 0.01$ ) are approximately 1 cM apart. Small values of  $\theta$  are equivalent to the actual map distance ( $w$ ) between loci, and thus recombination fractions are additive over small distances. The simplest case relating  $\theta$  to  $w$  occurs when it can be assumed that multiple crossovers between two loci do not occur when the distance is very small, then  $\theta = w$ .

For larger distances, recombination fractions are not additive because multiple crossovers occur. When this is the case, mapping functions must be used to translate  $\theta$  values into actual map distance. Some commonly used mapping functions are those of (Haldane, 1919; Kosambi, 1944). Haldane's mapping function assumes no interference, (i.e. crossing over is evenly distributed over the entire chromosome). In fact, the phenomenon known as interference inhibits the formation of crossovers in the vicinity of an existing crossover. The Kosambi mapping function takes interference into account and is the mapping function most often used in humans.

Linkage analyses are designed to test whether or not two genes transmitted from parents to children segregate in an independent fashion, and the statistical analyses are either parametric or nonparametric. Identification of large pedigrees with numerous affected individuals is desirable in order to maximise the power of the study to detect linkage. In practice, large pedigrees are often not available, particularly when studying recessive or late-onset disorders (such as PD). Smaller families may then be grouped together for linkage analysis, but locus heterogeneity (i.e. different disease genes resulting in indistinguishable phenotypes) is a commonly encountered problem which complicates this approach. Correct phenotypic designation of affected and unaffected status is an essential requirement for successful linkage analysis, and should be decided at the outset of the study in order to minimise bias. Linkage testing can fail as a consequence of insufficiently informative markers, markers that are insufficiently close to the locus of the disease (and thereby having a high rate of recombination between the marker and disease gene), and genetic heterogeneity, in which the wrong locus is tested for linkage.

### **1.6.3 The lod score method**

The lod score method (Morton, 1955), a maximum likelihood analysis, has been used successfully to map a substantial number of mendelian disease genes. Linkage calculates the probability that two loci are linked, expressed as a lod score which is a  $\log_{10}$  of the odds ratio favouring linkage. Convention dictates that a lod score  $>3$ , which indicates a probability in favour of linkage of 1000 to 1, is enough to establish linkage, and conversely a Lod score of  $-2$  indicating a probability against linkage of 100 to 1 excludes linkage between the two loci being tested. The odds ratio favouring linkage is defined as the likelihood that the two loci are linked at a specified recombination ( $\theta$ ) versus the likelihood that they are not linked,  $\theta + 0.5$ . Lod scores are calculated by computer programs over a range of values of  $\theta$ , facilitating the maximum probability of recombination (and hence genetic distance) between the two loci to be calculated. The application of the lod score method to complex traits is problematic, especially where a disease inheritance model cannot be specified with confidence (this issue is further detailed below (Martinez and L.R., 1989)).

Standard lod score (parametric) analysis requires a precise genetic model, detailing the mode of inheritance, gene frequencies and penetrance of each genotype. This requires

that reproducible diagnostic criteria are established so that segregation analysis can identify all of the above. Nonparametric linkage methods are alternatives to lod score analyses as specification of the disease inheritance model is not required. Multipoint linkage analysis quantitatively assesses the most likely location of a disease in a framework order of marker loci by moving the disease within the framework and calculating a location score, to express the likelihood that the disease locus is located within that particular part of the framework. A location score is defined as twice the natural log of the odds ratio (Strachan and Read, 1999).

#### *1.6.3.1 Two-point and multipoint analysis*

Two-point analysis simply involves simultaneous analysis of two loci. Multilocus analysis allows a more efficient use of the data obtained as more than two loci can be analyzed simultaneously. It is therefore particularly useful for establishing the chromosomal order of a set of linked loci. This can be done by analysis of multiple pairwise lod scores using computer programs such as the LINKMAP option in FASTLINK (Lathrop *et al.*, 1984; Schaffer *et al.*, 1994). Multilocus mapping also helps overcome problems caused by the limited informativeness of markers as simultaneous linkage analysis of a number of markers extracts the full information. Programs such as LINKMAP can notch the disease locus across the marker framework, calculating the overall likelihood of the pedigree data at each position. The result is a curve of likelihood against map location. Peak heights depend on precise distances between markers and on the mapping function and in reality these are seldom accurately known. The highest peak normally marks the most likely location (Strachan and Read, 1999).

#### **1.6.4 Markers for genetic mapping**

The tools needed for genetic mapping in humans have only become available during the last two decades. Markers used for mapping must fulfil three requirements: they must be polymorphic (two, or preferably more, common alleles in the population), their chromosomal location must be known, and it must be easy to type the marker (i.e. to distinguish between alleles). Initial attempts at mapping relied on the use of easily typed protein polymorphisms such as blood group proteins and classical HLA antigens, but as

the chromosomal location of these proteins was not known linkage groups could be established but the diseases could not be mapped to a specific autosome. It was not until the 1980s that a comprehensive array of human DNA markers, in the form of restriction fragment length polymorphisms (RFLPs), became available (Botstein *et al.*, 1980). The usefulness of RFLPs in genetic mapping is limited by their biallelic nature (i.e. the presence or absence of a restriction enzyme cleavage site) which results in low heterozygosity (50% maximum), and by the labour-intensive methodology required to type large numbers. Although RFLPs are still useful in genetic mapping, particularly when typed with the aid of the polymerase chain reaction (PCR), they have been largely replaced in linkage projects by variable number tandem repeat markers (VNTRs) such as microsatellites.

The use of panels of fluorescent tagged primers and microtitre plates for PCR as well as computerised semi-automated genotyping techniques have significantly increased the throughput accuracy of genotyping in recent years (Schwengel, 1994). A microsatellite marker comprises a pair of fluorescently labelled primers. Microsatellite markers are derived from polymorphic repetitive sequences, usually of variable numbers of dinucleotide CA repeats (mono-, di-, tri-, or tetranucleotide, repeated in multiple tandem copies). The number of repeat copies is usually highly variable between individuals in a population, relatively stable between generations, and inherited in a mendelian fashion. Microsatellites are abundant, occur throughout the genome, and may be easily typed by PCR (Silver, 1992; Weber and May, 1989).

### **1.6.5 Genotyping**

The technique of genotyping makes use of polymorphic microsatellite DNA markers to identify a chromosomal region segregating with the disease in one or more families. Multiple copies of a DNA fragment, which may be sized by electrophoresis on a high resolution polyacrylamide gel, are produced after PCR amplification of a short segment of DNA. Allele lengths for each marker are defined by their number of tandem repeat sequences, enabling family members to be genotyped according to the pattern observed. Segregation of a marker allele with the disease phenotype in a family suggests linkage between the microsatellite marker and a putative disease gene.

## **1.7 TOOLS FOR GENETIC LINKAGE**

### **1.7.1 The polymerase chain reaction (PCR)**

The polymerase chain reaction (PCR) enables the selective amplification of specific DNA sequences. Two oligonucleotides act as primers by hybridising to opposite strands of DNA flanking the target sequence during repeated cycles of DNA denaturation, primer annealing and fragment length extension by thermostable DNA polymerases. As the extension products of each cycle are used as templates in subsequent cycles, an exponential accumulation of target DNA occurs, further moving the reaction equilibrium towards target specificity. One primer may be labeled with a fluorescent dye detectable by laser on an automated DNA sequencer, or the products may be visualised under UV illumination after agarose electrophoresis in the presence of ethidium bromide.

### **1.7.2 Computer Data Analysis**

As identification of linkage for a mendelian disorder using a genome-wide search usually requires the generation of thousands of genotypes, such improvements are of great benefit in linkage studies. Linkage analysis in this thesis was performed using the computer programs MLINK, ILINK and LINKMAP from the LINKAGE package, version 5.1, FASTLINK (Lathrop *et al.*, 1984; Schaffer *et al.*, 1994), running via a remote telnet connection to the HGMP computer system (see Chapter 2, Materials & Methods). Disease frequency of familial PD was taken as  $10^{-3}$ , but varying this had no significant effect on the results of the linkage analysis. Allele frequencies for each marker were set as equal.

## **1.8 COMPLEX GENETIC ANALYSIS**

### **1.8.1 Genetic Analysis in Complex disease**

Genetic complexity may occur for one or more of the following reasons:

1. The disease may be aetiologically heterogeneous, with only a subset due to genes conferring high risk.
2. The disease may involve many different genetic loci that act together to cause disease.
3. A gene for the disease may predispose only in the presence of a particular environmental exposure.

Genetic studies of complex diseases such as PD face difficulties arising from uncertainties in diagnosis, disease definition and lack of understanding of its genetic transmission, although the several PD loci discovered to date have reduced this uncertainty. In view of the difficulties of assigning a precise genetic model and to avoid averages of the above variables being taken over a heterogeneous set of families, several approaches can be used. These include seeking a pool of families in which the disease segregates in a manner which would fit with autosomal dominant inheritance, using affected members of the pedigree only in parametric analysis and using a non-parametric (model-free) method of linkage analysis. The choice of strategy is based on its ability to detect susceptibility gene(s) of a given effect.

### **1.8.2 Linkage analysis in families with autosomal dominant inheritance with varying degrees of penetrance**

Despite the apparent genetic complexity of PD, rare families can be found with many affected members in a pattern consistent with autosomal dominant inheritance, although some have reduced penetrance. Each of the genetically defined familial disorders share clinical characteristics. These families can be used for a genome search using standard lod score analysis. PD is known to be genetically heterogeneous (Gasser *et al.*, 1997) but identifying the mendelian subset does not necessarily cast any light on the causes of non-mendelian disease. Several methods of analysis can be used and are described below.

### **1.8.3 Use of affecteds-only pedigree analysis**

This entails the use of a parametric method for analysis of only the affected members of

a kindred. The penetrance is irrelevant for affected people and unaffected members are scored as having an unknown disease phenotype. If the penetrance is low, unaffected people provide relatively little information but the genotypes of affected people can be inferred, as they must have the susceptibility allele. This strategy has been used during the course of this thesis and resulted in linkage to the known susceptibility loci described in PD being excluded in two dominant kindreds (see chapter 5). The risk of false positives is reduced if the analysis is restricted to checking a few candidate loci. Familial PD is also rare, minimising the risk of heterogeneity and of phenocopies (i.e. affected individuals expressing the disease but lacking the proposed causative gene mutation).

#### **1.8.4 Non-parametric linkage analysis**

An appropriate strategy for mapping complex disease traits involves the study of affected relative pairs. Large numbers of pairs are required to demonstrate linkage to anonymous polymorphic DNA markers. Methods of non-parametric linkage analysis ignore unaffected family members and look for alleles or chromosomal segments that are shared by affected individuals. However, the non-parametric sib-pair method has the additional disadvantage of poor localization capacity. Shared segment methods can be used within nuclear families (sib pair analysis), within extended families, or in whole populations. At the population level they constitute association studies.

#### **1.8.5 Identity by state and descent**

Whether a particular allele is inherited identically by state (IBS) or identically by descent (IBD) is of prime importance in establishing allelic ancestry. Alleles IBD are demonstrably copies of the same ancestral allele. For very rare alleles, two independent origins are unlikely, so IBS generally implies IBD, but this is not true for common alleles. Multiallele microsatellites are more efficient than two-allele markers for defining IBD, and multilocus multiallele haplotypes are better still as any one haplotype is likely to be rare. IBD is more powerful but requires parental samples, which are not necessarily available, particularly in the case of late-onset disorders like PD.

### 1.8.6 Advantages and Limitations of the classical ASP method

Affected sib pairs (ASPs) allow model-free analysis in nuclear families. If both sibs are affected by a genetic disease, then they are more likely to share whichever segment of chromosome carries the disease locus. This allows a simple form of linkage analysis. ASPs are typed for markers and chromosomal regions sought where the sharing is above the random 1:2:1 ratios of sharing 2, 1 or 0 haplotypes identical by descent. Sib pair analysis has been used as one of the main tools for seeking genes conferring susceptibility to common mendelian diseases (Jawaheer *et al.*, 2001) and played an important role in the study of type 1 diabetes where excess allele sharing confirmed the important role of HLA which is informative to such an extent that marker allele IBS is equivalent to IBD, although the inheritance pattern fits neither a simple dominant or recessive model.

There are clear limitations to the classical ASP method demanding that certain extensions are used to allow meaningful analysis of the data. These include: 1) the need for unambiguous assignment of IBD of the marker loci; 2) other affected relative pairs are excluded from the analysis; 3) marker information from unaffected relatives is not included, and 4) analysis is restricted to a single genetic marker. The power of ASP linkage mapping is related to the magnitude of the relative risk (RR), defined as the recurrence risk for a relative of an affected person divided by the risk for the general population (Risch, 1990a). For a given RR value, the power decreases as the recombination fraction ( $\theta$ ) between the marker and the disease locus increases, and the polymorphism information content (PIC) of the marker decreases (Risch, 1990d). Assuming a trait with an RR value of 3 (as in PD), 100 ASPs are required to achieve 80% power using completely informative markers (PIC=1) at  $\theta=0$ ; when  $\theta=0.05$ , the required sample size is threefold higher. Several non-parametric linkage methods have been developed to relax some of the limitations of the classical ASP method, with inclusion of other affected relative pairs (Risch, 1990a). However, for RR values of 3, affected pairs of distant relatives are probably no more powerful than affected sib pairs (Risch, 1990a). Furthermore, in the context of diseases with late onset such as PD, these analyses may be sensitive to incorrect specification of marker allele frequencies

(Babron, 1994). Other ASP extensions appear more suitable for linkage analysis in PD. The Holmans method was used for this project as it maximizes information from sibs with untyped parents (Holmans, 1993). The WPC-MP method relies on IBD scoring and is robust to incorrect marker allele assumptions. The WPC-MP and Holmans methods were used for analysis of data.

Candidate regions defined by ASP analysis are usually too large for positional cloning. Unlike the process of fine-mapping in mendelian disease that can be used to narrow down a candidate region, sib pair analysis will reveal a number of candidate regions. It is not likely that a chromosomal segment can be defined that is shared by all affected sib pairs, as the areas will be broad. This is understandable because, if a susceptibility factor is neither necessary nor sufficient for disease, then not all affected sib pairs will share the chromosomal segment that contains the susceptibility locus. Also, many ASPs share many segments by chance including, perhaps, segments that lie coincidentally close to a susceptibility locus.

### **1.8.7 The future of genetic analysis**

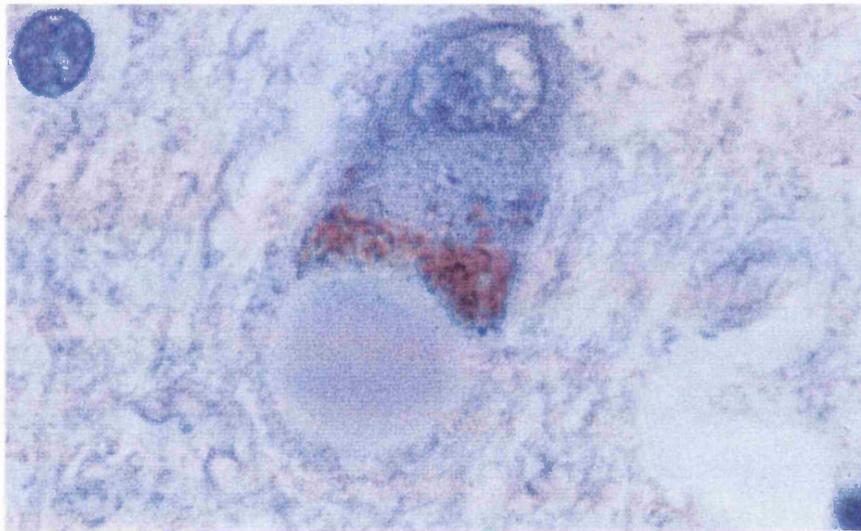
A third generation of markers known as single nucleotide polymorphisms (SNPs), are increasingly being used, allowing more rapid genome screening. SNPs are essentially single base pair differences occurring at the same position in different individuals. SNPs are highly abundant, occurring on average at least once per kilobase (Wang et al, 1998). When the two alternative bases both occur at appreciable frequency in the population SNPs may be used as biallelic genetic markers. The disadvantage of SNPs is that there can be a maximum of just four alleles in the population for any given SNP. One great advantage, however, is that SNPs can be screened by high throughput hybridization methods. DNA “chips” have been developed as part of hybridization technology. These comprise high density arrays of oligonucleotides capable of hybridizing or not hybridizing to certain SNPs. Thus, an individual’s total genomic DNA can be fluorescently labeled, momentarily placed on a chip to hybridize, and the resulting pattern of spots detected nearly immediately using light signals (Goffeau, 1997; Wang et al, 1998). Considerably more markers could be rapidly screened, although the information content per each marker would be less and sophisticated computational methods of analysis would be required to decode the information.

Although SNP maps are at an early stage of development they have the potential to supersede microsatellite markers for mapping.

## **1.9 IS THERE A GENETIC BASIS FOR PARKINSON'S DISEASE AND OTHER NEURODEGENERATIVE DISEASES?**

Idiopathic (or sporadic) PD is one of the commonest neurodegenerative disorders, with a clear age-dependent prevalence of 1 to 2 percent after the age of 65 years (De Rijk *et al.*, 1997a). PD has been found in every ethnic group and geographical area that has been studied, with the lowest prevalence in China, Japan and Africa (Zhang and Roman, 1993). Since its description by James Parkinson nearly 200 years ago (Parkinson, 1817), intense efforts have been made to establish its aetiology. The phenotype of idiopathic PD is characterised by resting tremor, rigidity and bradykinesia, which responds well to L-dopa (Levodopa) treatment. The pathological hallmarks of PD are the presence of Lewy bodies (Fig. 1.1) and massive loss of dopaminergic neurons in the *pars compacta* of the *substantia nigra*. Parkinsonism is the term used to describe this constellation of clinical signs but it also includes a number of other neurodegenerative diseases which are often less responsive to L-dopa and have different neuropathological findings. These diseases include multiple system atrophy (MSA), progressive supranuclear palsy (PSP) and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). They will be briefly covered here for the sake of completeness, but most of the genetic work described in this thesis relates to so-called 'familial' PD and the emphasis will be on this form of parkinsonism.

**Figure 1.1:** Histochemical staining of a typical Lewy body within the brain of a patient with classical Parkinson's Disease. The Lewy body typically comprises a relatively homogeneous core and a peripheral region containing aggregates of ubiquitin and several other proteins. These are now known to include  $\alpha$ -synuclein and Parkin, which are altered in some forms of familial PD.



### 1.9.1 Environmental factors

To date, the only unequivocally accepted risk factor for PD is increasing age but it remains far from clear how this can help to understand the cause of the disease, as striatal dopamine declines with normal ageing (Carlsson, 1995). In any case, the absence of a convenient biomarker for PD has meant that all epidemiological studies have tended to underestimate PD in a population. The discovery that N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can cause a clinical parkinsonian syndrome (Irwin *et al.*, 1987) suggested that toxins present in the environment could be responsible. There is also a fascinating apparent inverse relationship between smoking and the risk for PD (Morens *et al.*, 1994; Rybicki *et al.*, 1999). Two explanations have been offered for this observation. The first suggests that one or more compounds in cigarette smoke are protective against environmental exposure to an external trigger, the second that there could be an interaction between cigarette smoking and genetically determined risk factors. Gene-environment interactions are further explored below. Four lines of evidence have now emerged in favour of a genetic predisposition to PD: i) epidemiologic surveys, ii) twin data, iii) candidate gene studies, and iv) analysis of families with apparent dominant or recessive inheritance of a 'parkinsonian' phenotype.

### 1.10 EPIDEMIOLOGICAL STUDIES

The genetic contribution in Parkinson's disease has been debated for over a century, when Gowers noted that 15% of his patients had affected relatives (Gowers, 1893). Several case-control studies have consistently indicated that PD is more common among relatives of index patients with PD compared with a matched control population, as between 6 and 30 % of index patients had first- or second-degree affected relatives (De Michele *et al.*, 1996; Lazzarini *et al.*, 1994; Marder *et al.*, 1996; Payami *et al.*, 1994). These studies were subject to recall bias, but a large recent population-based case-control study also found that PD significantly aggregated in families, the strength of the association being age-dependent. This is in keeping with the fact that familial factors, which can be genetic, environmental, or both, play a role in PD (Elbaz *et al.*, 1998). Overall, the relative risk in first degree relatives of individuals with PD is in the range of 2 to 3, which is similar to that found in Alzheimer's Disease (Farrer *et al.*, 1989). All of these studies have rested on the diagnosis of PD by the traditional clinical

criteria of history and clinical examination without longitudinal follow-up. Since the pathology of the disease begins years before it produces signs or symptoms, clinical diagnosis currently has a low predictive value, particularly in those individuals with a relatively high prior risk (e.g. with a family history of PD).

### **1.10.1 Twin studies and the role of positron emission tomography (PET)**

Early twin studies found similar concordance rates between monozygotic (MZ) and dizygotic (DZ) twins and this evidence was originally used to argue against a significant genetic aetiology in PD. The aggregated MZ/DZ concordance ratio of 1.2:1 found in previous twin studies provided no evidence of a significant genetic risk (autosomal dominant or recessive models are 2:1 or 4:1 ratios) and was much more in keeping with an exogenous cause (Barucha *et al.*, 1986; Marsden, 1987; Tanner, 1992; Vieregge *et al.*, 1992; Ward *et al.*, 1983). Although the most recent twin study found similar overall concordance rates for PD in a total of 19842 white male twins, genetic factors appeared to be important when PD began at or before the age of 50 years (Tanner *et al.*, 1999). Concordance rates in twin pairs with PD were stratified by zygosity and age at diagnosis: similar rates led the authors to conclude that genetic factors do not play a major role in causing late-onset PD. However, subset analysis of 16 twin pairs with diagnosis at or before the age of 50 years showed a relative risk of 6 (95% confidence interval 1.69-21.26) suggesting that genetic factors appear to be important in this age group. All 4 of the MZ pairs were concordant from this sub-set, as compared to only 2 of 12 DZ pairs, supporting a primarily inherited cause of early-onset PD (Tanner *et al.*, 1999). The potential limitations of this study are: 1) it was cross-sectional and therefore bias could have been introduced as observations were made at a single time point, 2) diagnostic misclassification, 3) the cohort was exclusively white US males so the relevance of these findings to women and non-caucasian racial groups remains uncertain. The data obtained from these twin studies suggested that the genetic contribution to PD is much less over the age of 50 and it can be inferred that environmental factors are more significant in the development of PD in this age group (Tanner *et al.*, 1999).

### 1.10.2 Why did early twin studies show MZ/DZ concordance?

The reliability of twin studies in estimating the genetic component of a disease has been questioned, as a similar pattern of concordance in MZ pairs could be produced by shared environment. However, no consistent perinatal risk factor has been linked to the development of PD later in life. In addition, significant differences in the age of onset of PD symptoms were observed in previous twin studies, contributing to the low concordance rates. Cross-sectional studies do not allow detection of cases with subclinical disease and latencies for clinical concordance vary greatly within PD twin pairs, as illustrated by a previously reported pair who were separated by three decades (Gibb and Lees, 1988). Interestingly, even in rare families, such as the Contursi kindred (described below), which show clear evidence of autosomal dominant inheritance, there is a wide variation in the age of onset (Golbe *et al.*, 1990). In summary, the early twin studies cannot be viewed too simplistically. The evidence suggests that there are other, as yet unidentified, modifiers of the expression of PD as well as the presence of genetic factors but until these are identified a full interpretation of these results is not possible.

### 1.10.3 [<sup>18</sup>F]-DOPA PET studies

The critical pathology underlying the PD phenotype is neuronal degeneration in the *substantia nigra* resulting in dysfunction of the nigrostriatal dopaminergic pathway. The use of positron emission tomography (PET) has been put forward as a sensitive, specific way of detecting subclinical nigrostriatal dysfunction in PD *in vivo* (Burn *et al.*, 1992). Striatal uptake of [<sup>18</sup>F]-dopa is believed to reflect nigrostriatal nerve terminal aromatic amino acid decarboxylase activity (Hoshi *et al.*, 1993). Patients with typical PD display a characteristic reduction of striatal tracer uptake and the same alteration can be found in clinically unaffected members of PD kindreds. The basic assumption of PET studies in this context is that the dysfunction identified by [<sup>18</sup>F]-dopa PET represents a subclinical (or preclinical) manifestation of the disease, although the significance of finding reduced [<sup>18</sup>F]-dopa uptake in a clinically normal subject remains controversial (Piccini *et al.*, 1997). The disease process itself may be much more frequent than clinically overt PD and the inherited nature of the disorder may be masked by the low clinical penetrance of the mutations. A substantial role for genetic factors in PD was also reported after analysis of a longitudinal study of dopaminergic

function in twins, where the concordance levels for dopaminergic dysfunction were found to be significantly higher in MZ than in DZ twin pairs with no apparent family history (Piccini *et al.*, 1999). Nevertheless, as all of these twin pairs shared the same environment in early life, establishing a genetic component does not reduce the possibility of an important concomitant environmental factor (Golbe *et al.*, 1999b). Future studies which examine the aetiology of PD must consider not just susceptibility genes but also environmental factors and gene-environment interactions.

## **1.11 CANDIDATE GENE STUDIES FOR SUSCEPTIBILITY TO PD**

Nigral degeneration with Lewy body formation and the resulting clinical picture of PD may represent a final common pathway of a multifactorial disease process in which both environmental and genetic factors have a role. Many studies have examined the role of oxidative stress, xenobiotic toxicity, altered dopamine metabolism or impaired proteosomal degradation in PD, and therefore research has focused on candidate genes which might logically be involved in PD, such as the genes involved in dopamine synthesis and metabolism, cellular detoxification and mitochondrial function (Frim *et al.*, 1994; Agundez *et al.*, 1995; Bandmann, 1997; Bandmann *et al.*, 1995a; Bandmann *et al.*, 1996; Bandmann *et al.*, 1997a; Bandmann *et al.*, 1995b; Parboosingh *et al.*, 1995; Plante-Bordeneuve *et al.*, 1994a; Plante-Bordeneuve *et al.*, 1994b; Plante-Bordeneuve *et al.*, 1997; Riedl *et al.*, 1998). A number of approaches have been used over the last decade in an attempt to identify susceptibility genes in PD such as linkage studies in families, direct DNA sequence analysis of candidate genes, and allelic association studies in familial and/or sporadic cases. The major studies and their contribution to our knowledge of the pathogenesis of PD are outlined below.

### **1.11.1 Linkage analysis and exclusion of several candidate genes**

Seven candidate genes for proteins involved in cell protection were originally studied by genetic linkage in 8 autosomal dominant kindreds (Gasser *et al.*, 1994), as free radical toxicity is thought to play a role in the loss of nigral tissue by causing degeneration of dopaminergic neurons. Interestingly, four of these proteins (glutathione peroxidase (GPX1), catalase (CAT), copper-zinc (Cu/Zn) superoxide dismutase-1

(SOD1) and debrisoquine 4-hydroxylase (CYP2D6)) are involved in detoxification processes (see below). The other three candidates examined were tyrosine hydroxylase (TH), brain-derived neurotrophic factor (BDNF) and amyloid precursor protein (APP). Simple sequence tandem repeats (SSTRs, commonly called microsatellite markers) spanning the chromosomal regions for these candidate genes were chosen. Summed lod scores for all families excluded linkage to the genes GPX1, TH, APP, SOD1 and CYP2D6 as well as to the chromosomal region containing the genes CAT and BDNF (Gasser *et al.*, 1994). Additional studies excluded the genes for basic fibroblast growth factor (bFGF), the pre- and postsynaptic dopamine transporter, the aromatic hydrocarbon receptor and its nuclear translocating factor, as well as for superoxide dismutase-2 (SOD2) (Supala *et al.*, 1994).

### **1.11.2 Direct sequencing studies**

DNA sequencing is the most direct method for investigating possible candidate genes, particularly because most PD kindreds are too small for linkage analysis. In one study, sequencing of the SOD1 gene in 23 index cases of FPD failed to reveal any mutations in the coding region (Bandmann *et al.*, 1995a). Examination of the coding regions of SOD1, SOD2 and catalase genes using single stranded conformation analysis (SSCP) also failed to find any pathogenic mutations (Parboosingh *et al.*, 1995). Homozygous *weaver* mice, a mutant mouse strain, display progressive postnatal depletion of dopaminergic cells in the mesencephalon and have thus been proposed as an animal model for PD. A mutation in the putative G-protein inward rectifier potassium channel (mGIRK2) has now been identified as the causative gene in the *weaver* mouse (Patil *et al.*, 1995). However, analysis of the H5 pore region of the human homologue (hGIRK2) in human familial and sporadic cases of PD has so far failed to find any abnormal sequences (Bandmann *et al.*, 1996).

### **1.11.3 Allelic association studies**

Discovery of susceptibility loci can be important in understanding the causes of a disease, especially in genetically heterogeneous disorders like PD (Gasser *et al.*, 1997). Allelic association studies compare the frequency of a given candidate gene

polymorphism in patients with the disease of interest with those in controls and are aimed at detecting a genetic background predisposing to disease. However, the power of association studies depends mainly on: a) the cohort sizes used, and b) the contribution of a given factor to the disease state. In most cases the relationship of the factor to the disease state is unknown and therefore a negative study can only lend weight to the evidence rather than provide an absolute answer (Wood, 1998). While the characterisation of single genes involved in rare mendelian families with PD is likely to be significant in enhancing our understanding of the disease, association studies should be (if designed appropriately) the most powerful in trying to elucidate susceptibility genes, if present, in PD as a whole. In order to generate the most useful data, recent guidelines on the design of association studies have suggested that only associations that make biological sense should be reported. They should also contain an initial study as well as an example of independent replication and should be observed in family and population-based studies. For alleles with modest effects, which might depend on alleles at other genes, replication may be challenging. Several large, independent datasets, with family studies, should be used to confirm the validity of the association.

Two types of allelic association study have been used in PD. One analysed sequence variants without any known functional relevance (e.g. polymorphisms of tyrosine hydroxylase). The second examined functionally relevant genetic polymorphisms, such as the M1, M2 and M3 alleles of N-acetyltransferase-2 (NAT2) which lead to the slow acetylation phenotype (see chapter 3 below). There are several inherent problems with most of the association studies performed to date in PD, namely the type and appropriateness of controls, small sample sizes and different diagnostic criteria between studies. In PD, the presence of Lewy bodies in the brain is the ultimate diagnostic finding but Lewy bodies are found in only about 80% of all cases examined at autopsy (Hughes *et al.*, 1992). Furthermore, to minimise the effects of population stratification in association studies, the controls and study sample must be consistently matched for age, sex and ethnicity. Population stratification is another bias of association studies as subjects within a population tend to marry people like themselves (assortative mating) and even in highly mixed populations found in many countries, populations are still stratified. Unfortunately, robust association analyses such as the transmission disequilibrium test (Spielman and Ewens, 1998) are difficult to apply in late-onset disorders like PD, since parental marker information normally has to be extrapolated.

#### 1.11.4 Detoxification Enzymes and PD

As mentioned above, MPTP has been identified as an environmental substance which is toxic to dopaminergic neurons and therefore it may initiate nigral degeneration in genetically predisposed individuals (Langston *et al.*, 1983). Detoxification enzymes such as those involved in xenobiotic metabolism have been studied in PD because decreased hepatic metabolism of xenobiotics would probably increase their delivery (and therefore toxicity) to the central nervous system. If one or more of these enzymes carried a genetic mutation that led to defective enzyme production or function, then affected individuals might be more susceptible to injury on exposure to environmental toxins.

For example, the P450 enzyme system has a major role in the detoxification of xenobiotics (Tsuneoka *et al.*, 1993). Previously, six of the genes encoding P450 enzymes have been studied with respect to PD: CYP1A1, CYP2C9, CYP2C19, CYP1A2, CYP2E1 and CYP2D6. The normal CYP2D6 gene contains nine exons and encodes debrisoquine 4-hydroxylase, which has a number of functionally different isoforms. The CYP2D6A mutant allele contains a deletion in exon 3, while the CYP2D6B mutant allele contains a G-to-A transition at the intron3/exon4 junction. Polymorphisms in CYP2D6 can produce active protein products with alterations in catalytic function, protein stability or membrane integration (Riedl *et al.*, 1998; Tsuneoka *et al.*, 1993). CYP2D6B has been the most intensively studied mutant allele as it accounts for 75% of poor metabolisers. Several studies have revealed a positive association of the poor metaboliser CYP2D6 genotype with an increased risk of sporadic PD (Agundez *et al.*, 1995; Armstrong *et al.*, 1992) although other studies have failed to replicate these findings (Bordet *et al.*, 1994; Diederich *et al.*, 1996; Gasser *et al.*, 1996). The two most recent studies (Ho *et al.*, 1999; Sabbagh *et al.*, 1999) found no evidence for an association between CYP2D6 and PD suggesting that the CYP2D6 locus is not a major genetic determinant of the disease. Other genes have been studied, but the results so far are either conflicting, negative or await further confirmation. These include dopamine transporter genes and dopamine receptors (Higuchi *et al.*, 1995; Parboosingh *et al.*, 1995; Plante-Bordeneuve *et al.*, 1997) monoamine oxidase A&B (MAOA, MAOB) (Ho *et al.*, 1995; Hotamisligil *et al.*, 1994; Kurth *et al.*, 1993; Mellick *et al.*, 1999), tyrosine hydroxylase (Plante-Bordeneuve *et al.*, 1994b),

glutathione-S-transferase (GSTM1) (Nicholl *et al.*, 1999; Stroombergen *et al.*, 1996) and the tau gene A0 polymorphism (Pastor *et al.*, 2000).

#### **1.11.5 N-acetyltransferase 2 (NAT2)**

Over 40 years ago, neurological side-effects were observed when isoniazid (L-isonicotinyl hydrazide) was administered to patients with TB, which was apparently related to plasma concentrations of the unchanged drug (Evans *et al.* 1960). A wide variation of metabolism of this substance was found in human populations and can divide the population into fast or slow inactivators on the basis of excretion of the free form of the drug in the urine. The fact that certain populations have a higher proportion of fast inactivators relative to others and an early population genetics study (Knight *et al.*, 1959) suggested that this was determined by autosomal genetic factors and that slow inactivation was recessive to fast inactivation. This activity is now known to be due to variations in the levels N-acetyltransferase enzymes, responsible for arylamine N-acetylation (Grant *et al.*, 1990). As it has been suggested that poor acetylators may not eliminate neurotoxic metabolites efficiently, recent candidate gene studies for neurological diseases have looked at polymorphisms in the N-acetyltransferase-2 (NAT2) gene (Bandmann *et al.* 1997, 1999; Nicholl *et al.* 1997; Agundez *et al.*, 1998; Maraganore *et al.* 2000).

The degree of activity of NAT2 determines the rate of detoxification of aromatic amines and a slow acetylator genotype is defined by the presence of two mutant alleles. An association has been described between the slow acetylator genotype for NAT2 and familial PD (Bandmann *et al.*, 1997b). This study has more recently been confirmed using more precise genotyping methods (Bandmann *et al.*, 1999). The biologically plausible hypothesis is that slow acetylation could lead to impaired ability of patients with familial PD to handle neurotoxic substances.

To further investigate the preliminary findings, a study of 161 nuclear families with PD was undertaken as part of a family-based association study (see chapter 3). This represents a novel approach to evaluate the importance of these results.

### 1.11.6 Apolipoprotein E (APOE)

The partial overlap in the clinical phenotype and pathology of PD with Alzheimer's Disease (AD), as well as striking structural similarities of  $\alpha$ -synuclein (involved in rare forms of familial PD, see below) and apolipoprotein E (ApoE), prompted a recent report (Kruger *et al.*, 1999). The ApoE- $\epsilon$ 4 allele is an important susceptibility factor in late-onset AD (Roses and Strittmatter, 1996) but ApoE genotype analysis in PD was inconclusive. Combined data on a polymorphism in the promoter region of *SNCA* with the ApoE- $\epsilon$ 4 allele in 193 sporadic PD patients from Germany compared with 200 healthy controls matched for age, sex and origin showed a highly significant difference between PD patients and control individuals ( $P < 0.01$ ). The risk factors were independently associated with an increased risk of sporadic PD. This potentially important observation has not, however, been reproduced by other investigators (N Khan, personal communication). Interactions of ApoE- $\epsilon$ 4 with other loci have also been suggested by the observation that the ApoE- $\epsilon$ 4 genotype and gene polymorphisms of *CYP2D6* and *SNCA* are significantly associated with PD (Bon *et al.*, 1999). Another report found an association between *NACP-Rep1* and essential tremor (Tan *et al.*, 2000).

### 1.12 MITOCHONDRIA AND PD

The case for specific mitochondrial DNA mutations playing a role in PD is still unresolved (Bandmann *et al.*, 1997a). Mitochondrial respiratory failure and oxidative stress appear to be two major components in nigral neuronal death in PD and several groups have reported inhibition of mitochondrial respiratory chain function in PD patients. A complex 1 deficit has previously been noted in the *substantia nigra* (Schapira *et al.*, 1990) and platelets (Parker *et al.*, 1989). Human neuroblastoma cells were depleted of their own mtDNA and repopulated with mitochondria derived from platelets of PD or controls by means of a cell-hybrid system. A 20% reduction in complex 1 activity was observed along with increased toxin susceptibility and oxygen free-radical production in the cell hybrids containing the mtDNA of PD patients (Swerdlow *et al.*, 1996). These data suggest involvement of mtDNA in the development of complex 1 deficiency but direct genetic studies of mtDNA in PD have so far been negative. Other groups have speculated that although there is no evidence for increased

maternal transmission in PD, it is possible that complex heteroplasmic mitochondrial inheritance could play a role in a sub-group of PD patients and that nuclear gene disturbances may be important in another sub-group of PD patients. It is also possible that several DNA sequence variants may be necessary to cause mitochondrial dysfunction rather than a single point mutation (Bandmann *et al.*, 1997a). It is important to establish whether complex 1 deficiency is primary or secondary and whether it is localised to the nigrostriatal system only. It has been postulated that the primary cause of PD may be the combination of genetic background and putative nigral neurotoxins. Exposure of nigral neurons with their high dopamine content to these toxins may lead to oxidative damage thus causing the most serious complex 1 deficiency in nigral cells compared with systemic organs.

### **1.13 CONTRIBUTION OF CANDIDATE GENE LINKAGE AND FUNCTIONAL POLYMORPHISM ASSOCIATION STUDIES TO UNDERSTANDING PD**

Association studies are aimed at establishing the importance of the different gene polymorphisms in disease pathogenesis. Do these polymorphisms modify disease process or alter susceptibility to PD? Either or both of these possibilities may be true. Table 1.1 summarizes the major association studies undertaken on functional polymorphisms in PD. A recent paper attempted to assess the variability and validity of polymorphism association studies in PD (Tan, 2000). From 84 studies on 14 genes, those with four or more independent studies of a specific gene polymorphism were subjected to meta-analysis. Significant associations were found in polymorphisms of NAT2, MAOB, GSTT1 and tRNAGlu. These results, however, have to be interpreted with caution, as large study numbers are required as well as carefully matched controls in order to establish whether a particular association implies linkage disequilibrium with a disease-causing locus or if it is of functional importance in the disease process. Future studies should be designed to circumvent these limitations and improve confidence in genuine associations, as at present, despite the wealth of literature, testing for these polymorphisms in patients with PD is not of any real diagnostic or predictive value.

**Table 1.1:** Summary of the major association studies of functional polymorphisms in PD.

<b>Candidate Polymorphism</b>	<b>References (abbreviated)</b>
<b>CYP2D6</b>	Riedl <i>et al.</i> 1998; Tsuneoka <i>et al.</i> 1993
<b>CYP2E1</b>	Bandmann <i>et al.</i> 1997b
<b>ApoE 4</b>	Kruger <i>et al.</i> 1999, Bon <i>et al.</i> 1999
<b>MAO-A</b>	Hotamisligil <i>et al.</i> 1994
<b>MAO-B</b>	Kurth <i>et al.</i> 1993; Mellick <i>et al.</i> 1999
<b>Dopamine Transporter Protein</b>	Parboosingh <i>et al.</i> 1995
<b>Dopamine Receptor, DRD2</b>	Oliveri <i>et al.</i> 2000
<b>NADPH-menadione reductase, NQ1</b>	Bandmann <i>et al.</i> 1997b.
<b>Glutathione transferase, GSTM1, GSTP1, GSTZ1, GSTT1</b>	Bandmann <i>et al.</i> 1997b
<b>N-Acetyl Transferase 2 (NAT2)</b>	Bandmann <i>et al.</i> 1997b.
<b>Tyrosine Hydroxylase, TH</b>	Plante-Bordeneuve <i>et al.</i> 1997
<b>Mitochondrial DNA polymorphisms</b>	Bandmann <i>et al.</i> 1997a;
<b>NACP-1 Rep</b>	Tan <i>et al.</i> 2000

### 1.13.1 Autosomal Dominant PD kindreds

Although, several pedigrees had previously been described with Parkinsonian features (Allan, 1937; Bell and Clark, 1926; Spellman, 1962), often there was no pathological data, and it is only more recently that an increasing number of well-documented multi-generational Parkinsonian kindreds have been reported with evidence of autosomal

dominant inheritance with variable penetrance. However, only a few kindreds have been reported where the clinico-pathological features are indistinguishable from the sporadic form of the disease with a late age of onset, good Levo-dopa response and with typical Lewy body inclusions (Wszolek *et al.*, 1995). Others exhibit some atypical features, such as young age of onset and rapid disease course (Golbe *et al.*, 1996), marked cognitive decline with an atypical distribution of Lewy bodies (Muentert *et al.*, 1998), or atypical Parkinsonian features such as apathy, hypoventilation and scattered Lewy bodies (Perry *et al.*, 1975; Perry *et al.*, 1990). Finally, the chromosome 17 linked syndromes of pallido-ponto-nigral degeneration (Clark *et al.*, 1998; Wszolek *et al.*, 1992) and fronto-temporal dementia (Hutton *et al.*, 1998) can show Parkinsonian features as part of their rather broad phenotype.

The recent discoveries in mendelian PD families (see below) have firmly established the existence of a genetic component in the disease. Indeed, the elucidation of the molecular events leading to nigral degeneration in these inherited cases may help to identify the molecular pathogenesis of the more common forms of PD. At present however, there is no direct evidence that any of the genes for familial PD have a direct role in the aetiology of the common sporadic form of PD, or in those cases who have a limited number of affected family members, although circumstantial evidence (such as the presence of  $\alpha$ -synuclein and parkin in Lewy bodies (Shimura *et al.*, 1999; Spillantini *et al.*, 1997)) suggests that this is likely.

## **1.14 DISEASE CAUSING LOCI AND PARKINSON'S DISEASE GENES**

Traditional linkage analysis and positional cloning strategies in independent PD kindreds have identified the  $\alpha$ -synuclein gene, *SNCA* (OMIM 601508) (Polymeropoulos *et al.*, 1997) and three disease-causing gene loci on chromosomes 2p13, 4p14-16.3 and 1p35-p36 (Farrer *et al.*, 1999b, Valente *et al.*, 2001; Gasser *et al.*, 1998). Two further genes Parkin (Kitada *et al.*, 1998a) and UCH-L1 (Leroy *et al.*, 1998) were identified as a result of using a candidate approach in several families (see below). A further locus (PARK5) has been allocated according to HUGO, the nomenclature database (<http://www.gene.ucl.ac.uk>), but details of this locus are yet to be published. However, the two genes appear to have differing significance in the pathogenesis and overall cause of PD. Mutations in the Parkin gene are considered to be

a major cause of autosomal recessive familial early-onset and isolated juvenile-onset Parkinson's disease (AR-JP, OMIM 602544) (Kitada *et al.*, 1998b) but to date only one German kindred has been identified with the Ile93Met missense mutation in UCH-L1.

#### 1.14.1 Alpha Synuclein (PARK1)

The first definite evidence that a parkinsonian syndrome could be caused by a single gene came from linkage studies in Lewy body parkinsonism in an Italian family, the Contursi kindred. This is the largest PD pedigree characterised to date and consists of at least 60 affected members in 4 generations who originate from the village of Contursi in southern Italy. Some members had emigrated to America, so the family was initially described as an “Italian-American kindred” (Golbe *et al.*, 1990). Linkage of the disease locus to chromosome 4q21-q23 in this family (Polymeropoulos *et al.*, 1996) was followed by identification of a G>A transition at position 209 in exon 4 of the *SNCA* gene causing an alanine to threonine substitution in the  $\alpha$ -synuclein protein (Ala53Thr).

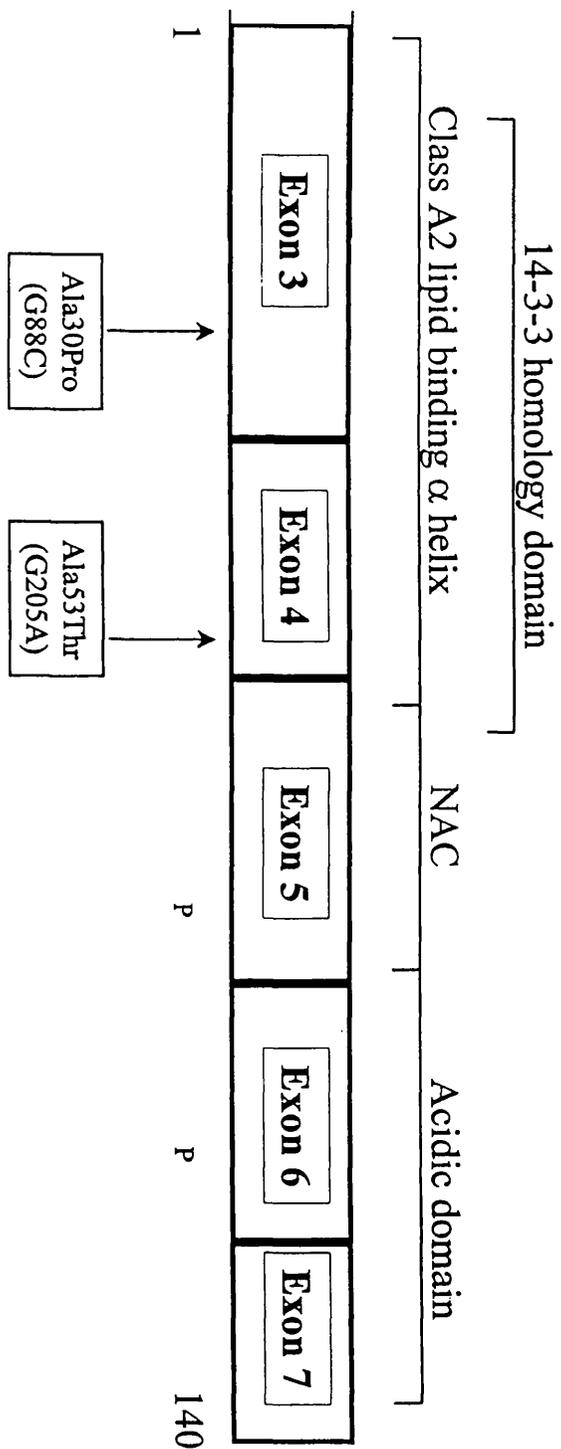
Designated “Parkinson’s disease type 1” (PARK1), the disorder in this family was to some extent typically parkinsonian, with Lewy bodies at post-mortem and a typical pattern of dopamine deficiency in PET studies indistinguishable from sporadic PD (Samii *et al.*, 1999). However, there were also some atypical features, such as a relatively early age of onset of illness at  $46 \pm 13$  years. In this family, the penetrance of the gene was estimated to be 85%. This mutation was also found in 3 other apparently unrelated Greek kindreds (Polymeropoulos *et al.*, 1997). Phenotypic similarity, a comparable age at onset and the fact that both kindreds originated from opposite ends of the same Mediterranean trade route, suggested that this mutation came from a single founder (Athanasidou *et al.*, 1999). Although this mutation is highly penetrant, there is considerable variation in expression as the oldest “carrier” is an otherwise asymptomatic 85 year-old with only mild rigidity on examination.

A mutation in a conserved region of exon 3 of *SNCA* has since been described in an unrelated German kindred (Kruger *et al.*, 1998). Mutation analysis of all 5 translated *SNCA* exons in an index case from an independent German familial PD kindred detected a G to C transversion at nucleotide 88 of the coding sequence in exon 3 of

SNCA causing the Ala30Pro substitution in the resulting protein (Fig. 1.2). The affected individual developed signs of progressive parkinsonism at age 52 concordant with an unusual family history, as his mother presented with symptoms at age 56 and died from the disease at age 60. A younger sib, aged 55, reported impaired motor function in the right arm and clinical examination was suggestive of Parkinson's disease. The 33-year-old child of the index patient and a 50-year-old sib were carriers of the mutation but, although both cases were clinically abnormal, the phenotype did not fulfil the diagnostic criteria of PD (Kruger *et al.*, 1998). The Ala30Pro substitution was not found in 1,140 chromosomes of control individuals leading the authors to conclude that SNCA mutations participate in the pathogenesis of only rare cases of Parkinson disease.

The PARK1 locus was examined in a series of autosomal dominant families collected by the EU consortium (GSPD) of which I analysed two UK kindreds (section 5.4.2) in order to detect the numerical importance of the locus-see appendix 3.3 (Gasser *et al.*, 1997). After the description of mutations in the SNCA gene in the Contursi kindred, access to a large number of EU sibling pairs and a series of autosomal dominant PD families enabled me to rapidly screen this series for the G209A and G88C coding mutations in the SNCA gene (section 4.3; Vaughan *et al.*, 1998a). Sequencing of SNCA for new coding mutations was also performed by Dr M Farrer (Vaughan *et al.*, 1998b) (section 4.3.2 and appendix 2.2 and 4.1).

**Fig. 1.2:** Schematic diagram of the *SNCA* gene, showing the two mutations (G88C and G209A) so far identified associated with familial PD.



### 1.14.2 The Synuclein family of proteins

Three distinct synucleins in human brain,  $\alpha$ -,  $\beta$ - and  $\gamma$ -synuclein, had been identified (Jakes *et al.*, 1994, Lavedan *et al.*, 1998). Alpha-synuclein was detected as a 19 kDa protein in the cytosolic fraction of brain homogenates and immunostaining of human brain sections showed that it was concentrated at presynaptic nerve terminals (Jakes *et al.*, 1994). A computer search of protein sequence databases found  $\alpha$ -synuclein shares 95% sequence homology with rat synuclein (Campion *et al.*, 1995; Maroteaux and Scheller, 1991), which is also expressed in the brain associated with synaptosomal membranes in neurons. A fragment of  $\alpha$ -synuclein forms the non- $\beta$  amyloid component (NAC) of amyloid plaques in AD (Ueda *et al.*, 1993) and secondary structure modelling predicted that this peptide has a strong tendency to form  $\beta$ -structures consistent with its association with amyloid. Campion *et al.*, (1995) mapped the  $\alpha$ -synuclein (*SNCA*) gene to chromosome 4 and sequenced the entire coding region. As it was a candidate for familial dementia, RT-PCR products from 26 unrelated patients with familial early-onset Alzheimer disease were sequenced, but no mutations were found (Higuchi *et al.*, 1998). However, 3 alternatively spliced transcripts were found in normal lymphocytes. Northern blotting showed that *SNCA* mRNA was principally expressed in brain but was also at low levels in all tissues except liver.

Shortly afterwards (Chen *et al.*, 1995) mapped the locus more precisely to 4q21.3-q22 by PCR-based analysis of human/rodent hybrid cells and by fluorescence *in situ* hybridisation (FISH). Alpha-synuclein also shares physical and functional homology with 14-3-3 proteins, which are a family of ubiquitous cytoplasmic chaperones: regions of alpha-synuclein and 14-3-3 proteins share over 40% homology (Ostrerova *et al.*, 1999). In addition, it has been suggested that synelfin, an orthologue of  $\alpha$ -synuclein in the zebra finch, may play a role in song learning, but the function of mammalian synucleins remains unclear. One hypothesis proposed that synuclein is involved in the turnover of pre-synaptic membranes and synaptic signalling, processes important in learning and memory (Clayton and George, 1998; Clayton and George, 1999). No mutations in beta or gamma synuclein have been found in PD subjects to date (Lavedan *et al.*, 1998)

### 1.14.3 Alpha Synuclein, the Lewy body and neurodegenerative disease

Despite the rarity of *SNCA* mutations in familial PD, the protein product,  $\alpha$ -synuclein, has become the centrepiece of a new understanding of the Lewy body and of a new hypothesis of the pathogenesis of PD. The wider importance of  $\alpha$ -synuclein in PD and related disorders such as diffuse Lewy body disease and dementia with Lewy bodies has since been recognized, as  $\alpha$ -synuclein protein is a major fibrillar component of Lewy bodies, Lewy neurites (Spillantini *et al.*, 1997) and the glial cell inclusion bodies (GCIs) of Multiple System Atrophy (MSA)(Tu *et al.*, 1998). Lewy bodies are roughly spherical structures (see Fig 1.1 above) comprising radially arrayed intraneuronal aggregations of at least 20 known antigenic components, including various proteins, fatty acids, sphingomyelin and polysaccharides. Alpha-synuclein and ubiquitin appear to be the major constituents of Lewy bodies, although the latter is not always present (Spillantini *et al.*, 1997). Indeed, immunostaining for  $\alpha$ -synuclein has now become diagnostic for Lewy bodies.

### 1.14.4 Other Lewy body proteins implicated in PD

Following the identification of  $\alpha$ -synuclein as the first single protein with a definite pathological link with PD, other Lewy body components, some of which interact with  $\alpha$ -synuclein, have been suggested as candidate genes in familial PD. Evidence that  $\alpha$ -synuclein may have an interacting role with other familial PD gene products has also been suggested from neuropathological studies of families linked to chromosome 4p15. For instance, (Engelender *et al.*, 1999) identified a novel  $\alpha$ -synuclein-interacting protein, synphilin-1, encoded by the gene *SNCAIP*. Synphilin-1 was present in many regions in brain, including *substantia nigra* and in Lewy bodies of PD patients (Wakabayashi *et al.*, 2000). Alpha-synuclein was found to interact with synphilin-1 in neurons *in vivo*. As Lewy bodies contain many multi-ubiquitinated chains arising from incomplete degradation of constituent proteins, a central role for ubiquitin in the proteasome pathway has been proposed and implicated ubiquitin as a potential candidate gene in Parkinson disease (Wilkinson *et al.*, 1989).

Ubiquitin C-terminal hydrolase L1 (UCH-L1) is a member of a gene family whose products hydrolyze small C-terminal adducts of ubiquitin to generate the ubiquitin

monomer. Expression of UCH-L1 (which represents 1 to 2% of total soluble brain protein) is highly specific to all neurons and to cells of the diffuse neuroendocrine system and their tumors (Doran *et al.*, 1983). UCH-L1 cDNA was cloned, the structure of the gene defined and the gene product was referred to as PGP9.5 (Day and Thompson, 1987, Day, 1990 #4755). UCH-L1 was mapped to chromosome 4 by PCR analysis of DNA from a panel of human/rodent somatic cell hybrids (Edwards *et al.*, 1991). By *in situ* hybridization, the assignment was regionalised to 4p14. Interestingly, an Ile93Met missense mutation in the ubiquitin C-terminal hydroxylase (*UCH-L1*) gene was identified in a German family with familial PD. Indeed, kinetic studies of Ile93Met UCH-L1 and wild-type enzymes showed that the mutant had nearly a 50% reduction in activity (Leroy *et al.*, 1998). Two models have been suggested which may explain the toxic effect of this missense mutation on the neuron and these are further discussed in chapter 6.

## **1.15 AUTOSOMAL RECESSIVE PD, PARK2 AND THE PARKIN GENE**

### **1.15.1 Background to the discovery of Parkin and linkage studies in AR-JP kindreds**

Autosomal recessive juvenile parkinsonism (AR-JP, PARK2, OMIM 602544) is one of the monogenic forms of Parkinson's disease (PD) initially described in Japan. AR-JP patients show the typical signs of PD, but they are associated with a) early onset, typically before the age of 40; b) dystonia at onset; c) diurnal fluctuations; d) slow disease progression; and e) early and severe L-dopa induced dyskinesias. The clinical features of 17 patients from 12 Japanese families with AR-JP have been described (Ishikawa and Tsuji, 1996.). In 11 of these families, affected members were products of consanguineous marriages with a mean age of onset of 27 (range 9 to 43) years. The most prominent symptoms were retropulsion, dystonia of the feet, and hyperreflexia with classic Parkinsonism. Symptoms of tremor, rigidity, and bradykinesia were mild. Patients responded to L-dopa but dopa-induced dyskinesias and wearing-off phenomena occurred frequently. A distinguishing feature of the phenotype was sleep benefit, with reduction of parkinsonian symptoms after awakening. AR-JP is pathologically characterised by highly selective degeneration of dopaminergic neurons in the *zona compacta* of the *substantia nigra* and the absence of Lewy bodies (Ishikawa *et al.*,

1996). Recently, the gene responsible for AR-JP was identified and designated Parkin following linkage studies and positional cloning techniques (see below).

### **1.15.2 Linkage Analysis of PARK 2 and positional cloning of the Parkin gene**

By linkage analysis using a diallelic polymorphism of the manganese superoxide dismutase gene (SOD2; OMIM 147460), perfect segregation of the disease was found at the SOD2 locus (Matsumine *et al.*, 1997). By extending the linkage analysis to 13 families with autosomal recessive juvenile Parkinsonism, they discovered strong evidence for the localization of the gene at 6q25.2-q27, including the SOD2 locus, with the maximum cumulative pairwise lod scores of 7.26 and 7.71 at D6S305 ( $\theta = 0.03$ ) and D6S253 ( $\theta = 0.02$ ) respectively. Linkage analysis was then performed on further families to narrow the region prior to restriction mapping and positional cloning to identify the gene responsible for AR-JP.

Non-Japanese PARK 2 families were first demonstrated in Europe, the United States and the Middle East. Homozygous deletions in 3 AR-JP families greatly reduced the initial 17cM candidate interval (Matsumine *et al.*, 1998, Tassin, 1998, Jones, 1998). Linkage of the gene for AR-JP to 6q25.2-q27 was described in 1 Algerian and 10 European multiplex families as part of the EU GSPD consortium (Tassin *et al.*, 1998). The clinical spectrum of the disease in these families was broader than reported previously, with age at onset up to 58 years and the presence of painful dystonia in some patients. In all patients examined, 2 of the 3 cardinal signs of PD (akinesia, rigidity, and tremor) were found. Marked improvement with L-dopa treatment occurred in all except 2 untreated secondary cases found in family studies. Age at onset was less than 40 years for at least 1 affected sib. Linkage to 6q25.2-q27 was also found in a group of 15 families from 4 distinct ethnic backgrounds (Jones *et al.*, 1998). A full genomic screen excluded other candidate regions. Detailed mapping of the linked region (including the position of the SOD2 gene) showed that recombination events restricted the AR-JP locus to a 6.9-cM region and excluded SOD2 (Jones *et al.*, 1998). Moreover, nucleotide sequence analysis of the coding regions of the SOD2 gene did not show causative mutations.

The gene responsible for AR-JP (Parkin) was isolated by positional cloning techniques in a Japanese patient with deletion of 6q (including the closely linked marker D6S305)

(PARK2; 602544, (Kitada *et al.*, 1998a)). The Parkin gene spans more than 500 kb and has 12 coding exons with an open reading frame of 1,395-bp. Five exons (exons 3-7) were deleted in the original patient. Four other AR-JP patients from 3 unrelated families had a deletion affecting exon 4 alone (Kitada *et al.*, 1998a). Alternative splicing of these exons produces different parkin transcripts in different tissues (Sunada *et al.*, 1998). The Parkin protein is composed of 465 amino acids with a moderate similarity to ubiquitin at the amino terminus and a RING-finger motif at the carboxy-terminus (Kitada *et al.*, 1998a). Detailed mutational analyses have now clearly shown that Parkin is numerically far more important than other genes so far described in PD (see chapter 4). Association studies of the Parkin gene in sporadic PD have been limited so far. Heterozygosity at codon 167 in the Parkin gene has been reported as a genetic risk factor for the development of spodic PD (Sato *et al.*, 1999) but this was not confirmed in a population of Central European origin (Klein *et al.*, 2000).

### **1.16 GENE LOCUS 2p13 (PARK 3)**

Six families of European origin with autosomal dominant PD were studied. Affected individuals in these families had a phenotype similar to idiopathic PD, with age of onset ranging from 37 to 89 years (mean between 54 and 63 years) (Gasser *et al.*, 1998). Dementia was prominent in addition to parkinsonism in two of the kindreds. Autopsy data from 3 of the families was consistent with Lewy body PD. Several markers spanning an area on chromosome 2p (2p13) showed consistently positive lod scores in two kindreds which held the greatest potential to show linkage. This region of 40cM was subsequently investigated more closely by typing additional markers in all six families in the study and a multipoint lod score over all six families was 3.96. Further refinement of the interval determined by linkage analysis identified a common disease haplotype over 7 markers spanning a 3.2 cM region in two kindreds which originated from the same area in northern Germany and southern Denmark. This was suggestive of a possible founder effect. Analysis of this haplotype in clinically unaffected members in two of the linked families over the age of onset (mean 56 years) gave an estimated penetrance of 40%. The authors speculated that as the disease expressed in these kindreds appeared very similar to sporadic PD any mutation found may shed light on the molecular aetiology in a significant proportion of the PD population as a whole. The founder haplotype has not been identified in other German pedigrees with familial PD

and a further study also failed to identify the haplotype in a population of patients from northern Germany with sporadic or familial Parkinson's disease (Klein *et al.*, 1999). The disease gene at this locus has yet to be identified, although sequencing of a biologically plausible candidate gene in the area, TGF- $\alpha$ , which supports dopaminergic neurons *in vitro*, did not reveal any mutations (T Gasser, personal communication). Further parkinsonian kindreds which show linkage to 2p13 have yet to be reported. This study highlighted the difficulties of using small kindreds and analysing them within the same study, as one family (family K) was uninformative due to its small size at both 4q21 (Gasser *et al.*, 1997) and 2p13.

## **1.17 GENE LOCUS 4p15 (PARK 4)**

### **1.17.1 Linkage in autosomal dominant PD**

Farrer and colleagues identified an 8.7cM haplotype of six adjacent markers (4p14-16.1) which segregated with autosomal dominant, early-onset parkinsonism and essential tremor (ET) in a North American kindred from Iowa (Farrer *et al.*, 1999b). This kindred had previously been reported in detail (Muentner *et al.*, 1998). Affected members of the family share many features with typical idiopathic PD, although the mean age of onset is considerably younger (mean 33.6 years), and several atypical features are present, such as early weight loss, dysautonomia and dementia. Neuropathological changes include, in addition to nigral degeneration and Lewy-body formation, conspicuous vacuoles in the hippocampus and several other brain areas. Multipoint linkage analysis at 4p14-16.1 yielded a lod score of 2.64 at  $\theta = 0$ , (D4S391) insufficient for statistical significance in a genome search. Interestingly, the disease-linked haplotype was also found in individuals in the pedigree who did not have clinical Lewy body parkinsonism but instead had a clinical phenotype of postural tremor. Rather than this being indicative of false positive linkage, it was suggested that in some circumstances tremor can be an alternative phenotype of the same pathogenic mutations as Lewy body parkinsonism (Farrer *et al.*, 1999b). More recently identified members of this kindred have allowed reanalysis at this locus such that the multipoint lod score is now above 3.0 (M Farrer, personal communication). UCH-L1 has been excluded as a cause of disease by both two-point linkage analysis and direct sequencing of an index member of the kindred (Farrer *et al.*, 1999b). Identification of additional

families linked to 4p15-linked families would provide statistical support for the Iowan haplotype and help refine a candidate region for positional cloning of 4p15. The results of an independent Italian family, examined where 4 affected individuals in 2 successive generations were screened for linkage to PARK 4 are described in chapter 3.

### **1.18 GENE LOCUS PARK 6 (1p35-p36)**

A large Sicilian family has been identified with four definitely affected members (referred to as the Marsala kindred). The phenotype was characterised by early onset (range 32-48 years) Parkinsonism with slow progression and a sustained response to L-dopa. Linkage of the disease to the Parkin gene was excluded. A genome wide homozygosity screen was performed in the family. Linkage analysis and haplotype construction allowed identification of a single region of homozygosity shared by all the affected members, spanning 12.5cM on the short arm of chromosome 1 (1p35-p36). This region contains a novel locus for autosomal recessive early onset Parkinsonism, PARK6. A maximum Lod score 4.01 at recombination fraction 0 was obtained for marker D1S199 (Valente *et al.*, 2001).

The study of other large dominant and recessive Parkinsonian kindreds will be important in furthering our understanding of the pathogenesis of Parkinson's disease. Unfortunately, such kindreds are both rare and of limited size, making linkage analysis to identify the gene loci problematic. The clinical and pathological features in 2 large unpublished kindreds (UK402, UK403) who are not linked to any of the PD loci described to date are presented in chapter 5.

### **1.19 OBJECTIVES OF THIS THESIS**

In this thesis, I have attempted to apply molecular genetic techniques to the study of a large population of sibling pairs and two large autosomal dominant PD kindreds (UK 402, UK 403) collected over a three year period as part of a genetic consortium formed to study genetic susceptibility in Parkinson's disease (GSPD). A range of techniques were used, which include linkage studies and mutation analysis of candidate and cloned genes. Where certain aspects of the work were undertaken by other partners, this is clearly indicated. For example, the methodology for gene sequencing is attached as an

appendix, as this was not carried out by myself. A brief introduction to each aspect of the thesis work is given in the individual chapters. Kindreds were first followed up and clinically ascertained by myself, and DNA collected for analysis (CHAPTER 3). Microsatellite markers at key loci, previously identified in familial PD, were analyzed by the author in the UK and Italian families (as part of GSPD- CHAPTER 3). An intra-familial association study was performed solely by myself to examine the role of NAT2 in familial PD based on the preliminary work described above (chapter 3). A comprehensive clinical and genetic study of the three genes described in familial PD to date was performed within the GSPD collaboration (CHAPTER 4). A clinico-genetic analysis of two major autosomal dominant kindreds is described in CHAPTER 5. Exclusion mapping at the PD loci on chromosomes 4q (PARK1), 6q (PARK2), 2 (PARK3) and 4p (PARK4) were performed in these families solely by myself. Sequencing of the 2 genes (UCH-L1 and SNCA) was performed elsewhere but the results are included for discussion purposes (CHAPTER 5). These studies were performed as part of an overall strategy to complete a genome screen on the two kindreds (which is still in progress). Potential future directions for this field are discussed in CHAPTER 6.

## **CHAPTER TWO: GENERAL MATERIALS AND METHODS**

## **2.1 OUTLINE OF CHAPTER**

This chapter describes the materials and experimental methodology employed in this study. The first part concerns the assessment and collection of affected sibling pairs and their unaffected relatives (to determine allele segregation in the families). Subjects were recruited as part of the EU collaboration, Genetic Susceptibility in Parkinson's disease (GSPD). They were all personally examined and videotaped using a standard protocol (all 70 UK cases were clinically examined and DNA collected by myself) and were only included in the study if they fulfilled rigorous diagnostic criteria for clinically definite PD (see Table 2.1 below). The methodology of linkage analysis is described here, from the extraction of DNA from blood, generation of DNA fragments using the polymerase chain reaction (PCR), separation of fragments by polyacrylamide gel electrophoresis (PAGE), scoring of genotypes and computational linkage analysis. DNA sequencing methodology for Parkin, SNCA and UCH-L1 has been included as appendix 2, as this was done by other laboratories.

## **2.2 DIAGNOSTIC CRITERIA FOR PD**

In order to carefully define PD consistently across the EU partnership, clear diagnostic criteria were established and agreed before the ascertainment and collection of families. This was to prevent any confusion from allied disorders such as MSA and PSP and to enable retrospective analysis of individuals or families, should interesting genetic associations or mutations be identified. These inclusion criteria are summarised in Table 2.1 below. They are similar to those previously proposed (Koller, 1992) but more rigorously exclude allied conditions.

**Table 2.1:** Clinical criteria used in this thesis and in the EU study to define PD and exclude differential diagnoses. Index patients and their relatives were included in the study if they had:

Pathologically proven PD (DNA obtained from frozen brain)	PM samples only
Bradykinesia, rigidity, rest tremor, asymmetry of symptoms/signs at onset	3 out of 4
Improvement at some stage with L-dopa therapy	over 50%
No supranuclear ophthalmoplegia	except 40% limitation of upgaze
No pyramidal or cerebellar signs or dyspraxia	
No severe, early (<1 yr from onset) loss of postural reflexes	
No prominent, early (<1 yr from onset) urinary symptoms)	(urgency, frequency, incontinence)
No significant postural hypotension	>30mm systolic BP
No mini-mental test score of less than 24/30 within 2 yrs of onset	
No neuroleptic drug ingestion in the 6 months prior to onset	
No encephalitis or possible toxic exposure in the 6 months prior to onset	

Exclusion criteria could be supplemented by investigative data, including cerebellar atrophy on imaging, denervation on external sphincter EMG. Wilson's disease or other metabolic causes of PD were screened if there was clinical suspicion (especially if PD began in the index case under the age of 40 years).

## **2.3 PATIENTS AND FAMILIES**

### **2.3.1 Ascertainment of affected sibling pairs with Parkinson's disease**

The methods of ascertainment varied between countries depending on the established links and communication networks. In the UK (and Ireland) all registered neurologists were circulated with a request to notify the study centre about families. The UK centre (based at the Institute of Neurology) used the Parkinson's Disease Society (a lay charitable association), the PD Research Group (an established network of clinicians interested in PD research) and the British Neurological Surveillance Unit which exists as a reporting resource for precisely this kind of project. In the UK, ascertainment of patients with familial PD had been in progress for several years and DNA from 24 sets of affected siblings was already available. The collaboration started with access to around 100 sibling pairs. Throughout the three years until August 1999, recruitment and collection of blood from identified subjects in Italy, Germany, Holland, France and the UK continued in parallel.

### **2.3.2 Assessment & collection of families**

#### *2.3.2.1 Ascertainment of index subjects*

In each centre, the index patients were examined using a standard protocol by a clinician with experience in the diagnosis of PD, either at home or in hospital. 30mls of blood was taken from each subject with informed consent. Each patient was videotaped using a standard format and inclusion was subject to the agreement of the examining clinician and two videotape reviewers experienced in the diagnosis of PD (only used in doubtful cases). This was done according to a standard protocol (see appendix 1). Index subjects were only included if they fulfilled rigorous diagnostic criteria for clinically definite PD (see below).

#### *2.3.2.2 Ascertainment of families*

Once an index subject was examined and found to fulfill the criteria for definite PD, their affected and unaffected siblings and parents (if available) were examined

according to the protocol described above. Parents and siblings were also allocated as clinically definite/probable/possible/non-PD or unknown 'PD'.

Samples were collected from subjects allocated as clinically probable and possible PD but were not included in the initial exclusion studies on known PD loci. Participating clinicians (including myself in the UK) also collected blood samples from parents and unaffected siblings if available (to aid in determining allele segregation in the families), the spouses of patients (to provide age, sex and ethnically matched control subjects for determining allele frequencies in the normal population), and larger PD families.

Information was also obtained on age, age of onset, sex and ethnic origin of patients and spouses. Patients without affected relatives (isolated cases) were sampled for future association studies.

### **2.3.3 Data management and analysis methods**

Three databases were designed for accurate data storage and transfer and for appropriate data analyses. All clinical information was stored in a standard database format. The central clinical and genotyping databases were stored in Paris (see appendix 1) to allow the study of phenotype/ genotype correlation. All core information on DNA samples and family trees was stored in the central databases in both London and Paris. These databases were used to perform linkage analyses and to control inter-lab consistency.

### **2.3.4 Geographical distribution and numbers collected**

Up to the end of 1999, 246 families with familial PD were recruited by all collaborators. Forty-three families had been excluded as they did not fulfill the rigorous inclusion criteria for the study (see Table 2.1) or they had one or more of the exclusion criteria. Many siblings had been seen at an early stage in their disease and therefore it was not possible to assess their response to L-dopa treatment. A total of 176 families were eligible for inclusion in a future genome screen after 27 families had been excluded because they had mutations in the parkin gene (Abbas *et al.*, 1999, Luecking, *et al.*, 2000). Summary table 2.2 shows the geographical origin and family statistics. Collection of these families allowed analysis of four distinct chromosomal regions.

A total of 85 sibling pairs were examined in the UK (by myself) and, of these, 49 fulfilled the rigorous inclusion criteria for the GSPD study. The pedigrees for all the UK kindreds are collated in appendix A3.5. There was a wide geographical dispersion of cases throughout the United Kingdom (see figure 2.1). An EU collaboration such as the one described has several strengths. Not only does it allow recruitment of large numbers of patients (necessary, as familial PD is rare with an incidence of 1:1000) but a systematic method of data collection was set up with standardised documentation and videotaping of individuals. The strict diagnostic criteria that were used and the international ongoing collaboration through which collection has taken place minimises the risk of mis-classification of PD in the absence of a biological marker (Hughes *et al.*, 1992). Ideally, perhaps, a more consistently accurate diagnosis would rely on neuropathological and clinical criteria. In this study, response to L-dopa was used as a major criterion: patients were excluded from the study if they did not demonstrate a sustained response of greater than 50% to L-dopa. Any doubtful cases of PD were reviewed by independent clinicians before a decision was made to include them in the study.

**Table 2.2:** Geographical origin of EU sib pairs used in the GSPD study. The “UK” group includes some Irish families, so is referred to here as “British Isles”.

Origin	No. of families with 2 affected sibs	No. of families with 3 affected sibs	Total no. of families	Total no. of affected sib pairs
Netherlands (NL)	26	2	28	32
Germany (DE)	21	1	22	24
France (FR)	59	2	61	65
Italy (IT)	29	0	29	29
British Isles (UK)	32	4	36	44
Total	167	9	176	194

**Fig. 2.1:** Map showing the distribution of sibling pairs involved in this thesis across the UK. Each sibling pair was visited, clinically examined and videotaped by myself, prior to the collection of blood samples for genomic DNA isolation.



## **2.4 DNA METHODS**

### **2.4.1 DNA extraction from blood**

Genomic DNA was extracted from venous blood samples using the Nucleon II kit (Scotlab) according to the manufacturers instructions. Essentially, 10mls of blood was added to 40mls of reagent A to lyse erythrocytes (see Buffers & Solutions below) and the mixture was shaken for four minutes before centrifugation (Beckman, model GS-6R centrifuge) at 2,600 rpm for five minutes to pellet the lymphocytes. The supernatant was discarded, 2 ml of reagent B added to lyse lymphocytes (see Buffers & Solutions below) and the pellet gently resuspended. 500 µl of 5 M sodium perchlorate was then added to remove proteins from the mixture and the mixture shaken for 10 minutes at room temperature and for 15 minutes in a 65<sup>0</sup>C water bath. Further proteins were removed by the addition of 2 ml of chloroform and the contents shaken for one minute before centrifugation for two minutes at 2,000 rpm (Beckman, model TJ-6 centrifuge). Nucleon suspension (300 µl) was added and the mixture centrifuged as above for five minutes. DNA was precipitated from the aqueous phase by the addition of two volumes of absolute ethanol and gentle inversion of the mixture. The DNA was then transferred into a tube containing 0.5 - 1 ml of sterile 1 x TE using a glass hook.

### **2.4.2 DNA extraction from human tissues**

Approximately 100mg of tissue was finely ground in liquid nitrogen in a pestle and mortar and lysed in 300µl of digestion buffer (68 mM NaCl /21 mM EDTA, pH 8; 0.5% SDS) plus 5µl of 10mg/ml proteinase K. Each lysis sample was incubated at 50°C for 2 hours followed by 37°C overnight. The following day, 300µl of 5M LiCl was added and samples inverted for 1 minute. Next, 600µl of chloroform was added to each sample and the tubes placed on a rotating wheel for 30 minutes. Samples were then centrifuged at 10,000 rpm for 15 minutes and the supernatants transferred to clean microcentrifuge tubes containing 2 volumes of absolute ethanol. These samples were gently inverted several times to precipitate genomic DNA and then spun at 10,000 rpm for 5 minutes. Ethanol was removed and DNA pellets briefly washed in 70% ethanol, followed by a further centrifugation at 10,000 rpm for 5 minutes. All ethanol was removed and pellets left to air dry for 10 minutes before being resuspended in 100-200µl of TE (10mM Tris-

HCl, 1mM EDTA, pH 7.5). A 1 $\mu$ l aliquot was run on a 1% agarose gel to check the DNA and the remainder evaluated by spectrophotometer.

### **2.4.3 Measurement of DNA concentration and dilution of DNA**

DNA concentration was estimated by measurement of optical density (OD) using a spectrophotometer (Cecil, model CE202) at a wavelength of 260 nm using quartz cuvettes calibrated against distilled water. Purity of the DNA was monitored by measuring the OD at 260/280 nm. DNA was considered to be of acceptable purity if the ratio was greater than 1.6. For linkage analysis using fluorescent tagged primers and microtitre plates, DNA was diluted to a concentration of 10 ng/ $\mu$ l in TE, and stored in covered deep-well microtitre plates (Beckman) at 4<sup>0</sup>C when in frequent use or at -20<sup>0</sup>C for longer term storage. Concentrations of all other DNA samples were adjusted to approximately 50 - 100ng/ $\mu$ l.

## **2.5 GENETIC LINKAGE ANALYSIS: METHODOLOGY**

### **2.5.1 Use of fluorescent-labeled primers for PCR of microsatellite markers**

PCR reactions using fluorescent-labeled primers were carried out in final reaction volumes of 20 $\mu$ l in 96-well microtitre plates (Micro Test III, Falcon) generally consisting of 0.2 mM each of dATP, dCTP, dGTP and dTTP (Promega); 2  $\mu$ l of GeneAmp 10 x magnesium-free PCR Buffer II (Perkin Elmer); 1.2  $\mu$ l of 25 mM MgCl<sub>2</sub> (1.5 mM MgCl<sub>2</sub>); 10 ng of each primer; autoclaved and filtered distilled water to make up reaction mixture volumes to 15 $\mu$ l; 0.5 units of DNA polymerase added last (AmpliTaq Gold<sup>TM</sup> 5units/ $\mu$ l, Perkin-Elmer). The reaction mixture was prepared at room temperature and aliquoted into microtitre plate wells using an eight-channel pipette (Scotlab). 50 ng (5  $\mu$ l) of template DNA was then added to each well and overlain with approximately 25 $\mu$ l of light paraffin oil to prevent evaporation. Microtitre plates were then centrifuged at 1000 rpm for 30 seconds (Beckman GS-6R centrifuge).

PCR reactions were performed using a Perkin Elmer 9600 thermal cycler. Reaction mixes were first heated to 95<sup>0</sup>C for 11 minutes to activate the AmpliTaq Gold™; subsequent cycling conditions were:

94<sup>0</sup>C (denaturation) 30 seconds; 45 - 57<sup>0</sup>C (annealing) 30 seconds;

72<sup>0</sup>C (extension) 30 seconds: (repeated for 32 - 40 cycles)

PCR conditions were optimised for each primer pair to determine the optimal annealing temperature and the number of cycles required to produce an approximately constant PCR yield. As PCR is a sensitive technique capable of amplifying very small quantities of template DNA, great care was taken to avoid contamination during set-up and all reagents and materials used were sterile. A negative control (omitting template DNA) was always included in experiments.

### **2.5.2 Oligonucleotide primers for microsatellite markers**

Microsatellite markers were used for exclusion mapping to investigate the main loci discovered to date in familial PD. A genomic screen was also started on the two UK autosomal dominant kindreds (see chapter 5), which utilised the Linkage mapping set described below. The ABI PRISM™ Linkage Mapping Set is composed of 400 markers that define a ~10cM resolution human index map. The loci have been selected from the Genethon linkage map, based on chromosomal locations and heterozygosity. The map of marker loci was generated from the same CEPH genotype data used for the 1996 Genethon map. The markers are organized into 28 panels containing between 10 & 20 fluorescent dye-labeled pairs that generate PCR products that can be pooled and detected in a single gel lane or capillary injection. Overlapping alleles are distinguished by labeling with 3 different fluorescent dyes, [FAM], [HEX], and [NED], which are displayed on the ABI PRISM 377 as blue, green and yellow respectively. The Linkage Mapping Set User's Manual (P/N 904999) gives comprehensive information on PCR amplification conditions, electrophoresis conditions, detection and data analysis (see Perkin Elmer website: [www.perkin-elmer.com/ab](http://www.perkin-elmer.com/ab)).

Additional microsatellite markers used for exclusion mapping of known familial PD loci (see chapter 1) were analysed using custom-made fluorescent-labeled primers, manufactured by Perkin-Elmer with a 5' 6-FAM, TET, NED or HEX dye on one of

each primer pair. Markers used were dinucleotide (CA)<sub>n</sub> repeats from the Généthon genetic map (Dib *et al.*, 1996), tetranucleotide repeats from the Utah marker development group (The Utah marker development group, 1995), and tri- and tetranucleotide repeats from the Cooperative Human Linkage Centre (CHLC) (Sheffield *et al.*, 1995). In all cases, markers with the highest possible heterozygosities were selected for use in order to maximise informativeness. Additional microsatellite markers were mostly from the Généthon map, details of which are available from the Genome Database (GDB) at <http://www.gdb.org>.

### **2.5.3 Agarose gel electrophoresis**

To check for the presence of a PCR product of the desired size and quantity, 5µl of reactions from four randomly selected wells plus the negative control were added to 2µl of agarose gel loading buffer and visualised by electrophoresis on 3.2% agarose mini-gels (Flowgen Instruments Ltd) stained with ethidium bromide (1mg/ml Sigma). Electrophoresis was performed at 50 V for 30 - 60 minutes with a 100 bp size standard (Gibco) (1µl) run alongside the PCR products to enable estimation of their size. Ethidium bromide staining of the agarose gel permitted direct visualisation of DNA products using transillumination with ultraviolet light.

### **2.5.4 Polyacrylamide gel electrophoresis of fluorescent-labeled PCR products**

Electrophoresis through a polyacrylamide gel is an effective means of separating small DNA fragments with high resolution, allowing fragments differing in size by as little as 1 bp to be separated. Denaturing polyacrylamide gels are polymerised in the presence of an agent such as urea which suppresses base pairing in nucleic acids. Denatured (single stranded) DNA migrates through these gels at a rate that is determined by fragment size and almost completely independent of base sequence and composition, permitting sizing of fragments according to distance traveled through the gel. Smaller fragments migrate further than larger ones because larger fragments are retarded more than smaller fragments by the pore size of the gel polymer.

#### *2.5.4.1 Polyacrylamide gel preparation*

36cm well-to-read glass plates were used with a Perkin Elmer 377 automated sequencer. Plates were cleaned with detergent and rinsed with distilled water. The dry plates were assembled in the 377 cassette prior to pouring the gel. The catalysts TEMED (Sigma) (35  $\mu$ l) and freshly prepared 10% ammonium persulphate solution (APS) (Sigma) (250  $\mu$ l) were added to 50 ml of 4% acrylamide gel mix to start polymerisation. The mix was then taken up into a 50 ml syringe and carefully introduced into the notch between the front and back plates, spreading evenly between the glass plates. A spacer was inserted into the upper notch between the plates and the gel left for two hours to polymerise. After polymerisation, the upper spacer was removed and a 48 or 64 well shark's tooth comb was carefully inserted in its place. The cassette and plates were then placed in the 377 sequencer and the plates checked for background fluorescence using Genescan (version 2.0.2) software (Applied Biosystems). Heating plate and buffer chambers were assembled and 1.3 L of 1 x TBE buffer added before pre-running the sequencer until the gel temperature reached 50°C. Samples were then loaded.

#### *2.5.4.2 Pooling of PCR products for loading*

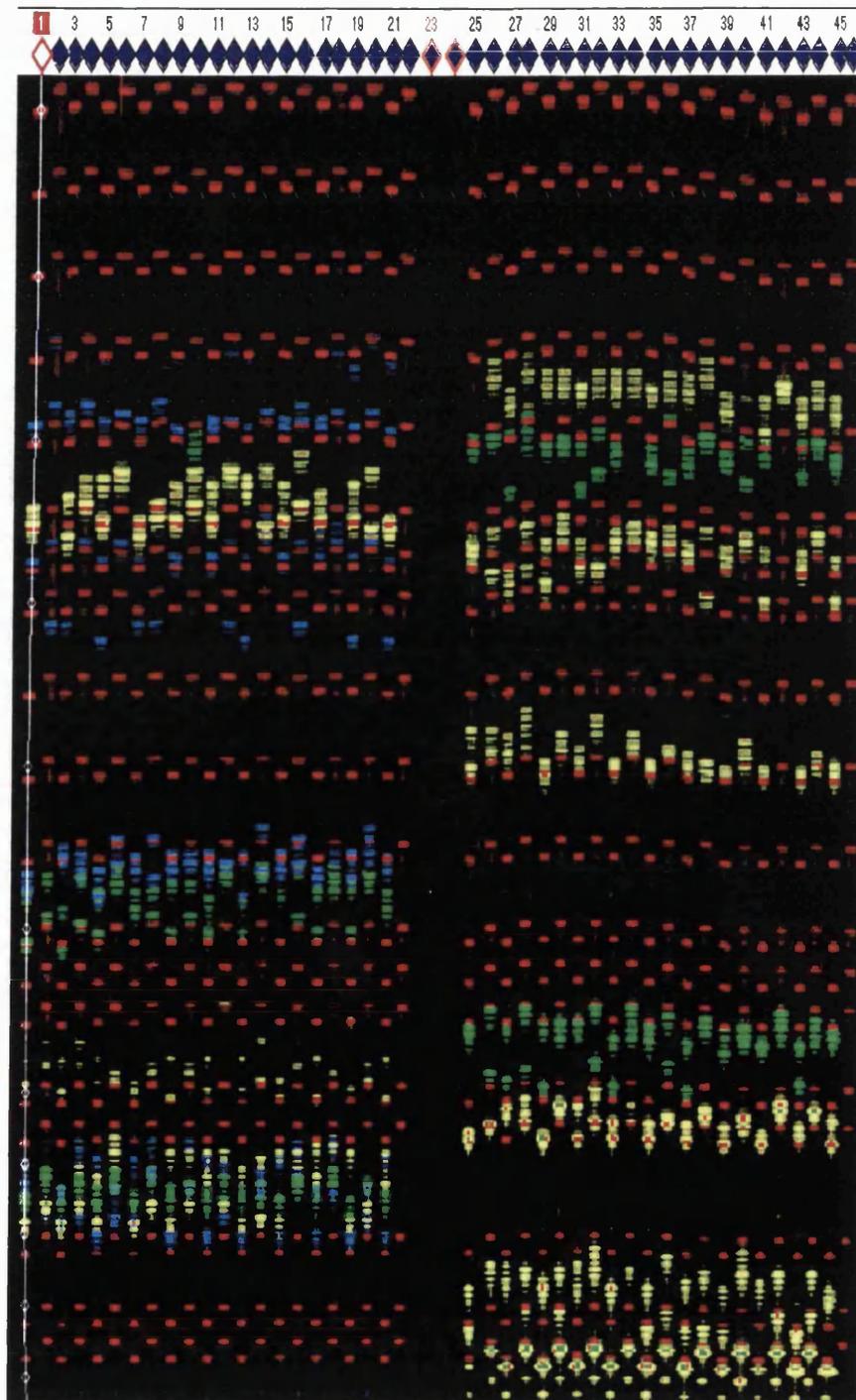
Up to 12 non-overlapping microsatellite markers, amplified from a single DNA sample, were run simultaneously in each lane (multipooled). PCR products from each DNA sample were first pooled according to the dye they contained as follows: 6-FAM - 4 $\mu$ l; TET - 4 $\mu$ l; NED - 4 $\mu$ l; HEX - 10 $\mu$ l. These volumes were adjusted according to the yield of the PCR reaction as determined from agarose gel electrophoresis. Pooling was performed in microtitre plates using an eight channel pipette. 2.5 $\mu$ l of pooled product from each well was then aliquoted into a fresh microtitre plate and an equal volume of loading mix added. The loading mix consisted of 100  $\mu$ l of deionised formamide, 20 $\mu$ l of loading buffer (blue dextran, 50mg/ml, EDTA 25mM, Perkin Elmer) and 24 $\mu$ l of Genescan 350-TAMRA/ 500-TAMRA 400 ROX (if NED-labeled primer used) size standard (depending on the anticipated size of the largest PCR fragment) (Applied Biosystems). The final mix of pooled product and loading mix was denatured at 95°C for 2 minutes in a Hybaid thermal cycler and then placed immediately onto ice before loading.

#### *2.5.4.3 Gel loading and electrophoresis conditions*

Wells were carefully flushed with 1 x TBE buffer immediately prior to loading. Alternate (odd-numbered) wells were loaded with 1.8µl of final mix using Sorenson MiniFlex 0.2mm flat tips (Anachem). Great care was taken to avoid spill-over into adjacent wells. Electrophoresis at 3,000 V for two minutes ensured that samples were run into the gel before even-numbered lanes were loaded. Loading of alternate lanes made it possible to distinguish adjacent lanes in the final gel image and improved the ability of the software to track lanes correctly. Total run time was two hours. A maximum of 36 samples could be run in adjacent lanes. Where more than 36 DNA samples from a single family were genotyped for a given marker (necessitating two electrophoresis runs), five samples from the first run were included in the second run as a control, to ensure gel-to-gel consistency of scoring.

PCR products produced using fluorescent-tagged primers were sized by electrophoresis through a denaturing 4 % polyacrylamide gel in an automated DNA sequencer (Applied Biosystems, model 377). During electrophoresis, a section of the gel furthest from the loading comb is scanned by a laser causing each dye moiety (attached to one oligonucleotide primer incorporated into PCR fragments) to emit light of a known wavelength as it migrates past the laser. A size standard consisting of DNA fragments of known size, labeled with the fluorescent dyes TAMRA or HEX, is run in each lane to allow accurate sizing of PCR fragments. This method of DNA sizing has the great advantage over radioactive methods in that markers of non-overlapping size and dye composition may be multiplexed in each lane, maximising efficiency and increasing sample throughput. As many as 24 microsatellites may be run in each lane although in practice a maximum of 20 markers were run simultaneously during this study.

**Figure 2.2:** An example of a fluorescent marker gel image analysed using GeneScan. PCR products amplified by fluorescent-labelled primers are detected by a laser detection device as they run through a polyacrylamide gel inserted in an ABI377 automated sequencer and are analysed by the associated computer software, GeneScan.



## **2.6 DATA ANALYSIS FOR FLUORESCENT-LABELED PCR PRODUCTS**

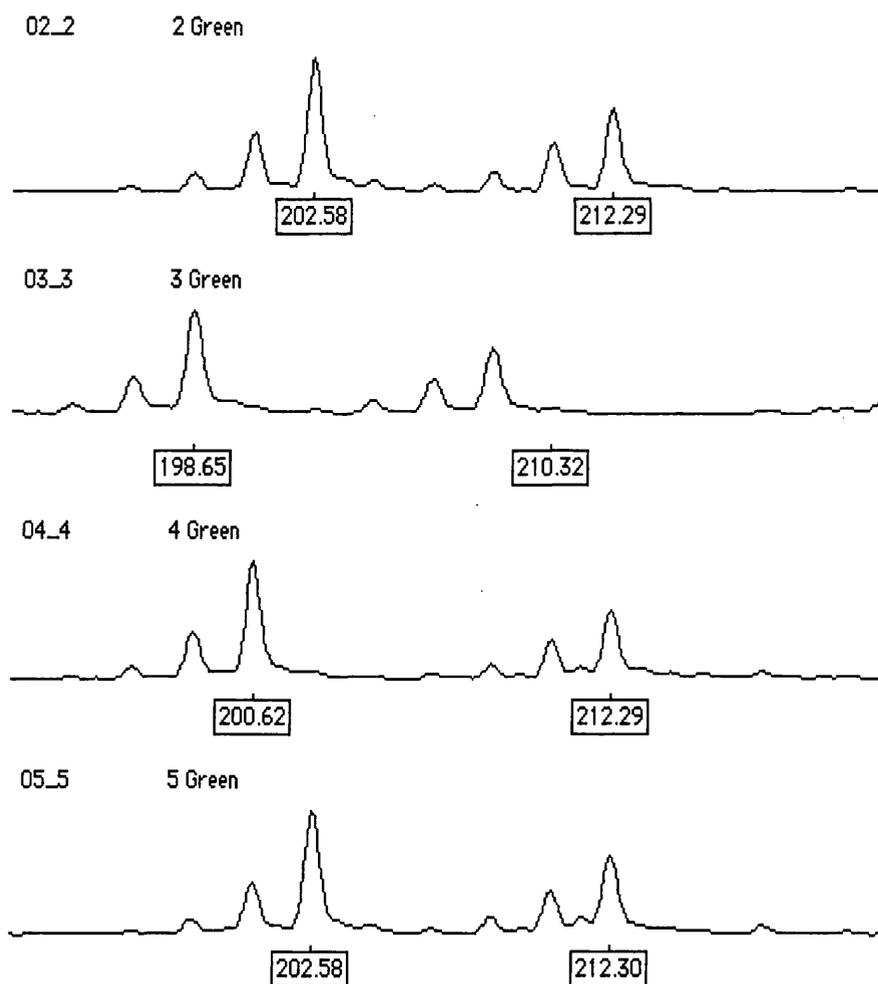
### **2.6.1 Initial data processing**

Data collected during the electrophoresis run were analysed automatically using the GeneScan program in order to size DNA fragments separated by electrophoresis. Automatic lane tracking was checked using the gel image, and adjusted lane by lane where necessary. To ensure accurate sizing, the automatic designation of peak sizes for internal lane size standards was manually checked in each lane. Markers were only sized if there were two size standard bands of greater size, and two of smaller size, present in the lane. An example of a typical GeneScan run is shown in Figure 2.2 below.

### **2.6.2 Genotyping using the Genotyper programme**

Fragment size data collected using the GeneScan program were then analysed using the Genotyper (version 2.0) program (Applied Biosystems) as described in the program manual. The Genotyper program labels fluorescent peaks with fragment size (to 0.01 of a base) and filters out background peaks. Manual adjustments are required by scrolling through all electrophoretograms to ensure that alleles are correctly labeled. Although time-consuming, this step is extremely important as labeling of incorrect (non-allele) peaks is a major source of genotyping error if not manually checked. Peaks in each marker range are grouped into discrete alleles and sequentially numbered from smallest to largest. Genotypes were scored blind without reference to the family pedigree to minimise bias. An example of allele sizing of a microsatellite marker using the Genotyper programme is illustrated in Figure 2.3 (below).

**Fig. 2.3:** Example of allele sizing of a microsatellite marker using the Genotyper program. As control size markers are run alongside all samples on the polyacrylamide gel, precise sizes can be assigned to PCR products as well as the signal intensity at each position. Genotyper interprets these data and generates graphs as shown here.



## **2.7 GENOMIC SCREENING METHODS**

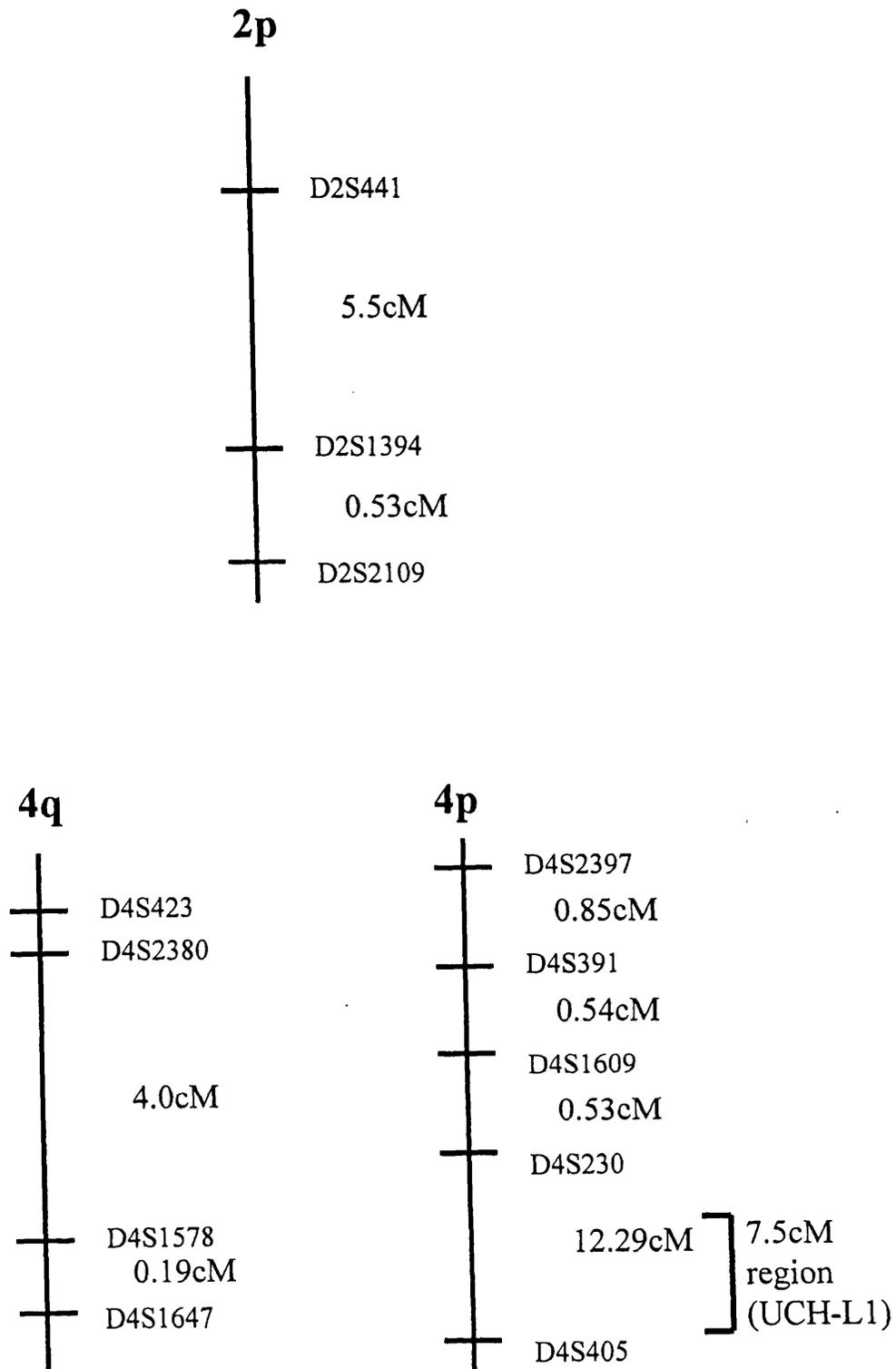
### **2.7.1 Genotyping**

Four partners from the European collaboration (Paris, Rotterdam, Munich and London) participated in laboratory work. DNA was extracted from venous blood at each centre (including the two Italian centres, Naples and Rome). Aliquots of DNA from all centres were sent to London. Each laboratory partner analysed three regions on chromosome 2p, 4p and 4q (see Figure 2.4 and Table 2.3 below). A total of 125 highly informative families with affected sib pairs (ASPs) were further analysed. My own role was to be responsible for experimental analysis (PCR, gene-scanning and genotyping) of all sibling pairs contributed from the UK and IT for the three dominant familial PD loci (PARK1: 4q21-q23; PARK3: 2p13; and PARK4: 4p15-p16.1).

**Table 2.3:** The markers from the Genethon map used to analyse each region. In all families, DNA of parents or unaffected siblings was available to determine allele segregation.

<b>CHROMOSOMAL LOCATION</b>	<b>MARKERS</b>	<b>HETEROZYGOSITY</b>	<b>ANNEALING TEMPERATURE</b>
4q21-23 (PARK 1)	D4S2380	0.77	45°C
4q21-23 (PARK 1)	D4S1647	0.65	47°C
4q21-23 (PARK 1)	D4S1578	0.77	52°C
2p13 (PARK 3)	D2S441	N/A	45°C
2p13 (PARK 3)	D2S2109	0.74	52°C
2p13 (PARK 3)	D2S1394	N/A	52°C
4p15 (PARK 4)	D4S2397	N/A	49°C
4p15 (PARK 4)	D4S391	0.86	52°C
4p15 (PARK 4)	D4S1609	0.67	52°C
4p15 (PARK 4)	D4S230	0.85	49°C
4p15 (UCH-L1)	D4S3350	N/A	55°C
4p15 (UCH-L1)	D4S405	0.87	55°C

**Figure 2.4.** Schematic diagram of chromosomes 2p (PARK3), 4p (PARK4) and 4q (PARK1) indicating the location of Genethon markers used for genotyping.



## 2.7.2 Analysis programs

Multipoint linkage analyses of the 3 UK kindreds (UK401, 402, 403) with familial PD were performed using the LINKMAP program of FASTLINK (Cottingham *et al.*, 1993; Lathrop *et al.*, 1984; Schaffer *et al.*, 1994). Power simulation studies were performed using SLINK (Ott, 1989). A conservative ‘affecteds-only’ methodology was used for the exclusion studies in the two kindreds described in chapter 5, in order to avoid bias resulting from inclusion of possibly affected individuals or incorrect estimation of penetrance or age of onset.

The FASTLINK 3.0 version of the MLINK linkage program was used to calculate paired scores for each marker, assuming an autosomal dominant mode of inheritance with a disease allele frequency set at 0.0001 (Cottingham *et al.*, 1993; Lathrop *et al.*, 1984; Schaffer *et al.*, 1994). The gene frequency of hereditary PD was estimated to be 0.0001 for the purposes of linkage analysis in this study. Genetic penetrance was set at 100% for these calculations to provide a more conservative means of assessing linkage with LOD scores for selected markers. Marker allele frequencies were assumed to be equal. All map distances are derived from the Marshfield Linkage Maps, URL address: ([http://www.research.marshfieldclinics/Map\\_Markers/data/Maps/](http://www.research.marshfieldclinics/Map_Markers/data/Maps/)).

## 2.8 MUTATION SCREENING

Mutation analysis of several genes identified to date in familial PD were undertaken. In chronological order as described in this thesis they are as follows: N-acetyl transferase 2 (NAT2),  $\alpha$ -synuclein (SNCA), ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) and Parkin. Mutation screening of the NAT2 gene and for the two known mutations in SNCA (G209A, exon 4 and G88C exon 3 (Polymeropoulos *et al.*, 1997, Kruger, 1998 #3815)) was undertaken solely by myself. Sequencing of SNCA and UCH-L1 was undertaken by Dr S J Lincoln and Dr M Farrer (Florida, USA). Mutation screening of UCH-L1 was undertaken by Dr S J Lincoln and Dr M Farrer. Screening for mutations in the Parkin gene was undertaken by Dr N Abbas and Dr C Luecking (Paris, France). Methodology for any work not solely performed by myself is attached as an appendix for information and referenced as such below.

All mutation screening involved initial amplification of exons by PCR following DNA extraction. A control containing uncut PCR product (1µl of water substituted for the restriction enzyme in each case) was always run with digested products and a digested sample of control (wild type) DNA was always included.

### 2.8.1 Analysis N-acetyl transferase 2 (NAT2)

All PCR reactions were started with an initial denaturing step of 94°C for 5 minutes. The restriction enzyme analysis was performed according to the protocol supplied by the manufacturer (Promega/New England Biolab). Following digestion [*KpnI*, *BamHI* at 37°C, *TaqI* at 65°C] fragments were separated on an agarose gel, stained with ethidium bromide and visualised using UV light. The NAT2 typing method used in this study is based on methods described elsewhere (Cascorbi *et al.*, 1995, Bell, 1993 #1473). Initially, all samples were amplified using the primers 5'GGA ACA AAT TGG ACT TGG and 5' TCT AGC ATG AAT CAC TCT GC. Each polymerase chain reaction (PCR) reaction contained 100ng of genomic DNA, 10pmol of each primer, 200nM dNTPs, 19mmol/L Tris-HCl (pH 8.3), 2mmol/L MgCl<sub>2</sub>, and 2.0U of Taq polymerase with a final volume of 50µl. The PCR conditions were: 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 90 s followed by a final extension time of 5 min.

The genotype of N-acetyltransferase was analysed for the wild-type allele and the three mutant alleles after restriction enzyme digest of all PCR products with the enzymes *KpnI*, *TaqI* and *BamHI* for the detection of the following polymorphisms: C481T, G590A and G857A (mutant alleles *M1*, *M2* and *M3* respectively). The distinct sequence variants encoding the different mutant alleles result in a loss of *KpnI*, restriction site for *M1*, a *TaqI* restriction site for *M2* and a *BamHI* restriction site for *M3*. The presence of any two mutant alleles defines the slow acetylator genotype, fast acetylators have one or two wild type alleles. The restriction enzyme digest resulted in a characteristic fragment pattern for each of the different polymorphisms (see Chapter 3, Fig. 3.4).

## 2.8.2 Analysis of Alpha-Synuclein

PCR was performed using 75ng of genomic DNA per reaction, 10pmol of each primer, 10 mM Tris-HCL (pH 8.3), 50mM KCL, 1.5 mM MgCl and 0.5 units of Amplitaq Gold DNA polymerase (Perkin Elmer) in a final volume of 20 µl. For mutation analysis genomic DNA was amplified using a Hybaid thermal cycler as follows:

95°C x 11 min, followed by 35 cycles of: 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 seconds. A single 7 minute extension period at 72°C was included at the end.

**Table 2.4:** The two known mutations in *SNCA* were suitable for analysis by restriction digestion of PCR products.

	Exon 3 (Kruger et al., 1998)	Exon 4 (Polymeropoulos et al., 1998)
Mutation	G88C Ala30Pro	G209A Ala53Thr
Restriction enzyme	<i>Mva I</i>	<i>Tsp45 I</i>
Enzyme recognition site	5' CC(A/T)GG 3'	5' GT(C/G)AC 3'
Buffer	NE Buffer	Buffer K
Incubation Temp	37°C	65°C

### 2.8.2.1 Exon 4 PCR for G209A mutation

This G to A transversion results in an alanine to threonine exchange at amino acid 53 (Ala53Thr) creating a novel *Tsp45 I* restriction site in exon 4 (Table 2.4). Restriction digestion of PCR products was performed with *Tsp45 I* at 65°C according to the manufacturer's protocol (New England Biolabs, Beverly, MA, USA) and the digested PCR products separated by electrophoresis on a 3.2 % agarose gel, stained with ethidium bromide and visualised using UV light. DNA from a member of the kindred described carrying the G209A mutation was used as the positive control.

To ensure the correct fragment was being amplified, PCR products from 3 index cases were directly sequenced with the Perkin-Elmer dye terminator cycle sequencing kit on an ABI 373 fluorescent sequencer. In addition, in order to further confirm that the amplicons were correct, 10 more PCR products were digested with three different enzymes, *Hinf I*, *Alu I* and *DdeI* at 37°C, and the restriction maps obtained were compared with the known sequence.

#### 2.8.2.2 Exon 3 G88C mutation of SNCA

This G to C transversion results in an Alanine to Proline change at amino acid 30 (Ala30Pro) creating a novel *MvaI* restriction site in exon 3 (Table 2.3). Restriction digestion of PCR products was performed with *MvaI* at 37°C according to the manufacturer's protocol (MBI) and the digested PCR products separated by electrophoresis on a 3.2 % agarose gel, stained with ethidium bromide and visualised using UV light. DNA from a member of the kindred described carrying the G88C mutation was used as the positive control.

### 2.8.3 Sequencing SNCA

#### 2.8.3.1 Sequencing of the $\alpha$ -synuclein gene in 30 autosomal dominant PD kindreds

This was done by Dr M Farrer of the Mayo Clinic, Jacksonville, Florida. Sequencing methodology is supplied as appendix 2.

### 2.8.4 Sequencing Parkin

#### 2.8.4.1 PCR amplification and sequence analysis

The sequencing and analysis of Parkin in both studies were all performed at INSERM U289 in Paris as part of the EU consortium and therefore the full methodology is provided in the appendices (appendix 2) and referenced (Abbas *et al.*, 1999, Luecking *et al.*, 2000).

## 2.8.5 Sequencing of UCH-L1

UCH-L1 exons were amplified from genomic DNA with primers designed to flank intronic sequence (appendix 2). This was done by Dr M Farrer of the Mayo Clinic, Jacksonville, Florida. Sequencing methodology is supplied as attached appendix 2.

## 2.9 BUFFERS AND SOLUTIONS

Unless stated otherwise, buffers and solutions were prepared using distilled water. All autoclaving was carried out at 121°C, 15 lbs/square inch for 30 minutes. All chemicals were of analytical grade and purchased from Merck (BDH) unless stated otherwise. They are organised according to procedures.

### 2.9.1 Genomic DNA extraction

The Nucleon DNA extraction kit (Scotlab) was used for preparing genomic DNA according to its instructions. Reagents involved:

**Phosphate buffered saline (PBS):** 130 mM NaCl, 2 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>,

1 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4

**Lysis buffer, Reagent A (blood leucocytes):** 10 mM Tris/HCl pH 7.5, 5 mM MgCl<sub>2</sub>,

0.32 M sucrose, 1% (v/v) Triton X-100

**Lysis buffer, Reagent B:** 10 ml 75 mM NaCl/24 mM EDTA, pH 8; 1 ml 0.5% SDS

**Nucleon resin** (composition unknown)

**10x TE**

**100mM Tris/10mM EDTA (pH 8.0)**

*For 1 litre*

12.11g Trizma Base

3.72g EDTA (Adjust pH to 8.0 with conc.HCl)

### **2.9.2 Polymerase Chain Reaction (PCR)**

*dNTP solution for PCR (10X):* 10 mM each dNTP (dATP, dCTP, dTTP, dGTP)

*PCR Buffer 10x:* 0.5 M KCl, 0.1 M Tris/HCl pH 8.4, 1-3mM MgCl<sub>2</sub>

### **2.9.3 Restriction Analysis**

All enzymes for molecular biology were supplied by Promega except for *Tsp45I* and *MvaI*, supplied by New England Biolabs. Each enzyme was used with the buffers supplied by the manufacturer.

### **2.9.4 Agarose gels**

*6x Agarose gel loading buffer*

40% (w/v) sucrose in water

0.25% (w/v) bromophenol blue

*10 x TBE (1 L)*

(0.89 M Tris-HCl, pH 8; 0.89 M Boric acid; 20 mM EDTA)

Tris base (Trizma, Sigma) 121.1 g

Boric acid (anhydrous) 61.8 g

EDTA (Sigma) 7.4 g

## 2.9.5 Acrylamide gels (for sequencing/Genescan)

### *4% acrylamide gel mix for 377 sequencer (50 ml)*

Urea (Fison's) 18 g

Nanopure water 27.5 ml

40% acrylamide solution (Biorad, 19:1 acrylamide:bisacrylamide) 5 ml

Amberlite resin (Sigma, deionising) 0.5 g

10 x TBE 5 ml

The TBE was filtered through Whatman filter system (0.45 µm filter). The remaining constituents were mixed on a magnetic stirrer until dissolved. They were then filtered onto TBE.

**2x Formamide loading buffer:** 95% (v/v) deionised formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol.

**CHAPTER 3: GENETIC LINKAGE AND ASSOCIATION STUDIES IN PD ON  
A LARGE EUROPEAN POPULATION OF AFFECTED SIBLING PAIRS**

### **3.1 OUTLINE OF CHAPTER**

During the course of experimental work for this thesis, four loci (Farrer *et al.*, 1999b; Gasser *et al.*, 1998; Matsumine *et al.*, 1997; Polymeropoulos *et al.*, 1996), and three genes (Kitada *et al.*, 1998a; Leroy *et al.*, 1998; Polymeropoulos *et al.*, 1997) have been implicated in familial PD, as described in Chapter 1. PARK 6 (1p35-p36, no gene yet identified) (Valente *et al.*, 2001) was described only very recently, so no experimental work was performed on this locus. The linkage studies described in this chapter examined a large European population of affected sib pairs for each of the autosomal dominant PD-associated loci reported to date in order to assess their contribution and significance to the aetiology of PD. A detailed study of linkage analysis of the PARK 4 locus in a two generational Italian kindred is presented as part of this work. Studies on PARK 2 and Parkin are described in chapter 4. The EU sibling pair series was used to perform an intra-familial association study of N-acetyltransferase2 (NAT2). The significance of the results presented in this chapter are discussed and future directions of study suggested.

### **3.2 RESULTS OF EU AFFECTED SIBLING PAIR STUDY OF THREE DOMINANT LOCI DESCRIBED IN FAMILIAL PD**

#### **3.2.1 Clinical analysis of EU sibling pairs**

These are summarised in the following Tables 3.1, 3.2 and as Figure 3.1 over the following pages. The motor scale of the UPDRS (Unified Parkinson's Disease Rating Scale) assesses 27 motor functions of patients with Parkinson's disease. Each function is scored from 0 (no impairment) to 4 (severe impairment), resulting in a total score ranging from 0 to 108 (Fahn and Elton, 1987). B) Hoehn and Yahr stages describe functional disability of patients with Parkinson's disease. The scale ranges from mild unilateral symptoms (stage 1) to severely disabling disease: patient still able to stand and walk unassisted but markedly incapacitated (stage 4) or patients confined to wheelchair or bed unless aided (stage 5) (Hoehn and Yahr, 1967). C) The Mini-Mental State examination tests orientation, naming, copying, short term memory, attention, reading, writing, spatial and constructive capacities in 30 tasks, all scored with 1 (succeeded) or 0 (failed). The maximum score is 30, dementia was considered for scores below 24.

**Table 3.1:** Summary of clinical characteristics of 361 patients with definite Parkinson’s disease gathered as part of the EU collaboration, GSPD, including 194 sib pairs.

Abbreviations: UPDRS, Unified Parkinson’s Disease Rating Scale; on, under treatment; off, without treatment; MMSE, Mini Mental State Examination.

<b>Number of Affected sibpairs</b>		<b>194</b>
<b>Number of patients</b>		<b>361</b>
Women : Men		176 : 185
Mean age at onset in years (range)		57.9 ± 11.2 (24 – 84)
Mean disease duration in years (range)		9.8 ± 6.7 (0 – 34)
<b>Clinical signs</b>		
at onset	Micrography (%)	48
	Bradykinesia (%)	73
	Tremor (%)	74
	Asymmetric signs (%)	76
at examination	Bradykinesia (%)	98
	Rigidity (%)	95
	Rest tremor (%)	84
	Urinary urgency (%)	30
	Urinary incontinence (%)	19
	UPDRS off	48 ± 25 (n = 73)
	UPDRS on	29 ± 17 (n = 282)
	Hoehn and Yahr	2.8 ± 1.4
	MMSE	25 ± 9
on treatment	Improvement with levodopa (%)	59 ± 19 (30 - 100)
	Mean daily dose of levodopa	560 ± 350
	Duration of levodopa treatment in months	85 (7) ± 86 (7)
	Dyskinesias (%)	46

**Table 3.2:** Comparison of clinical characteristics of the PD sib-pair families in the EU study expressed as mean ( $\pm$ SD), range, and median values of all measurables.

Parameter	NL	DE	FR	IT	UK	TOTAL
<b>Mean age at study <math>\pm</math>SD</b>	67.4 $\pm$ 10.2	63.9 $\pm$ 10.8	63.5 $\pm$ 12.7	66.1 $\pm$ 10.6	68.5 $\pm$ 8.4	66.0 $\pm$ 10.8
<b>Range</b>	44-89	41-79	32-86	37-86	48-86	32-89
<b>Median</b>	68.5	66.5	66	68	69.5	67
<b>Mean age at onset <math>\pm</math>SD</b>	59.6 $\pm$ 10.9	54.3 $\pm$ 12.0	52.9 $\pm$ 13.9	54.0 $\pm$ 12.9	56.9 $\pm$ 10.5	55.4 $\pm$ 12.3
<b>Range</b>	39-84	34-76	24-80	21-84	31-80	21-84
<b>Median</b>	60	55.5	52	57	58	57
<b>Mean MMSE <math>\pm</math>SD</b>	25.9 $\pm$ 3.8	-	28.4 $\pm$ 2.5	26.4 $\pm$ 3.0	29.5 $\pm$ 2.1	27.5 $\pm$ 3.3
<b>Mean UPDRS off <math>\pm</math>SD</b>	0	45.9 $\pm$ 17.2	32.8 $\pm$ 31.0	54.3 $\pm$ 21.9	60.4 $\pm$ 23.6	46.8 $\pm$ 26.7
<b>Mean UPDRS on <math>\pm</math>SD</b>	25.3 $\pm$ 7.8	25.9 $\pm$ 12.4	24.2 $\pm$ 16.5	31.5 $\pm$ 15.8	44.4 $\pm$ 21.7	30.0 $\pm$ 16.8
<b>Mean Hoehn-Yahr <math>\pm</math>SD</b>	2.67 $\pm$ 0.88	2.7 $\pm$ 0.76	2.54 $\pm$ 1.16	2.99 $\pm$ 0.96	3.09 $\pm$ 1.08	2.82 $\pm$ 1.01
<b>Mean daily dose L-dopa <math>\pm</math>SD</b>	392 $\pm$ 190.2	515.2 $\pm$ 264	506 $\pm$ 276	572.2 $\pm$ 347.7	759.4 $\pm$ 314.5	562.7 $\pm$ 370
<b>Range</b>	150-900	100-1400	100-1500	62-1850	62.5-3250	62-3250
<b>Median</b>	375	500	450	500	673.75	500
<b>Mean duration on L-dopa <math>\pm</math>SD</b>	66.1 $\pm$ 42.6	73.6 $\pm$ 47.3	89.9 $\pm$ 90	104.3 $\pm$ 71.3	113.6 $\pm$ 105.2	92.3 $\pm$ 79.7
<b>Range</b>	2-158	1-168	4-324	3-336	6-660	1-660
<b>Median</b>	60	60	48	90	96	72
<b>Initial mean per cent improvement on L-dopa <math>\pm</math>SD</b>	50 $\pm$	50 $\pm$	61.7 $\pm$ 18.4	47.9 $\pm$ 7.9	58.5 $\pm$ 16.6	53.8 $\pm$ 13.3
<b>Range</b>	50-50	50-50	30-100	30-70	30-100	30-100
<b>Median</b>	50	50	50	50	60	50

**Fig. 3.1, A-F** Comparisons of PD patients between EU countries in the GSPD consortium and against the full database (Total). Mean values were used to generate each column and error bars correspond to standard deviations from the mean. Abbreviations: NL, Netherlands; DE, Germany; FR, France; IT, Italy; UK, United Kingdom; TOT, total.

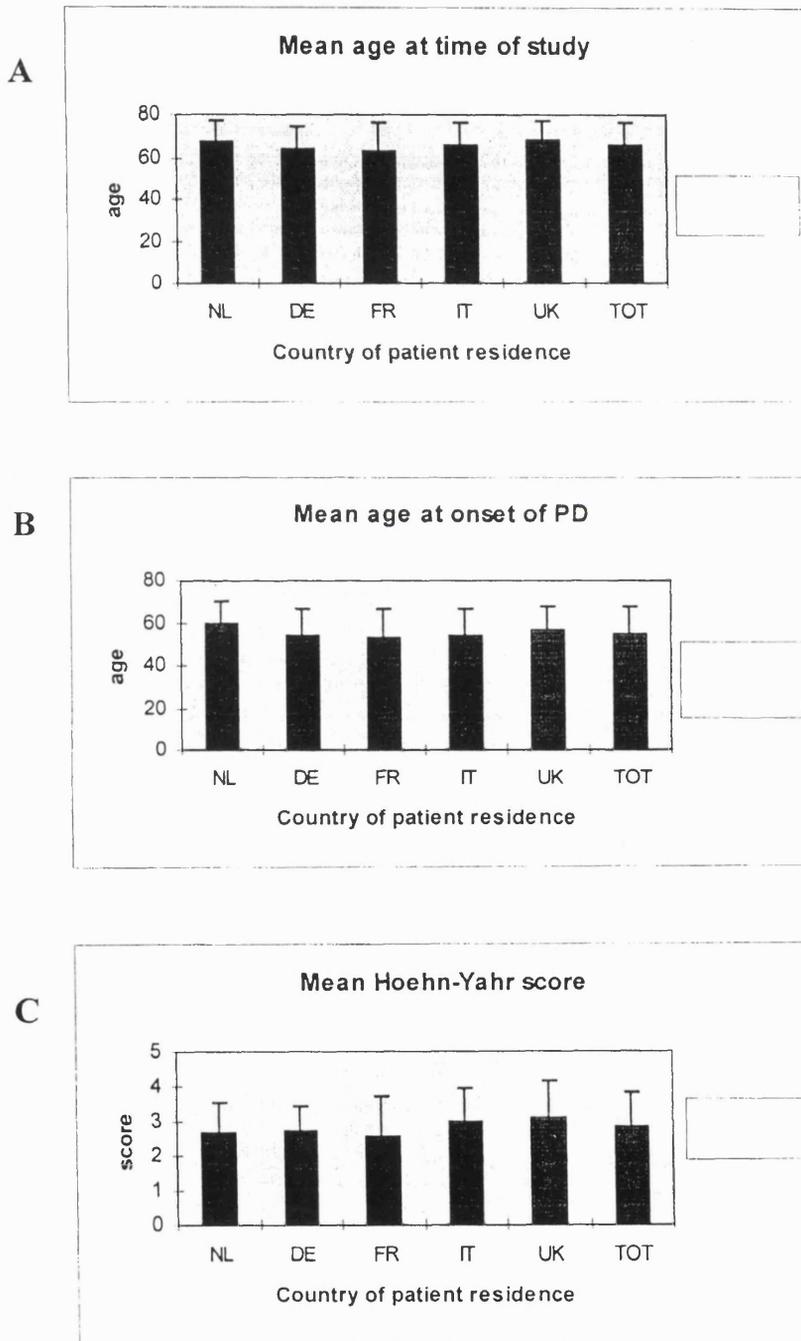
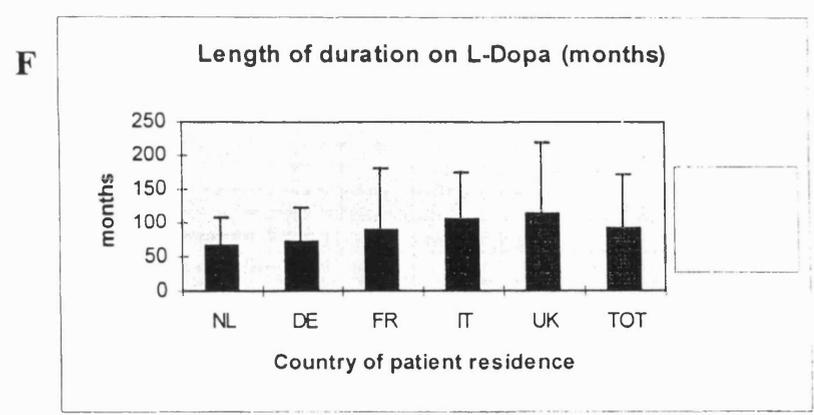
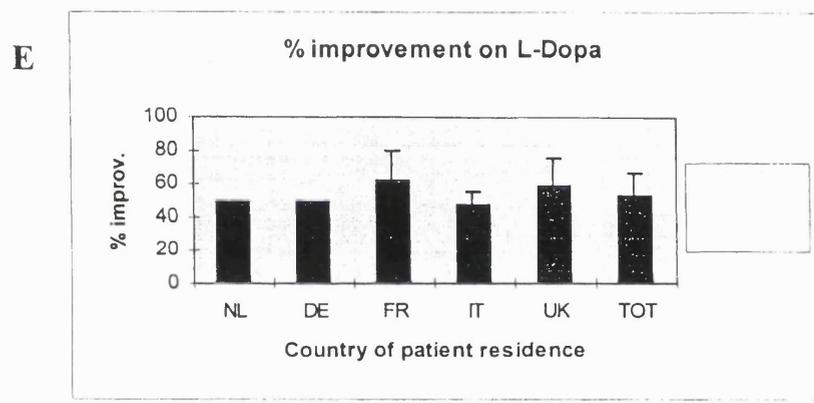
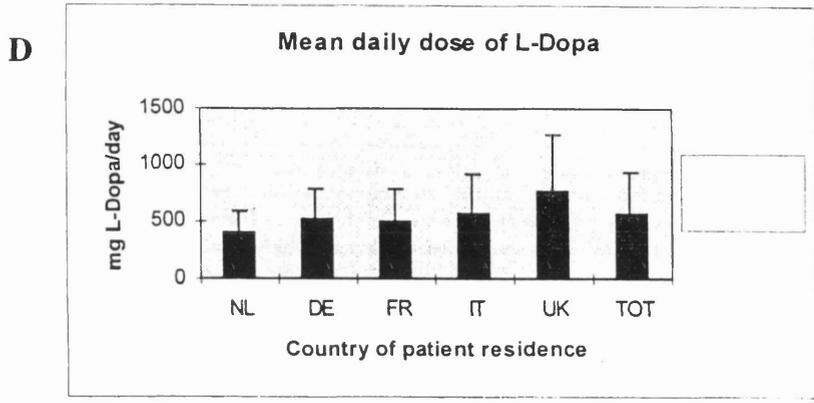


Fig. 3.1, D-F: (continued)



### **3.3 LINKAGE STUDIES IN EU AFFECTED SIBLING PAIRS OF THREE DOMINANT LOCI (PARK 1, 3 AND 4) IN FAMILIAL PD**

#### **3.3.1 Linkage analysis of the major loci in PD in a large population of affected sibling pairs**

Linkage analysis of the three major loci described to date (PARK1 (Polymeropoulos *et al.*, 1996), PARK 3 (Gasser *et al.*, 1998) and PARK 4 (Farrer *et al.*, 1999b)) in autosomal dominant PD (ADPD) was undertaken in one hundred and ninety-four strictly diagnosed sibling pairs (see Section 2.3.2). This was done as part of the European-based consortium (GSPD). PCR and genotyping of all sibling pairs of UK and Italian origin was performed primarily by myself at the Institute of Neurology, Queen Square, London. Fluorescent polymorphic markers spanning each described locus were run in this cohort (see Section 2.5.1) and the genotypes analysed in Paris using a non-parametric linkage method, assuming the level of significance to exclude linkage to be set at  $p < 0.05$ .

#### **3.3.2 EU sib pair statistical data analysis at dominant PD loci (PARK1, 3 AND 4)**

All of the statistical analysis was performed by Dr Maria Martinez, INSERM, Paris. Allele frequencies for markers tested at each of the three loci (see Table 2.3) were set to their maximum likelihood values estimated in the data with the computer program VITESSE (O'Connell and Weeks, 1997). Pairwise and multipoint model free linkage analyses were conducted with the SIBPAIR and MLBGH programs, respectively. To test for linkage, the likelihood of association over all families were maximised as a function of the rate of marker alleles identical by descent (IBD- $y$ ) among affected siblings, and the likelihood ratio test statistic was calculated against the null hypothesis of no linkage ( $y=0.5$ ). The statistic follows a chi-square distribution with one degree of freedom (df) and can thus be expressed as a lod score (see Table 3.3 below).

The above programs provide a model-free likelihood-based test statistic for linkage, which applies to the whole sibship of affected siblings. A model-free linkage method was used which was based on a likelihood ratio as in the MLS (maximum lod score) method (Risch, 1990b). Both tests can be expressed as a LOD score ( $\log_{10}$  of the ratio). The MLS is maximised as a function of 2 parameters and it applies to sibling pairs only.

The LOD is maximized as a function of 1 parameter (thus, for a given significance level it requires a lower criterion than that of MLS). The LOD method used here can analyze each sibship as a whole and not as just sibling pairs (its statistical distribution is thus not affected by non-independence).

**Table 3.3:** Overall pairwise 2 point LOD scores for each marker on chromosomes 2p13 (PARK3), 4p14-15 (PARK4 & UCH-L1) and 4q21-23 (PARK1).

2p13		4p15		4q21-23	
marker	LOD score	marker	LOD score	marker	LOD score
D2S441	0.09	D4S2397	0.06	D4S1647	0
D2S2109	0.13	D4S391	0.05	D4S1578	0.006
D2S1394	1.61	D4S1609	0.14	D4S2380	0
		D4S230	0.02		
		D4S405	0.04		
		D4S3350	0.012		

### 3.4 RESULTS OF LINKAGE ANALYSIS AT PARK 1, 3 AND 4

#### 3.4.1 Pairwise Linkage Analysis

The genotyping of the regions 2p13 (PARK3) , 4p15 (PARK4) and 4q21-23 (PARK1) in 125 families (see table 3.3) revealed one marker on chromosome 2p, D2S1394, with an overall pairwise LOD=1.61 (nominal p-value=0.003). For the other markers overall LODs were <0.14 (Table 3.3). When the data were analysed in subsets of specific countries, the pairwise linkage analysis showed p-values smaller than 5% for marker D4S405 (UCH-L1) in German families (LOD=1.22; p=0.01), marker D2S1394 in

German families (LOD=0.71; p=0.04) and British families (LOD=0.91; p=0.02) and marker D4S1647 in Italian families (LOD=0.67; p=0.04). However, when these country specific results were adjusted for multiple comparisons (by multiplying the p-values with the number of comparisons: n=6; one overall and five country specific comparisons), no marker retained p-values smaller than 5%, therefore providing no evidence of positive linkage.

### **3.4.2 Multipoint LOD score results**

Multipoint LOD scores were all zero except at the PARK 3 locus (2p13). At PARK 3, max LOD = 0.79 at D2S1394, thus the evidence for linkage with this marker is lower than with the overall pairwise LOD score (1.61)-see table 3.3. Multipoint analyses were run using inter-marker genetic distances estimated in the data as this is not always the same as true genetic order. Use of data-estimated inter-marker genetic distances is the more conservative approach.

## **3.5 EVIDENCE FOR A CHROMOSOME 4p14-15 (PARK4) HAPLOTYPE SEGREGATING WITH PARKINSON'S DISEASE IN A KINDRED OF ITALIAN DESCENT (ITNA035)**

### **3.5.1 Aims and design of the study**

A chromosome 4p14-15 haplotype segregating with Parkinson's disease and postural tremor has previously been identified as the PARK4 locus for Lewy body parkinsonism in an Iowan kindred (Farrer *et al.*, 1999b, Spellman, 1962 #1136, Waters, 1994 #1076, Muenter *et al.*, 1998). As part of the linkage studies described in Section 3.3 markers linked to the PARK4 locus were analysed. Markers linked to the PARK4 locus (as shown in table 3.3) were initially analysed by myself in London on all families. Fine typing of the region in the single multiplex Italian family identified (ITNA 035) as a result of this large study was performed by Dr Matt Farrer (Mayo Clinic, Jacksonville, USA). Results from this combined study are as shown in Figure 3.2. All individual family pedigrees were examined in the EU sibling pair series to determine if any shared a similar segregating disease PARK4 haplotype to that originally described (Farrer *et*

*al.*, 1999b). One multi-generational Italian family (ITNA 035) was identified with 4 affected members sharing a linked haplotype at PARK4. A clinico-genetic analysis of the kindred is described in the following sections.

### **3.5.2 Clinical description of the kindred**

This Italian family (ITNA 035= EU central database code for the kindred) was ascertained at the Movement Disorders Clinic of the Federico II University of Naples. The pedigree of this family is shown in Figure 3.2.

#### *3.5.2.1 Individual 033*

The proband (033) is a 54 year old woman who presented with right arm motor difficulties and resting tremor at age 49. Tests for secondary causes of parkinsonism were negative (including a CT head which was normal). She was diagnosed with PD and treated with carbidopa/L-dopa (L-dopa) with a good response. The dosage was titrated up to 700 mg of L-dopa per day (in divided doses). At the age of 51 years she developed depression which was effectively treated with fluoxetine. Concurrently she gave a history of early morning akinesia, wearing off and peak-dose dyskinesias. Pergolide, 3 mg daily, was added. Neurological examination at age 52, during the “on” state, revealed a Mini-Mental Status Examination score of 27/30 (Folstein *et al.*, 1983). The patient had normal speech, slight hypomimia, minimally slowed gait, infrequent resting tremor of the right hand, slight rigidity of the right upper extremity, moderate slowing and breakdown of movement with rapid alternating movements, moderate diffuse dyskinesias, and right arm dystonia. Pertinent negative findings included normal eye movements and no dysautonomia. Her UPDRS score was 25.

#### *3.5.2.2 Individual 037*

This patient developed activation tremor in all limbs and gait impairment at the age of 36 years. Initially symptoms were thought to be psychogenic and she was treated with levosulpiride, which caused marked worsening of her parkinsonism. A neurological examination at age 38 revealed hypomimia, normal eye movements and tremulous speech. She had activation tremor in all limbs which was high frequency and somewhat

irregular, and there was marked rigidity at the neck and the limbs, with brisk tendon reflexes. The subject required assistance with ambulation. Raynaud syndrome and tachyarrhythmia (105-125 bpm) were also noted. Marked weight loss had occurred (10 kg in about 1 year). Past medical history was significant for febrile seizures from 6 months until to 6 years of age. As an infant, mental development was reportedly slow but she was able to become fully independent and lead a normal adult life. A CT scan showed mild cortical atrophy. She was treated with benserazide/L-dopa (600 mg per day in divided doses) with improvement. For example, a single dose of L-dopa allowed independent walking for about 2 hours. However, peak dose dyskinesias, mainly of the trunk and the lower limbs, and motor fluctuations appeared within the first months of treatment. During “off” periods she complained of painful neck and foot dystonias. The disease course was rapidly progressive with increasing mental and motor deterioration. She was wheelchair bound at age 41. The treatment with L-dopa was discontinued at age 45 due to intractable dyskinesias. Neurological examination at age 50 showed that the subject was unable to ambulate, and was anarthric. She suffered from prominent drooling, marked hypomimia, seborrhea, marked axial rigidity, generalized limb flexion with ankylosis, weak tendon reflexes, perioral tremor, She had a positive snout reflex and glabellar tap. She died at the age of 50.

### *3.5.2.3 Individual 035*

This 81 year old woman noted the insidious onset of bilateral upper extremity resting tremor at 68 years of age. Carbidopa/L-dopa was administered but treatment was withdrawn after a few days because of gastrointestinal side effects. She was subsequently treated with anticholinergic drugs (bornaprine 4mg three times per day) with slight benefit. Neurological examination at age 78 revealed a Folstein MMSE score of 28/30. She had normal speech, slight hypomimia, mild bilateral upper extremity resting tremor, and rigidity which was mild in the neck and upper extremities and moderate in both lower extremities. Posture and gait were abnormal but she could walk without assistance. Rapid alternating movements were moderately impaired, and somewhat worse on the right than on the left (H&Y stage 3). She died at 82 years.

#### 3.5.2.4 Individual 034

At age 54 this patient noted stiffness and reduced dexterity of the right hand. Treatment with L-dopa/carbidopa was started and gave improvement. L-dopa dosage was progressively increased by the patient because of motor fluctuations and when she was seen, at age 74, she was taking Sinemet 25/250, one tablet eight times daily. On examination she had unintelligible speech, moderate hypomimia, no tremor, marked neck rigidity, moderate to marked limb rigidity, severely stooped posture. She was very unstable and gait was impossible. Hand and leg movements were severely impaired. Choreic diffuse dyskinesias were present. UPDRS motor score was 53. Sphincter function was normal, tendon reflexes brisk, plantar responses flexor. Mild cognitive impairment was found (MMSE score 23). She died two years after examination at the age of 76 years.

#### 3.5.3 Power Analysis of ITNA 035

This pedigree was evaluated for its power to detect linkage using the SIMLINK program version 4.12 (Ploughman and Boehnke, 1989). Marker frequencies were set at 0.40, 0.30, 0.20 and 0.10 and 500 replicates were performed. Genotype data was managed and re-coded for linkage and haplotype analysis using Cyrillic 2.1.3 (Cherwell Scientific, Inc) and MEGA2 (<http://watson.hgen.pitt.edu/docs/mega.html>). SIMLINK and MEGA 2 analysis was performed by Dr Matt Farrer, Mayo Clinic, Jacksonville, Florida. Two-point linkage analysis for chromosome 4p14-p15.3 markers was performed by myself using MLINK (Terwilliger and Ott, 1994).

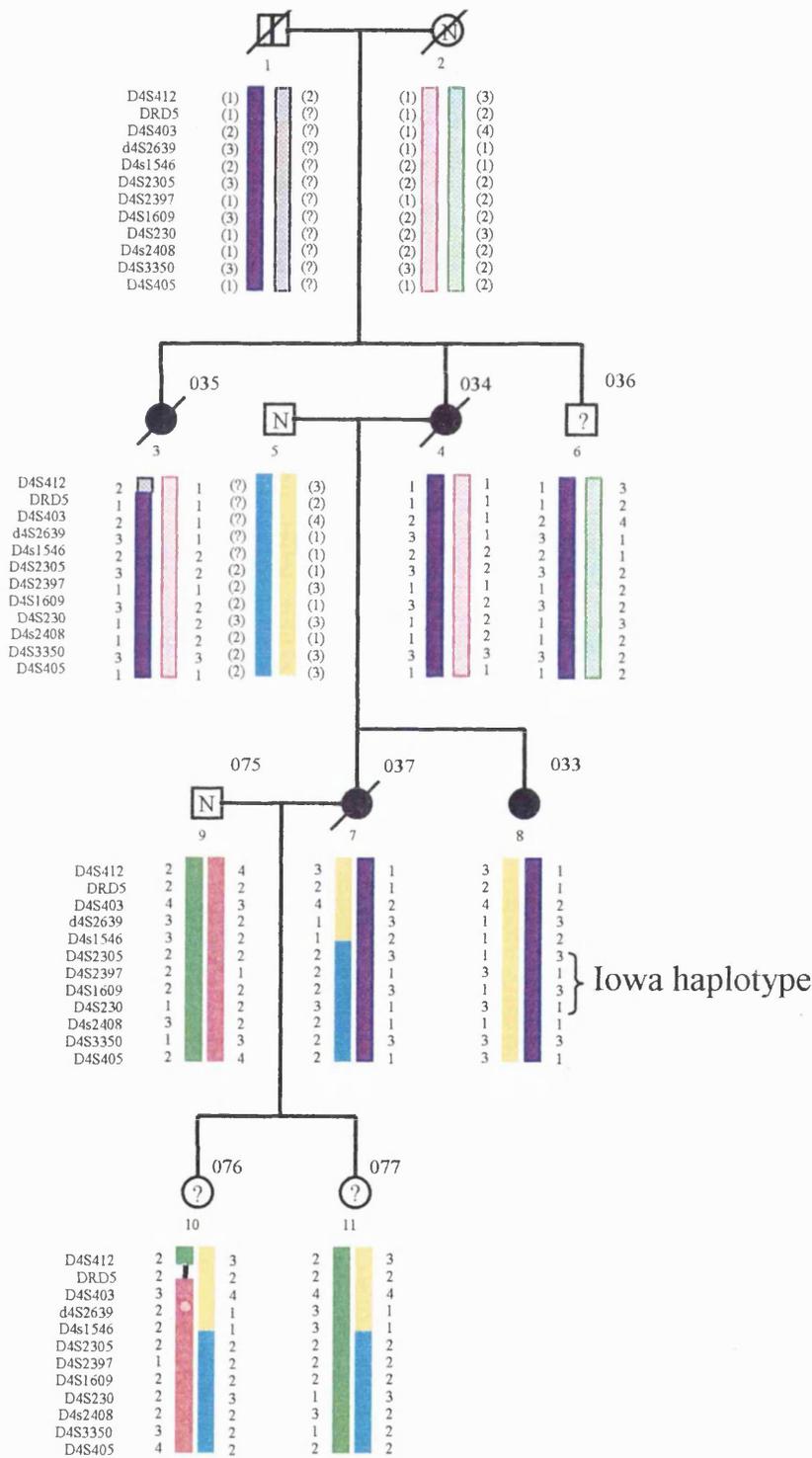
As the disease appeared to segregate in an autosomal dominant fashion penetrance for homo- and heterozygotes was set equal. Affecteds-only analysis was applied given the wide range of onset age and as disease penetrance may be uncertain. Married individuals were considered unaffected. The disease allele frequency was set at 0.0001, given the population prevalence of familial Parkinsonism (Morgante *et al.*, 1992). Intermarker distances and frequencies were taken from the most recent version of the Marshfield map (<http://www.marshmed.org/genetics>). SIMLINK analysis involved simulating a marker segregating within the pedigree structure with a frequency of 0.4, 0.3, 0.2 and 0.1 (with an average assumed heterozygosity of ~75%). Linkage analysis is performed on all the simulations obtained (500 in this case, using a disease allele

frequency of 0.0003), and an average of the lod score obtained (Elod). Power analysis suggested that lod scores generated on the kindred would not be conclusive: observed z values ( $\theta=0$ ) from two-point MLINK analysis were positive but equivocal, consistent with *a priori* power analysis (Table 3.4).

**Table 3.4:** Two-point linkage analysis and p values for sharing of chromosome 4p markers. Chromosome 4p loci are shown in order from the telomere to centromere. Only two-point lod scores at  $\theta=0$  are shown, p values derived from cluster analysis are those most appropriate for an autosomal dominant trait. P value was generated by permutation. Z score is the LOD, p is p-value generated by permutation.

PARK4 locus	cM	Lod Z ( $\theta=0$ )	P
4p14-p15.1			
DRD5	31.05	0.27	0.27
D4S403	N/A	0.57	0.28
D4S2639	N/A	0.40	0.28
D4S1546	N/A	0.23	0.26
D4S2305	39.3	0.27	0.27
D4S2397	45.20	0.23	0.27
D4S1609	46.60	0.26	0.27
D4S230	47.20	0.48	0.27
D4S2408	48.40	0.27	0.27
D4S3350	53.70	0.22	0.27
D4S405	N/A	0.25	0.25

**Fig. 3.2:** Pedigree of an Italian family with familial PD with haplotypes of marker loci spanning the linked region (PARK4) on chromosome 4p14-p15.3 (indicated as a dark column and the marker order is shown alongside). The haplotype shared with the Iowa kindred linked to PARK4 (Farrer *et al.*, 1999b) is also indicated. Inferred haplotypes (DNA not available) are shown in brackets.



### **3.5.4 Haplotype and linkage analysis**

Three recombinant haplotypes were observed in persons 035, 037 and 076 (see Figure 3.2), in good agreement with expectation for the size of the interval and number of meioses examined. The haplotypes were generated by Monte Carlo simulations (Dr M Farrer) which take into account both the genetic distances between markers and the number of meioses. Expected numbers of meioses are calculated and compared with the observed number in a repetitive/reiterative way (maybe 10,000 times) to generate both the most parsimonious solution and an empirical distribution for the p-value.

Individuals with Parkinson's disease within ITNA 035, all share a chromosome 4p haplotype from DRD5 to D4S405, spanning a genetic distance of 31.05cM. Person 036 also shares the haplotype yet is not affected by disease (Figure 3.2). Notably, allele sizes for four adjacent markers are shared in common with the Iowa haplotype: D4S2305 (417bp), D4S2397 (138bp), D4S1609 (171bp) and D4S230 (195bp) with allele frequencies of 0.167, 0.267, 0.250 and 0.240, spanning 5.36cM with intermarker distances of 3.44, 1.39 and 0.53cM. These marker frequencies were obtained from CEPH (allele frequencies were assumed to be similar in the French and Italian populations) as allele frequencies were not available from a comparable Italian population.

## **3.6 CANDIDATE GENE STUDIES IN FPD: SLOW ACETYLATOR N-ACETYLTRANSFERASE 2 (NAT 2) GENOTYPE: AN INDICATOR OF SUSCEPTIBILITY TO FAMILIAL PARKINSON'S DISEASE?**

### **3.6.1 Aims and design of the study**

Preliminary data as part of a case-control study had suggested a highly significant association between the slow acetylator genotype for N-acetyltransferase2 (NAT2) and familial PD (Bandmann *et al.*, 1997b). The degree of activity of NAT2 determines the rate of detoxification of aromatic amines and a slow acetylator genotype is defined by the presence of two mutant alleles. The biologically plausible hypothesis is that slow acetylation could lead to impaired ability of patients with familial PD to handle neurotoxic substances (see section 1.11.5).

A large intra-familial association study of EU sibling pairs was performed by myself to determine if this original finding was reproducible. A total of 161 family units (affected sibling pairs with all available unaffected relatives) were typed for the four most frequent alleles of NAT2 in a given population (see Table 3.5).

### **3.6.2 Genotyping for N-acetyltransferase 2 (NAT-2)**

The alleles of NAT2 are as outlined in chapter 2 and illustrated below in Figure 3.3. Restriction digestion of NAT2 PCR products was used to genotype individuals as previously described in chapter 2 and illustrated below in Figure 3.4. Table 3.5 shows the population frequencies of the commonest NAT2 alleles.

### **3.6.3 Statistical analysis**

Statistical analysis was carried out by Dr Peter Holmans, Neuropsychiatric Genetics Unit, University of Wales College of Medicine, Cardiff, UK. Analyses were done using two different approaches, allelic association and matched case-control study. Analyses for the M1, M2 and M3 alleles only were done as they account for most slow acetylators among white patients (Lin *et al.*, 1993).

#### *3.6.3.1. Allelic Association Study*

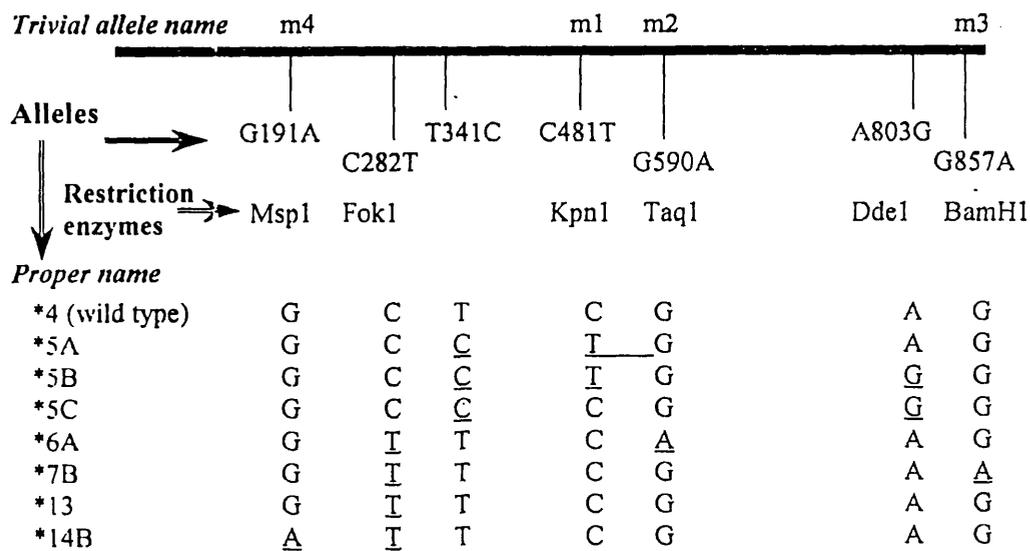
Each allele was considered separately to see whether any particular allele was under-represented in a given sample. The data was parameterised in terms of the likely relative risk to homozygote and heterozygote carriers of the “disease” allele, and a likelihood-ratio and chi-square calculated assuming heterozygote and homozygote risks were equal.

#### *3.6.3.2. Matched case-control analysis*

All individuals were classified according to whether they were homozygotes for M1, M2 or M3 or who carried any combination of two of these three alleles (and were therefore slow acetylators). They were contrasted with the remaining subjects, fast acetylators, containing at least one wild-type allele (matched case-control analysis). Slow acetylators were tested against other genotypes using a matched case-control

analysis with the affected sibs in each sibship as cases and the unaffected sibs as controls. The relative risk was estimated through calculation of the odds ratio (OR). For each odds ratio, a 95% confidence interval (CI) was computed and statistical tests were performed by computing two-sided p values for which values less than 0.05 were considered statistically significant.

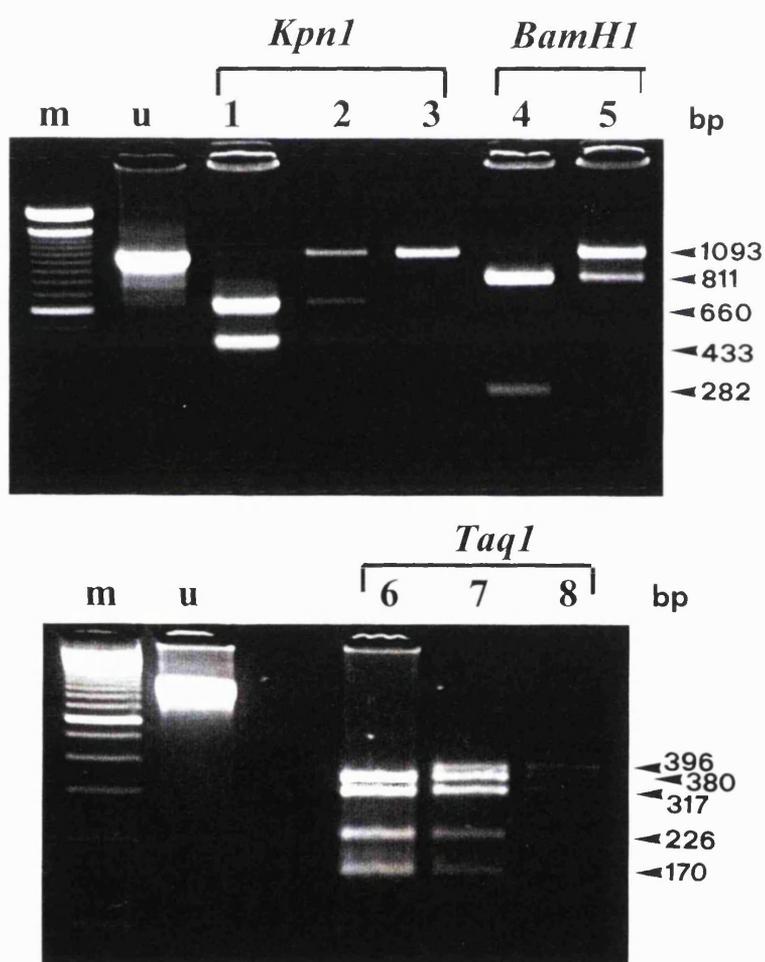
**Fig. 3.3:** Structure of the NAT2 gene and illustration of its alleles, showing the nucleotide differences responsible for restriction site changes in each allele.



Underlined nucleotides indicate mutant alleles  
(After: Cascorbi et al., 1995)

**Figure 3.4:** Genotyping for NAT2 by restriction analysis of PCR products. Mutant alleles (M1, M2, or M3) can be distinguished from wild type (wt) by the creation or loss of particular restriction enzyme recognition sites for *KpnI*, *BamHI* or *TaqI*. Key: Mw, molecular weight marker; U, uncut PCR product; lane 1, wt/wt; lane 2, wt/m1; lane 3, m1/m1; lane 4, wt/wt; lane 5, wt/m3; lane 6, wt/wt; lane 7, wt/m2; lane 8, m2/m2.

### Restriction digests of NAT2 PCR products



**Table 3.5:** Population frequencies of NAT2 alleles in 372 Caucasian-Americans & 129 African-Americans (Bell *et al.*, 1993).

Allele	Population frequency of allele	Population frequency of allele
	Caucasian-American (372)	African-American (129)
Allele 1 (WT, wild type, fast acetylator)	0.25	0.36
Allele 2 (M1-slow acetylator)	0.45	0.3
Allele 3 (M2-slow acetylator)	0.28	0.22
Allele 4 (M3-slow acetylator)	0.02	0.02
Allele 5 (M4-slow acetylator)	0	0.09

### 3.6.4 Allelic association study (sib-TDT analyses using all 4 alleles) using TRANSMIT

#### 3.6.4.1 SIB-TDT Results.

All affecteds were used in the analysis, so positive results would be due to linkage rather than association. (see Table 3.6).

All five samples showed an excess of allele 2 (M2), although this is minimal in the Dutch and Italian samples. The distribution of the alleles in the four groups from the total population of the 161 nuclear families studied is shown in table 3.6.

All p-values are obtained by TRANSMIT using bootstrap simulation (see section 3.7.8.1) and are therefore valid for any number of affected siblings per independent family.

**Table 3.6:** Allele-specific TDT-style analyses (using TRANSMIT) for each of the 4 alleles used for typing NAT2.

Allele	Observed	Expected	Chi-sq (1 df)	p-value
1 (wt)	162	169.18	1.33	0.23
2 (m1)	291	286.53	0.38	0.52
3 (m2)	207	200.82	0.93	0.34
4 (m3)	18	21.47	1.68	0.09

#### Overall Chi-squared test

All 4 alleles simultaneously chi-sq = 3.37 on 3df. The simulated p-value from TRANSMIT is 0.26.

**Table 3.7:** Matched case-control analysis to compare slow versus fast acetylators

Slow acetylators (2 non-WT alleles) compared with the rest:

Type of analysis	Relative risk to slow acetylators	95% CI	Chi-sq	p-value
Study 1	1.71	1.04-3.01	4.08	0.025 <p< 0.05
Study 2	1.75	1.08-3.19	4.01	0.024 <p<0.05

**Study 1:**Total population of affected sibling pairs (n=161). **Study 2:**Population of sibling pairs with the index case included in the original pilot study (Bandmann et al 1997) removed from analysis (n=161).

The results of study 1 show that confirm the previously described association between NAT2 slow acetylator genotype and familial PD (Bandmann *et al.*, 1997b; Bandmann *et al.*, 2000). Reanalysis of this data having removed the original index case included in the pilot study (Bandmann *et al.*, 1997b) showed that the result was still significant (a total of 46 families were included with just one affected sibling in study 2).

## **3.7 DISCUSSION**

### **3.7.1 Clinical data analysis on EU sibling pairs**

Some interesting features of the above data are worthy of note (Tables 3.1 and 3.2; Figure 3.1). Firstly, although the clinicians responsible for collecting clinical data in each country were different, there is generally a remarkable concordance between the countries. In particular, the mean age of individuals at the time of the study and the mean age at onset of PD are very similar, although cases from the Netherlands (NL) were a little older at onset (t-test,  $p < 0.01$ ) and cases from France (FR) tended to be younger at onset (t-test,  $p < 0.05$ ). MMSE values obtained from Netherlands cases were significantly different from other countries (t-test,  $p < 0.0005$ ), but this could be put down to differences in the clinical judgement of the individual observer. Of particular interest, however, is the difference between the mean daily dose of L-dopa in the UK cases relative to the rest of Europe, as UK doses were significantly higher than all countries except Italy (t-test,  $p < 0.0005$ ). Doses of L-dopa given to patients in the Netherlands are particularly low relative to the rest of Europe, indicating different prescribing habits here. Interestingly, the initial percentage improvement on L-dopa noted from UK patients was significantly higher than in all other countries except France, perhaps reflecting the higher mean dose. However, not all countries measured this parameter as precise percentages. The mean length of duration of patients on L-dopa was also generally longer in the UK cohort, although France and Italy were longer than the Netherlands and Germany. In summary, it is encouraging to observe essential similarities between countries as it strengthens the value of the pooled data from the EU consortium. Differences between countries are also of interest as they may indicate important differences in clinical practice across Europe.

### **3.7.2 Linkage analysis of the major dominant loci described in PD to date in a large population of European affected sibling pairs (ASPs)**

PD is now considered to be a genetically heterogeneous disease with complex inheritance although, at the start of my studies, the role of genetic factors in the aetiology of PD was controversial (see chapter 1). The establishment of a European consortium (GSPD) allowed recruitment, analysis and screening of affected sib pairs for a genome-wide screen of susceptibility genes in sib pairs with PD. Future work with

this invaluable resource will allow completion of this genome screen as well as further the opportunity to test the significance of published genetic factors responsible for PD.

During the course of this thesis work, 194 strictly diagnosed ASPs without known PD mutations were identified by myself (in the UK) and other clinicians in the consortium. A total of 246 families were collected up to the end of 1999, each family having at least 2 affected siblings. All the information was collected, analysed and stored on centralised databases. The initial genotyping screen on a highly informative subset of 125 families with ASPs focused on chromosomal regions 4q 21-23 (PARK1), 2p13 (PARK2), 4p15-16.1 (PARK4) and 4p14 (UCH-L1). Three additional markers gave initial p-values <0.05 in country-specific analyses (D4S1647 in Italian families (4q21-q23), D2S1394 in German and British families (2p13) and D4S405 in German families (4p14)), although the findings did not reach significance at the level of 5% after correction for multiple comparisons.

### 3.7.2.1 *PARK 1 (4q 21-23)*

Genetic markers D4S2380 and D4S1647 showed no obligate recombination events in the affected individuals ( $Z_{\max}=5.22$  at D4s1647) in the Contursi kindred (a large family of Italian descent) first linked to the PARK1 locus (Polymeropoulos *et al.*, 1996). The original G209A mutation in exon 4 of SNCA was then described in the same family in affected individuals with parkinsonism (Polymeropoulos *et al.*, 1997). The only other families described as having the same mutation originate from the area around a common trade route between Italy and Greece. Affected members in both the Italian and Greek families may all be descendents of a single founder as they share the same background haplotype (Athanasiadou *et al.*, 1999). Interestingly, it was the Italian subset of families which showed putative evidence for linkage at D4S1647. In order to further investigate these results, the entire coding region of the SNCA gene was also sequenced (by collaborators at the Mayo clinic, Jacksonville, Florida) in an index case from each of the GSPD Italian families included in the original study and no mutations found in the coding region (Dr M Farrer, personal communication). Thus, although these families were from the same country of origin (Italy) further SNCA mutations were not found, confirming the rarity of mutations in this gene as a cause of familial PD (Vaughan *et al.*, 1998a; Vaughan *et al.*, 1998b). Despite this, D4S1647 remains an interesting marker, since a polymorphism in the promoter region of SNCA (NACP-Rep 1), as well of the closely linked markers D4S1647 and D4S1628 marker has

been reported to be associated with PD (Kruger *et al.*, 1999). Linkage disequilibrium between NACP-Rep1 and the D4S1647 marker was identified in PD cases, which might include the presence of a susceptibility gene. However, this association has yet to be reproduced (Khan *et al.*, 2001a).

### 3.7.2.2 *PARK 3 (2p13)*

The only marker for which a pairwise lod score was found with a P value of <0.05 in the overall analysis, D2S1394, is localised near to the *PARK3* locus (Gasser *et al.*, 1998). In the initial description of this locus, a significant maximum two-point LOD score for D2S1394 was not described (Gasser *et al.*, 1998), yet the marker was part of a common haplotype spanning an interval of 3.2cM, segregating with PD in two families originating from neighbouring regions of southern Denmark and northern Germany. The presence of a founder mutation was proposed for this, although no genealogical link between the families has been detected (Gasser *et al.*, 1998). Although an initial nominal p-value of 0.04 was found in the subset of ASPs collected from Germany in our study, it is too early to comment on whether this finding strengthens the role of the *PARK3* locus as a susceptibility factor for PD: further cohorts of ASPs should be examined to determine if this is a reproducible result.

### 3.7.2.3 *PARK4 (4p14-16.3)*

Analysis of the linkage data at the *PARK 4* locus did not reveal significant linkage, excluding *PARK 4* as a major susceptibility locus in this population of affected sibling pairs. However, familial analysis did indicate one dominant Italian kindred with an identical shared *PARK4* haplotype to that described in an Iowan kindred (Vaughan *et al.*, 1999) (see below). The D4S405 marker (4p14), which is closely localised to the *UCH-L1* gene, showed some weak evidence for linkage in German families. The mutation in the *UCH-L1* gene that causes PD was originally detected in a German family (Gasser *et al.*, 1998). However, as described in chapter 4, no coding mutations in *UCH-L1* were found in a sample of index cases from the GSPD series which shared an affected haplotype at 4p14-16.3 (Harhangi *et al.*, 1999, Lincoln, 1999 #4356).

### 3.7.3 Affected sibling pair analysis and complex disease

The strengths of this ASP study include the systematic data collection with standardised documentation and videotaping of the affected individuals, the strict diagnostic criteria that were used and the international ongoing collaboration through which a large number of families with PD have been collected. The ASP method is a very appropriate approach to map genes in a complex disease trait such as PD because prior assumptions about the mode of inheritance do not have to be made. At present, with the exception of a minority of dominant kindreds and mainly young onset cases with Parkin mutations (see chapter 4), the mode of inheritance of the majority of PD is unknown. However, a major disadvantage of the use of ASPs in genetic studies is the large numbers of families required (especially to perform a future genome-wide search). Power in the current GSPD sample is low due to the absence of parental haplotype information (only 10% of the sibling pair families had one or both parents available for sampling). Clinically unaffected siblings were also not available in all families (due to reluctance to consent, death or intercurrent illness). The other major problem to overcome in genome-wide ASP studies is genetic heterogeneity as the proportion of ASPs that harbour the same genetic mutation determines the power of the study (see section 1.8.6). Analyses become even more difficult for the geneticist in sibling pair studies if susceptibility in a particular disease is based, not on a large number of the affecteds harbouring mutations from a small number of different genes, but a large number of susceptibility loci, acting in concert to cause the disease. Acting alone these loci may be insufficient to give rise to the affected phenotype. The problem here is that it would be impossible to detect positive linkage to an area (power would be too low) or to exclude an unlinked area with sufficient confidence. Power is also usually low, in particular for susceptibility loci with a low relative risk or low frequency of the risk allele.

Problems with ascertainment, diagnostic criteria, aetiological heterogeneity and failure to correct for the testing of multiple genetic models, have all been blamed for failure to replicate linkages in other family datasets using ASPs (Davies *et al.*, 1994). It has been shown (Risch, 1990b; Risch, 1990c) that if a complex trait is caused by, for example, six loci all with equal effects, then the number of families required to achieve replication (i.e. after an original genome screen has revealed several areas of interest, and a further cohort of ASPs is needed to test these areas) is approximately five times the number of families required for the first detection ( $N-1$ , where  $N$  is the number of

loci). Although strict inclusion criteria were used to define cases, including “response to L-dopa” as a major criterion, and any doubtful cases were reviewed by other experts via videotape, misclassification of PD could have occurred in interpreting the data because there is no biological marker for PD and a definite diagnosis of PD rests on post mortem neuropathological findings.

Lod scores at the dominant loci in this study were generally below 1 for the reasons discussed above which reduce the power of the sample. Follow-up studies are needed to see if these findings are reproducible, both to investigate if the positive lod score at PARK3 (D2S1394) is a biologically significant finding and because, for all the data, significance tests are not very reliable in evaluation of extremely small P values. Replication of linkage findings /exclusion of linkage in complex genetic diseases has often proved unreliable, resulting in confusion as to which linkages are true- or false-positive findings. Reasons for this include use of insufficiently stringent thresholds for identification of loci, genetic heterogeneity and inadequate power of replication studies. It has been estimated that to achieve 80% power to detect a locus of magnitude  $\lambda=1.8$  in a complex genetic disease, even at low significance threshold of LOD 1.0, will require studying 200 affected sibling pairs (with both parents available for genotyping, using a 10-cM marker map). The low power of linkage studies to replicate small genetic effects must be considered in comparisons between the results of future genetic studies (Laval *et al.*, 2001).

These preliminary results show that a European Consortium (GSPD) has been successfully established and recruited a large number of affected sib pairs with PD for a genome wide search for susceptibility genes in PD. Evaluating the significance of the currently described dominant susceptibility regions in PD (PARK 1, PARK3 and PARK 4) further will require their examination in a further cohort of sibling pairs. GSPD studies on PARK2 have been described in chapter 4. PARK 6 has only recently been published and therefore was not investigated as part of the experimental work of this thesis.

### **3.7.4 Future work of the GSPD consortium**

Genome-wide screening did not form a major part of this thesis and therefore comment will be brief. Just prior to the submission of this thesis, the first results of American

analysis of ASPs has been published in abstract form (full report in press (Scott *et al.*, 2001)). Five major susceptibility regions were isolated from the first cohort analysed. One hundred and seventy-four caucasian 174 Caucasian multiplex (2 or more sampled PD patients) containing 870 sampled members (378 affected), 185 sampled affected sibling pairs and 70 other sampled affected relative pairs were ascertained as part of a multi-center genetic linkage study. Mean age at onset was  $59.9 \pm 12.6$  years. Recruitment guidelines were similar to that of GSPD (see table 2.1). Marker genotypes were obtained on 344 microsatellite markers (average spacing 10 cM). Families were studied for linkage using a multi-analytical approach consisting of two-point parametric (MLOD) and multipoint non-parametric (LOD\*) methods. To identify potential genetic heterogeneity by age at onset, 18 families having at least one family member with onset prior to age 40 were considered separately from the remaining late-onset families (n=156). Six regions were found to generate interesting lod scores. The strongest results overall (MLOD or LOD\* > 2) were obtained for markers on chromosomes 5q, 8p, 9q, and 17p-17q. Additional regions of interest (MLOD or LOD\* > 1.5) were 14q and Xq. Results in the late-onset subset were similar to those obtained overall. In contrast, analysis of the 18 early-onset families detected significant evidence for linkage to chromosome 6q (MLOD and LOD\* > 5), in the vicinity of the parkin gene. Regions of interest on 5q, 8q, 9q, and 17P-17q are currently being evaluated to identify positional and functional candidate genes in these regions (Scott *et al.*, 2001). These new areas also confirm the genetic heterogeneity of PD (Gasser *et al.*, 1997; Scott *et al.*, 2001). They will need to be investigated in the current cohort of EU sibling pairs.

The final issue that these studies may help to address is whether all the genes responsible for PD or parkinsonism represent a spectrum of one clinicopathologic entity or that they each reflect a different disease entity is not clear and thus the subject of debate. From genotype-phenotype correlation studies, it is known that there are clinical differences between the responsible gene and corresponding disease status. However, in many patients, the phenotype is often indistinguishable from that of sporadic PD (Abbas *et al.*, 1999). Strategies to increase homogeneity by studying a founder population may increase the chances of success as well as the use of a subset of larger families in which the mode of inheritance is clear. Individual susceptibility loci detected in sibling pair studies can be tested in these larger families which, on their own, may not have enough power to generate sufficient odds for linkage. The collection of a large number of dominant kindreds and extension of the sibling pair families already collected should

allow this approach to be feasible in future work involving genome-wide screening.

### **3.7.5 Evidence for a chromosome 4p14-15 haplotype segregating with PD in a kindred of Italian descent**

As described in section 3.5, a segregating haplotype at 4p14.1-16.3 (PARK4 locus) with Parkinson's disease and postural tremor was detected in an Italian sibling pair (4 affected individuals in two generations). These data are consistent with linkage but not conclusive. No mutations were found in the coding region of UCH-L1 in sequencing an index case from both the Spellman-Muenter Waters-Miller (Iowa kindred) and the Italian sibling pairs (M Farrer, personal communication). Positional cloning strategies are currently being employed to find the causative gene at this locus (Farrer *et al.*, 1999b) but the Italian GSPD kindred may represent an important link in narrowing down the PARK 4 candidate region (4p14-16.3).

### **3.7.6 Phenotypic comparison of both kindreds**

Disease onset in the Iowa family is in the fourth decade. Invariably, affecteds are L-dopa-responsive at first but the clinical course is rapidly progressive, with the development of dementia noted in some affected family members in the terminal stages (Muenter *et al.*, 1998). While affecteds-only linkage analysis of early onset disease in the Iowa kindred did not achieve a lod score of >3.0, mean lod scores generated were consistent for the size of the kindred. In addition, eight Iowa family members, without parkinsonism but with postural tremor, and three asymptomatic individuals have subsequently been noted to share the haplotype. How may these differences in expression and the variability in age of disease onset and duration between the Iowa and Italian families be explained? At the present time, without having identified the underlying molecular defect, speculation only is possible. While disease transmission within either family appears autosomal dominant and highly penetrant, disease segregation is likely to be influenced by bias in ascertainment with family branches predominantly traced through affected family members.

The age of onset within both Italian and Iowa kindreds is variable (Iowa  $33 \pm 8.5$  years,

Italian  $52 \pm 16$  years). Individual 036 in Fig 3.2 shares the affected haplotype, but clinical examination at the age of 72 years was normal. Both non-penetrance and postural tremor in disease haplotype carriers have been described (Farrer *et al.*, 1999b; Polymeropoulos *et al.*, 1997). The age of onset in the Contursi kindred, where the underlying Ala53Thr mutation in SNCA is known, is also variable as members of the Contursi family with the mutation may be affected as early as 32, and yet some reach 85 years without any manifestation of disease. Assessment of expression of the G209A allele in lymphoblastoid cell lines led authors to conclude that the lack of, or significantly reduced expression of, the G209A allele in affected heterozygotes may indicate that the timing of reduced expression may be critical for disease onset. If so, the parkinsonian phenotype in the Contursi kindred may arise from haploinsufficiency at the alpha-synuclein gene at a time point before symptom onset (Markopoulou *et al.*, 1999). Non-penetrance and variable expressivity of disease haplotypes in the Italian and other kindreds with parkinsonism probably reflect the sum of both environmental and genetic interactions with genetic mutations of major effect (Farrer *et al.*, 1999b). The phenotypic differences between the Italian (ITNA 035) and Iowan kindreds (Farrer *et al.*, 1999b) do not exclude the fact that the affecteds in each family may harbour the same disease gene mutation. Rather, they serve to illustrate the fact that autosomal dominant PD appears to have variable expressivity for the reasons outlined above.

### **3.7.7 ITNA 035: Haplotype and power analysis at the PARK4 locus**

Power and linkage data on the family ITNA 035 was not informative given the individuals close relationship, small size of the kindred and the lack of information on phase (when DNA was not available, individual haplotypes were inferred). This may have led to some inaccuracies in haplotyping and phase of some individuals as no DNA was available on the oldest members of the kindred. The inferred haplotypes shown in fig 3.2 are the best fit for the data. As stated in section 3.5.4, four affected individuals in two successive generations of an independent Italian kindred (ITNA035) all appear to share part of the 'Iowa haplotype' (Farrer *et al.*, 1999b) for four adjacent, telomeric markers, D4S2305, D4S2397, D4S1609 and D4S230 (same allele sizes in both families). The 'Iowa' haplotype shows no evidence for recombination. Three recombinant haplotypes in the Italian family were observed. Assuming inter-marker linkage equilibrium, the region of shared haplotype between the Iowa and Italian

families spans 5.36cM and has a frequency of 1/374 (according to the CEPH population). Use of CEPH controls here may be an incorrect assumption. The GSPD consortium is currently collecting unaffected controls from similar geographical areas to the affected sibling pair population. These controls will have an important future role as part of the collaborative genome screen.

Within the Italian family, the size of the interval shared is not unexpected given that individuals were first degree relatives and only 15 meioses were considered within the pedigree. According to Figure 3.2, person 036 also shares the haplotype yet is not affected by parkinsonism (see section 3.5.2). In contrast, the expanded Iowa haplotype spans the interval from D4S1551 to D4S3350, a genetic distance of 10.7cM (based on the most recent sex-averaged Marshfield map (K)) and nine recombinant haplotypes were observed in the 84 meioses considered (Farrer *et al.*, 1999b). Further studies to assess the frequency of the Iowa haplotype in the Northern European data set are needed as identifying a family with parkinsonism with the haplotype may simply be due to chance.

These findings support the assignment of PARK4 as a locus for Lewy body parkinsonism but are not conclusive due to the small size of the Italian kindred. To ultimately determine if this is a real finding and has not occurred by chance, a common founder for both families or the mutation in the disease gene at the PARK4 locus would need to be discovered. A genealogical link has yet to be established between the two kindreds and despite several candidate genes being sequenced in the region, the disease gene is yet to be identified (M Farrer, personal communication). To date no genealogical link has been made between the two families, although the Iowa family can only be traced to European immigrants who arrived in American in the 1820's. Little more is known about this multiplex Italian kindred beyond recent ancestry in Naples. Although the high degree of similarity in allele sizes between Italian and Iowa kindreds for more telomeric 4p15 markers, may imply the alternate and perhaps more plausible conclusion of a common founder, unless one is demonstrated between the two kindreds the family cannot be used with confidence to narrow the 4p14-16.3 candidate region (PARK 4) (Farrer *et al.*, 1999b; Vaughan *et al.*, 1999)

Additional kindreds with parkinsonism, linked to 4p14-16.3, are required to confirm the PARK4 locus. Identification of a segregating UCH-L1 mutation in a PARK4- linked family would confirm the gene's pathogenic assignment in Parkinson's disease,

although sequencing of UCH-L1 in an index case from the Iowan kindred failed to reveal any coding mutations (Farrer *et al.*, 1999b). Alternatively, identification of additional 4p15 linked families, without mutations in UCH-L1, would provide support for the PARK4 locus and help refine a candidate region for positional cloning.

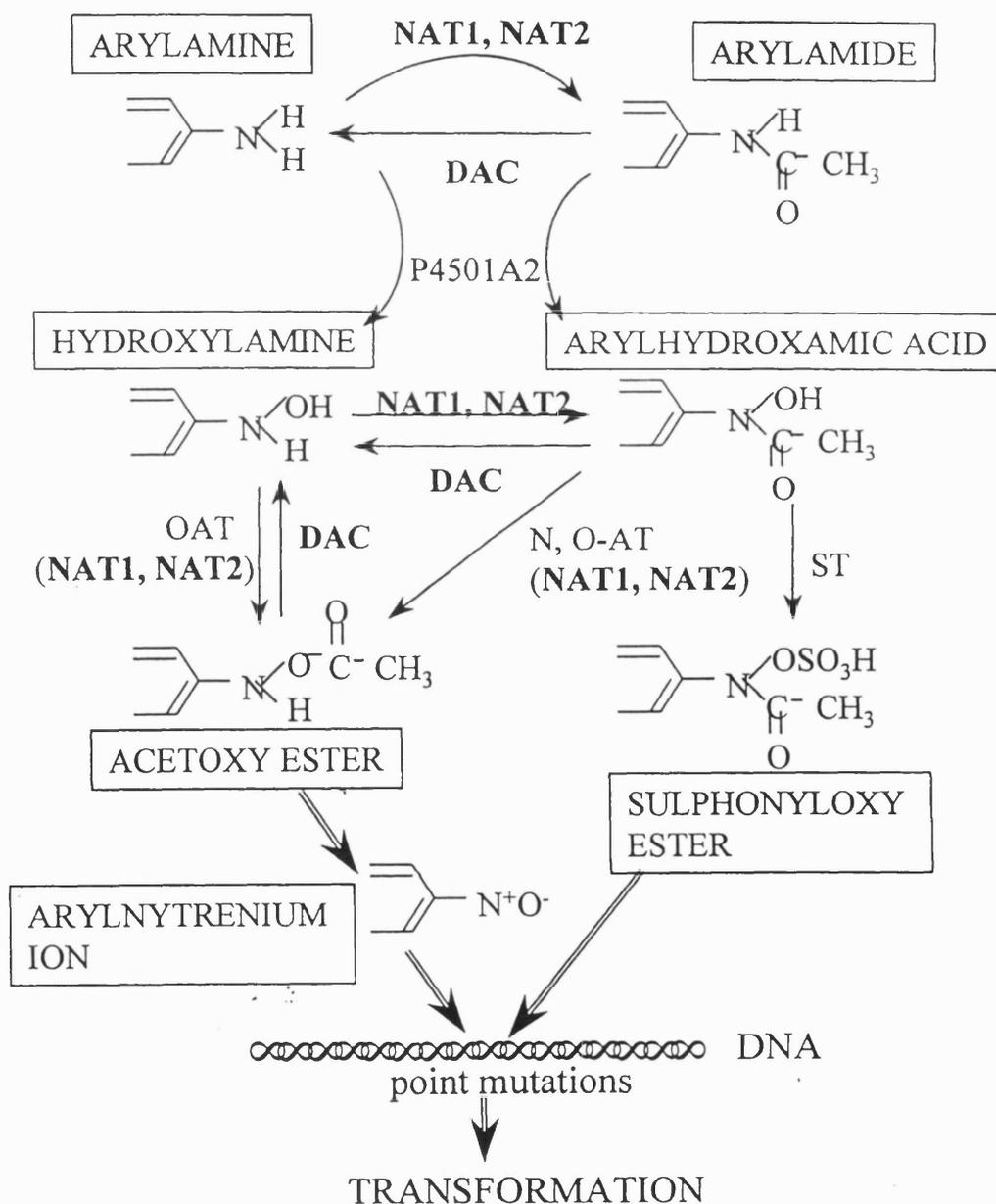
### **3.7.8 N-acetyltransferase 2, familial PD and association studies**

Discovery of susceptibility loci can be important in understanding the causes of a disease. PD is known to be genetically heterogeneous. While the characterisation of single genes involved in rare mendelian families with PD have been significant in enhancing our understanding of the disease, association studies should be (if designed appropriately) the most powerful in trying to elucidate susceptibility genes, if present, in PD as a whole. Recent guidelines (Anon, 1999) have suggested that association studies should report associations that make biological sense and alleles that affect the gene product in a physiologically meaningful way. They should also contain an initial study as well as an independent replication and should be observed in family and population-based studies. For alleles with modest effects, which might depend on alleles at other genes, replication may be challenging. Several large, independent datasets, with family studies, should be used to confirm the validity of the association.

For NAT2, a number of biological and genetic studies had already been performed. Indeed, preliminary data had suggested a highly significant association between the slow acetylator genotype for N-acetyltransferase2 (NAT2) and familial PD. A large intra-familial association study of EU sibling pairs found that the slow acetylator genotype for N-acetyltransferase 2 (NAT2) was over-represented in familial PD. The results in this study confirmed a significant association between the slow acetylator genotype for NAT2 and familial PD. Several functional polymorphisms in xenobiotic metabolism have been studied in relation to patients with PD but none with consistent results, although there is a biologically plausible hypothesis for the role of slow acetylation in PD. N-acetylation is involved in a number of detoxification processes. NAT1 and NAT2 (encoded by separate genes located at chromosome 8p21.3-23.1 (Hickman *et al.*, 1994)) both catalyse the transfer of an acetyl group from the co-factor acetyl coenzyme A to the amine nitrogen atom of aromatic amines and hydrazines (Fig. 3.5 below). The different isoforms due to polymorphisms/mutations in the NAT2 gene

could feasibly increase susceptibility to toxic damage and this could affect some cell types (such as the neurons of the substantia nigra) more than others.

**Fig.3.5:** Possible metabolic activation and deactivation pathways for arylamine carcinogens, showing a central role for N-acetyl-transferase 2 (NAT2). Abbreviations: P4501A2, cytochrome P450 1 A2; DAC, microsomal deacetylases; OAT, O-acetyltransferase (mediated by NAT); N,OAT: intramolecular N, O-acetyltransferase (mediated by NAT); ST, sulphotransferase. (After Grant, D.M., 1993, Molecular genetics of the N-acetyltransferases. Pharmacogenetics 3:45-50).



It may be that differences in translational efficiency or protein stability affect levels of NAT2 protein. In turn, these differences could affect susceptibility to aromatic amine-induced toxicity. The precise substrates are important to identify in order to evaluate toxicological risks associated with diseases such as PD and cancer. One such mechanism could be by the production of reactive hydroxylamines which may in turn be metabolised to form DNA-binding electrophiles. If these affected genes controlling cell growth characteristics, they could lead to malignancy. Alternatively, if certain cell types were more susceptible to levels of acetylation metabolism, such as neuronal populations in the substantia nigra, an increased susceptibility to more focal disease such as PD could occur.

In the original study, the slow acetylator genotype for NAT-2 was more common in the familial PD (69%) than in all controls (31%) producing an odds ratio of 3.79 (95% CI 2.08-6.9). There was also evidence for a highly significant trend for slow-acetylator genotype from controls through sporadics to familial cases ( $P=0.000002$ ), indicating that the genetic loading for this factor is highest in familial PD (Bandmann *et al.*, 1997b). This association was subsequently confirmed by typing of all the NAT-2 alleles more precisely using a method which detected all mutant NAT2 alleles with a frequency of  $>1\%$  in the Caucasian population. The slow acetylator genotype remained considerably more common amongst FPD (73%) than normal controls (NPC, 43%) (Bandmann *et al.*, 2000). The results of this study confirmed the original association found between the NAT2 slow acetylator genotype and familial PD in a sample population and also suggested that the wild type allele 4 confers a protective effect against the development of PD in patients with a positive family history for that disorder.

This finding has since been examined by a number of groups but attempts by other groups at replication of the original study have so far yielded inconsistent results. One group failed to replicate the association of slow acetylation and PD in their own study which included a larger number of controls, but a smaller number of patients with familial PD (Nicholl *et al.*, 1997). However, the inclusion of more than one affected member of their 30 families in the chi square analysis was not statistically sound and no precise details were given for age, sex, ethnicity and region of the patients and controls. Thus, their negative results based on the analysis of a total of 46 affected family members are questionable. A comparison between the NAT2 slow acetylator frequency

amongst our familial PD cases and the controls used by Nicholl and colleagues (Nicholl *et al.*, 1997) continued to give a statistically significant difference ( $p < 0.025$ ).

A smaller study detected a statistically significant higher frequency of slow-acetylation genotypes in patients with early-onset sporadic PD (onset before the age of 50) compared with both healthy control subjects and with late-onset sporadic PD (Agundez *et al.*, 1998). However, only a small number of early-onset cases were included and this data must therefore be interpreted with considerable caution. In another study, a sample of 139 unrelated patients with PD and 113 control subjects the NAT2 M3 allele was found to be associated with PD (OR =7.9; 95% confidence interval=1.7-36.3). Case-control analyses for CYP2D6, APOE and NAT2 and M1 or M2 did not show a significant association (Maraganore *et al.*, 1999). These findings conflict with our original study as the association was originally described between the slow acetylator genotype for NAT2 and familial PD. It was argued that this result was less likely to represent linkage disequilibrium between one of the alleles and another gene in the immediate vicinity and that the association implied direct involvement of NAT2 on the assumption that both alleles are inherited independently. The findings of Maraganore are not directly comparable because the majority of their cases had sporadic PD. Analyses restricted to familial PD cases were not possible because only 19 cases were familial. As only one of three slow acetylator alleles was associated with PD in this study, linkage disequilibrium of NAT2 with another, as yet unknown susceptibility gene cannot be excluded.

A similar study by a Rotterdam group investigated the three mutant alleles (M1, M2 and M3) of the NAT2 gene in 80 patients with sporadic PD and 161 age-matched randomly selected controls from a prospective-based cohort study. The allelic frequencies and genotype distributions in cases were very similar to those found in controls arguing against the slow acetylator genotype being a significant susceptibility factor in PD. However, these were sporadic cases and this association has to date mainly been significant in familial PD. To date, three metabolic studies investigating *in vivo* acetylation in patients with PD have been reported. Familial cases were specifically excluded from two studies and no information on family history was given in the remaining study (Ladero *et al.*, 1989, Igbokwe, 1993 #3913, Peters, 1994 #3914). The largest study on acetylation in European Caucasians with PD found a higher

proportion of slow acetylators amongst patients (n=100) in comparison with controls (n= 93), but this did not reach statistical significance (Ladero *et al.*, 1989).

### 3.7.8.1 Statistical Analysis

Explanations for the different findings in each of these studies relate to the usual reasons for lack of reproducibility in association studies as a whole (Anon., 1999). The matched case-control used as part of the analysis of the intra-familial association study may not be robust to linkage. If there are 2 or more affected sibs in a sibship and there is linkage to the locus being tested then these members may not be treated as independent with respect to the transmitted alleles/genotypes. This is because there is a likelihood that they could be the same and cannot be distinguished from genuine association by the analysis. This would also be a problem for TRANSMIT except that p values can be obtained by bootstrap simulation. Bootstrap simulation is a way of assessing the distribution of an estimated quantity (the genotypic relative risk in this study) without relying on assumptions like asymptotic normality (which fail here due to the non-independence of allele transmissions to multiple affected sibs from the same sibship). Obviously, if the study was repeated several times, the distribution could be observed but this was not practical.

The basic idea of bootstrap simulation is to assume that the actual dataset is representative of the population from which it was drawn, and thus to construct a large number of replicate samples by sampling families at random from the original sample, with replacement (which means that a family can be selected more than once (otherwise all the replicate samples would be identical to the observed one)). The quantity can be estimated from each sample and the resulting distribution used to construct confidence intervals for the observed value and/or p-values for hypothesis tests. The 95% confidence intervals can be estimated in order of magnitude by picking the 2.5 and 97.5 percentiles.

How a "genetic defect" such as slow acetylation, present in a considerable proportion of the general population, should make some people more prone to develop dopaminergic cell death, whilst others live healthily to old age without developing PD may be explained in terms of "susceptibility genes". It is not the gene in isolation, but rather an interaction between genetically determined endogenous factors and exogenous influences such as exposure to particular environmental toxins which leads to the

development of the disease. The biologically plausible hypothesis is that slow acetylation could lead to impaired ability of patients with familial PD to handle neurotoxic substances and therefore identifying the naturally occurring substrates for NAT-2 would be an important step. Slow acetylators are known to be more susceptible to low-level environmental exposure to carcinogens (Vineis *et al.*, 1994). Similarly, slow acetylators might also be more susceptible to low exposure of neurotoxins. In rabbits, hydrazine-induced central nervous system toxicity is related to the acetylator genotype, slow acetylators showing greater susceptibility with increased irritability, seizures and early death (Hein and Weber, 1984). Other hypotheses to explain the observed effect have been discussed previously (Bandmann *et al.*, 1999).

### 3.8 SUMMARY & CONCLUSIONS

The studies described in this chapter were performed on a large population of affected sibling pairs with PD. Their great value as a starting point for analysing whether there are genetic variations which contribute to the development of PD has been illustrated. A segregating disease haplotype at the PARK 4 locus has been described in an independent Italian kindred although the two-point lod score analyses were not sufficiently informative to show definite linkage to PARK4. These studies have also illustrated the potential and pitfalls of association studies for complex genetic diseases, such as PD. They may be valuable in testing plausible hypotheses of aetiology of PD, such as the role of genetic predisposition to environmental toxins due to different genotypes of the N-acetyl transferase gene. Association studies, however, rely on large numbers of affected patients in families and are also highly dependent on careful choices of controls. Nevertheless, they may generate data with application to the more common forms of PD.

In particular, an association has been confirmed between familial PD and slow acetylator status for NAT2 using a family-based association study. Data from the present family-based study confirmed an excess risk to slow acetylators but this effect was only just significant at the 5% level in contrast to the low p-value in the original study (Bandmann *et al.*, 1997b). A discrepancy in significance levels between these data may have arisen from the different choice of control groups, but the fact that this has association has now been replicated in both population- and family-based studies

lends weight to the validity of the association. Further work on NAT2 in PD should not only include an attempt to replicate these findings in a different population, but also aim to investigate the biological relevance of NAT2 status on the handling of identified dopaminergic neurotoxins such as MPTP and possibly also further examine the influence of NAT2 status on gene expression in the basal ganglia.

The following chapter describes studies on three genes which have been identified in familial PD cases to date, SNCA, UCH-L1 and Parkin and discusses their possible epidemiological and biological relevance to PD.

## **CHAPTER 4: GENES AND PARKINSON'S DISEASE**

## 4.1 OUTLINE OF CHAPTER

This chapter describes a series of studies following the discovery of a mutation in exon 4 of the human  $\alpha$ -synuclein gene (SNCA) in 1997 (Polymeropoulos *et al.*, 1997), (see section 1.14). The mutation was initially reported in 4 families with autosomal dominant inheritance of PD and a further mutation was later described in an independent German kindred. In order to examine whether mutations in these exons are commonly found in familial PD, 230 European familial index cases of PD and 100 cases of MSA were also screened for the Ala53Thr mutation in exon 4 and the Ala30Pro mutation in exon 3 of the SNCA gene. A second part of the study involved screening a series of cases of Multiple System Atrophy (MSA) for these 2 known coding mutations in SNCA as antibodies raised against  $\alpha$ -synuclein protein have been found to stain the characteristic pathological bodies seen in MSA, glial cell inclusions (GCI) (Tu *et al.*, 1998).

Work on other genes implicated in the pathogenesis of PD are also described, including studies on the Parkin gene implicated in autosomal recessive PD (Kitada *et al.*, 1998a) and UCH-L1, a further gene implicated in autosomal dominant PD (Leroy *et al.*, 1998). The EU consortium, GSPD, undertook two major studies on Parkin of which I was one of the UK clinical collaborators. The first study involved analysis of the 12 coding exons of the Parkin gene in 35 families, mostly European, with early onset autosomal recessive Parkinsonism. All sequencing work was done by investigators at INSERM, Paris, so any results of Parkin mutations are provided for the purposes of discussion. The collection and clinical analysis of young-onset sibling pairs was performed by myself. The second study involved examination of the frequency of Parkin gene mutations and its associated phenotype in a large series of EU patients. Other clinical collaborators for this study in the UK were Professor NP Quinn (NPQ), Dr A Schrag (AS) and Dr DJ Nicholl (DJN). Finally, a neuropathological study was performed on a series of 15 brains with a young-onset parkinsonism to screen for mutations in Parkin and the pathological features of the first and only known UK brain from a patient with a Parkin mutation are described. The histological analysis was performed at the Parkinson's Disease Society Brain Bank, UK. Collaborators for this project were Dr S Daniel (SED), Dr A Kingsbury (AK) and Dr C Luecking (CL).

## 4.2 ESTABLISHING THE RARITY OF $\alpha$ -SYNUCLEIN MUTATIONS AS A CAUSE OF FAMILIAL PD

### 4.2.1 Aims and design of the study

To assess the significance of the first mutation to be described in familial PD, 230 cases from families recruited as part of the sibling pair EU study (153 independent pairs), multiplex family and two generational PD study (77 independent families) were screened by myself at the Institute of Neurology for the Ala53Thr mutation in  $\alpha$ -synuclein. In the sibling pair study there were at least 2 individuals in each family, fulfilling the diagnostic criteria of idiopathic PD (Maraganore *et al.*, 1991). These cases were drawn from the same population of ASPs available for PARK1 linkage screening as described in section 3.3. Table 4.1 shows a demographic study of the cases studied. PARK 1 linkage studies have been presented in sections 3.3 and 3.4 and did not exclude linkage to this locus. *SNCA* mutation screening in these sibling pairs then became a direct way of analysing large numbers of affected cases very quickly. Twenty-five index cases from the multiplex and two generational PD study who did not completely fulfil these criteria (including little or unknown response to L-dopa, severe postural hypotension and relative paucity of tremor) were also examined but classified as atypical. The initial mutation screening for the G209A mutation in *SNCA* was carried out before publication of the G88C mutation in *SNCA*.

**Table 4.1:** Demographic and clinical characteristics of index cases of familial PD.

Origin	No. of cases	Mean age at onset	Range	Standard deviation	Sex ratio M:F
France	96	55.7	27-87	13.6	53:43
Germany	30	57.4	35-78	11.5	18:12
UK	69	56.5	31-71	11.14	36:33
Italy	35	51.3	34-73	11.3	18:17

### **4.3 SCREENING FOR THE 2 KNOWN SNCA MUTATIONS IN A LARGE POPULATION OF AFFECTED SIBLING PAIRS & SEQUENCING OF ALL 7 CODING EXONS IN A SERIES OF KINDREDS WITH FAMILIAL PD**

#### **4.3.1 Mutation screening by restriction digest of PCR products**

##### *4.3.1.1 G209A mutation in exon 4 SNCA*

A specific band of 216 bp was detected in all samples tested and the sequence found to be identical to the published data (Polymeropoulos *et al.*, 1997). Digestion of the positive control (obtained from a member of the Contursi kindred) with *Tsp45 I* generated products of 88bp, 128bp (mutant allele), and 216 bp (normal allele) as published, due to the novel restriction site created by the G209A mutation in one of the alleles. None of the 230 cases which we screened by *Tsp45 I* digestion generated a restriction pattern which differed from unaffected controls (Fig. 4.1), enabling us to conclude that this particular mutation was not present in this population.

##### *4.3.1.2 G88C mutation in exon 3 SNCA*

This G to C substitution creates a new *Mva1* restriction site. Digestion of PCR products from mutation carriers generate fragment sizes of 192bp (normal allele) and 136 bp and 56 bp (mutant allele) (Kruger *et al.*, 1998). No mutations of this product were detected by *Mva1* digestion in any of the 230 cases screened (Fig. 4.2).

#### **4.3.2. Sequencing of the SNCA gene in a series of autosomal dominant PD kindreds**

In order to examine whether mutations in SNCA are commonly found in familial PD, all 7 exons of the SNCA gene were amplified by PCR from index cases of 30 European Caucasian kindreds affected with familial Parkinson's Disease. Each product was directly sequenced and examined for mutations in the open reading frame. No mutations were found in any of the samples examined, although mutations in the regulatory or intron regions of the gene were not excluded by this study. As this work was largely done by Dr M Farrer at the Mayo clinic, Jacksonville, USA, the methodology is included in appendix 2 (Vaughan *et al.*, 1998b) and the results described here for discussion purposes. Table 4.2 shows a clinical summary of the families sequenced.

**Table 4.2.** Clinical characteristics of 16 of the European and American families. All affected members exhibited at least two out of four of the cardinal parkinsonian signs (akinesia, resting tremor, rigidity, postural instability), and improvement on L-dopa.

Family	Number of affecteds	Number affecteds examined	Region of Origin	Mean age of onset	Atypical features	Reference
A	12	4	German-Canadian	51 (35-60)	Amyotrophy/ dementia (some)	Wszolek, 1993
B	8	4	Danish-American	62 (51-82)	Dementia (some)	Wszolek, 1993
C	11	4	German-American	60 (55-66)	None	Wszolek, 1993
D	18	5	English-American	63 (48-78)	None	Wszolek, 1995
G	11	4	German-American	56 (48-74)	Dementia (some)	Denson, 1997
IT-027	9	4	Italian	55 (46-67)	None	unpublished
IT-1	6	4	Italian	54 (36-89)	None	Bonifati, 1996
IT-0	3	2	Italian	56 (28-74)	None	Bonifati, 1994
K	4	4	German	56 (45-63)	None	unpublished
L	6	2	English	60 (52-66)	None	unpublished
M	3	3	English	45 (30-55)	None	unpublished
UK 402	16	8	English	53 (42-70)	None	unpublished
UK 401	4	4	Irish	35 (31-41)	None	Sawle, 1990
UK 403	7	3	English	64 (58-70)	None	unpublished
Q	3	3	Welsh	63 (59-65)	None	unpublished
R	8	6	English	64 (42-75)	None	unpublished

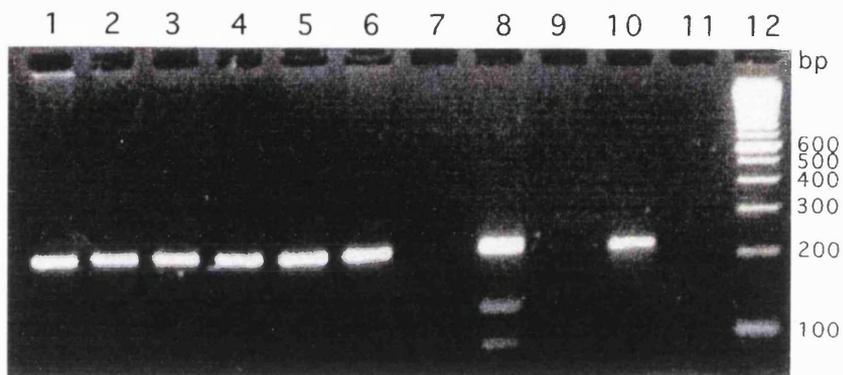
### 4.3.3 Identification of a new probable branch of the Contursi kindred

Twenty-seven Italian PD probands with an autosomal dominant inheritance of PD were screened for the presence of the Ala53Thr mutation by restriction digestion of PCR products with *Tsp45I*. One of these cases was shown to carry this mutation (data of this individual case are not shown, but the appearance was identical to the positive control from a member of the Contursi kindred, see lane 8 of Figure 4.1 below). This case was not included in the original series of Italian sibling pairs which were putatively linked to PARK1 (see section 3.5) as the case was not part of an affected sibling pair. This individual, a 34 year old man at the time of the study, developed symptoms of parkinsonism at the age of 32. A history of other members of his family revealed that his father and four more distant relatives had also been affected. The family originated from near to Salerno, southern Italy, in a region close to the Contursi family, suggesting a genealogical link.

### 4.3.4 Results Summary

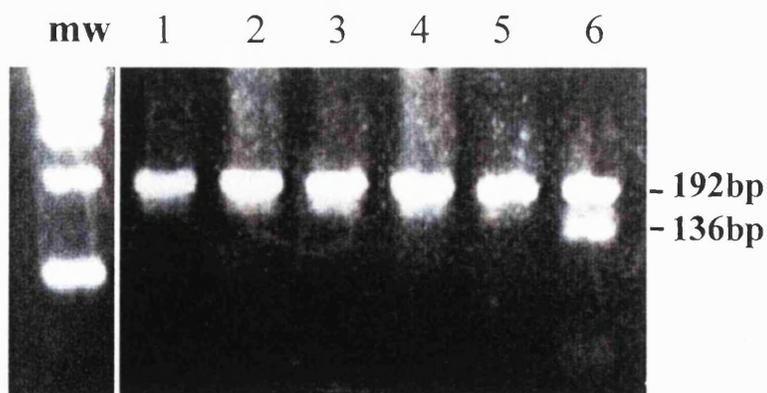
These data confirm that the two known mutations in *SNCA* are a very rare cause of familial PD in a large population of European sibling pairs, two generational families and 30 cases of MSA. Novel coding mutations in *SNCA* were also excluded in index cases from 30 autosomal dominant families further confirming the rarity of *SNCA* mutations in familial PD as part of a collaborative effort (Vaughan *et al.*, 1998b) (see appendix 4). A new branch of the Contursi kindred was identified as a result of a specific collaboration with the Italian group of GSPD who provided the clinical case.

**Fig. 4.1:** *Tsp45 I* digestion of a sample of 230 cases of familial PD. A selected sample of UK familial PD patients is shown, none of which showed a double product after digestion of the *SNCA* PCR product for exon 4 with *Tsp45 I*. A positive control from a known member of the Contursi kindred (bearing the G209A mutation) was run alongside (lane 8).



**Fig. 4.2:** *MvaI* digestion of PCR products obtained from a sample of 230 familial PD cases. A selected sample of UK familial PD patients is shown on this agarose gel, none of which showed a double product after digestion of the *SNCA* PCR product with *MvaI*. A positive control from a known member of the German kindred with the G88C mutation was run alongside (lane 6).

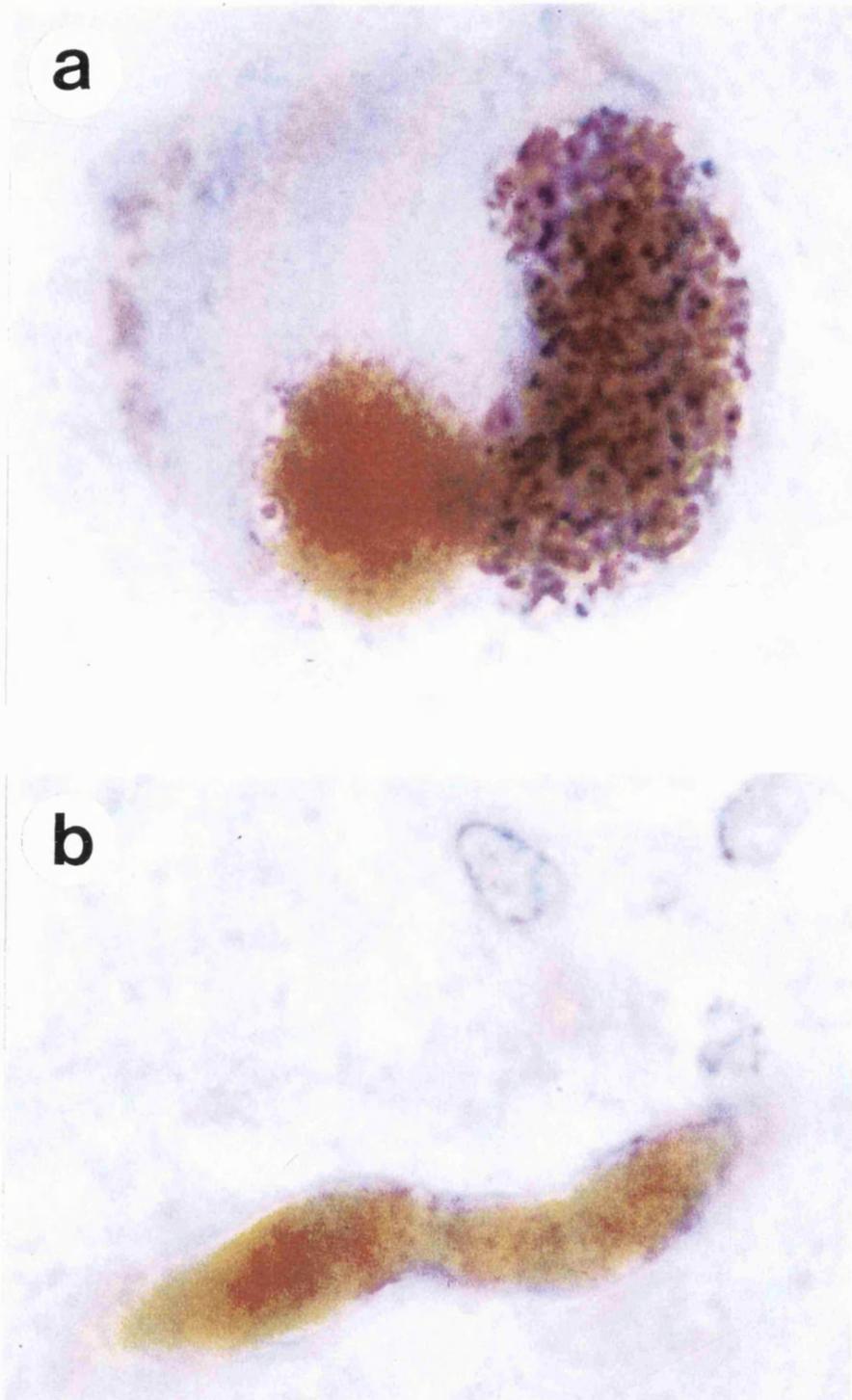
#### Identification of the G88C mutation in *SNCA* by *MvaI*



#### 4.4 STUDIES IN FAMILIAL PD AND MULTIPLE SYSTEM ATROPHY

In collaboration with the Parkinson's Disease Society Brain Research Centre, I was supervised by Dr S Daniel (SED), and examined the *substantia nigra* in a series of 20 cases of pathologically proven familial PD. In addition, cortical sections from 10 cases of pathologically proven MSA (4 OPCA, 6 SND) were examined in order to evaluate the specificity of neuropathological features in familial PD and the corresponding expression of  $\alpha$ -synuclein. The average age of onset of familial PD was 64 (range 44-77 years) and in the MSA cases it was 55 (48-67). Details of family history of PD in most cases were by history and the criteria for inclusion as "a family history" was the presence of PD in a closely affected relative (Parent/sibling/cousin/aunt/uncle). Six cases of MSA were classified histologically as striato-nigral degeneration (SND) and four as olivoponto cerebellar atrophy (OPCA). Alpha-synuclein staining of Lewy bodies was positive in all the cases of familial PD. The GCIs in all 10 cases of MSA were also positive for  $\alpha$ -synuclein protein as originally described elsewhere (Tu *et al.*, 1998). All cases were screened for the two coding mutations G209A and G88C in *SNCA* in 30 cases by restriction digestion of PCR products as described above. The coding region of 4 of the cases of pathologically proven MSA were sequenced by another investigator (SD) as part of this project but no pathological mutations were detected (see appendix 2 for sequencing methodology). Figure 4.3 below shows  $\alpha$ -synuclein staining of Lewy bodies and Lewy neurites in sections of brain from a PD patient in this study. Methodology for immunostaining is described in appendix 4.3, as this was done by SED and Dr Ann Kingsbury of the PDS Brain Bank.

**Fig. 4.3:** Alpha-synuclein staining of a) Lewy bodies, and b) Lewy neurites found in human PD brain. Peroxidase detection of a rabbit polyclonal anti-synuclein antibody forms a brown deposit at the site of positive antibody binding.



## **4.5 CLINICO-GENETIC STUDIES OF THE PARKIN GENE IN A LARGE EU SERIES**

Two studies (study 1 and study 2) were performed with the author (myself) as a clinical collaborator to investigate the numerical importance of Parkin (Kitada *et al.*, 1998a) in a central European population. Study 1 was an initial study performed as soon as Parkin was cloned (Kitada *et al.*, 1998a). Study 2 was performed later on a larger familial series of patient and also included sporadic and juvenile-onset cases (Luecking *et al.*, 2000).

### **4.5.1 Study 1: the first study of Parkin in an EU series with PD**

The study described below is briefly summarized and the sequencing results included for discussion purposes, as it was a clinical collaborative study involving all the EU partners (including myself in the UK). Sequencing of the Parkin gene in this study, however, was done solely by Dr N Abbas, INSERM, Paris (Abbas *et al.*, 1999). From the GSPD collection of affected sibling pairs, thirty-eight families were selected according to the following criteria: i) presence of parkinsonism with good response to L-dopa (>30% improvement) in at least 2 siblings, and absence of excluding criteria such as extensor plantar reflexes, ophthalmoplegia, early (after 2 years of disease evolution) dementia or autonomic failure; ii) onset < 45 years in at least one of sibling; iii) inheritance compatible with autosomal recessive transmission (several patients in a single generation with or without known consanguinity). The families originated from France (n=12), Italy (n=10), Germany (n=7), Great Britain (n=4), Algeria (n=1), Morocco (n=1), The Netherlands (n=1), Portugal (n=1), Vietnam (n=1). Four families from Algeria, France, Italy and Portugal were excluded from sequence analysis because they were found to carry homozygous deletions of either exons 3 or 8-9 (18) or exon 4 (family IT-005).

The patients and unaffected relatives were examined by the designated GSPD investigator in each country (myself in the UK) according to a standardized protocol using the agreed inclusion and exclusion criteria detailed in section 2.2. All patients were videotaped and the clinical data were centralized. Blood samples were taken with informed consent from the patients and their first degree relatives. In order to determine the frequency and diversity of mutations in the Parkin gene as a cause for the AR-JP phenotype in Europe, the 12 coding exons of the Parkin gene were amplified in 35

families with autosomal recessive early onset Parkinsonism (see appendix 2.2 and 4.2). Patients were only sequenced if screening for homozygous deletions in Parkin proved negative. Only the results of the UK families found to be positive for a mutation in the Parkin gene are shown in detail below. Figure 4.5 below shows pedigrees for all UK families with mutations in Parkin from both studies. A clinical summary and composite figure of the mutations detected in Parkin in the whole of the EU series of which this family was a part (table 4.10, figure 4.9) is given for discussion purposes only below as well as.

#### **4.5.2 Clinical phenotype of first UK kindred (UK 086) described with a Parkin mutation**

**II:1** This case was first seen aged 60 years of age with a one year history of gait disturbance, left- sided bradykinesia, rigidity and loss of arm swing and micrographia. Since the age of 53 she had been taking *Procyclidine* for “incoordination & tremor” from her GP. A CT head scan at the time was reported as normal as was a caeruloplasmin level (to exclude Wilson’s disease, a rare but important differential diagnosis of young-onset parkinsonism). At the time of diagnosis of “idiopathic PD” she was started on Sinemet LS. Eighteen months later she began to complain of on-off fluctuations. She developed marked dyskinesias 30 months after her original diagnosis. Surgery for the severe dyskinesias has been declined. Clinically her reflexes were normal.

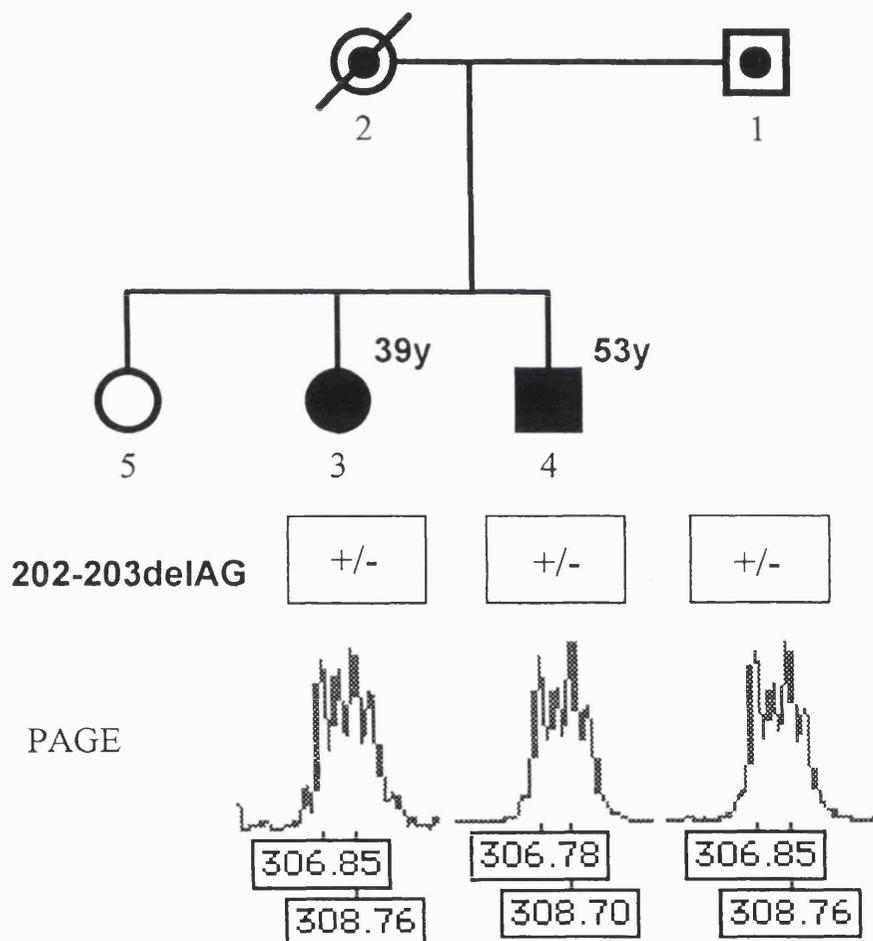
**II:2** The brother of individual II.1 developed an asymmetrical right-sided resting and postural tremor at the age of 42. Ten years later, when first clinically examined by myself, he had developed bilateral signs and described a 2 year history of a shuffling gait and freezing when walking. His illness had always been exquisitely L-dopa responsive (Madopar 125mg TDS) and characterised by early onset marked fluctuations and a non-painful foot dystonia. Clinically his reflexes were normal.

**I:1** The father (not clinically examined) was said to be asymptomatic as was the mother who had died of other causes at the age of 50.

### 4.5.3 Results of Parkin sequencing in UK 086

A pedigree for this family (UK 086) and details of the mutation are given in Figure 4.4.

**Figure 4.4:** Pedigree of 'Parkin' family UK086, the first UK family to show linkage to PARK2. Sequencing of the Parkin gene in this family revealed a heterozygous Gln34Arg change in affected family members as shown.



Sequencing of Parkin was performed by Dr N Abbas at INSERM, Paris as part of the EU consortium and therefore full methodology is provided in appendix 2. Segregation studies on the PARK2 region were also performed in Paris. The results of these studies are therefore provided for discussion purposes. A novel mutation in the Parkin gene was described: a Gln34Arg (stop 37) which is a 2bp deletion in exon 2 resulting in a truncated protein after 3 missense amino acids. The mutation was not homozygous (Abbas *et al.*, 1999). Segregation analysis indicated a mutation on the other allele, suggesting that these patients were compound heterozygotes (figure 4.4). However, the second mutation has not been identified. During the same study this mutation was described in one other Italian family suggesting that it had occurred independently.

In the families UK-086, DE-012 and IT-015, no second heterozygous mutation could be detected by sequencing (Abbas *et al.*, 1999). The rest of the EU Parkin screening results are as published (Abbas *et al.*, 1999). Table 4.3 summarises the EU series (study 1) including the UK kindreds in terms of clinical characteristics and mutation analysis. The entire UK series of families (study 1 and study 2) with mutations in the Parkin gene are clinically summarised in tables 4.4-4.9.

**Table 4.3:** Clinical characteristics of patients from 12 families with Parkin mutations  
(Abbas *et al.*, 1999)

	<b>Deletion</b>	<b>Truncating</b>	<b>Missense</b>	<b>Total</b>
Families (patients)	4 (11)	4 (9)	4(11)	12 (31)
Mean age at onset (range)	33.9 ±16.3 (7-58)	38.2 ± 8.0 (27- 53)	42.5 ± 8.5 (30-56)	38.1 ± 12.1 (7-58)
Mean disease duration (range)	14.8 ± 6.5 (3-26)	16.3 ± 9.4 (4-29)	16.3 ± 8.9 (0.5-31)	15.8 ± 8.0 (0.5-31)
Hoehn and Yahr score	3.4 ± 1.1	2.2 ± 0.9	2.8 ± 0.9	2.8 ± 1.0
Bradykinesia	11/11	8/9	11/11	97%
Rigidity	10/11	9/9	11/11	97%
Tremor	6/11	8/9	7/11	68%
Dystonia	6/11	1/7	0/5	30%
Good response to L-dopa (de novo cases)	10/10 (1)	9/9	9/9 (2)	100%
Dyskinesia	6/10	4/9	8/9	71%
Fluctuations	5/10	3/6	ND	50%
Brisk reflexes lower limbs	4/11	0/6	3/4	33%

#### **4.5.4 Study 2: Further Parkin studies on a sporadic and familial series of UK cases as part of an EU collaboration**

The whole series described below represents the collaborative work achieved on Parkin analysis by the GSPD consortium. Seventy-three families (152 patients with PD and 53 unaffected relatives) were selected according to the following criteria: presence of parkinsonism with  $\geq 30$  percent improvement in response to L-dopa, except for 3 untreated patients; inheritance compatible with autosomal-recessive transmission (affected siblings without affected parents); onset  $\leq 45$  years in at least one of the affected sibs; absence of extensor plantar reflexes, ophthalmoplegia, early dementia or early autonomic failure. The families originated from Italy (n=20), France (n=14), Great Britain (n=12), The Netherlands (n=10), Germany (n=9), Portugal (n=2), and one each from Spain, Algeria, Morocco, Argentina, India and Vietnam. Eight of the families were consanguineous, and 12 were previously reported (Abbas *et al.*, 1999, Lucking, 1998 #4373).

In addition, 100 patients were studied with sporadic PD (8 untreated), mostly European, including 8 from consanguineous marriages, selected according to the same clinical criteria and without family history of PD. The collection of patient samples was in random order to avoid any bias. For each patient, clinical information was collected from the patient or the patient's records and peripheral blood samples obtained for DNA analysis. DNA was extracted from peripheral blood leukocytes according to standard procedures (see section 2.4.1). The above cases with early onset Parkinsonism were screened for Parkin gene mutations using a newly established semiquantitative multiplex PCR protocol followed by genomic sequencing of the coding exons in a subset of patients (see appendix 2.2).

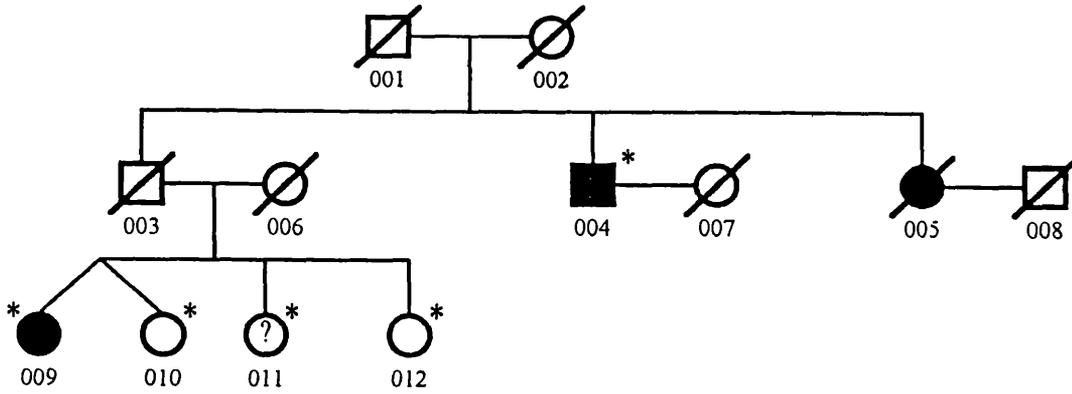
#### **4.5.5 UK Patients and families**

The clinical details of the individuals where DNA was available for sequencing from UK families are summarised below and in tables 4.4 & 4.5 below and comprise a series of isolated and familial cases. All pedigrees of the familial cases collected by myself with mutations in the Parkin gene are shown in figure 4.5. Tables 4.4 & 4.5 only include clinical details on UK 001,040,057 & 088 as UK 086 is described in section

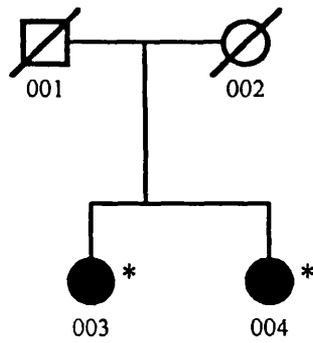
4.5.3 above and UK 401 is described in chapter 5 in more detail as it is one of the major kindreds studied by the author (see section 5.3). I am grateful to AS and NPQ for clinical contribution during the collection of the series of sporadic and juvenile-onset cases (summarised below and in tables 4.6 and 4.7). All of these cases were sequenced for mutations in the Parkin gene at INSERM, Paris by Dr C Luecking. Only the UK results are presented but a brief summary table of results is shown for the EU series for discussion purposes only (Table 4.10).

**Fig. 4.5:** Pedigrees of UK sibling pairs with mutations in the Parkin gene

**UK001**



**UK040**



**UK057**

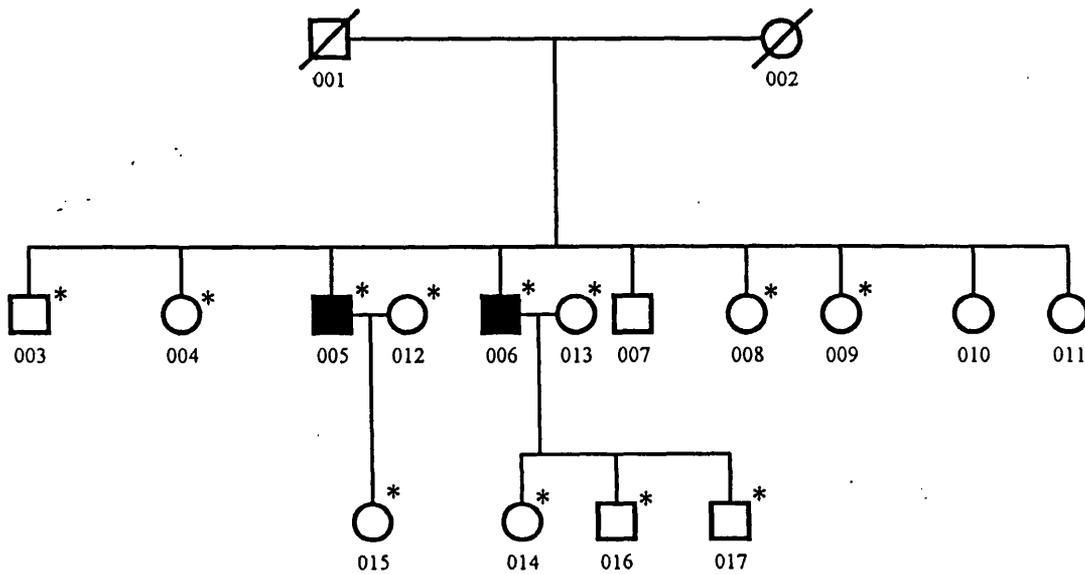
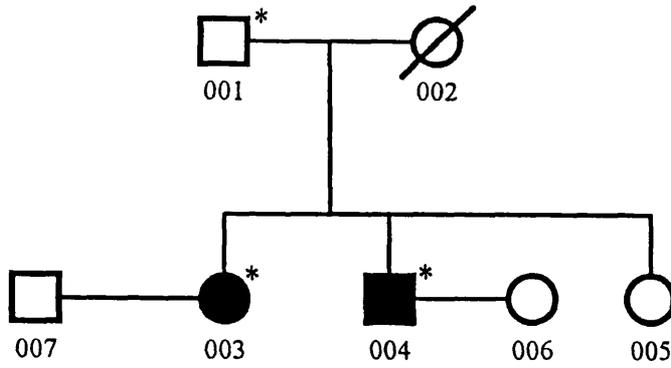
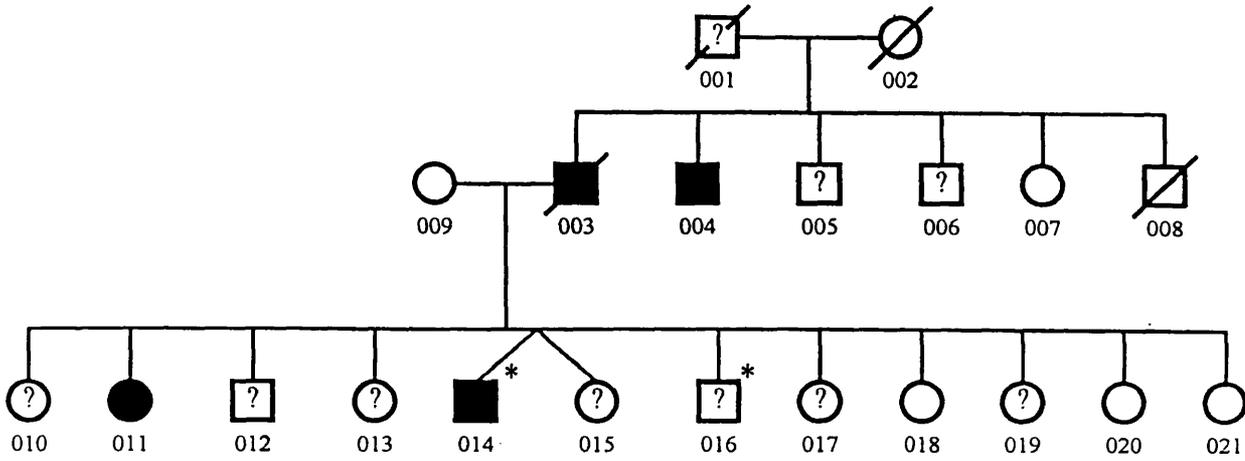


Figure 4.5 (contd.)

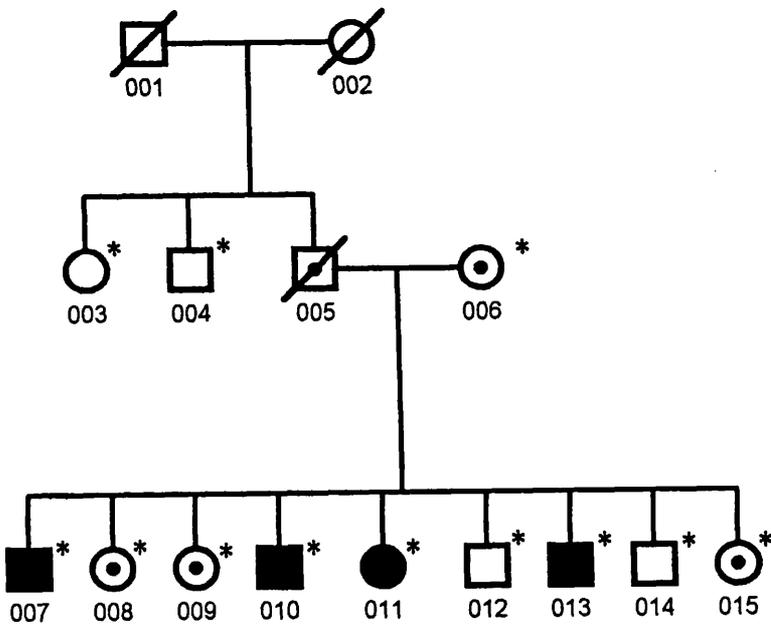
UK086



UK088



UK401



#### *4.5.5.1 Clinical summary of familial cases (sibling pairs) with Parkin mutations*

##### **UK 001**

This family is interesting, not only because of the affected sibling pair of ID 009 and 011 but also the history of parkinsonism in individuals 004 and 005. Individuals 009 and 010 (010 is clinically normal) are dizygotic twins. The index case (009) first developed an asymmetrical rest tremor and poor balance. She then described tremulousness of head, arms and legs, urinary dysfunction and progressive rigidity. Two thalamotomies were performed 11 years after disease onset which improved her tremor. Clinical examination revealed dystonic inturning of both feet in addition to parkinsonism. Urodynamic tests showed a significant residual volume as well as an unstable bladder. Autonomic function tests were normal. PET scanning in this patient revealed low F-Dopa uptake into the putamen and caudate, the caudate being less affected. Individual 011 described a state of “tremulousness” since the age of 20 and had never been treated with L-dopa. Clinical examination at the age of 36 revealed a rapid, fine rest and postural tremor with mild right upper limb cogwheel rigidity. She had poor postural reflexes, a shuffling gait and rigidity which did respond partially to treatment with Pergolide.

Individual 004 developed parkinsonism at the age of 25 after an episode of “encephalitis” which he was told about but did not recall. At onset he developed and insidious, asymmetrical rest tremor involving the right hand and leg which did not progress throughout the course of his illness. Fifty years after this diagnosis he denied symptoms of gait disturbance, rigidity or bradykinesia and was only taking a total dose of 187.5 mg of L-DOPA. He denied diplopia and any history of oculogyric crises. A trial off L-dopa for 3 days resulted in resurgence of tremor, rigidity and a stooped gait. Examination findings revealed micrographia, a prominent rest and postural tremor of the upper limbs. He had a generalised chorea attributable to long-term use of L-dopa. There was no evidence of gait disturbance or bradykinesia. Individual 005 developed upper limb tremor, rigidity and a bradykinetic gait at the age of 65 which was L-dopa responsive. The disease course seems to have been different to that of individual 004 (brother) as at death, 12 years later, family members report that this patient was unable to move out of a chair and that her disease appeared less responsive to L-dopa. The Parkin status on ID 004 is unknown. ID 005 was not clinically examined before death.

### ***UK 040***

The clinical details on this family are as detailed in Table 4.4 & 4.5.

### ***UK 057***

This family of Dutch origin had a total of 3 affected members. Only individuals 005 and 006 were clinically examined. Historical and clinical report from the elder sister (living abroad) revealed symptoms and signs of a parkinsonian phenotype from the age of 38.

### ***UK 086***

See sections 4.5.2 and 4.5.3.

### ***UK 088***

This kindred, of Irish descent had many members describing a tremor but only 2 with definite signs and symptoms of parkinsonism (individuals 011 and 014). At the age of 9 this patient noted dystonic twisting of his right foot whilst playing football. From the age of 12 he developed a tremor in all 4 limbs which was increased on action. From the age of 19 he described painful spasms in the right arm and leg. Wilson's disease was excluded and L-dopa produced an almost complete remission of tremor and dystonic movements of his right limbs. He appeared to be quite susceptible to hypomanic episodes which were attributed to L-dopa. After a disease duration of 30 years he developed 'wearing off' phenomena and a peak dose dystonic walk manifested by twisting movements of the lower back and dystonia of both legs. As well as the other affected sibling (011) other members of the family have a history of tremor and stutter (individuals 010, 012, 013, 015, 016, 017 and 019). Clinical findings are summarised in tables 4.4 and 4.5.

**Table 4.4 (i):** Clinical characteristics of UK familial PD cases with Parkin mutations (A). [Key: ND =No data; 1=symptom present; 0=symptom absent; Micro=Micrographia; LD=L-dopa; Brady = Bradykinesia; MMS= Minimal mental score; UPDRS=Universal PD Ratings System; H&Y=Hoehn-Yahr score; Prob=probably present.

Code	UK001	UK001	UK040 ID 003	UK040 ID 004	UK057 ID 005	UK057 ID 006	UK088 ID 014
Sex	F	F	F	M	M	M	M
Onset	17	30	30	32	32	39	9
Disease Dur.	14	6	8	12	40	31	33
Bradykinesia	0	0	1	0	1	1	1
Rigidity	1	0	1	0	1	1	1
Rest tremor	1	1	0	1	1	1	1
%improv LD	60	ND	50	50	80	80	70
Asymmetry	L	R	R	R	ND	L	R
Onset micro	1	1	1	0	1	1	1
onset brady	1	0	1	0	1	1	
onset tremor	1	1	1	1	1	1	1
MMS	30	30	30	30	25	30	30
Dose LD	500	ND	600	1400	375	ND	468.5
Duration LD	48	ND	8	36	480	240	216
Dyskinesia	1	ND	0	1	1	1	1
UPDRS off	ND	ND	ND	10	ND	ND	ND
UPDRS on	ND	ND	21	ND	42	73	ND
HY (on)	4	0	2	1	3.5	4	ND
HY (off)	ND	2	ND	ND	ND	ND	ND

**Table 4.5 (ii):** Clinical characteristics of UK familial PD cases with Parkin mutations (B). [Key: ND =No data; 1=symptom present; 0=symptom absent; Micro=Micrographia; LD=L-dopa; Brady = Bradykinesia; MMS=Minimental score; UPDRS =Universal PD Ratings System; H&Y= Hoehn-Yahr score; Prob=probably present.

Symptom	UK001 ID 009	UK001 ID 011	UK040 ID 003	UK040 ID 004	UK057 ID 005	UK057 ID 006	UK088 ID 014
Onset Dystonia	1	1	0	0	0	1	1
Site onset dystonia	R foot	L foot	0	0	0	L foot	L leg
Reflexes Brisk	0	1	0	0	1	1	0
Delay Dyskinesia	ND	1	0	0	1	1	1
Fluctuations	ND	1	0	1	0	1	1
Delay fluctuations	ND	ND	0	ND	0	1	1
Dystonia Now	1	1	0	0	0	0	1
Progression	1	2	1	1	1	1	1
Action tremor	1	1	0	ND	1	1	1
Ancestry	English	English	Indian	Indian	Dutch	Dutch	Irish
Consanguinity	0	0	0	0	prob	Prob	0

#### *4.5.5.2 Clinical Summary of sporadic and juvenile onset cases (SJO 1-5)*

In addition to the details below, the clinical summaries are set out in tables 4.6 and 4.7.

##### ***SJO1***

This patient was of Japanese origin. Clinical details are summarised in tables 4.6 and 4.7.

##### ***SJO2***

As well as the clinical details listed in table 4.6, other features of this patients illness were episodic hypomania (attributed to L-dopa). 'Off' pain in the legs was a significant feature.

##### ***SJO3***

Dysphoria was a feature in this patient but it did not manifest as acute hypomania. Prominent motor fluctuations were a feature 15-20 years after taking L-dopa.

##### ***SJO 4***

This patient developed shaking of the arms at 13 as an initial symptom followed 3 years later by becoming generally slow, a tendency to fall backwards and micrographia. A year later he appeared to develop autonomic symptoms of postural faintness, depression and urinary frequency. Clinical examination revealed axial parkinsonism with tremor of the arms and legs at rest. He had a mild sensory neuropathy. Urinary sphincter EMGs were normal but autonomic function tests confirmed autonomic failure with markedly abnormal sympathetic function and relatively normal sympathetic function. There was no clear evidence of central impairment. EMGs revealed pathologically small sural nerve action potentials at 3 and 4 microvolts respectively. The rest of the EMG nerve study was normal compatible with axonal sensory neuropathy. Nerve biopsy confirmed this. Brainstem auditory evoked potentials were compatible with brainstem abnormalities, although an EEG was normal. Brain PET scanning showed marked loss of F-DOPA uptake in both caudate and putamen. The parkinsonism was clearly L-dopa responsive.

**Table 4.6 (i):** Clinical characteristics of sporadic and juvenile onset UK cases with Parkin mutations Key: ND =No data; 1=symptom present; 0=symptom absent; Micro=Micrographia; LD=L-dopa; Brady = Bradykinesia; MMS= Minimental score; UPDRS=Universal PD Ratings System; H&Y=Hoehn-Yahr score; Prob=probably present.

Code	SJO1	SJO2	SJO3	SJO4	SJO5
Sex	M	M	M	M	M
Onset	23	29	24	13	17
Duration of disease	11	24	24	11	24
Bradykinesia	1	1	1	1	1
Rigidity	ND	1	1	1	ND
Rest tremor	1	1	1	1	0
% Improvement with L-dopa	100	100	100	20	70
Asymmetry	1	1	1	1	ND
Onset micrographia	ND	ND	1	ND	ND
Onset bradykinesia	1	1	1	1	ND
Onset tremor	1 (Rt leg)	1	1 (Rt hand)	3 (arms & head)	1
Minimental Score	30	29	30	30	ND
Dose L-dopa	312.5	1500	Nil	Nil	ND
Duration L-dopa	72	240	72	0	240
Dyskinesia	1	1	1	ND	1
H-Y (on)	ND	2	2	ND	ND
H-Y (off)	ND	4	2.5	3	ND

**Table 4.7(ii):** Clinical characteristics of sporadic and juvenile-onset UK cases with Parkin mutations (B). Key: ND =No data; 1=symptom present; 0=symptom absent; Micro=Micrographia; LD=L-dopa; Brady = Bradykinesia; MMS= Minimental score; UPDRS=Universal PD Ratings System; H&Y=Hoehn-Yahr score; Prob=probably present.

Code	SJO1	SJO2	SJO3	SJO4	SJO5
Onset dystonia	1	1	1	1	0
Site onset dystonia	R foot	Both feet	ND	R foot	ND
Reflexes ? brisk	0	0	0	0	ND
Delay dyskinesias	1	1	1	ND	1
Fluctuations	1	1	1	ND	1
Delay fluctuations	1	1	1	1	1
Dystonia	1	1	1	1	ND
Progression	1	2	1	ND	ND
Action tremor	1	ND	ND	ND	ND
Ancestry	Japanese	English	English	English	Portugese
Consanguinity	ND	0	0	0	1

**Table 4.8:** Parkin mutations found in UK families

Family	Allele 1	Allele 2	Type of mutation
UK 001	Cys/Stop268	.	Single heterozygous point mutation
UK 040	Cys334/Cys334	TCTGCdel hom	Homozygous Point mutation
UK 057	Ex3 heterozygous deletion	Ex 4 heterozygous deletion	Compound deletion
UK 088	Arg275Trp heterozygous	Gly430Asp heterozygous	Compound point mutation
UK 401	Ex 8 heterozygous deletion	In 5, splice-site	Compound point mutation & heterozygous deletion

**Table 4.9:** Parkin mutations found in UK sporadic and juvenile-onset cases

Isolated /juvenile cases	Allele 1	Allele 2	Type of mutation
SJO 1	Ex 6 het del	Ex 7 het del	Heterozygous deletion
SJO2	202-203 del AG hom		Homozygous point mutation
SJO3	Arg275Trphet	Ex 5+6 het del	Compound point mutation +deletion
SJO4	Ex3hetdel	Gly430het	Compound point mutation +deletion
SJO5	255delAhom		Homozygous point mutation

**Table 4.10 (& overleaf):** Clinical characteristics of patients with PD due to Parkin mutations (familial and isolated) compared with patients without Parkin mutations (Luecking *et al.*, 2000). Values are means ( $\pm$  SD). P-values are given for the comparison between patients with and without Parkin mutations. A).

		Patients	
		With <i>parkin</i> mutations	without <i>parkin</i> mutations
Number of patients		101	85
Women : Men		49:52	31:54
Age at onset in years		32 $\pm$ 11	42 $\pm$ 11 (P<0.001)
Disease duration in years		17 $\pm$ 11	13 $\pm$ 11 (P=0.002)
<i>Clinical signs</i>			
<b>At onset</b>	Micrography (%)	30	47 (P=0.02)
	Bradykinesia (%)	63	65
	Tremor (%)	65	75
	Dystonia (%)	42	22 (P=0.02)
	Asymmetric signs (%)	89	98 (P=0.02)
<b>At examination</b>	Bradykinesia (%)	95	98
	Rigidity (%)	92	99
	Rest tremor (%)	74	80
	Postural tremor (%)	54	47
	Urinary urgency (%)	11	25 (P=0.01)
	Brisk reflexes (%)	44	21 (P=0.04)
	No or slow progression (%)	88	72
	UPDRS <sup>a</sup> , no treatment	41 $\pm$ 22	43 $\pm$ 16
	UPDRS <sup>a</sup> , during treatment	23 $\pm$ 18	26 $\pm$ 15

		Patients	
		with <i>parkin</i> mutations	without <i>parkin</i> mutations
	Mean Hoehn and Yahr <sup>b</sup> (HY), no treatment	3.2±1	3.1±0.8
	Years to reach HY stage 2, no treatment	11±9	5±3
	Years to reach HY stage 3,	19±10	17±8
	Years to reach HY stage 4,	26±8	33±2
	Years to reach HY stage 5,	40±19	44
	Mini-Mental-State <sup>c</sup>	29±3	28±2
<b>On treatment</b>	Improvement with L-dopa	72±20	64±17 (P=0.03)
	Daily dose of L-dopa	500±340	600±400
(Months)	Duration of L-dopa	123±102	111±99
	Dyskinesias (%)	77	63 (P=0.04)
	Fluctuations (%)	79	65
	(months of treatment)	55±61	61±54
	Dystonia (%)	58	45
	(months of treatment)	65±72	54±40

#### **4.5.6 Analysis of Study 2**

Statistical analysis of the clinical features of patients with and without Parkin gene mutations were compared. A summary paragraph and table of results (table 4.10) is included below for discussion purposes. A summary of the EU series of Parkin mutations found in this study is provided for discussion purposes (see Figure 4.9).

#### **4.5.7 Results summary for the EU series (study 2)**

Among the families with early-onset Parkinson's disease, 36 (49 percent) had Parkin gene mutations. The age at onset ranged from 7 to 58 years. Among the patients with isolated Parkinson's disease, mutations were detected in 10 of 13 patients (77 percent) with onset  $\leq 20$  years, but only in 2 of 64 patients (3 percent) with onset  $> 30$  years. The age at onset in the patients with Parkin gene mutations was lower ( $32 \pm 11$  vs  $42 \pm 11$  years,  $P < 0.001$ ) and, clinically, they more often had symmetric involvement and dystonia at onset, more often hyper-reflexia, responded better to L-dopa and had more frequent dyskinesias during treatment as compared with patients with no mutations. Nineteen different exon rearrangements (deletions and multiplications) were detected, and 16 different point mutations (Luecking *et al.*, 2000).

### **4.6 NEUROPATHOLOGY OF THE FIRST UK BRAIN POSITIVE FOR A PARKIN MUTATION**

#### **4.6.1 Background and patient selection**

This study was undertaken in collaboration with Dr C Luecking, INSERM, Paris (sequencing of Parkin) and Dr S Daniel of the PDS Brain Bank (neuropathological analysis). My own role was responsibility for the clinical collation of the series actually screened for Parkin mutations and extraction of the DNA from each fresh frozen frontal cortex used as part of the series and scientific analysis of the results. AR-JP has been pathologically characterised by highly selective degeneration of dopaminergic neurons in the *zona compacta* of the *substantia nigra* and the absence of Lewy bodies (Ishikawa *et al.*, 1996, Hayashi 2000, Mori 1998, Van de Warrenberg 2001, Yamamura 1993/5). Changes were confined to the *substantia nigra pars compacta* (SNPC) and *locus ceruleus*. The former region showed obvious neuronal loss and gliosis in the medial and

ventrolateral regions. In the remainder of that region and in the *locus ceruleus*, the population of neurons was reduced and there was low melanin content in most of the neurons but no detectable gliosis or extraneuronal free melanin pigment suggestive of a neurodegenerative process (Takahashi *et al.*, 1994). Pathological changes are further discussed in section 4.9.6.

To further investigate the pathology of young-onset PD, a clinico-pathological genetic analysis was undertaken on a series of 16 patients who developed PD below the age of 40, with or without a family history of the disease. All patients had been consented and donated brain tissue to the Parkinson's Disease Society Brain Bank, London. Extraction of genomic DNA from frontal cortex of flash-frozen brain was performed according to the methods described in section 2.4.2. Table 4.11 shows a clinical summary of the patients studied. The criteria used for inclusion were as given in section 2.2.

**Table 4.11:** Clinical characteristics of patients studied in which pathological sections were available for Parkin gene analysis.

Index case	1	2	3	4	5	6	8	9	10	11	12	13	14	15	16
Sex	M	M	F	F	F	F	M	M	F	M	M	M	F	M	M
Age at death	59	75	82	71	82	78	66	80	79	60	77	45	69	66	65
Age at onset	29	42	32	30	38	43	41	46	40	39	52	42	42	36	53
Bradykinesia	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rigidity	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rest tremor	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1
% Improvement	80	N	50	50	50	50	60	50	70	80	60	50	80	70	80
Asymmetry	R	L	R	N	L	R	R	N	L	L	N	R	L	0	L
Onset micrographia	N	N	N	N	N	N	1	N	N	1	N	1	1	1	1
Onset bradykinesia	1	1	1	1	1	1	1	N	1	1	1	1	1	1	1
Onset tremor	1	1	0	1	1	1	0	1	1	0	1	1	1	1	1
Minimal state	30	30	30	30	30	28	28	28	30	23	30	30	30	30	30
Dose L-dopa	82	N	10	N	N	37	82	37	37	10	11	N	15	62	22
Duration L-dopa	30	N	30	24	28	33	21	10	10	18	24	N	27	37	14
Dyskinesias	1	N	1	1	N	1	N	N	N	1	1	N	1	1	0
Hoehn-Yahr	N	N	4	N	N	5	N	N	4	4	N	5	4	4	4
Fluctuations	N	N	N	N	N	N	N	N	N	1	1	N	1	1	1
Delay-fluctuations	N	N	N	N	N	N	N	N	N	1	1	N	1	1	1
Dystonia	1	N	1	N	N	N	N	N	N	1	0	N	N	0	0
Reflex change	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Delay-dyskinesias	N	N	N	N	N	N	N	N	N	1	1	N	1	1	0
Progression	2	1	1	1	1	3	2	2	2	2	2	3	2	2	2

Key to Table 4.11: ND =No data; 1=symptom present; 0=symptom absent

#### **4.6.2 Screening for mutations in the Parkin gene**

Screening for Parkin gene mutations was performed by Dr C Luecking (INSERM 289, Paris) after I had collated the clinical series and extracted genomic DNA from flash frozen frontal cortex using standard techniques. Screening for Parkin gene mutations was first performed in a series of fifteen brains from which genomic DNA had been extracted. Exon dosage was first performed by Dr C Luecking to screen for deletions in the Parkin gene. Parkin gene sequencing was then undertaken on any subject where a detected exon dosage was abnormal. Primers were as published by (Kitada *et al.*, 1998a).

#### **4.6.3 Results of mutation screen**

All pathological observations are summarised in table 4.12. One subject analysed was found to be heterozygous for the known mutation Arg275Trp in exon 7 of the Parkin gene. There was no second mutation detected. An extensive clinico-pathological analysis of the first UK patient found to be heterozygous for the known mutation Arg275Trp in the Parkin gene is described below. Exon dosage screening of all other brains in the series was normal (Dr C Luecking, personal communication).

**Table 4.12:** Pathological summary of UK brains examined for Parkin mutations

country	Family	code	Pathology
UK	1	P12/96	LB, NFT
UK	2	P34/92	NFT
UK	3	P40/98	NFT (possible post-encephalitic PD)
UK	4	P3/91	LBS
UK	5	P35/93	Probable post-encephalitic
UK	6	P23/98	LB, NFT
UK	8	P50/98	LB
UK	9	P3/98	NFT
UK	10	P37/89	SND,NFT
UK	11	P27/96	A few LBs only
UK	12	P7/97	LB
UK	13	P22/91	Severe neuronal loss, gliosis of striatum.No LBs
UK	14	P3/96	LB
UK	15	P72/97	LB
UK	16	P61/87	LB, NFT

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Key: LB= Lewy bodies, NFT=Neurofibrillary tangles,SND=Striatonigral degeneration

#### **4.6.4 Clinical evaluation of a case of PD with a Parkin mutation**

The index case developed PD at the age of 32 with an abrupt onset of oculogyric crises. There was no known family history of Parkinson's disease or past history of tremor. Interestingly the patient was able to recall almost to the day, the exact date of onset of oculogyric crises and denied a preceding history of a 'flu-like' illness or sleep reversal. These crises occurred approximately once per week, responded somewhat to Benzhexol and lasted up to 30 minutes and continued for several years. She then developed a mild

bradykinetic- rigid syndrome without tremor which progressed very slowly. The oculogyric spasms reduced in frequency. There was no significant history of motor fluctuations. Thirteen years after the onset of symptoms, she was started on L-dopa with the early appearance of dyskinesias leading to dose reduction.

When seen 46 years after the onset of her symptoms (aged 78) she was taking 175 mg of L-dopa and bromocriptine 2.5 mg t.d.s. Examination revealed hypomimia, markedly hypometric, slowed saccadic eye movements, occasional jaw tremor, nocturnal jaw spasms and very quiet speech. Tone was mildly increased in her upper limbs, more on the left than the right, with no rest tremor. Postural reflexes were absent and she had started to fall on turning. She was bradykinetic with a shuffling, festinant gait and stooped posture, and had difficulties getting out of chairs. Reduced arm swing (bilateral) was clinically present. She had a Hoehn & Yahr grade IV and UPDRS motor score of 27 several hours after taking L-dopa. Investigations at the time showed a positive apomorphine challenge test and normal CAT scan of the head.

Her clinical course in the final 5 years was characterised by a more rapid progression of parkinsonism: increasing difficulty with gait and balance as well as swallowing and speech difficulties (requiring a percutaneous gastrostomy aged 81). Two falls resulted in bilateral fractured neck of femur. At this time she was taking 300mgs of L-dopa and 5mgs of selegiline daily. Involuntary movements were recorded during the early part of her disease but it was unclear if they represented dyskinesias. Despite her complaint of involuntary nocturnal jaw-clenching (thought to be dyskinesias) widespread involuntary movements were not a feature of her late disease. Post-encephalitic Parkinson's syndrome was felt to be the most probable diagnosis despite the absence of a definite history of encephalitis. She ultimately developed pneumococcal pneumonia and died two years later.

## **4.6.5 Pathology (report courtesy of Dr S Daniel, PDS Brain Bank)**

### *4.6.5.1 Macroscopic examination*

This did not reveal significant cerebral atrophy with no base abnormality of the cranial nerves or major vessels. There was very severe loss of pigmented nigral cells. The locus ceruleus was not identified macroscopically.

### *4.6.5.2 Histological examination*

Examination of sections of cerebrum, brain stem and cerebellum was performed using routine histological staining. The predominant pathology was of neurofibrillary tangles, moderate in number in the cerebral cortex but less frequent in the subcortical nuclei and brain stem sites (Fig. 4.6). In the midbrain, few nerve cells remained in the medial region and occasional neurons were observed to contain tangles. Similar appearances were found in the locus ceruleus. The frontal region and parahippocampus had the most extensive tangle involvement. Occasional tangles were identified in the striatum and pallidum. There were diffuse senile plaques in the striatum. No Lewy body inclusions were present. Tangle pathology was less prevalent in additional sites of predilection in the brainstem and absent from the cerebellar dentate nucleus. Ischaemic damage was present in *basis pontis*.

### *4.6.5.3 Immunohistochemistry for Parkin protein*

Sections of human brain from this PD patient were also immunostained using an anti-Parkin antibody (Figure 4.7, A-D below and see appendix 4.3 for methodology). Particularly striking staining with this antibody was observed in the nuclei of oligodendrocytes from the cerebellum and substantia nigra and in cerebellar neuritic processes.

**Figure 4.6:** Tangle pathology in a section of post-mortem brain from one of the UK familial PD cases (P40/98, index case 3). The section was incubated with an anti-parkin antibody, then peroxidase stained, and counterstained with haematoxylin. Analysis of the Parkin gene in this subject revealed a single Arg275Trp mutation in exon 7.



**Figure 4.7:** Immunostaining for Parkin in human brain from an autopsied case with a Parkin mutation: a) Purkinje cells in cerebellum showing nuclear localisation of Parkin; b) oligodendrocytes of the substantia nigra with nuclear localisation of Parkin; c) Parkin staining of neurites in cerebellum; d) Parkin aggregates in oligodendrocytes of cerebellum.

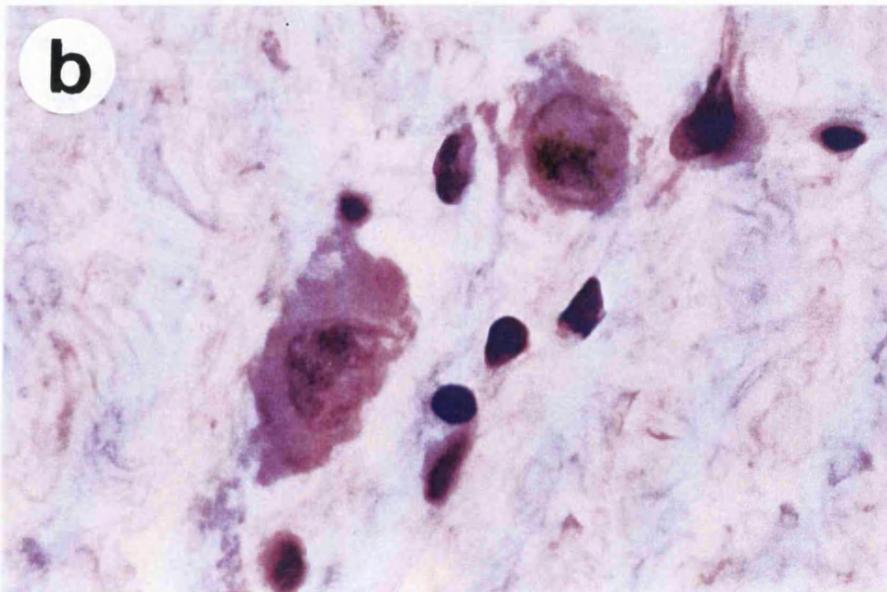
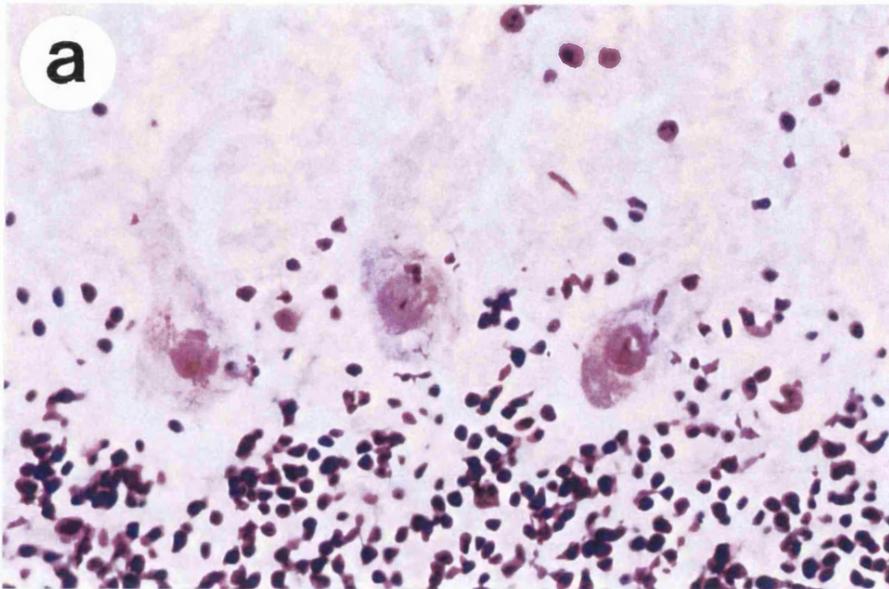
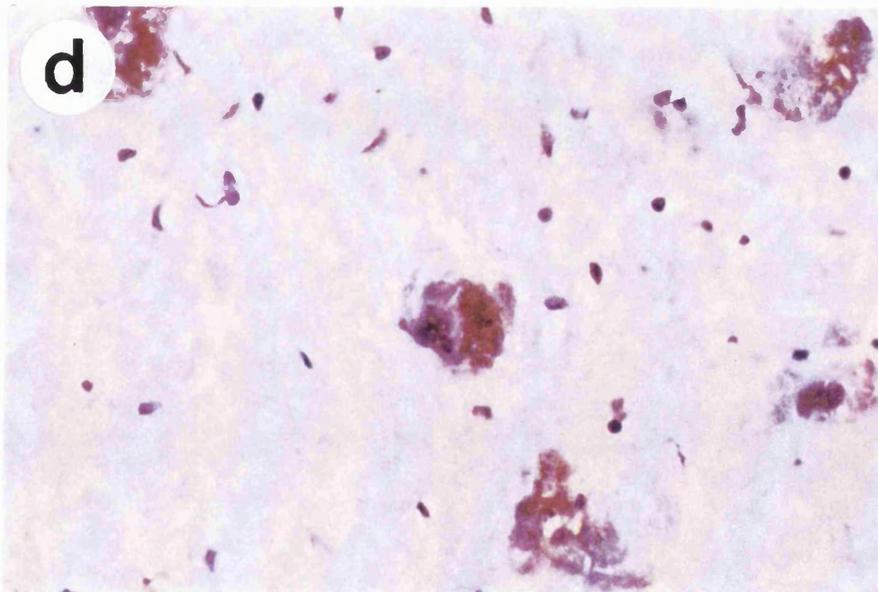
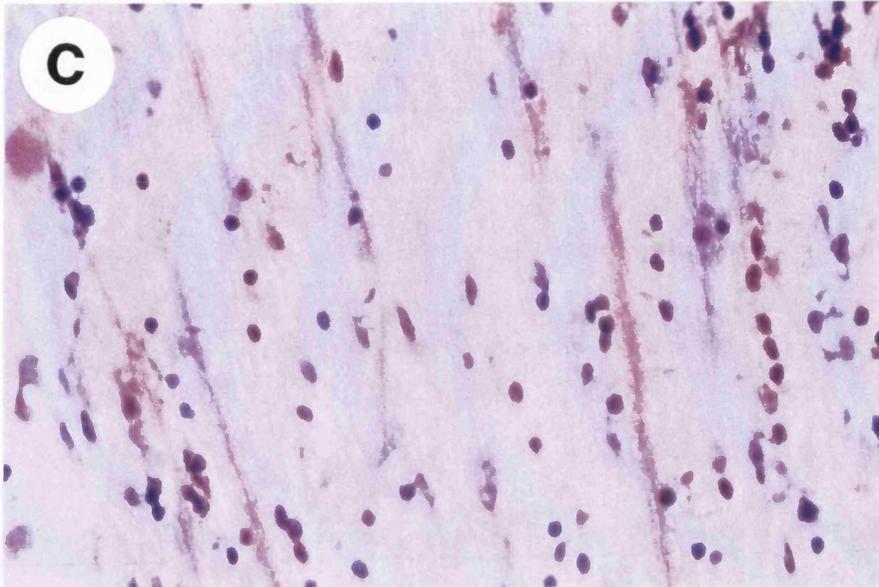


Figure 4.7 (contd.)



## **4.7 UBIQUITIN C-TERMINAL HYDROLASE L1 (UCH-L1)**

### **4.7.1 Aims and design of the study**

A mutation in the UCH-L1 gene has been described in a kindred of German descent (Leroy *et al.*, 1998). The role of UCH-L1 has been described in section 1.14.4. To examine the importance of the Ile93Met mutation in the UCH-L1 gene, two types of study were performed. The first study involved sequencing an index case from families with 3 or more affecteds and a history of PD in either parent. The sequencing of UCH-L1 was solely performed by Dr M Farrer and Dr SJ Lincoln, Jacksonville, (see appendix 4). I was responsible for the clinical collection of seven of the eleven families. Disease segregation within each family was compatible with autosomal dominant transmission. The second study involved sequencing an index case from an affected sibling pair which had been collected as part of the GSPD study. Not all families could be sequenced for UCH-L1 due to resource and time restrictions, therefore the above analysis at 4p14-16.1 was performed by myself to ensure that before UCH-L1 was sequenced in an affected case, segregation of a shared affected haplotype at PARK 4 and a marker linked to UCH-L1 was present in both affecteds from each individual family. UK 401 was screened for mutations in UCH-L1 before it was sequenced for Parkin. The clinical characteristics of all families are described below and the sequencing results briefly summarised for discussion purposes. Appendix 4.1 includes a list of UK families in whom UCH-L1 was sequenced.

### **4.7.2 Sequencing study of UCH-L1 in an index case from 13 autosomal dominant PD families (study 1)**

The clinical details of the eleven families with 3 or more affecteds and a history of PD in either parent are described below. None of these families had a mutation within the SNCA (Farrer *et al.*, 1998, Vaughan *et al.*, 1998a). UK families are coded in a standard way. Any families not of UK origin are described and referenced.

**Family 3** (Lincoln *et al.*, 1999)

This family, of English/Irish descent, had 21 individuals in five generations affected with parkinsonism. Age of onset ranged from the third to the fifth decade, and was typically that of rigidity of a limb and/or resting tremor. L-dopa-responsiveness was usually excellent for approximately five years, after which severe "on/off" fluctuations, dyskinesias, autonomic dysfunction, and marked gait freezing developed. Cognitive dysfunction was variable. Lewy bodies in the *substantia nigra* were confirmed on autopsy in an affected member.

**Family 4** (Lincoln *et al.*, 1999)

The family was of African-American origin, and affected individuals include a brother and sister who developed parkinsonism in their 50s. The disease began in most individuals with resting tremor in an upper extremity and subsequent insidious progression of parkinsonism, including increasing bradykinesia and gait difficulty. Although L-dopa therapy was initially beneficial, dyskinesias developed after about 7 years.

**Family UK 301**

Members of this family presented with a L-dopa-responsive parkinsonian syndrome between the ages of 19 and 71 with age of onset lower in successive generations. All individuals presented with unilateral resting tremor of an upper extremity, with subsequent rapid progression to bilateral involvement. Subjects in later generations developed early dysarthria and upper limb rigidity in addition to the resting tremor.

**UK 401**

This kindred of Irish descent has 5 out of 10 siblings who developed L-dopa-responsive parkinsonian syndrome in the third and fourth decade. The index case presented with a stiff left leg at 32 years. He then became rapidly bradykinetic and suffered falls

secondary to poor balance. F-dopa PET studies on the proband revealed profound impairment of F-dopa re-uptake in the striatum.

### ***UK 003***

A total of 6 affected members, of Welsh descent, developed a parkinsonian syndrome responsive to L-dopa between 50 and 63 years of age. At onset the common presenting symptom was a unilateral resting tremor. Progression of the syndrome was characterised by increasing gait difficulty and later falls.

### ***UK 402 (see section 5.3.2)***

16 of 32 members of this English family developed a L-dopa-responsive parkinsonian syndrome. Age of onset ranged from 42-70 years. The usual mode of presentation was that of asymmetrical resting tremor. Dyskinesias typically developed after 5-7 years. Severely affected members of the kindred had motor fluctuations and gait disturbance.

### ***Family UK 062***

A total of 4 affected members were characterised in this family. The presenting feature was micrographia with subsequent development of a complete L-dopa-responsive parkinsonian syndrome.

### ***FamilyUK 074***

A total of 6 individuals in this English family were affected. The parkinsonian syndrome was L-dopa-responsive. Progression was characterised by motor fluctuations and severe gait difficulties.

### ***Family UK 305***

This family, of English descent included three of four additional siblings with essential tremor. A total of 3 affected individuals in this family had a L-dopa-responsive parkinsonian syndrome.

### ***Family MI*** (Lincoln *et al.*, 1999)

This family of English/Dutch descent had a typical age of onset between 50 and 70 years. Manifestations in five affected members included resting tremor, rigidity, and postural instability. Initially carbidopa/L-dopa had excellent results, but wearing off and dyskinesias developed around eight years later. Three individuals separately suffered from essential tremor.

### ***Family MN*** (Lincoln *et al.*, 1999)

This family, of Dutch/Norwegian descent, typically had an age of onset ranging from 60 to 75 years. A total of five individuals in two generations had PD. The findings on examination included resting tremor, micrographia, and later, gait difficulties. L-dopa responsiveness persisted throughout the typical course of 15 years, with some wearing off phenomena and dyskinesias. Dementia was not seen.

### **4.7.3 PARK4 analysis and sequencing study of UCH-L1 in an index case from 11 affected sibling pairs (study 2)**

European Caucasian families with PD were included in the study. In all families at least 2 affected sibs in each family were present. PD was diagnosed using the rigorous criteria of idiopathic PD according to a similar study design as described elsewhere (Maraganore *et al.*, 1991). All patients gave their informed consent according to the declaration of Helsinki. As part of the ongoing total genome screen in families with PD, 96 affected families were tested for allele sharing on chromosome 4p by myself. The markers used to test for allele sharing on chromosome 4p were D4S230, D4S1609,

D4S391, D4S2397 and D4S405 (UCH-L1), spanning 12.4cM telomeric to centromeric on the Genethon map.

#### **4.7.4 Mutation analysis of UCH-L1 (study 1)**

No mutations in the coding region of the UCH-L1 gene were found in 11 families with PD in which it was sequenced. However, one major coding polymorphism (S18Y) and several common, non-coding, promotor (-16(Y)/-24(R)) and intronic variants (Ex2+6(Y) and +19(M)) were found in several affected individuals (these included one Dutch and one Italian affected sibling) (Lincoln *et al.*, 1999). The frequency of the S18Y allele was 23% from study 1, estimated from 110 individuals without documented movement disorder. It was lower in the small population of affected sibling pairs screened (see study 2 below). This difference is probably a chance finding due to small sample size. The alternative explanation is that the observation is secondary to a genetic background with different allele frequencies (Harhangi *et al.*, 1999).

#### **4.7.5 Mutation analysis of UCH-L1 (study 2)**

Twenty-nine out of 96 GSPD families showed a pattern of inheritance consistent with autosomal dominant transmission (as in the original report (Leroy *et al.*, 1998)). Mutation analysis (see appendix 2) was performed on an index case, by our collaborators at The Mayo Clinic, from those families in which 2 affected siblings shared a haplotype for all five markers (a total of 11 out of the remaining 29 families). The characteristics and country of origin of the individuals tested for mutations in UCH-L1 are listed in Table 4.13 for discussion purposes. In total 11 index cases were sequenced for mutations in UCH-L1 gene from GSPD affected sib pair families. In the affected sibling pairs, the overall mean age at onset of PD was 55.2 $\pm$  8.1 years with a mean duration of illness of 10.9 $\pm$  9.4 years.

**Table 4.13:** Characteristics and origin of the individuals sequenced for the UCH-L1 gene

Origin	No. of families	Individuals sequenced	Mean age of onset years (SD)	Mean duration of illness years (SD)
French	4	4	57.0 (9.3)	12.8 (14.5)
German	1	1	51.0	18.0
Italian	4	4	56.5 (7.3)	7.5 (5.8)
Dutch	2	2	51.0 (12.7)	10.5 (6.4)

## 4.8 DISCUSSION: GSPD COLLABORATIVE ANALYSIS OF THE SNCA, UCH-L1 AND PARKIN GENES

### 4.8.1 Evaluation of the importance of single genes in familial PD

The collection of patient samples as part of the EU consortium allowed the significance of mutations described in SNCA (PARK1), Parkin (PARK2), and UCH-L1 (4p14) to be evaluated in a large population of EU sibling pairs and a series of autosomal dominant PD kindreds. Clearly, for some rare PD kindreds, SNCA mutations are solely causative for the disease. In section 4.3, I described how this gene was screened in an index case from each sibling pair included in this study and how none of the published mutations were found in any of our families. Hence, we concluded that mutations in SNCA (PARK1) were likely to be a very rare cause of familial PD, and consequently that familial PD is genetically heterogeneous (Gasser *et al.*, 1997). Several families reported in this original linkage paper (Gasser *et al.*, 1997) were subsequently sequenced for mutations in the entire SNCA coding region, but no mutations were found (Vaughan *et al.*, 1998b, see appendix 3.3). It could be argued that sequencing a gene in the region of PARK1 in families where linkage to this locus had been excluded was experimentally flawed. However, many of these families were small in size and therefore the power to generate odds for linkage small. One of the German families (family K) actually

showed a lod score of 1.5 at the PARK1 locus (Gasser *et al.*, 1997), but no mutations in SNCA were found in this family (Vaughan *et al.*, 1998b).

#### 4.8.2 The initial controversy surrounding the SNCA gene

In the absence of haplotype data, the case for the first mutation described in SNCA (G209A in exon 4) being causative for the parkinsonian phenotype in the Contursi kindred was controversial. While the authors reported the same amino acid change in the Contursi kindred and in three apparently unrelated Greek kindreds, it remained a possibility that the families may have shared a common origin as the two geographical locations (southern Italy and the Peloponnese) were formerly connected by a trade route (Polymeropoulos *et al.*, 1997). A founder effect therefore could not be excluded (Vaughan *et al.*, 1998b). Indeed, this was reported in subsequent studies of the Greek kindreds (Athanasiadou *et al.*, 1999). This latter author studied 10 polymorphic markers linked to the PARK1 locus and found a shared haplotype indicating a founder chromosome shared by the southern Italian family (Contursi kindred) originally reported by (Polymeropoulos *et al.*, 1997) and the 7 Greek families that carried the Ala53Thr mutation. Clinically, the Ala53Thr cases, in addition to early age at onset, showed prominent bradykinesia and muscular rigidity but rarely had tremor, i.e. a similar phenotype to the Italian cases. All 7 Greek families with PD originated from 3 villages of the northern Peloponnese in Greece and 6 of the families were from 2 villages only 17 km apart.

Numerous investigators, including ourselves, have failed to detect the G209A (Ala53Thr) mutation in SNCA in patients with sporadic or familial PD (Bennett and Nicholl, 1998, Farrer *et al.*, 1998, Vaughan *et al.*, 1998a, Vaughan *et al.*, 1998b, Zarepari *et al.*, 1998). In addition, this amino acid at position 53 is not conserved between mammalian species in contrast with neighbouring residues and, in rats, a threonine at position 53 is the native sequence. The zebra finch homologue, synelfin, also carries a threonine at position 53 whereas both *Bos taurus* and *Torpedo californica* do not (Maroteaux and Scheller, 1991). There are no reports that suggest the presence of Lewy bodies in the brains of the rat or zebra finch or a phenotype resembling that of PD. Various reasons for this lack of phenotype have been proposed, including the short life-span of rodents and the need for an interaction with other cellular components not

present in these animals (Polymeropoulos *et al.*, 1997). To test whether the alanine/threonine substitution at this position in the Contursi kindred was a neutral (perhaps ancestral) variant, fellow investigators at the Mayo clinic (MF, SL) sequenced exons 3 to 5 of African Green monkey SNCA cDNA and found that monkey and man have an identical sequence (S.J. Lincoln, personal communication). Therefore, the Contursi Ala53Thr mutation does not reflect the ancestral human gene.

The possibility that SNCA was in linkage disequilibrium with the true causative gene defect was also considered before publication of the G88C mutation in exon 3. In support of this, a previously reported German family (Family K) had a lod score of 1.5 at the PARK1 locus, which is close to the theoretical maximum for this small family (Gasser *et al.*, 1997), yet in the present study no mutations were identified in the exons of SNCA when this family was sequenced (see Fig. 4.2). This was used to argue the case initially that other genes in the region might be responsible for the parkinsonian syndrome in the Contursi kindred (Vaughan *et al.*, 1998b). The fact that histopathological studies of brainstem and cortex from patients with sporadic PD and dementia with Lewy bodies showed that Lewy bodies are strongly immunoreactive for  $\alpha$ -synuclein (Spillantini *et al.*, 1997) helped argue the case for SNCA being the causative gene. Nevertheless, as Lewy bodies are composed of many different proteins, the presence of  $\alpha$ -synuclein in these structures did not necessarily implicate it in their formation. After the second SNCA mutation (G88C in exon 3) was confirmed in an independent German kindred, it seemed more likely that alpha-synuclein is involved in the pathogenesis of PD, although SNCA mutations as a cause of familial PD remain apparently very rare (Kruger *et al.*, 1998).

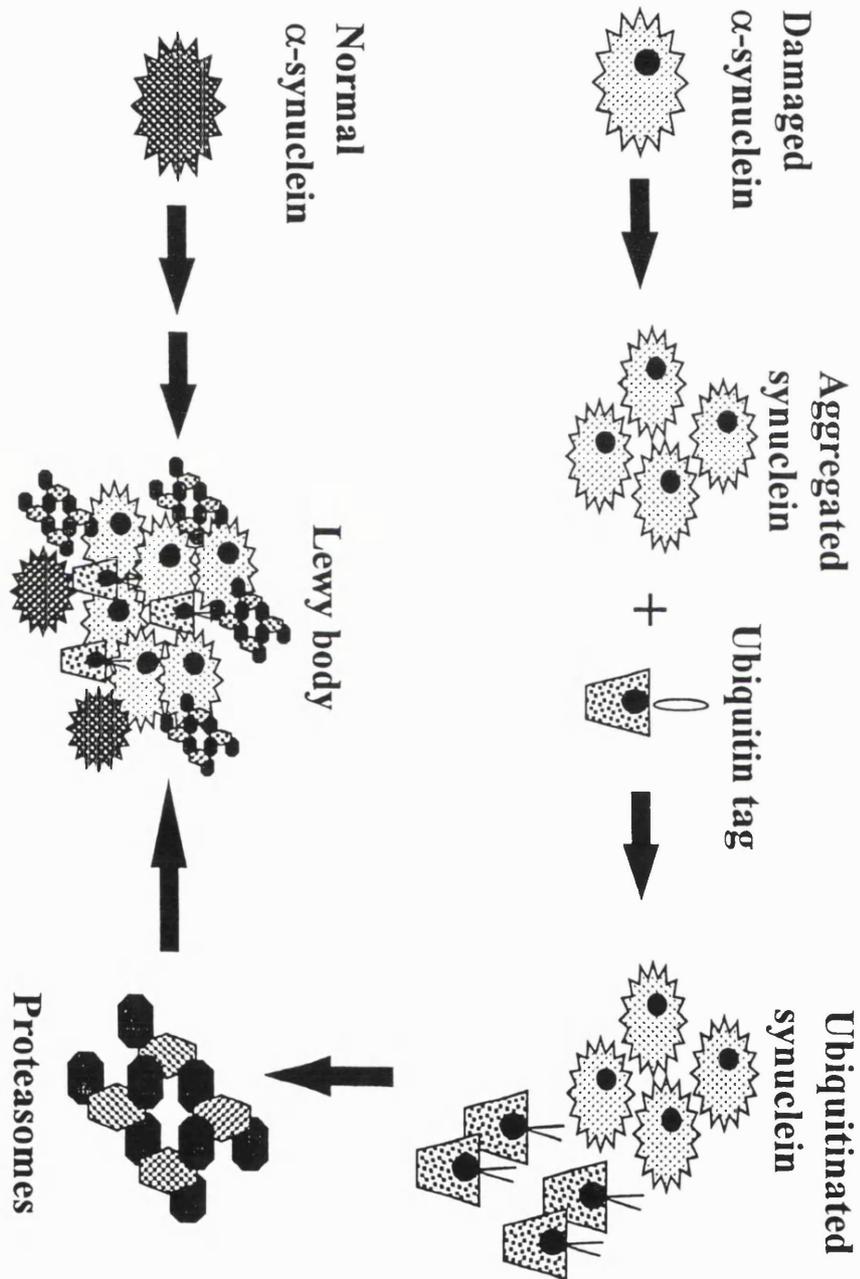
#### **4.8.3 The role of $\alpha$ -synuclein in Parkinson's disease**

It was previously unclear whether Lewy body formation represented a primary step in the pathogenesis of PD or if it was simply a collection of proteins which reflected the final part of a complex disease process, thereby acting as a secondary marker for PD. It is clear that Lewy body formation is not an essential feature accompanying dopaminergic neuronal death, but the mechanism underlying the selective neuronal loss and Lewy body formation remains unknown (Hattori and Mizuno, 1999). Nevertheless, the discovery of mutations in SNCA and the association of  $\alpha$ -synuclein with brain

stem-type and cortical Lewy bodies in Parkinson disease and Lewy body dementia (Spillantini *et al.*, 1997) may indicate a primary role for  $\alpha$ -synuclein (Fig. 4.3). Analysis of the staining pattern of  $\alpha$ -synuclein in Lewy bodies led investigators to propose that intact  $\alpha$ -synuclein is a major structural component of LBs and that abnormal aggregation of  $\alpha$ -synuclein into filamentous structures may actually precede aggregation of neurofilaments and ubiquitination. This is the basis of a new model for the pathogenesis of PD (Figure 4.8 below) (Spillantini *et al.*, 1997).

Conformational abnormalities leading to aggregation and deposition of proteins are a central feature in several neurodegenerative disorders (Tran and Miller, 1999). It is generally thought that aggregation of mutated proteins leads to the accumulation of deposits which are toxic to cell physiology leading to death of the affected neurons. Indeed, this is emerging as a common, but poorly understood pathogenic theme in both sporadic and hereditary neurodegenerative diseases. Alpha-synuclein appears to be natively unfolded and therefore lacks defined secondary structure.

**Figure 4.8:** A model of how both normal and abnormal  $\alpha$ -synuclein proteins could lead to Lewy body formation in PD.



In both the G209A and G88C mutations in SNCA, a hydrophobic residue substitutes for alanine. Both mutations occur in a coding region linking repeats of a consensus sequence of amino acids, KTKEGV. The substitution in the Contursi kindred lies between repeats 3 and 4, whereas that of the German kindred occurs between repeats 2 and 3. The two protein defects might therefore produce a similar change in the structure and/or interactions of  $\alpha$ -synuclein (Golbe *et al.*, 1999a). For instance, expression of the mutant protein may cause disruption of the alpha helix, resulting in the extension of beta sheet structure. This may then lead to increased self-aggregation of protein, abnormal transport, and accumulation of synaptic proteins. Expression studies with  $\alpha$ -synuclein in the rat optic system have shown that a portion of  $\alpha$ -synuclein is carried by the vesicle-moving fast component of axonal transport and that it binds to rat brain vesicles through its amino-terminal repeat region. Alpha-synuclein with the Ala30Pro mutation was found to be devoid of vesicle-binding activity, leading the authors to speculate that it is then prone to assembly into Lewy body filaments (Jensen *et al.*, 1998).

Some groups have suggested that abnormalities of  $\alpha$ -synuclein metabolism may have a direct toxic effect, as over-expression of wild-type and mutant forms of  $\alpha$ -synuclein in cultured neurons causes apoptosis (Saha *et al.*, 2000). Interestingly, unusual high molecular weight  $\alpha$ -synuclein-immunoreactive proteins have been described in brain homogenates from an affected family covering a broad clinical phenotype, ranging from PD to dementia with psychosis. Distinctive histopathology was revealed with  $\alpha$ -synuclein immunostaining (see also Figure 4.3), including pleomorphic Lewy bodies, synuclein-positive glial cell inclusions and widespread, severe neuritic dystrophy (Gwinn-Hardy *et al.*, 2000a). Many investigators have suggested that  $\alpha$ -synuclein may provide a link between PD and Alzheimer's disease, as well as other neurodegenerative diseases (Heintz and Zoghbi, 1997). To date, no patients with sporadic PD have been found to carry the Ala53Thr mutation (Vaughan *et al.*, 1998a).

#### **4.8.4 Alpha-synuclein and Multiple System Atrophy**

Alpha-synuclein has also been found to be a major component of glial cell inclusions in multiple system atrophy (MSA) (Spillantini *et al.*, 1997; Tu *et al.*, 1998). Subsequent to our study a further report found that alpha-synuclein was specifically localized to the

filaments in GCIs and that insoluble  $\alpha$ -synuclein accumulated selectively in MSA white matter. The general conclusion from our study and this further report is that a reduction in the solubility of  $\alpha$ -synuclein may induce this protein to form filaments that aggregate into cytoplasmic inclusions, which contribute to the dysfunction or death of glial cells as well as neurons in neurodegenerative disorders with different phenotypes. Given what is known about  $\alpha$ -synuclein expression, the abundance of  $\alpha$ -synuclein in GCIs is surprising. This may point to a selective upregulation in the expression of  $\alpha$ -synuclein in oligodendrocytes in MSA or to an impairment in the ability of these cells to degrade  $\alpha$ -synuclein, which they may produce at very low levels. Separate other conclusions can be drawn from two populations. In the familial PD sample other genes may interact with wild-type  $\alpha$ -synuclein to induce Lewy body formation.

MSA is considered to be a sporadic disease in view of the rarity of a family history, age at onset. No SNCA mutations were found in the MSA cases. One can conclude that either non-coding mutations in SNCA are responsible, or that post-translational modifications may alter the biophysical properties of  $\alpha$ -synuclein and play a mechanistic role in the formation of Lewy bodies and GCIs.

#### **4.8.5 In vitro studies of the $\alpha$ -synuclein mutants**

The formation of  $\alpha$ -synuclein aggregates could be a critical step in the pathogenesis of PD, which is accelerated by mutations. (Narhi *et al.*, 1999) presented evidence related to the pathogenic mechanism of Parkinson disease caused by the 2 known mutant proteins, Ala30Pro and Ala53Thr. They showed that both wild type and mutant  $\alpha$ -synuclein form insoluble fibrillar aggregates with antiparallel beta-sheet structure upon incubation at physiological temperature *in vitro*. Their studies showed that the lag time for the formation of aggregates was about 280 hours for the wild type protein, 180 hours for the Ala30Pro mutant protein, and only 100 hours for the Ala53Thr mutant protein, i.e. aggregate formation appeared to be accelerated by both Parkinson disease-linked mutations. In another *in vitro* study, (Conway *et al.*, 2000) compared the rates of disappearance of monomeric  $\alpha$ -synuclein and appearance of fibrillar  $\alpha$ -synuclein for the wild type and 2 mutant proteins, Ala53Thr and Ala 30 Pro, as well as equimolar mixtures that may model heterozygous Parkinson disease patients. Whereas Ala53Thr and an equimolar mixture of Ala53Thr and wild type fibrillised more rapidly than wild

type  $\alpha$ -synuclein, the Ala30Pro mutation and its corresponding equimolar mixture with wild type formed fibrils more slowly. However, under conditions that ultimately produced fibrils, the Ala30Pro monomer was consumed at a comparable rate or slightly more rapidly than the wild type monomer, whereas Ala53Thr was consumed most rapidly. The difference between these trends suggested the existence of nonfibrillar  $\alpha$ -synuclein oligomers. Conway *et al.*, (2000) concluded that drug candidates that inhibit  $\alpha$ -synuclein fibrillisation but do not block its oligomerisation could mimic the Ala30Pro mutation and may therefore accelerate disease progression. The PD-associated mutations (Ala30Pro and Ala53Thr) have no effect on membrane interaction, but the Ala30Pro mutation is thought to alter the three-dimensional conformation of  $\alpha$ -synuclein (McLean PJ *et al.*, 2000).

To study the mechanism of action through which mutant  $\alpha$ -synuclein toxicity is mediated, cell models expressing wild or mutant (G209A)  $\alpha$ -synuclein were examined (Tabrizi *et al.*, 2000). Increased expression of either wild-type or mutant  $\alpha$ -synuclein resulted in the formation of cytoplasmic aggregates which were associated with the vesicular compartment. Expression of mutant  $\alpha$ -synuclein was found to cause increased susceptibility to dopamine toxicity compared with wild-type protein expression. The authors speculated that similar mechanisms may underlie or contribute to cell death in sporadic PD (Tabrizi *et al.*, 2000).

Further experimental studies have examined the potential link between aggregated  $\alpha$ -synuclein proteins forming brain lesions in neurodegenerative synucleinopathies, and oxidative stress, implicated in the pathogenesis of some of these disorders. (Giasson *et al.*, 1999) used antibodies to specific nitrated tyrosine residues in  $\alpha$ -synuclein to demonstrate extensive and widespread accumulation of nitrated  $\alpha$ -synuclein in the signature inclusions of PD, dementia with Lewy bodies, the Lewy body variant of Alzheimer disease, and multiple system atrophy brains. The authors also showed that nitrated  $\alpha$ -synuclein is present in the major filamentous building blocks of these inclusions, as well as in the insoluble fractions of affected brain regions of synucleinopathies. The selected and specific nitration of  $\alpha$ -synuclein in these disorders provides evidence to directly link oxidative and nitrative damage to the onset and progression of neurodegenerative synucleinopathies.

#### 4.8.6 Animal models

Lewy pathology, neuronal degeneration and motor deficits have been observed in transgenic mouse models expressing Ala53Thr mutant human  $\alpha$ -synuclein despite a lack of transgene expression in dopaminergic neurons of the *substantia nigra* (van der Putten, 2000).

Evidence that  $\alpha$ -synuclein is an essential presynaptic, activity-dependent negative regulator of dopamine neurotransmission was reported in studies of homozygous mouse models deleted for  $\alpha$ -synuclein by targeted disruption. Alpha-synuclein knockout (-/-) mice exhibited intact brain architecture with a normal number of dopaminergic cell bodies, fibers, and synapses (Abeliovich *et al.*, 2000). Nigrostriatal terminals of  $\alpha$ -synuclein<sup>-/-</sup> mice displayed a standard pattern of dopamine discharge and reuptake in response to simple electrical stimulation. However, they exhibited an increased release with paired stimuli that could be mimicked by elevated calcium. Concurrent with the altered dopamine release,  $\alpha$ -synuclein<sup>-/-</sup> mice displayed a reduction in striatal dopamine and an attenuation of dopamine-dependent locomotor response to amphetamine.

Transgenic mice that expressed wild type  $\alpha$ -synuclein under the control of the PDGF- $\beta$  promoter (expressed in all neurons) were developed by Masliah *et al.*, 2000. Neuronal expression of human  $\alpha$ -synuclein resulted in progressive accumulation of  $\alpha$ -synuclein and ubiquitin-immunoreactive inclusions in neurons in the *neocortex*, *hippocampus*, and *substantia nigra*. Ultrastructural analysis revealed both electron-dense intranuclear deposits and cytoplasmic inclusions. These alterations were associated with loss of dopaminergic terminals in the basal ganglia and with motor impairments. Masliah *et al.*, 2000 concluded that accumulation of wild type  $\alpha$ -synuclein may play a causal role in Parkinson disease and related conditions.

Feany and Bender, 2000 produced transgenic fly lines that produced normal human  $\alpha$ -synuclein and separate lines with each of the 2 mutant proteins linked to familial Parkinson disease, Ala30Pro and Ala53Thr  $\alpha$ -synuclein. Pan-neural expression of human  $\alpha$ -synuclein resulted in adult-onset loss of dopaminergic neurons, filamentous intraneuronal inclusions containing  $\alpha$ -synuclein reminiscent of Lewy bodies, and locomotor dysfunction. *Drosophila* expressing the Ala30Pro  $\alpha$ -synuclein lost their climbing ability earlier than flies expressing wild type or Ala53Thr  $\alpha$ -synuclein. However, all transgenic flies showed premature loss of climbing ability. In addition to

degenerative changes in the brain, retinal degeneration also occurred when  $\alpha$ -synuclein was expressed specifically in the eye. Expression of wild type or mutant  $\alpha$ -synuclein during development of the eye produced no effect. However, continued expression of  $\alpha$ -synuclein in the adult produced retinal degeneration that was detectable by 10 days and marked at 30 days in transgenic flies expressing wild type, Ala30Pro, or Ala53Thr  $\alpha$ -synuclein.

It has been proposed that MSA and familial PD may all be part of a growing list of disorders “the  $\alpha$ -synucleinopathies”. While direct mutations of  $\alpha$ -synuclein may be involved in the formation of Lewy bodies, wild-type  $\alpha$ -synuclein expression and function could also be modified by as yet undefined mutations in other genes, such as UCH-L1 and SNCAIP. The involvement of UCH-L1 and Parkin in familial PD is discussed below prior to a discussion of how interactions of all three genes may be involved in the pathogenesis of PD.

#### **4.9 WHAT CAN PARKIN TELL US ABOUT THE AETIOLOGY OF PD?**

In the studies described in section 4.5 above, it was found that mutations in Parkin are a common cause of autosomal recessive young-onset PD in our large population of EU families. Interestingly, parkinsonism results from a wide range of mutations in the Parkin gene.

##### **4.9.1 GSPD mutations in Parkin: Study 1**

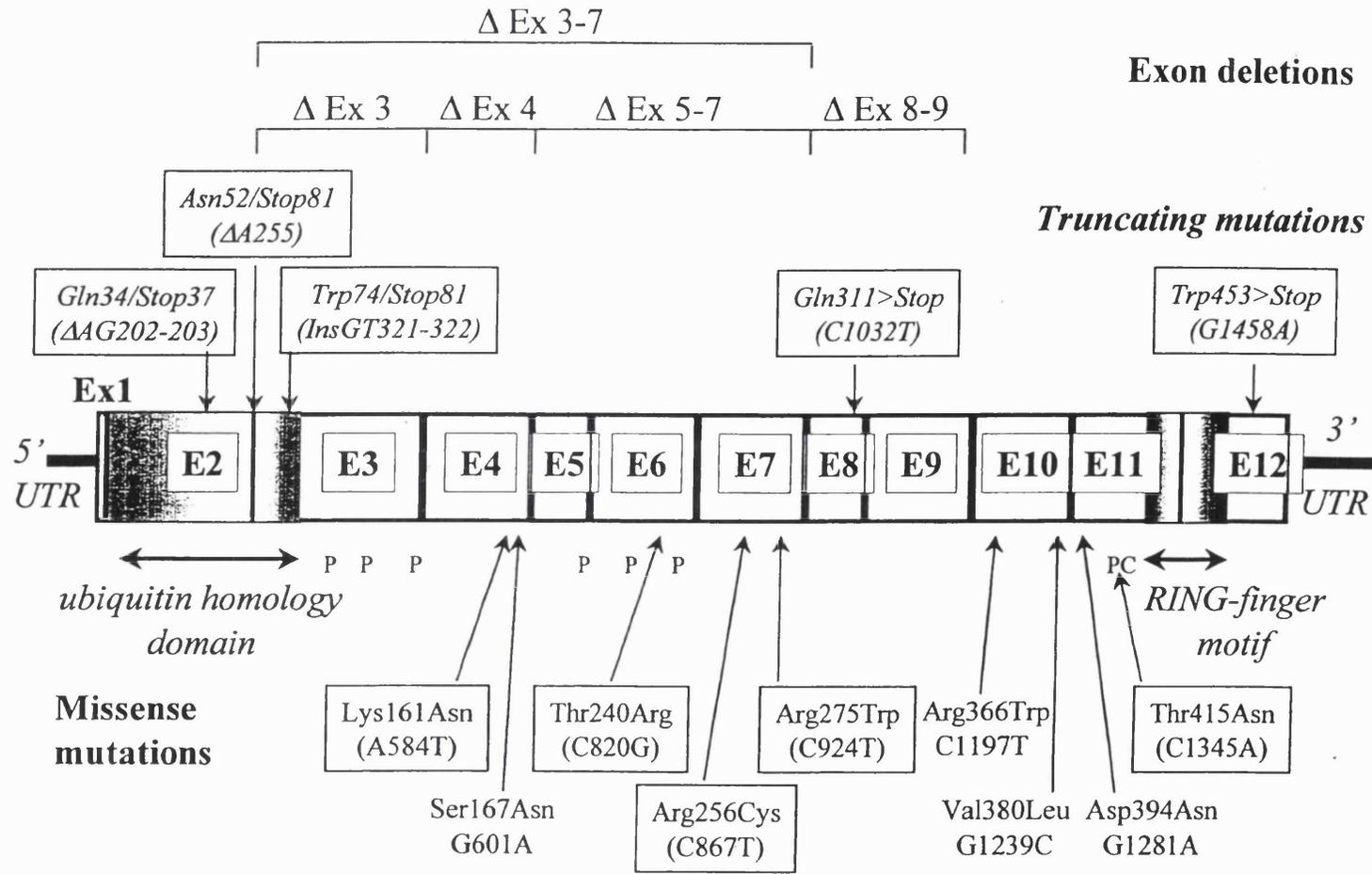
As part of study 1 the Parkin gene was examined in a series of patients from the UK collected by myself as part of the GSPD consortium. Secondly, analysis of the EU series (done in collaboration with Dr N Abbas, INSERM, Paris) showed that various point mutations in the Parkin gene are a common cause of autosomal recessive parkinsonism in Europe and seem to be more frequent than the exon deletions so far described. Furthermore, mutations in the Parkin gene are associated with a wide range of ages at onset as well as of clinical signs and can result in familial parkinsonism clinically indistinguishable from idiopathic PD. The mutations detected are diverse in their effect on the Parkin protein (i.e. deletions, missense, and nonsense mutations) as

well as in their localisation within the gene. The diversity of mutations and the absence of a mutational hot spot will complicate molecular diagnosis, but the relative importance of the altered amino acids should help to analyse the function, still unknown, of the Parkin protein.

While the mean age at onset was 38, typical of early onset PD, onset occurred after age 40 in 13 patients. Dystonia or brisk reflexes were less frequent than previously reported (10) (Hattori *et al.*, 1998c, Ishikawa, 1996. #4749). Overall, the phenotypes of patients classified according to the effect of the mutations on the Parkin protein were similar, although the earlier ages at onset, 7 to 18 years, were observed in the Algerian family with deletion of exons 8 and 9 (Lucking *et al.*, 1998). Similarly, early onset was also observed in the Japanese family with deletion of exon 3-7 (Kitada *et al.*, 1998a) as well as in the patient with the Gln311Stop mutation (Hattori *et al.*, 1998c), raising the question of the functional consequences of exon deletions and truncating events in specific regions of the Parkin protein, especially as onset within the group with truncating mutations was later (between 27 and 53 years).

Different deletions of the Parkin gene can cause AR-JP (see Figure 4.9 below), with age at onset as late as 58, as observed in the Italian family IT-005. Despite the fact that initial reports described homozygous deletions of exon 4 or 3-7 in 4 Japanese families (Kitada *et al.*, 1998a), we found homozygous deletions of exon 3 or 8-9 in two European and one Algerian family in this study (Lucking *et al.*, 1998) and the newly identified exon 4 deletion in an Italian family, as well as concluding that point mutations are a more common cause of AR-JP (Abbas *et al.*, 1999). Previously reported point mutations include two homozygous single base pair substitutions in two consanguineous Turkish families which resulted in a Thr240Arg missense mutation in exon 6 and an Ala311Stop nonsense mutation in exon 8 (Hattori *et al.*, 1998b). Taken together, 3 families with previously reported exon deletions (Lucking *et al.*, 1998), and the 9 families with mutations detected in the present study demonstrate that mutations in the Parkin gene are the cause of the disease in approximately 30% of families with AR-JP analysed (12/38), including 11 of the 35 from Europe. Point mutations were detected in 8 out of 12 families, and thus seem to be more frequent than homozygous exon deletions (4 of 12 families). All the point mutations described are novel and show that a wide variety of different mutations in the Parkin gene can account for the disease.

**Figure 4.9:** Schematic illustration of mutations reported to date across the coding region of the Parkin gene. (P represents putative phosphorylation sites on the protein).



#### 4.9.2 Pathogenic role of Parkin mutations (study 1)

The pathogenic role of the point mutations was shown by: i) co-segregation of the mutations with the disorder in the families and ii) their absence in a large number of control chromosomes (110 to 166 depending on the mutation). All the point mutations identified in the EU series are likely to have major functional consequences. For the purposes of discussion, the analysis described in this section will include comment on all of the EU series, although my sole contribution was just the UK series.

The four truncating mutations (202-203delAG, 255delA, 321-322insGT and Trp453Stop), which were detected in homozygous state in 3 of 5 families, clearly cause a loss of function of the Parkin protein, compatible with the recessive mode of inheritance. Three of the missense mutations result in non-conservative amino acid changes (Lys161Asn, Arg256Cys and Arg275Trp). In family IT-020, the 202-203delAG frameshift mutation on one allele, which already results in a loss of function, is associated with an apparently deleterious Lys161Asn missense mutation on the other allele. The conservative Thr415Asn amino acid change, that involves neutral amino acids with different polar side chains, homozygous in all five patients of family IT-014, is located within two consensus sequences of different protein kinases (cAMP- and cGMP-dependent protein kinases and protein kinase C), and might alter post-translational modifications. In addition, codon 415 is located very close to the first cysteine of the RING-finger motif (codon 418) and could affect its structure. Two missense mutations (Arg256Cys and Arg275Trp in patients of the families DE-012 and IT-015, respectively) and the truncating mutation 202-203delAG in the patients of the family UK-086 were heterozygous. No mutations were detected by sequencing on the other allele, suggesting that there may be a mutation in other regions of the gene that were not sequenced (e.g. the promotor region) or a heterozygous exon deletion that could not be detected by the techniques used in this study. This hypothesis would be in line with the fact that AR-JP appears to be an autosomal recessive disease. An alternative hypothesis could be that the parkinsonian phenotype observed in these particular families is due to reduced levels of functional Parkin protein derived from only one allele. The exon 2 truncating mutation in the British family UK-086 was also found in another Italian family (IT-020), but in this case a missense mutation was found on the other allele. Nevertheless, this may also support the hypothesis that reduced

levels of a part-functional protein may lead to parkinsonism. Indeed, it would appear that more recent studies bear this out (Dr Matt Farrer, personal communication).

#### **4.9.3 No obvious mutational hotspot in the Parkin gene (study 1)**

Mutations which had different effects on the Parkin protein were distributed over 6 exons, currently excluding a mutation hot spot. Only one of the point mutations, the 202-203delAG frameshift, was found in two families of different geographical origins (IT020 and UK086). Two explanations for this are (i) that the mutation occurred independently (a recurrent *de novo* mutational event), or (ii) that an ancient common founder is responsible. While sharing two frequent alleles for the PARK2 D6S411 (allele 2 = 59%) and D6S1550 (allele 2 = 68%), the patients in these 2 families had different alleles for two other tightly linked markers, D6S305 and D6S1579 (data not shown), suggestive of a recurrent *de novo* mutational event. However, in view of the small numbers of families in this study, it is probably more likely that an ancient founder effect associated with recombinations or mutations involving tightly linked markers is actually responsible. More recent studies on these same families (Periquet *et al.*, 2001) have concluded that exon rearrangements in the Parkin gene occur independently, whereas some point mutations, found in families from different geographic origins, may have been transmitted by a common founder. 11

#### **4.9.4 Parkin analysis shows lack of genotype-phenotype correlation**

In contrast to the reported Gln311Stop mutation (Hattori *et al.*, 1998c), the truncating point mutations identified in this study correspond to the N- and C-terminal regions of the Parkin protein (Ubiquitin-like and RING-finger motif, respectively), whereas the missense mutations affect the more central regions of the protein, as does the Thr240Arg mutation. The absence of significant clinical differences in this study among the patients with different types of mutations suggests that the modified amino acids are all of functional importance and that their replacement, like the truncating mutations, causes loss of function. The location of the mutations may therefore point to unknown functional domains although further studies with larger numbers are the only way to investigate this further.

#### 4.9.5 Screening for Parkin mutations: Study 2

In this study, Parkin gene mutations were detected in almost half of the families with autosomal-recessive Parkinson's disease in which at least one patient had age at onset  $\leq 45$  years. Parkin gene mutations were not, however, detected in a comparable proportion of patients with sporadic early-onset Parkinson's disease. On average, patients with Parkin gene mutations had the onset of symptoms in their early thirties, but the age at onset ranged widely from 7 years to 58 years. Early-onset Parkinsonism is clearly genetically heterogeneous as sporadic cases of Parkinsonism with an age of onset over 30 were more likely to be due to other gene/environmental interactions. The phenotype of patients with Parkin gene mutations cannot be clearly distinguished from other patients with early-onset PD. However, Parkin patients tend to have earlier onset, more frequent dystonia and less asymmetry at onset, more frequent hyper-reflexia, and better response to L-dopa, although they have a greater frequency of dyskinesias during treatment. Furthermore, the clinical manifestations of the disease were similar, regardless of the age at onset. In addition, patients with late-onset disease who have mutations can be difficult to distinguish from those with idiopathic PD. In general, however, disease progresses slowly in the patients with mutations.

Despite long disease durations, the patients with Parkin gene mutations responded remarkably well to low doses of L-dopa. Previous literature has reported the development of early L-dopa-induced dyskinesias (Hattori *et al.*, 1998a; Kitada *et al.*, 1998a), but in this study, the mean delay was about 5 years, with a maximum of 20 years. This was similar to patients without Parkin mutations. Finally, dementia was rare among the patients with mutations. This might be explained by less widespread neuronal loss in patients with mutations, in whom the *substantia nigra* and to a lesser extent the *locus coeruleus* are selectively affected, as compared with patients with idiopathic Parkinson's disease (Mori *et al.*, 1998; Takahashi *et al.*, 1994). However, the low frequency of dementia in the patients with mutations could be due to a younger mean age at examination or the exclusion of patients who became demented early in the course of the disease. There were no clinical differences between patients with missense mutations and truncating mutations. This was surprising, since missense mutations might be expected to interfere less with the function of the Parkin protein than truncating mutations, and result in a milder phenotype. This may be explained by the fact that the 10 conserved amino acids that were affected by the missense mutations are

of crucial importance for the function of the protein or that their modification results in decreased protein synthesis or more rapid degradation (Bross *et al.*, 1999). In addition, the wide range of clinical signs, even within single families with mutations (e.g. onset variation of up to 20 years) suggests that additional factors contribute to the phenotype.

The position of the mutations probably indicates functionally important protein regions, as does conservation of the respective amino acids in mice and rats (Gu *et al.*, 2000).

The observation that 13 of the mutations were found repeatedly in as many as 10 families raises the question of common founders. However, many of the mutations were found in families from different European countries, suggesting that these alterations are recurrent. The point mutations that accounted for the disease in approximately 40 percent of our patients, seem to be less frequent among Japanese patients (Hattori *et al.*, 1998a). Finally, the identification of 15 index patients with single heterozygous mutations indicates that other mutations remain to be discovered. Mutations in non-coding regions of the Parkin gene can be envisaged (this has recently been described in UK 401, see section 5.4.1).

The second study showed that mutations in the Parkin gene are a major cause of autosomal recessive familial early onset (45 years) and isolated juvenile onset (20 years) parkinsonism, but accurate diagnosis cannot be made on the basis of the clinical group differences alone. However, unlike late onset PD, progression is slow, the response to L-dopa sustained and the frequency of dementia low. A Japanese study found that deletions in the Parkin gene were found in only 2.2% of 184 Japanese patients with Parkinson's disease. However, deletions were present in 25.0% and 40.0% of the patients with juvenile-onset (these authors defined this as < 40 years) with a family history. Deletions were not found in any adult-onset cases (>40 years). Half the patients with Parkin gene-related PD lacked both heredity and consanguinity (Ujike *et al.*, 2001).

#### **4.9.6 Pathological studies of Parkin patients**

There are several novel and potentially important findings in our own study. First, we have identified a patient with a mutation in the Parkin gene which may be responsible for the distinct pathology (tau-positive neurofibrillary tangles, NFTs). A second mutation has not yet been detected. There have been very few pathologic reports of

Parkin gene-related parkinsonism. With one exception (Chen *et al.*, 2000), Lewy bodies have not been reported in any of these cases. Pathological analysis on a Japanese brain with a homozygous deletion in exon 4 of the the Parkin gene showed NFTs with a relative paucity of senile plaques. Argyrophilic astrocytes and threads were also reported (Mori *et al.*, 1998). This case also showed previously unreported neuronal loss and fibrillary gliosis in the *substantia nigra pars reticulata* (SNPR). The remaining neurons of the *substantia nigra pars compacta* (SNPC) had low melanin content which has been consistently reported in other cases (Takahashi *et al.*, 1994, Gibb, 1991).

The authors speculated that this may be a feature of hereditary juvenile Parkinsonism and might be related to the individual causative gene abnormalities (Hayashi *et al.*, 2000).

It has been generally accepted that Parkin loss of function leads to nigral neuronal loss, without Lewy body pathology. Subsequent analysis of Parkin protein in a Japanese study indicated that it was absent in all regions of the brains of patients with AR-JP but was not decreased in the brains of sporadic PD patients (Shimura *et al.*, 2000).

Immunoreactivity was detected also in a few Lewy bodies. Parkin protein was located in the Golgi complex and the cytosol (Shimura *et al.*, 1999). Tau-positive, thorn shaped argyrophilic astrocytes have been reported in a Dutch family with autosomal recessive early-onset parkinsonism with a heterozygous exon deletion in the Parkin gene, although NFTs were absent (van de Warrenburg *et al.*, 2001). These reports show the pathological heterogeneity found in Parkin patients at post-mortem, which has not been explained.

A kindred has been recently reported with a novel mutation in the Parkin gene, with Lewy bodies at post-mortem (Matt Farrer, personal communication, 2001). Data from analysis of two kindreds in this report led the authors to conclude that compound heterozygous parkin mutations and loss of Parkin protein may lead to early-onset parkinsonism with Lewy body pathology, while hemizygous mutations may confer an increased susceptibility to typical PD and possibly post-encephalitic PD. The authors speculated that hemizygous loss of Parkin function confers susceptibility to parkinsonism, but on its own is not sufficient to cause the disease. They speculate that further characterisation of Parkin gene splicing and the pathological function of deleted parkin isoforms may help to understand the mechanism underlying neuronal degeneration in Parkin-associated disease.

The Parkin protein has an amino terminus with similarity to ubiquitin and a carboxy-terminus with a RING-finger motif, which may regulate cell-growth and differentiation. The moderate homology of parkin to ubiquitin may suggest a role in the regulation of proteosomal degradation of proteins. Zhang et al. (2000) proposed that Parkin ubiquitinates itself and promotes its own degradation, and acts as an E2-dependent ubiquitin-protein ligase, promoting degradation of synaptic vesicle-coated protein CDCrel-1, which then interacts with parkin through its two RING finger domains. Interestingly, several pathogenic point mutations occur in one or other of these RING finger domains. This was confirmed by Choi *et al.* (2000) who observed a di-ubiquitinated parkin on Western blots after treatment of neuroblastoma cells with the proteosomal inhibitor MG132. It has since been proposed that Parkin ubiquitinates a subset/fraction of SNCA, which then acts as a seed for Lewy body formation (M Farrer, personal communication).

Parkin protein may be expressed as different isoforms in human brain. Most Japanese patients with AR-JP have deletions spanning one or several consecutive exons, whereas a few have one base microdeletion in exon 5. These deletion-prone exons are alternatively spliced out in the normal leucocyte Parkin transcript. Although the physiological diversity of Parkin remains to be elucidated, characterization of the functional diversity of the three different Parkin isoforms in different tissues may help understand how the Parkin gene defect causes selective degeneration of dopaminergic neurons in the *substantia nigra* in AR-JP (Sunada *et al.*, 2001). Loss of exon 7 (encoding the RING finger motif) may lead to the formation of Lewy bodies, possibly via a synuclein pathway, whereas splice-site or promoter mutations in Parkin protein may result in the formation of tangle pathology. For instance, the Arg275Trp mutation may confer a dominant negative gain of function, and in conjunction with aberrantly spliced or processed Parkin, lead to either tangle pathology or Lewy body formation. These findings may 'map' functional epitopes responsible for tau versus synuclein interactions.

#### **4.9.7 Immunostaining for Parkin and Tau: evidence of epitope masking?**

Parkin antibody staining was clearly observed in the cytoplasm and nucleus from sections examined in this case. Different antibodies raised against different regions of

the Parkin protein have been reported to give different localisation on immunostaining. Immunoblotting studies describe parkin distribution found in rat, mouse, bird, frog, and *Drosophila* brains (Horowitz *et al.*, 2001), where it was widely distributed though the neuraxis. However, immunolocalisation appeared to vary with antibody specificity. A polyclonal anti-human parkin antibody raised to an epitope conserved in the rodent (AB5112, Chemicon), gave predominant cytoplasmic labeling of rodent neurons, whereas another polyclonal raised to part of the human protein with some divergence to the rodent homologue (Ab-1, Oncogene Sciences) generated nuclear labeling in rat but not mouse brains. Nevertheless, nuclear labeling was observed in mouse after epitope unmasking protocols were performed. Human brain staining for parkin was also nuclear. Gu *et al.* (2000) cloned rat parkin cDNA and examined its distribution in rat brain. Moreover, they found that it had 85% amino acid identity to human parkin and 95% identity to the mouse. A novel antibody ASP5p was generated to an epitope identical in all three species and showed a mainly cytoplasmic localisation in rat brain neurons in those areas principally affected in parkinsonism (*substantia nigra*) as well as in some unaffected areas (cerebellum, glial cells).

The above may suggest epitope masking due to conformational differences of the Parkin protein in the nucleus and the cytoplasm. Moreover, alternative splicing may lead to different protein isoforms which localize to either cytoplasm or nucleus. The possible functional significance of these findings remain unclear.

#### **4.9.8 Conclusions on Parkin from studies 1 & 2 and neuropathology**

The pathological study described here (section 4.6) is the first clinico-pathological analysis of a European patient with a mutation in the Parkin gene. Parkin is expressed in neurons, and its pattern of expression closely follows that of  $\alpha$ -synuclein, although Parkin expression is not tightly correlated with regions of the brain in which Lewy bodies have been found in PD or in diffuse Lewy body disease, although the Parkin transcript is abundant in brain (including the *substantia nigra*). In Japanese patients with AR-JP, Parkin deletions are typically seen in exons 3-7 of the Parkin gene (Kitada *et al.*, 1998b). Alternative splicing of these exons from the Parkin gene produces different Parkin transcripts in different tissues (Sunada *et al.*, 1998). Pathological studies of the brains of Japanese patients with AR-JP showed absence of Parkin protein,

although Parkin protein was not decreased in the brains of sporadic PD patients (Shimura *et al.*, 1999).

Until a recent report (see below), the major histopathological differences between patients with Parkin mutations and idiopathic Parkinson's disease were considered to be absence of Lewy bodies and the restriction of cell death to the *substantia nigra* and the *locus coeruleus* (Mori *et al.*, 1998). Thus, Parkin gene mutations may be responsible for a particularly selective cell death, the mechanism of which might differ from that in idiopathic Parkinson's disease. However, recent findings suggest that compound heterozygous Parkin mutations and loss of Parkin protein may lead to early-onset parkinsonism, that in some instances can be associated with Lewy body pathology (M Farrer, personal communication). This finding shows that the role of the Parkin gene should not only be further investigated in AR-JP by looking at the pathological function of deleted Parkin isoforms, but it should also be examined in younger patients with typical PD.

In conclusion, Parkin mutations are an important cause of early-onset autosomal recessive familial PD (38 +/- 12 years, range 7-58 years) and isolated juvenile-onset parkinsonism (at or before the age of 20 years) (Abbas *et al.*, 1999; Luecking *et al.*, 2000). Although dystonia at onset, hyperreflexia and slow disease progression are characteristic features of patients with Parkin mutations, there are no specific clinical signs that distinguish these patients from others. Exon deletions and mutations are reported to result in parkin protein truncation or amino acid substitution. The presence of both deletions and multiplications of some exons (e.g. exon 2 and 3) have also been detected leading the authors to suggest that this may be due to unequal recombination (Luecking *et al.*, 2000). Single heterozygotes in 2 families with autosomal recessive early-onset PD have been described (Abbas *et al.*, 1999). Either this represents failure to detect the second mutation or it indicates that a single mutation in parkin may be sufficient to cause the phenotype. The age-of-onset distribution for the different mutations in parkin has been similar, suggesting that the substitutions of amino acids resulting from missense mutations are as essential for parkin protein function as the truncation and deletion mutations.

Although Parkin is generally considered to be an autosomal recessive disease, a kindred has been recently reported with a novel mutation in the Parkin gene exhibiting autosomal dominant inheritance of PD with Lewy bodies (Chen *et al.*, 2000). However, the main histopathological difference between patients with Parkin mutations and those with sporadic PD are the general absence of Lewy bodies (despite Chen *et al.* 2000) and the restriction of neuronal loss to the *substantia nigra* and *locus coeruleus* in patients with Parkin mutations (Mori *et al.*, 1998). Pathological studies of the brains of patients with AR-JP showed absence of Parkin protein, (Shimura *et al.*, 1999). It has been hypothesised that accumulation of (as yet unidentified) proteins cause a selective neuronal cell death in AR-JP without formation of Lewy bodies. The mechanism of this remains unclear despite the identification of the function of Parkin protein (Shimura *et al.*, 2000). The Parkin gene product is a ubiquitin-protein ligase involved in protein degradation as it collaborates with the ubiquitin-conjugating enzyme UbcH7 (UBE2L3; 603721). Mutations in the Parkin gene are therefore considered to cause loss of ubiquitin-protein ligase activity (Shimura *et al.*, 2000). How Parkin may interact with other PD genes is discussed in section 4.11.1.

#### **4.10 THE ROLE OF THE UBIQUITIN CARBOXY-TERMINAL HYDROLASE (UCH-L1) GENE (4p14) IN PD**

A missense mutation in the UCH-L1 gene, Ile93Met, which caused a partial loss of the catalytic activity of this thiol protease was identified in a German family with familial PD (Leroy *et al.*, 1998), see Section 1.14.4. An affected German sibling pair were found to have an Ile93Met mutation. Affected members of this kindred became symptomatic with a resting tremor at the age of 51 and 49 years respectively, progressing to rigidity, bradykinesia, and postural instability. Both individuals showed a beneficial response to L-dopa replacement therapy. A paternal uncle and the paternal grandmother were also affected, although, with the exception of the 2 sibs, all other affected individuals in this pedigree were deceased. The lack of the affected phenotype in the father of the 2 patients showed that the mutation had incomplete penetrance in the family. Analysis of 500 chromosomes from control individuals of different ethnic backgrounds showed no example of the Ile93Met mutation. The high cross-species conservation of isoleucine93 in human, rat, mouse and yeast indicates its importance in UCH-L1 structure and function.

#### 4.10.1 Collaborative GSPD study on UCH-L1

To examine the importance of the Ile93Met mutation in UCH-L1, sequencing of the entire coding region of UCH-L1 was performed in 11 GSPD autosomal dominant families (MF, Jacksonville) as well as 29 smaller families with at least 2 affected sib pairs. The families were all collected as part of GSPD (UK families by myself) and the sequencing results are included here for discussion purposes (see appendix 4). No mutations were detected in the UCH-L1 gene, although mutations in the regulatory or intronic regions of the gene were not sequenced. It was concluded that the Ile93Met variant must either be a rare cause of disease or a harmless substitution whose occurrence in the original family described reflects a chance co-occurrence.

A recent American study investigated the association of PD with this mutation and with a common polymorphism of the same gene (S18Y). The Ile93Met mutation was not identified in either cases or controls but those with the S18Y polymorphism had a significantly lower risk of PD (Maraganore *et al.*, 1999). Analysis of the S18Y polymorphism in a large German sample of sporadic and familial PD cases also suggested this variant had a protective effect on the pathogenesis of sporadic PD (Wintermeyer *et al.*, 2000). However, a further study failed to confirm the influence of the S18Y polymorphism on the risk of developing PD (Mellick and Silburn, 2000). Overall, the evidence for or against UCH-L1 in familial PD is very weak and requires further work. The fact that other studies have also failed to detect the Ile93Met mutation as a cause of familial PD allow two possible conclusions to be drawn: the first is that the Ile93Met is indeed a rare cause of PD, the second is that the mutation has no effect on one's risk of developing PD and its occurrence in two affected sibs is just an accidental occurrence (Leroy *et al.*, 1998, Harhangi *et al.*, 1999; Lincoln *et al.*, 1999). These two possibilities can only be discriminated by the identification of the same or other UCH-L1 polymorphisms/mutations that segregate with familial PD or through the observation of this polymorphism in unaffected individuals.

The work reported here in section 4.7 has shown that the Ile93Met mutation is not a major cause of familial PD in Europe. Mutations in the regulatory or intronic regions were not excluded. Other studies have not reproduced the original findings. The frequency of the S18Y polymorphism was reported in 313 patients with sporadic PD

and 302 control subjects (Japanese and Caucasians). The frequency of the mutant allele (Y) was significantly higher in Japanese control subjects (51.2%) than in Japanese PD patients (43.4%) ( $\chi^2=3.917$ ,  $p=0.048<0.05$ ). It appears that this polymorphism has a weak protective factor against PD in at least the Japanese population. The frequencies of Y allele and S/Y and Y/Y genotypes in the PD patients and the controls were significantly higher in Japanese than in Caucasian population ( $p<0.0001$ ). The authors speculated that the role of this polymorphism in PD may be different between Caucasian and Japanese populations. (Zhang *et al.*, 2000). A further study in German Parkinson's disease patients showed that although sequence variants in the coding region of UCH-L1 are a rare event a protective effect of the S18Y polymorphism may exist (Wintermeyer *et al.*, 2000). However, a further Australian case-control study using patients with idiopathic PD found that the S18Y polymorphism did not confer protection against developing idiopathic PD (Mellick and Silburn, 2000).

Nevertheless, UCH-L1 is a thiol protease and there is a plausible mechanism for its involvement in neurodegeneration. In particular, the Ile93Met mutation is considered to cause a partial loss of the catalytic activity of the enzyme leading to aberrations in the proteolytic pathway and aggregation of proteins (Leroy *et al.*, 1998). Enzyme kinetic studies of the mutant and wild-type proteins showed that the Ile93Met UCH-L1 had nearly a 50% reduction in activity than the wild-type protein. The natural substrate for UCH-L1 is unknown, so although this result must therefore be treated with caution, two models have been suggested which may explain the toxic effect of this missense mutation on the neuron. The first model proposes that the mutant protein is less soluble, therefore it cannot be degraded by the normal processes and simply accumulates. In the second model, the catalytic enzyme activity of UCH-L1 is altered by the amino acid substitution, leading to altered turnover of the unknown substrate, which by its accumulation acts as a "seed" for other proteins (Leroy *et al.*, 1998). Lewy bodies are strongly ubiquitinated and immunoreactive with neurospecific UCH-L1 and some subunits of the 26S proteasome. Although these results do not confirm the involvement of the UCH-L1 gene in the pathogenesis of PD, several reports have examined the formation of intraneuronal inclusion bodies and proposed that dysfunction of the ubiquitin-dependent proteolytic pathway may be responsible (Lowe *et al.*, 1990).

## 4.11 FUTURE DIRECTIONS OF STUDY

### 4.11.1 Alpha synuclein, Parkin and UCH-L1-are they all involved in a common pathway in PD?

Several investigators have speculated on the fact that Parkin,  $\alpha$ -synuclein and UCH-L1 may be interacting proteins in a common pathway. Mutations in these genes encode altered proteins which may then give rise to the clinical phenotype of PD. Environmental factors may also play a possible role in gene expression. Studies in normal human brain show that expression of each of these three genes was predominantly neuronal. Alpha-synuclein and Parkin mRNAs were expressed in a restricted number of brain regions, whereas UCH-L1 mRNA was more uniformly expressed throughout the brain. The melanin-containing dopamine neurons of the *substantia nigra* had particularly robust expression. The expression pattern of  $\alpha$ -synuclein and Parkin mRNAs were similar, suggesting that the two proteins may be involved in common pathways contributing to the pathophysiology of PD such as ubiquitination (Solano *et al.*, 2000). UCH-L1 mutations may alter ubiquitin recycling.

### 4.11.2 Genes, the ubiquitin-proteasome pathway and nigral degeneration

Ubiquitin, a small protein consisting of 76 amino acids, has been found in all eukaryotic cells studied. It is one of the most conserved proteins known. Ubiquitin is required for ATP-dependent, non-lysosomal intracellular protein degradation, which eliminates most intracellular defective proteins as well as normal proteins with a rapid turnover (Baker and Board, 1987). Degradation involves covalent binding of ubiquitin to the protein to be degraded, through isopeptide bonds. The function of ubiquitin is presumed to be labeling of the protein for disposal by intracellular proteases. Ubiquitinated histones are present primarily in actively transcribed chromosomal regions, so ubiquitin may play a role in regulation of gene expression (Baker and Board, 1987).

Animal models of neurodegenerative diseases have also been associated with progressive accumulation of ubiquitinated protein conjugates (Yamazaki *et al.*, 1988). Findings suggested that altered function of the ubiquitin system may directly cause neurodegeneration (MacDonald, 1999). The role of ubiquitin in a variety of neuropathological conditions including PD, Pick's disease, and Alzheimer disease is

widely supported (Lowe *et al.*, 1988). Since most misfolded proteins are degraded via the ubiquitin-proteasome pathway, defects in this pathway may have a central role in neuronal degeneration. Specifically, impaired proteosomal degradation of abnormal proteins, such as alpha-synuclein and Parkin, or defects in the pathway by, for instance, mutant UCH-L1, may underlie the pathogenesis of PD.

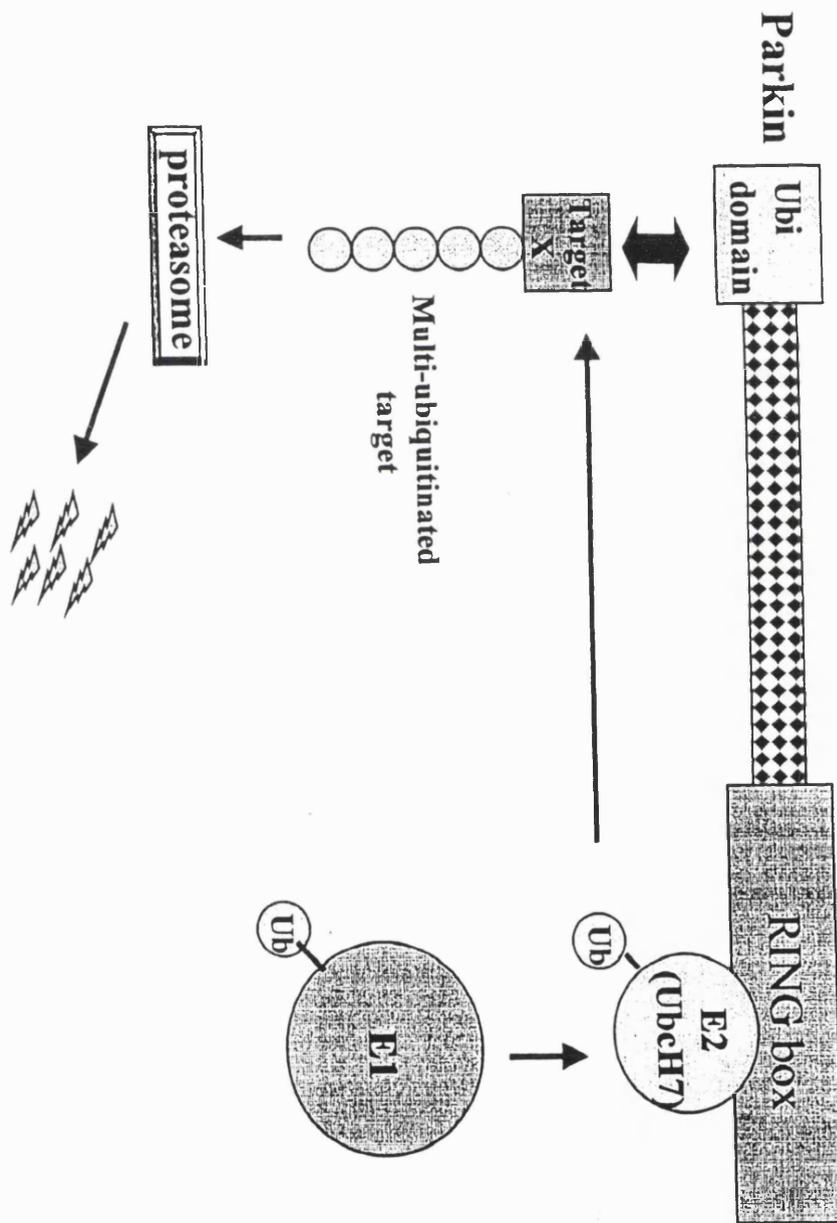
Shimura *et al.*, 2001 showed evidence of a functional link between mutant parkin and  $\alpha$ -synuclein in the cell via the process of ubiquitination, an intracellular mechanism for targeting proteins for degeneration. They proposed that an accumulation of non-ubiquitinated  $\alpha$ -synuclein aSp22 may lead to cellular toxicity. If nigral neurons are particularly sensitive to these accumulations, the end result could be nigral degeneration and PD. A summary of the main evidence provided is as follows: a) Parkin is an E3 ubiquitin ligase, recruiting ubiquitin conjugating enzymes UbcH7 and UbcH8 as well as recognising the target for ubiquitination; b) Lewy bodies contain Parkin, ubiquitin and polyubiquitinated  $\alpha$ -synuclein, but LBs are not found in brains of patients with parkin mutations; c) Parkin colocalises with  $\alpha$ -synuclein and UbcH7 in purified presynaptic fractions of brainstem; d) Parkin co-immunoprecipitates (co-IPs) UbcH7 and alpha-synuclein, including a novel glycosylated 22kDa isoform of  $\alpha$ -synuclein (aSp22) in these fractions, but does not co-IP these substrates in homogenates of ARPD brains. Therefore, the authors proposed that mutant Parkin either: a) fails to recognise and bind aSp22 at parkin's N-terminal Ub1 domain, or b) fails to recruit UbcH7 via parkin's RING box. The result is an accumulation of nonubiquitinated aSp22 leading to accelerated neural toxicity. In other forms of PD, functional Parkin mediates ubiquitination of aSp22 and other substrates, but these fail to be appropriately targeted to the proteasome (for other genetic or environmental causes, such as mutant  $\alpha$ -synuclein) and accumulate in Lewy bodies. Although there are likely to be additional targets for Parkin in brain, it seems to show remarkable specificity, as beta-synuclein is not targeted for ubiquitination. Other E2 proteins may also be recruited by Parkin, as a Parkin pool is found at postsynaptic terminals which do not contain UbcH7 or  $\alpha$ -synuclein (Shimura *et al.*, 2001). Mutations in the ubiquitin C-terminal hydroxylase, UCH-L1 may also cause perturbations in this pathway. Figure 4.10 shows the hypothetical pathway for the proposed interaction of Parkin with Ubiquitin. It is proposed that the C-terminal RING-box domain of the Parkin protein recruits a specific E2 enzyme (UbcH7) and the N-terminal Ub1 domain required for recognition of the

target protein, designated 'X', for ubiquitination before proteosomal degradation (see Figure 4.10).

Synuclein pathology appears to be a prominent feature of some neurodegenerative diseases (such as PD, MSA), yet a secondary pathology in others (such as AD). Lewy bodies occur in families with either amyloid precursor protein (APP) or presenilin mutations (Lippa *et al.*, 1998). This surprising observation has been extended to prion diseases where Lewy bodies have been described in association with tangle/tau pathology (Piccardo *et al.*, 1998). Farrer *et al.*, 1999 suggested that, with respect to AD at least, it seems as if the tangle/tau pathology of the Lewy body/synuclein pathology are alternative responses to the primary lesion, because cases of AD with little tau pathology have much synuclein pathology and vice versa. From the limited components identified to date, it is possible to speculate that these proteins may functionally overlap in a common pathway of cytoskeletal maintenance and intracellular vesicle transport. The normal function of  $\alpha$ -synuclein may depend on an ability to undergo a conformational change in the presence of specific phospholipids (Perrin *et al.*, 2000). Nitrated  $\alpha$ -synuclein is present in the major filamentous building blocks of Lewy bodies, as well as in the insoluble fractions of affected brain regions of synucleinopathies. The selective and specific nitration of  $\alpha$ -synuclein in these disorders provides evidence to directly link oxidative and nitrative damage to the onset and progression of neurodegenerative synucleinopathies (Giasson *et al.*, 1999).

Despite experimental progress, how  $\alpha$ -synuclein, Parkin and UCH-L1 interact remains largely speculative at present although the discovery of mutations in all three genes, other PD loci and recent biochemical studies have enhanced molecular exploration of PD and may also lead to a future revolution in our understanding of other neurodegenerative diseases that are characterized by involvement of abnormal protein handling including MSA, AD, other tau-opathies, CAG repeat disorders and amyotrophic lateral sclerosis. Novel genes are still to be discovered which will further enhance our study of the aetiology of PD as described in chapter 5.

**Figure 4.10** Model of the involvement of Parkin in the ubiquitination pathway. Several other proteins are also likely to be involved. *Key:* Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; Ubl, ubiquitin-like domain. “X” is a putative target protein.



**CHAPTER 5: A CLINICO- GENETIC AND PATHOLOGICAL STUDY OF  
THREE LARGE KINDREDS WITH FAMILIAL PARKINSON'S DISEASE**

## 5.1 OUTLINE OF CHAPTER

This chapter describes a series of clinical, molecular and pathological studies that were performed on 3 large PD kindreds (UK 401, 402, 403) identified as a result of the collection of the large population of affected sibling pairs (see Section 3.2). Exclusion mapping studies of the known dominant and recessive loci in all three kindreds are described. The PARK 6 locus (Valente *et al.*, 2001) was unpublished at the time of these studies and was therefore not included. PET studies and gene sequencing results of Parkin and SNCA are included for discussion purposes, as they were done as part of collaborations (collaborators are acknowledged in each section). All the clinical and linkage analyses on UK 401, UK 402 and UK 403 were performed by myself except where stated. A clinical description of UK 401 has been published previously (Sawle *et al.*, 1992). Dr David J Nicholl was a clinical collaborator for UK 403.

## 5.2 AIMS AND DESIGN OF THE STUDY

### 5.2.1 Patients

The families were recruited as part of an ongoing European study of familial PD (GSPD). The diagnosis of Parkinson's disease was made using (i) a pathologically proven diagnosis according to UK Parkinson's Disease Society Brain Bank criteria (Hughes *et al.*, 1992) or (ii) a clinical diagnosis of idiopathic PD using a similar study design on familial PD (Maraganore *et al.*, 1991) with at least two of the three cardinal signs present: tremor, rigidity and bradykinesia; responsiveness to L-dopa and unilateral/asymmetric symptoms at onset; and no atypical features (see Section 2.2). One affected member from each kindred was scanned with  $^{18}\text{F}$ -dopa PET (PET scan results are shown in appendix 5, as the PET study was performed by Dr P. Piccini, MRC Cyclotron Unit, Hammersmith Hospital). In some cases, a retrospective diagnosis of PD was made in deceased family members via a review of medical records, family documentation and videos, where at least two of the three above cardinal signs were present. A diagnosis of possible PD was based on the historical account from other family members if there was insufficient information to make a reliable diagnosis of PD based on the above criteria. Genealogical data were collected via civil and church records of births, deaths and marriages. Familial lineages had been extensively traced by a distant member of one kindred for reasons unrelated to this study. An extensive

clinico-genetic analysis was undertaken on kindreds UK 410-403. The results of collaborative pathological and PET studies are also described in the relevant sections.

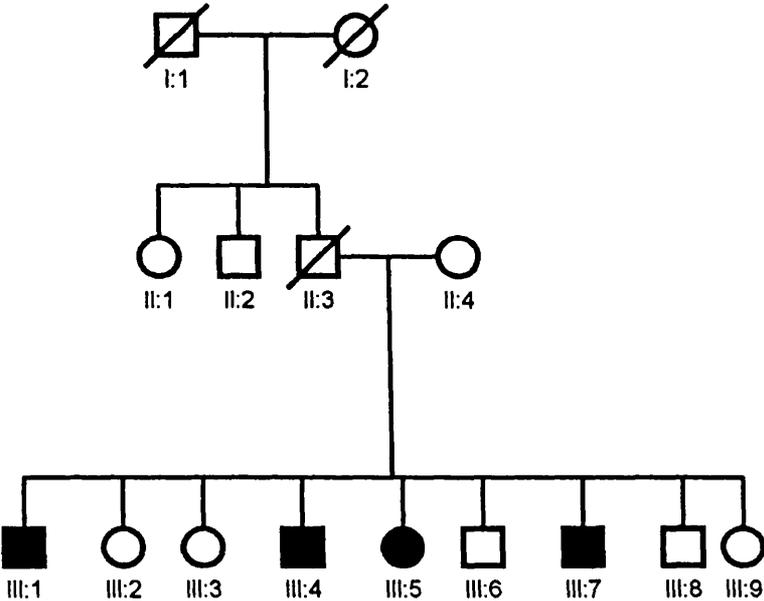
Informed written consent was obtained from all subjects, and the study received approval from the ethical committees of the University Hospital NHS Trust, Birmingham; National Hospital for Neurology & Neurosurgery, London and the Hammersmith Hospitals Trust Research Ethics Committee. Approval to administer radiolabel ligands was obtained from the Administration of Radioactive Substances Advisory Committee of the United Kingdom (Dr P Piccini).

### **5.3 CLINICAL DESCRIPTION OF THE KINDREDS UK 401, 402 & 403**

#### **5.3.1 Clinical Analysis UK 401**

This kindred originated from a small village in Southern Ireland and was not known to be consanguineous (Sawle *et al.*, 1992). The pedigree is shown in Figure 5.1. Analysis of the three generations show that the parents (II.3 and II.4) did not have parkinsonism although II.3 died at the age of 44 with a heart condition and was said to have symptoms of stiffness and poor mobility which had been attributed to arthritis. Individuals II.1 and II.2 aged 60-66 when examined in 1990 were clinically normal and asymptomatic. Parkinsonism was not reported in other members of the family. There were four affected siblings (3 male and 1 female), the mode of transmission was consistent with autosomal recessive or dominant inheritance. I personally clinically examined the case (III.4) and an apparently unaffected member of the family (III.8). The rest of the clinical descriptions here are based on published data (Sawle *et al.*, 1992). The mean age of the onset of symptoms of tremor, rigidity or bradykinesia was 35 years (range 31- 41 years) and the mean disease duration in the known affected individuals was 20 years (range 15-28 years). No patient had abnormal eye movements, ataxia, dementia, pyramidal features or autonomic dysfunction. All affected siblings (III.1, III.4, III.5 and III.7) responded well to L-dopa therapy (see Table 5.1). The neurological examination was normal in siblings III.2 (age 58), III.3 (aged 57), III.6 (aged 51), III.8 (aged 48) and III.9 (aged 46).

Figure 5.1: Pedigree of family UK401 showing multiple members affected by PD.



#### 5.3.1.1 UK 401, individual III.1 (the index case)

This 60 year old man reported initial symptoms of fatigue, lower limb stiffness, tendency to limp from the age of 32 years. Since the age of 41 he complained of feeling slowed up, a poor balance and frequent falls. At 42 developed an upper limb tremor and difficulty turning in bed. He was hardly able to walk by the time Parkinson's disease was diagnosed at the age of 46. Clinically he exhibited the classical triad of tremor, rigidity and bradykinesia. Within a few days of starting L-dopa (Sinemet) he was able to resume a fully active life. He gave a positive family history of three other siblings with parkinsonism who were subsequently investigated. Screening for Wilson's disease and acanthocytosis was negative. A CT head, neuropsychological, and autonomic testing were normal (Sawle *et al.*, 1992).

#### 5.3.1.2 UK401, other individuals

Clinically III: 8, after the first scan, complained of generalised aches and pains and an inner tremulousness, but no symptoms of definite parkinsonism. He was reported to have mild facial hypomimia, poor right arm swing and slowed alternating fine finger movements. An Apomorphine challenge test was negative and he was commenced on Selegiline. When examined by myself 10 years later, his aches and pains had settled, as had the inner tremulousness. He had normal gait and armswing with no clinical evidence of bradykinesia or rigidity. He had stopped taking selegiline several years before as it had had no demonstrable effect on his clinical symptoms.

The daughter of I11:5 at 19 years of age (individual not shown on fig 5.1) had a mild postural tremor, but no other symptoms or signs and was not included in the original genetic analyses. The clinical presentation of the patients was comparable to that of young-onset PD cases however their phenotype was indistinguishable from idiopathic Parkinson's disease with a common feature of severe resting leg tremor. All four cases showed slow clinical progression, drug-induced dyskinesia and fluctuations (Sawle *et al.*, 1992) (see Table 5.1). The condition progressed only slowly: even the index patient with disease of longest duration remained quite active.

**Table 5.1.** Clinical summary of affected members of UK 401

<b>Case</b>	<b>III:1</b>	<b>III:4</b>	<b>III:5</b>	<b>III:7</b>
<b>Sex</b>	M	M	F	M
<b>Age at onset</b>	32	37	39	31
<b>Disease duration (yrs)</b>	28	18	14	20
<b>First symptoms</b>	RIGIDITY	REST TREMOR	BRADYKINESIA	REST TREMOR
<b>Bradykinesia</b>	+	+	+	+
<b>Rigidity</b>	+	+	+	+
<b>Rest tremor</b>	+	+	+	+
<b>Postural instability</b>	+	-	-	-
<b>Other features</b>				GENERALISED TREMOR, DYSTONIC POSTURING
<b>Response to L-dopa</b>	++	++	++	++
<b>Source</b>	Sawle, 1992	Sawle, 1992	Sawle, 1992	Sawle, 1992

### 5.3.2 Clinical analysis of UK 402

The pedigree of this kindred (UK 402) is shown in Figure 5.2. The clinical description of this kindred has not previously been published, although the linkage data on this family was summarised in an earlier publication (Family UK-A) (Gasser *et al.*, 1997). The kindred originated from Lincolnshire. Fifteen affected members were identified (7 Male; 8 Female). All six living affected members and nine unaffected members were personally examined (by myself, NLK, DJN and GGL). Two further members were examined (by myself, GGL) in which the presence of PD could not be unequivocally confirmed. Asymmetrical rest tremor was the most common initial presentation, with a good L-dopa response followed by development of motor fluctuations and dyskinesias. The clinical course of the disease was similar to that of sporadic PD but with a lower age of onset. Clinical status is known for four of the most recent generations. Segregation ratios were based on generation III as these were the only individuals for which complete medical information is known and who have lived long enough to fully acquire their PD risk. The mean age of onset for the kindred is 57 years old (44-72;  $SD \pm 13.2$ ). The mode of inheritance was consistent with autosomal dominant inheritance with reduced penetrance. There were no obligate heterozygotes. No post-mortem data was available on this kindred. Table 5.2 (overleaf) summarises the clinical details of UK 402.

#### 5.3.2.1 UK 402, individual III:17 (the index case)

III:17 developed an asymmetrical rest and slight upper limb tremor with bradykinesia of the right arm at age of 58. At presentation, he also had a flexed posture and gait disturbance years. He started on L-dopa with good effect aged 59 years. His parkinsonism slowly progressed and at last assessment was Hoehn and Yahr stage 2.0. Fifteen affected members were identified (7 Male; 8 Female). All 6 living affected members and 9 unaffected members were personally examined (by myself, Dr N Khan and Dr G Lennox). Two further members were examined (by myself and Dr G Lennox) in which it could not be stated whether they were actually displaying signs of early PD or not. The mode of inheritance was consistent with autosomal dominant inheritance with full penetrance. There were no obligate heterozygotes. No post-mortem data was available on this kindred.

Figure 5.2: Pedigree of UK402 showing multiple individuals with PD

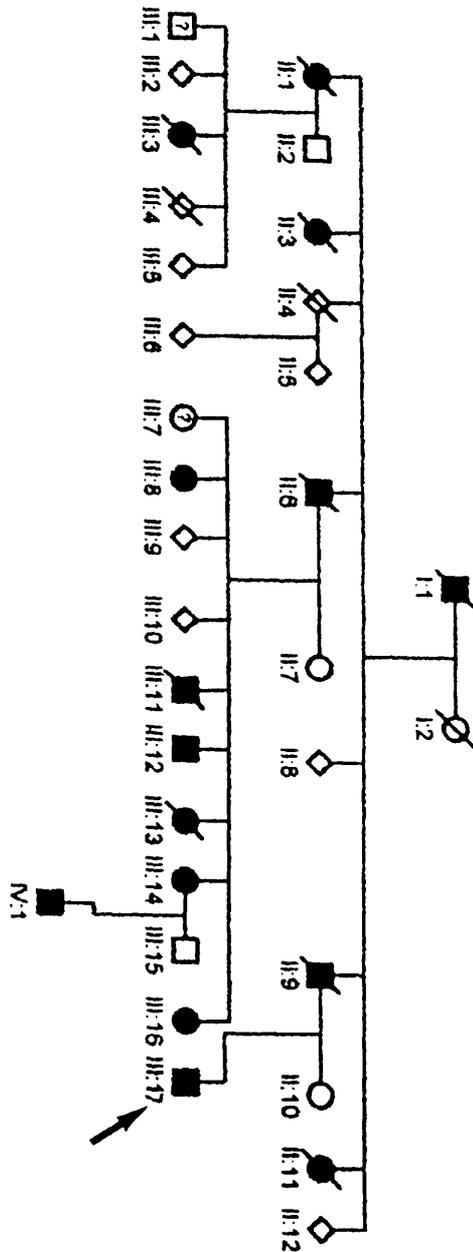


Table 5.2 Clinical summary of affected members of UK 402

Case (individuals in bold correspond with 'affecteds' in fig 4 )	Sex	Age at onset	Disease duration (& current status)	First symptoms	Bradykinesia	Rigidity	Rest Tremor	Postural instability	Other features	Response to L-dopa	Source
I:1	M	65	10 (dead)	Asymmetric RT	+	+	+			+	H
II:1	F	78	8 (dead)	Asymmetric RT	+	+	+			+	H
II:3	F	40	12 (dead)	Asymmetric RT	+	+	+	+		Not given	H
II:6	M	60	10 (dead)	Asymmetric RT	+	+	+	+		+	H
II:9	M	55	15 (dead)	Asymmetric RT	+	+	+	+		+	H
II:11	F	69	10 (dead)	Asymmetric RT	+	+	+	+		Not given	H
III:1	M	-	80y old at time of examination		-	-	-	-	Facial hypomimia & stooped gait only. Not progressed over 2 years	Not given	PE
III:3	F	48	9 (dead)	Asymmetric RT	+	+	+			+	H
III:7	F	-	78y old at examination	-	-	-	-	+	Slight unsteadiness of gait	Not given	PE
III:8	F	57	19 (alive)	Asymmetric RT	+	+	+		O-OF, D, lower limb dystonia	+	PE
III:11	M	47	8 (dead)	Asymmetric RT	+	+	+		Psychosis	+	H
III:12	M	64	10 (alive)	Asymmetric RT	+	+	+			+	H
III:13	F	48	11 (dead)	Asymmetric RT	+	+	+		rapid progression	+	H
III:14	F	54	15(alive)	Asymmetric RT	+	+	+		O-OF, D, lower limb dystonia	+	PE
III:16	F	44	19 (alive)	Asymmetric RT	+	+	+		Severe 'yes-yes head tremor; O-OF, D, lower limb dystonia	+	PE
III:17	M	58	8(alive)	Right- sided B & tremor	+	+	+/-		Slight upper limb tremor	+	PE
IV:1	M	44	1	Asymmetric RT	-	+	+	-	Pyramidal signs R leg	+	Review of medical records

RT= Rest Tremor; O-OF= on-off fluctuations; D= dyskinesias; PE= personal examination; V= review of family video; H= historical report from family members

### 5.3.3 Clinical analysis of UK 403

A total of 8 affected members were identified (Figure 5.3, Table 5.3) including one subject (XIV:21) who was examined neuropathologically (4 males and 4 females). Six cases (XV:25, XV:34, XIV:4, XIV:6, XIV:21, XIV:23) were personally examined (by DJN, SLH and myself), along with 22 other unaffected family members. Two cases were designated as affected based on interviews with multiple first-degree relatives. One case (XV:26) was examined (DJN) and it was unclear whether there were early signs of PD or not. Assuming that a single gene locus was responsible for the Parkinsonism in this family, the mode of transmission was consistent with autosomal dominant inheritance with reduced penetrance. Based on deceased family members, who had lived to the age of 75, XIII-1 to XIII-9 and XIV-14 to XIV-XIV:25 (as these were the only individuals for which complete medical information was known and who had lived long enough to fully acquire their PD risk), some 36% (4/11) of individuals were affected. A summary of the clinical phenotype and response to L-dopa are shown in Table 5.3 (below) whilst a more detailed description of the proband and autopsied case follows. Clinical status was known for only the three most recent generations (XIV, XV and XVI), but none of generation XVI have fully realised their PD risk.

The mean age of onset for family A was 64 years old (42-75;  $SD \pm 11.2$ ). At least two individuals were obligate heterozygotes with no evidence of PD based on interviews with their relatives: XIII:14 died aged 78 years old of "bronchitis" and his daughter (XIV:25) died aged 82 years of "probable heart disease". Other disorders that were noted in members of this kindred include XV:11, who died of amyotrophic lateral sclerosis aged 53 years, and XV:26 who had an asymmetrical postural and action tremor for the last three years. Dementia has not been a feature in any of the examined affected members.

#### 5.3.3.1 UK 403, individual XV:34 (the index case)

XV:34 developed an asymmetrical rest tremor and bradykinesia of her right arm at the age of 65 years. She was put on L-dopa with good effect aged 66 years. Parkinsonism slowly progressed and at last assessment was Hoehn and Yahr stage 2.5 with some postural instability.

### 5.3.3.2 UK 403, individual XIV:21 (the autopsied case)

XIV:21 initially presented with a rest tremor in her left arm, first noted whilst holding a pair of binoculars aged 65 years. Her symptoms gradually progressed with subsequent development of bradykinesia and a hesitant gait. She was put on L-dopa and remained on it for at least a further 16 years. There was a good response with L-dopa (Sinemet) and Pergolide throughout. She subsequently developed marked motor fluctuations and dyskinesias. These symptoms slowly progressed over the following 20 years and she was bed-bound for last 18 months of her life. She had been treated intermittently for depression, but there was no evidence of dementia. She died aged 85 years old of septicaemia.

### 5.3.4 Genealogical studies of UK 403

The kindred had approximately 10,000 members in some 250 branches, but only the two branches of this pedigree where Parkinson's disease was known to be a feature and with a definite common ancestor are shown in Figure 5.4. The founding couple were both born in a village in Suffolk in the 15<sup>th</sup> century (I:1 born 1450; I:2 born 1480). The kindred shared an unusual family name, with only 1 in 6000 UK families sharing this name (Telecommunications, 1998). The origins of all living individuals with this family name can be traced back to this village in Suffolk. Reviewing the genealogical data, it is apparent that several members of this kindred migrated to London in the late 17th and early 18th century (for example, XI:20 shown in figure 6.x), as many did during the Industrial Revolution. The numbers of individuals who settled in and around Shoreditch in the early part of the 18th century is worthy of comment as several direct descendants of the founding couple were baptised, married or buried in the East End of London during this time. James Parkinson lived all his life (1755-1824) and also attended St Leonard's church in Shoreditch regularly where he was secretary of the Sunday School (Critchley, 1955). Given that he rarely travelled outside of London (Roberts *et al.*, 1997), and three of the 6 patients he originally described he viewed in the street, rather than by formal examination (Parkinson, 1817), it is possible that he may have met some members of this kindred. Unfortunately, this has proved impossible to substantiate as there are no records of the patients he described and, similarly, there are no medical records of the Suffolk kindred for this period, thus this must remain highly speculative.

During the recruitment for this study, a number of other PD kindreds were identified where family members originated from Suffolk. An example of one such kindred, UK 403A is shown in Figure 5.5 and originates from the same village as UK 403. There was strong circumstantial evidence to suggest a genealogical link between UK 403 and UK 403A (i) the graves of the two families were intermingled in the same village graveyard; (ii) there were at least four marriages which have taken place between members of UK 403 and persons bearing the same surname as UK 403A in the 15<sup>th</sup> and 16<sup>th</sup> century (Figure 5.5, II:4, III:2, IV:3 and V:6) . The origins of UK 403A were traced back to the late 18<sup>th</sup> century, but no firm genealogical link between UK 403 and UK 403A could be made.

### **5.3.5 Clinical description of UK 403A**

Family B have 4 affected members (Table 5.3 and Figure 5.4): III:3 and III:6 have L-dopa responsive typical PD (examined by DJN and myself); II:4 and II:8 have PD based on family report. In addition, there were other members of UK 403A who appeared to have either essential tremor (I:1, II:6; based on family report) or highly atypical Parkinsonism (III:4; examined by DJN) rather than PD. No post-mortem data were available on this kindred.

Figure 5.3: Pedigree of UK403 with autosomal dominant PD.

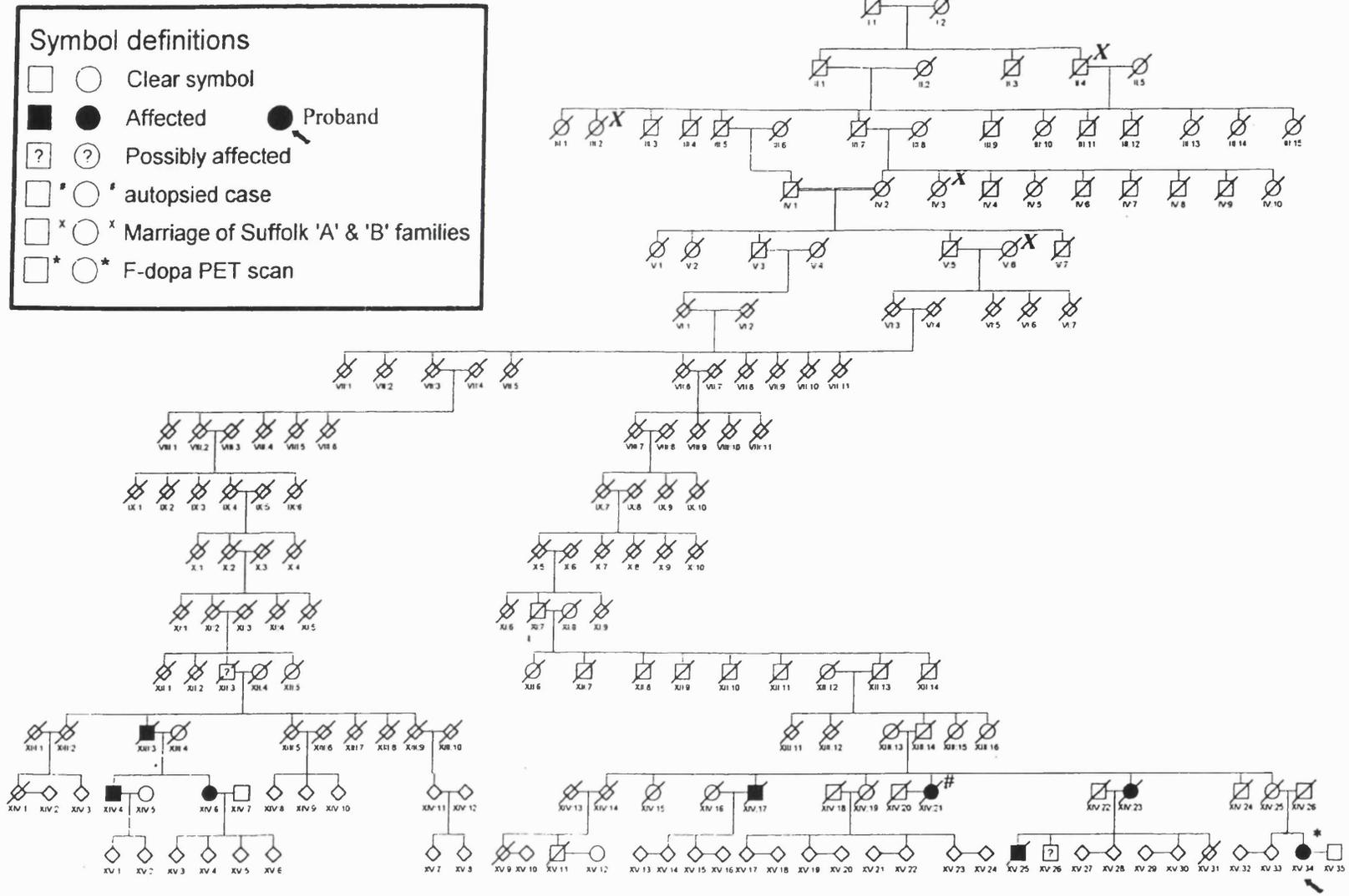
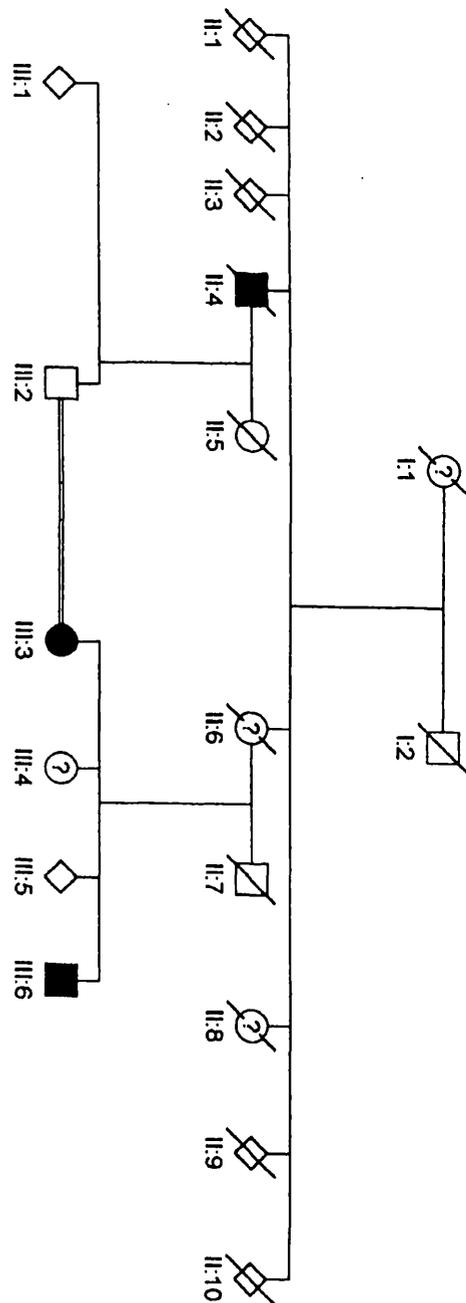


Figure 5.4: Pedigree of UK403A with autosomal dominant PD.



**Table 5.3:** A clinical summary of affected members of family UK403 & UK403A

UK 403											
Case (individuals in bold correspond with 'affecteds' in figs 1A & 1B )	Sex	Age at onset	Disease duration (yrs) (& current status)	First symptoms	Bradykinesia	Rigidity	Rest Tremor	Postural instability	Other features	Response to L-dopa	Source
<b>XV:34</b>	F	65	7 (alive)	Asymmetric RT	+	+	+	+	O-OF;D	+	PE
<b>XIV:21</b>	F	65	20(dead)	Asymmetric RT	+	+	+	+	O-OF;D; autopsied	+	PE
<b>XIV:23</b>	F	75	5 (dead)	Asymmetric RT	+	+	+	+		+	PE
<b>XV:25</b>	M	42	11(dead)	Asymmetric B	+	+	+	+	Painful dystonias; panic attacks	+	PE
<b>XIV:17</b>	M	73	10(dead)	B	+		+			Not given	V;H
<b>XIV:6</b>	F	57	14(alive)	Asymmetric RT	+	+	+	-	D	+	PE
<b>XIV:4</b>	M	68	6 (alive)	Asymmetric RT	+	+	+	+	O-OF, D	+	PE
<b>XIII:3</b>	M	91	2 (dead)	RT	+	+				Not given	H
<b>XV:26</b>	M	54	3 (alive)	Asymmetric Tremor	-	-	-	-	Asymmetrical action tremor; impassive face	Unclear	PE
<b>III:3</b>	F	58	16(alive)	Asymmetric RT/B	+	+	+	+	O-OF;D	+	PE
<b>III:6</b>	M	73	5 (alive)	Asymmetric B/RT	+	+	-	-	O-OF;D	+	PE
UK 403A											
<b>I:1</b>	F	76	10 (dead)	Tremor							H
<b>II:4</b>	M	Late 50s	~10 (dead)	Asymmetric B/RT	+		+			Not given	H
<b>II:6</b>	F	85	10 (dead)	Tremor							H
<b>II:8</b>	F	72	~10 (alive)	Tremor	+						H
<b>III:3</b>	F	58	15 (alive)	Asymmetric B/RT	+	+	+	+	Micrographia	+	PE
<b>III:4</b>	F	75	7 (alive)		+	+	-		Probable neuroleptic induced PD, akathisia; dementia		PE
<b>III:6</b>	M	73	5 (alive)	Asymmetric B/RT	+	+	+	+		+	PE

RT= Rest Tremor; O-OF= on-off fluctuations; D= dyskinesias; PE= personal examination; V= review of family video; H= historical report from family members

### 5.3.6 Neuropathology of case XIV:21 (Courtesy of Dr S.E Daniel, PDS brain bank)

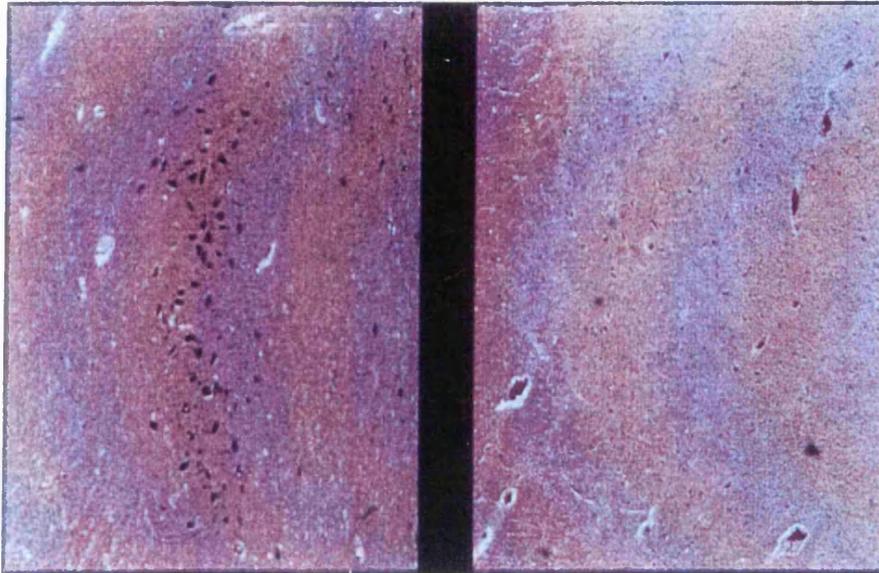
The whole brain was fixed in 10% neutral formalin for 6 weeks prior to cutting. Tissue blocks were taken from the frontal [anterior (Brodmann area 9) and precentral region (Brodmann area 4)], temporal, parietal and occipital cortex, hippocampus, parahippocampus, striatum, thalamus, hypothalamus, subthalamus, *substantia innominata*, cerebellar vermis and hemisphere, midbrain, pons and medulla. Under light microscopy sections of cerebrum, brain stem and cerebellum were examined using haematoxylin eosin, Luxol fast blue nissl, Bielschowsky silver impregnation and immunocytochemistry for GFAP (dilution 1:400), ubiquitin (dilution 1:150), and  $\alpha$ -synuclein (dilution 1:2000).

Macroscopically, there was mild atrophy involving the posterior frontal region with slight dilatation of the lateral ventricle. Pigment was markedly depleted in *substantia nigra* and *locus ceruleus*. Under light microscopy, pigmented neurons of the *substantia nigra* and *locus ceruleus* were moderately depleted with several surviving nerve cells containing Lewy bodies (Figure 5.3). Above the brain stem, Lewy bodies were also identified in *nucleus basalis* of Meynert and amygdaloid nuclear complex. The caudate nucleus showed slightly increased gliosis, but there were no significant abnormalities of thalamus, subthalamus, putamen, pallidum or claustrum.

In cerebral cortex occasional Lewy bodies were found in anterior cingulate gyrus, parahippocampus, frontal and temporal neocortex. Lewy body score according to consensus guide lines (McKeith *et al.*, 1996) area 1=0, area 2 (anterior cingulate gyrus)=13, area 3=1, area 4 (parahippocampus)=3, area 5=0. Lewy-neurites were few in number in CA2/3 region.

All Lewy body pathology was immunoreactive with anti-ubiquitin and anti- $\alpha$ -synuclein (see Chapter 4, Figure 4.3) and was more easily visualised using these techniques. There were additional age-related cortical changes of mature senile plaques, which were moderate in number in frontal cortex. Neurofibrillary tangles were inconspicuous except in hippocampus and parahippocampus. The overall appearances were characteristic of idiopathic Parkinson's disease.

**Figure 5.5:** Neuropathology in case XIV:21 of UK 403.



**A. NORMAL MIDBRAIN**

**B. MIDBRAIN FROM  
AUTOPSIED CASE**

### 5.3.7 PET studies on UK 402 and UK 403

All PET scans were performed and analyzed by a single operator (Dr Paola Piccini [PP], Hammersmith Hospital, London). An example PET scan of the index case in UK 402 (III:17) is shown in appendix 5 and results provided below for discussion purposes. An affected member from each kindred was scanned using an ECAT EXACT3D (CTI/Siemens 966) 3D-only PET tomograph after intravenous injection of 3.5-4.5 mCi of  $^{18}\text{F}$ -dopa. Analysis of data was performed using in-house software written in IDL (Research Systems, Inc, Boulder, Colorado). Region of interest analysis was performed using a standard template as previously described (Rakshi *et al.*, 1996).  $^{18}\text{F}$ -dopa influx constants ( $K_i \text{ min}^{-1}$  values) were calculated for right and left caudate and putamen using the multiple time graphical analysis approach with occipital activity as a reference tissue.

#### 5.3.7.1 UK 402, individual III.17

The  $^{18}\text{F}$ -dopa  $K_i$  values in this subject were: Left Caudate=0.0072, Right Caudate=0.0074, Left Putamen=0.0054, Right Putamen=0.0057  $\text{min}^{-1}$ . In this subject also the pattern of striatal  $^{18}\text{F}$ -dopa reduction is characteristic of idiopathic disease.

#### 5.3.7.2 UK 403, individual XV:34

The  $^{18}\text{F}$ -dopa  $K_i$  values in this subject were: Left Caudate=0.0068, Right Caudate=0.0071, Left Putamen=0.0047, Right Putamen=0.0052  $\text{min}^{-1}$  ( $^{18}\text{F}$ -dopa  $K_i$  values for 12 normal volunteers matched for age: R and L Caudate=0.0145, R and L Putamen=0.0150). The observed pattern of striatal  $^{18}\text{F}$ -dopa reduction is characteristic of idiopathic disease, uptake in the putamen being affected more than in the caudate (Brooks *et al.*, 1990a).

## 5.4 MOLECULAR INVESTIGATIONS

Genomic DNA was extracted from peripheral blood using standard techniques (see section 2.4.1) and the PCR products analysed on an ABI 377 automated sequencer (ABI, San Francisco, CA, USA) using Genescan 2.1 and Genotyper 2.1 software (see section 2.6). Linkage/haplotype analysis was performed of the known loci for

parkinsonism was performed. Genotype data from the markers shown in Figures 5.6-5.14 (below) were managed and recoded for linkage analysis using Cyrillic 2.1.3. LOD scores were generated using the FASTLINK version of the MLINK program (Cottingham *et al.*, 1993; Dwarkadas *et al.*, 1994)) under an “affecteds-only” analysis. This included only clinically definite PD as indicated in the pedigrees shown in figures 5.1-5.3. Affecteds-only analysis was applied given the wide range of disease onset. Penetrance may be uncertain, as has been observed in other loci predisposing to familial PD (Polymeropoulos *et al.*, 1996). Parkinsonism was treated as a dichotomous, autosomal dominant trait with the disease allele frequency set at 0.0001-the population prevalence of familial parkinsonism (De Rijk *et al.*, 1997b) and marker allele frequencies were set equal. A phenocopy rate for PD in the general population was set at 1.5% (De Rijk *et al.*, 1997b). In the multipoint analyses, all markers were included in a single two-point linkage calculation apart from the 2p13 (PARK3) locus, where three overlapping partial linkage analyses were performed. Intermarker distances were taken from the Marshfield map (<http://www.marshmed.org/genetics/>) and are given in Kosambi sex-averaged centiMorgans.

Two-point linkage analysis and multipoint analysis was performed using two linked markers to the PARK1 locus on chromosome 4q21-q23 as previously described (Polymeropoulos *et al.*, 1996). D4S2380 co-localises with D4S423 which flanks the alpha-synuclein gene (SNCA). Exclusion analysis with four polymorphic markers (D6S1550, D6S305, D6S411, D6S1579) spanning the PARK2 locus was performed (Matsumine *et al.*, 1997). These markers co-localise on the Marshfield map and therefore two-point lod scores for chromosome 6 markers were calculated. Multipoint analysis for the PARK3 locus was performed using data from eight polymorphic markers spanning the region from D2S2320 to D2S286 (PARK3 locus) (Gasser *et al.*, 1998) and from four markers linked to PARK 4 (Farrer *et al.*, 1999b). Multipoint linkage analysis in this area also included examination of D4S405, a marker 12.29 cM distal to the PARK4 locus (4p14-the location of the gene ubiquitin C-terminal hydrolase isoenzyme L1 (UCHL-1, PGP9.5)). Where multipoint data was not conclusive across the region (i.e. lod scores were not  $<-2.0$ , the accepted criteria for exclusion), the relevant gene was sequenced in an index case from that kindred. In each region, where two point analyses indicated that a particular marker was not informative, it was not used in multipoint analyses.

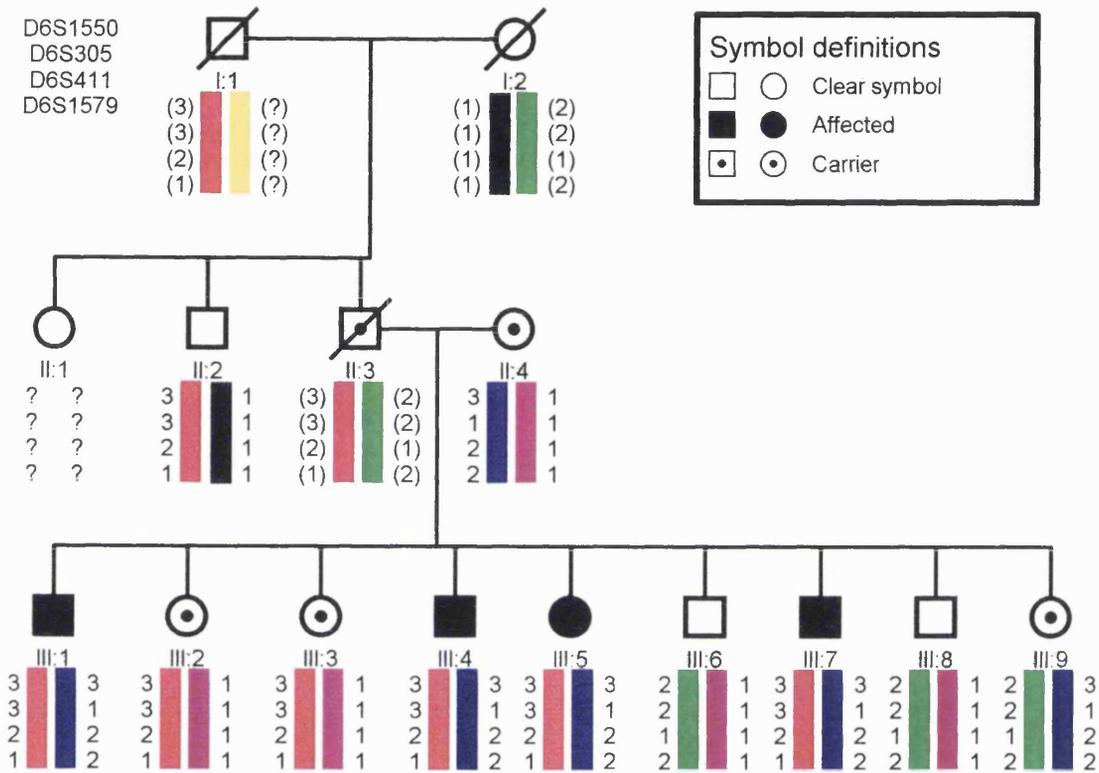
### 5.4.1 UK 401: Molecular Analysis of the PARK2 locus

The inheritance pattern observed in UK 401 is consistent with an autosomal recessive model. Linkage studies at the PARK 2 locus showed a segregating haplotype in affected members (D6S1550, D6S305 D6S411, 6S1579—see Figure 5.6 below). Analysis of haplotypes in the kindred showed two parkin haplotypes 3-3-2-1 and 3-1-2-2 (presumed mutant alleles segregating with the disease). These haplotypes were heterozygous in the clinically affected individuals suggesting they were compound heterozygotes (later confirmed in sequencing studies). LOD scores generated from linkage data presented at the PARK2 locus in this Irish kindred (Table 5.4) at the four markers is not conclusive due to the small size of the kindred, but it was suggestive of linkage to PARK2. Sequencing of the coding region of the Parkin gene by collaborators revealed a compound point mutation (Intron 5, splice-site, Dr M Farrer, personal communication) and a heterozygous deletion (Exon 8) (Luecking *et al.*, 2000). Other loci were not examined in this kindred due to these results.

**Table 5.4:** Two-point LOD scores at the PARK2 locus for UK 401. These markers form a segregating haplotype and co-localise on the genetic map at 6q25-27.

Marker	Recombination Fraction							
	0.0	0.001	0.01	0.05	0.1	0.2	0.3	0.4
<b>D6S1550</b>	1.81	1.80	1.77	1.63	1.44	1.03	0.60	0.19
<b>D6S411</b>	0.90	0.90	0.89	0.81	0.72	0.52	0.30	0.09
<b>D6S305</b>	1.81	1.80	1.77	1.63	1.44	1.03	0.60	0.19
<b>D6S1579</b>	0.9	0.9	0.89	0.81	0.72	0.52	0.30	0.09

**Figure 5.6:** Haplotype analysis of UK 401 at the PARK2 locus. Markers used for the genotyping are given in the adjacent panel.



## 5.4.2 UK 402 & 403: linkage analysis of candidate regions

### 5.4.2.1 PARK1 locus 4q21-23 (SNCA)

Two-point analysis excluded linkage to the two polymorphic markers most closely linked to PARK 1 (D4S2380 and D4S1647) in UK 402 and UK 403, although the multipoint exclusion data was not conclusive across the region (Figures 5.7 and 5.8 below). For this reason, the entire coding region of SNCA was sequenced in an index case in each family and no mutations were found (Vaughan *et al.*, 1998b).

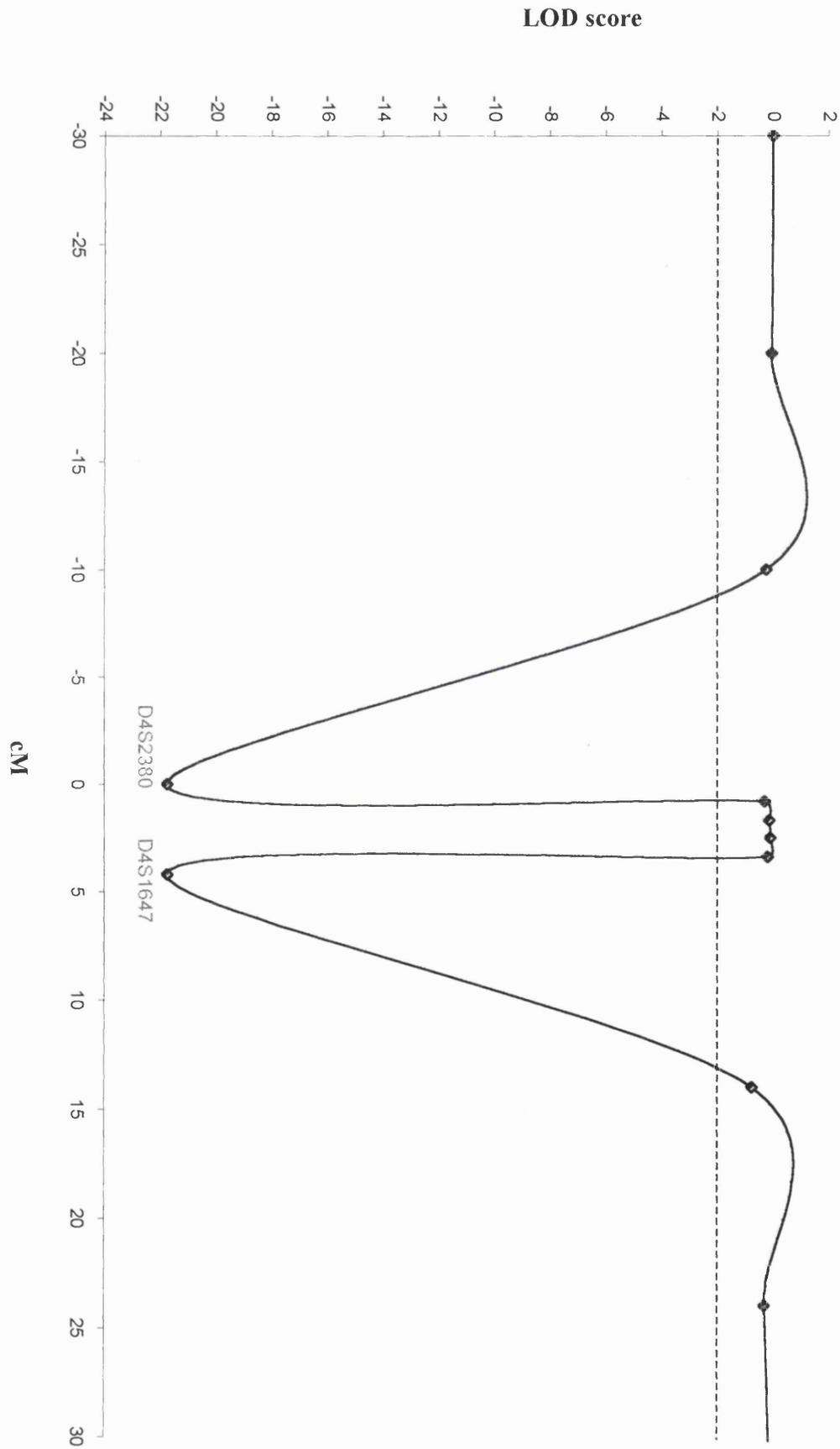
**Table 5.5:** Two-point LOD scores between parkinsonism in UK 402 and microsatellite markers linked to the PARK1 locus

Marker	Recombination Fraction							
	0.0	0.001	0.01	0.05	0.1	0.2	0.3	0.4
<b>D4S2380</b>	$-\infty$	-2.13	-1.14	-0.48	-0.23	-0.1	-0.01	-0.0010
<b>D4S1647</b>	$-\infty$	-4.59	-2.60	-1.27	-0.75	-0.1	-0.01	-0.0010

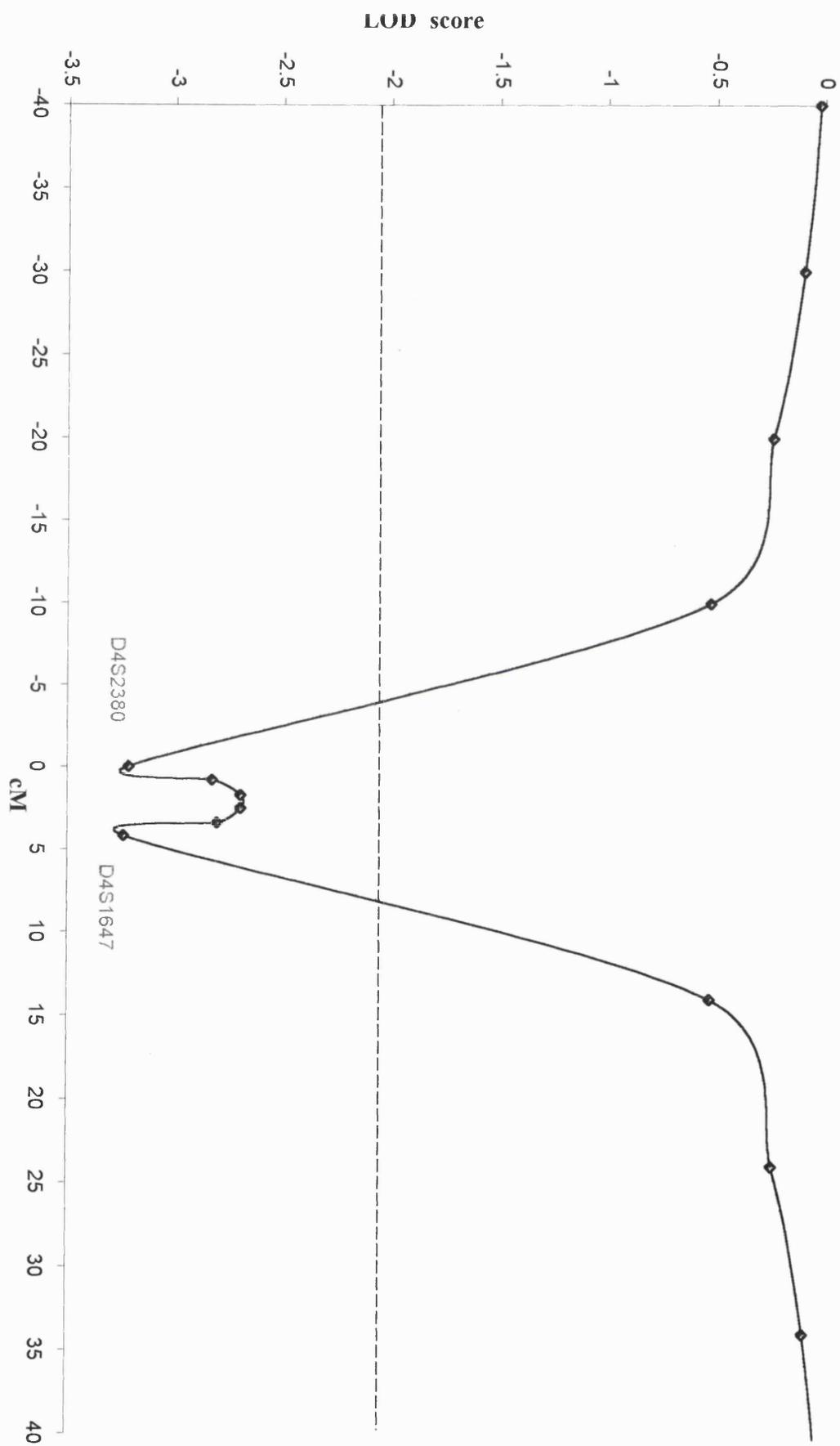
**Table 5.6:** Two-point LOD scores between parkinsonism in UK 403 and microsatellite markers linked to the PARK1 locus

Marker	Recombination Fraction							
	0.0	0.001	0.01	0.05	0.1	0.2	0.3	0.4
<b>D4S2380</b>	-6.59	-4.76	-2.89	-1.53	-0.95	-0.45	-0.2	-0.1
<b>D4S1647</b>	-3.18	-2.41	-1.48	-0.81	-0.52	-0.2	-0.1	-0.01

**Figure 5.7.** Multipoint LOD score graph for selected markers at the PARK 1 locus in UK 402



**Figure 5.8:** Multipoint LOD score graph for selected markers at the PARK 1 locus in UK 403



5.4.2.2. *PARK2* locus 6q 25.2-27 (*Parkin*)

Most families with mutations in the *Parkin* gene described to date show a recessive model of inheritance. For UK 402 and 403, two point lod scores for markers linked to the *PARK2* locus were calculated assuming an autosomal dominant model, as this was the pattern of inheritance (figs 5.2 and 5.3) observed in these kindreds (see tables 5.7 and 5.8) but genotypes are shown in Figures 5.9 and 5.10 (below) to illustrate that neither haplotypes nor consanguineous genotypes indicate linkage to the *PARK2* locus in UK 402 and UK 403. Alleles numbered 0 indicate that no score for this marker was obtained for this individual due to an unreliable or uninterpretable PCR product.

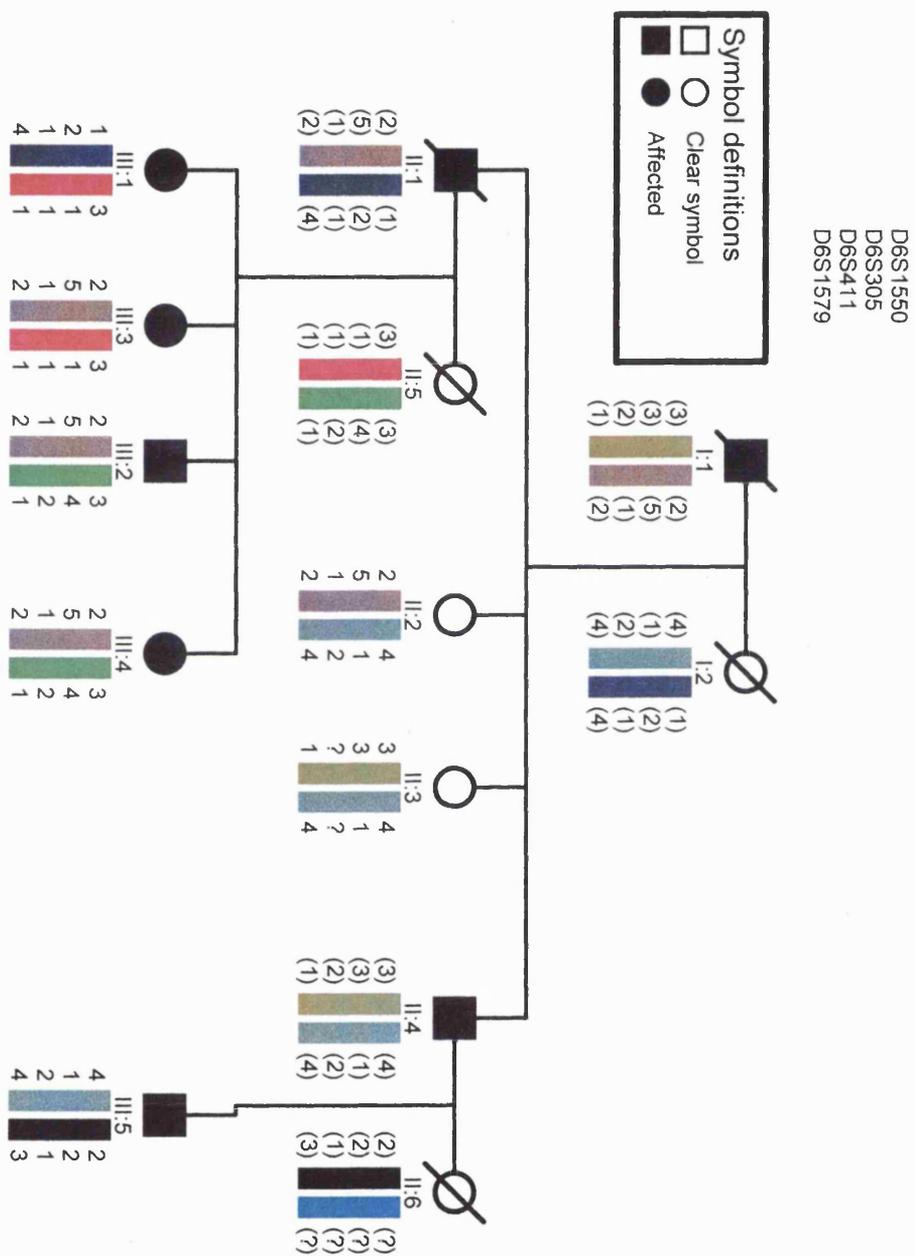
**Table 5.7:** Two-point LOD scores between parkinsonism in UK 402 and microsatellite markers linked to the *PARK2* locus

Marker	Recombination Fraction							
	0.0	0.001	0.01	0.05	0.1	0.2	0.3	0.4
<b>D6S1550</b>	-∞	-1.82	-0.84	-0.22	-0.02	-0.07	-0.06	-0.02
<b>D6S305</b>	-∞	-4.66	-2.67	-1.33	-0.79	-0.33	-0.12	-0.03
<b>D6S411</b>	0.36	0.36	0.34	0.27	0.19	0.08	0.02	0
<b>D6S1579</b>	-∞	-2.3	-1.3	-0.62	-0.35	-0.12	-0.04	-0.01

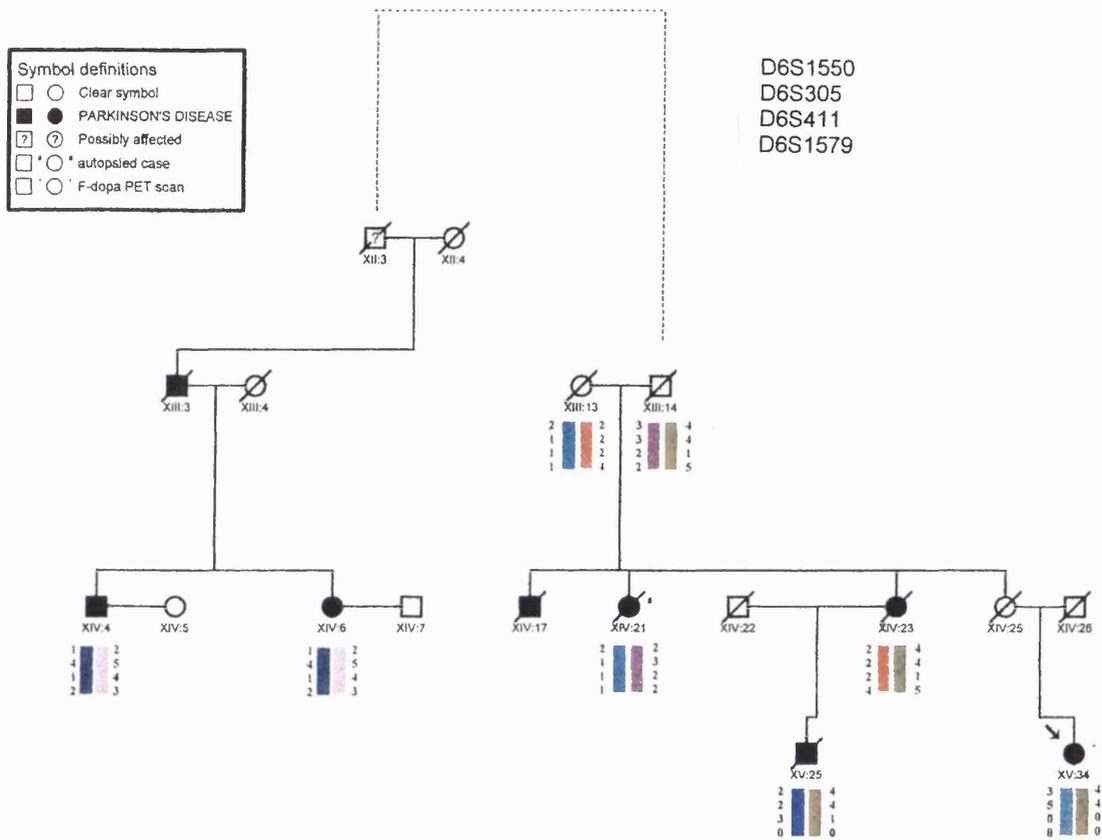
**Table 5.8:** Two-point LOD scores between parkinsonism in UK 403 and microsatellite markers linked to the *PARK2* locus

Marker	Recombination Fraction							
	0.0	0.001	0.01	0.05	0.1	0.2	0.3	0.4
<b>D6S1550</b>	-2.90	-2.04	-1.11	-0.47	-0.23	-0.06	-0.01	0
<b>D6S305</b>	-2.88	-2.1	-1.18	-0.54	-0.3	-0.11	-0.03	-0.01
<b>D6S411</b>	0.2	0.19	0.19	0.16	0.13	0.08	0.04	0.01
<b>D6S1579</b>	-2.89	-2.11	-1.19	-0.54	-0.3	-0.11	-0.03	-0.01

**Figure 5.9.** Simplified pedigree of UK 402 and haplotypes of markers spanning the PARK2 locus



**Figure 5.10** Simplified pedigree of UK 403 and haplotypes of markers spanning the PARK2 locus



### 5.4.2.3 PARK3 locus (2p13)

Linkage of UK 402 and UK 403 to PARK3 was excluded using two point and multipoint analysis. Multipoint analysis results are shown in Tables 5.9 and 5.10 and in Figures 5.11 and 5.12. The dots correspond to the lod score at each individual marker. Not all markers are labelled as such for figure clarity purposes. The markers labelled are the ones at which maximum informativeness was obtained. This corresponds to D2S2320-1.8cM-D2S134-2.6cM-D2S441-0.8cM-D2S358-0.8cM-D2S2115-0cM-D2S2113-2.6cM-D2S2110-1.3cM-D2S1394-2.1cM-D2S286. The data also include markers linked to the segregating haplotype (3.2 cM) identified for 2p13 (markers underlined) (Gasser *et al.*, 1998).

**Table 5.9:** Two-point LOD scores between parkinsonism in UK 402 and microsatellite markers linked to the PARK3 locus

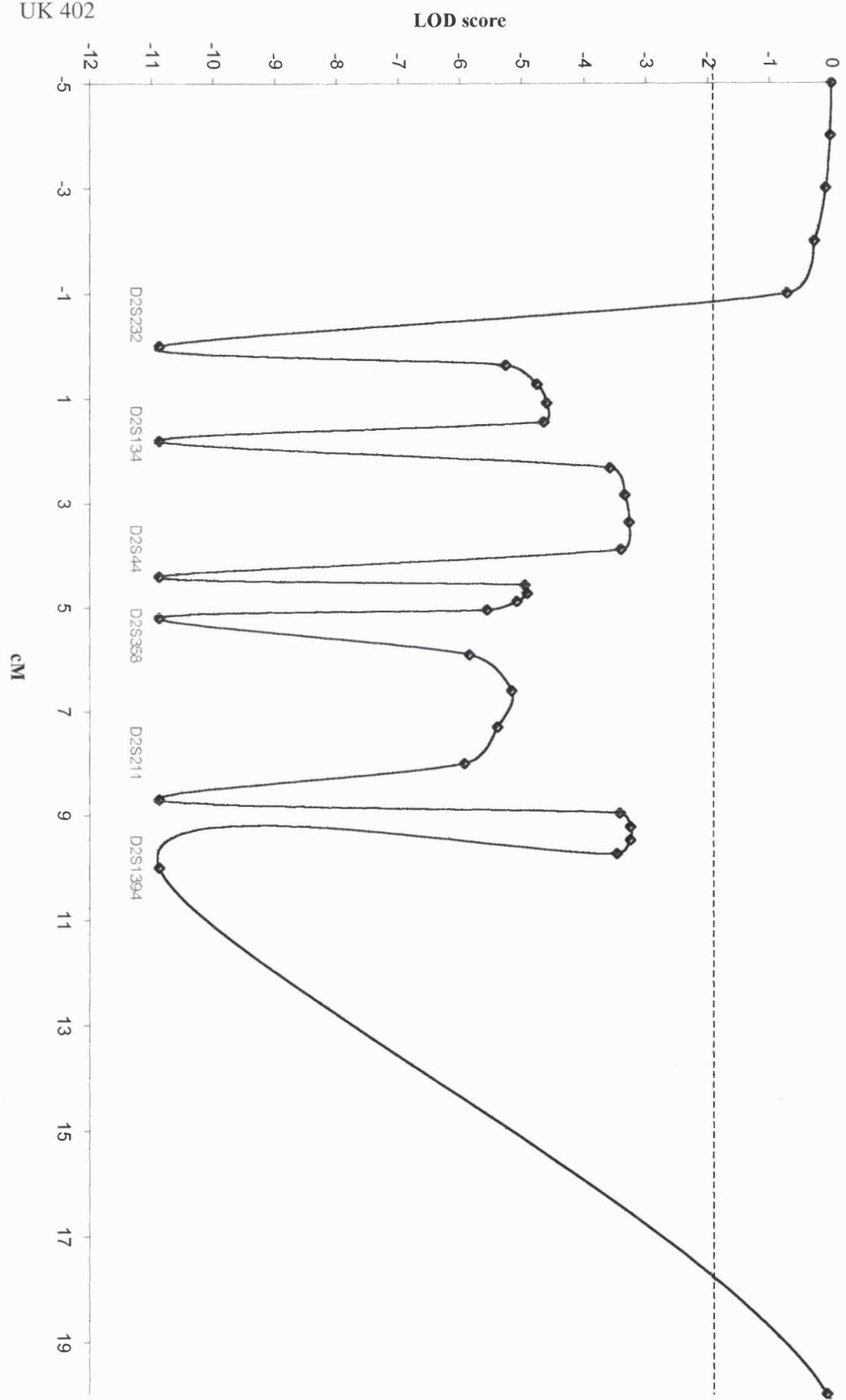
Marker	Recombination Fraction							
	0.0	0.001	0.01	0.05	0.1	0.2	0.3	0.4
<b>D2S2320</b>	-∞	-7.62	-4.49	-2.42	-1.49	-0.64	-0.24	-0.06
<b>D2S134</b>	-∞	-7.0	-4.02	-2.0	-1.19	-0.49	-0.18	-0.04
<b>D2S441</b>	-∞	-7.68	-4.63	-2.41	-1.46	-0.61	-0.22	-0.05
<b>D2S358</b>	-∞	-4.33	-2.35	-1.05	-0.56	-0.18	-0.06	-0.01
<b>D2S2110</b>	-∞	-6.89	-3.91	-1.91	-1.11	-0.44	-0.15	-0.03
<b>D2S1394</b>	-∞	-7.35	-4.35	-2.28	-1.42	-0.62	-0.24	-0.06

**Table 5.10:** Two-point LOD scores between parkinsonism in UK 403 and microsatellite markers linked to the PARK3 locus

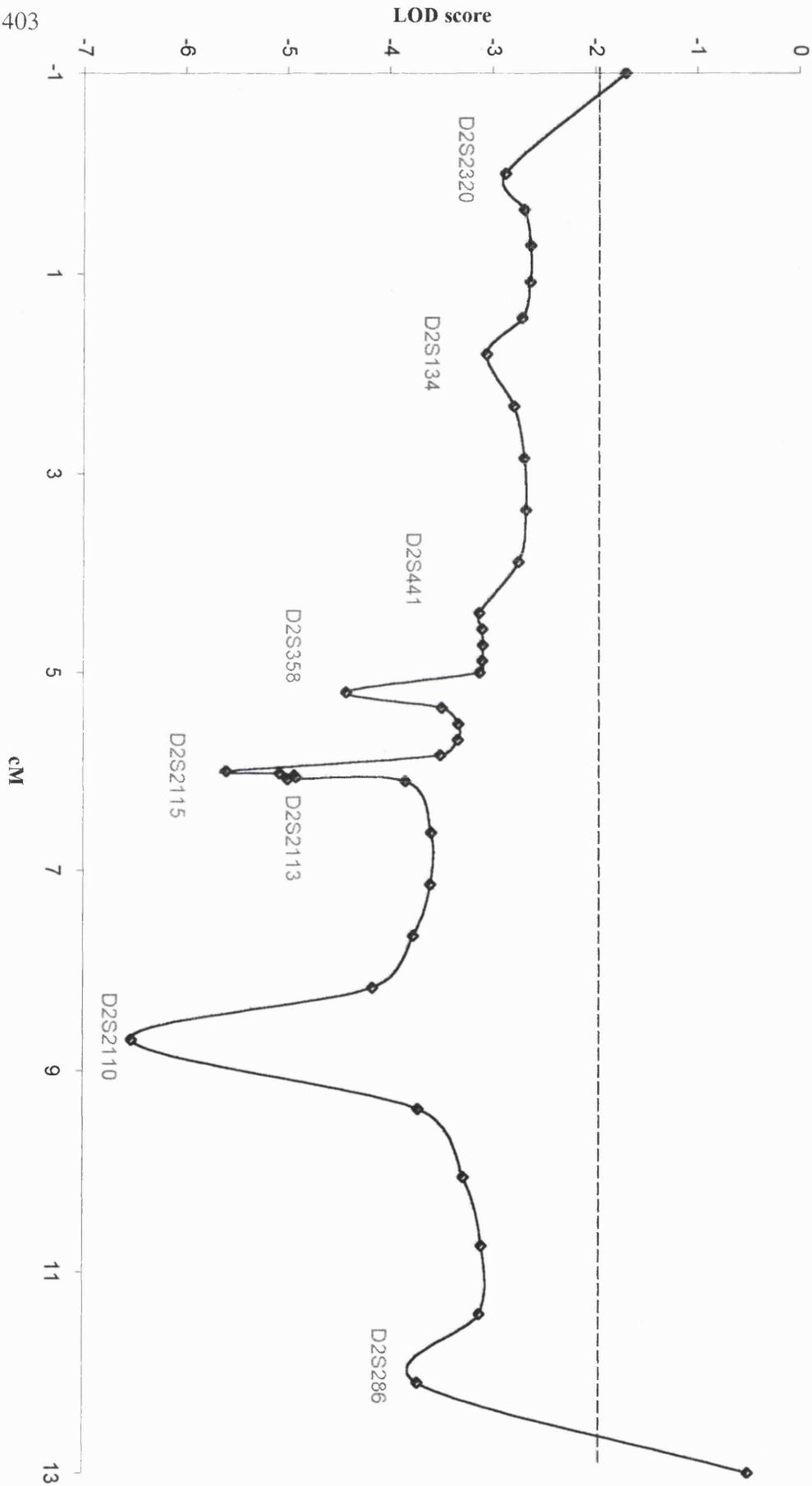
Marker	Recombination Fraction							
	0.0	0.001	0.01	0.05	0.1	0.2	0.3	0.4
<b>D2S2320</b>	-2.92	-2.25	-1.35	-0.67	-0.38	-0.15	-0.05	-0.01
<b>D2S134</b>	-3.11	-2.34	-1.41	-0.74	-0.46	-0.20	-0.08	-0.01
<b>D2S441</b>	-2.88	-2.1	-1.88	-0.54	-0.3	-0.11	-0.03	-0.01
<b>D2S358</b>	-3.22	-2.45	-1.51	-0.82	-0.52	-0.23	-0.09	-0.01
<b>D2S2115</b>	-3.27	-2.23	-1.28	-0.62	-0.37	-0.15	-0.06	-0.01
<b>D2S2113</b>	-2.94	-2.08	-1.15	-0.51	-0.27	-0.09	-0.02	-0.01
<b>D2S2110</b>	-6.48	-4.49	-2.58	-1.25	-0.73	-0.28	-0.09	-0.01
<b>D2S286</b>	-3.28	-2.24	-1.29	-0.63	-0.37	-0.15	-0.05	-0.01

Figure 5.11. Multipoint LOD score graph for selected markers at the PARK 3 locus in

UK 402



**Figure 5.12:** Multipoint LOD score graph for selected markers at the PARK 3 locus in UK 403



5.4.2.4 PARK 4( locus 4p15)

Multipoint and two-point analyses excluded linkage to PARK4 (4p15) and UCH-L1 (4p14). The marker map in this region corresponds to D4S2397-3.0cM-D4S391-0.6cM-D4S1609-0.6cM-D4S230-12.29cM-D4S405.

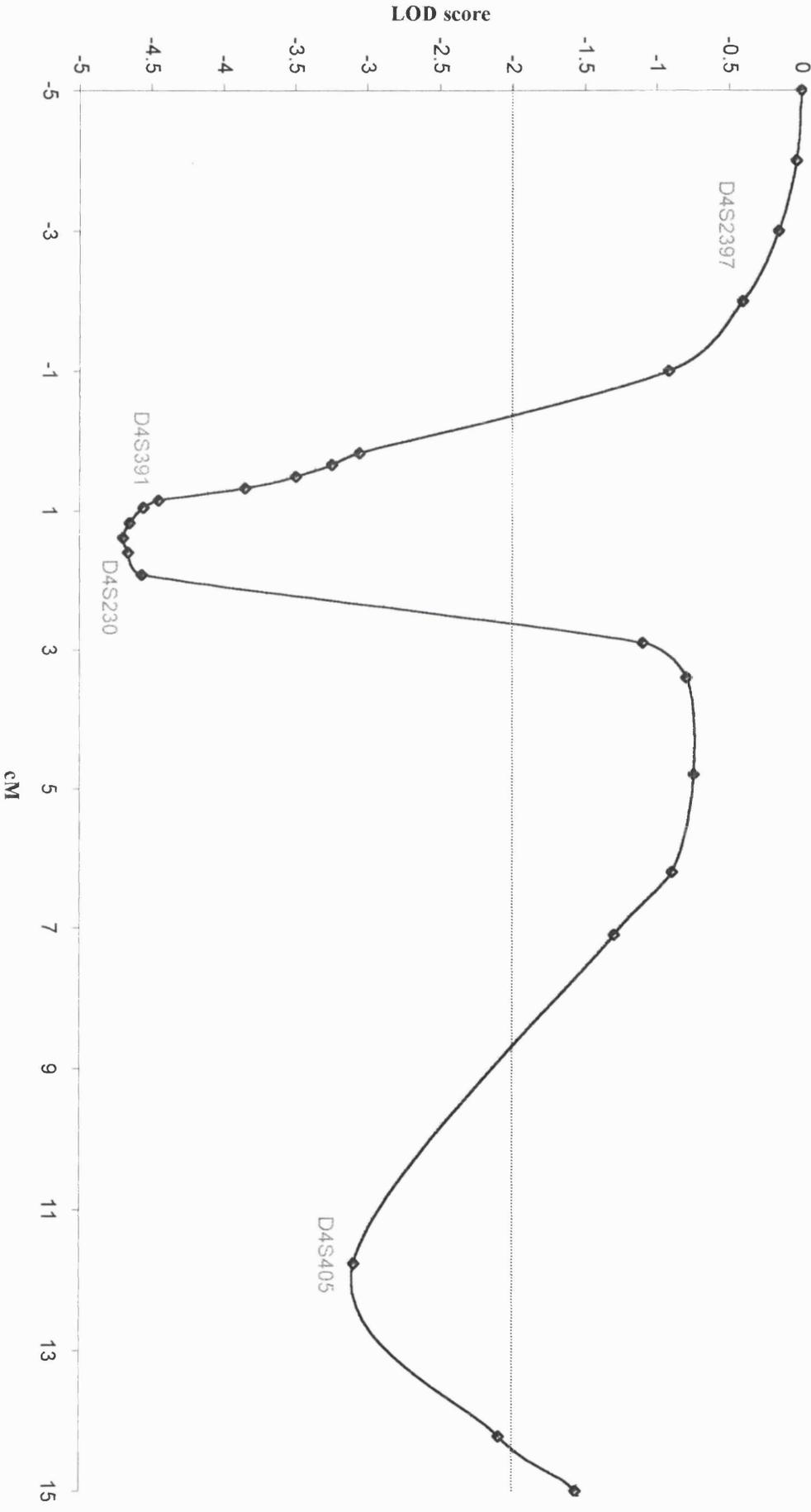
**Table 5.11:** Two-point LOD scores between parkinsonism in UK 402 and microsatellite markers linked to the PARK4 locus and 4p14 (UCH-L1)

Marker	Recombination Fraction							
	0.0	0.001	0.01	0.05	0.1	0.2	0.3	0.4
D4S2397	-0.21	-0.21	-0.2	-0.16	-0.12	-0.06	-0.03	-0.01
D4S391	-3.04	-2.00	-1.05	-0.42	-0.20	-0.06	-0.02	-0.00
D4S1609	0.11	0.11	0.10	0.09	0.07	0.04	0.02	0.01
D4S230	-2.98	-2.13	-1.20	-0.55	-0.31	-0.12	-0.04	-0.01
D4S405	-3.61	-2.83	-1.85	-1.00	-0.61	-0.25	-0.09	-0.02

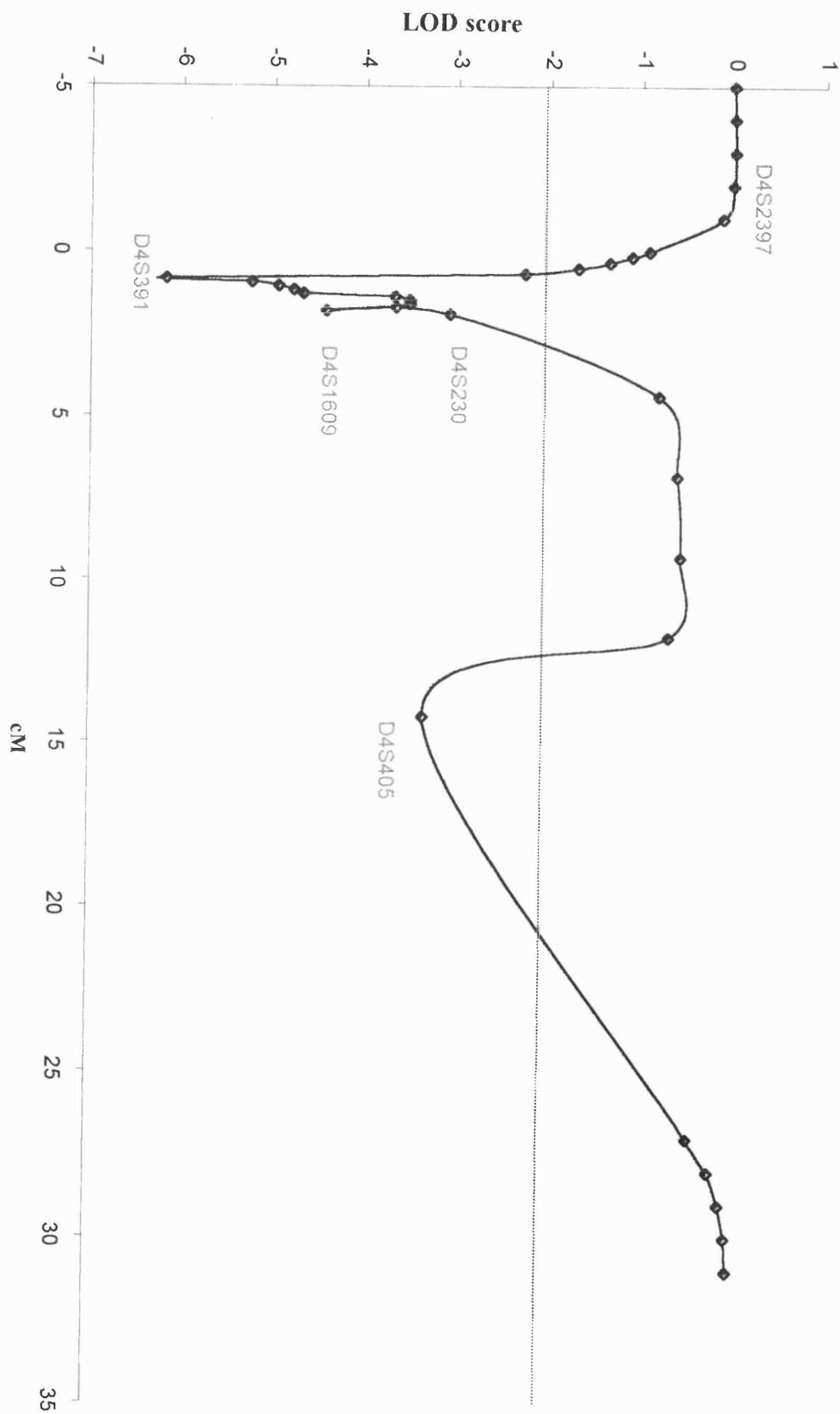
**Table 5.12:** Two-point LOD scores between parkinsonism in UK 403 and microsatellite markers linked to the PARK4 locus and 4p14 (UCH-L1)

Marker	Recombination Fraction							
	0.0	0.001	0.01	0.05	0.1	0.2	0.3	0.4
D4S2397	0.2	0.2	0.19	0.17	0.14	0.08	0.04	0.01
D4S391	-6.26	-4.42	-2.57	-1.24	-0.72	-0.29	-0.10	-0.02
D4S1609	-3.08	-2.11	-1.17	-0.52	-0.28	-0.10	-0.03	-0.01
D4S230	-2.88	-2.10	-1.18	-0.54	-0.30	-0.11	-0.05	-0.01
D4S405	-3.09	-2.05	-1.11	-0.48	-0.26	-0.11	-0.05	-0.01

**Figure 5.13.** Multipoint LOD score graph for selected informative markers at the PARK 4 locus and 4p14 (UCH-L1) in UK 402



**Figure 5.14:** Multipoint LOD score graph for selected informative markers at the PARK 4 locus and 4p14 (UCH-L1) in UK 403



## 5.5 DISCUSSION

### 5.5.1 Parkin sequencing and retrospective PET analysis in UK 401

At the time the linkage studies were performed on this kindred (prior to the cloning of the Parkin gene), the PARK 2 locus had been refined to an 11.3-cM region between the locus defined by the markers D6S411/D6S1550/D6S1579 and marker D6S1719. A deletion in a German kindred in this paper had restricted the region further to one containing D6S411 and D6S1550 and flanked by D6S1579 and D6S305, which colocalise (Tassin *et al.*, 1998). For this reason, these four markers were chosen to try to detect an affected segregating haplotype at the PARK2 locus. Originally one parental haplotype was inferred for the father (deceased) and the assumption was made that each parent carried a mutant allele.

Subsequently, UK 401 was linked to PARK2 by myself and, after the Parkin gene was cloned (Kitada *et al.*, 1998a), this family was found to have a single heterozygous deletion in affected members (Luecking *et al.*, 2000). In addition, further sequencing of the proband has revealed a splice-site mutation in exon 5 (Dr M Farrer, personal communication). As several members of this family underwent extensive PET scans six years before this study (Sawle *et al.*, 1992) the subsequent molecular analysis in has raised interesting questions about the interpretation of the original PET data.

PET with [<sup>18</sup>F]-DOPA has been used to identify pre-clinical parkinsonism in at-risk subjects (Brooks, 1991). All of the affected members of UK 401 underwent PET with [<sup>18</sup>F]-DOPA in 1990 (Sawle *et al.*, 1992). For each subject, the caudate and putamen *K<sub>i</sub>* values were all lower than normal values and comparable with those measured in patients with sporadic PD. In PD the earliest changes are often asymmetrical, targeting the posterior putamen with relative preservation of the caudate (Kish *et al.*, 1988). In progressive supranuclear palsy the pattern is usually symmetrical, affecting both caudate and putamen early in the disease (Brooks *et al.*, 1990b). Although the putaminal [<sup>18</sup>F]-DOPA uptake was similar in affected members to that in patients with sporadic PD, the reduction of caudate [<sup>18</sup>F]-DOPA uptake in all 4 patients was striking. Therefore, [<sup>18</sup>F]-DOPA uptake in this original study appeared to be symmetrically reduced in both caudate and putamen, suggesting a less selective neurodegenerative process than that observed in sporadic PD. Clinically unaffected members of UK 401 who underwent [<sup>18</sup>F]-DOPA PET scans in 1990 include individuals III:3 and III:8. III:3

was said to have an abnormal PET scan (Sawle *et al.*, 1992) with normal caudate *Ki* levels but with putamen levels just below the normal range. The explanation given for this was that it represented a focal subclinical deficit of the nigrostriatal pathway, as although this individual had been taking chlorpromazine, the [<sup>18</sup>F]-DOPA PET appearances in patients taking high dose neuroleptics are usually normal (Brooks, 1991). The unaffected daughter of III:5 (data not shown) was scanned at 19 years. Clinically she had a postural tremor and was not genotyped as part of this study. Her [<sup>18</sup>F]-DOPA PET scan in 1990 showed normal caudate uptake but putamen uptake in the range intermediate between normality and Parkinson's disease (Sawle *et al.*, 1992). Individual III:8 underwent [<sup>18</sup>F]-DOPA PET scanning on two occasions in 1990/1991, thirteen months apart. At initial scanning he was said to be clinically asymptomatic. [<sup>18</sup>F]-DOPA PET scanning was abnormal on both scans but there was no progression between scans.

Genotyping of the PARK2 locus in this family (see figure 5.6) showed that individual III:8 did not inherit either mutant allele and therefore it can be concluded that either III:8 was a phenocopy or the original clinical signs observed and [<sup>18</sup>F]-DOPA PET scan results were misleading. Given the authors clinical examination findings 10 years later and the fact that Parkin sequencing of III:8 did not reveal a mutation on either allele reinforces a cautious interpretation of [<sup>18</sup>F]-DOPA PET scanning results in isolation.

Sequencing of Parkin in UK 401 revealed a compound point mutation (intron 5, splice-site) and heterozygous deletion (exon 8). The exon 8 deletion in this kindred causes a frameshift mutation in the Parkin gene. Sequencing of Parkin is currently on-going to determine which parent (II:3 or II:4) is responsible for the transmission of the individual mutations. Mutations in the Parkin gene are considered to cause loss of function in Parkin protein, (compatible with a recessive disease) affecting its ability to act as a ubiquitin protein ligase in proteosomal degradation (Shimura *et al.*, 2000). One can speculate that this loss of function in Parkin only becomes clinically apparent in individuals who have two mutant alleles (although this is based solely on one abnormal PET scan (III:3) in the limited number of asymptomatic members scanned to date). The alternative explanation is that cautious interpretation of the [<sup>18</sup>F]-DOPA PET of individual III:3 is necessary, although genotyping studies in Figure 5.6 indicate that III:3 carries one of the mutant alleles (3-3-2-1). Few PET studies have been performed

on Parkin positive patients. (Broussolle *et al.*, 2000) found a profound decrease of [<sup>18</sup>F]-DOPA uptake, representing 28% of putamen and 44% of caudate nucleus control subject values similar to that described in sporadic PD. A kindred from South Tyrol (northern Italy) with familial PD was found to have mutations in the Parkin gene. PET data provided evidence that parkinsonism in this family was associated with presynaptic dopaminergic dysfunction similar to sporadic PD pathophysiology, along with alterations at the postsynaptic D2 receptor level. In asymptomatic carriers of a single Parkin mutation with an apparently normal allele, a mild but statistically significant decrease of mean [<sup>18</sup>F]-DOPA uptake compared to controls was found in all striatal regions indicating a preclinical disease process (Hilker, 2001). This would confirm the hypothesis in UK 401 that III:3 has preclinical disease as shown by the [<sup>18</sup>F]-DOPA PET performed in 1990 (Sawle *et al.*, 1992). This hypothesis should be tested by analysing all members of the kindred by further [<sup>18</sup>F]-DOPA PET and by completion of Parkin gene sequencing for all members of UK 401.

### 5.5.2 Identification of two large UK kindreds with a novel PD locus

Affected members of UK 402 and UK 403 were indistinguishable clinically and pathologically from the sporadic PD. The fact that no linkage has been shown to three major loci or markers linked to UCH-L1 (Farrer *et al.*, 1999b; Gasser *et al.*, 1998; Leroy *et al.*, 1998; Polymeropoulos *et al.*, 1996) in UK 402 and UK 403 indicates that at least one (if not two) further loci are yet to be described. No mutations were found in the coding region of the SNCA gene (Polymeropoulos *et al.*, 1997) in sequencing studies performed on an index case from each kindred (Vaughan *et al.*, 1998b) as the PARK1 region was not completely excluded by linkage studies. Both the mode of inheritance, two point LOD scores and haplotype analysis appear to exclude linkage to PARK 2 (Matsumine *et al.*, 1997). The pedigrees UK 402 and UK 403 were conservatively evaluated for their power to detect linkage using the SLINK program (Ott, 1989; Weeks *et al.*, 1990). This revealed that for a linked marker UK403 may generate a maximum 2-point lod score of 1.24 at  $\theta=0$ . In UK402, a linked marker may generate a maximum two point lod score of only 0.65 at  $\theta=0$  but if the two pedigrees were linked to the same genetic locus this generated a maximum combined 2-point lod score of 1.88 at  $\theta=0$ . Theoretical modeling assuming that the UK402 and the UK403 kindreds do share the same genetic locus revealed families would have a 40%

probability of observing a lod score of  $> 1.0$ ,  $\theta=0$ . However, only at  $\theta=0$  are lod scores likely to reach  $-2.0$ , the accepted criteria for exclusion. Genome screening of both kindreds, may demonstrate linkage to a novel autosomal dominant PD locus.

Further linkage and PET studies are currently ongoing to identify a novel familial PD locus in these individuals including a genome-wide screen (see section 5.5.3 below).

In both UK 402 and UK 403, the initial clinical presentation with an asymmetrical rest tremor, followed by the subsequent development of the other features of L-dopa responsive parkinsonism was indistinguishable from the sporadic form of PD (Hughes *et al.*, 1992), with a comparable age of onset. Likewise, in UK 403, the neuro-pathological appearances with pigment depletion in *substantia nigra* and adjacent structures, with typical Lewy bodies in surviving neurons, were identical to those found in sporadic PD. This differs from the majority of the published Parkinsonian kindreds, which apart from notable exceptions (Gwinn-Hardy *et al.*, 2000b; Wszolek *et al.*, 1995), have often had atypical features. In spite of the clinical similarities and the physical proximity between UK403 (Suffolk) and UK 402 (Lincolnshire), this author is unaware of any genealogical links between the two kindreds. Thus, the genetic basis of parkinsonism in the two kindreds may differ. Aggregation of PD in current generations along with historic evidence of PD in deceased family members in both kindreds suggested a genetic trait consistent with autosomal dominant inheritance of a major gene with reduced penetrance. In UK 403, although there was strong circumstantial evidence that kindreds 403 and 403A are related (section 5.3.4), and several other phenotypically similar PD kindreds originate from this region (Maraganore *et al.*, 1991), no direct genealogical link was made between these various families.

Other important factors to consider include the size of the Suffolk kindred. Assuming a prevalence of PD of 2% in those aged over 65 (De Rijk *et al.*, 1997b) there are likely to be individuals who have had sporadic PD (phenocopies). One potential example of this was a member of UK 403 who was a distant relative of the individuals shown in figure 5.4. This patient had neuropathologically confirmed typical Lewy body PD (S Daniel, personal communication). This individual had no family history of PD, yet shared the same family name and was traced back to an ancestor who lived in 1400 to a parish

adjacent to the founding Suffolk village. No common environmental factor was identified which could explain the occurrence of PD in UK 402 and UK 403: both Suffolk and Lincolnshire are predominantly rural counties with a large proportion of the population involved in agriculture, the affected members have lived in both urban and rural areas throughout the United Kingdom with no consistent environmental exposure that could have accounted for their parkinsonism. Likewise, no conjugal PD cases were identified which would be more suggestive of an environmental cluster, rather than a genetic effect.

Since there was no medical information regarding the earlier generations of UK 403, the possibility of more than one PD locus in this family could not be excluded, although this is probably unlikely given the similar phenotypes and age of onset of the two branches of UK 403. Nonetheless, it was impossible to exclude the possibility that parkinsonism in UK 403 was due to more than one gene since no medical information is available prior to generation XIII. This has presented problems in other kindreds where a single gene locus has been assumed (for example, in a large Amish kindred, bipolar depression appeared to be inherited as a complex trait even though all the affected members could be traced back to a single founder around 1750, and segregation analysis suggested dominant inheritance (Ginns *et al.*, 1996; Risch and Botstein, 1996). An example of this problem in UK 403 is shown by considering the analysis of the PARK2 locus (Matsumine *et al.*, 1997). Affected siblings XIV:4 and XIV:6 shared the same genotypes for markers run in this region, yet there was not a linked haplotype in this region when all affecteds were considered and therefore, under both a recessive and autosomal dominant model, the family did not appear to be linked to PARK2. Clearly, ASPs have a 50% probability of sharing alleles but it is theoretically possible that a mutation in the parkin gene is present in this sibling pair (XIV:4 and XIV:6) despite the dominant family history, and that more than one locus is responsible for the phenotype of parkinsonism in this kindred. It must be borne in mind that the ancestral link is tenuous although historically documented through intense research: there remains a lot of missing genetic information from intervening generations. However, mutations in the Parkin gene are unlikely, given that XIII:3 (father) was also affected and most Parkin-induced disease appears to be autosomal recessive, but there are exceptions to this

(Chen *et al.*, 2000). Parkin (Kitada *et al.*, 1998a) sequencing is underway in XIV:6 to exclude this possibility.

Non-penetrance and variable expressivity of disease haplotypes in this and other kindreds with parkinsonism probably reflect the sum of both environmental and genetic interactions, with genetic mutations having major effect. Analysis using age-associated liability classes takes advantage of all the genetic information in the kindred for phase and haplotype, construction, albeit that some individuals, especially those within two standard deviations of the mean onset age for the disease, must be considered at risk. However, affecteds-only analysis was used because the penetrance was uncertain, as evidenced by at least one obligate heterozygote (XIV:25 in UK 403, fig 5.4) as has proven the case with other loci predisposing to familial PD (Gasser *et al.*, 1998; Polymeropoulos *et al.*, 1996). One pitfall of model-based approaches is the assumptions underlying disease transmission, which could invalidate a model-based method if misspecified. It could be argued that multipoint analysis of a complex trait under an autosomal dominant model is inappropriate because there is an increased propensity for false-negative results. Considering the pedigree, autosomal dominant inheritance is most likely but this is not completely certain.

### 5.5.3 Future directions of study

Subsequent analysis of UK 401 (Khan *et al.*, 2001b) has enabled measurement of the progression of nigrostriatal dopaminergic dysfunction in a kindred with mutations in the Parkin gene. The rate of disease progression appears slower than that of idiopathic PD and similarly affects both caudate and putamen storage. Additionally, a group of 4 asymptomatic carriers of a parkin haplotype showed significant nigrostriatal dysfunction indicating that ubiquitin ligase activity in carriers may cause only subclinical nigro striatal dysfunction (Khan *et al.*, 2001b). The analysis of known PD loci in UK 402 and UK 403 suggest that there are other, as yet unmapped, loci for this disorder. Other kindreds have been published which harbour novel loci (Gwinn-Hardy *et al.*, 2000b). Genome screening in both UK 402 and UK 403 is currently an on-going research effort. Neither kindred by itself is of sufficient power to detect linkage (see SLINK studies in section 5.5.2). However, both kindreds are amongst the largest in Europe available at the present time and [<sup>18</sup>F]-DOPA PET studies are also being used to

assess pre-clinical disease in a number of the currently clinically unaffected individuals.  
It is hoped that this will increase the power to detect linkage in both kindreds.

## **CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS OF STUDY**

## 6.1 GENERAL CONCLUSIONS

The identification of genetic forms of PD in the last four years has transformed our understanding of the pathogenesis of parkinsonism. The two genes so far discovered in autosomal dominant PD families, SNCA (Polymeropoulos *et al.*, 1997) and UCH-L1 (Leroy *et al.*, 1998), other dominant loci PARK3 (Gasser *et al.*, 1998) and PARK4 (Farrer *et al.*, 1999), and Parkin (Kitada *et al.*, 1998), the first recessive PD gene cloned, emphasized the importance of molecular genetics in the aetiology of PD. A further recessive locus (PARK6) has just been published (Valente *et al.*, 2001). Genetic studies on these rare families are already providing important clues to understanding of PD although their importance in the aetiology of the sporadic form of the disease is currently less certain.

This thesis described a range of studies which explored genetic susceptibility in Parkinson's disease. The studies include a number of different methodologies, such as the use of association studies and linkage studies in sibling pairs (chapter 3). The relative importance of the various genes in PD were described using sequence analysis (collaborative studies) as well as direct screening for known mutations in a large population of EU affected sibling pairs (chapter 4). The description of two kindreds with familial PD in chapter 5 showed that further loci are still to be discovered. This final chapter briefly summarises the main findings of the studies contained within this thesis, how this work has contributed to the field, and briefly discusses future directions of study.

### 6.1.1 Linkage studies on the known autosomal dominant PD loci from a large population of affected sibling pairs

The study of affected relative PD by the formation of the GSPD enabled the clinical assessment, collection and analysis of DNA samples from a large number of affected sibling pairs (ASPs). A total of 246 families were collected up to the end of 1999 as part of the GSPD consortium with at least 2 affected sibling pairs and all the information collected, analysed and stored on centralized databases. I was responsible for the total collection of UK ASPs. An average of 125 ASPs were genotyped for evidence of linkage to the three autosomal dominant PD loci described to date (PARK1, 3 and 4). For three markers D4S1647 (alpha-synuclein (SNCA)), D4S405 (Ubiquitin-C-terminal

hydrolase, (UCHL-1)) and D2S1394 (PARK2, (locus at 2p.13)), nominal p-values of 0.05 or lower were observed suggesting the presence of susceptibility regions.

Evaluating the significance of these regions further will require their examination in a further cohort of sibling pairs. Genome-wide linkage studies on this cohort of ASPs is currently on-going as an extension of the collaborative work of GSPD. A PARK4 (Farrer *et al.*, 1999) (locus 4p15) haplotype segregating with Parkinson's disease and postural tremor was detected in an independent Italian family as a consequence of these Linkage studies although the current data are supportive of evidence for linkage but not conclusive. Positional cloning strategies will have to be employed to find the causative gene at this locus but this kindred may represent an important link in narrowing down the 4p candidate region (Vaughan *et al.*, 1999).

### **6.1.2 Slow Acetylation and familial PD**

Preliminary data suggested a highly significant association between the slow acetylator genotype for N-acetyltransferase2 (NAT2) and familial PD (Bandmann *et al.*, 1997). A large intra-familial association study performed by the author in EU ASPs found that the slow acetylator genotype for N-acetyltransferase 2 (NAT2) was over-represented in familial PD.

### **6.1.3 GSPD collaborative analysis of the SNCA, UCH-L1 and Parkin genes**

No mutations were found by collaborative direct sequencing of the coding region of SNCA (Vaughan *et al.*, 1998b) and UCH-L1 (Lincoln *et al.*, 1999) in index cases from a series of autosomal dominant PD kindreds or screening of 230 index familial PD cases for the two known mutations in SNCA (Vaughan *et al.*, 1998a). Hence, although of great interest, mutations in these genes are a very rare cause of familial PD. In contrast, the author linked a new kindred to the PARK2 locus (UK 401) and was responsible for the analysis and collection of UK families subsequently found to have mutations in the Parkin gene. These results contributed to a major EU study which showed that mutations in the Parkin gene are a common cause of autosomal recessive parkinsonism in Europe (Luecking *et al.*, 2000). The first neuropathological case of UK

Parkin-induced disease was identified by myself in collaboration with Dr C Luecking, Dr SE Daniel and Professor AJ Lees.

#### **6.1.4 Identification of two large UK PD kindreds with a novel PD locus**

Two large kindreds with familial PD were identified during the author's collection of affected sibling pairs. These families were found to be indistinguishable clinically and pathologically from the sporadic PD. No mutations were found in the SNCA gene. Linkage studies to the other dominant familial PD loci were also negative. Genome-wide linkage and PET studies are ongoing to identify a novel familial PD locus in these individuals.

#### **6.2 CONCLUSION AND OUTLOOK**

The challenges facing research into the genetic basis of PD are to establish how the mutant proteins cause disease, to identify further genes and to establish how these proteins interact with as yet unknown environmental factors. The fact that non-penetrance has been described in all three of the parkinsonian dominant loci described to date suggests that other factors, genetic or environmental, may further modify expression at each locus. This can be inferred, despite the fact that approximately a 70% loss of nigral neurons are required to express the parkinsonian phenotype (Pakkenberg *et al.*, 1991). The biological and numerical importance of the genetic contribution to PD remains uncertain. The evidence to date suggests that the majority of PD is not inherited in a mendelian manner. It is clear that there are PD families which do not show linkage to any of the disease-causing loci, neither do they have mutations in any of the PD genes described.

Knowledge of the gene products encoded by the three genes in PD described to date should enhance the exploration of the molecular mechanisms of neurodegeneration in PD, as well as in other neurodegenerative diseases that are characterised by involvement of abnormal protein handling, such as AD and MSA. Results of allelic association studies in sporadic cases have either been inconclusive or have only tentatively excluded the involvement of various candidate genes, as these results are dependent on the power of the study. There are also studies currently awaiting

replication, which may prove to be important in sporadic PD. Twin studies suggest that the genetic contribution is much less over the age of 50 and it must therefore be inferred that environmental factors are more significant in the development of PD in this age group. Genetic factors are probably more significant in familial and young-onset PD. In most cases of Parkinson's disease, however, a genetic contribution (if any) remains largely obscure.

The majority of PD appears to reflect a complex disease trait in which the extent to which the genetic component contributes to the manifestation of clinical disease depends upon the penetrance of the susceptibility allele, the presence or absence of endogenous and exogenous toxins and the role of as yet unidentified factors which modify gene expression. Further functional studies of the currently identified factors and their biological pathways (such as ubiquitination) are necessary. Factors which modify gene expression may explain the discrepancy in the variable age of onset of MZ twins and the wide range of onset of PD in kindreds known to have a genetic mutation thought to cause PD, such as the Contursi kindred (Golbe *et al.*, 1990). Mitochondrial mutations and other gene-gene interactions may also explain the variable expressivity of PD and the complex nature of its heritability. Identification of the specific mutations via linkage analysis of large multiplex kindreds, large sibling pair studies, allelic association studies and mitochondrial gene analysis are all underway and should be expanded. Herbicide/pesticide exposure, rural living, minor head trauma and nonsmoking remain the most significant exogenous factors which should be considered in parallel with the emerging expansion of knowledge of the role genetic factors in PD. Hence after over 180 years, molecular analysis of familial parkinsonism will finally allow us to refine the disease that James Parkinson so carefully documented.

**APPENDIX 1**

**A1.1 Standard patient consent form used during clinical collection of affected sibling pairs and their relatives**

NATIONAL HOSPITAL FOR NEUROLOGY AND NEUROSURGERY

Tel: 071-837 3611

RESEARCH ON HUMAN VOLUNTEERS

Subject/Patient Consent Form

Brief description of Project: \_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

Consultant(s) in charge/Director of project: \_\_\_\_\_  
\_\_\_\_\_

The subject/patient (Name): \_\_\_\_\_ Hosp No \_\_\_\_\_

has given his/her consent participate in the above named study.

The nature, purpose and possible consequences of the procedures involved have been explained to me by:

Name: \_\_\_\_\_

Position: \_\_\_\_\_

Signature: \_\_\_\_\_ Date \_\_\_\_\_

and Witnessed by:

Name of witness: \_\_\_\_\_

Position: \_\_\_\_\_

Address: \_\_\_\_\_  
\_\_\_\_\_

Signature: \_\_\_\_\_ Date \_\_\_\_\_

Signature Subject/Patient/~~Guardian~~: \_\_\_\_\_ Date \_\_\_\_\_

Address: \_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

Please return this form to:

PATIENT SERVICES MANAGER  
National Hospital for  
Neurology and Neurosurgery  
Queen Square  
LONDON WC1N 3BG

IT IS A REQUIREMENT OF THE JOINT MEDICAL COMMITTEE THAT ANY ADVERSE EFFECTS WHICH MAY OCCUR DURING A CLINICAL TRIAL ARE REPORTED TO THE PATIENT SERVICES MANAGER IMMEDIATELY

## A1.2 Forms used to record clinical examination findings of affected sibling pairs and for generating a clinical database of PD patients

### Familial Parkinson's Disease

PATIENTS CODE \_\_\_\_\_

Index case      yes            (DE, FR, IT, NL, UK)      no            (Family number i.e. no!)      (DNA code from each laboratory)

Date of birth \_\_\_\_\_

Sex      female            male     

Initial examination      yes            no     

Neurologist \_\_\_\_\_

Date at examination \_\_\_\_\_

#### I Diagnostic criteria

	yes	no
1. bradykinesia	<input type="checkbox"/>	<input type="checkbox"/>
2. rigidity	<input type="checkbox"/>	<input type="checkbox"/>
3. rest tremor	<input type="checkbox"/>	<input type="checkbox"/>
4. improvement with L-Dopa at least $\geq 30\%$	<input type="checkbox"/>	<input type="checkbox"/>
5. asymmetry of signs at onset	<input type="checkbox"/>	<input type="checkbox"/>

#### II Exclusion criteria

##### A At examination

1. supranuclear ophthalmoplegia	<input type="checkbox"/>	<input type="checkbox"/>
2. pyramidal syndrome	<input type="checkbox"/>	<input type="checkbox"/>
3. cerebellar syndrome	<input type="checkbox"/>	<input type="checkbox"/>
4. dyspraxia	<input type="checkbox"/>	<input type="checkbox"/>
5. severe early (<1 year) loss of postural reflexes	<input type="checkbox"/>	<input type="checkbox"/>

##### B Medical history of

1. use of neuroleptic drugs in the last 6 months	<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/> if yes, please specify date, duration, type and dose:		
2. cerebral ischemia	<input type="checkbox"/>	<input type="checkbox"/>
3. encephalitis	<input type="checkbox"/>	<input type="checkbox"/>
4. intoxication	<input type="checkbox"/>	<input type="checkbox"/>
5. other, please specify:	<input type="checkbox"/>	<input type="checkbox"/>

##### C Investigations

	not done	normal	abnormal
1. cerebral CT scan or MRI	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/> if abnormal, please specify:			
2. before the age of 40 yrs			
serum copper	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
urine copper	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
ceruloplasmine	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3. acanthocytes	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

#### III Clinical information

##### A Origin

	place of birth	origin
patient	_____	_____
parents :		
father	_____	_____
mother	_____	_____

*B Clinical history*

1. age at onset (yrs) \_\_\_\_\_
2. disease duration (yrs) \_\_\_\_\_
3. signs at onset:
- |              | yes                      | no                       |
|--------------|--------------------------|--------------------------|
| micrographia | <input type="checkbox"/> | <input type="checkbox"/> |
| bradykinesia | <input type="checkbox"/> | <input type="checkbox"/> |
| tremor       | <input type="checkbox"/> | <input type="checkbox"/> |
- if yes, please specify localisation:*
4. history of
- |   |                          |                          |
|---|--------------------------|--------------------------|
| dystonia in childhood, or before L-Dopa | <input type="checkbox"/> | <input type="checkbox"/> |
| postural tremor                         | <input type="checkbox"/> | <input type="checkbox"/> |
5. associated signs:
- |                         |                          |                          |
|-------------------------|--------------------------|--------------------------|
| intellectual impairment | <input type="checkbox"/> | <input type="checkbox"/> |
|-------------------------|--------------------------|--------------------------|
- please, specify the MMS score:*
- sphincter disturbances
- |              |                          |                          |
|--------------|--------------------------|--------------------------|
| incontinence | <input type="checkbox"/> | <input type="checkbox"/> |
| urgency      | <input type="checkbox"/> | <input type="checkbox"/> |
| impotence    | <input type="checkbox"/> | <input type="checkbox"/> |
- orthostatic hypotension  
(= a decrease of more than 30 mmHg after standing for 3 min )
- if yes, please specify:*
6. family history of
- |                     |                          |                          |
|---------------------|--------------------------|--------------------------|
| essential tremor    | <input type="checkbox"/> | <input type="checkbox"/> |
| Alzheimer's disease | <input type="checkbox"/> | <input type="checkbox"/> |
| other:              | <input type="checkbox"/> | <input type="checkbox"/> |

*C Treatment*

1. daily dose of L-Dopa (mg) \_\_\_\_\_  
how many times per day \_\_\_\_\_  
duration of treatment (months) \_\_\_\_\_
2. side effects
- |                        |                          |                          |
|------------------------|--------------------------|--------------------------|
| dyskinesias            | <input type="checkbox"/> | <input type="checkbox"/> |
| motor fluctuations     | <input type="checkbox"/> | <input type="checkbox"/> |
| early-morning dystonia | <input type="checkbox"/> | <input type="checkbox"/> |
3. other treatments:

*D Motor scale UPDRS (appendix 1)*

on-score: \_\_\_\_\_  
off-score: \_\_\_\_\_

*E Hoehn and Yahr (appendix 2)*

score: \_\_\_\_\_

*F Videa (protocol appendix 2)*

date \_\_\_\_\_  
last dose of L-Dopa (mg) \_\_\_\_\_  
time elapsed since the last dose of L-Dopa \_\_\_\_\_

#### IV Diagnostic evaluation

- Definite PD = 3 out of 5 diagnostic criteria, including response to L-Dopa  
and absence of all exclusion criteria  
or neuropathological confirmation
- Probable PD = at least 2 out of 5 diagnostic criteria  
and absence of all exclusion criteria
- Possible PD = 1 out of 5 diagnostic criteria  
or poor response to L-Dopa  
or clinical history reported by relatives
- Clinical evaluation impossible (presence of other neurological signs...)
- other than PD (please specify)

#### V Remarks

Motor examination of the Unified Parkinson's disease Rating Scale (UPDRS)

	ON	OFF
Speech		
Facial expression		
Tremor at rest	face, lips, chin	
	R/L hands	
	R/L feet	
Action tremor R/L		
Rigidity	neck	
	R/L upper extremity	
	R/L lower extremity	
Finger taps R/L		
Hand grips R/L		
Hand Pronation-Supination R/L		
Leg agility R/L		
Arise from chair		
Posture		
Postural stability		
Gait		
Body bradykinesia		
<b>Total</b>		

Speech	<ul style="list-style-type: none"> <li>0= Normal</li> <li>1= Slight loss of expression, diction and/or volume</li> <li>2= Monotone, slurred but understandable, moderately impaired</li> <li>3= Marked impairment, difficult to understand</li> <li>4= Unintelligible</li> </ul>	Leg agility	<ul style="list-style-type: none"> <li>0= Normal</li> <li>1= Mild slowing and/or reduction in amplitude</li> <li>2= Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.</li> <li>3= Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.</li> <li>4= Can barely perform the task</li> </ul>
Facial expression	<ul style="list-style-type: none"> <li>0= Normal</li> <li>1= Minimal hypomimia, could be normal "Poker face"</li> <li>2= Slight but definitely abnormal diminution of facial expression</li> <li>3= Moderate hypomimia, lips parted some of the time</li> <li>4= Masked or fixed facies with severe or complete loss of facial expression, lips parted 1/4 inch or more</li> </ul>	Arising from chair	<ul style="list-style-type: none"> <li>0= Normal</li> <li>1= Slow, or may need more than one attempt</li> <li>2= Pushes self up from arms of seat</li> <li>3= Tends to fall back and may have to try more than one time, but can get up without help</li> <li>4= Unable to rise without help</li> </ul>
Tremor at rest	<ul style="list-style-type: none"> <li>0= Absent</li> <li>1= Slight and infrequently present</li> <li>2= Mild in amplitude and persistent. Or moderate in amplitude, but only intermittent present</li> <li>3= Moderate in amplitude and present most of the time</li> <li>4= Marked in amplitude and present most of the time</li> </ul>	Posture	<ul style="list-style-type: none"> <li>0= Normal erect</li> <li>1= Not quite erect, slightly stooped posture, could be normal for older person</li> <li>2= Moderately stooped posture, definitely abnormal; can be slightly leaning to one side</li> <li>3= Severely stooped posture with kyphosis; can be moderately leaning to one side</li> <li>4= Marked flexion with extreme abnormality of posture</li> </ul>
Action or postural tremor of hands	<ul style="list-style-type: none"> <li>0= Absent</li> <li>1= Slight, present with action</li> <li>2= Moderate in amplitude, present with action</li> <li>3= Moderate in amplitude with posture holding as well as in action</li> <li>4= Marked in amplitude</li> </ul>	Gait	<ul style="list-style-type: none"> <li>0= Normal</li> <li>1= Walks slowly, may shuffle with short steps, but no festination or propulsion</li> <li>2= Walks with difficulty, but requires little or no assistance; may have some festination, short steps or propulsion</li> <li>3= Severe disturbance of gait requiring assistance</li> <li>4= Cannot walk at all, even with assistance</li> </ul>
Rigidity	<ul style="list-style-type: none"> <li>0= Absent</li> <li>1= Slight or detectable only when activated by mirror or other movements</li> <li>2= Mild to moderate</li> <li>3= Marked but full range of motion easily achieved</li> <li>4= Severe range of motion achieved with difficulty</li> </ul>	Postural stability	<ul style="list-style-type: none"> <li>0= Normal</li> <li>1= Retropulsion, but recovers unaided</li> <li>2= Absence of postural response; would fall if not caught by examiner</li> <li>3= Very unstable, tends to loose balance spontaneously</li> <li>4= Unable to stand without assistance</li> </ul>
Finger taps	<ul style="list-style-type: none"> <li>0= Normal</li> <li>1= Mild slowing and/or reduction in amplitude</li> <li>2= Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.</li> <li>3= Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.</li> <li>4= Can barely perform the task</li> </ul>	Body bradykinesia	<ul style="list-style-type: none"> <li>0= None</li> <li>1= Minimal slowness, giving movement a deliberate character; could be normal for some persons. Possibly reduced amplitude</li> <li>2= Mild degree of slowness and poverty of movement which is definitely abnormal. Alternatively some reduced amplitude</li> <li>3= Moderate slowness, poverty or small amplitude of movement</li> <li>4= Marked slowness, poverty or small amplitude of movement</li> </ul>
Hand movements (each hand separately)	<ul style="list-style-type: none"> <li>0= Normal</li> <li>1= Mild slowing and/or reduction in amplitude</li> <li>2= Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.</li> <li>3= Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.</li> <li>4= Can barely perform the task</li> </ul>		
Rapid alternating movements of hands, both simultaneously	<ul style="list-style-type: none"> <li>0= Normal</li> <li>1= Mild slowing and/or reduction in amplitude</li> <li>2= Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.</li> <li>3= Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.</li> <li>4= Can barely perform the task</li> </ul>		

### Modified Hoehn and Yahr Staging

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Stage 0	= No signs of the disease.
Stage 1	= Unilateral disease.
Stage 1.5	= Unilateral plus axial involvement.
Stage 2	= Bilateral disease, without impairment of balance.
Stage 2.5	= Mild bilateral disease, with recovery on pull test.
Stage 3	= Mild to moderate bilateral disease, some postural instability; physically independent.
Stage 4	= Severe disability; still able to walk or stand unassisted.
Stage 5	= Wheelchair bound or bedridden unless aided.

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### Video protocol

- + sitting on a chair with both hands on the knees, counting backwards
  - + hands held horizontally, palms turned towards the face
  - + rapidly alternating hand movements : pronation-supination (> 10 times)
  - + opening-closing of the hands (> 10 times for each hand)
  - + finger taps
  - + foot taps (lifting the whole foot)
  - + getting up from chair with arms crossed at first try
  - + walking (4 to 5 lengths)
  - + postural stability
- ⇒ if clinical idiosyncracies please videotape

Family tree with at least all 1<sup>st</sup> degree relatives

FAMILY CODE \_\_\_\_\_

Please specify each individual by symbol:  ● = affected  ○ = unaffected,  ◐ = probable or possible (please specify), \* = blood sampled, → = index case  
Please note for each individual: DNA code, date of birth, date or age at death (cause if available), age at onset

**Items for the clinical data base (Excel) in familial Parkinson's disease  
according to the diagnostic procedure on paper (enclosed)**

center	DE, FR, IT, NL, UK
family	number of each family according to each laboratory
code	your own DNA code
index case	1: yes, 0: no
patients origin	p.e. Normandie, Wales, Calabria, Bayern...
death mother/father	age at death of the mother/father
status	1: definite PD 2: probable PD 3: possible PD 0 normal
sex	1: female, 2: male
age	age at time of examination
onset	age at onset of the first symptoms
bradykinesia, rigidity, rest tremor	1: present, 0: absent
%improvLD	percentage of L-Dopa improvement in the beginning of treatment
asymmetry	asymmetric onset of signs R: Right, L: Left, 0: none
onset micro	micrography at onset, 1: present, 0: absent
onset brady	bradykinesia at onset, 1: present, 0: absent
onset tremor	rest tremor at onset, 1: present, 0: absent
MMS	score of Mini Mental State
incontinence, urgency, impotence	1: present, 0: absent
hypotension	orthostatic hypotension= more than 30mmHg decrease after standing for 3 minutes 1: present, 0: absent
dose LD	daily treatment with L-Dopa given in mg
duration LD	since how many months there is a daily treatment with L-Dopa
dyskinesias	1: present, 0: absent
other treat	name the drug
UPDRS off, on	off and on scores of the UPDRS scale
Hoehn-Yahr	Hoehn and Yahr score
exclusion criteria	0: absent 1: supranuclear ophthalmoplegia 2: pyramidal syndrome 3: cerebellar syndrome 4: dyspraxia 5: severe and early loss of postural reflexes 7: others
video	1: done, 0: not done

center	family	code	index case	patients origin	mothers origin	death mother	fathers origin	death father	status	sex
FR	85	SAL-LEB-085-006	1	normandie	normandie	82	normandie	80	1	2
FR	85	SAL-LEB-085-007	0	normandie	normandie	82	normandie	80	1	2

age	onset	bradykinesia	rigidity	rest tremor	%improv LD	asymmetry	onset micro	onset brady	onset tremor	MMS	incontinence
66	60	1	1	1	50	R	0	1	1	27	0
66	58	1	1	1	40	R	0	0	1	24	1

urgency	impotence	hypotension	dose LD	duration LD	dyskinesia	other treat	UPDRS off	UPDRS on	Hoehn-Yahr	video
0	0	0	375	6	1			46	4	1
0	0	1	300	5	1		71		4	1

exclusion criteria	remarks
0 monozygotic twin	
0 monozygotic twin/halluzinations	

ADDENDUM for the clinical data base (Excel) in familial Parkinson's disease (August 1998)

- attitude/action tremor: 0: absent, 1: present, 2: atypical (please specify localization in remarks)
- onset tremor 0: absent,  
1 rest tremor,  
2: attitude or action tremor,  
3: both (please specify localization in remarks)
- onset dystonia 0: absent  
1: present
- site dystonia at onset give localization of dystonia at onset
- fluctuations 0: absent, 1: present
- delay fluctuations delay in month between the beginning of levodopa treatment and onset of fluctuations
- dystonia 0: absent, 1: present (please indicate if painful in remarks)
- delay dystonia delay in month between the beginning of levodopa treatment and onset of dystonia
- reflexes LL reflexes in the lower limbs: 0: normal  
1: increased  
2: decreased or absent
- delay dyskinesias delay in month between the beginning of levodopa treatment and onset of dyskinesias
- Hoehn-Yahr off if available
- progression 0: no progression of the disease  
1: slow progression  
2: moderate progression  
3: rapid progression  
/ if unknown , or not done, or not relevant

**APPENDIX 2: Sequencing methodology used to examine SNCA ( $\alpha$ -synuclein),  
Parkin and UCH-L1 genes**

**A2.1: Sequencing SNCA**

*A2.1.1 Sequencing of the  $\alpha$ -synuclein gene in 30 autosomal dominant PD kindreds*

Primers were designed to human genomic non-A $\beta$  component of Alzheimer's disease amyloid precursor protein (NACP/synuclein) sequences submitted to NCBI database (accession no.'s U46896 to U46901). Exon 4 primers were taken published data (Polymeropoulos *et al.*, 1997). The 5' flanking intron of exon 7 was sequenced to provide an intronic sense primer for exon 7 (sequence available on request). PCR conditions were denaturation at 94°C (3min), followed by 35 cycles of 94°C (20s), 55°C (30s), 72°C (45s), with a final extension at 72°C (10min). PCR products were purified using Qiaquick columns prior to sequencing using dRhodamine terminators on an ABI377. Sequence chromatograms were analysed using PolyPhredPhrap (Nickerson *et al.*, 1997).

**Table A2.1.** PCR primers used for sequencing exons of the SNCA gene

SNCA Exon	Primers 5'/3', F & R	Product size (bp)
Exons 1 & 2	GAGAAGGAGGAGGACTAGGAGG	499
Exon 3	GTCTCACACTTTGGAGGGTTTC	395
Exon 4	GCTAATCAGCAATTTAAGGCTAG	215
Exon 5	CGATGGCTAGTGGAAGTGG	325
Exon 6	CGGAGGCATTGTGGAGTTTAG	373
Exon 7	GACTGGGCACATTGGAAGTGG	189

## A2.2 Sequencing Parkin

### A2.2.1 PCR amplification and sequence analysis

The sequencing and analysis of Parkin in both studies was all performed at INSERM U289 in Paris as part of the EU consortium and therefore the full methodology is included here (Abbas *et al.*, 1999, Lucking *et al.*, 2000). Screening for Parkin mutations was first performed in all index cases (except those with previously shown homozygous or compound heterozygous mutations) by a newly established, semi-quantitative PCR protocol for the detection of rearrangements of Parkin exons. Three different exon combinations, covering exons 2-12, were amplified by multiplex PCR: exons 4, 7, 8, 10; exons 5, 6, 8, 11; exons 2, 3, 9, 12 and an external control C328, a 328 bp sequence of the *transthyretine* gene on chromosome 18.

**Table A2.2.** PCR primers used for sequencing exons of the Parkin gene

Parkin Exon	Primers 5'/3', F & R	Product size (bp)
Exon 1	GCGCGGCTGGCGCCGCTGCGCGCA	112
Exon 2	ATGTTGCTATCACCATTTAAGGG	308
Exon 3	ACATGTCACCTTTTGCTTCCCT	427
Exon 4 (inner)	AGGTAGATCAATCTACAACAGCT	121
Exon 4 (outer)	ACAAGCTTTTAAAGAGTTTCTTGT	261
Exon 5	ACATGTCTTAAGGAGTACATTT	227
Exon 6	AGAGATTGTTTACTGTGGAAACA	268
Exon J-17	GAGCCCCGTCCTGGTTTTCC	137
Exon J-17	TGCCTTTCCACACTGACAGGTACT	239
Exon 8	TGATAGTCATAACTGTGTGTAAG	206
Exon 9	GGGTGAAATTTGCAGTCAGT	278
Exon 10	ATTGCCAAATGCAACCTTGTC	165
Exon 11	ACAGGGAACATAAACTCTGATCC	303
Exon 12	GTTTGGGAATGCGTGTTTT	255

Primers were as published by Kitada *et al.* 1998 except for exon 3, for which exonic primers were used: Ex3iFor :5'-AATTGTGACCTGGATCAGC-3' and Ex3iRev :5'-CTGGACTTCCAGCTGGTGGTGAG-3'. C328 primers were TTRForHex : 5'-ACGTTCTGATAATGGGATC-3' and TTR328Rev : 5' – CCTCTCTCTACCAAGTGAGG-3'. All forward primers were fluorescently labeled with Hex. The PCR products (2.5  $\mu$ l) were analyzed on a 5% denaturing polyacrylamide gel using an automated Sequencer ABI 377 and GeneScan 3.1 and Genotyper 1.1.1 software (Applied Biosystems). All reactions were done twice. A case with a known heterozygous exon deletion was always processed in parallel as a positive control. Ratios of the peak heights of each multiplex reaction were calculated and compared to the ratios of a normal control processed in parallel. Comparison of the case and control ratios yielded a factor, that was interpreted as follows: heterozygous exon deletion for values  $\leq 0.6$ ; normal for values between 0.8 and 1.2; heterozygous exon duplications for values between 1.3 and 1.7; homozygous duplication or heterozygous triplication for values between 1.8 and 2.3; homozygous triplication for values above 2.6. Exon rearrangements were deduced whenever all factors concerning one or several exons were abnormal. The consequence of the observed rearrangements at the protein level (frameshift vs. in-frame rearrangement) was deduced from the exon sequence published by Kitada *et al.* (DDBJ accession number: AB009973). PCR conditions were 40 ng of DNA in a total volume of 25  $\mu$ l with 3 mM MgCl<sub>2</sub>, 0.2 mM dNTP and 1U Taq Polymerase. Primer concentrations that yielded similar peak heights within one multiplex reaction were: exon 2 (0.8  $\mu$ M), exon 3 (0.4  $\mu$ M), exon 4 (1.0  $\mu$ M), exon 5 (0.6  $\mu$ M), exon 6 (1.4  $\mu$ M), exon 7 (0.44  $\mu$ M), exon 8 (1.0  $\mu$ M in combination 1 and 0.8  $\mu$ M in comb 2), exon 9 (0.4  $\mu$ M), exon 10 (1.04  $\mu$ M), exon 11 (0.8  $\mu$ M), exon 12 (1.2  $\mu$ M) and C328 (1.92  $\mu$ M). A 95°C denaturation (5 min) was followed by 23 cycles of 95°C (30 sec), 53°C (45 sec) and 68°C (2,5 min), with a final extension at 68°C (5 min). Under these conditions, PCR amplification was exponential for all combinations (data not shown). Base deletions and insertions could be deduced from the size of the PCR products.

In all index cases that were not explained by exon rearrangements or by base deletions/insertions (except 5 familial as well as 48 isolated cases), the entire coding region of the Parkin gene was sequenced as described.

To verify their pathogeneity, all new Parkin variants were analyzed on available DNA for cosegregation in the families and for their absence on control chromosomes, by restriction assays, polyacrylamide gel electrophoresis or the exon dosage technique. Controls were tested for exon combination 3. For 2 point mutations (939G>A and 1101C>T), mismatch reverse primers were used in order to create a restriction site (table 1): 5'-GGCAGGGAGTAGCCAAGTTGAGGAT-3' for the Alw-I-digestion and 5'-AGCCCCGCTCCACAGCCAGCGC-3' for the BstU-I-digestion (the underlined nucleotide is different from the wild type sequence).

### A2.3 Sequencing of UCH-L1

UCH-L1 exons were amplified from genomic DNA with primers designed to flank intronic sequence. PCR reactions contained a final concentration of 0.8µM for each primer and 1 unit of *Taq* polymerase and 5µl of Q solution (Qiagen). Amplification was done using a 65-55°C touchdown protocol over 35 cycles with a final extension of 72°C for 10 min. PCR products were purified using Qiagen PCR kit and their concentration estimated on an agarose gel. DNA (100ng) for each exon was sequenced on both strands using the Rhodamine dye terminator cycle sequencing kit (Perkin Elmer) and relevant PCR primers. Sequencing was performed on an ABI377 automated sequencer. Heterozygote base calls were made using Factura software (Perkin Elmer) and sequence alignment was performed with Sequence Navigator (Perkin Elmer).

**Table A2.3.** PCR primers used for sequencing exons of the UCH-L1 gene

UCH-	Primers 5'/3', F & R	Product size (bp)
Exons 1 & 2	CTCCCCCTGCACAGGCCTCA	353
Exon 3	CTCCTCCCAGGCTCGGGT	307
Exon 4	TGCACTCTCATTCTGAGATG	228
Exons 5 & 6	AGGTTGCTCAGCATGTTTCAG	364
Exon 7	CTTAGTGGGCTTAGAATAGG	372
Exon 8	ATCTAGGCTAGGTAAGCACG	271
Exon 9	GGAGCCTTCCCTATGTGAC	465

## APPENDIX 3

### A3.1 List of UK affected sibling pair families and large kindreds with PD

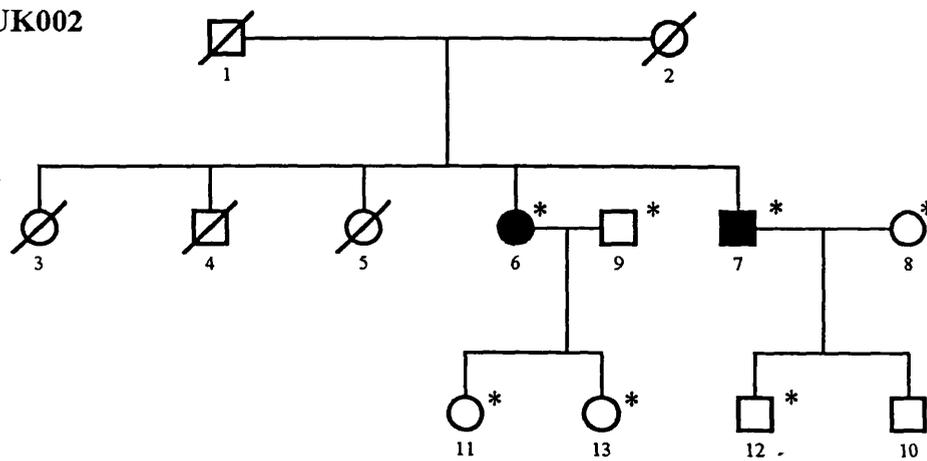
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UK013	UK063	
UK017	UK064	
UK018	UK073	
UK024	UK074	
UK025		
UK028	UK082	
UK034	UK084	
UK035	UK085	
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UK040	UK087	
UK041	UK089	
UK045	UK092	
UK046	UK093	
UK047	UK095	

#### List of large UK families

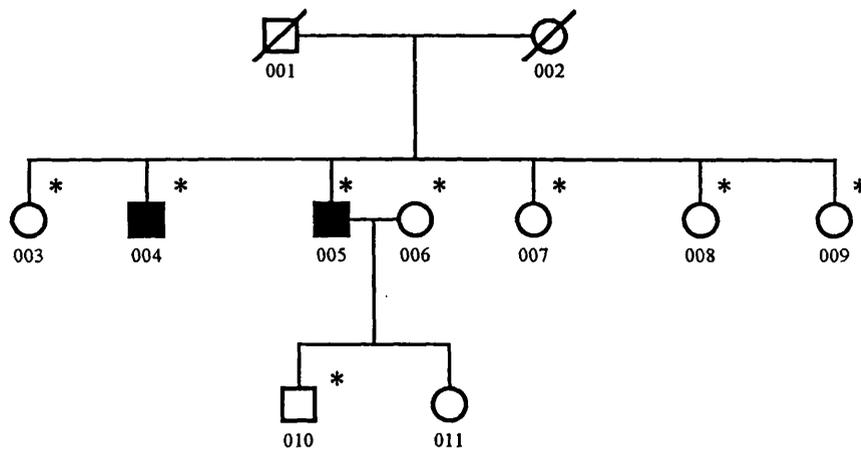
UK 401	UK 402	UK403
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**A3.2 Pedigrees of all UK affected sibling pairs (ASPs):**

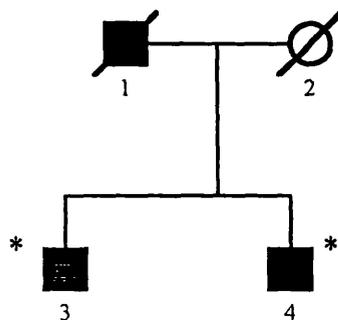
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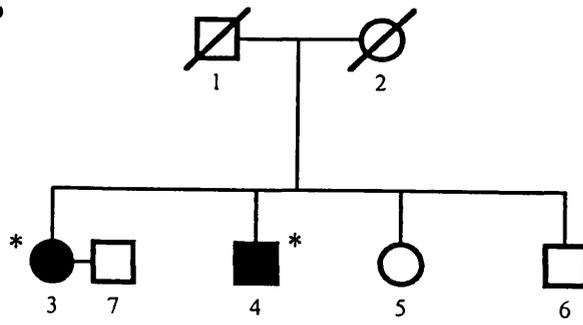
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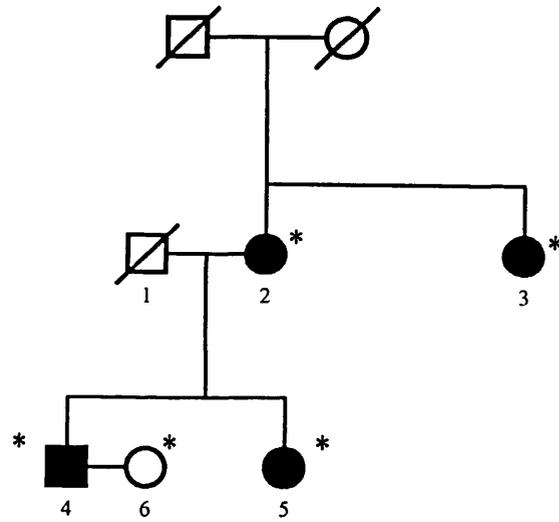
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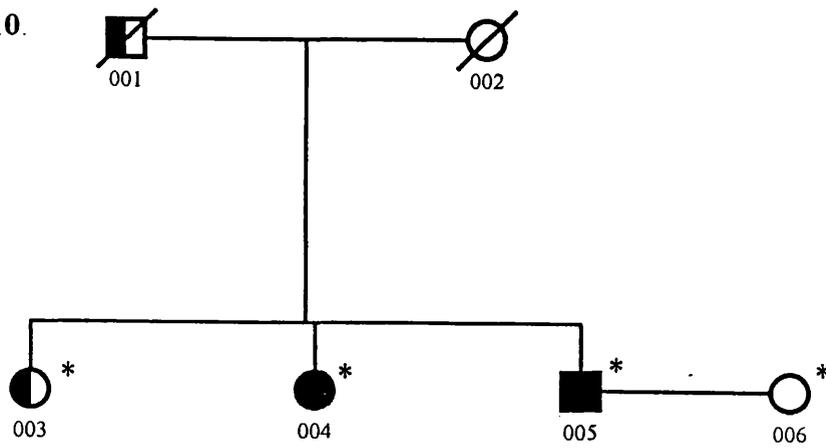
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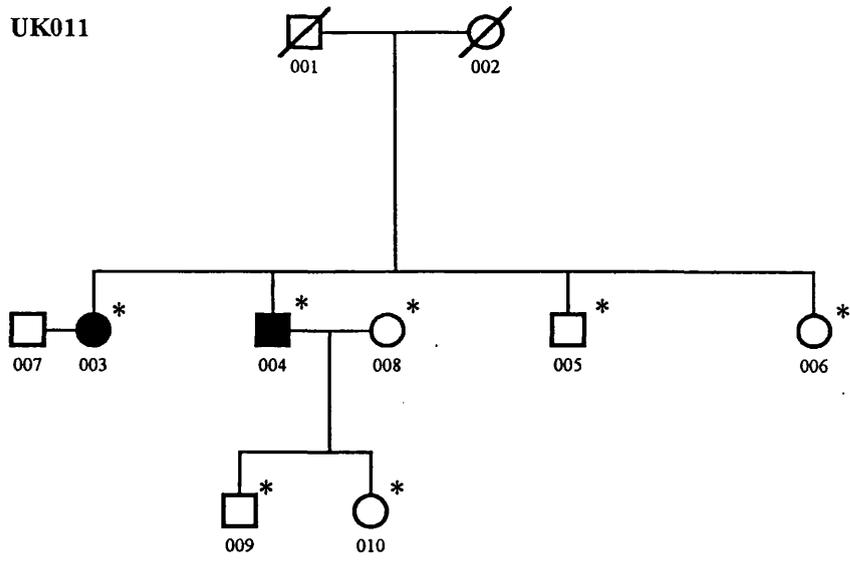
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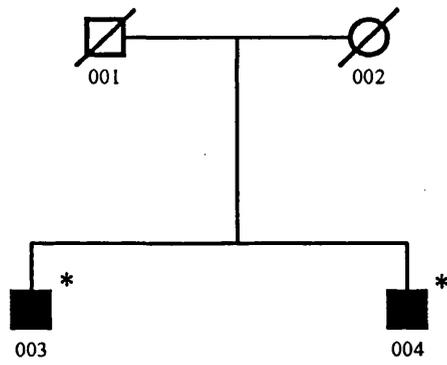
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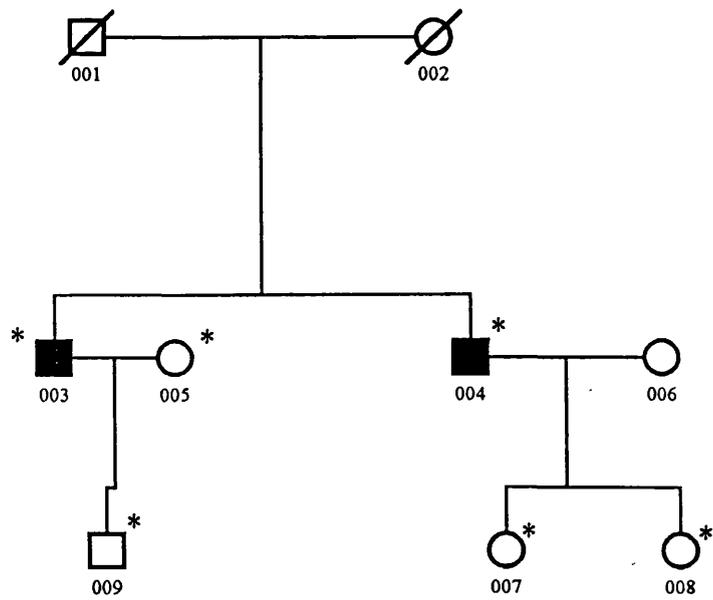
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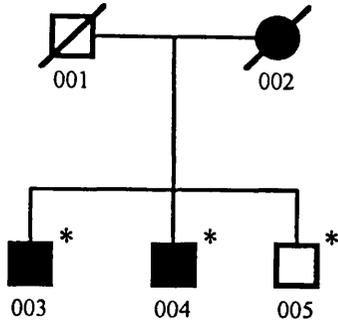
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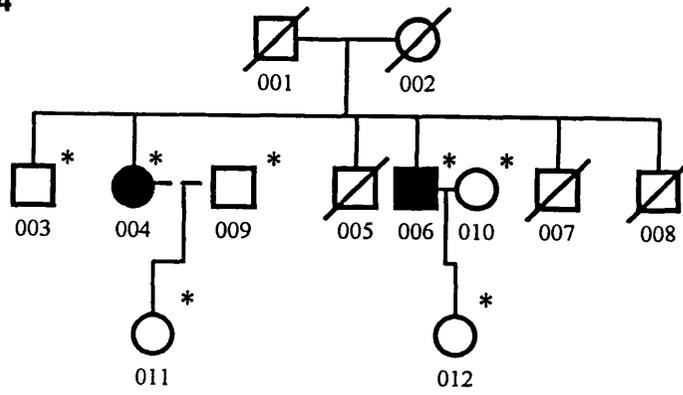
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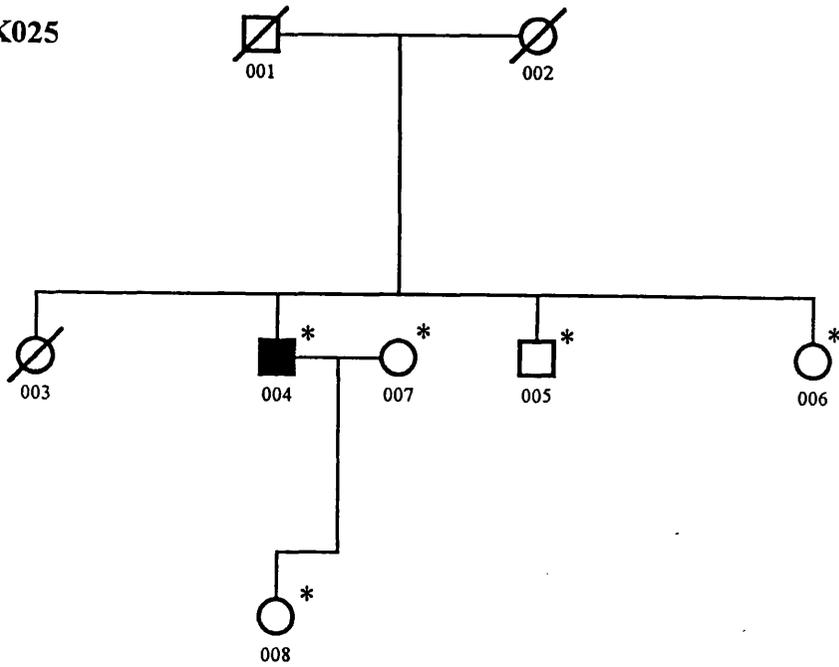
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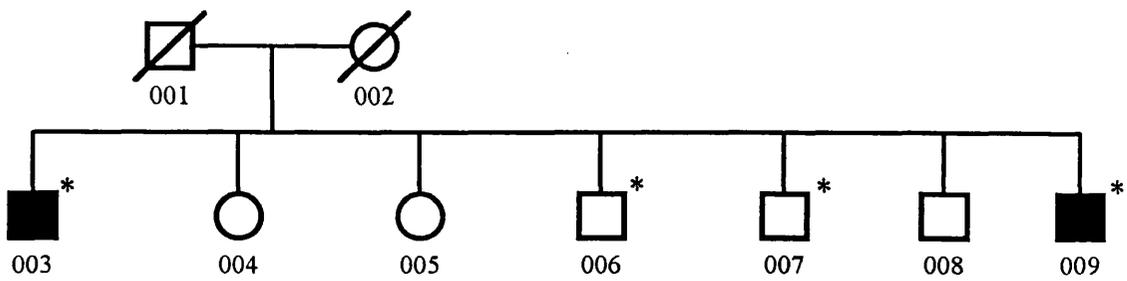
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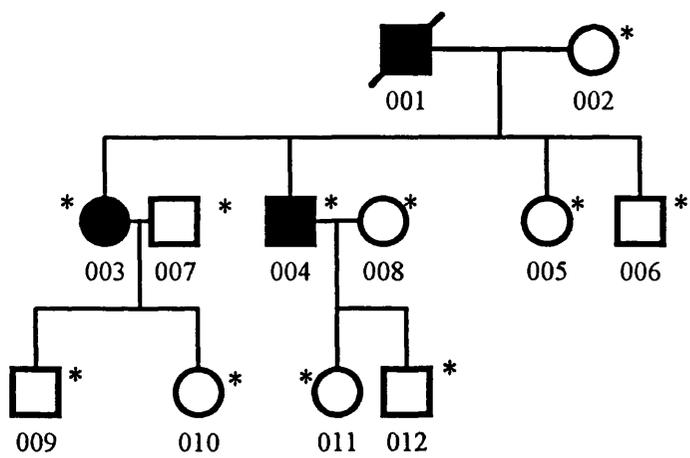
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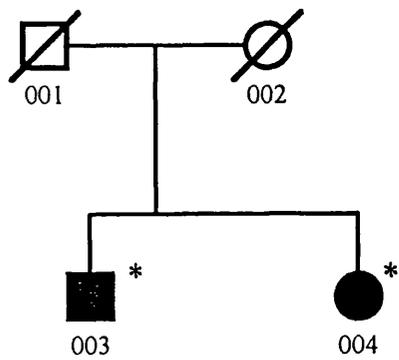
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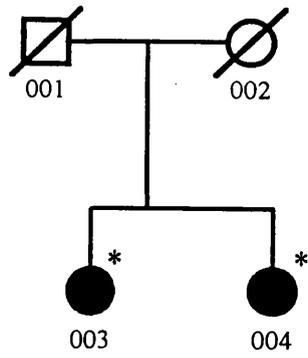
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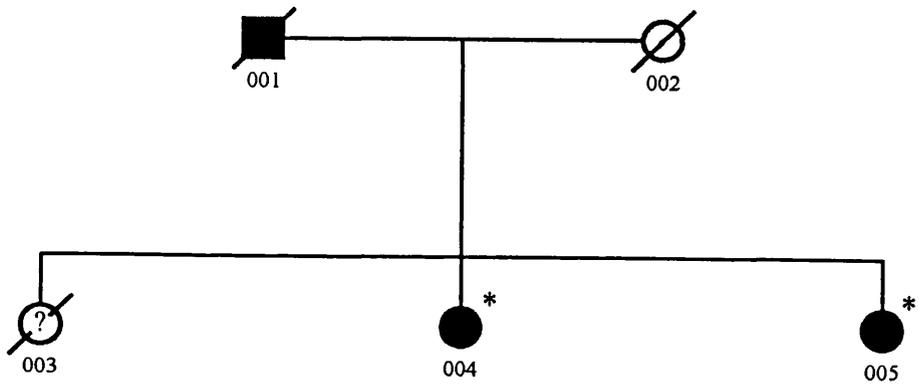
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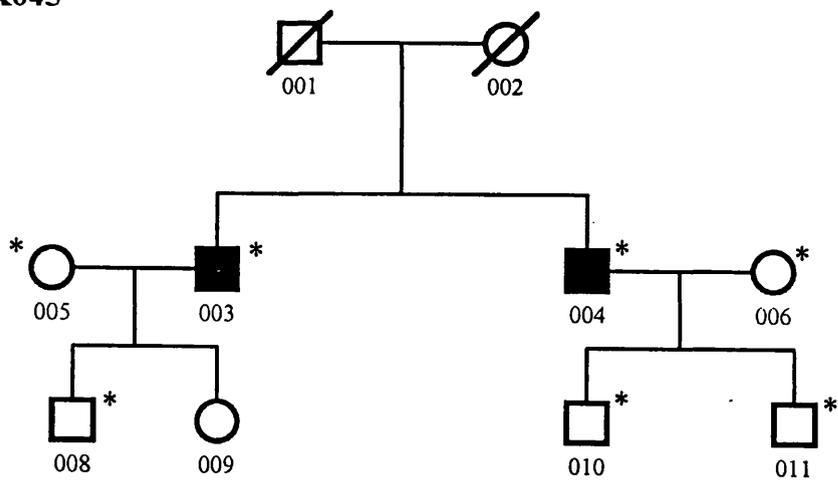
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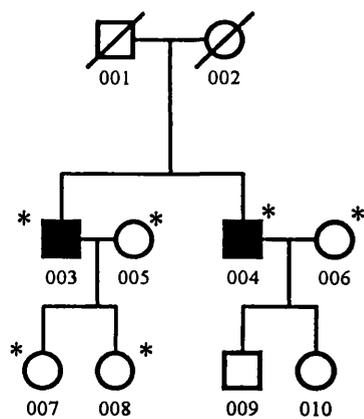
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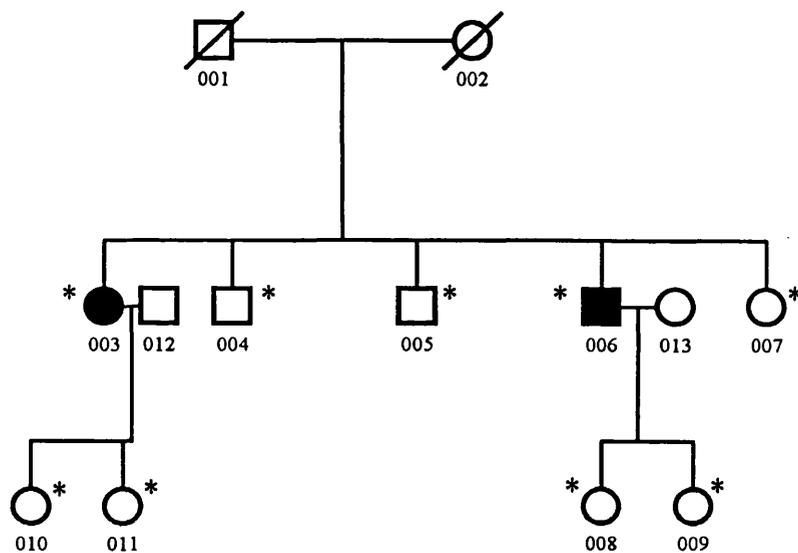
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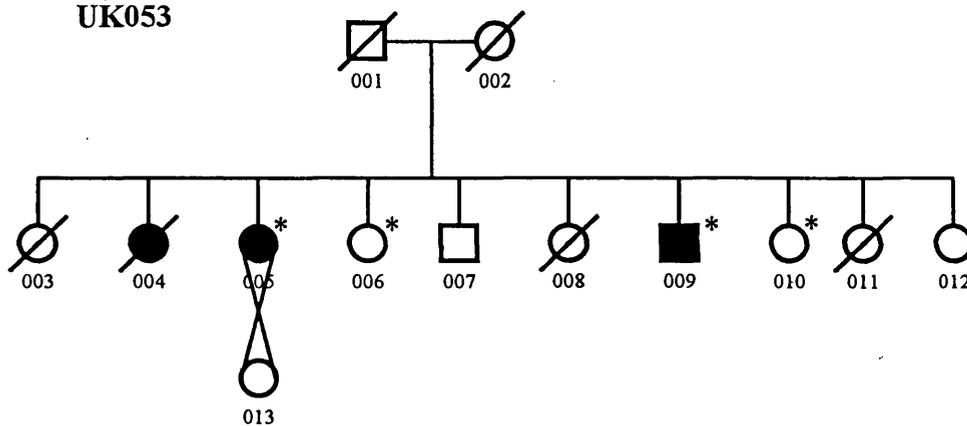
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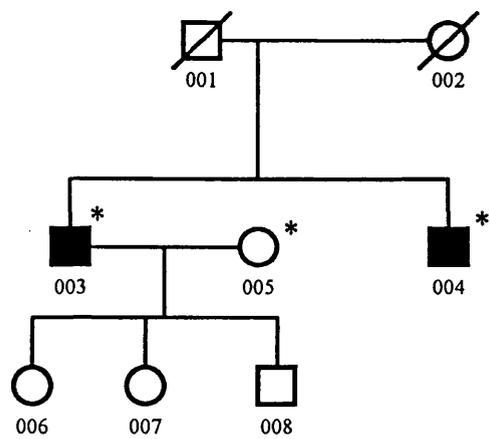
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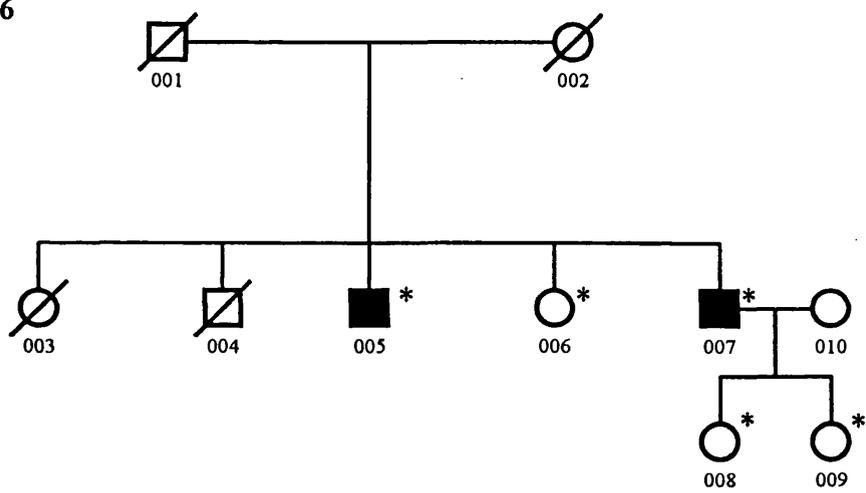
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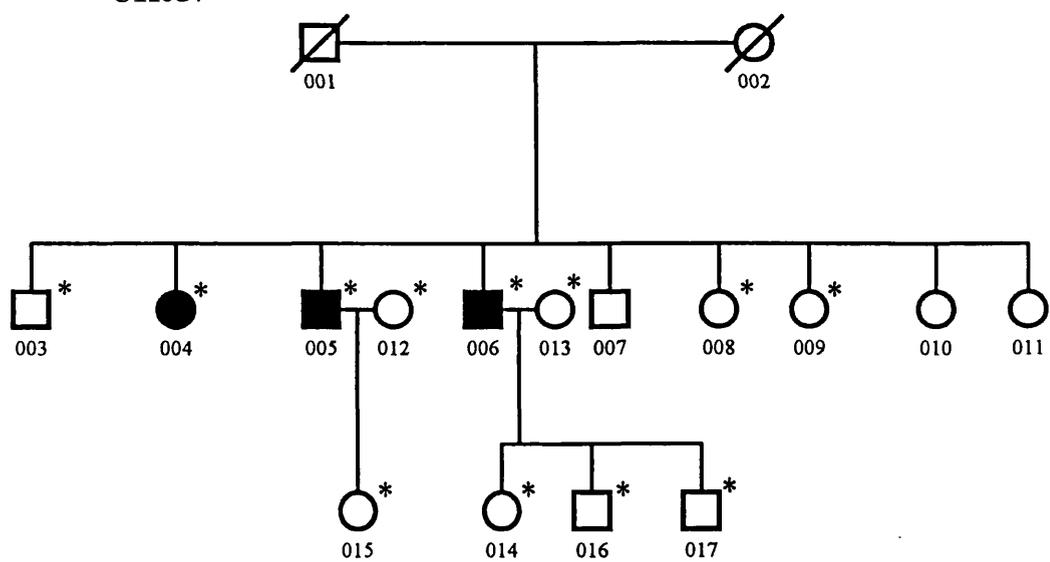
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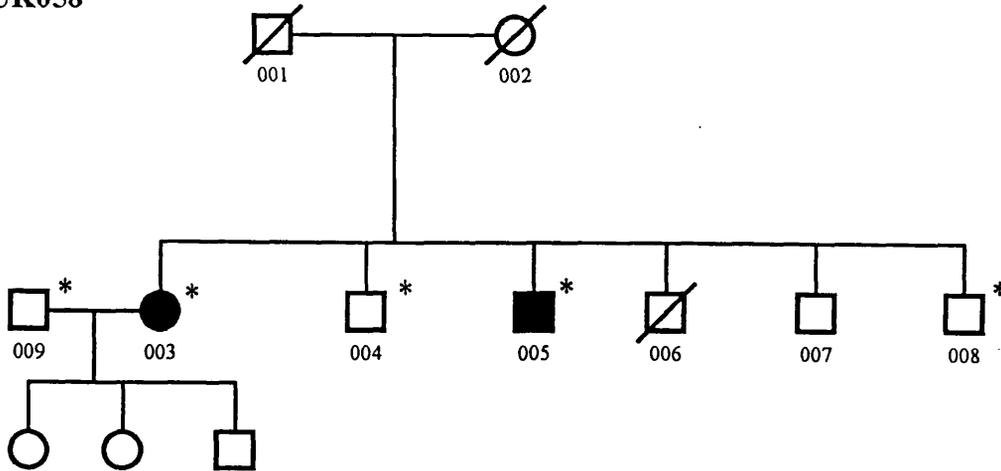
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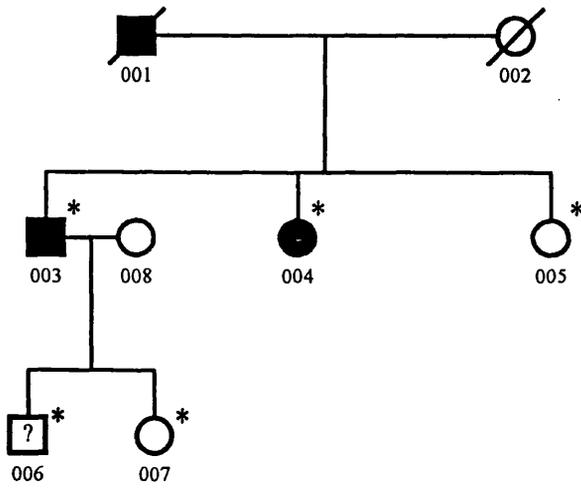
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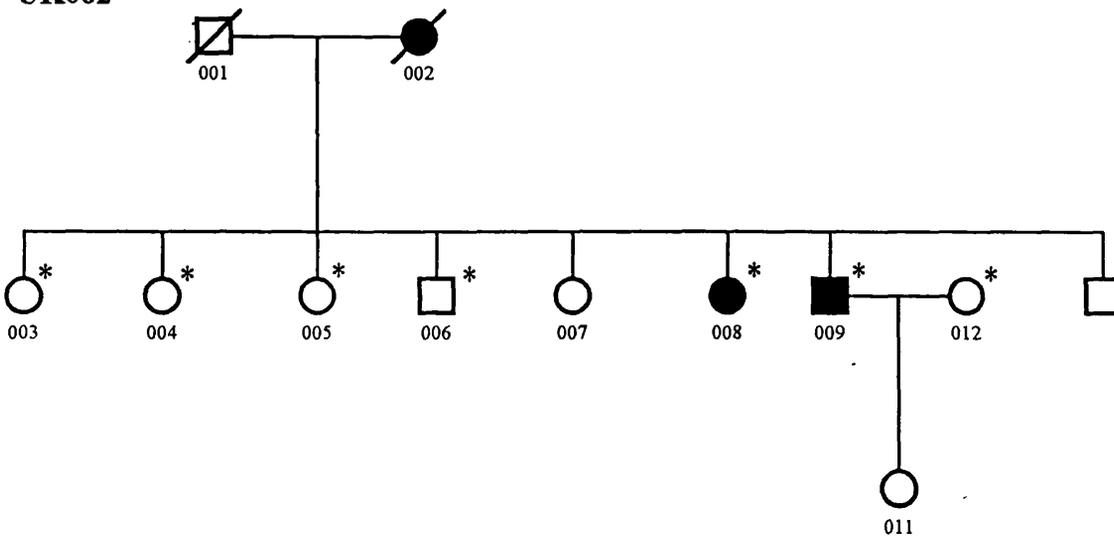
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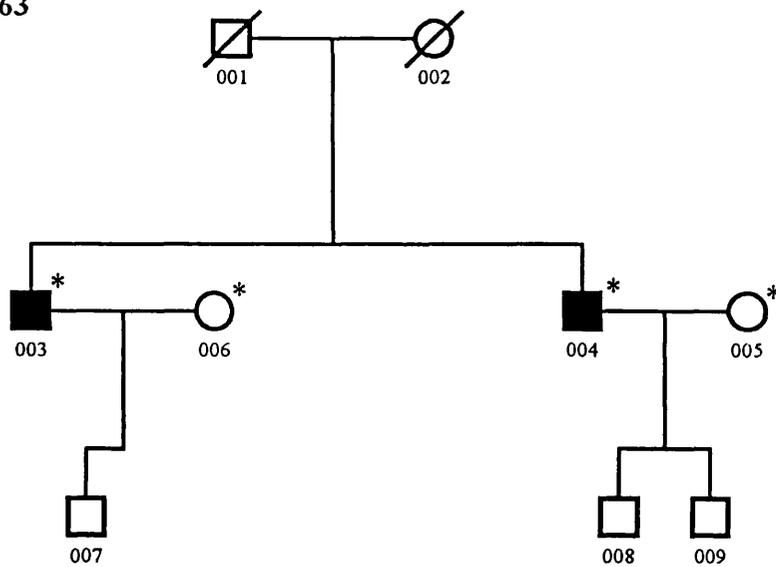
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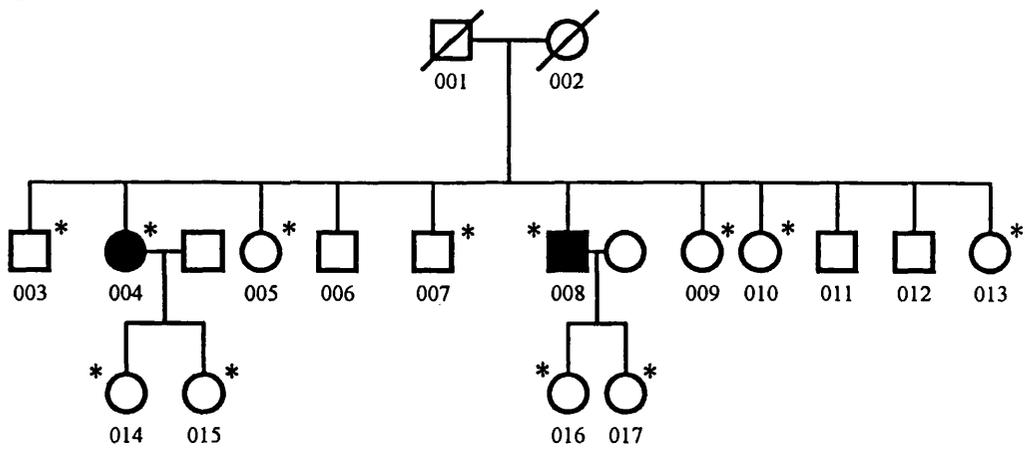
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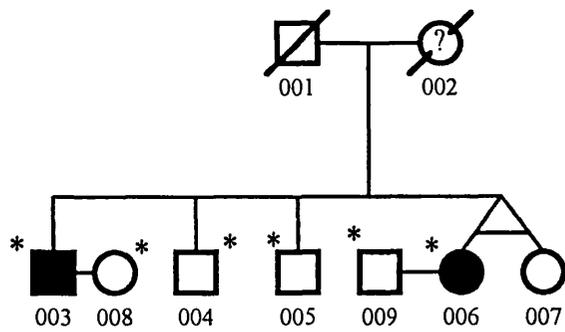
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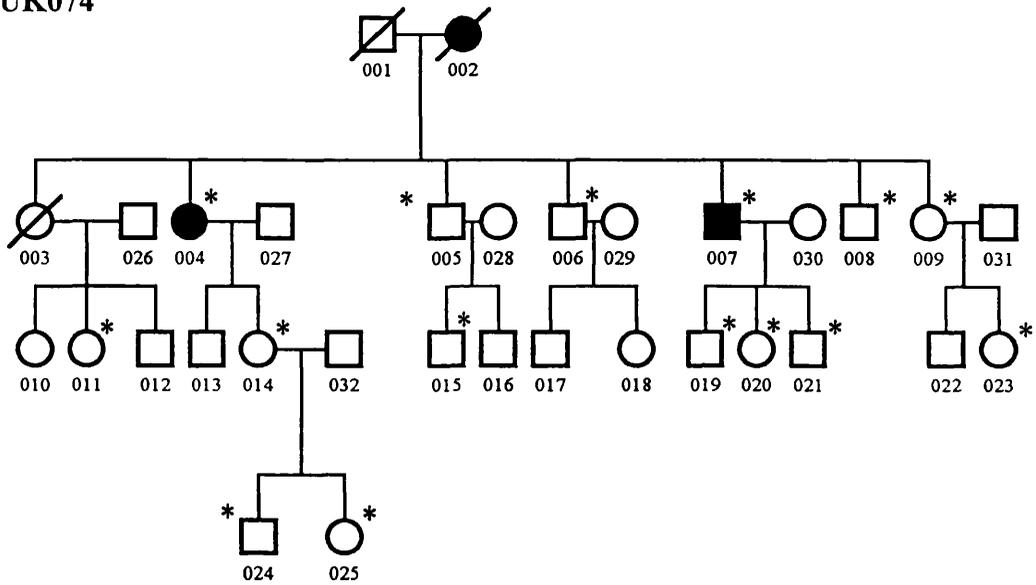
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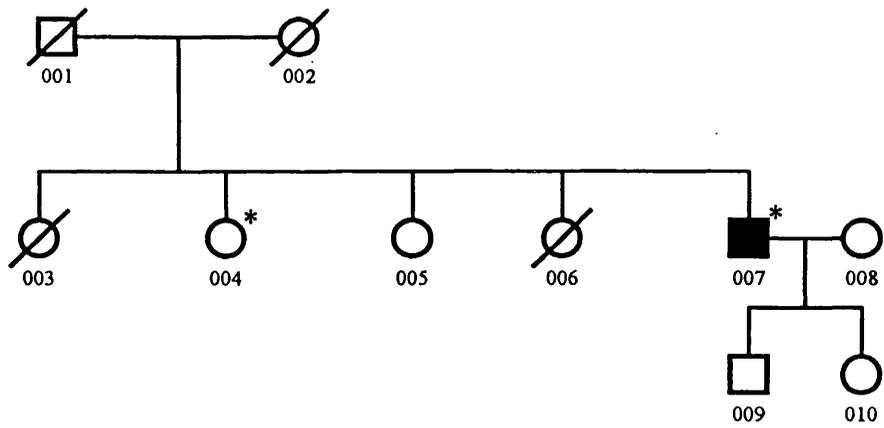
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UK074



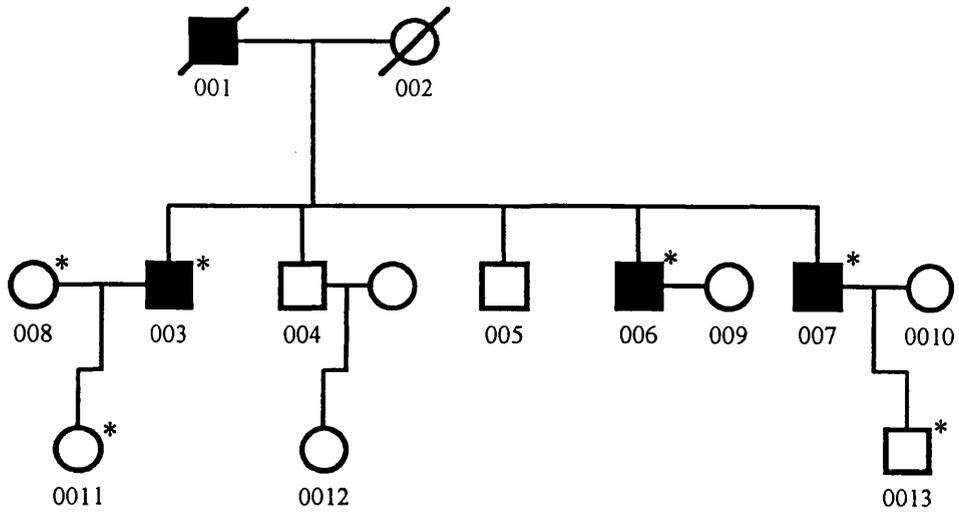
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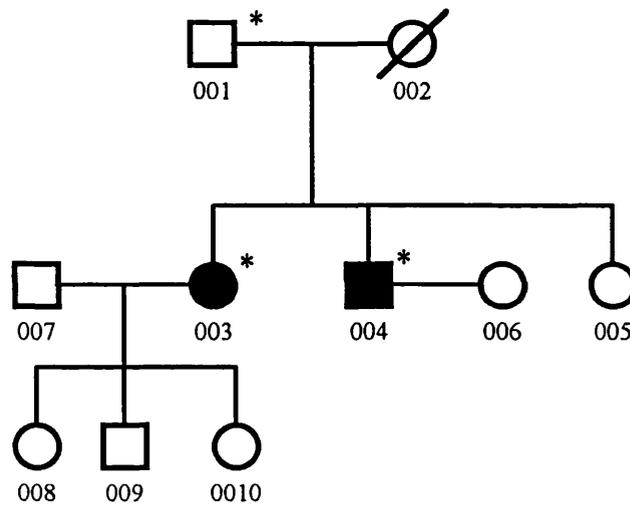
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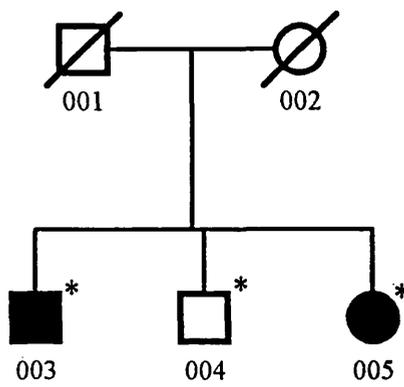
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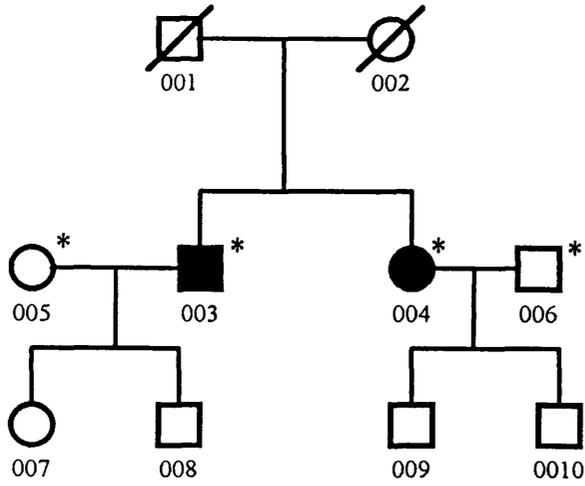
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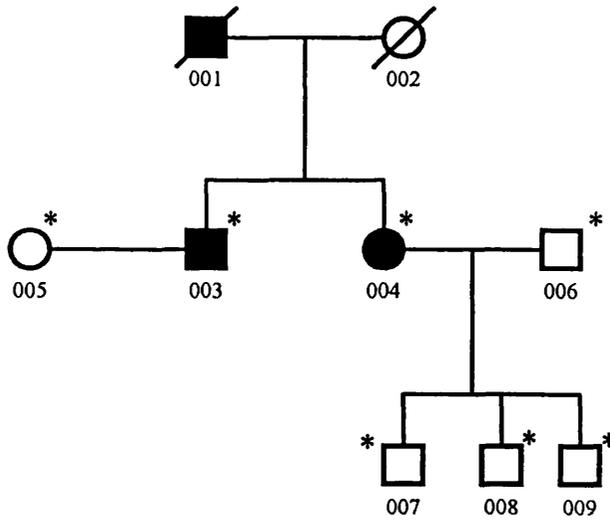
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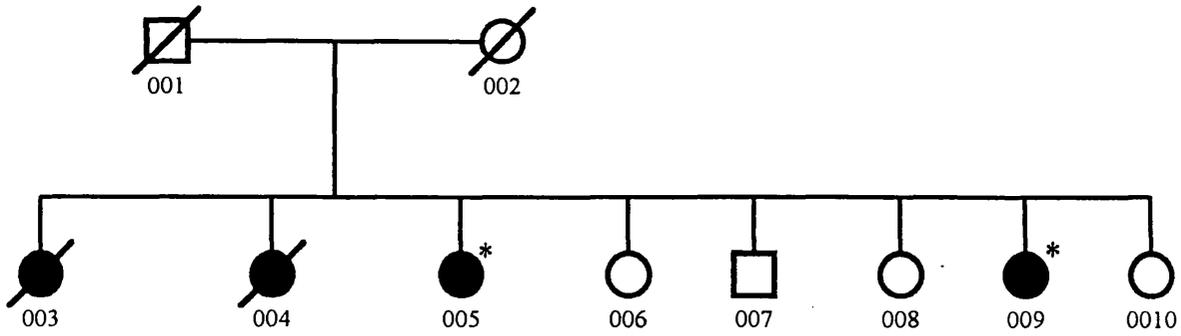
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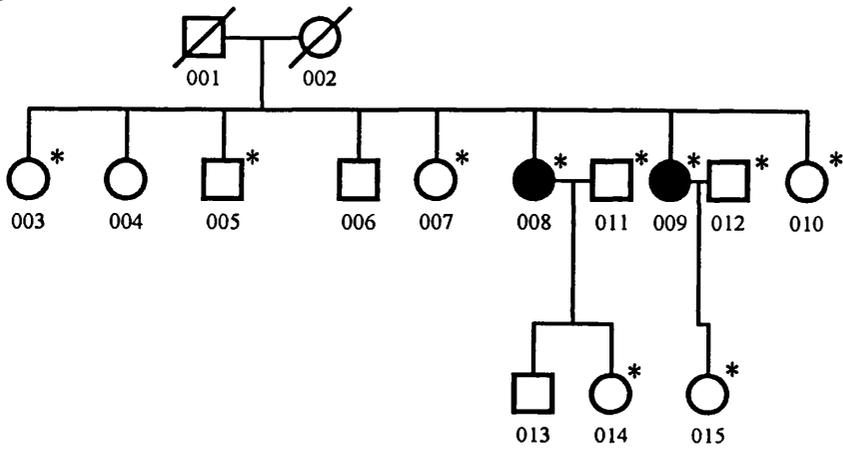
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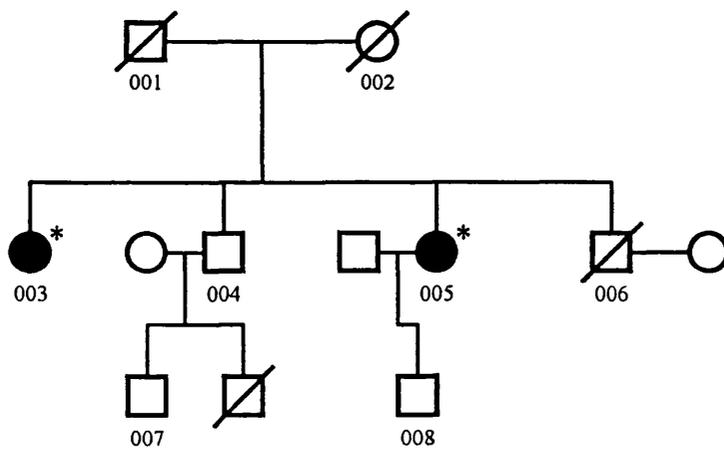
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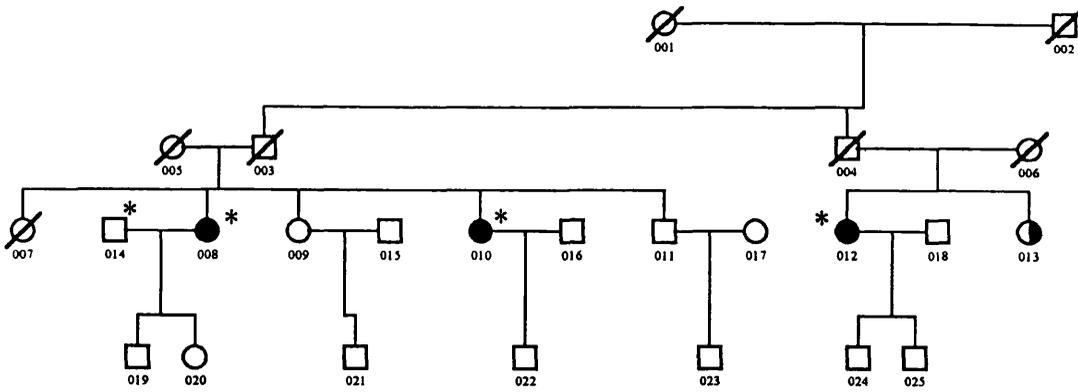
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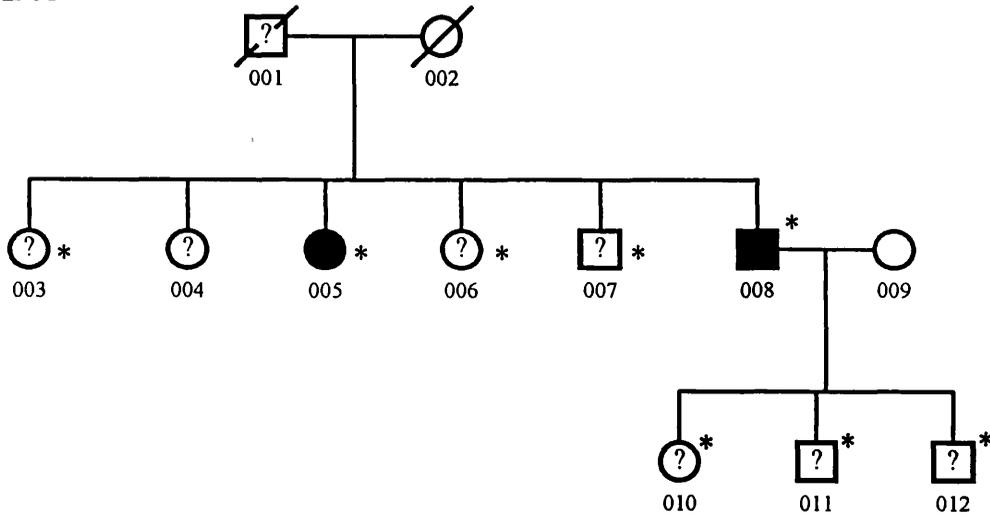
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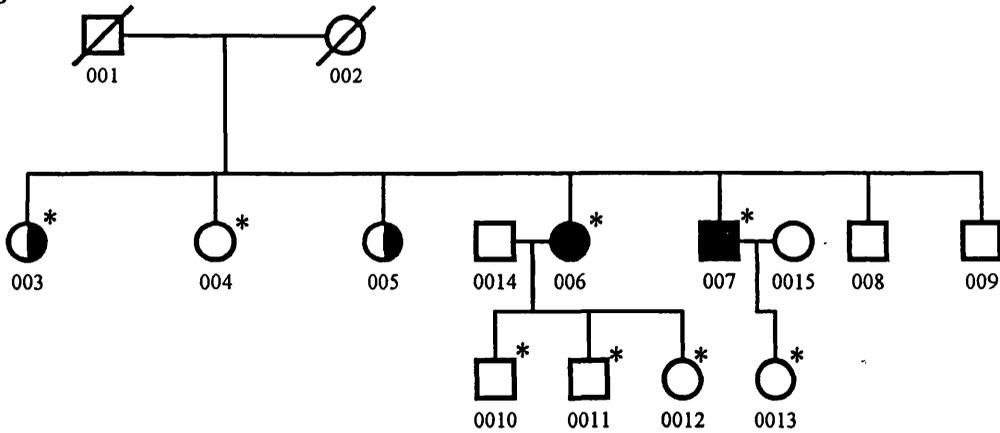
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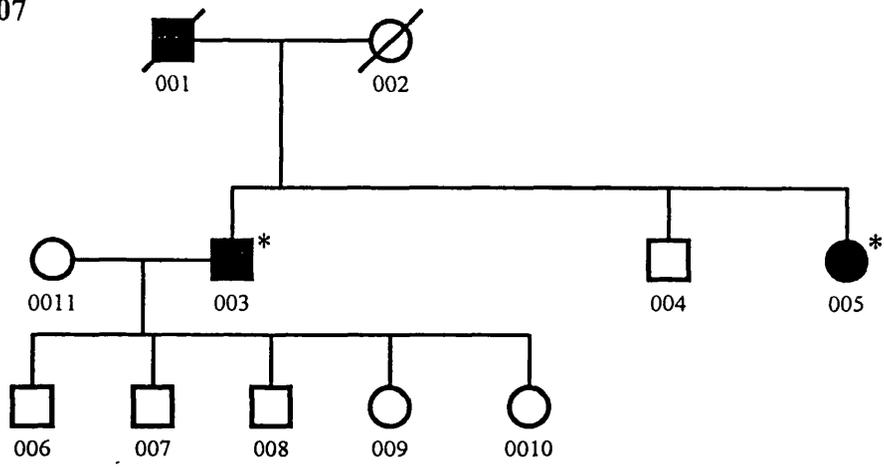
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UK306

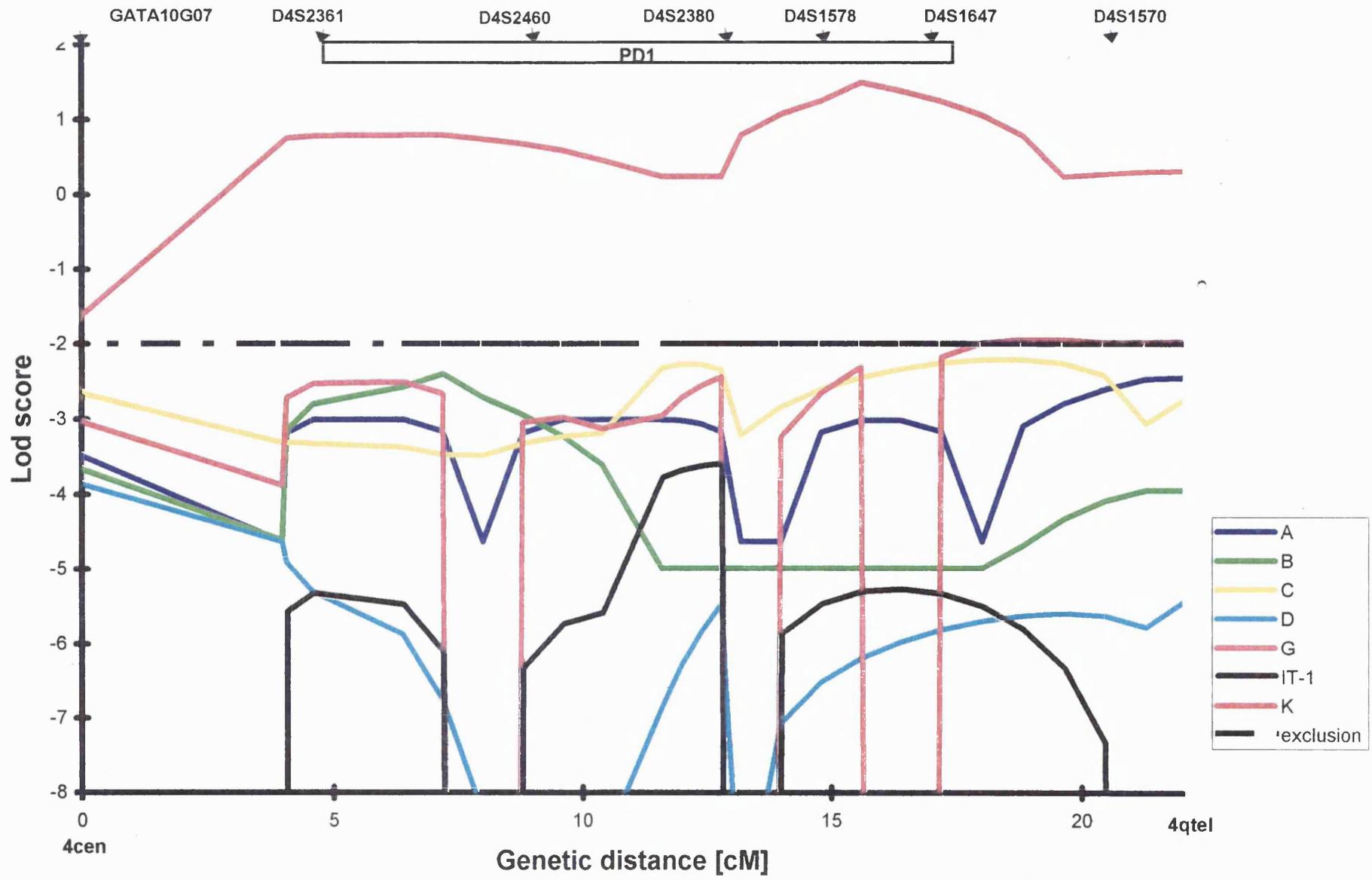


UK307





**A3.3: Multipoint analysis of PARK1 locus in EU multigenerational families**  
 (Gasser *et al.* 1997)



## **APPENDIX 4:**

**A4.1 List of large UK families sequenced for coding mutations in SNCA, and UCH-L1. No mutations were detected.**

*List of UK families sequenced for mutations in alpha-synuclein gene (SNCA)*

UK401

UK402

UK403

*List of UK families sequenced for mutations in the UCH-L1 gene*

UK301

UK003

UK402

UK062

UK074

UK305

UK401

## **A4.2 List of UK families, sporadic and juvenile-onset cases with coding mutations in Parkin**

### *List of UK families with mutations in the Parkin gene*

UK001

UK040

UK057

UK086

UK088

UK401

### *List of individuals with sporadic or juvenile onset PD with mutations in the Parkin gene*

SJO 01

SJO 02

SJO 03

SJO 04

SJO 05

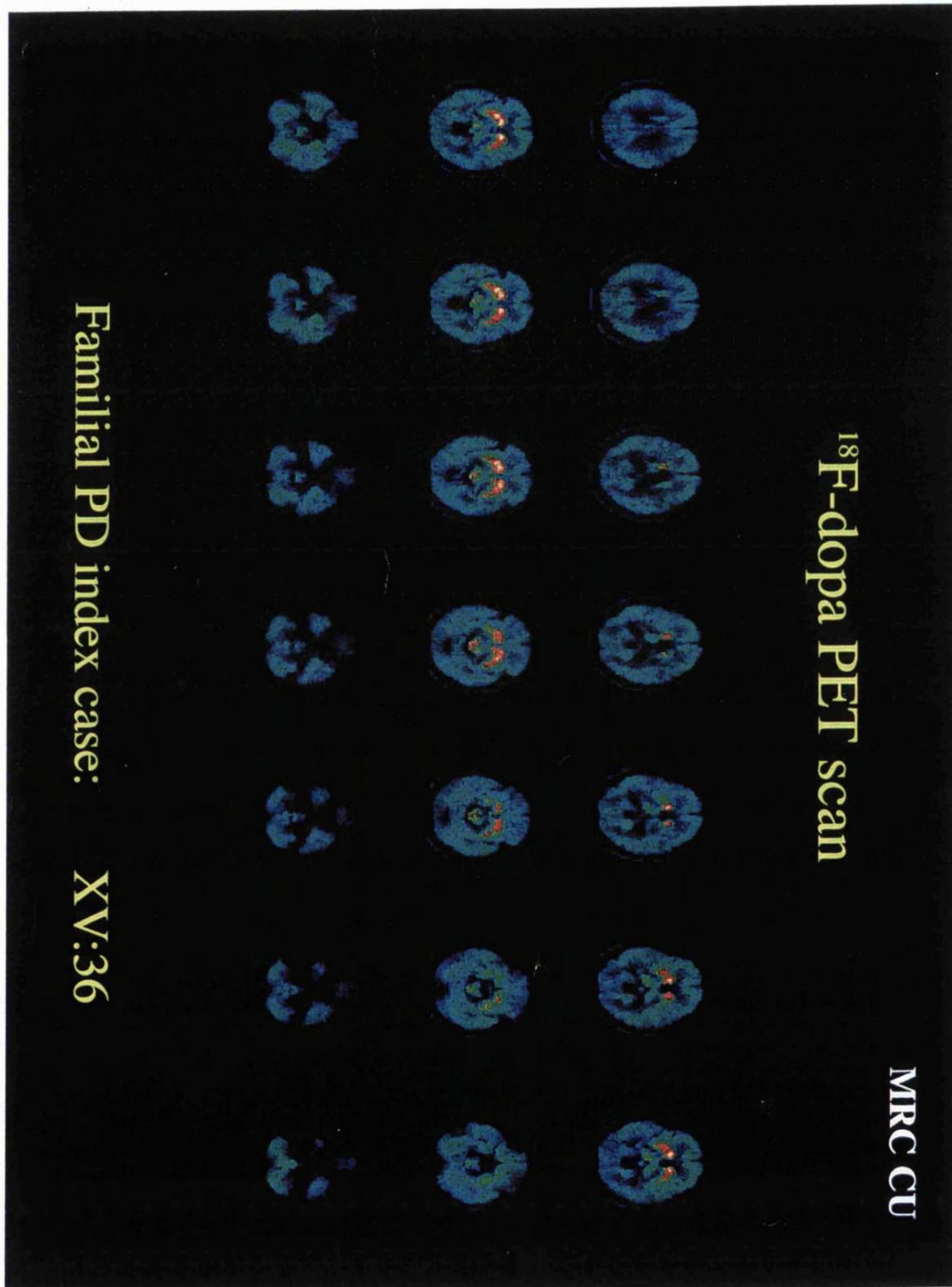
### **A4.3 Immunocytochemistry for $\alpha$ -synuclein and Parkin in frozen sections of human brain**

Snap frozen samples of human brain taken from medulla, pons, midbrain, cerebellum, striatum P2, striatum A1 and frontal cortex were sectioned by cryostat then fixed in 4% paraformaldehyde/0.5% glutaraldehyde at 0°C for 10 minutes. Sections were washed twice in PBS followed by a further fixation in methanol at -20°C for 2 minutes.

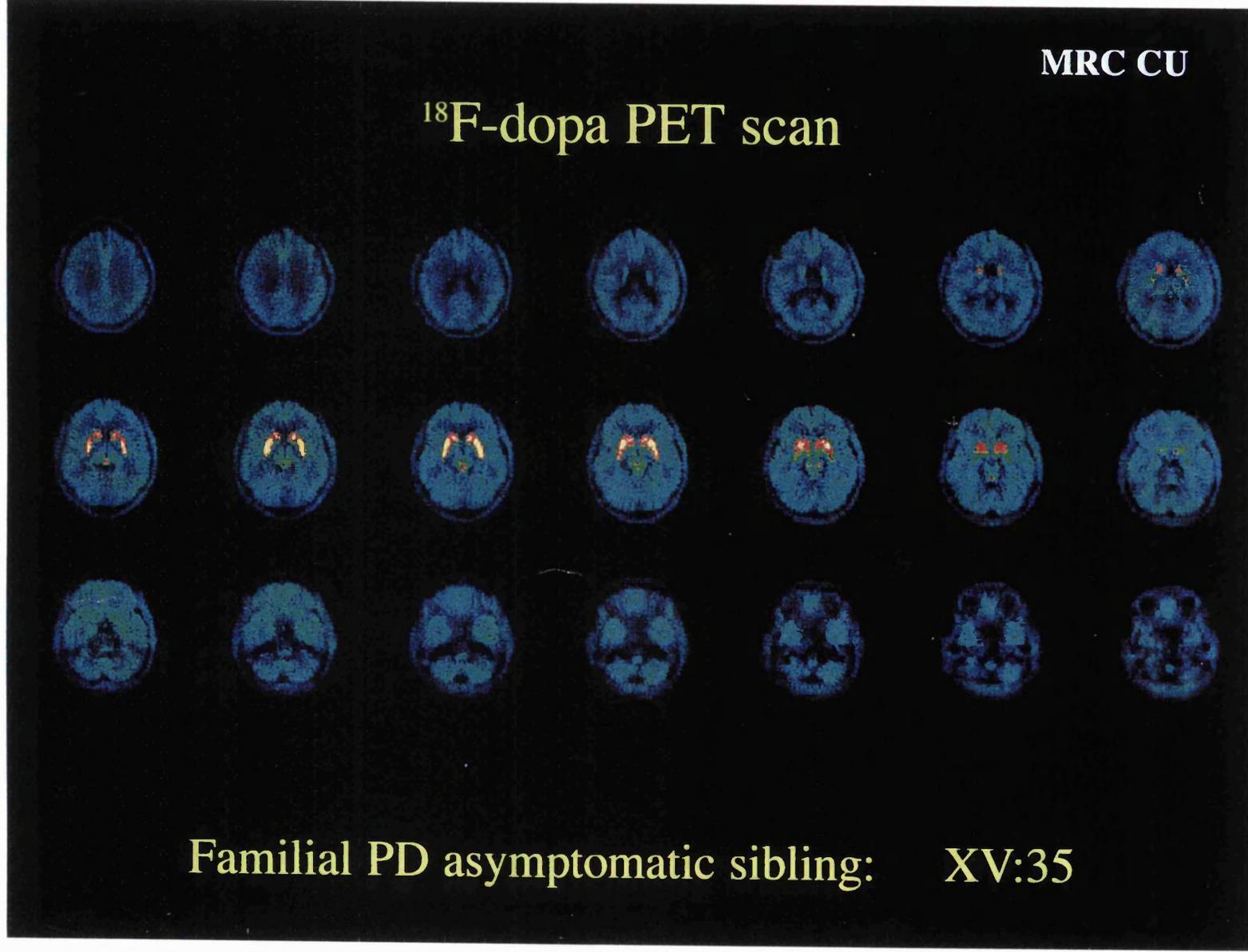
Endogenous reactions on fixed sections were exhausted by incubation in 1% H<sub>2</sub>O<sub>2</sub> in methanol for 10 minutes at room temperature then sections rehydrated through 95% and 70% ethanol to PBS. Endogenous immunoglobulins were blocked in 1% normal goat serum in PBS for 10 minutes at room temperature. Sections were incubated in either a) a commercially available rabbit anti-parkin polyclonal IgG antibody (Oncogene Sciences, Inc.) diluted 1:150 in PBS, or b) a commercially available  $\alpha$ -synuclein antibody, for 2 hours at room temperature, followed by two 5 minute washes in PBS. Next, a secondary biotinylated goat anti-rabbit secondary antibody diluted at 1:150 was incubated with the sections for 30 minutes at room temperature followed by two 5 minute washes in PBS. A streptavidin/biotin complex was formed by incubating sections in the Vectastain ABC reagents for 30 minutes at room temperature, followed by one 5 minute wash in PBS. Finally, staining was detected by incubation in DAB for 10 minutes at room temperature followed by brief counterstaining in haematoxylin, rinsed in tap water for 10 minutes. Sections were dehydrated and finally mounted under coverslips in DPX.

APPENDIX 5

A5.1  $^{18}\text{F}$ -DOPA PET scan of index case XV:36 (UK 403) with familial PD



A5.2  $^{18}\text{F}$ -DOPA PET scan of an asymptomatic sibling XV:35 from a kindred with familial PD



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2. Bandmann O, Vaughan JR, Holmans P, Marsden CD and Wood NW. (1997). The slow acetylator genotype for N-acetyltransferase 2 is associated with familial Parkinson's disease. *Lancet* 350:1136-9.
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13. Periquet M, Luecking CB, Vaughan JR, *et al.* (2001). Origin of the mutations in the Parkin gene in Europe: exon rearrangements are independent recurrent events, whereas point mutations may result from founder effects. *Am. J. Hum. Genet.* 68:617-626.
14. Khan N, Graham E, Dixon P, Morris C, Mander A, Clayton D, Vaughan J, *et al.* (2001). Parkinson's disease is not associated with the combined  $\alpha$ -synuclein/apolipoprotein E susceptibility genotype. *Ann. Neurol.* 49:665-668.

## **BOOK CHAPTERS**

15. Vaughan JR and Wood NW. Candidate genes and Parkinson's disease. A molecular approach to Parkinson's disease (1999).

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competitor DNA were reduced from 100 ng and 200 µg to 10 ng and 20 µg, respectively. The composition of buffer B was 1.2 M sorbitol and 10 mM K<sub>2</sub>HPO<sub>4</sub> at pH 7.5. After the second posthybridization wash in 40% formamide and 2× standard saline citrate (SSC), a 15-min wash at room temperature in 2× SSC and 0.1% Triton X-100 was done. The oligonucleotide probes were as follows: 5'-GCTTGCCTTGTGAATTCTGTGAATTGCCTGGTGTAAATGAGGAAATGG-3', 5'-GATGCCTTGTGATGGTGGCTTGTGTGGGCGCTCCGGTCTCTTAGATTA-3', 5'-GGA-ACCTTGGACGACCTAGTGGATTCCTTCCCAATTCTTGGCCTAATGAAACTAT-3', 5'-ATGGTCTAATTGGTGGTGGACTCATCGGCGGTGTGACGGAGGAGTAATTA-3', 5'-AAGCTTGGAACTGTTTCGTCTTTTGTGACTGGCATTGGCATGGGAAATG-3', and 5'-GTGGAGAGCAATCTATGATAAATGGGACCTTGGGCTGGAGTGTATGC-3'. The probes were directly labeled with a Cy3 fluorochrome at amino-modified thymidine residues indicated by the asterisks (19). To detect poly(A)<sup>+</sup> RNA, FISH was performed with T43 labeled with

fluorescein isothiocyanate (20). In formamide-containing solutions, the concentration was reduced to 10% for poly(A)<sup>+</sup> RNA detection. Images were taken with an Olympus IX70 inverted epifluorescence microscope and Oncor (Gaithersburg, MD) imaging software, version 2.0.5.

24. Strain K5552, which encodes an epitope-tagged version of Ash1p (Ash1p-myc9), was grown to midlogarithmic phase, fixed, and processed for simultaneous FISH and immunofluorescence. After FISH, immunofluorescence was performed as described previously (21) with the following alterations. Antibody to myc was diluted 1:5 into a solution of 1× phosphate-buffered saline, 0.1% bovine serum albumin, 20 mM vanadyl ribonucleoside complex, and ribonuclease inhibitor (40 U/µl). The secondary antibody, goat antibody to mouse immunoglobulin G, conjugated to dichlorotriazinyl amino fluorescein (Jackson Laboratories), was diluted 1:50 into the same solution.
25. Plasmid C3431 is a derivative of YEplac195 (17) carrying a Sal I-Sac I ASH1 fragment.
26. Plasmid pHZ18-poly(A) containing the ADHIII 3'-UTR

has been described (5). Plasmid pXMRS25 was constructed from pHZ18 (22) by insertion of an ASH1 fragment generated by the polymerase chain reaction (PCR). The PCR product contained the last five amino acid codons of ASH1 and extended 250 nucleotides beyond the stop codon. The ASH1 fragment was subcloned into the Sac I site of pHZ18 by the inclusion of a Sac I restriction site in the PCR primers. The primers for PCR were 5'-GGGCCGAGCTCGAGACAGTAGAGAATTGATACATG-3' and 5'-GGGCCGAGCTCATCAGGATGACCAATCTATGGCG-3'. To verify that no mutations were introduced by PCR, the ASH1 region of plasmid pXMRS25 was confirmed by DNA sequencing.

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TECHNICAL COMMENTS

Genetic Complexity and Parkinson's Disease

Mihael H. Polymeropoulos *et al.* describe the genetic linkage of a large Parkinson's disease (PD) pedigree to chromosome 4q21-q23 (1). In this study, which affirms a long hypothesized genetic component to the disease, linkage was detected in a single large family with the use of an autosomal dominant model with 99% penetrance of the disease trait. The clinical presentation in this family, however, may differ from typical idiopathic PD because of the apparent autosomal dominant transmission, early onset, rapid course, and less frequent occurrence of tremor as a significant sign (2). Thus, it is unclear whether the putative PD locus identified by Polymeropoulos *et al.* (which they termed PD1) is responsible for the majority of familial idiopathic PD cases.

As part of an ongoing multicenter study of the genetics of idiopathic PD, we have ascertained 94 Caucasian families (a total of 213 affected relatives sampled: 108 affected sibpairs and 31 affected relative pairs) with at least two individuals in each family meeting clinical criteria for idiopathic PD (3). We have identified approximately 200 multiplex idiopathic PD families to ascertain for a genomic screen. The 94 families discussed here were those completely ascertained, with DNA sampled, at the time of the analysis. Linkage analysis of chromosome 4q21-q23 markers in these idiopathic PD families did not reveal evidence for linkage of an autosomal dominant, highly penetrant gene, as was described by Polymeropoulos *et al.* (1, 4). We determined two-point log odds (lod) scores, with the use of the model of Polymeropoulos *et al.* as well as a low penetrance "affecteds-only"

autosomal dominant model. These lod scores were strongly negative for markers D4S2361, D4S2409, D4S2380, D4S1647, and D4S2623. Multipoint analysis of the genetic map D4S2361-17cM-D4S1647-10.5cM-D4S2623 supported these findings for both models, excluding the entire candidate region. We found no evidence for heterogeneity of either the two-point ( $P > 0.20$ ) or multipoint ( $\ln$  likelihood = 1) lod scores (5). Because the power of the parametric lod score method suffers when the genetic model is misspecified, we also used nonparametric analyses of affected relative pairs (6). As with the parametric lod score analysis, we found no significant evidence for linkage using either two-point or multipoint analysis; in this data set, the multipoint location scores (MLS) exclude the entire 27.5 cM region for recurrence risks to siblings as low as 2.5 (Fig. 1). Because

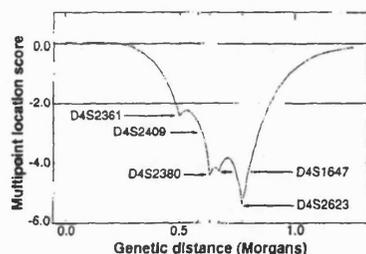


Fig. 1. Multipoint exclusion map for chromosome 4q21-q23 markers. The multipoint lod scores (MLS) within the region are all less than -2.0 at  $\lambda_s = 2.5$ , excluding the entire candidate region identified by Polymeropoulos *et al.* (1). Arrows indicate chromosome markers.

the pedigree analyzed by Polymeropoulos *et al.* contained many younger onset cases (mean age at onset of the disease was 46), we repeated our analysis in the 22 families with at least one affected individual with an onset earlier than age 45; the analysis in the subset supported the results from the full sample (7).

The absence of linkage to chromosome 4q21-q23 in our dataset indicates that there is genetic heterogeneity in PD. It is possible that the region identified by Polymeropoulos *et al.* harbors a disease locus responsible only for a rare autosomal dominant form of PD. Such a situation would be analogous to the genetics of Alzheimer's disease (AD), where mutations (in the amyloid precursor protein and the presenilin 1 and presenilin 2 genes) that cause autosomal dominant AD are responsible for less than 2% of all cases (8). Therefore, although the report by Polymeropoulos *et al.* is a first step in unraveling the genetic etiology of PD, other independent genetic effects likely remain to be discovered.

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3. The families enrolled in this study were ascertained in the following manner. Each of the principal investigators of the 12 study sites identified idiopathic PD patients with one or more first-degree relatives with PD. All 94 families included in the analysis were responsive to levodopa. Specifically excluded were patients with a history of encephalitis, neuroleptic therapy within the year before diagnosis, evidence of

normal pressure hydrocephalus, or a clinical course with atypical features, suggesting secondary Parkinsonism. All first-degree relatives of these patients who consented to participate in this study were subsequently examined and queried regarding the above exclusion criteria and atypical clinical features. Each of these individuals was assigned a status based on their history and the number of the following clinical signs that were present: resting tremor, bradykinesia, and rigidity. Individuals were coded as "affected" if their examination demonstrated at least two of the signs and had no other etiologies for parkinsonism or atypical clinical features, "unclear" if they had only one sign but may have had a history of atypical clinical features, and "at risk" if they had no signs. Mean age at onset of PD symptoms in affected individuals was 61.4 years (SD, 13.1 years). Mean age at examination in affected individuals was 71.5 years (SD, 10.2 years) and in unclear or at risk individuals was 68.4 years (SD, 14.5 years).

- Microsatellite markers spanning the region defined by Polymeropoulos *et al.* (1) were selected for the analysis; the resulting genetic map was: D4S2361-7.7cM-D4S2409-5.3cM-D4S2380-4cM-D4S1647-10.5cM-D4S2623 (Cooperative Human Linkage Center, database is online at www.chlc.org). Two-point and multipoint lod scores were calculated with the use of the VITESSE software package [J. R. O'Connell and D. E. Weeks, *Nature Genet.* 11, 402 (1995)], we assumed, as did Polymeropoulos *et al.*, autosomal dominant inheritance, a disease allele frequency of 0.001, and 99% penetrance. A low penetrance, "affecteds only" analysis was also performed. Allele frequencies were estimated from 75 unrelated Caucasian controls.
- Heterogeneity analysis of two-point and multipoint lod scores was performed using the admixture test, implemented in the HOMOG software package [J. Ott, *Analysis of Human Genetic Linkage*, revised ed. (Johns Hopkins Press, Baltimore, MD, 1991)]. The asymptotic chi-square test is not valid for multipoint lod scores; therefore, a log likelihood comparison is used to assess heterogeneity.
- Two-point affected-relative-pair analysis was performed using the SimIBD software package [S. Davis, M. Schroeder, L. R. Goldin, D. E. Weeks, *Am. J. Hum. Genet.* 58, 867 (1996)]. Multipoint affected sibpair exclusion mapping was performed with the use of ASPEX [available from N. Risch, Stanford University, and based on N. Risch, *Am. J. Hum. Genet.* 46, 229 (1990)] with the use of all five microsatellite markers. Estimates of the recurrence risk to siblings ( $\lambda_s$ ) range from 3 to 150, with the majority of studies supporting a  $\lambda_s$  between 10 and 20 [R. C. Duvoisin, *Adv. Neurol.* 60, 306 (1993); K. Kondo and K. Watanabe, *ibid.*, p. 346]. Exclusion mapping with the recurrence risk set at the low end of this range determined that the region can be excluded for  $\lambda_s \geq 2.5$ .
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- The Deane Laboratory Parkinson's Disease Research Group is a multicenter study of the genetics of idiopathic Parkinson Disease coordinated by the Center for Human Genetics, Duke University Medical Center. The collaborating sites and principal investigators are: University of Minnesota, M. Nance; Ohio State University, J. Hubble; University of Kansas Medical Center, W. Koller; University of Pennsylvania Graduate Neurological Center, M. B. Stern and A. Colcher; Emory University School of Medicine, R. L. Watts; Rush Presbyterian-St. Luke's Hospital, C. Goetz and E. Pappert; Carolina Neurological Clinic, F. H. Allen Jr.; Baylor College of Medicine, J. Jankovic and W. Ondo; Marshfield Clinic, B. C. Hiner; University of California, San Francisco, M. Aminoff and G. Dowling; University of California, Los Angeles, G. W. Small.
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Polymeropoulos *et al.* present results of a genome-wide screen for genetic linkage in a large family with autosomal-dominantly inherited L-Dopa-responsive parkinsonism with Lewy-body pathology (1). They convincingly demonstrate linkage with polymorphic markers on chromosome 4q21-4q23, with a maximum two-point lod score of 6.00 for marker D4S2380. The locus was termed PD1. The role of the PD1 locus in other families with inherited parkinsonism and in sporadic PD remains to be investigated.

We have examined polymorphic markers closely linked to PD1 in 13 multigenerational families with inherited parkinsonism (Table 1). Affected members in all families exhibited at least two of the three cardinal clinical signs of PD (akinesia, rigidity, and resting tremor), as well as asymmetry at onset and a marked improvement

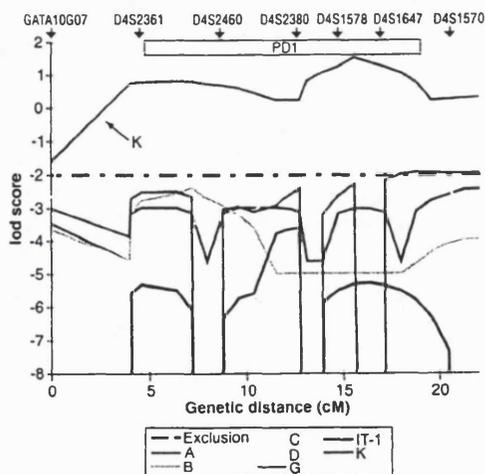
on L-Dopa treatment. Rigorous exclusion criteria were applied (supranuclear ophthalmoplegia, cerebellar or pyramidal signs, and severe autonomic or postural disturbance within 2 years of onset). The wide range of age at onset and spectrum of clinical features, including the presence of dementia in addition to parkinsonism in some affected individuals, was similar to that observed in the family studied by Polymeropoulos *et al.* (1). No additional neurologic deficit was observed except for amyotrophy in one affected of family A. Multipoint analysis with eight polymorphic markers spanning the region from GATA 10G07 to D4S2623 excluded the entire 17 cM region likely to contain PD1 in five of the families (families A, B, C, D, and IT-1). In one additional family (G), the major portion of the critical region was also excluded, with lod scores between -1.9 and -2 for the remainder of

**Table 1.** Demographic and clinical characteristics in 13 families with inherited parkinsonism.

Family	Number of affected relatives	Affected relatives examined	Age at onset* (years)	L-Dopa response	Atypical features	Reference†
1 A	12	4	51 (35 to 60)	Positive	Amyotrophy and dementia in some	(2, 3)
2 B	8	4	62 (51 to 82)	Positive	Dementia in some	(2, 3)
3 C	11	4	60 (55 to 66)	Positive	None	(2, 3)
4 D	18	5	63 (48 to 78)	Positive	None	(3, 4)
5 G	11	4	56 (48 to 74)	Positive	Dementia in some	(3)
6 IT-1	5	4	54 (36 to 89)	Positive	None	(5)
7 K	4	4	45 (37 to 63)	Positive	None	-
8 FR-041	3	3	63 (60 to 65)	Positive	None	-
9 FR-722	8	5	59 (53 to 64)	Positive	None	(6)
10 FR-727	4	3	41 (31 to 52)	Positive	None	-
11 FR-755	4	3	38 (29 to 52)	Positive	None	-
12 UK-A	14	5	53 (42 to 70)	Positive	None	-
13 UK-B	5	5	37 (31 to 41)	Positive	None	(7)

\*Mean and range. †Unpublished (-).

**Fig. 1.** Multipoint linkage analysis of the PD1-region on chromosome 4q21-q23 in seven families with familial parkinsonism. Polymorphic DNA fragments were amplified by PCR with the use of published primer sequences and a standard protocol. Multipoint analysis was performed using GENEHUNTER (8), and two-point analysis was done using VITESSE (9). An autosomal dominant model with an age-dependent penetrance was assumed. As was done by Polymeropoulos *et al.* (1), unaffected individuals were set to be unaffected only when they were older than the mean age of onset in the respective families; all other unaffected individuals were treated as unknown. Frequency of the disease allele was set to 0.001. Marker allele frequencies were set to be equal for all alleles. Estimating marker allele frequencies from founders in the pedigrees did not alter multipoint lod scores significantly.



the interval (Fig. 1). Data from one (previously unpublished) family of southern Bavarian origin showed positive lod scores with a maximum multipoint score of 1.5 (family K, Fig. 1). This lod score is close to the theoretical maximum in this relatively small family.

In six families (FR-041, FR-722, FR-727, FR-755, UK-A, and UK-B), only the two polymorphic markers most closely linked to PD1 (D4S1647 and D4S2380) have been analysed. Obligate recombinations (no allele shared by all affecteds) were observed in five of these families either for each of the markers individually (three families), or for the haplotype of both markers (two families), again strongly arguing against linkage with the PD1 locus. In one family (FR-041), a positive pairwise lod score was obtained for D4S2380 (0.29 at Theta = 0). Positive lod scores in families K and FR-041 may reflect true linkage, but they may also be a result of random fluctuations, because the relatively small size of these families precludes definite proof of linkage.

We conclude that mutations at the PD1 locus are probably a rare cause of autosomal-dominant parkinsonism. The role of the PD-1 gene in sporadic PD is still to be determined.

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Response: Scott et al. and Gasser et al. are discussing genetic studies of families with PD that are designed to examine whether a locus that we previously reported (1) on chromosome 4q21-q23 is operating in their sample. The results of Scott et al. in 94 Caucasian families do not demonstrate linkage even when the 22 families with earlier onset are examined separately. Similarly, Gasser et al. exclude linkage in 13 multigenerational families with Parkinson's disease, with the exception of one family for which they achieved a maximum multipoint lod score of 1.5 for genetic markers in the 4q21-q23 region. Cumulatively, these comments suggest that the chromosome 4 locus will not account for the majority of familial Parkinson's disease and will be expected to operate only in a small percentage

of families with the illness.

We have recently demonstrated that a mutation in the alpha synuclein gene is responsible for the phenotype in four families with early onset Parkinson's disease (2). Because the mutation was not detected in 50 individuals with sporadic PD, or in two other families with late onset of the illness, we concluded that mutations in the alpha synuclein gene will not account for the majority of the genetic factors of PD, but rather for a proportion of those families with an early onset autosomal dominant form of the illness. These results are in agreement with the observation of Scott et al. and Gasser et al., and suggest that the understanding of genetic complexity of Parkinson's disease is just beginning to take shape.

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Experiments in a Parkinson's Rat Model

Derek L. Choi-Lundberg et al. present evidence (1) that a replication-defective adenoviral (Ad) vector that encodes human glial cell line-derived neurotrophic factor (GDNF) protects dopaminergic neurons in substantia nigra (SN) in rats from progressive degeneration induced by the neurotoxin 6-hydroxydopamine (6-OHDA) that has been injected into the striatum. These results are important because of possible applications of Ad vector-mediated GDNF gene therapy in patients with Parkinson's disease. The experimental design used by Choi-Lundberg et al., however, raises some concerns.

Choi-Lundberg et al. (1) injected 6-OHDA into the striatum of rats 7 days after labeling SN neurons with the retrograde fluorescent tracer fluorogold (FG). Thus, the neurotoxin acted mainly on SN neurons that were loaded with FG. Because of neuronal death and membrane disruption, the fluorescent tracer diffused in the extracellular space, from where it might have been incorporated by other cells. That such an uptake of tracer really occurred in the experiment by Choi-Lundberg et al. is demonstrated by figure 2, C through G, in their report, showing that microglia and other non-neuronal cells in

the SN have been labeled with FG. Similar to non-neuronal cells, SN neurons that survived the neurotoxin might have incorporated the tracer through their cell membranes (2).

To conclude, the finding (1) of a reduced loss of FG-labeled neurons in the SN of GDNF-treated rats does not necessarily imply a neuroprotective action of GDNF. A control in which the injection of FG is made after the complete or nearly complete degeneration of the SN neurons would seem to be necessary to definitely support the conclusions made by Choi-Lundberg et al.

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## Early report

## Association of slow acetylator genotype for N-acetyltransferase 2 with familial Parkinson's disease

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### Summary

**Background** Epidemiological studies have identified positive family history and exposure to environmental toxins as risk factors for Parkinson's disease (PD). An inherited defect of xenobiotic metabolism could result in increased susceptibility to such toxins. We investigated the frequency of functionally relevant polymorphisms in six detoxification enzymes among patients with PD to elucidate the relation between these polymorphisms and the disease.

**Methods** We obtained brain-tissue samples from 100 patients with apparently sporadic PD and blood samples from 100 living patients with familial PD. For the control group, we extracted DNA from the tissue of 100 pathologically normal brains. The six enzymes analysed in these three groups were: CYP2D6, CYP2E1, NAD(P)H-menadione reductase, glutathione transferases M1 and T1, and N-acetyltransferase 2. We also investigated N-acetyltransferase 2 in 100 blood samples from patients with genetically proven Huntington's disease. We used PCR-based methods and restriction-enzyme analysis to detect polymorphisms.

**Findings** The slow acetylator genotype for N-acetyltransferase 2 was more common in the familial PD group (69%) than in all controls (37%). Even after correction for multiple comparisons, this result remained highly significant ( $p=0.002$ ) for familial PD compared with normal controls (odds ratio 3.79 [95% CI 2.08–6.90]) and compared with Huntington's disease (2.45 [1.37–4.38],  $p=0.004$ ). The slow acetylator frequency for N-acetyltransferase 2 for sporadic PD was between that for Huntington's disease and familial PD. The frequencies of all the other polymorphisms were similar in the two study groups and the normal control group.

**Interpretation** We found an association between the slow acetylator genotype for N-acetyltransferase 2 and familial PD. Further studies are needed to investigate the biological relevance of these findings, but slow acetylation could lead to impaired ability of patients with familial PD to handle neurotoxic substances.

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### Introduction

Parkinson's disease (PD) is common among the elderly.<sup>1</sup> Its pathological hallmark is prominent loss of dopaminergic neurons in the substantia nigra.<sup>2</sup> There is increasing evidence for involvement of genetic factors in the pathogenesis of PD: several large families with autosomal dominantly inherited PD have now been described, and the first genetic locus for PD has been identified in one of these families.<sup>3</sup> Relatives of patients with PD are at a greater risk of developing the disease than are controls, and PD seems to be more common among monozygotic than dizygotic twins of affected parents.<sup>4</sup> A further identified risk factor is exposure to environmental toxins, such as organochlorines and alkylated phosphates.<sup>5</sup> A genetic predisposition might make patients with PD less able to inactivate endogenous or exogenous toxins, leading to increased radical stress or inhibition of mitochondrial function.<sup>6,7</sup> Other exogenous factors, such as smoking, may have a protective effect against the development of PD.<sup>8</sup> We investigated the possible involvement of six detoxification enzymes in the pathogenesis of PD. Only genetic polymorphisms with a well-characterised influence on the biological activity of these enzymes were analysed.

We investigated the enzymes N-acetyltransferase 2, CYP2D6, CYP2E1, NAD(P)H-menadione reductase (NQ1), and glutathione transferases M1 and T1. N-acetyltransferase 2 catalyses the transfer of an acetyl group from the cofactor acetyl coenzyme A to the amine nitrogen atom of aromatic amines and hydrazines. The degree of activity of this enzyme determines the rate of detoxification of aromatic amines. The three mutant alleles *M1*, *M2*, and *M3* account for most slow acetylators among white patients.<sup>9</sup> The A, B, and L polymorphisms of CYP2D6 have been associated with PD.<sup>10–12</sup> However, subsequent studies failed to replicate the original findings for the A or B alleles.<sup>13</sup> CYP2E1 is a potent producer of reactive oxygen species.<sup>14</sup> Induction of this enzyme causes oxidative stress in astrocytes in vitro, leading to increased concentrations of malonaldehyde—a late-stage marker for lipid oxidation—and decreased concentrations of glutathione.<sup>14</sup> Similar changes have been seen in PD.<sup>15,16</sup> A C→T polymorphism in the promoter region is associated with ten-fold higher concentrations of CYP2E1 transcription in vitro.<sup>17</sup> NQ1 is a flavoprotein that catalyses a two-electron reduction. A C→T polymorphism in exon 6 of the *NQ1* gene abolishes enzymatic activity of NQ1.<sup>18</sup> Glutathione transferases M1 and T1 have important roles in free-radical scavenging via their glutathione-dependent peroxidase activities, but they are also capable of metabolising and, therefore, detoxifying various structurally diverse substrates.<sup>19</sup> Whole-gene deletion of glutathione transferase M1 or T1 leads to abolition of enzymatic function.

Enzyme	Controls	Treatment group					
		Sporadic PD		Familial PD		Huntington's disease	
		Number alleles/total	Odds ratio (95% CI)	Number alleles/total	Odds ratio (95% CI)	Number alleles/total	Odds ratio (95% CI)
N-acetyltransferase 2 (SA)*	37/100	59/100	2.45 (1.37-4.38)	69/100	3.79 (2.08-6.90)	48/100	1.57 (0.88-2.80)
CYP2D6A†	3/200	0/200	..	3/200	1.00 (0.19-5.18)	..	..
CYP2D6B†	34/200	49/200	1.58 (0.96-2.61)	46/200	1.45 (0.88-2.42)	..	..
CYP2D6L†	65/200	65/200	1.00 (0.65-1.54)	54/200	0.77 (0.50-1.19)	..	..
CYP2D6 (PM)‡	4/100	9/100	2.37 (0.68-8.18)	4/100	1.00 (0.24-4.23)	..	..
CYP2E1†	6/200	10/200	1.70 (0.59-4.88)	9/200	1.52 (0.52-4.46)	..	..
NQ1†	29/200	35/200	1.25 (0.72-2.16)	37/200	1.34 (0.78-2.30)	..	..
Glutathione transferase M1§	58/100	60/100	1.09 (0.61-1.93)	55/100	0.89 (0.50-1.57)	..	..
Glutathione transferase T1§	17/100	18/100	1.07 (0.51-2.26)	21/100	1.30 (0.63-2.68)	..	..
Glutathione transferase M1+T1 (combined deletion)§	11/100	10/100	0.90 (0.36-2.26)	12/100	1.10 (0.45-2.68)	..	..

\*Frequency of slow acetylator genotype (carrying any two of mutant alleles M1, M2, and M3). †Number deleted alleles with investigated polymorphism vs total number alleles analysed. ‡Frequency of poor metaboliser phenotype (carrying mutant alleles CYP2D6A or CYP2D6B). §Frequency for homozygous deletion for both alleles of each gene.

Table 1: Summary of investigated polymorphisms

Research into the aetiology of PD is hampered by about 20% of patients with the clinical diagnosis of PD having a different underlying pathology at necropsy.<sup>20</sup> Therefore, to avoid this pitfall, we used only DNA samples extracted from brain tissue with pathological diagnosis of PD for the sporadic PD group. We analysed separately blood samples from patients with familial PD, assuming that there could be a stronger genetic component in this group. Incidental Lewy body disease with subclinical damage to the nigrostriatal system may be substantially more common than clinically manifest PD and share some biochemical abnormalities with PD.<sup>21</sup> Therefore, we used as the initial controls only DNA samples from brains with no histopathological evidence of nigral-cell loss. We used 100 blood samples from patients with genetically proven Huntington's disease as a second control group for N-acetyltransferase 2.

### Patients and methods

We included only white European patients and controls in this study. We initially obtained 100 samples of brain tissue from sporadic PD cases for another study.<sup>22</sup> To enable comparison in the present study we included 100 samples in all other groups. The control brain-tissue samples came from cases with similar distribution for sex and age to cases in the study group, which was achieved by obtaining the samples from the UK Parkinson's Disease Brain Bank and the brain bank at the Institute of Psychiatry, London, UK. All blood samples from patients with genetically proven Huntington's disease were obtained from the diagnostic service laboratory of the Institute of Neurology, London, UK. The project had the approval of the local ethics committee.

The defining characteristics on histology for sporadic PD were depletion of pigmented neurons in substantia nigra and locus ceruleus, with Lewy bodies in some remaining nerve cells and elsewhere in the nervous system. The striatum was normal; there were no glial cytoplasmic inclusions or additional pathology to account for symptoms of parkinsonism. The mean age at death was 76.7 (SD 7.9) and the group consisted of samples from 65 men and 35 women.

We identified 100 families with at least two living individuals who met the diagnostic criteria for idiopathic PD from a similar study by Maraganore and colleagues.<sup>23</sup> Most familial cases of PD were identified by a nationwide search through the British Neurological Surveillance Unit. In addition, patients with a positive family history notified us themselves after reading advertisements in the UK Parkinson's Disease Society Newsletter. All patients (55 men and 45 women, mean age 68.4 years [7.7]) came from different families and blood samples from the index cases and affected relatives were assessed individually.

The normal control group consisted of 100 DNA samples

taken from brain tissue with no pathological changes. All controls (65 men and 35 women) were aged 60 years or older when they died (mean 77.1 [8.8]).

We obtained blood samples from 100 Huntington's disease patients (65 men, 35 women, mean age 52.6 years [13.4]). All cases had a pathological trinucleotide repeat expansion with more than 40 repeats in the Huntington's disease gene.

We extracted DNA from the brain-tissue and took blood samples by standard techniques. Each PCR contained 100 ng genomic DNA, 10 pmol of each primer, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L potassium chloride, 1.5 mmol/L magnesium chloride, and 1.25 U of *Taq* polymerase, with a final volume of 25  $\mu$ L. When appropriate, PCRs were digested with the respective enzyme, according to the protocol supplied by the manufacturer (Promega UK Ltd, Hitchin, New England Biolab, Southampton, UK). After digestion, fragments were separated on an agarose gel, stained with ethidium bromide, and visualised with ultraviolet light. We analysed the A, B, and L polymorphisms of CYP2D6. The base-pair deletion of the allele CYP2D6A creates a restriction site for *HpaII* and the G-A polymorphism of the allele CYP2D6B results in a loss of a restriction site for *BstNI*.<sup>10</sup> Slow metabolisers carry two copies of CYP2D6A or CYP2D6B. The C→T polymorphism of the allele CYP2D6L results in a loss of a *CfoI* restriction site.<sup>24</sup> For CYP2E1, the C→T polymorphism in the 5' flanking region results in loss of a restriction site for *RsaI*.<sup>25</sup> The C→T polymorphism in exon 6 of NQ1 creates a restriction site for *HinI*.<sup>26</sup> Glutathione transferase M1 and T1 polymorphisms were characterised simultaneously with a multiplex PCR.<sup>27</sup> We analysed genotype of N-acetyltransferase 2 for the wild-type allele and the three mutant alleles M1, M2, and M3.<sup>28</sup> Briefly, after initial amplification, the PCR product was cut with three different enzymes, *KpnI*, *TaqI*, and *BamHI*. The distinct sequence variants encoding the different mutant alleles result in the loss of a *KpnI* restriction site for M1, a *TaqI* restriction site for M2, and a *BamHI* restriction site for M3. The presence of any two mutant alleles defines the slow acetylator genotype, since fast acetylators have one or two wild-type alleles.

We used  $\chi^2$  analysis to compare the frequency of ten variables separately between the sporadic PD and normal control groups, and the familial PD and normal control groups. We made 20 comparisons and corrected the p values from the initial comparison for multiple comparison by multiplying the initial p value by a factor of 20. We used Yates' correction for all tests and calculated odds ratios with 95% CIs for each variable. The power of the sample to give a significant result (corrected for multiple comparison), given the observed values of the odds ratio and the frequency of the variable in the controls, was also calculated. Since the proportions for the four groups were evenly spaced, we tested for trend for the slow acetylator frequency across the groups (table 1) to test the hypothesis that the data for the Huntington's disease group would be intermediate between the normal control and the sporadic PD group, assuming a linear trend across the four groups.

Allele	Familial PD	Sporadic PD	Huntington's disease	Controls
Wild type	33	43	54	68
M1	89	91	91	61
M2	72	58	53	66
M3	6	1	2	5

Table 2: Frequency of wild-type allele and mutant alleles *M1*, *M2*, and *M3* among patients and controls

To assess whether a particular mutant allele of N-acetyltransferase 2 was over-represented in the familial or sporadic PD groups, we used the relative predispositional effects method described by Payami and colleagues.<sup>29</sup> Briefly, the overall  $\chi^2$  was calculated first for the wild-type, and *M1*, *M2*, and *M3* alleles. If the result was significant, the wild-type allele was removed to allow for differences in its frequency, and  $\chi^2$  was calculated for just *M1*, *M2*, or *M3*. A significant result suggested an association between a particular allele and the disease; a negative result suggested an association between the slow acetylator genotype, resulting from different allele combinations, and the disease.

## Results

The proportion of slow acetylators was substantially higher among patients with familial PD (69%) than among normal controls (37%,  $p < 0.001$ ; table 1). This difference remained highly significant after correction for multiple comparison ( $p = 0.002$ ). The proportion of the sporadic PD group with slow acetylators was also higher (59%). However, this initially significant result ( $p = 0.003$ ) was no longer significant after correction for multiple comparison ( $p = 0.06$ ). Comparison of the slow acetylator genotype frequency between the two PD groups and the Huntington's disease group showed a highly significant difference between the familial PD and Huntington's disease groups (odds ratio 2.41 [95% CI 1.34–4.35],  $p = 0.004$ ), but not between the sporadic PD and the Huntington's disease groups (1.56 [0.88–2.76],  $p = 0.16$ ). The slow acetylator frequency for N-acetyltransferase 2 in the sporadic PD group was between that of the Huntington's disease group and the familial PD group, and the odds ratio (2.45 [1.37–4.38]) for the comparison of sporadic PD with controls also suggested an involvement of N-acetyltransferase 2 in sporadic PD. Therefore, we tested for trend of N-acetyltransferase 2 slow acetylator frequency across the four groups. The result was highly significant ( $p = 0.000002$ ).

In a second analysis, we compared the frequency of the wild-type allele and the mutant alleles *M1*, *M2*, and *M3* between the different groups (table 2). As expected, the frequency of the wild-type allele was lower in both PD groups than in the Huntington's disease or normal control group. We found a significant association between the slow acetylator phenotype and familial PD before removal of the wild-type allele ( $p = 0.0005$ ). After we removed the wild-type allele from the model, the result was no longer significant, which suggests that the familial PD association is with all three slow acetylator alleles, rather than one allele in particular. Similarly, a comparison between all N-acetyltransferase 2 alleles in Huntington's disease and familial PD was significant ( $p = 0.019$ ), but not after removal of the wild-type allele ( $p = 0.172$ ). No significant differences were found for any comparison of the other polymorphisms between sporadic PD and controls or familial PD and controls (table 1).

## Discussion

The interest in possible exogenous causes of PD was stimulated by the discovery that exposure to the pyridine derivative N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

(MPTP) can result in parkinsonism with dopaminergic-cell loss in the substantia nigra. However, at least 400 people are known to have self-administered MPTP, but only a few have developed symptoms of parkinsonism.<sup>30</sup> Therefore, a genetic predisposition may exist that increases susceptibility to the neurotoxic effects of MPTP and, possibly to other toxins. We analysed the genetic polymorphisms of six different detoxification enzymes and found a significantly increased frequency of slow acetylators for N-acetyltransferase 2 among patients with familial PD. This difference was apparent not only when we compared the familial PD group with the normal control group but also for the comparison with the Huntington's disease group. We added the Huntington's disease group as a second control to validate our findings from comparison of the familial PD group and the normal controls. Huntington's disease, like PD, affects the basal ganglia but is, unlike PD, a genetically well-defined, single-gene disorder and, therefore, we thought, particularly suitable as a disease control.

The largest metabolic study on acetylation in PD found a higher proportion of slow acetylators among patients than controls, but this difference was not significant.<sup>31</sup> None of the metabolic studies analysed familial and sporadic PD separately.<sup>31–33</sup> Our data also show a higher proportion of slow acetylators among sporadic PD patients than among either of the control groups. The trend analysis supported the hypothesis of an involvement of N-acetyltransferase 2 in sporadic cases with a highly significant linear relation from normal controls and Huntington's disease to sporadic and familial PD. This trend could be explained in two ways: first, a subgroup of apparently sporadic PD patients are familial cases, but a positive family history could not be obtained. Information on family history is not always complete for samples obtained from a brain bank and the slow acetylator frequency in the sporadic PD group may be due only to contamination of this group with unrecognised familial cases. Second, analysis for trend suggested that the N-acetyltransferase 2 slow acetylator genotype predisposes familial and sporadic patients to develop PD, but this effect is stronger in familial cases.

The results for the Huntington's disease and normal control groups are similar to the PCR-based data from a previous UK study, with a frequency of 44% slow acetylators.<sup>34</sup> Other studies have found a higher proportion of the slow acetylator genotype among white patients, but included patients with other diseases as well as normal controls, or analysed the frequency of the slow acetylator genotype in the general population rather than only controls aged 60 years or older. If fast acetylation conferred some protection against the development of PD, exclusion of controls with any damage to the nigrostriatal system in this trial might have contributed to the low frequency of slow acetylators in the normal control group.

The DNA sequence variants that occur with the two common mutant alleles *M1* and *M2* have been functionally characterised: *M1* causes a decrease of the N-acetyltransferase 2 protein in the liver by defective translation, whereas *M2* produces an unstable enzyme.<sup>35</sup> There was no consistent rise in the proportion of one particular mutant allele in the familial PD group, which could indicate that the detected association between the slow acetylator genotype and familial PD is not because of linkage disequilibrium between one of the alleles and

another gene in the immediate vicinity. Rather, the association implies the direct involvement of N-acetyltransferase 2, on the assumption that both alleles are inherited independently. Slow acetylators are more susceptible to low-level environmental exposure to carcinogens.<sup>36</sup> Similarly, slow acetylators might also be more susceptible to low exposure to neurotoxins, but this hypothesis needs to be investigated in metabolic studies.

We found no significant differences between the patient and control groups for any of the other analysed polymorphisms. However, the power of our study to detect an effect of these polymorphisms was low because of their rarity and the number of patients studied. The similar frequency of all polymorphisms other than N-acetyltransferase 2 in the two PD groups and the normal control group makes it unlikely that the detected differences in N-acetyltransferase 2 frequency are due to differences in genetic background of the familial PD group and the normal controls or Huntington's disease patients.

Further studies are needed to clarify the biological importance of these data and the possible involvement of N-acetyltransferase 2 in apparently sporadic PD.

#### Contributors

O Bandmann designed the study and did the experimental work, J Vaughan identified most of the patients with familial PD, P Holmans undertook the statistical analysis, C D Marsden and N W Wood jointly supervised the project.

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# Sequencing of the $\alpha$ -synuclein gene in a large series of cases of familial Parkinson's disease fails to reveal any further mutations

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**A mutation in exon 4 of the human  $\alpha$ -synuclein gene was reported recently in four families with autosomal dominant Parkinson's disease (PD). In order to examine whether mutations in this exon or elsewhere in the gene are common in familial PD, all seven exons of the  $\alpha$ -synuclein gene were amplified by PCR from index cases of 30 European and American Caucasian kindreds affected with autosomal dominant PD. Each product was sequenced directly and examined for mutations in the open reading frame. No mutations were found in any of the samples examined. We conclude that the A53T change described in the  $\alpha$ -synuclein gene is a rare cause of PD or may even be a rare variant. Mutations in the regulatory or intronic regions of the gene were not excluded by this study.**

## INTRODUCTION

A recent report described an American-Italian kindred (Contursi kindred) with autosomal dominant inheritance of a levodopa-responsive parkinsonian syndrome. Affected patients exhibited the core triad of tremor, rigidity and bradykinesia characteristic of classical Parkinson's disease (PD) as well as pathological evidence of Lewy bodies. Genomic analysis of this family ultimately led to mapping of a locus to chromosome 4q21–23, designated PD-1 (1). These authors have since identified a G→A transition at position 209 in exon 4 of the  $\alpha$ -synuclein gene causing an alanine to threonine substitution at position 53 [Ala53Thr (2)] and proposed this as the causative mutation necessary for the development of the parkinsonian phenotype in the kindred. Support for this hypothesis was given by the finding of the same mutation in affected members

of three apparently unrelated Greek families. However, shortly before identification of the  $\alpha$ -synuclein gene mutation, polymorphic markers spanning the locus PD-1 were examined in 13 European multigenerational PD families with an autosomal dominant pattern of inheritance, and showed no linkage in 11 out of the 13 families (3). In two small families, positive lod scores were obtained, indicating the possibility of linkage.

Following identification of the Ala53Thr mutation in the  $\alpha$ -synuclein gene, we sequenced all seven exons of  $\alpha$ -synuclein in 30 index cases of familial PD. Some of these families were found previously to be unlinked to the PD-1 locus (3). Sequencing of the  $\alpha$ -synuclein gene would allow us to confirm or refute the linkage data obtained and, more importantly, to estimate the numerical importance of the  $\alpha$ -synuclein gene in autosomal dominant PD.

## RESULTS AND DISCUSSION

Polymerase chain reaction (PCR) products were generated from genomic DNA from 30 index cases of familial PD using a panel of primer pairs flanking each exon of the  $\alpha$ -synuclein gene. Each of these products were of a size consistent with published data (2). These were then sequenced directly in order to screen for the presence of polymorphisms and/or mutations, but no coding base pair differences were found relative to the published sequence in any of the 30 families.

The recent description of a mutation in the  $\alpha$ -synuclein gene in four families with autosomal dominant PD may aid in the understanding of the genetic basis of familial PD. The mutation, if it is indeed pathogenic, is a rare cause of PD (4,5). While these authors reported the same amino acid change in the Contursi kindred and in three apparently unrelated Greek kindreds, it remains a possibility that they may share a common origin as the two locations formerly were connected by a trade route. Thus, in the absence of haplotype data, a founder effect cannot be excluded.

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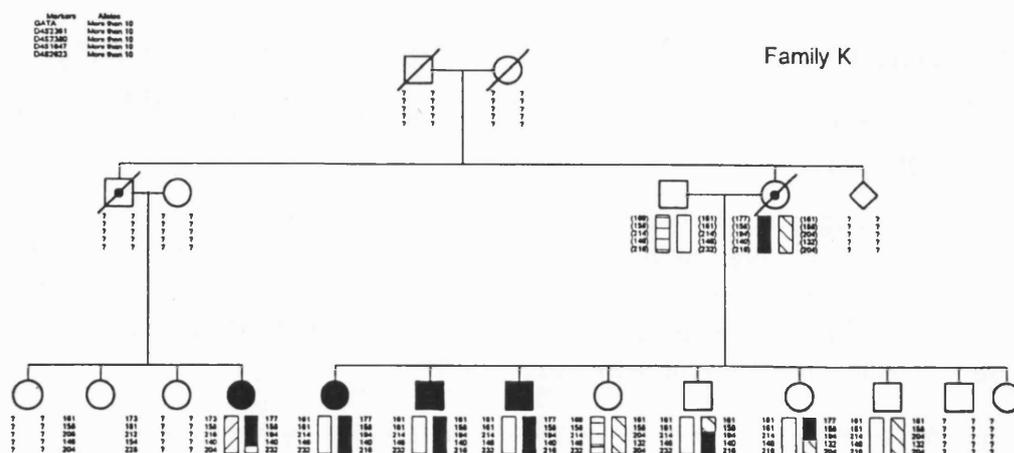


Figure 1. Haplotype data obtained from polymorphic markers spanning the PD-1 locus on chromosome 4q21-q23 in family K.

Table 1. Clinical characteristics of 16 of the families

Family	No. of affecteds	No. of affecteds examined	Region of origin	Mean age at onset	Atypical features	Reference
A	12	4	German-Canadian	51 (35-60)	Amyotrophy/dementia	(9,10)
B	8	4	Danish-American	62 (51-82)	Dementia (some)	(9,10)
C	11	4	German-American	60 (55-66)	None	(9,10)
D	18	5	English-American	63 (48-78)	None	(10,11)
G	11	4	German-American	56 (48-74)	Dementia (some)	(12)
IT-027	9	4	Italian	55 (46-67)	None	unpublished
IT-1	6	4	Italian	54 (36-89)	None	(13)
IT-0	3	2	Italian	56 (28-74)	None	(14)
K	4	4	German	56 (45-63)	None	unpublished
L	6	2	English	60 (52-66)	None	unpublished
M	3	3	English	45 (30-55)	None	unpublished
N	16	8	English	53 (42-70)	None	unpublished
O	4	4	English	48 (38-55)	None	unpublished
P	7	3	English	64 (58-70)	None	unpublished
Q	3	3	Welsh	63 (59-65)	None	unpublished
R	8	6	English	64 (42-75)	None	unpublished

All affected members exhibited at least two out of four of the cardinal parkinsonian signs (akinesia, resting tremor, rigidity, postural instability), and improvement on l-Dopa therapy.

To date, no patients with sporadic PD have been found to carry the Ala53Thr  $\alpha$ -synuclein mutation (2). In addition, the amino acid at position 53 is not conserved between mammalian species, in contrast with neighbouring residues. For instance, in rats, a threonine at position 53 is the native sequence. To test whether the alanine/threonine substitution at this position in the Contursi kindred was a neutral, perhaps ancestral, variant we also sequenced exons 3-5 of African green monkey  $\alpha$ -synuclein cDNA and found that monkey and man have an identical sequence (unpublished data). Therefore, the Contursi Ala53Thr mutation does not reflect the ancestral human gene.

The possibility that the  $\alpha$ -synuclein gene is in linkage disequilibrium with the true causative gene defect should also be considered. In support of this, we previously reported a lod score of 1.5 at the PD-1 locus for family K, close to the theoretical maximum for this small family (3), yet in the present study no

mutations were identified in the exons of  $\alpha$ -synuclein. This does not exclude other candidates in the region as responsible for the parkinsonian syndrome in this kindred. The pedigree shown in Figure 1 gives haplotype data obtained for markers spanning the PD-1 locus in family K.

Histopathological studies of brainstem and cortex from patients with sporadic PD and dementia with Lewy bodies have shown that Lewy bodies are strongly immunoreactive for  $\alpha$ -synuclein (6). However, as Lewy bodies are composed of many different proteins, the presence of  $\alpha$ -synuclein in these structures does not necessarily implicate it in their formation.

If mutations in the  $\alpha$ -synuclein gene rarely cause PD, an understanding of its biochemical function and interactions may suggest logical candidate genes for investigation, as mutations in heterogeneous gene products in a biochemical pathway may ultimately lead to a similar parkinsonian phenotype. However,

until further novel mutations in the  $\alpha$ -synuclein gene are found, the assignment of the  $\alpha$ -synuclein gene as the PD-1 locus must be regarded as provisional rather than certain.

**Table 2.** PCR primers for the exonic amplification of  $\alpha$ -synuclein

NACP exon	Primers 5'/3', forward and reverse	Product size (bp)
Exons 1 and 2	GAGAAGGAGGAGGACTAGGAGG CGGCGTTCTCCAGGATTC	499
Exon 3	GTCTCACACTTTGGAGGGTTTC CACCTACCTACACATACCTCTGACTC	395
Exon 4	GCTAATCAGCAAITTAAGGCTAG GATATGTTCTTAGATGCTCAG	215
Exon 5	CGATGGCTAGTGGAAAGTGG CGATGGCTAGTGGAAAGTGG	325
Exon 6	CGGAGGCATTGTGGAGTTTATG CCACGTAATGAGCATGTAGAGAGC	373
Exon 7	GACTGGGCACATTGGAAGTCTGAG GCTGTGAGTGTGATGCGTAATTG	189

## MATERIALS AND METHODS

### Patients

Thirty index cases from Caucasian families exhibiting apparent autosomal dominant inheritance were examined. In these families, at least two affected individuals in each family were personally examined in order to ensure fulfilment of the diagnostic criteria of idiopathic PD, in accordance with a similar study design (7). Post-mortem information was available from members of two kindreds, and this confirmed the presence of Lewy bodies in a typical distribution. Three members of family K, which previously had given a maximum multipoint lod score of 1.5 at locus PD-1, were included in the study (3). Clinical characteristics of 16 kindreds are shown (Table 1). Index cases of seven American Caucasian families were ascertained by The Mayo Clinic (onset ages of the affecteds sequenced were 69, 75, 53, 72, 67 and 45 years), and seven further families are also referenced for information (5).

### Laboratory methods

Primers were designed to human genomic non-A $\beta$  component of Alzheimer's disease amyloid precursor protein, NACP/synuclein (sequences submitted to NCBI database; accession nos U46896–U46901). They were designed to amplify sequences flanking each exon at ~50–100 bp proximal and distal to the coding sequence. The exact exon–intron organisation of the human  $\alpha$ -synuclein gene is not yet known. Primers were designed using Gene Runner 3.05, Hastings Software, Inc. Exons 1 and 2 (untranslated) were amplified together as one product. The 5' intron sequence flanking exon 7 is not known. Thus intron 6–7 was PCR amplified using exon 6F and exon 7R primers and Expand Long Template PCR system (Boehringer Mannheim) (for primers, see Table 2). A 2.8 kb PCR fragment was excised from an agarose gel, TA cloned (Invitrogen) and sequenced. Exon 4 primers used were as published (2). PCR conditions were denaturation at 94°C (3 min), followed by 35 cycles of 94°C (20 s), 55°C (30 s), 72°C (45 s), with a final extension at 72°C (10 min). PCR products were purified using QIAquick columns prior to sequencing using

dRhodamine terminators on an ABI377. Sequence chromatograms were analysed using PolyPhredPhrap (8).

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# The $\alpha$ -Synuclein Ala53Thr Mutation Is Not a Common Cause of Familial Parkinson's Disease: A Study of 230 European Cases

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We report the results of a screen of 230 European familial index cases of Parkinson's disease for the recently described Ala53Thr mutation in the  $\alpha$ -synuclein gene in an autosomal dominant Parkinson's disease kindred. No mutations were found from this broad white population, and we therefore conclude that although of great interest, this mutation is a very rare cause of familial Parkinson's disease.

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The  $\alpha$ -synuclein Ala53Thr mutation is not a  
common cause of familial Parkinson's disease:  
a study of 230 European cases.  
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Parkinson's disease (PD) is a common progressive neurodegenerative disorder, with a prevalence in the United Kingdom of 164 per 100,000.<sup>1</sup> Its clinical characteristics include resting tremor, bradykinesia, and rigidity. The Lewy body, an intracytoplasmic inclusion, is considered to be the pathological hallmark of the disease, although it is not entirely specific to PD. To date, the etiology of PD remains obscure. Four lines of evidence have been proposed in favor of a genetic predisposition to PD. First, analysis of 100 consecutive cases of PD revealed that 24% had a positive family history.<sup>2</sup> Second, the similar concordance rates previously found in twin studies<sup>3,4</sup> may reflect a clinical underestimation of the incidence of PD, as recent studies using positron emission tomography of clinically unaffected cotwins suggest that a number may have presymptomatic PD.<sup>5,6</sup> Third, an increasing number of families are being reported who show apparent autosomal dominant inheritance of PD.<sup>7,8</sup> A variety of association studies using polymorphisms of candidate genes have been performed. For instance, an excess of mutant cytochrome P450 alleles has been found in PD patients<sup>9,10</sup> and overrepresentation of the slow acetylator genotype for *N*-acetyltransferase 2 (NAT2) has been reported in familial PD,<sup>11</sup> although further association studies are needed to fully assess the significance of these findings.

The investigation of the Contursi kindred, showing autosomal dominant inheritance of parkinsonism with Lewy body pathology, ultimately led to mapping of a locus to chromosome 4q21-23, designated PD-1.<sup>12</sup> These authors have since identified a G  $\rightarrow$  A transition at position 209 in exon 4, causing an alanine-to-threonine substitution in the  $\alpha$ -synuclein gene<sup>13</sup> and proposed this as the causative mutation necessary for the development of this syndrome. Polymorphic markers closely linked to PD-1 have previously been examined in 13 European multigenerational families with autosomal dominant PD.<sup>14</sup> In two small families, slightly positive linkage was found (LOD score, 1.5; but due to limited information, it was impossible to confirm true linkage to PD-1). In 11 of 13 families, no linkage was found, confirming that familial PD exhibits genetic heterogeneity. We report the results of a screen of 230 European familial index cases for the Ala53Thr  $\alpha$ -synuclein mutation to assess its significance in a wider population of cases of familial PD.

## Materials and Methods

### Patients

Only white European patients were included in this study. Two hundred thirty index cases were analyzed (Table) from families recruited as part of a sibling pair (153 independent pairs), and multiplex family and two-generational PD study (77 independent families). In the sibling pair study, there were at least 2 individuals in each family, both personally examined.

fulfilling the diagnostic criteria of idiopathic PD according to a similar study design initially described by Maraganore and colleagues.<sup>15</sup> Twenty-five index cases from the multiplex and two-generational PD study were also examined who did not completely fulfill these criteria (including little or unknown response to levodopa, severe postural hypotension, and relative paucity of tremor), and they were therefore classified as atypical. All index cases were identified by a neurological specialist in their country. Postmortem information was available from members of three kindreds and confirmed the presence of Lewy bodies in a typical distribution.

#### Mutation Analysis

DNA was extracted from blood by using standard techniques. Polymerase chain reaction (PCR) was performed by using 75 ng of genomic DNA per reaction, 10 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 0.5 U of Amplitaq Gold DNA polymerase (Perkin-Elmer) in a final volume of 20 L. For mutation analysis, genomic DNA was amplified by using a Hybaid thermal cycler as follows: 95°C for 11 minutes, followed by 35 cycles of 94°C for 30 seconds, 48°C for 30 seconds, and 72°C for 30 seconds.

The G209A mutation results in an alanine-to-threonine substitution at position 53 of the amino acid sequence, creating a novel *Tsp451* restriction site. Restriction digestion of PCR products was performed with *Tsp451* at 65°C according to the manufacturer's protocol (New England Biolabs, Beverly, MA) and the digested PCR products were separated by electrophoresis on a 3.2% agarose gel, stained with ethidium bromide, and visualized by using ultraviolet light. DNA from a member of the kindred described,<sup>12</sup> carrying the Ala53Thr mutation, was used as the positive control.

To ensure the correct fragment was being amplified, PCR products from 3 index cases were directly sequenced with the Perkin-Elmer dye terminator cycle sequencing kit on an ABI 373 fluorescent sequencer. In addition, to further confirm that the amplicons were correct, 10 more PCR products were digested with three different enzymes, *Hinf*I, *Alu*I, and *Dde*I, at 37°C, and the restriction maps obtained were compatible with the known sequence.<sup>13</sup>

#### Results

A specific of 216 bp was detected in all samples tested and the sequence was identical to the published data.<sup>13</sup> Digestion of the positive control with *Tsp451* generated products of 88, 128, and 216 bp due to the novel *Tsp451* restriction site created by the Ala53Thr mutation in one of the alleles (Fig). However, no mutations

of this product were detected in any of the 230 cases screened after *Tsp451* digestion.

#### Discussion

Assessing the significance of the Ala53Thr mutation in the  $\alpha$ -synuclein gene to the etiology of PD must include an examination of a wide number of families, particularly those of a similar phenotype. It has been acknowledged that the phenotype of the Contursi kindred has atypical features, notably the rather aggressive course (an average of 9 years between onset and death) and a young age at onset.<sup>12</sup> Most cases in our study had a classic PD phenotype, but we have also included some with phenotypes very similar to that of the Italian kindred. The present study therefore excluded the Ala53Thr mutation in  $\alpha$ -synuclein in a wide range of index cases of atypical and typical familial PD.

The description of a mutation in the  $\alpha$ -synuclein gene is of great interest because it is the first genetic defect described in a family presenting with a parkinsonian syndrome with Lewy bodies post mortem. A strong case has been proposed for the pathogenic role of the Ala53Thr mutation in the  $\alpha$ -synuclein gene. With one exception, the Ala53Thr mutation segregated with the syndrome of parkinsonism in the Contursi kindred. The lone exception was an asymptomatic woman with mild rigidity on examination. This mutation was also found in affected individuals in three smaller Greek families. It was absent from 314 control chromosomes from the two populations, so it is not thought to be just a rare polymorphism.<sup>13</sup> The mutant protein is assumed to be expressed and it has been proposed that it may cause disruption of the  $\alpha$  helix, resulting in the extension of  $\beta$ -sheet structure. This may then lead to increased self-aggregation of protein, resulting in amyloid-type structures.<sup>13</sup>

Recent evidence has shown that brainstem and cortical Lewy bodies are strongly immunoreactive for  $\alpha$ -synuclein in sporadic PD and dementia with Lewy bodies.<sup>16</sup> However, to date, no patients with sporadic PD have been found to carry the Ala53Thr  $\alpha$ -synuclein mutation.<sup>13</sup> Thus, although direct mutations of  $\alpha$ -synuclein may be involved in the formation of Lewy bodies,  $\alpha$ -synuclein expression and function could also be modified by, as yet, undefined mutations

Table. Demographic and Clinical Characteristics of Index Cases of Parkinson's Disease

Origin	No. of Cases	Mean Age (yr) of Onset	Age Range (yr)	SD	Sex Ratio M/F
France	96	55.7	27-87	13.6	53/43
Germany	30	57.4	35-78	11.5	18/12
UK	69	56.5	31-71	11.14	36/33
Italy	35	51.3	34-73	11.3	18/17

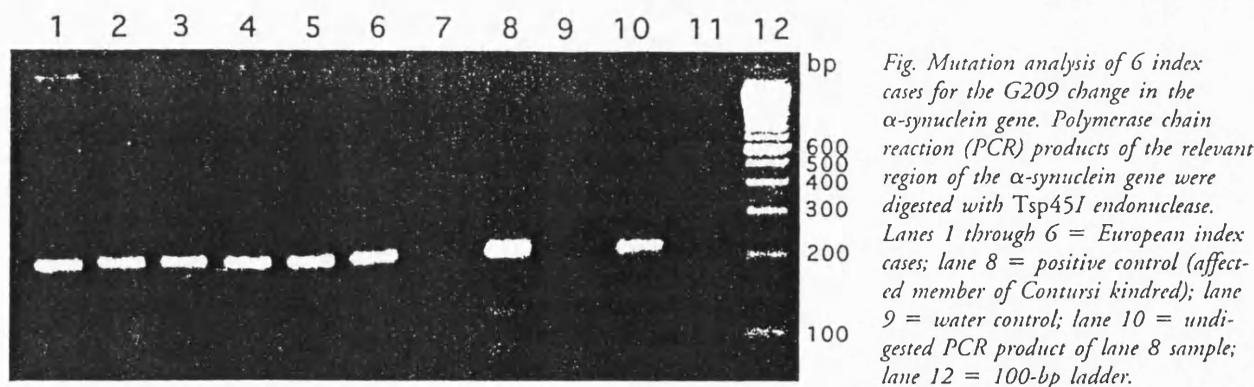


Fig. Mutation analysis of 6 index cases for the G209 change in the  $\alpha$ -synuclein gene. Polymerase chain reaction (PCR) products of the relevant region of the  $\alpha$ -synuclein gene were digested with Tsp45I endonuclease. Lanes 1 through 6 = European index cases; lane 8 = positive control (affected member of Contursi kindred); lane 9 = water control; lane 10 = undigested PCR product of lane 8 sample; lane 12 = 100-bp ladder.

in other genes. The link between  $\alpha$ -synuclein staining of Lewy bodies in sporadic cases of PD and the proven Ala53Thr mutation in a small number of familial cases needs further evaluation.

The Ala53Thr transition in exon 4 of  $\alpha$ -synuclein was the only mutation screened for in this study, and the possibility exists that these subjects have other mutations in this gene. To investigate this, all seven exons of the  $\alpha$ -synuclein gene in index cases from 30 European and American white kindreds affected with autosomal dominant PD were amplified by PCR, sequenced, and examined for mutations in the open reading frame. Fourteen index cases were taken from families screened in this study. No mutations were found in any of the samples examined, although mutations in the regulatory or intronic regions of the gene were not excluded.<sup>17</sup>

Until a direct relationship between mutant  $\alpha$ -synuclein expression, the presence of Lewy bodies, and the syndrome of parkinsonism has been shown, a cautious interpretation is still required. The possibility that the  $\alpha$ -synuclein gene is in linkage disequilibrium with the true causative gene defect should also be considered. Rats have a threonine at the same position in their homologue of the human  $\alpha$ -synuclein gene, yet there are no reports of the presence of Lewy bodies in the brains of rats.<sup>18</sup> Finally, despite the fact that the mutation was found in three apparently unrelated Greek kindreds, in the absence of haplotype data there remains the possibility of a founder effect.

We therefore conclude that the Ala53Thr mutation found in the  $\alpha$ -synuclein gene is a very rare cause of familial PD. Although the evidence that  $\alpha$ -synuclein is a gene involved in PD is now strong, more biochemical studies are needed to define its functional role in healthy and parkinsonian brains. Complete mapping of the genes responsible for putative genetic susceptibility in a large cohort of familial PD patients will help identify more precisely the causes of PD and may ultimately lead to more effective treatments in the future.

## Appendix

The European Consortium on Genetic Susceptibility in Parkinson's Disease (GSPD) comprises N. W. Wood and J. R. Vaughan (UK); A. Brice, A. Durr, J. Tassin, M. Martinez, J. Feingold, and Y. Agid (France); T. Gasser and B. Bereznaï (Germany); M. Breteler, S. Harhangi, and B. Oostra (The Netherlands); V. Bonifati, E. Fabrizio, G. Meo, G. De Michele, G. Volpe, and G. Campanella (Italy). In addition, we would like to thank the following: S. Medjbeur, M. Vidailhet, A.-M. Bonnet, M. Borg, E. Broussolle, A. D'Este, F. Durif, G. Fènelon, J.-R. Fève, P. Pollak, O. Rascol, F. Tison, C. Tranchant, J.-M. Warter, M. Vérin, and F. Viallet (The French Parkinson's Disease Genetics Study Group).

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## In Vivo Differentiation of Astrocytic Brain Tumors and Isolated Demyelinating Lesions of the Type Seen in Multiple Sclerosis Using $^1\text{H}$ Magnetic Resonance Spectroscopic Imaging

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Jack P. Antel, MD,\* and Douglas L. Arnold, MD\*

We used computer pattern recognition of proton magnetic resonance spectroscopic image data to differentiate between brain tumors and large, isolated, demyelinating lesions of the type seen in multiple sclerosis. Leave-one-out linear discriminant analyses correctly classified resonance profiles from five acute demyelinating lesions, 20 low-grade astrocytomas, 22 anaplastic astrocytomas, and 24 glioblastomas. Classification of nonacute lesions will require further development, as the metabolic profiles of demyelinating lesions evolve over time.

De Stefano N, Caramanos Z, Preul MC, Francis G, Antel JP, Arnold DL. In vivo differentiation of astrocytic brain tumors and isolated demyelinating lesions of the type seen in multiple sclerosis using  $^1\text{H}$  magnetic resonance spectroscopic imaging. *Ann Neurol* 1998;44:273-278

Increased sensitivity of conventional magnetic resonance imaging (MRI) in detecting multiple sites of demyelination in the central nervous system has made it easier to distinguish demyelinating disease (DD) from brain tumors. Nevertheless, differential diagnosis between malignant gliomas and large, demyelinating brain lesions may be impossible based solely on clinical and neuroradiological grounds. Although abnormalities in cerebrospinal fluid can be seen in patients with DD, these abnormalities are not omnipresent in, or specific to, this condition.<sup>1</sup> Thus, in the presence of a large

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## ARTICLE

# A wide variety of mutations in the *parkin* gene are responsible for autosomal recessive parkinsonism in Europe

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Autosomal recessive juvenile parkinsonism (AR-JP, PARK2; OMIM 602544), one of the monogenic forms of Parkinson's disease (PD), was initially described in Japan. It is characterized by early onset (before age 40), marked response to levodopa treatment and levodopa-induced dyskinesias. The gene responsible for AR-JP was recently identified and designated *parkin*. We have analysed the 12 coding exons of the *parkin* gene in 35 mostly European families with early onset autosomal recessive parkinsonism. In one family, a homozygous deletion of exon 4 could be demonstrated. By direct sequencing of the exons in the index patients of the remaining 34 families, eight previously undescribed point mutations (homozygous or heterozygous) were detected in eight families that included 20 patients. The mutations segregated with the disease in the families and were not detected on 110–166 control chromosomes. Four mutations caused truncation of the parkin protein. Three were frameshifts (202–203delAG, 255delA and 321–322insGT) and one a nonsense mutation (Trp453Stop). The other four were missense mutations (Lys161Asn, Arg256Cys, Arg275Trp and Thr415Asn) that probably affect amino acids that are important for the function of the parkin protein, since they result in the same phenotype as truncating mutations or homozygous exon deletions. Mean age at onset was  $38 \pm 12$  years, but onset up to age

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58 was observed. Mutations in the *parkin* gene are therefore not invariably associated with early onset parkinsonism. In many patients, the phenotype is indistinguishable from that of idiopathic PD. This study has shown that a wide variety of different mutations in the *parkin* gene are a common cause of autosomal recessive parkinsonism in Europe and that different types of point mutations seem to be more frequently responsible for the disease phenotype than are deletions.

## INTRODUCTION

Parkinson's disease (PD) is a frequent neurodegenerative disorder with a prevalence close to 2% after age 65 (1). The major signs of the disease are tremor, rigidity and bradykinesia associated with a good response to treatment with levodopa. The disorder is caused by a massive loss of dopaminergic neurons in the pars compacta of the substantia nigra. The aetiology of the disease is still unknown, but the existence of genetic susceptibility factors is strongly suspected (2). Several familial forms of PD with autosomal dominant transmission have been reported. Mutations in the  $\alpha$ -synuclein gene on chromosome 4q21-q23 were described in several PD families with early onset and rapid disease progression (3,4). However,  $\alpha$ -synuclein is a minor locus, found only in a subset of families with dominant transmission (5-7). A second susceptibility locus was localized on chromosome 2p13 in German families with autosomal dominant PD (8). Autosomal recessive juvenile parkinsonism (AR-JP, PARK2; OMIM 602544) was first described in Japan (9,10). AR-JP patients show the typical signs of PD, but they are associated with: (i) early onset, typically before the age of 40; (ii) dystonia at onset; (iii) diurnal fluctuations; (iv) slow disease progression; and (v) early and severe levodopa-induced dyskinesias. In a few cases, neuropathological examination has shown a massive loss of dopaminergic neurons in the pars compacta of the substantia nigra, but the absence of Lewy bodies, the histopathological hallmark of PD (9,11,12). Recently, the AR-JP locus, designated PARK2, was mapped to chromosome 6q25.2-27 in consanguineous Japanese families (13). Subsequently, linkage analyses, our own (14) and others' (15), have demonstrated the existence of non-Japanese PARK2 families in Europe, the USA and the Middle East. Homozygous deletions of one or more microsatellite markers in three AR-JP families greatly reduced the initial 17 cM candidate interval (14-16). Very recently, Kitada *et al.* identified a novel gene, designated *parkin*, in which homozygous deletions of either exon 4 or exons 3-7 were detected in four Japanese families with AR-JP (17). The *parkin* gene has an estimated genomic size of 500 kb and consists of 12 coding exons with an open reading frame of 1395 bp. The corresponding protein, parkin, composed of 465 amino acids, shows moderate homology to ubiquitin at the N-terminus and contains a RING-finger motif at the C-terminus (17). Subsequently, homozygous deletions of exon 3 were found in two European families and of exons 8-9 in one Algerian family (18). To date, two point mutations in the *parkin* gene have been reported in two Turkish AR-JP families (19).

In order to determine the frequency and the diversity of mutations in the *parkin* gene as a cause for the AR-JP phenotype in Europe, we amplified the 12 coding exons of the *parkin* gene in 35 families with autosomal recessive early onset parkinsonism and sequenced the exons in the patients that did not show homozygous exon deletions.

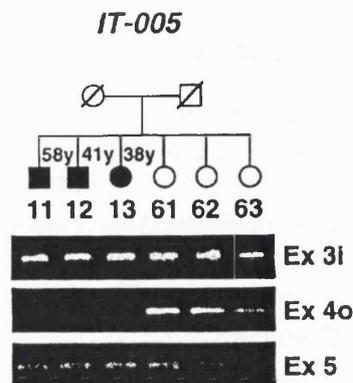


Figure 1. Homozygous exon 4 deletion in the patients of the Italian family IT-005. Bands (PCR products of the indicated exons) correspond to the numbered individuals. Black squares (men) and circles (women) represent affected individuals. Age at onset (in years) is given above the patient's symbol. Slashed symbols indicate deceased individuals. Ex, exon; i, inner; o, outer.

## RESULTS

### A new family with an exon 4 deletion

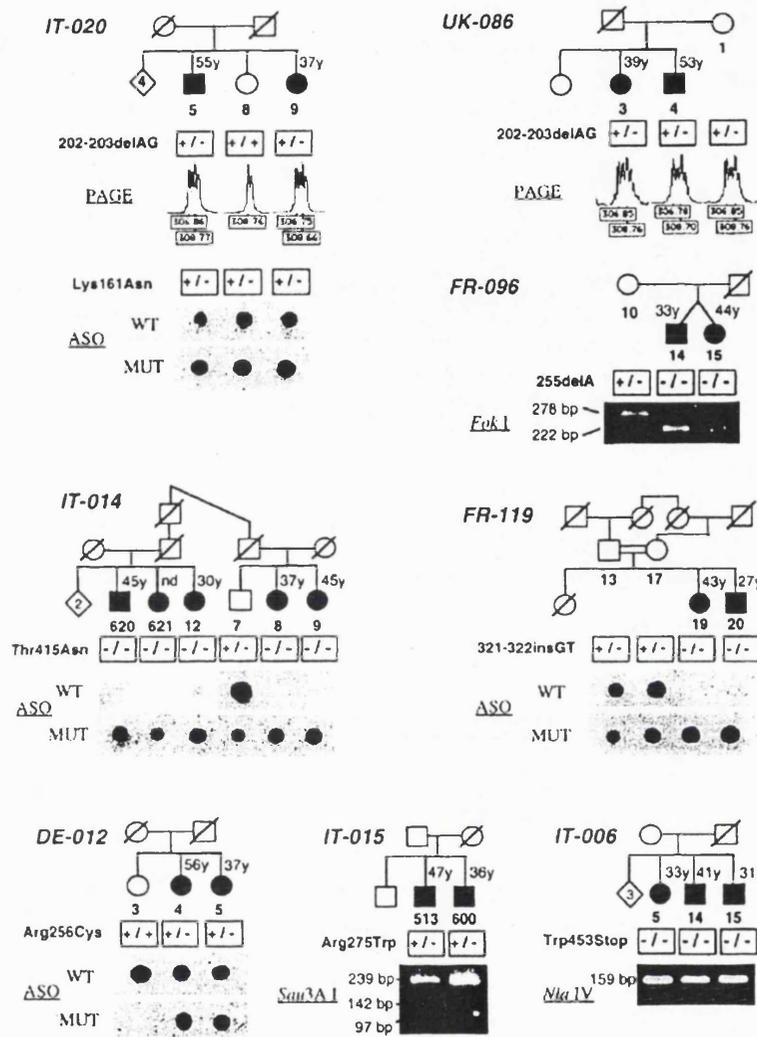
A homozygous deletion of exon 4 was detected in all three patients of an Italian family (IT-005), previously shown to be homozygous for four markers at the disease locus (14; Fig. 1).

### Point mutations in the *parkin* gene

In the index patients from the 34 families that did not show homozygous exon deletions, sequence analysis of all coding exons, including the exon-intron boundaries, revealed 11 sequence variations in exons and three in introns (Table 1). Eight of the exonic variations co-segregated with the disorder in the families (Fig. 2) and were not detected in 110-166 control chromosomes (Table 1). They are therefore most probably causative mutations (Fig. 3).

Four mutations resulted in truncated proteins: 202-203delAG in exon 2 of families IT-020 and UK-086 (from the UK); 255delA in exon 2 of family FR-096 (from France); 321-322insGT in exon 3 of family FR-119; 1459G→A (Trp453Stop) in exon 12 of family IT-006. These mutations were homozygous in patients, except for the 202-203delAG mutation in families IT-020 and UK-086.

Four missense mutations were observed. In family IT-020, a heterozygous 584A→T transversion (Lys161Asn) in exon 4 was detected in addition to the 202-203delAG mutation described above. Segregation analysis showed that the two mutations were located on different alleles and that all patients were compound heterozygotes (Fig. 2). Two other heterozygous, non-conservative amino acid changes were observed in exon 7 of patients from families DE-012 (from Germany) and IT-015: 867C→T (Arg256Cys) and 924C→T (Arg275Trp), respectively. Finally, a



**Figure 2.** Families with point mutations in the *parkin* gene. Complete co-segregation of the mutations with the disease is shown. Black squares (men) and circles (women) represent affected individuals with age at onset (in years) indicated above the patient's symbol. Slashed symbols indicate deceased individuals. The numbers of unaffected sibs not analysed are given in diamonds. For each mutation the genotype of the family member is indicated (+/+, homozygous wild-type; +/-, heterozygous for the mutation; -/-, homozygous for the mutation). Under each genotype, the detection data are shown. PAGE: electrophoregrams with allele sizes in bp; ASO: autoradiograms of mutated and wild-type allele; restriction assay: PCR products after digestion with the appropriate restriction enzyme. Fragment lengths in bp are indicated. Mut, mutated; nd, age at onset not determined, as patient is unaware of her clinical signs.

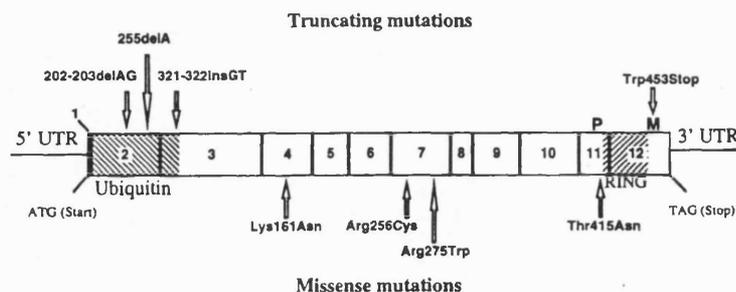
homozygous 1345C→A transversion in exon 11 of all patients from family IT-014 resulted in a conservative (Thr415Asn) amino acid change.

In three of the families with heterozygous mutations (UK-086, DE-012 and IT-015), no complementary heterozygous mutation could be detected by sequencing.

### Polymorphisms

Three of the 11 exonic sequence variations were detected in control chromosomes and were thus classified as polymorphisms (Table 1). They resulted in two conservative and one non-conservative amino acid change. A 601G→A transition (Ser167Asn) was heterozygous

in only one of the two affected members of family FR-730 and on 1% of the control chromosomes (2/166). In addition, it was found in all sibs, including an unaffected, of family IT-020, in which the disease is already explained by the compound heterozygosity of two causative mutations (see above). A 1239G→C transversion (Val380Leu) in exon 10 was either heterozygous or homozygous in 11 families (IT-014, IT-020, IT-058, FR-017, FR-029, FR-038, FR-096, FR-431, UK-006, UK-086 and DE-022). It did not segregate with the disease and was found on 16% of control chromosomes. A 1281G→A transition (causing the non-conservative Asp394Asn change) in exon 11 was heterozygous in the index patient of family UK-046 and heterozygous or homozygous on 7% of control chromosomes.



**Figure 3.** Point mutations in the *parkin* gene. The coding sequence of the gene with its 12 exons is represented as a bar. Exons are numbered 1–12. The eight causative point mutations are positioned according to their effect on the protein (truncating versus missense). The ubiquitin-like domain and the RING-finger motif are hatched. The mutations Thr415Asn and Trp453Stop the putative phosphorylation sites (P) and the *N*-myristoylation site (M), respectively, are indicated. UTR, untranslated region.

**Table 1.** Variants of the *parkin* gene

	Nucleotide change <sup>a</sup>	Amino acid change/ stop codon position <sup>a</sup>	Mutation type	Detection technique	Expected fragment length (bp)	No. of controls	Variant frequency (%)
Exonic							
Ex2	202–203delAG	Gln34/Stop37	Frameshift	PAGE	WT: 308 V: 306	55	0
Ex2	255delA	Asn52/Stop81	Frameshift	FokI Creation of site	WT: 278+30 V: 222+57+30	83	0
Ex3	321–322insGT	Trp74/Stop81	Frameshift	ASO		83	0
Ex4	584A→T	Lys161Asn	Missense	ASO		83	0
Ex4	601G→A	Ser167Asn	Missense	AlwNI Loss of site	WT: 164+97 V: 261	83	1
Ex7	867C→T	Arg256Cys	Missense	ASO		83	0
Ex7	924C→T	Arg275Trp	Missense	Sau3AI Loss of site	WT: 142+97 V: 239	83	0
Ex10	1239G→C	Val380Leu	Missense	ASO		45	16
Ex11	1281G→A	Asp394Asn	Missense	TaqI Loss of site	WT: 221+84 V: 303	90	7
Ex11	1345C→A	Thr415Asn	Missense	ASO		83	0
Ex12	1459G→A	Trp453Stop	Nonsense	NlaIV Loss of site	WT: 142+17+35+61 V: 159+35+61	83	0
Intronic							
Intron2	IVS2+25T→C			BstNI Creation of site	WT: 308 V: 264+44	45	19
Intron3	IVS3–20C→T			MnII Loss of site	WT: 201+60 V: 261	45	10
Intron7	IVS7–35A→G			MaeIII Creation of site	WT: 206 V: 159+47	36	27

The nucleotide numbers are given according to the cDNA sequence published in the DNA Data Bank of Japan (DDBJ accession no. AB009973).

Ex, exon; PAGE, polyacrylamide gel electrophoresis; ASO, allele-specific oligonucleotide.

<sup>a</sup>Causative mutations are given in italic.

Variants in introns 2, 3 and 7 (IVS2+25T→C, IVS3–20C→T, IVS7–35A→G) were located at a distance from the splice sites. These polymorphisms were detected on control chromosomes at a frequency of 19, 10 and 27%, respectively (Table 1). Finally, a homozygous 768C→T transition (Pro223Ser) was found in all individuals sequenced, suggesting that 768C was an error in the initial cDNA sequence (17).

#### Functional domains of the parkin protein

According to the PROSITE program, the conservative amino acid change Thr415Asn is located within the consensus sequence of putative phosphorylation sites of cAMP- and cGMP-dependent protein kinases (KKTT) and protein kinase C (TTK). The truncating nonsense mutation Trp453Stop is located within the putative *N*-myristoylation site (GCEWNR).

**Table 2.** Clinical characteristics of 31 patients from 12 families according to the type of mutation in the *parkin* gene

Clinical characteristics	Mutation type			
	Deletion	Truncating	Missense	Total
No. of families (patients)	4 (11)	4 (9)	4 (11)	12 (31)
Mean age at onset in years (range)	33.9 ± 16.3 (7–58)	38.2 ± 8.0 (27–53)	42.5 ± 8.5 (30–56)	38.1 ± 12.1 (7–58)
Mean disease duration in years (range)	14.8 ± 6.5 (3–26)	16.3 ± 9.4 (4–29)	16.3 ± 8.9 (0.5–31)	15.8 ± 8.0 (0.5–31)
Hoehn and Yahr score	3.4 ± 1.1	2.2 ± 0.9	2.8 ± 0.9	2.8 ± 1.0
Bradykinesia	11/11	8/9	11/11	97%
Rigidity	10/11	9/9	11/11	97%
Tremor	6/11	8/9	7/11	68%
Dystonia at onset	6/11	1/7	0/5	30%
Good response to levodopa ( <i>de novo</i> cases)	10/10 (1)	9/9	9/9 (2)	100%
Dyskinesia	6/10	4/9	8/9	71%
Fluctuations	5/10	3/6	ND	50%
Brisk reflexes lower limbs	4/11	0/6	3/4	33%

### Genotype–phenotype correlations

Point mutations were present in eight families, that included 20 patients (Table 2). The patients of family IT-020 with a truncating mutation on one allele and a missense mutation on the other allele were classified in the missense mutation group, assuming that missense mutations may be less deleterious if they result in only partial loss of function of the parkin protein, thereby determining the severity of the illness. Accordingly, the patients of the families for whom only one heterozygous mutation could be detected were included among the truncating (UK-086) or missense mutations (DE-012 and IT-015). The 11 patients with homozygous exon deletions of the *parkin* gene [three previously published families (18) and family IT-005], the 11 patients with missense mutations (four families) and the nine patients with truncating mutations (four families) showed an overall phenotype of early onset parkinsonism (Table 2). Mean age at onset was 38 ± 12, ranging from 7 to 58. Parkinsonian rigidity (97%) and bradykinesia (97%) were almost always present, whereas tremor was observed in only 68%. These features were associated with dystonia at onset in 30% and with brisk reflexes in the lower limbs without a Babinski sign in 33%. After a mean disease duration of 16 ± 8 years, dyskinesias and fluctuations were present in 71 and 50%, respectively. The effect of the mutations on age at onset, severity or frequency of signs did not differ significantly among the patients carrying different types of mutations.

### DISCUSSION

The initial report of Kitada *et al.* describing homozygous deletions of exon 4 or exons 3–7 in four Japanese families (17), our own observation of homozygous deletions of exon 3 or exons 8–9 in two European and one Algerian families (18) and the newly identified exon 4 deletion in an Italian family show that a variety of deletions in the *parkin* gene can cause autosomal recessive parkinsonism, with age at onset as late as 58, as observed in the Italian family IT-005 (Fig. 1). However, it was suspected that other types of mutations (e.g. point mutations) might be a more common cause of the disease (18). The only point mutations reported to date were two homozygous single

base pair substitutions in two consanguineous Turkish families, which resulted in a Thr240Arg missense mutation in exon 6 and an Ala311Stop nonsense mutation in exon 8. Until now, however, it could not be determined whether point mutations in the *parkin* gene account for a significant proportion of autosomal recessive early onset parkinsonism.

Taken together, our three families with previously reported exon deletions (18) and the nine families with mutations detected in the present study demonstrate that mutations in the *parkin* gene are the cause of the disease in ~30% of the families with autosomal recessive parkinsonism analysed (12/38), including 11 of 35 from Europe. Point mutations were detected in two-thirds of our families (eight of 12 families) and thus seem to be more frequent than homozygous exon deletions (four of 12 families).

The fact that a second mutation could not be detected in families DE-012, IT-015 and UK-086 (which were heterozygous for only one mutation) suggests the presence of a complementary mutation that could not be detected by the techniques used in this study. This second mutation might be located in regions of the gene that were not sequenced (e.g. the promoter region) or might be present as a heterozygous exon deletion that cannot be analysed by simple PCR amplification. However, in order to estimate the frequency of compound heterozygotes resulting from the combination of a point mutation and an exon deletion or of two different exon deletions, we compared the frequencies of the homozygous exon deletions ( $n = 4$ ) and the homozygous point mutations ( $n = 4$ ) in eight families with known or suspected consanguinity. The frequency for the two types of homozygous mutations was 50% each in this sample. The frequency of compound heterozygotes for each of the two combinations (deletion + point mutation or deletion + deletion) could therefore reach up to 25%. However, the small sample size precludes accurate estimation of these frequencies.

Only one of the point mutations, the 202–203delAG frameshift, was found in two families of different origins (Italy and UK), suggesting that the mutation occurred independently. Although the patients from these families shared two frequent alleles for the PARK2 markers D6S411 (allele 2 = 59%) and D6S1550 (allele 2 = 68%), the alleles for two other tightly linked markers, D6S305

and D6S1579, were different (data not shown), indicating that a recent common founder effect is unlikely.

All the point mutations are novel and show that a wide variety of different mutations in the *parkin* gene can account for the disease. The pathogenic role of the point mutations was shown by: (i) co-segregation of the mutations with the disorder in the families; and (ii) their absence in a large number of control chromosomes (110–166 depending on the mutation). All the point mutations identified are likely to have major functional consequences. The four truncating mutations (202–203delAG, 255delA, 321–322insGT and Trp453Stop), which were detected in the homozygous state in three of five families, clearly cause a loss of function of the parkin protein, compatible with a recessive mode of inheritance. Three of the missense mutations result in non-conservative amino acid changes (Lys161Asn, Arg256Cys and Arg275Trp). In family IT-020, the 202–203delAG frameshift mutation on one allele, which results in a loss of function, is associated with an apparently deleterious Lys161Asn missense mutation on the other allele. The conservative Thr415Asn amino acid change, which involves neutral amino acids with different polar side chains, homozygous in all five patients of family IT-014, is located within two consensus sequences of different protein kinases (cAMP- and cGMP-dependent protein kinases and protein kinase C) and might alter post-translational modifications. In addition, codon 415 is located very close to the first cysteine of the RING-finger motif (codon 418) and could affect its structure.

The mutations, which had different effects on the parkin protein, were distributed over six exons, excluding a mutational hotspot. It is interesting to note that, in contrast to the reported Gln311Stop mutation (19), the truncating point mutations identified in this study correspond to the N- and C-terminal regions of the parkin protein (ubiquitin-like and RING-finger motif, respectively), whereas the missense mutations affect the more central regions of the protein, as does the Thr240Arg mutation (19). The previously described ubiquitin-like and RING-finger domains were not affected by the missense mutations. The C-terminal region appears to be extremely important since a homozygous Trp453Stop nonsense mutation, which only removes the last 12 C-terminal residues, causes the disease, perhaps by altering an *N*-myristoylation site between codons 450 and 455, which prevents a necessary post-translational modification of the protein. The absence of significant clinical differences in this study among the patients with different types of mutations suggests that the modified amino acids are all of functional importance and that their replacement, like the truncating mutations, causes loss of function. The location of the mutations may therefore point to unknown functional domains.

The phenotype of the families with point mutations in the *parkin* gene and those with exon deletions covered a clinical spectrum that was broader than in the Japanese families originally described (17,19) and is often very close to that of idiopathic PD (Table 2). Mean age at onset (38 years) was that of early onset PD. In 13 patients, however, onset occurred after age 40. Dystonia or brisk reflexes were less frequent than previously reported (10,19). Overall, the phenotypes of patients classified according to the effect of the mutations on the parkin protein were similar, although earlier ages at onset (7–18 years) were observed in the Algerian family with deletion of exons 8 and 9 (18). Similar early onset was also observed in the Japanese family with deletion of exons 3–7 (17) as well as in the patient with the Gln311Stop

mutation (19), raising the question of the functional consequences of exon deletions and truncating events in specific regions of the parkin protein, especially as onset within our patients group with truncating mutations was later (between 27 and 53 years).

Finally, three exonic variants (Ser167Asn, Val380Leu and Asp394Asn) were classified as polymorphisms, since they were detected at different frequencies (between 1 and 16%) on control chromosomes. In some families, they did not segregate with the disease. Furthermore, they were found in a family in which the disease was explained by other mutations (the polymorphism Ser167Asn in family IT-020) or were homozygous in healthy controls (Val380Leu and Asp394Asn). They are therefore insufficient to cause the disease by themselves. They might, however, alter the function of the parkin protein and contribute to the pathogenesis of idiopathic PD. Association studies will help to clarify this question.

In conclusion, this study shows that point mutations in the *parkin* gene are a common cause of autosomal recessive parkinsonism in Europe and seem to be more frequent than the exon deletions so far described. Furthermore, the mutations in the *parkin* gene are associated with a wide range of ages at onset as well as of clinical signs and can result in familial parkinsonism clinically indistinguishable from idiopathic PD. The mutations detected are diverse in their effects on the parkin protein as well as in their localization within the gene. The diversity of the mutations and the absence of a mutational hotspot will complicate molecular diagnosis, but the evident importance of the amino acids affected will help to analyse the function, still unknown, of the parkin protein.

## MATERIALS AND METHODS

### Patients and statistical analysis

Thirty-eight families were selected according to the following criteria: (i) presence of parkinsonism with good response to levodopa ( $\geq 30\%$  improvement) in at least two siblings and absence of excluding criteria such as extensor plantar reflexes, ophthalmoplegia, early (after 2 years of disease evolution) dementia or autonomic failure; (ii) onset at  $\leq 45$  years in at least one of the siblings; and (iii) inheritance compatible with autosomal recessive transmission (several patients in a single generation with or without known consanguinity). The families originated from France ( $n = 12$ ), Italy ( $n = 10$ ), Germany ( $n = 7$ ), Great Britain ( $n = 4$ ), Algeria ( $n = 1$ ), Morocco ( $n = 1$ ), The Netherlands ( $n = 1$ ), Portugal ( $n = 1$ ) and Vietnam ( $n = 1$ ). Four families from Algeria, France, Italy and Portugal were excluded from sequence analysis because they were found to carry homozygous deletions of either exon 3 or exons 8–9 (18) or exon 4 (family IT-005).

The patients and unaffected relatives were examined by us in one of the movement disorder clinics of the European Consortium on Genetic Susceptibility in Parkinson's Disease or the French Parkinson's Disease Genetics Study Group, according to a standardized protocol using the same inclusion and exclusion criteria. All patients were videotaped and the clinical data were centralized. The Kruskal–Wallis test was used for comparisons of means and the  $\chi^2$  test for comparison of frequencies (Yates corrected). Blood samples were taken with informed consent from the patients and their first degree relatives.

### PCR amplification and sequence analysis

The 12 coding exons of the *parkin* gene from 35 index patients were amplified from genomic DNA by PCR as described by Kitada *et al.* (17). In family IT-005, an additional pair of exonic primers was used for exon 3 (Ex3iFor, 5'-AATTGTGAC-CTGGATCAGC-3'; Ex3iRev, 5'-CTGGACTTCCAGCTGG-TGGTGAG-3'). For exons 4 and 7 only the intronic primer pairs were used. The same primers were used for the sequencing of the PCR products of the 12 exons on both strands using the Big Dye Terminator Cycle Sequencing Ready Reaction DNA Sequencing kit (Applied Biosystems, Foster City, CA), according to the manufacturer's recommendations, on an ABI 377 automated sequencer with the Sequence Analysis v.3.0 (Applied Biosystems) software.

Each time a nucleotide variant was identified in an index case, its co-segregation with the disease was established in the patient's family and its frequency was determined in 36–90 controls (with or without other neurological diseases). Three different techniques were used to detect mutations in the PCR products corresponding to the different exons: (i) allele-specific oligonucleotides (ASOs) to detect the wild-type and the variant sequence; (ii) digestion with the appropriate restriction endonuclease; or (iii) PAGE (Table 1). Nucleotide positions were determined according to the cDNA sequence published in the DNA Data Bank of Japan (DDBJ accession no. AB009973).

### ASOs

Ten microlitres of the PCR product were blotted onto Hybond N<sup>+</sup> nylon membranes (Amersham Life Science, Little Chalfont, UK) after denaturation at 95°C for 5 min and fixed in a microwave oven at 600 W for 2 min. For exon 3, the primers Ex3iFor and Ex3iRev were used. The oligonucleotides (Table 3), labeled with [<sup>32</sup>P]CTP using the Terminal Transferase kit (Boehringer Mannheim, Mannheim, Germany), were hybridized to the membrane at 44°C overnight in a buffer consisting of 5× SSPE, 5× Denhardt's and 0.1% SDS. The membranes were washed twice for 30 min in 2× SSC at 59°C and exposed to MP film (Amersham) for 3–6 h.

Table 3. Oligonucleotides used for the ASO technique

Location	Nucleotide change	Oligonucleotide sequence
Ex3	321–322insGT	WT: 5'-TGCAGAGACC--GTGGAGAAAA-3'
		V: 5'-GCAGAGACCGTGTGGAGAAAA-3'
Ex4	584A→T	WT: 5'-GCCGGGAAAΔCTCAGGGTA-3'
		V: 5'-GCCGGGAAAICTCAGGGTA-3'
Ex7	867C→T	WT: 5'-TGCAACTCCCGCCACGTGA-3'
		V: 5'-TGCAACTCCIGCCACGTGA-3'
Ex10	1239G→C	WT: 5'-TGCAAGTCCGCTATTTGAAG-3'
		V: 5'-TGCAAGTCCCTATTTGAAG-3'
Ex11	1345C→A	WT: 5'-AGAAAACAACAAGCCCTG-3'
		V: 5'-AGAAAACAACAAGCCCTG-3'

The nucleotide change in the oligonucleotides is underlined. WT, wild-type; V, variant.

### Restriction assay

Fifteen microlitres of PCR product were digested with restriction enzymes according to the manufacturer's recommendations. The expected fragment lengths are given in Table 1.

### Polyacrylamide gel electrophoresis

A 5'-fluorescent (Hex) forward primer (17) was used to amplify exon 2. The presence of the 202–203delAG variant, resulting in a shorter PCR product (306 versus 308 bp), was established by fragment size measurement using an ABI 377 automated sequencer with the Genescan v.2.0.2 and Genotyper v.1.1.1 software (Applied Biosystems).

### Determination of additional functional domains in the parkin protein

The Internet web site PROSITE (<http://expasy.hcuge.ch/cgi-bin/scanprosite?1>) was used to determine additional functional domains of the parkin protein.

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### ABBREVIATIONS

AR-JP, autosomal recessive juvenile parkinsonism; ASO, allele-specific oligonucleotide; DE, German family; FR, French family; IT, Italian family; PD, Parkinson's disease; UK, British family; V, variant; WT, wild-type.

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A coding substitution (I93M) in the ubiquitin carboxy-terminal L1 (UCH-L1) gene has recently been identified in a German family with Parkinson's disease. We have sequenced the entire coding region of the gene in 11 families who have a pattern of disease consistent with autosomal dominant inheritance. We found a polymorphism (S18Y) in exon 3, two polymorphisms in the 5' non-coding region, upstream of the transcription start, and an insertion/deletion polymorphism in intron 4. The S18Y allele is present on ~20% of chromosomes in a Caucasian population. These changes are, therefore, unlikely to be pathogenic. We conclude that the I93M variant must either be a rare cause of disease or a harmless substitution whose occurrence in the family reflects a chance co-occurrence. *NeuroReport* 10:427-429 © 1999 Lippincott Williams & Wilkins.

## Low frequency of pathogenic mutations in the ubiquitin carboxy-terminal hydrolase gene in familial Parkinson's disease

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**Key words:** Genetics; Parkinson's disease; Ubiquitin carboxy-terminal hydrolase L1

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### Introduction

Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) represents 1-2% of total soluble brain protein and is thought to hydrolyze polymeric ubiquitin and ubiquitin conjugates to monomeric ubiquitin [1]. Its occurrence in Lewy bodies and its function in the proteasome pathway make it a compelling candidate gene in Parkinson's disease (PD) [2]. Leroy and colleagues recently reported an exon 4 UCH-L1 I93M substitution in an affected sib-pair, with loss of enzyme activity and suggested inheritance of this allele is associated with disease [3]. With this background, we have chosen to sequence the entire UCH-L1 gene in 11 families with two or more affected individuals with a history of PD in either parent (Table 1). None of these families has a mutation within the  $\alpha$ -synuclein gene [4,5]. Disease segregation within each family is compatible with autosomal dominant transmission.

### Patients and Methods

*Autosomal dominant PD (see Table 1):* Family 3, of English/Irish descent, has 21 individuals in five generations who have been affected with parkinsonism. Age of onset has ranged from the third to the fifth decade, and is typically that of rigidity of a limb and/or resting tremor. Levodopa responsiveness is usually excellent for ~ 5 years, after which severe 'on/off' fluctuations, dyskinesias, autonomic dysfunction and marked gait freezing may develop. Cognitive dysfunction is seen variably. Lewy bodies in the substantia nigra have been confirmed on autopsy.

Family 4 is of African-American origin, and affected individuals include a brother and sister who developed parkinsonism in their 50s. The disease usually begins with resting tremor in an upper extremity with subsequent insidious progression of parkinsonism, including increasing bradykinesia and gait difficulty. Although levodopa

Table 1. Familial Parkinson's disease.

Family	No. affected	Origin	Age of onset (range)	Polymorphisms	
				Exonic	Intronic
3	21	English and Irish	24-47		Ex2+6(Y)/+19(M)*
4	3	African-American	40-65		-16(Y)/-24(R) <sup>†</sup>
9465	4	English	19-71	S18Y	+T
1447	5	Irish	31-41		
12189	6	Welsh	50-63		
12028	16	English	42-70		
12349	4	English	49-52		-16(Y)/-24(R) <sup>†</sup>
11693	6	English	47-60		
2001	3	English	47-52		
MI	7	English and Dutch	45-72		Ex2+6(Y) <sup>†</sup>
MN	5	Dutch and Norwegian	60-75		

\*Intronic polymorphisms between exons 2 and 3.

<sup>†</sup>Polymorphisms 5' to the translational start site.

+T, additional thymidine in polypyrimidine tract of intron 4

therapy is initially beneficial, dyskinesias develop after about 7 years.

Members of family 9465 present with a levodopa-responsive parkinsonian syndrome between the ages of 19 and 71 with age of onset lower in successive generations. All individuals presented with unilateral resting tremor of an upper extremity, with subsequent rapid progression to bilateral involvement. Subjects in later generations developed early dysarthria and upper limb rigidity in addition to the resting tremor.

In family 1447, a kindred of Irish descent, five of 10 siblings developed levodopa-responsive parkinsonian syndrome in the third and fourth decade. The index case presented with a stiff left leg at 32 years. He then became rapidly bradykinetic and suffered falls secondary to poor balance within 5 years of onset. F-dopa PET studies on the proband revealed profound impairment of F-dopa re-uptake in the striatum.

Family 12189, of Welsh descent, has a total of six affected members who developed a parkinsonian syndrome responsive to levodopa between 50 and 63 years of age. At onset the common presenting symptom was a unilateral resting tremor. Progression of the syndrome was characterized by increasing gait difficulty and later falls.

Sixteen of 32 members of the English family 12028 developed a levodopa-responsive parkinsonian syndrome. Age of onset ranged from 42 to 70 years. The usual mode of presentation is that of asymmetrical resting tremor. Dyskinesias typically develop after 5-7 years. Severely affected members of the kindred have on/off phenomena and gait disturbances.

Family 12349 is of English origin and contains four affected members. The presenting feature is

micrographia with subsequent development of a complete levodopa-responsive parkinsonian syndrome.

Family 11693 is also English, and six members are affected. The parkinsonian syndrome is levodopa responsive. Progression is characterized by on/off phenomena and severe gait difficulties.

Family 2001 is of English descent. Three of four additional siblings suffer from essential tremor. A total of three affected individuals in this family have a levodopa-responsive parkinsonian syndrome.

The MI kindred is of English/Dutch descent, and affected members have a typical age of onset between 50 and 70 years. Manifestations in five affected members include resting tremor, rigidity and postural instability. Initially carbidopa/levodopa had excellent results, but wearing off and dyskinesias develop around 8 years later. Three individuals separately suffer from essential tremor.

The MN kindred, of Dutch/Norwegian descent, typically has an age of onset ranging from 60 to 75 years. A total of five individuals in two generations have been diagnosed with PD. The findings include resting tremor, micrographia and, later, gait difficulties. Levodopa responsiveness persists throughout the typical course of 15 years, with some wearing off phenomenon and dyskinesias. Dementia is not seen.

In addition to these well characterized subjects in families where the disease occurrence is compatible with autosomal dominant transmission, 10 sporadic Parkinson's disease patients (PD) (age at onset 45-75), three multiple system atrophy patients (MSA) (age at onset 60-71) and one patient with parkinsonism and peripheral neuropathy (age at onset 60) were sampled and sequenced for any UCH-1 mutations. The origin of these patients included Austria-

Table 2. UCH-L1 primer sequences and product size.

Exon	Primer sequences	Product size (bp)
1 and 2	CTCCCCCTGCACAGGCTCA; GTCCTGCCAGCAGCCGGAA	353
3	CTCCTCCAGGCTCGGGT; CTCAGCTGGGGAGCGGC	307
4	TGCACTCTCATTCTGAGATG; GATGGGTGGCCTCTAAACCC	228
5 and 6	AGGTTGCTCAGCATGTTTCA; CAGTAGAAACACAGATGGC	364
7	CTTAGTGGGCTTAGAATAGG; AAGTGCCCTCATGAGAATAC	372
8	ATCTAGGCTAGGTAAGCACG; TGCTGGCTATACTGGAAGAG	271
9	GGAGCCTTCCCTATGTGAC; ACCACATCCAAGGTCTTAAC	465

Hungary, Canada, China, England, Germany, Italy, Lithuania, the Philippines, Poland, Puerto Rico, Russia, Spain and Sweden. The S18Y polymorphism was found in four of the PD cases and one of those with MSA. The Ex2 +6/+19 intronic polymorphism was found in two with PD. An additional thymidine in the polypyrimidine tract of intron 4 was found in one patient with PD and one with MSA.

**Sequencing and mutation screening:** UCH-L1 exons were amplified from genomic DNA with primers designed to flank intronic sequence (Table 2). PCRs contained a final concentration of 0.8  $\mu$ M of each primer, 1 unit Taq polymerase and 5  $\mu$ l of Q solution (Qiagen). Amplification was performed using a 65–55°C touchdown protocol over 35 cycles with a final extension at 72°C for 10 min. PCR products were purified using Qiagen PCR kit and their concentration estimated on an agarose gel. DNA (100 ng) for each exon was sequenced on both strands using the Rhodamine dye terminator cycle sequencing kit (Perkin Elmer) and relevant PCR primers. Sequencing was performed on an ABI377 automated sequencer. Heterozygote base calls were made using Factura software (Perkin Elmer) and sequence alignment was performed with Sequence Navigator (Perkin Elmer). A C54A transversion in codon 18 which creates an *Rsa*I site and results in a serine to tryptophan substitution was identified. Screening for this variant in a series of control individuals, without neurological illness, revealed an allele frequency of 23% in Hardy-Weinberg equilibrium.

## Results and Discussion

We failed to find any mutations in the UCH-L1 gene in 11 families with PD in which it was sequenced, or in sporadic PD, MSA or parkinsonism with peripheral neuropathy. However, we have described one major coding polymorphism (S18Y) and several common, non-coding, promoter (-16(Y)/-24(R)) and intronic variants (Ex2+6(Y) and +19(M)). The frequency of the S18Y allele was 23%, estimated from 110 individuals without documented movement disorder.

There are two possible conclusions from our work: the first is that the I93M is indeed a rare cause of PD, as Leroy and colleagues have proposed [3]. The second is that the I93M mutation has no effect on the risk of developing PD, and its occurrence in two affected sibs is just an accidental occurrence. These two possibilities can only be discriminated by the identification of the same or other UCH-L1 polymorphisms that segregate with familial PD or through the observation of this polymorphism in unaffected individuals.

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## The Ile93Met mutation in the ubiquitin carboxy-terminal-hydrolase-L1 gene is not observed in European cases with familial Parkinson's disease<sup>☆</sup>

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### Abstract

Recently an Ile93Met mutation in the ubiquitin-carboxy-terminal-hydrolase-L1 gene (UCH-L1) has been described in a German family with Parkinson's disease (PD). The authors showed that this mutation is responsible for an impaired proteolytic activity of the UCH-L1 protein and may lead to an abnormal aggregation of proteins in the brain. In order to determine the importance of this or any other mutation in the coding region of the UCH-L1 gene in PD, we performed mutation analysis on Caucasian families with at least two affected sibs. We did not detect any mutations in the UCH-L1 gene, however, we cannot exclude mutations in the regulatory or intronic regions of the UCH-L1 gene since these regions were not sequenced. We conclude that the UCH-L1 gene is not a major gene responsible for familial PD. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Parkinson's disease; Ubiquitin-carboxy-terminal-hydrolase-L1; Genetics; Candidate gene; Family studies

Parkinson's disease (PD) is one of the most common neurodegenerative disorders. The prevalence of PD in Europe in subjects aged 55 years and older is 1.6% [2,3]. PD is characterized by a progressive neuronal degeneration, which mainly affects the dopaminergic neurons in the nigrostriatal system but also other regions of the brain [7]. The presence of intraneuronal inclusions, known as Lewy bodies in the pars compacta of the substantia nigra, is gener-

ally considered as the pathologic hallmark of PD, albeit not totally specific [7]. The major clinical signs of the disease include resting tremor, rigidity, bradykinesia and postural disturbances with a good response to treatment with levodopa. The cause(s) of PD are still largely unknown. In the vast majority, PD occurs in a sporadic form. However, in the last few years there is increasing evidence that some forms of familial PD are genetically determined [1,6,8,9,17,19]. At present, it is generally accepted that PD is a genetically heterogeneous disorder. It is known that mutations in the  $\alpha$ -synuclein gene are responsible for a small minority of autosomal dominant inherited PD [9,17], whereas mutations in Parkin are responsible for some cases of autosomal recessive inherited parkinsonism [1,8,19]. Recently, a missense mutation in the ubiquitin-carboxy-terminal-hydrolase-L1

<sup>☆</sup> The French Parkinson's Disease Genetics Study Group, the Italian Parkinson Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease also contributed to this work.

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**Table 1**  
Characteristics and origin of the individuals sequenced for the UCH-L1 gene

Origin	Number of families	Individuals sequenced	Mean age of onset years (SD)	Mean duration of illness years (SD)
French	4	4	57.0 (9.3)	12.8 (14.5)
German	1	1	51.0	18.0
Italian	4	4	56.5 (7.3)	7.5 (5.8)
Dutch	2	2	51.0 (12.7)	10.5 (6.4)

(UCH-L1) gene encoding for the UCH-L1 protein has been reported in relation to PD [11]. The authors showed that this Ile93Met mutation is responsible for an, in vitro, partial loss of the catalytic thiol protease activity of the UCH-L1 protein, which may lead to aberrations in the proteolytic pathway and aggregation of proteins [11]. This missense mutation was found in two affected sibs of a German family in which two other members also were affected with PD. It was suggested that the disease appeared to be autosomal dominant with incomplete penetrance [11]. The gene for human UCH-L1 has been mapped to chromosome 4p14 [5]. However, a large family with levodopa-responsive Lewy body parkinsonism, in which a chromosome 4p14 haplotype segregates with disease, has also been described. UCH-L1 was excluded as a candidate gene by both gene sequencing, for coding exons, and as a heterozygous coding variant (S18Y) in two affected individuals demonstrated expression of the gene from both chromosomes [6]. We present here the results of mutation analysis on the complete coding region of the UCH-L1 gene in families with PD, that were recruited as part of the study of the European Consortium on Genetic Susceptibility in Parkinson's disease [20]. All these families were previously tested for allele sharing on chromosome 4p (unpublished data).

In this study, we only included European Caucasian families with PD. In all families, at least two affected sibs in each family were present. PD was diagnosed using the rigorous criteria of idiopathic PD according to a similar study design as has been described by Maraganore et al. [15]. All patients gave their informed consent according to the Declaration of Helsinki. As part of the ongoing total

genome screen in families with PD, up to present, 96 affected families with PD were tested for allele sharing on chromosome 4p. The markers used to test for allele sharing on chromosome 4p are D4S230, D4S1609, D4S391, D4S2397 and D4S405, spanning 12.4cM telomeric to centromeric on the Genethon Map. Since the family described by Leroy et al. [11] showed an apparent autosomal dominant (AD) inheritance pattern, only those families, that showed an inheritance pattern compatible with AD transmission, were eligible for mutation analysis for the UCH-L1 gene. Mutation analysis was performed on the complete coding region of the UCH-L1 gene in one randomly selected sibling from those families in which two affected siblings shared a haplotype for all five markers. The characteristics and country of origin of the individuals tested for mutations in the UCH-L1 gene are listed in Table 1. DNA was extracted from blood using standard techniques. All coding exons of the UCH-L1 gene, including flanking intronic sequences, were amplified from genomic DNA using specific primers. The sequences of the primers are shown in Table 2. The PCR reactions and product sequencing were done similarly to the study of Lincoln et al. [12].

In total, 29 out of the 96 families showed an inheritance pattern compatible with autosomal dominant transmission. Of these 29 families, 11 affected siblings were sequenced for mutations in the UCH-L1 gene because they shared a haplotype for all five markers. The overall mean age at onset of PD was  $55.2 \pm 8.1$  years with a mean duration of illness of  $10.9 \pm 9.4$  years. All individuals were analyzed for mutations in any coding region of the UCH-L1 gene. First, we searched for the Ile93Met alteration at nucleotide position 277 as described previously [11]. We did not observe this mutation in the UCH-L1 gene in the 11 affected siblings that were sequenced. To further detect mutations in the UCH-L1 gene we systematically performed sequence analysis of coding exons 1-9. Subsequently we identified the C54A variant in exon 3 in one Dutch sibling and one Italian sibling. This creates a novel RsaI restriction site and is responsible for a serine to tyrosine substitution at codon 18 (S18Y) (Table 2). The allele frequency we found for this polymorphism was somewhat lower as compared with other studies [6,12]. This could easily be a chance finding since our sample size was relatively small. Alternatively the

**Table 2**  
Primer sequences and PCR product size of the UCH-L1 gene

Exon	Primer	Forward (5'-3')	Reverse (3'-5')	Product size (bp)
1 + 2	Ex 1 + 2	CTCCCCTGCACAGGCCTCA	GTCCCTGCCAGCAGCCGGAA	353
3	Ex 3	CTCCTCCCAGGCTCGGGT	CTCAAGCTGGGGAGCGGC	307
4	Ex 4	TGCACTCTCATTCTGAGATG	GATGGGTGGCCTCTAAACCC	228
5 + 6	Ex 5 + 6	AGGTTGCTCAGCATGTTCCAG	CAGTAGAAACACAGATGGC	364
7	Ex 7	CTTAGTGGGCTTAGAATAGG	AAGTGCCTCATGAGAATAC	372
8	Ex 8	ATCTAGGCTAGGTAAGCACG	TGCTGGCTATACTGGAAGAG	271
9	Ex 9	GGAGCCTTTCCTATGTGAC	ACCACATCCAAGGTCTTAAC	465

difference could be explained by the difference in genetic background with different allele frequencies.

The lack of the Ile93Met mutation or any other mutations in the coding region of the UCH-L1 gene in our study population suggests that a mutation in the UCH-L1 gene is not a major cause of familial PD in Europe. However, we cannot exclude mutations in the regulatory or intronic regions, as these regions were not sequenced. Since the prevalence of the Ile93Met mutation is currently unknown and no mutations were found in 500 control individuals of different ethnic background [11], it could be hypothesized that the mutation found in the German family may be unique in this family. This explanation is supported by the fact that yet no other mutations in the UCH-L1 gene were found in the present study, which included German families. Although our results and very recently another report [12] do not support the involvement of the UCH-L1 gene in the pathogenesis of PD, the malfunctioning of the ubiquitin-dependent proteolytic system is known to be involved in the formation of intraneuronal inclusion bodies [13,14,16]. Like  $\alpha$ -synuclein, UCH-L1 is a constituent of Lewy bodies. It is thought to play a role in the cleavage of polymeric ubiquitin into monomers and may as well hydrolyze C-terminal esters and amides of ubiquitin [10]. Lewy bodies are strongly ubiquitinated and immunoreactive with neuro-specific ubiquitin carboxy terminal hydrolase and some subunits of the 26S proteasome. The reports of mutations in the  $\alpha$ -synuclein gene [9,17] and the observation that  $\alpha$ -synuclein is a major component of Lewy bodies [18] has accelerated research in Lewy body formation and deposition. Alpha-synuclein antibodies have enabled novel, occult pathology to be described in both neurites in idiopathic PD and glia in multiple system atrophy (MSA) [4,18]. Recently, another gene-defect, initially described in Japan and associated with an autosomal recessive juvenile parkinsonism (AR-JP), has been mapped to chromosome 6q25.2-q27 [8]. Mutations in this gene, named Parkin, have now also been reported in European and Algerian families [1,19]. The similarity of the Parkin protein [8] to the ubiquitin protein family suggests that the ubiquitin-dependent proteolytic system may play an important role in the pathogenesis of PD, but the lack of Lewy bodies in AR-JP suggests that not only Lewy body formation but also other factors involved in neurodegeneration like oxidative stress and specific neurotoxins may be involved in the pathogenesis of PD. At present, it is generally accepted that a genetic contribution in the etiology to PD is of major importance and therefore research should be focussed on other yet unidentified susceptibility genes in PD.

We conclude that the UCH-L1 gene is not a major gene responsible for familial PD.

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## Mutations in the gene encoding human persyn are not associated with amyotrophic lateral sclerosis or familial Parkinson's disease

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### Abstract

The synucleins are a family of small proteins expressed in nervous tissue, which have been implicated in neurodegeneration. Using single strand conformation polymorphism analysis we screened for polymorphisms and mutations in the gene encoding human persyn, a recently discovered member of the synuclein family, in controls, patients with sporadic or familial amyotrophic lateral sclerosis (ALS) or familial Parkinson's disease (PD). Six polymorphisms in the genomic sequence of persyn were detected; A<sub>590</sub>C (5' untranslated region), G<sub>1943</sub>C (exon 3), G<sub>2049</sub>A (intron 3), T<sub>4502</sub>C (intron 3), T<sub>4552</sub>A (exon 4) and C<sub>5018</sub>T (3' untranslated region). However no associations with disease state were found in our sample group. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Human persyn gene; Polymorphisms; Amyotrophic lateral sclerosis; Familial Parkinson's disease

The synucleins are a family of small cytosolic proteins, abundant in nervous tissue, whose exact function has yet to be elucidated but which have been implicated in neurodegeneration.  $\alpha$ -Synuclein has been identified as the precursor of the non-A (component of amyloid (NACP) in Alzheimer's disease (AD) [21]. More recently two point mutations in the  $\alpha$ -synuclein gene have been identified in subjects with familial Parkinson's disease (PD) [11,16]. Further support for a role for  $\alpha$ -synuclein in neurodegeneration stems from the observation that both the Lewy bodies [2,18–20] and glial cytoplasmic inclusions [22] of PD and multiple systems atrophy (MSA), respectively, are immunoreactive for  $\alpha$ -synuclein.

Persyn (also known as BCSG-1 or gamma synuclein) is a recently identified member of the synuclein family which has been implicated as a marker of disease progression in breast cancer [9,15]. Persyn protein shows sequence homology with both  $\alpha$ - and  $\beta$ -synuclein, especially at the amino terminal, where the first 70 amino acids are approximately 75% identical. However it demonstrates an expression

pattern distinct from the other synucleins, being found predominantly in the motor and sensory neurons of the peripheral nervous system [4]. Expression has been shown to increase in the aging cerebral cortex and forebrain structures of mice [4]. At the cellular level persyn immunoreactivity is diffusely distributed throughout the cell body and axons of peripheral neurons [3] whilst  $\alpha$ - and  $\beta$ -synuclein are pre-synaptic proteins [7,8,14]. Over-expression of persyn in cultured sensory neurons results in a marked reduction in neurofilament (NF-H, NF-M and NF-L) protein staining, associated with a selective increase in neurofilament susceptibility to calcium dependent proteases [3]. This suggests that persyn plays a part in controlling the neurofilament network.

Amyotrophic lateral sclerosis (ALS) is a condition characterized by upper and lower motor neuron degeneration. A striking feature of the molecular pathology of ALS is accumulation of neurofilaments in the proximal axons (spheroids) and anterior horn cells of motor neurons and occasionally in the cortical pyramidal neurons [5,12].

The persyn gene is thus a strong candidate gene for ALS since mutations in the persyn gene may result in altered control of the neurofilament network (possibly through reduced susceptibility to calcium dependent proteases) lead-

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Table 1  
Persyn polymorphism allele frequencies<sup>a</sup>

Persyn polymorphism (genomic sequence Position)	Allele frequency
590	<sup>C</sup> (A = 0.46 C = 0.54) <sup>F</sup> (A = 0.52 C = 0.48) <sup>F</sup> (A = 0.56 C = 0.44) <sup>S</sup> (A = 0.52 C = 0.48)
1943	<sup>C</sup> (G = 0.25 C = 0.75) <sup>F</sup> (G = 0.22 C = 0.78) <sup>F</sup> (G = 0.27 C = 0.73) <sup>S</sup> (G = 0.22 C = 0.78)
2049	<sup>C</sup> (G = 0.98 A = 0.02) <sup>F</sup> (G = 1.0 A = 0) <sup>F</sup> (G = 1.0 A = 0) <sup>S</sup> (G = 1.0 A = 0)
4552	<sup>C</sup> (T = 0.25 A = 0.75) <sup>F</sup> (T = 0.22 A = 0.78) <sup>F</sup> (T = 0.25 A = 0.75) <sup>S</sup> (T = 0.22 A = 0.78)
5019	<sup>C</sup> (C = 0.96 T = 0.04) <sup>F</sup> (C = 0.90 T = 0.10) <sup>F</sup> (C = 0.96 T = 0.04) <sup>S</sup> (C = 0.95 T = 0.05)

<sup>a</sup> (n = 55); <sup>F</sup> familial PD cases (n = 25); <sup>F</sup> familial ALS cases (n = 26); <sup>S</sup> sporadic ALS cases (n = 66). Alleles = bases A, T, C and G. One familial ALS sample only was heterozygous for the T<sub>4502</sub>C polymorphism. In all cases comparison of allele frequencies in cases and controls by  $\chi^2$  yielded P-values >0.1.

ing to the neurofilamentous accumulations which may contribute to the neuronal damage in ALS [10]. The detection of the related  $\alpha$ -synuclein in Lewy bodies [2,18–20] and the observation that neurofilaments are also a component of the Lewy body [6], make persyn a candidate for PD. We therefore screened for polymorphisms or mutations in the human persyn gene in familial or sporadic ALS and familial PD.

We analyzed DNA from 26 index cases with familial ALS, 66 cases of sporadic ALS, 55 neurologically normal (genetically unrelated) controls and 25 index cases with familial Parkinson's disease (PD) subjects. Prior informed consent had been obtained in all cases. 'Familial' ALS was defined as one or more affected 1st degree relatives. All 25 PD cases were index cases from an affected sibling pair. All ALS cases used had been previously clinically classified by El Escorial criteria as 'definite' or 'probable'. Familial ALS cases had been previously shown not to have exonic mutations in the superoxide dismutase 1 (SOD-1) gene [17]. All familial PD cases had satisfied the UK brain bank clinical criteria for diagnosis of PD and showed no 'atypical features' and had been previously screened and were negative for the  $\alpha$ -synuclein Ala 53 Thr and Ala 30 Pro mutations. The mean ages of onset were 56.5 years (65.2% male, 34.8% female) for the 66 sporadic ALS cases, 52.8 years (65.4% male and 34.6% female) in 16/26 familial ALS cases where age of onset was known and 56.8 years (64% male and 36% female) for the 25 familial PD cases. The mean age of the 55 controls was 54.8 years (47% male, 43% female).

Genomic DNA was extracted from peripheral blood leucocytes by standard techniques using a commercially available Nucleon II kit. Five oligonucleotide primer pairs (designated 1–5) were designed to amplify fragments from persyn's five exons (DDBJ/EMBL/GenBank accession nos. AF017256 & AF037207) using an HGMP primer design program (Primer version 5.0). Primer design was such that the amplified products contained the corresponding coding region (Exons 1–5) and some exon/intron boundary or 5'/3' untranslated DNA. Primer pair 1 (amplification bases 567–729) forward primer TCATCGCGTCAATAG-

GAG, reverse primer TACCCACATACATGACCCC. Primer pair 2 (amplification bases 1544–1585) forward primer TTCTGTTGTGTTTGTCCCTGACC, reverse primer TGGGGCTTCTCACCTGAG. Primer pair 3 (amplification bases 1912–2039) forward primer ATAGTGGCCGAGAA-GACCAA, reverse primer TGGGGTGTGGAGTGGAGT. Primer pair 4 (amplification bases 4515–4586) forward primer ATTCTCCACATGGTCTCATGC, reverse primer GGGGAGCAGTGGCTCTGT. Primer pair 5 (amplification bases 4876–5169) forward primer GGGTGTGCAGGT-CATTCTCT, reverse primer CCCTCCTCAAGCTACACAGC. For each primer pair, 25 ng of genomic DNA was used as a template for amplification by polymerase chain reaction (PCR). A 25  $\mu$ l PCR reaction with a final concentration of 1 $\times$  PCR buffer, 1.5 mM Mg<sup>2+</sup>, 0.2 mM dNTPs, 0.5  $\mu$ M for each primer using Taq polymerase was performed. DNA was initially denatured for 2 min at 95°C and then subject to 30 cycles of: heating to 95°C for 1 min, annealing (at temperature specific to the primer pair used: 1 = 55°C, 2 = 57.5°C, 3 = 61°C, 4 = 57.5°C and 5 = 58.5°C) and extension at 72°C for 1 min. This was followed by a prolonged extension at 72°C for 10 min.

All PCR products were initially analyzed by single strand conformation polymorphism (SSCP) detection; 8  $\mu$ l of PCR product was mixed with an equal volume of formamide SSCP dye and denatured by heating at 95°C for 5 min, the mixture was then snap chilled on ice prior to loading all of the mix on to a 10% polyacrylamide gel. The running times varied according to the size of the PCR product. Selected samples showing different conformations on electrophoresis sequenced using an ABI 377 system. Where the identified polymorphism resulted in a change in the restriction site map for the amplified region, the relevant restriction endonuclease was used to confirm sample genotype. In the case of the polymorphism in the 5' untranslated region there was a change in the restriction site map but none of the enzymes were commercially available and therefore all subsequent genotyping was performed by SSCP. The following restriction endonucleases were utilized for polymorphism detection; Aki I (exon 3), Msp I (intron 3), Hph I (exon 4) and Bbv II (3' untranslated region). After digestion of the PCR

products with the appropriate restriction enzyme, fragment sizes were analyzed by polyacrylamide gel electrophoresis to allow for sample genotyping.

Allele frequencies were calculated for the different sample groups. For each polymorphism, case (familial ALS, sporadic ALS and familial PD groups) and control allele frequencies were compared using the chi squared  $\chi^2$  test.

Six polymorphisms in the human genomic persyn sequence were detected: A<sub>590</sub>C (5'untranslated region), G<sub>1943</sub>C (exon 3), G<sub>2049</sub>A (intron 3), T<sub>4502</sub>C (intron 3), T<sub>4552</sub>A (exon 4) and C<sub>5019</sub>T (3'untranslated region). The polymorphism T<sub>4502</sub>C (intron 3) was detected by SSCP (and subsequent sequencing) in one familial ALS case only. The nucleotide substitution at position 4552 results in an amino acid change, val110glu. Three of the polymorphisms described here have been previously reported, those in exons 3 and 4 by Ninkina et al. [15] in persyn cloned from a single human juvenile brain-stem and that in the 3'untranslated region in GenBank (Ac AF037207). The polymorphisms in exons 3 and 4 were found to be associated with each other in all but three cases, in which it is likely recombination events had occurred. The allele frequencies for each of the described polymorphisms in the ALS, PD and control sample groups are shown in Table 1. Comparison of allele frequencies between the cases and the controls by chi squared analysis revealed no significant differences.

Here we report the results of a study to screen for polymorphisms and mutations in the gene encoding human persyn, a synuclein, in two neurodegenerative diseases, ALS and familial PD. Persyn was chosen as a candidate because not only is it closely structurally related to  $\alpha$ -synuclein, which has been implicated in the pathogenesis of a number of neurodegenerative disorders (Lewy body diseases, AD and MSA) [2,18–20,22] but it is expressed in motor neurons and has been shown to affect the neurofilament network. The latter is of significance because neurofilamentous accumulations in the anterior horn cells and cortical motor neurons are one of the pathological hallmarks of ALS [5,12]. In addition rare mutations in the neurofilament heavy chain have been identified in apparently sporadic ALS and in one family with ALS [1], although the pathogenic significance of these mutations remains uncertain. Furthermore, both neurofilament [6] and  $\alpha$ -synuclein [2,18–20] antigens are detectable in Lewy bodies.

In our study six different polymorphisms in the genomic sequence of persyn were detected; A<sub>590</sub>C (5'untranslated region), G<sub>1943</sub>C (exon 3), G<sub>2049</sub>A (intron 3), T<sub>4502</sub>C (intron 3), T<sub>4552</sub>A (exon 4) and C<sub>5019</sub>T (3'untranslated region) of which three (C<sub>5019</sub>T, G<sub>1943</sub>C and T<sub>4552</sub>A) have been previously described [15], GenBank (Ac AF037207). However none of the polymorphisms we describe were found to associate with ALS or familial PD. The failure to find mutations in persyn in familial PD cases supports a study by Lincoln et al. [13]. Hence we conclude that persyn

gene mutations are not likely to play a major role in the pathogenesis of ALS or familial PD, although our results cannot exclude the possibility of a rare causative association between persyn gene mutations and familial ALS or PD.

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## Detailed Genotyping Demonstrates Association Between the Slow Acetylator Genotype for N-Acetyltransferase 2 (NAT2) and Familial Parkinson's Disease

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**Summary:** In a preliminary report we demonstrated an association between the slow acetylator genotype of N-acetyltransferase 2 (NAT2) and familial cases of Parkinson's disease (FPD). Using a considerably more precise NAT2 typing method, which detects all mutant NAT2 alleles with a frequency of >1% in the white population, we have now retyped all the original patients and control subjects to investigate the reliability of our initial findings. The slow acetylator genotype remained considerably more common among FPD (73%) than normal control subjects (NPC, 43%) or the disease (Huntington's disease [HD]) control group (52%) with an odds ratio

(OR) of 3.58 (95% confidence interval (CI): 1.96–6.56;  $p = 0.00003$ ) for FPD versus NPC and an OR of 2.50 (95% CI: 1.37–4.56,  $p = 0.003$ ) for FPD versus HD. Furthermore, the wild-type allele 4 conferred a protective effect with an OR of 0.39 (95% CI: 0.23–0.64;  $p = 0.0025$ ) for FPD versus NPC and an OR of 0.50 (95% CI: 0.30–0.85,  $p = 0.01$ ) for FPD versus HD. The results of this study support an association between the NAT2 slow acetylator genotype and FPD in our population. **Key Words:** Genetics—Familial Parkinson's disease—N-acetyltransferase 2—Detoxification.

Parkinson's disease (PD) is characterized by cell death of dopaminergic neurons in the substantia nigra. The cause for this cell death is unknown, but there is growing evidence for the contribution of genetic factors. Recently, the first genetic defect, a point mutation in the alpha-synuclein gene, has been identified in a large family with autosomal-dominantly inherited PD and linkage to a second locus has been described in other kinships.<sup>1,2</sup> However, large families with autosomal-dominantly inherited PD amenable to linkage analysis are extremely rare. It also remains to be proven that the pathogenesis of PD in these families is related to the pathogenesis in the more common sporadic cases of PD (SPD) or in those cases that have a limited number of affected family members. Recent epidemiologic studies have nevertheless identified a genetic predisposition to PD even in

such cases. A further established risk factor is exposure to environmental toxins.<sup>3–5</sup> An obvious, although still theoretical, overlap between these two pathogenic concepts is the model of a genetic predisposition to defective handling of endogenous or exogenous toxins.<sup>6</sup>

We have previously reported preliminary data suggesting a highly significant association between the slow acetylator genotype for N-acetyltransferase 2 (NAT2) and familial Parkinson's disease (FPD), the slow acetylator genotype being defined by the presence of two mutant alleles.<sup>7</sup> N-acetylation is involved in a wide variety of detoxification processes. The enzymatic activity of NAT2 is genetically determined and influences the detoxification rate of aromatic amines.

In our initial study, a wide range of sequence variants in six different detoxification enzymes (NAT2, CYP2D6, CYP2E1, glutathione transferase M1 and T1, NADPH-menadione reductase) were analyzed for possible association with PD.<sup>7</sup> Therefore, we first undertook limited NAT2 typing only using a previously described, relatively simple method as a "screening method."<sup>8</sup>

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Compared with more precise methods, the limited typing in our initial study leads to an overall underestimation of the NAT2 slow acetylator genotype.<sup>9</sup> In addition, it wrongly types different alleles as the same (see Table 1) and is not in accordance with the new international terminology of NAT2 alleles.<sup>10</sup>

Using a more precise typing method known to detect all mutant alleles with a frequency of >1% in the white population,<sup>9</sup> we have now retyped all patients and control subjects included in our first study to validate the previously reported association between the slow acetylator genotype and FPD. Confirmation of the initial findings would not only support the possible relevance of these findings, but also facilitate the replication of our study in other populations, because other groups could then rely on the "screening method" again for first assessment of the slow acetylator genotype frequency in their population.

In addition, the more detailed genotyping should allow a more precise assessment of the question as to whether the association detected is likely to be the result of linkage disequilibrium between a particular NAT2 allele and FPD or true association between FPD and the NAT2 slow acetylator genotype.

We also investigated separately whether the wild-type allele 4 has a protective effect against the development of PD. This would further support our hypothesis that the high proportion of NAT2 slow acetylators among FPD is the result of a greater susceptibility to neurotoxic substances in these slow acetylators.

**METHODS**

**Patients and Control Subjects**

Only European patients and control subjects were included in this study. Details have been described elsewhere.<sup>7</sup> In brief, brain tissue was ascertained from the UK Parkinson's Disease Society Brain Bank, Institute of Neurology, Queen Square, London and the brain bank of

the Institute of Psychiatry, Denmark Hill, London for the apparently sporadic PD group (n = 100) and the control group (n = 100). The histopathologic diagnosis of PD was made according to standard criteria, whereas the control brains had no pathologic changes. A clinical study provided the familial cases. One hundred families were identified in whom there were at least two living individuals fulfilling the diagnostic criteria of idiopathic PD following a similar study design initially described by Maraganore et al.<sup>11</sup> All cases with genetically proven Huntington's disease (HD, n = 100) had a pathologic trinucleotide repeat expansion with more than 40 repeats in the HD gene. The project had approval of the local ethics committee.

**Genotyping**

DNA was extracted from brain tissue (SPD and normal control subjects) and blood (FPD and HD) using standard techniques. When appropriate, PCR reactions were digested with the respective enzyme. All PCRs were started with an initial denaturing step of 94°C for 5 minutes. The restriction enzyme analysis was performed according to the protocol supplied by the manufacturer (Promega/New England Biolab, Southampton, U.K.). Following digestion, fragments were separated on an agarose gel, stained with ethidium bromide, and visualized using ultraviolet light.

The NAT2 typing method used in this study is based on the method initially described by Cascorbi et al., but also on methods first described by Bell et al.<sup>8,9</sup> Initially, all samples were amplified using the primers 5'GGA ACA AAT TGG ACT TGG and 5'TCT AGC ATG AAT CAC TCT GC.<sup>8</sup> Each polymerase chain reaction (PCR) reaction contained 100 ng genomic DNA, 5 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl, and 1.25 U Taq polymerase with a final volume of 50 µl. The PCR conditions were: 94°C for 3 min followed by 30 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec followed by a final extension time of

**TABLE 1.** Characteristic linkages of various point mutations defining NAT2 alleles and the relationship between the precise, new typing nomenclature for NAT2 alleles (far left column) and the old terminology (far right column) as used for the initial typing

Allele	191	282	341	481	590	803	857	Old nomenclature
*4	G	C	C	G	A	A	G	Wild-type allele
*5A	G	C	T	G	A	A	G	M1 allele
*5B	G	C	T	G	G	G	G	M1 allele
*5C	G	C	C	G	G	G	G	—
*6A	G	T	C	A	A	A	G	M2 allele
*7B	G	T	C	G	A	A	A	M3 allele
*13	G	T	C	G	A	A	G	—
*14B	A	T	C	G	A	A	G	—

Adapted from Cascorbi et al., 1995. Reproduced with permission from Bandmann O, Vaughan J, Holmans P, Marsden CD, Wood NW. The slow acetylator genotype for N-acetyltransferase 2 is associated with familial Parkinson's disease. *Lancet* 1997;350:1136-1139.

5 min. All PCR products were then digested with the enzymes *MspI/AluI*, *FokI*, *KpnI*, *TaqI*, and *BamHI* for the detection of the following polymorphisms: G191A, C282T, C481T, G590A, and G857A. The restriction enzyme digest resulted in a characteristic fragment pattern for each of the different polymorphisms.<sup>9</sup>

To detect the A803G polymorphism, a nested PCR was carried out using the primers 5'GTG GGC TTC ATC CTC ACC TA and 5'GTG GGC TTC ATC CTC ACC TA.<sup>9</sup> Part of the original template (0.2  $\mu$ l) was used for the second PCR with a volume of 25  $\mu$ l and the following PCR conditions: 15 cycles of 94°C for 30 sec, 60°C for 60 sec, and 72°C for 60 sec. Subsequently, restriction enzyme analysis was carried out using the enzyme *DdeI*.

Finally, a second nested, allele-specific PCR was performed to determine the C341T polymorphism. Part of the initial PCR product (0.2  $\mu$ l) was used as a template for this PCR with a volume of 25  $\mu$ l using the antisense strand primer of the first PCR (5'GTG GGC TTC ATC CTC ACC TA) with either the wild-type primer (341T) 5'CCT GCA GGT GAC CAT or the mutation primer (341C) 5'CCT GCA GGT GAC CAC.<sup>9</sup>

The presence of any two mutant alleles defines the slow acetylator genotype; fast acetylators have one or two wild-type alleles.

#### Statistics

A chi-square test was used to compare the frequency of the slow acetylator genotype between sporadic PD and control subjects as well as FPD and control subjects. All tests were performed with Yates correction. Odds ratios with 95% confidence intervals (95% CI) were calculated for each variable. The power of the sample to give a significant result given the observed values of the odds ratio (OR) and the frequency of the variable in the control subjects was also calculated. Because the proportions for the four groups (FPD, SPD, HD, and control subjects) were evenly spaced, a test for trend was performed for the slow acetylator frequency across the groups to test for the hypothesis that the data for the HD group are

intermediate between the control subjects and the sporadic PD group, assuming that the frequencies varied in a linear way across the four groups.

To assess whether a particular mutant allele of NAT2 is overrepresented in FPD or SPD, the relative predispositional effects method was used as described by Payami et al.<sup>12</sup> In brief, the overall  $\chi^2$  is calculated first using all seven alleles (allele 4, 5A, 5B, 5C, 6A, 7B, 13). If this gives a significant result, the wild-type allele 4 is removed to allow for differences in the wild-type allele frequency and  $\chi^2$  is calculated using just the mutant alleles (allele 5A, 5B, 5C, 6A, 7B, 13). A significant result in this calculation suggests an association between a particular allele and the disease; a negative result suggests an association between the slow acetylator genotype resulting from different allele combinations and the disease.

Finally, a 2  $\times$  2 table comparison was undertaken comparing the frequency of allele 4 versus all the other alleles taken together. An OR was calculated to investigate whether allele 4 has a protective effect.

#### RESULTS

A total of 400 samples were typed for eight different alleles of NAT2. The distribution of the alleles in the four groups studied, FPD, SPD, normal control subjects, and HD, is shown in Table 2. Compared with the previous results based on the typing of the alleles M1, M2, M3, and the wt allele, the proportion of slow acetylators increased in all four groups: there were 73 of 100 slow acetylators in the FPD group (previously 69 of 100), 60 in the SPD group (59 of 100), 52 in the HD group (48 of 100) and 43 in the control group (39 of 100). There was no significant difference between the previous results based on the limited typing and the current results. The different ORs for the comparison of the slow acetylator genotype frequency between the different groups and the respective p values are listed in Table 3. The comparison of the slow acetylator genotype frequency in the FPD group with both the NPC group as well as with the HD group gave highly significant results with an OR of 3.58 (95% CI: 1.96–6.56; p = 0.00003) for FPD versus NPC and an OR of 2.50 (95% CI: 1.37–4.56, p = 0.003) for FPD versus HD.

There were also significantly more slow acetylators among patients with SPD than among NPC (p = 0.024). However, comparison of the frequency of the slow acetylator genotype between SPD and the patient control group of HD failed to reach statistical significance (see Table 3). Trend analysis across the different groups (FPD  $\rightarrow$  SPD  $\rightarrow$  HD  $\rightarrow$  NPC) gave a highly significant result ( $\chi^2$  = 19.59 [df = 1], p = 0.00001).

TABLE 2. Distribution of alleles in the four different groups

	FPD	SPD	HD	NPC
Allele 4	28 (14%)	42 (21%)	49 (24.5%)	59 (29.5%)
Allele 5A	7 (3.5%)	7 (3.5%)	6 (3%)	3 (1.5%)
Allele 5B	84 (42%)	91 (45.5%)	85 (42.5%)	61 (30.5%)
Allele 5C	2 (1%)	—	4 (2%)	5 (2.5%)
Allele 6A	70 (35%)	58 (29%)	53 (26.5%)	66 (33%)
Allele 7B	6 (3%)	1 (0.5%)	2 (1%)	5 (2.5%)
Allele 13	3 (1.5%)	1 (0.5%)	1 (0.5%)	1 (0.5%)
Allele 14B	—	—	—	—

**TABLE 3.** Chi square, p value, odds ratios, and 95% confidence intervals for the comparison of the slow acetylator frequency between the different patient and control groups

	Chi square	p value	Odds ratio	95% CI
FPD vs NPC	17.26 (1 df)	0.00003	3.58	1.96–6.56
FPD vs HD	8.53 (1 df)	0.003	2.50	1.37–4.56
FPD vs SPD	3.23 (1 df)	0.07	1.80	0.98–3.31
SPD vs NPC	5.12 (1 df)	0.024	1.99	1.11–3.53
SPD vs HD	0.99	0.32	1.38	0.78–2.45
HD vs NPC	1.28 (1 df)	0.26	1.44	0.81–2.54

CI, confidence interval; FPD, familial cases of Parkinson's disease; NPC, normal control subjects; HD, Huntington's disease; SPD, sporadic cases of Parkinson's disease.

Using the relative predispositional effects method described by Payami et al., it was investigated whether a particular mutant allele was overrepresented in the FPD group. A highly significant result was obtained for the comparison of all alleles in the FPD versus the NPC group ( $\chi^2$ : 19.01 [df = 6]  $p = 0.004$ ), but the result remained no longer significant after the wild-type allele was removed ( $\chi^2 = 4.86$  [df = 5],  $p = 0.32$ ). The comparison between the frequency of all alleles of the FPD group and the HD group approached significance ( $\chi^2 = 11.83$  [df = 6],  $p = 0.066$ ); if the (wild-type) allele 4 is left out of FPD versus HD, chi square is 4.75 (df = 5,  $p = 0.45$ ), suggesting that the differences between FPD and HD are mainly the result of differences in the frequency of allele 4 (see also *Discussion*).

Finally, a  $2 \times 2$  table comparison for allele 4 versus all the other alleles together was undertaken to investigate whether allele 4 has a protective effect. As shown in Table 4, there was a significant protective effect of allele 4 in the FPD group only with an OR of 0.39 (95% CI: 0.23–0.64;  $p = 0.0025$ ) for FPD versus NPC and an OR of 0.50 (95% CI: 0.30–0.85;  $p = 0.01$ ). The other comparisons were nonsignificant (see Table 4).

### DISCUSSION

Using a precise genetic typing method with previously proven excellent correlation to the NAT2 genotype,<sup>9</sup> we have confirmed the association between the slow acetylator genotype of NAT2 and FPD. We were also able to establish that the association between the slow acetylator genotype and FPD does not appear to be the result of linkage with a particular mutant allele. In addition, we have shown that the wild-type allele 4 confers a protective effect against the development of PD in patients with a positive family history for that disorder.

These data confirm the validity of our initial findings. Furthermore, they establish the usefulness of the initial,

comparatively quick "screening method" for the detection of relevant differences in the NAT2 slow acetylator frequency between patient and control groups, facilitating the investigation of additional patients and control subjects in our own or other populations. This might be particularly useful if the more detailed typing method cannot be undertaken as a result of a shortage of money or time. A group of patients with HD was included solely as a second genetically well-defined control group. Erroneously, the proportion of slow acetylators based on the "screening method" was given as 37 of 100 in our previous publication,<sup>7</sup> reanalysis of the data revealed that the correct figure is 39 of 100. The percentage of slow acetylators among our normal control group obtained by the more detailed typing method (43 of 100) is virtually identical to the figures reported in a previous study (44%) but lower than in others.<sup>9,13</sup> However, comparison between the slow acetylator frequency among our FPD cases and our second, patient control group (HD) as well as with a large group of British control subjects from a different study still results in a statistically significant difference (see above and reference 14). The overall frequency of slow acetylators might have increased even further if rare polymorphisms had been typed as well. However, this is unlikely to have changed the observed slow acetylator frequency significantly, because all polymorphisms with a frequency of >1% have been typed for this study.

A study published subsequently to our initial publication failed to replicate our results in their own study, which included a large number of control subjects but a smaller number of patients.<sup>14</sup> However, Nicholl and Bennett's inclusion of more than one affected member of their 30 families in the chi-square analysis was not statistically sound. Thus, their negative results based on the analysis of a total of 46 affected family members are questionable. As mentioned above, a comparison between the NAT2 slow acetylator frequency among our FPD cases and their control subjects continued to give a

**TABLE 4.**  $2 \times 2$  table comparison for allele 4 versus all the other alleles taken together

	Chi square	p value	Odds ratio	95% CI
FPD vs NPC	13.42 (1 df)	0.0025	0.39	0.23–0.64
FPD vs HD	6.43 (1 df)	0.01	0.50	0.30–0.85
FPD vs SPD	2.92 (1 df)	0.087	0.61	0.36–1.05
SPD vs NPC	3.50 (1 df)	0.061	0.63	0.40–1.00
SPD vs HD	0.51 (1 df)	0.47	0.82	0.51–1.32
HD vs NPC	1.09 (1 df)	0.30	0.77	0.49–1.21

CI, confidence interval; FPD, familial cases of Parkinson's disease; NPC, normal control subjects; HD, Huntington's disease; SPD, sporadic cases of Parkinson's disease.

statistically significant difference ( $p < 0.025$ ). Unfortunately, no precise details were given for age, sex, ethnicity, and region of the patients and control subjects analyzed by Nicholl and Bennett.

To date, three metabolic studies investigating *in vivo* acetylation in patients with PD have been reported. Familial cases were specifically excluded from two studies and no information on family history was given in the remaining study.<sup>15-17</sup> The largest study on acetylation in European whites with PD found a higher proportion of slow acetylators among patients in comparison with control subjects but this did not reach statistical significance.<sup>15</sup> Rather than relying on metabolic tests, we investigated the possible involvement of NAT2 at the molecular genetic level and found marked differences between our patients with FPD and two control groups. There was also a difference between the SPD group and both control groups but this did not reach statistical significance. A larger number of patients has to be investigated to determine the possible role of slow acetylation in SPD.

It may be difficult to understand how a "genetic defect," slow acetylation, present in a considerable proportion of the general population should make some people more prone to develop dopaminergic cell death, whereas others live a healthy life to old age without developing PD or even subclinical damage to the substantia nigra and incidental Lewy body disease. The theory of "susceptibility genes" addresses this problem: it is not the gene in isolation, but rather an interaction between genetically determined endogenous factors and exogenous influences such as exposure to particular environmental toxins which leads to the development of the disease. Several hypotheses can be considered. The most obvious explanation is that the acetylator genotype only becomes relevant after exposure to neurotoxins. Slow excretion of a particular substance might lead to a gradual accumulation in the body, finally resulting in damage to the substantia nigra. Circumstantial evidence for this hypothesis comes from the observation of differing clinical outcome after exposure to MPTP: at least 400 people are known to have self-administered MPTP but only a few have developed parkinsonian symptoms.<sup>18</sup> This may reflect a difference in genetically determined detoxification capacity between the subjects who remained clinically unaffected and those who developed parkinsonism. Slow acetylators are more susceptible to low-level environmental exposure to carcinogens.<sup>19</sup> Similarly, slow acetylators might also be more susceptible to low exposure of neurotoxins. In rabbits, hydrazine-induced central nervous system toxicity is related to the acetylator genotype,

slow acetylators showing greater susceptibility with increased irritability, seizures, and early death.<sup>20</sup>

An alternative hypothesis invokes a more direct influence of NAT2 acetylator status on basic biologic mechanisms in dopaminergic cells such as gene expression.<sup>21</sup> Rather than influencing the rate of RNA expression, acetylation can also activate or deactivate proteins directly<sup>22</sup> and could be of importance in the protection against cell death as a result of its influence on the synthesis of polyamines.<sup>23</sup> This could be of relevance because polyamines have been shown to be neuroprotective in at least one study.<sup>24</sup>

In summary, we have confirmed a strong association between FPD and slow acetylator status for NAT2 using a precise typing method which is in accordance with the new international nomenclature for the different NAT2 alleles and has previously been shown to be in accordance with the biochemically determined N-acetylation genotype.<sup>9,10</sup> Further work on NAT2 in PD should not only include an attempt to replicate our findings in a different population, but also aim to investigate the biologic relevance of NAT2 status on the handling of identified dopaminergic neurotoxins such as MPTP, and possibly also further examine the influence of NAT2 status on gene expression in the basal ganglia.

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ASSOCIATION BETWEEN EARLY-ONSET PARKINSON'S DISEASE  
AND MUTATIONS IN THE *PARKIN* GENE

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ABSTRACT

**Background** Mutations in the *parkin* gene have recently been identified in patients with early-onset Parkinson's disease, but the frequency of the mutations and the associated phenotype have not been assessed in a large series of patients.

**Methods** We studied 73 families in which at least one of the affected family members was affected at or before the age of 45 years and had parents who were not affected, as well as 100 patients with isolated Parkinson's disease that began at or before the age of 45 years. All subjects were screened for mutations in the *parkin* gene with use of a semiquantitative polymerase-chain-reaction assay that simultaneously amplified several exons. We sequenced the coding exons in a subgroup of patients. We also compared the clinical features of patients with *parkin* mutations and those without mutations.

**Results** Among the families with early-onset Parkinson's disease, 36 (49 percent) had *parkin* mutations. The age at onset ranged from 7 to 58 years. Among the patients with isolated Parkinson's disease, mutations were detected in 10 of 13 patients (77 percent) with an age at onset of 20 years or younger, but in only 2 of 64 patients (3 percent) with an age at onset of more than 30 years. The mean ( $\pm$ SD) age at onset in the patients with *parkin* mutations was younger than that in those without mutations ( $32\pm 11$  vs.  $42\pm 11$  years,  $P<0.001$ ), and they were more likely to have symmetric involvement and dystonia at onset, to have hyperreflexia at onset or later, to have a good response to levodopa therapy, and to have levodopa-induced dyskinesias during treatment. Nineteen different rearrangements of exons (deletions and multiplications) and 16 different point mutations were detected.

**Conclusions** Mutations in the *parkin* gene are a major cause of early-onset autosomal recessive familial Parkinson's disease and isolated juvenile-onset Parkinson's disease (at or before the age of 20 years). Accurate diagnosis of these cases cannot be based only on the clinical manifestations of the disease. (N Engl J Med 2000;342:1560-7.)

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PARKINSON'S disease is one of the most frequent neurodegenerative disorders, with a prevalence of 1 to 2 percent among persons older than 65 years of age.<sup>1</sup> It is characterized by resting tremor, rigidity, and bradykinesia, all of which respond well to treatment with levodopa. The pathological hallmarks are the presence of Lewy bodies (cytoplasmic eosinophilic hyaline inclusions) and massive loss of dopaminergic neurons in the pars compacta of the substantia nigra.<sup>2</sup> The cause of the disease is still unknown, but the existence of genetic susceptibility factors is strongly suspected.<sup>3,4</sup> Two genes ( $\alpha$ -synuclein<sup>5</sup> and ubiquitin carboxy-terminal hydrolase L1 [*UCH-L1*]<sup>6</sup>) and two gene loci (on chromosomes 2p13 and 4p14-16.3, respectively<sup>7,8</sup>) have been implicated in the pathogenesis of autosomal dominant Parkinson's disease, but they seem to account for the cases in only a few families. In contrast, mutations in the gene designated *parkin* have recently been identified in several families with autosomal recessive early-onset parkinsonism.<sup>9-14</sup> However, the frequency of mutations in this gene in familial and isolated cases of early-onset parkinsonism has not yet been assessed in large series of patients.

The phenotype associated with mutations in the *parkin* gene has not been clearly established, making the selection of patients for genetic testing difficult. In Japanese patients with *parkin* mutations, the disease is characterized by an early onset, dystonia at

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onset, hyperreflexia, early complications resulting from levodopa treatment, and slow progression.<sup>9-11</sup> In contrast, the clinical characteristics of some European and North African patients with *parkin* mutations were indistinguishable from those of patients with idiopathic Parkinson's disease, with an age at onset of up to 58 years.<sup>13,14</sup> The number of families analyzed so far is small, however, and the correlations between genotype and phenotype are uncertain. Brain tissue from the patients studied did not contain Lewy bodies, suggesting that the pathologic process might differ from that of idiopathic Parkinson's disease.<sup>15,16</sup>

We performed a clinical and molecular study of 73 families with early-onset autosomal recessive Parkinson's disease, including 152 affected family members, and 100 patients with early-onset isolated Parkinson's disease. They were screened for mutations in the *parkin* gene by a semiquantitative polymerase-chain-reaction (PCR) assay designed to detect exon rearrangements (deletions and multiplications) and by genomic sequencing. Correlations between genotype and phenotype were assessed both in patients with *parkin* mutations and in those without such mutations.

## METHODS

### Patients and Families

We studied 73 families (152 patients with Parkinson's disease and 53 unaffected relatives) that met the following criteria: symptoms of parkinsonism in affected family members that were reduced by at least 30 percent by treatment with levodopa (the response could not be assessed in 3 untreated patients); a mode of inheritance compatible with autosomal recessive transmission (affected siblings without affected parents); an age at onset of 45 years or younger in at least one of the affected siblings; and the absence of extensor plantar reflexes, ophthalmoplegia, early dementia, or early autonomic failure in the family members we examined. Twenty of the families originated from Italy, 14 from France, 12 from Great Britain, 10 from the Netherlands, 9 from Germany, 2 from Portugal, and 1 each from Spain, Algeria, Morocco, Argentina, India, and Vietnam. Eight of the families were consanguineous, and 12 have been described previously.<sup>13,14</sup> In addition, we studied 100 patients with isolated Parkinson's disease with an age at onset of 45 years or younger, most of whom were European, and 8 of whom were from consanguineous marriages. These 100 patients were selected according to the same clinical criteria used for the patients with familial disease but who had no family history of Parkinson's disease. Eight of these 100 patients had never received treatment.

The enrollment of the subjects was random. For each subject, we obtained clinical information from the subject or the subject's records and peripheral blood for DNA analysis. DNA was extracted from peripheral-blood leukocytes according to standard procedures. The study was approved by ethics committees in the countries of all the participating investigators, and written informed consent was obtained from all the study subjects.

### Molecular Analysis

Screening for mutations in the *parkin* gene was performed in all index patients (except those known to be homozygous or to have compound heterozygosity for such mutations<sup>13,14</sup>) with the use of a semiquantitative PCR assay for the detection of rearrangements of *parkin* exons. Exons 2 through 12 were amplified simultaneously in three groups by PCR (multiplex PCR): group 1 consisted of exons 4, 7, 8, and 11; group 2 consisted of exons 5, 6, 8, and 10; and group 3 consisted of exons 2, 3, 9, and 12 and an external control,

C328, a 328-bp sequence of the transthyretin gene on chromosome 18. The primers used were the same as those described by Kitada et al.<sup>9</sup> except for the primer for exon 3, for which exonic primers were used: 5'AATTGTGACCTGGATCAGC3' (Ex3iFor) as the forward primer and 5'CTGGACTTCCAGCTGGTGGTGGAG3' (Ex3iRev) as the reverse primer. The C328 forward primer was 5'ACGTTCTGATAATGGGATC3' (TTRForHex), and the reverse primer was 5'CCTCTCTCTACCAAGTGAGG3' (TTR328Rev). All forward primers were fluorescently labeled with HEX-phosphoramidite. The PCR products (2.5  $\mu$ l) were analyzed by 5 percent denaturing polyacrylamide-gel electrophoresis with an automated sequencer (model ABI 377) and GeneScan version 3.1 and Genotyper version 1.1.1 software (all from Applied Biosystems). All reactions were performed at least twice. The DNA from a patient known to have a heterozygous deletion of exons 8 and 9 was always processed in parallel as an internal control.

We calculated the ratios of all the peak heights in a given reaction and then compared the ratios with the ratios measured in a specimen from a normal subject. This comparison yielded the following rules: values of 0.6 or less were interpreted as indicating 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 indicating a heterozygous duplication of an exon; values of 1.8 to 2.3 were interpreted as indicating a homozygous duplication or heterozygous triplication of an exon; and values of more than 2.6 were interpreted as indicating a homozygous triplication of an exon. An exon rearrangement was confirmed only if all the ratios concerning this exon were abnormal. The consequence of the rearrangements at the protein level (a frame shift vs. an in-frame rearrangement) was deduced from the exon sequences published by Kitada et al.<sup>9</sup> (DNA Data Bank of Japan accession number AB009973).

Each PCR reaction involved, in a total volume of 25  $\mu$ l, 40 ng of DNA, with 3 mM magnesium chloride, 0.2 mM of each deoxynucleoside triphosphate, and 1 U of *Taq* polymerase. Denaturation for 5 minutes at 95°C was followed by 23 cycles consisting of 30 seconds of denaturation at 95°C, 45 seconds of annealing at 53°C, and 2.5 minutes of extension at 68°C, with a final period of extension at 68°C for 5 minutes. Primer concentrations were chosen to yield — within the exponential phase of the PCR (data not shown) — similar peak heights in each multiplex reaction and ranged from 0.4 to 1.9  $\mu$ M. Deletions and insertions of bases were deduced from the size of the PCR products.

In the case of 54 index patients with familial disease and 91 index patients with isolated disease, exon rearrangements or deletions or insertions of bases were not found on both chromosomes and thus did not account for the phenotype. Therefore, the entire coding region of the *parkin* gene (including exon-intron boundaries) was sequenced as described previously<sup>14</sup> in 53 index patients with familial disease and 50 patients with isolated disease.

Whether the *parkin* variants we identified cosegregated with the disease was assessed by genotyping of all available members, affected or not, of the respective families. In addition, 114 chromosomes from mostly European, unrelated controls who had no movement disorders were analyzed for the point mutations and the rearrangements of the exons represented in multiplex group 3. The techniques used were restriction assays, polyacrylamide-gel electrophoresis, and the semiquantitative PCR assay. For two point mutations — the substitution of A for G at nucleotide 939 of complementary DNA (cDNA) and the substitution of T for C at nucleotide 1101 of cDNA — mismatched reverse primers were used in order to create a restriction site that depended on the point mutation being looked for: 5'GGCAGGGAGTAGCCAAGTTG-AGGAT3' for digestion with *Aha*I and 5'AGCCCCGCTCCACAGCCAGCGC3' for digestion with *Bst*UI (in each primer, the underlined nucleotide differs from the wild-type sequence).

### Statistical Analysis

Means ( $\pm$ SD) were compared with the nonparametric Mann-Whitney U test. Frequencies were compared with the chi-square test, with Yates' correction when appropriate.

RESULTS

Frequency of Mutations in the *parkin* Gene

Twenty-five families (56 patients) with autosomal recessive Parkinson's disease had homozygous or compound heterozygous mutations on each allele of the *parkin* gene (Table 1). In addition, 11 families (27 patients) with a mutation in one allele were considered to have *parkin*-related disease, on the basis of the assumption that the second mutation was not detected by the methods used in this study. Thus, mutations in the *parkin* gene were detected in 36 of 73 families (49 percent), including 12 previously described families.<sup>13,14</sup> Among the 100 patients with isolated Parkinson's disease, 18 (18 percent) had *parkin* mutations. The frequency of mutations among consanguineous patients with isolated Parkinson's disease, a pattern that is suggestive of autosomal recessive inheritance, was similar to that among consanguineous patients with familial disease (50 percent vs. 62 percent). The frequency of mutations in the patients with isolated Parkinson's disease decreased significantly with increasing age at onset: mutations were detected in 10 of 13 patients (77 percent) with an age at onset of disease of 20 years or younger, but only in 2 of 64 patients (3 percent) with an age at onset of 31 to 45 years (Table 2). Sequencing of the *parkin* gene in 22 of the 64 patients with isolated Parkinson's disease who were older than 30 years at the onset of symptoms revealed a point mutation in only 1 patient. In 14 families in which the affected family members had *parkin* mutations on both chromosomes, none of 28 unaffected siblings had two *parkin* mutations, indicating the high penetrance of the mutations.

Clinical Studies

The 36 families with Parkinson's disease and *parkin* mutations and the 18 patients with isolated Parkinson's disease and *parkin* mutations came from a variety of regions: France (in 15 cases), Italy (in 13), Great Britain (in 7), the Netherlands (in 3), Spain (in 3), Germany (in 3), Portugal (in 2), Algeria (in 2) and Lebanon, India, Pakistan, Vietnam, Japan, and Argentina (in 1 case each).

As a group, the 100 patients with *parkin* mutations had a mean ( $\pm$ SD) age at onset of  $32\pm 11$  years (range, 7 to 58); the age at onset was not known for 1 patient (Table 3). Among the patients with an age at onset of 45 years or younger, the onset of the disease was earlier in the 18 patients with isolated Parkinson's disease and *parkin* mutations than in the 75 patients with familial Parkinson's disease and mutations (mean age,  $21\pm 9$  vs.  $32\pm 9$  years; median, 20 vs. 33 years;  $P<0.001$ ). This difference was not due to selection bias, because the mean ages at onset were similar in the two groups when all initially included patients with an age at onset of 45 years or younger were compared, whether or not they had *parkin* mu-

TABLE 1. FREQUENCY OF MUTATIONS IN THE *PARKIN* GENE IN 73 FAMILIES WITH AUTOSOMAL RECESSIVE EARLY-ONSET PARKINSON'S DISEASE AND 100 PATIENTS WITH ISOLATED EARLY-ONSET PARKINSON'S DISEASE.\*

NO. OF MUTATIONS	FAMILIES WITH AUTOSOMAL RECESSIVE DISEASE (N=73)		PATIENTS WITH ISOLATED CASES (N=100)	
	NO. OF FAMILIES	NO. OF CONSANGUINEOUS FAMILIES	NO. OF PATIENTS	NO. OF CONSANGUINEOUS PATIENTS
Two	25	5	14	4
One	11†	0	4	0
None identified	37	3	82‡	4
Total no. with mutations (%)	36 (49)	5 (62)	18 (18)	4 (50)

\*Early onset was defined as an onset at or before 45 years of age (in at least one of the affected siblings in affected families).

†The *parkin* gene was not sequenced in one family.

‡The *parkin* gene was not sequenced in 41 patients in whom the onset of disease was after 30 years of age.

TABLE 2. FREQUENCY OF MUTATIONS IN THE *PARKIN* GENE IN 100 PATIENTS WITH ISOLATED EARLY-ONSET PARKINSON'S DISEASE, ACCORDING TO THE AGE AT ONSET.

AGE AT ONSET	PATIENTS WITH HOMOZYGOUS OR HETEROZYGOUS MUTATIONS	CONSANGUINEOUS PATIENTS WITH HOMOZYGOUS OR HETEROZYGOUS MUTATIONS
	no. of patients/total no. (%)	
$\leq 20$ yr	10/13 (77)*	2/3 (67)
21-30 yr	6/23 (26)†	2/2 (100)
31-40 yr	1/49 (2)‡	0/2
41-45 yr	1/15 (7)§	0/1
Total no. of patients (%)	18/100 (18)	4/8 (50)

\* $P=0.003$  for the comparison with patients with an age at onset of 21 to 30 years.

† $P=0.005$  for the comparison with patients with an age at onset of 31 to 40 years.

‡The *parkin* gene was not sequenced in 35 patients.

§The *parkin* gene was not sequenced in six patients.

tations (age at onset in 118 patients with familial disease,  $34\pm 9$  years; in 100 patients with isolated disease,  $32\pm 9$  years). The mean age at onset was significantly younger in the patients with *parkin* mutations than in those without mutations, both in the total sample (Table 3) and in the group with familial cases alone ( $34\pm 10$  years for 82 patients with familial disease and mutations and  $43\pm 12$  years for 65 patients with familial disease but without mutations;  $P<0.001$ ).

The initial manifestations of the disease in most patients with *parkin* mutations were tremor (65 per-

ASSOCIATION BETWEEN EARLY-ONSET PARKINSON'S DISEASE AND MUTATIONS IN THE *PARKIN* GENE

TABLE 3. CHARACTERISTICS OF PATIENTS WITH PARKINSON'S DISEASE ACCORDING TO THE PRESENCE OR ABSENCE OF MUTATIONS IN THE *PARKIN* GENE.\*

CHARACTERISTIC	PATIENTS WITH <i>PARKIN</i> MUTATIONS (N=101)	PATIENTS WITHOUT <i>PARKIN</i> MUTATIONS (N=85)	P VALUE
Sex (F/M)	49/52	31/54	
Age at onset (yr)	32±11	42±11	<0.001
Duration of disease (yr)	17±11	13±11	0.002
Clinical signs at onset (%)			
Micrographia	30	47	0.02
Bradykinesia	63	65	
Tremor	65	75	
Dystonia	42	22	0.02
Asymmetric signs	89	98	0.02
Clinical signs at examination			
Bradykinesia (%)	95	98	
Rigidity (%)	92	99	
Resting tremor (%)	74	80	
Postural tremor (%)	54	47	
Urinary urgency (%)	11	25	0.01
Hyperreflexia (%)	44	21	0.04
No progression or slow rate of progression (%)	88	72	
Motor scale of UPDRS score†			
Without treatment	41±22	43±16	
During treatment	23±18	26±15	
Hoehn-Yahr assessment without treatment‡			
Mean stage	3.2±1.0	3.1±0.8	
Interval from onset to stage 2 (yr)	11±9	5±3	
Interval from onset to stage 3 (yr)	19±10	17±8	
Interval from onset to stage 4 (yr)	26±8	33±2	
Interval from onset to stage 5 (yr)	40±19	44	
Mini-Mental State Examination score§	29±3	28±2	
Clinical signs during treatment			
Percent improvement with levodopa	72±20	64±17	0.03
Daily dose of levodopa (mg)	500±340	600±400	
Duration of levodopa treatment			
Months	123±102	111±99	
Years	10	9	
Levodopa-induced dyskinesia			
Percentage of patients	77	63	0.04
Months of treatment	64±65	60±55	
Levodopa-induced fluctuations in symptoms			
Percentage of patients	79	65	
Months of treatment	64±61	61±54	
Dystonia			
Percentage of patients	58	45	
Months of treatment	65±72	54±40	

\*Plus-minus values are means ±SD. Among the patients with *parkin* mutations, 83 had familial disease and 18 had isolated disease. Among the patients without *parkin* mutations, 57 had familial disease and 28 had isolated disease.

†The motor scale of the Unified Parkinson's Disease Rating Scale (UPDRS)<sup>17</sup> assesses 14 motor functions of patients with Parkinson's disease. Some of the functions were tested separately for each side of the body, the arms and legs, the face, and the trunk, resulting in 27 subtests. Each subtest was scored on a scale from 0 (no impairment) to 4 (severe impairment), resulting in a total score ranging from 0 to 108.

‡The Hoehn and Yahr stages are used to describe the degree of functional disability of patients with Parkinson's disease. Stage 1 indicates mild unilateral symptoms, stage 2 bilateral or axial symptoms, stage 3 impairment of postural reflexes, stage 4 strongly disabling disease (the patient is able to stand and walk unassisted but is markedly incapacitated), and stage 5 severely disabling disease (the patient cannot stand or walk without assistance and is therefore confined to a wheelchair or bed).<sup>18</sup>

§The Mini-Mental State examination assesses orientation; short-term memory; attention span; and naming, copying, reading, writing, spatial, and constructive capacities with respect to 30 tasks, all scored as either 1 (succeeded) or 0 (failed). The maximal score is 30; dementia was considered to be present if the score was below 24.

cent) and bradykinesia (63 percent) (Table 3). The patients with *parkin* mutations had significantly higher frequencies of dystonia and symmetric symptoms at onset and of hyperreflexia at onset or later, as well as a better response to levodopa despite having had the disease for a longer period (Table 3) than those with no *parkin* mutations. Dystonia began in the lower limbs in 28 of 31 patients with mutations, but 2 patients first had torticollis and 1 had right-arm dystonia. Dyskinesia as a result of levodopa treatment was significantly more common in patients with mutations than in those with no mutations, but such dyskinesia occurred in both groups, on average, after nearly 5 years of treatment (range, 1 month to 20 years). There were no significant differences between the 24 patients with at least one missense mutation and the 52 patients with two truncating mutations; the 25 patients with single heterozygous truncating mutations were not assigned to either group, since the nature of the suspected second mutation was unknown.

Nineteen different homozygous and heterozygous exon rearrangements were found in 35 index patients, including 4 from previously described families with homozygous deletions of exons (Table 4 and Fig. 1A).<sup>13,14</sup> In addition to identifying the suspected deletions of an exon, our approach provided evidence of four duplications of an exon and one triplication of an exon. The results were highly reproducible and confirmed by cosegregation analysis. Rearrangements of exons 2, 3, 9, and 12 were not found in the controls.

Sixteen different exonic point mutations were found in 28 index patients, including 8 from previously described families<sup>14</sup> (Table 4 and Fig. 1B). In addition, an intronic deletion of 5 bp (IVS8 -21 to -17del) was detected. All point mutations cosegregated with the disease, and none were found in any control. The amino acids modified by mutations were conserved in the *parkin* orthologues in rats<sup>20</sup> and mice (Gene Bank accession numbers AF210434 and AB019558, respectively). However, in two patients from one family, the homozygous point mutation Arg334Cys was associated with the homozygous intronic 5-bp deletion and the heterozygous Asp280Asn mutation, so that the pathogenicity of the latter two mutations cannot be ascertained.

Many of the exon rearrangements were found repeatedly among the index patients, particularly deletions of exon 3 (in 10 patients), exon 2 (in 4), exon 4 (in 4), and exons 3 and 4 (in 4) (Fig. 1A). Six point mutations were found in more than one index patient: the deletion of A at nucleotide 255 of cDNA (in six index patients), the deletion of A and G at nucleotide 202 to 203 of cDNA (in five), Arg275Trp (in five), the insertion of G and T between nucleotide 321 and nucleotide 322 of cDNA (in two), Lys211Asn (in two), and Gly430Asp (in two) (Fig. 1).

TABLE 4. FREQUENCY OF HOMOZYGOUS, COMPOUND HETEROZYGOUS, AND SINGLE HETEROZYGOUS MUTATIONS AMONG 45 INDEX PATIENTS WITH FAMILIAL OR ISOLATED PARKINSON'S DISEASE, ACCORDING TO THE TYPE OF MUTATION.

TYPE OF MUTATION	EXON REARRANGEMENT	POINT MUTATION	EXON REARRANGEMENT PLUS A POINT MUTATION*	TOTAL No. (%)
Homozygous	9 (6)	10 (3)	NA	19 (35)
Compound heterozygous	8	3	9	20 (37)
Single heterozygous	9†	6	NA	15 (28)

\*NA denotes not applicable.

†The *parkin* gene was not sequenced in one index patient.

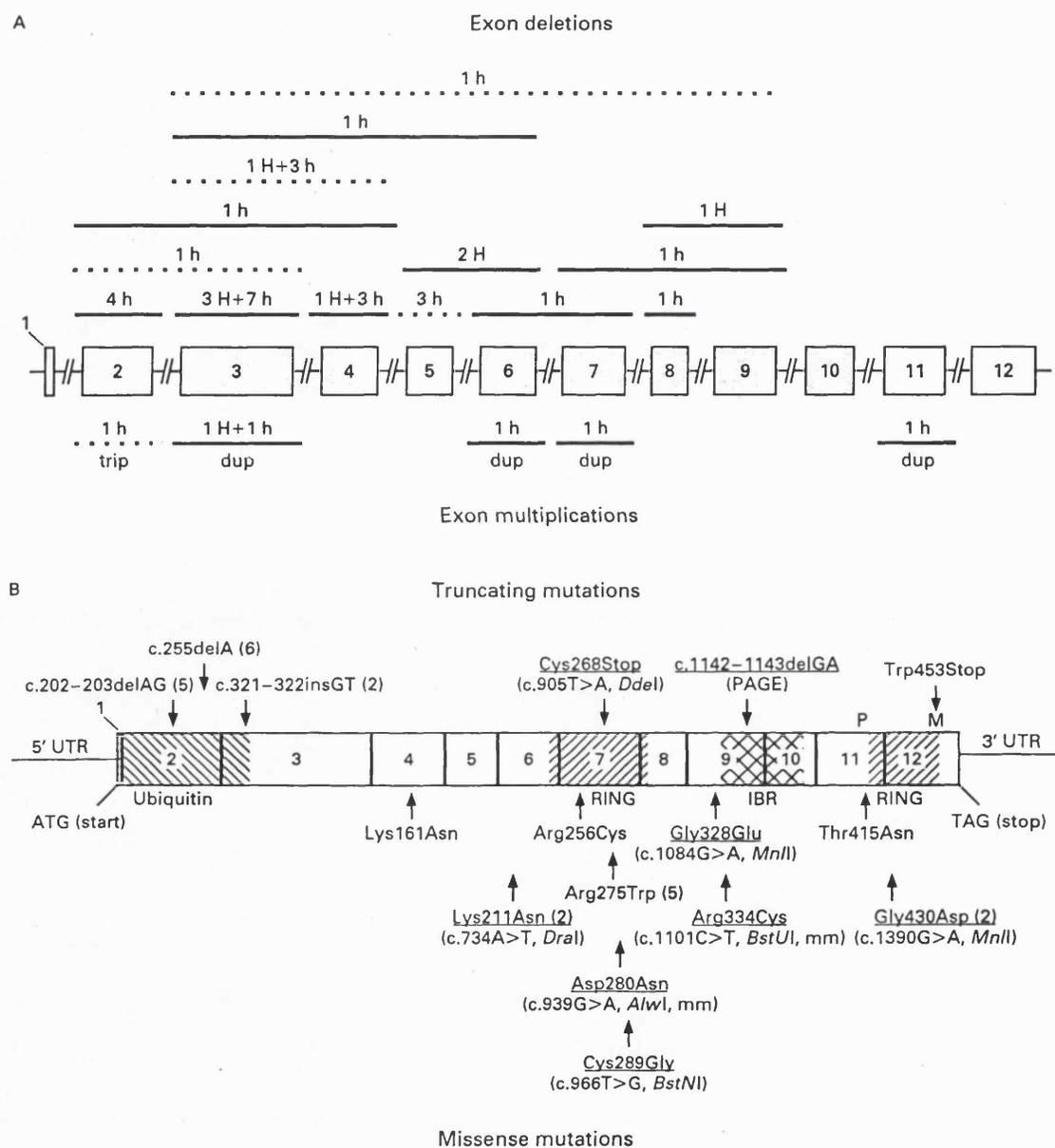
## DISCUSSION

We detected mutations in the *parkin* gene in almost half the families with autosomal recessive Parkinson's disease in which at least one affected member was 45 years of age or younger at the onset of symptoms. The frequency of such mutations was lower in a group of patients with isolated early-onset Parkinson's disease.

On average, patients with *parkin* mutations began to have symptoms in their early 30s, but the age at onset ranged widely, from 7 to 58 years. The fact that the onset occurred at an earlier age in patients with isolated Parkinson's disease and *parkin* mutations than in those with familial Parkinson's disease and *parkin* mutations suggests that among patients who are older than 30 years at the onset of isolated Parkinson's disease, the disease is mainly due to causes other than *parkin* mutations.

Can patients with *parkin* mutations be distinguished clinically from patients with early-onset Parkinson's disease from other causes? As a group, those with *parkin* mutations had an earlier onset of disease, were more likely to have dystonia and symmetric signs at onset, as well as hyperreflexia at onset or later, and were more likely to have a better response to levodopa, but were also more likely to have dyskinesia during treatment, than were patients without *parkin* mutations. These signs were less frequent, however, than in previous reports<sup>11,21</sup> and could not be used specifically to identify patients with mutations. Furthermore, the clinical manifestations of the *parkin* mutations were independent of the age at onset.

In addition, patients with late-onset disease who have mutations can be difficult to distinguish from those with idiopathic Parkinson's disease. In general, however, the disease progressed slowly in the patients with mutations. Despite having had symptoms for



**Figure 1.** Mutations in the *parkin* Gene.

Panel A shows the exon rearrangements identified. Deletions are indicated above the sequence, and duplications (dup) and triplications (trip) are indicated below the sequence. Their deduced effect on the protein is represented by a dotted line for in-frame rearrangements and by a solid line for frame-shift rearrangements. The number of index patients with the rearrangement and the type of mutation — heterozygous (h) or homozygous (H) — are indicated above each mutation. Panel B shows the point mutations resulting in truncation of the sequence of 12 exons or in a missense mutation. The hatched regions indicate the ubiquitin-like domain and the RING-IBR-RING finger motif.<sup>19</sup> The 6 truncating mutations are indicated above the sequence, and the 10 missense mutations are indicated below the sequence. For mutations identified in more than one index patient, the number of index patients with the mutation is given in parentheses. The nucleotide change and the restriction enzymes used to screen family members and unrelated control subjects without movement disorders are given in parentheses below the mutation (mm denotes the mismatch primer used for the PCR, and PAGE polyacrylamide-gel electrophoresis). Mutations that were not based on published sequences<sup>14</sup> are underlined. Nucleotides prefaced by a small c indicate the numbers in the complementary DNA sequences described by Kitada et al.<sup>9</sup> The ATG of the initiator methionine codon begins at nucleotide 102. The putative site of phosphorylation (P) and an *N*-myristoylation site (M) affected by the Thr415Asn and Trp453Stop mutations, respectively, are indicated. UTR denotes untranslated region.

many years, the majority of patients with *parkin* mutations had good responses to low doses of levodopa. Although levodopa-induced dyskinesia was reported to develop early,<sup>9,11,21</sup> the mean delay in our patients was about 5 years, with a maximum of 20 years. This time frame was similar to that for the patients without *parkin* mutations.

Finally, dementia was rare among the patients with mutations. This might be explained by a less widespread neuronal loss in patients with mutations, in whom the substantia nigra and, to a lesser extent, the locus caeruleus are selectively affected, as compared with patients with idiopathic Parkinson's disease.<sup>15,16</sup> However, the low frequency of dementia in the patients with mutations could also be due to a younger mean age at examination or to the exclusion of patients who had dementia early in the course of the disease.

There were no clinical differences between patients with missense mutations and those with truncating mutations. This finding was surprising, since missense mutations might be expected to interfere less with the function of the *parkin* protein than truncating mutations and therefore to result in a milder phenotype. We therefore assume that the 10 conserved amino acids that were affected by the missense mutations are of crucial importance for the function of the protein or that their modification results in decreased protein synthesis or more rapid degradation.<sup>22</sup> In addition, the wide range of clinical signs, even within single families with mutations (e.g., variation of up to 20 years in the age at onset) suggests that additional factors contribute to the phenotype.

The chief histopathological differences between patients with *parkin* mutations and those with idiopathic Parkinson's disease that have been detected so far are the absence of Lewy bodies and the restriction of neuronal cell loss to the substantia nigra and the locus caeruleus in the patients with *parkin* mutations.<sup>16</sup> Thus, *parkin* gene mutations are responsible for the death of selective cells, the mechanism of which might differ from that in idiopathic Parkinson's disease.

The PCR-based technique that we used revealed numerous rearrangements of exons, including those identified in eight families in which no mutations were found by direct sequencing.<sup>14</sup> In combination with genomic sequencing, this technique greatly improves the sensitivity of the molecular diagnosis in patients with *parkin* gene mutations. The various combinations of exon deletions, the exon duplications, and the newly identified point mutations increase the already wide variety of disease-related mutations identified in the *parkin* gene. The position of the mutations indicates functionally important protein regions such as the RING-IBR-RING domain, as does conservation of the corresponding amino acids in mice and rats.<sup>20</sup>

The presence of both deletions and duplications of some exons (e.g., exon 2 and 3) suggests that a mechanism such as unequal recombination might be involved. The observation that 13 of the mutations were found repeatedly in as many as 10 families raises the possibility of a founder effect. However, many of the mutations were found in families from different European countries, suggesting that these alterations are recurrent. The point mutations that accounted for the disease in approximately 40 percent of our patients seem to be less frequent among Japanese patients.<sup>11</sup> Finally, the identification of 15 index patients with single heterozygous mutations indicates that other mutations remain to be discovered, perhaps in non-coding regions of the *parkin* gene.

In conclusion, mutations of the *parkin* gene are frequent among patients with autosomal recessive Parkinson's disease. Although dystonia at the onset of disease, hyperreflexia, and a slow rate of disease progression are characteristic features of patients with *parkin* mutations, there are no specific clinical signs that distinguish these patients from patients with other causes of Parkinson's disease. The wide spectrum of mutations in the *parkin* gene renders molecular diagnosis difficult, but the relatively simple semiquantitative PCR method that we used detected approximately 70 percent of the mutations found in this series of patients.

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#### APPENDIX

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## Origin of the Mutations in the *parkin* Gene in Europe: Exon Rearrangements Are Independent Recurrent Events, whereas Point Mutations May Result from Founder Effects

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A wide variety of mutations in the *parkin* gene, including exon deletions and duplications, as well as point mutations, result in autosomal recessive early-onset parkinsonism. Interestingly, several of these anomalies were found repeatedly in unrelated patients and may therefore result from recurrent, de novo mutational events or from founder effects. In the present study, haplotype analysis, using 10 microsatellite markers covering a 4.7-cM region known to contain the *parkin* gene, was performed in 48 families, mostly from European countries, with early-onset autosomal recessive parkinsonism. The patients carried 14 distinct mutations in the *parkin* gene, and each mutation was detected in more than one family. Our results support the hypothesis that exon rearrangements occurred independently, whereas some point mutations, found in families from different geographic origins, may have been transmitted by a common founder.

### Introduction

Parkinson's disease (PD) is a frequent neurodegenerative disorder with a prevalence of ~2% in persons >65 years old (Elbaz et al. 1999). The main clinical features are rigidity, bradykinesia, and tremor, associated with a good response to levodopa. The disorder is caused by a massive loss of dopaminergic neurons in the pars compacta of the substantia nigra and is characterized by the presence of Lewy bodies (cytoplasmic eosinophilic hyaline inclusions) (Fearnley and Lees 1991). Genetic risk factors are probably involved in the pathogenesis of Par-

kinson's disease (de Silva et al. 2000), and several families with clearly established monogenic inheritance have been reported. The majority of such cases are caused by mutations in the *parkin* gene, which result in autosomal recessive early-onset parkinsonism (MIM 600116) (Hattori et al. 1998a, 1998b; Kitada et al. 1998; Leroy et al. 1998a; Lücking et al. 1998, 2000; Abbas et al. 1999). The number of patients with mutations in the  $\alpha$ -synuclein (Polymeropoulos et al. 1997) or ubiquitin carboxy-terminal hydrolase (UCH)-L1 genes (Leroy et al. 1998b) is much smaller. The phenotype associated with *parkin* gene mutations is variable but is usually characterized by early-onset parkinsonism and slow disease progression (Ishikawa and Tsuji 1996; Lücking et al. 2000). Postmortem examinations reveal massive loss of dopaminergic neurons in the substantia nigra pars compacta and the absence of Lewy bodies, results suggesting that the pathologic process may differ from that of idiopathic Parkinson's disease (Takahashi et al. 1994; Mori et al. 1998; van de Warrenburg, in press). A wide variety of mutations in the *parkin* gene have been detected, including exon deletions and duplications, as well as point mutation (Hattori et al. 1998a, 1998b; Kitada et al.

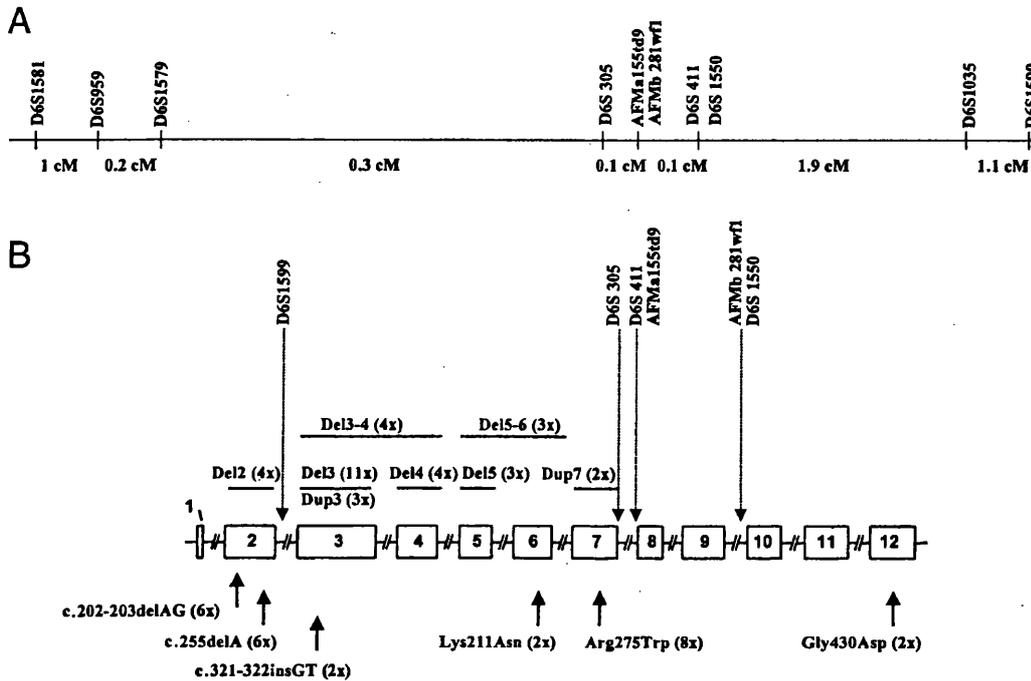
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**Figure 1** Recurrent mutations in the *parkin* gene and localization of the intragenic microsatellites, showing genetic map of the 10 microsatellites studied (A), exon rearrangements (B, top), and point mutations (B, bottom) that were detected more than once. Exon rearrangements are represented as bars corresponding to their size and position, and they are divided into deletions (del) and duplications (dup). The positions of point mutations are indicated by arrows. The ATG of the initiator methionine codon begins at nucleotide 102 of the published cDNA (Kitada et al. 1998). The number of index patients with the same mutation is indicated in parentheses. The positions of the microsatellites in the *parkin* gene are indicated by dotted arrows. Note the difference, arising from recent results of chromosome 6 sequencing, between the genetic map and physical map.

1998; Leroy et al. 1998a; Lücking et al. 1998, 2000; Abbas et al. 1999; Klein et al. 2000; Maruyama et al. 2000; Muñoz et al. 2000; Yamamura et al. 2000). The frequency of these mutations in Europe was estimated at 50% in families with early-onset parkinsonism that could have been autosomal recessive inheritance and at 18% in patients who had isolated parkinsonism with onset at age  $\leq 45$  years (Lücking et al. 2000).

Interestingly, several mutations were found repeatedly in index patients (Hattori et al. 1998a; Lücking et al. 1998, 2000; Abbas et al. 1999). In our series of patients, the deletion of exon 3 ( $n = 11$ ), the Arg275Trp mutation ( $n = 8$ ), the c.202-203 delAG mutation ( $n = 6$ ), and the c.255delA mutation ( $n = 6$ ) were found repeatedly. They may therefore result from recurrent, de novo mutational events or from founder effects. Divergent alleles of markers closely linked to the *parkin* locus would suggest independent de novo mutations, whereas conservation of alleles would support the hypothesis of a founder effect.

In the present study, haplotype analysis was performed with 10 microsatellite markers covering a 4.7-

cM region that contains the *parkin* gene, which is localized on chromosome 6q25.2-q27. The subjects were members of 48 families with early-onset autosomal recessive parkinsonism who carried 14 different mutations of the *parkin* gene found in more than one family. Our results support the hypothesis that exon rearrangements occurred independently, whereas there is evidence of founder effects in some families with point mutations.

## Patients and Methods

### Patients

Forty-eight families with early-onset parkinsonism caused by mutations in the *parkin* gene, including 69 patients and 49 unaffected relatives, were studied. All but four families (families TRUS and KUZ from Russia, families EGPD 25-95 and PW from Germany) have been described elsewhere (Lücking et al. 1998, 2000; Tassin et al. 1998; Abbas et al. 1999). The families were selected for *parkin* analysis according to the following criteria: (1) symptoms of parkinsonism (akinesia, rigidity, or

**Table 1**Number and Origin of Index Patients with *parkin* Mutations Detected More than Once

MUTATION <sup>a</sup>	NO. OF INDEX PATIENTS <sup>b</sup>		NO. OF HAPLOTYPES	COUNTRY OF ORIGIN <sup>c</sup> (No.)
	Familial	Isolated		
Del2	4	0	3	It (3), F (1)
Del3	9	2	8	F (3), N(3), UK (1), It (2), Po (1), R (1)
Del3-4	3	1	2	It (2), F (1), G (1)
Del4	4	0	4	F (1), It (1), UK (1), G (1)
Del5	3	0	3	F (1), It (1), V (1)
Del5-6	0	3	2	L (1), Pa (1), UK (1)
Dup3	2	1	2	It (1), Al (1), S (1)
Dup7	2	0	1	Ar (1), N (1)
Lys211Asn	2	0	2	N (2)
c.321-322insGT	2	0	1	F (1), G (1)
Gly430Asp	1	1	1	UK (1), Ir (1)
c.255delA	4	2	5	F (4), S (1), Po (1)
Arg275Trp	4	4	6	F (2), It (2), Ir (1), UK (1), G (1), G/Ir (1)
c.202-203delAG	3	3	4	UK (2), F (1), N (1), It (1), R (1)

<sup>a</sup> Del = deletion; Dup = duplication.<sup>b</sup> Because index patients were counted separately for the various repeated mutations, the total number ( $n = 60$ ) is greater than the number of families studied ( $n = 48$ ).<sup>c</sup> Al = Algeria; Ar = Argentina; F = France; G = Germany; Ir = Ireland; It = Italy; L = Lebanon; N = The Netherlands; Pa = Pakistan; Po = Portugal; R = Russia; UK = United Kingdom; V = Vietnam.

tremor), (2) marked improvement resulting from levodopa treatment, (3) age at onset  $\leq 45$  years for at least one affected sib, and (4) family history compatible with autosomal recessive inheritance (except family PW). In 13 of the 48 families, only one member was affected (referred to as isolated cases). These individuals were selected according to the same clinical criteria but had no family history of parkinsonism. They carried *parkin* gene mutations, on one or both alleles, that were detected at least twice in unrelated index patients (fig. 1B). The numbers and origins of the patients are summarized in table 1.

### Genotyping

Blood samples were taken, after written informed consent was obtained, from 69 patients and 49 unaffected relatives, and genomic DNA was extracted using standard procedures. Genotyping was performed by PCR, using the primers specified in the Genome Database (GDB), with the following microsatellite DNA markers: D6S1581 (1 cM), D6S959 (0.2 cM), D6S1579 (0.3 cM), D6S305 (0.1 cM), AFMa155td9 (0 cM), AFMb281wf1 (0.1 cM), D6S411 (0 cM), D6S1550 (1.9 cM), D6S1035 (1.1 cM), and D6S1599 (fig. 1A) (genetic distances according to the Whitehead Institute for Biomedical Research). The physical positions of markers D6S411, AFMa155td9, AFMb281wf1, D6S1550, and D6S1599 differ partly from the genetic map and were determined with the use of clones from the Sanger Center (PAC 292F10 and RP1-45F6). Marker D6S305 was positioned, on the basis of PCR results, in patients with various exon deletions (Leroy et al. 1998b; Lücking et

al. 2000). Marker D6S1599 was amplified with the following primers: D6S1599 forward, 5'-GGG TGT GCT TGG ATT CCT TCA TG-3', and D6S1599 reverse, 5'-TAG CAT GTG GAC TGC ATA TCA AC-3'. The primers were labeled by fluorescence, and PCR products were analyzed on an ABI 377 automated sequencer with the GENESCAN 3.1 and GENOTYPER 1.1.1 software pro-

**Table 2**Genotypes and Haplotypes of Index Patients with an Exon 2 Deletion in the *parkin* Gene

	IT31 (F)	IT22 (F)	F155 (F)	IT67 (F)
Origin	Italy	Italy	France	Italy
Consanguinity	No	No	No	No
Mutations	del2/del2-4	del2/del3	del2/del3	del2/ND
Marker:				
D6S1581	<u>7</u> 1	<u>8</u> 3	<u>7</u> 7	[3 7]
D6S959	<u>1</u> 1	<u>2</u> 2	<u>1</u> 2	2 2
D6S1579	<u>2</u> 5	<u>7</u> 2	<u>5</u> 5	<u>2</u> 2
D6S1599	<u>del</u> /del	<u>del</u> /1	<u>del</u> /12	[3 13]
D6S305	<u>7</u> 1	<u>3</u> 1	<u>9</u> 7	[1 3]
D6S411	<u>3</u> 4	<u>3</u> 3	<u>4</u> 3	<u>3</u> 3
AFMa155td9	<u>1</u> 3	<u>1</u> 1	<u>2</u> 2	[1 2]
AFMb281wf1	<u>3</u> 3	<u>3</u> 2	<u>3</u> 3	<u>3</u> 3
D6S1550	<u>2</u> 3	<u>4</u> 1	<u>4</u> 4	[2 3]
D6S1035	<u>2</u> 6	<u>5</u> 4	<u>4</u> 3	[4 5]

NOTE.—Reconstructed disease haplotypes bearing the analyzed mutation are underlined. The marker closest to the mutation is also underlined. When phase transmission was unknown, genotypes of the index patients are indicated in brackets. The haplotypes reconstructed in families with known consanguinity were considered as a single haplotype for statistical analysis. del = deletion; dup = duplication; F = familial parkinsonism; I = isolated parkinsonism; ND = not determined.

Table 3

Genotypes and Haplotypes of Index Patients with an Exon 3 Deletion in the *parkin* Gene

	F141 (F)	F155 (F)	F431 (F)	F192 (F)	F195 (F)	UK57 (F)	F711 (F)	IT22 (F)	KUS (F)	JMP10 (I)	UK10551 (I)
Origin	France	France	France	N	N	N	Portugal	Italy	Russia	Italy	UK
Consanguinity	Yes	No	No	No	No	No	Yes	No	No	No	No
Mutations	Del3/ Del3	Del3/ Del2	Del3/ ND	Del3/ Dup7	Del3/ Lys211Asn	Del3/ Del4	Del3/ Del3	Del3/ Del2	Del3/ /Del7	Del3/ Del2-3	Del3/ Gly430Asp
Marker:											
D6S1581	3 4	7 7	3 7	1 8	8 3	7 7	7 7	3 8	[7 8]	[1 7]	3 3
D6S959	1 2	2 1	2 2	2 2	3 5	2 2	1 1	2 2	[1 2]	[2 5]	[2 3]
D6S1579	2 2	5 5	2 2	5 2	2 2	2 2	2 2	2 7	[2 5]	[2 6]	[2 5]
D6S1599	2 2	12/del	3 2	2 3	3 2	3 2	2 2	1/del	[4 12]	13/del	2 2
D6S305	1 8	7 9	1 4	7 6	7 3	3 7	3 3	1 3	3 3	1 1	[3 7]
D6S411	3 3	3 4	3 5	3 3	3 4	3 3	3 3	3 3	3 3	[2 5]	3 3
AFMa155td9	1 1	2 2	2 2	2 2	2 2	1 2	2 2	1 1	1 1	[1 2]	[1 2]
AFMb281wf1	2 2	3 3	3 3	3 3	2 3	2 3	3 3	2 3	3 3	3 3	[2 3]
D6S1550	2 2	4 4	2 4	2 2	2 2	1 2	2 2	1 4	[2 4]	[3 4]	2 2
D6S1035	3 3	3 4	3 5	6 4	4 4	5 2	4 4	4 5	[3 5]	[3 5]	[2 4]

NOTE.—Data are as defined in Note to table 2. N = The Netherlands; UK = United Kingdom.

grams (all from Applied Biosystems). To ensure accurate sizing of the alleles, a control DNA sample of individual 1347.02, from the Centre d'Etude du Polymorphisme Humain, was tested for each marker, and allele numbers were assigned in increasing order from the smallest to the largest PCR product.

#### Haplotypes and Linkage Disequilibrium

Haplotypes were constructed manually to include a minimum number of recombinations. For each mutation, the disease-associated haplotypes (DHs) were compared among families, to detect (1) common *parkin* haplotypes that would indicate that the families were related and (2) a common ancestral DH. The haplotypes constructed in families with known consanguinity were considered as a single haplotype for statistical analysis.

The difference in allele distribution between normal and carrier chromosomes was evaluated by  $\chi^2$  and two-tailed Fisher's exact tests, with Yates correction when appropriate. The most frequent allele on disease-bearing chromosomes was defined as a single allele, and the others were pooled to form a second allele. The presence of linkage disequilibrium was tested by  $D = \sqrt{\chi^2/N}$ , where  $N$  is the total number of DHs and control chromosomes used. A  $P$  value  $<.01$  was considered to be statistically significant. The proportion of carrier chromosomes bearing the original associated allele was calculated with the equation  $\delta = (P_D - P_N)/(1 - P_N)$ , where  $P_D$  and  $P_N$  are the frequencies of carrier and normal chromosomes, respectively. The control population comprised 140 chromosomes from normal, white subjects.

#### Results

The origins of the 69 patients and 49 relatives from 48 families with early-onset parkinsonism are shown in

table 1. DHs were constructed in the 38 families in which unaffected relatives were available (tables 2-4).

#### Exon Rearrangements

Genotypes or haplotypes of patients with deletion of exon 2, 3, or 4 are shown in tables 2, 3, and 4, respectively. Patients with exon 2 deletions did not share common haplotypes, and alleles at markers D6S1579 and D6S305, which flank the deletion, also differed (table 2). Although allele 3 at marker AFMb281wf1 was present on 100% of the disease-causing chromosomes, the association was not statistically significant, because this allele was present in 80% of the control population. Interestingly, marker D6S1599, located in intron 2, was deleted with exon 2 in 3 of 4 families (table 2). This observation indicated the existence of at least two distinct breakpoints and supported the hypothesis of independent mutational events. Haplotypes of the two patients with deletions of exons 3 and 4, only one of which was associated with a deletion of marker D6S1599, also differed (data not shown).

Four of the five haplotypes that segregated with exon 4 deletions were identical at markers AFMa155td9, AFMb281wf1, and D6S1550 (table 4). However, the association was not statistically significant, because these were the most frequent alleles in the control population (50%, 82%, and 46%, respectively). Furthermore, the haplotypes for the markers closest to the deletion (D6S1599 and D6S305) differed, suggesting independent recurrent mutations (table 4). However patient IT05, who was homozygous for an exon 4 deletion and who had no known consanguinity, was homozygous for markers D6S1599, D6S305, and D6S411, which flank the deletion. Although the patient's parents were not

**Table 4**  
Genotypes and Haplotypes of Index Patients with an Exon 4 Deletion in the *parkin* Gene

	F29 (F)	DE25 (F)	UK57 (F)	IT05 (F)
Origin	France	Germany	United Kingdom	Italy
Consanguinity	No	No	No	No
Mutations	del4/ND	del4/ND	del4/del3	del4/del4
Marker:				
D6S1581	3 3	8 4	7 7	9 3
D6S959	2 5	6 1	2 2	5 1
D6S1579	2 3	2 3	2 2	2 5
D6S1599	10 3	4 1	2 3	3 3
D6S305	3 5	3 3	7 3	1 1
D6S411	3 3	3 3	3 3	4 4
AFMa155td9	2 1	2 2	2 1	3 2
AFMb281wf1	3 3	3 3	3 2	1 3
D6S1550	2 4	2 3	2 1	2 2
D6S1035	2 5	3 3	2 5	6 2

NOTE.—Data are as defined in Note to table 2.

known to be related, a common founder for the exon 4 deletion on both chromosomes may be suspected.

Eleven index patients had exon 3 deletions, the most frequent mutation in the *parkin* gene, but no significant association was observed between allele markers and the DHs (table 3). Allele 2 at marker D6S1599, the marker closest to the mutation, was present on 4 of 10 disease-bearing chromosomes but was also the most frequent allele in the control population (43%). Furthermore, patients F155 and JMP10 carried alleles 12 and 13, respectively, (whose sizes were very different from those of alleles 2 and 3), which probably arose from independent events. Patients F141 and F711 shared alleles 1-2-2 at markers D6S959, D6S1579, and D6S1599, but this association was not statistically significant, because these alleles were very frequent in the control population (32%, 50%, and 43%, respectively). This is also the case for patients F431 and UK57, who shared alleles 2-

2-3 for the same markers, and for patient F195, who had alleles 2-3 for markers D6S1579 and D6S1599. Alleles 2-2-3 were present in 57%, 50%, and 24% of the control population, respectively. Patients F155 and KUS shared alleles 2-5-12 at markers D6S959, D6S1579, and D6S1599; however, because the phase transmission for patient KUS was not known, we do not know whether these alleles were carried by the chromosome associated with the mutation. Because alleles 5 and 12 are rare in the control population (8% and 6%, respectively), these two patients may share a common haplotype, but no definite conclusion can be made.

No common haplotype, or even common marker alleles, were observed for the other exon deletions (exons 3 and 4, exons 5 and 6, exon 5) or duplications (exon 3 and exon 7) (data not shown).

*Point Mutations*

Genotypes or haplotypes of patients with the c.255delA, c.202-203delAG, and Arg275Trp mutations are shown in tables 5, 6, and 7, respectively. It is possible that F744 and S70 (alleles 3-2-2-7 at markers D6S1581, D6S959, D6S1579, and D6S1599) or S70 and UK17275 (alleles 2-7-3-3-1 at markers D6S1579, D6S1599, D6S305, D6S411, and AFMa155td9) share a common haplotype for the c.255delA mutation. No definite conclusion can be drawn, however, because the phase of transmission could not be determined for all markers or patients (table 5). However, genotype analysis revealed that, in most cases, the mutation segregated with allele 7 of marker D6S1599 (table 5), which is located 24 kb downstream of exon 2. This allele was not found in the 62 patients who did not carry the c.255delA mutation (not determined in one patient) (6/6 vs. 0/62; *P* < .0001).

Regarding allele frequencies (independent of the trans-

**Table 5**  
Genotypes and Haplotypes of Index Patients with c.255delA Mutations in the *parkin* Gene

	F171 (F)	F744 (F)	F96 (F)	S70 (I)	UK17275 (F)	S74 (I)
Origin	France	France	France	Spain	Portugal	France
Consanguinity	No	No	No	No	Yes	No
Mutations	c.255delA/Dup6	c.255delA/Del5	c.255delA/c.255delA	c.255delA/c.255delA	c.255delA/c.255delA	c.255delA/Del3-4
Marker:						
D6S1581	3 8	3 3	3 3	[3 8]	[3 8]	[1 7]
D6S959	1 5	2 1	1 2	2 2	[1 6]	2 2
D6S1579	3 6	2 5	7 7	[2 3]	[2 3]	[2 5]
D6S1599	7 2	7 4	7 9	[7 9]	7 7	[2 7]
D6S305	8 9	6 3	7 1	[3 8]	[3 6]	3 3
D6S411	4 3	3 3	4 3	3 3	3 3	[3 4]
AFMa155td9	2 1	2 2	[1 2]	1 1	1 1	2 2
AFMb281wf1	3 3	2 3	3 3	[2 3]	3 3	2 2
D6S1550	4 2	2 4	3 2	[2 4]	[1 2]	[2 3]
D6S1035	5 4	5 2	3 2	[3 4]	[3 4]	[3 4]

NOTE.—Data are as defined in Note to table 2.

**Table 6**  
**Genotypes and Haplotypes of Index Patients with the c.202-203delAG Mutation in the parkin Gene**

	IT20 (F)	UK86 (F)	NL24 (F)	TRUZ (F)	S749 (I)	UK4720 (I)
Origin	Italy	United Kingdom	Netherlands	Russia	France	United Kingdom
Consanguinity	No	No	No	No	Yes	No
Mutations	c.202-203delAG/ Lys161Asn	c.202-203delAG/ ND	c.202-203delAG/ Lys211Asn	c.202-203delAG/ ND	c.202-203delAG/ c.202-203delAG	c.202-203delAG/ c.202-203delAG
Marker:						
D6S1581	3 3	4 4	3 1	3 3	3 3	3 3
D6S959	[1 2]	6 1	2 5	[1 2]	[1 2]	[1 2]
D6S1579	[2 5]	3 4	2 2	[6 8]	4 4	3 3
D6S1599	[2 3]	3 2	2 3	[2 4]	2 2	2 2
D6S305	[3 9]	8 8	9 8	[5 6]	3 3	[1 4]
D6S411	3 3	3 1	3 3	3 3	[3 4]	3 3
AFMa155td9	2 2	[1 2]	2 1	2 2	[1 2]	[1 2]
AFMb281wf1	3 3	3 3	3 2	3 3	[2 3]	[2 3]
D6S1550	2 2	1 4	2 2	2 2	[2 4]	2 2
D6S1035	[3 6]	[3 5]	3 4	[2 3]	[4 5]	[2 5]

NOTE.—Data are as defined in Note to table 2.

mission phase), all index patients, whether homozygous or heterozygous for the c.202-203delAG mutation in exon 2, carried allele 2 at marker D6S1599 (table 6), but about half the patients with other mutations also carried this allele (6/6 vs. 29/62;  $P > .01$ ). Furthermore, in family UK86, allele 2 did not segregate with the c.202-203delAG mutation (table 6). Patients IT20, NL24, and TRUZ shared alleles 3-2-3-2 at markers D6S411, AFMa155td9, AFMb281wf1, and D6S1550, but this association was not significant because of the high frequency of these alleles in the control population (59%, 50%, 82%, and 46%, respectively). Although it cannot be proved, IT20 and NL24 may share a common haplotype over the entire region.

At marker D6S305, in intron 7, all patients with the Arg275Trp mutation in exon 7 carried allele 4 (table 7). This allele was observed in three other patients who did not carry the Arg275Trp mutation (8/8, vs. 3/61;  $P < .0001$ ). Furthermore, a common haplotype was observed between patients IT15 and IT63, who shared alleles 2-2-4-3 at markers D6S1579, D6S1599, D6S305, and D6S411; however, these alleles were the most frequent alleles in the control population (50%, 43%, and 59%, for D6S1579, D6S1599, and D6S411, respectively), except for allele 4 at marker D6S305, which was observed in only 9% in the control population. A common haplotype was also observed in UK2329 and S96 who shared alleles 3-1-2 at markers D6S411, AFMa155td9, and AFMb281wf1, with allele frequencies of 59%, 38%, and 9%, respectively. Patients IT15, IT63, S92, and UK4823 may share this haplotype, but the phase transmission was unknown for these markers.

For the other point mutations (Lys211Asn, c.321-322 insGT, and Gly430Asp), no common haplotypes or allelic associations were observed (data not shown).

#### Linkage Disequilibrium

We observed a strong linkage disequilibrium (LD) of 0.75 between the c.255delA mutation and allele 7 of marker D6S1599 (table 8). Allele 7 was present on five of seven independent chromosomes associated with the mutation, in patients from France, Portugal, and Spain (table 5, excluding patient S74, in whom the allele cannot be attributed to the mutation) but was absent from the control population ( $P < .0001$ ) (table 8). However, patient F96 (from France) and patient S70 (from Spain), both of whom were homozygous for the c.255delA mutation, carry alleles 7 and 9 at marker D6S1599 (table 5). This observation could be explained by the existence of two distinct founder mutations or by a recombination between the mutation and marker D6S1599. Alternatively, a mutation in marker D6S1599 could have changed allele 7 to 9.

At locus D6S1599, allele 2 was present on 4 of 5 of the chromosomes associated with the c.202-203delAG mutation for whom the phase transmission was known. Because allele 2 of marker D6S1599 was also very frequent in the control population (43%), this association was not statistically significant ( $P > .01$ ) (table 8).

At locus D6S305, (LD = 0.24) was observed between allele 4 and the Arg275Trp mutation (table 8). Allele 4 of marker D6S305 was present in five of five patients from France, Germany, and Italy on chromosomes carrying the mutation for which the phase transmission was known (table 7). This association was statistically significant since allele 4 was present in only 28% of the control chromosomes ( $P < .003$ ). LD for marker AFMb281wf1 could not be tested, because the phase transmission was known in only three subjects. However, the frequency of allele 2 was much higher in the

Table 7

## Genotypes and Haplotypes of Index Patients with the Arg275Trp Mutation

	IT15 (F)	IT63 (F)	UK 2329 (I)	S92 (I)	S96 (I)	UK 4823 (I)	PW (F)	EGPD 25-95 (F)
Origin	Italy	Italy	Ireland	France	France	United Kingdom	Germany	Germany/Ireland
Consanguinity	No	No	No	No	No	No	No	No
Mutation	Arg275Trp/ ND	Arg275Trp/ ND	Arg275Trp/ Gly430	Arg275Trp/ del3-6	Arg275Trp/ ND	Arg275Trp/ del5-6	Arg275Trp/ 40pbdel3	Arg275Trp/ ND
Marker:								
D6S1581	4 8	7 3	3 8	3 3	3 3	3 3	3 3	7 3
D6S959	[1 2]	1 1	2 5	2 1	[1 2]	[1 2]	ND	1 2
D6S1579	2 5	2 7	2 5	2 2	[2 5]	3 3	2 2	ND
D6S1599	2 2	2 4	10 13	4 4	[3 4]	2 2	ND	3 2
D6S305	4 8	4 3	[4 3]	4 8	[4 5]	[4 1]	4 3	4 9
D6S411	3 3	3 3	3 3	3 3	3 3	3 3	2 1	[4 6]
AFMa155td9	[1 2]	[1 2]	1 1	[1 2]	1 1	[1 2]	3 2	1 1
AFMb281wfl	[2 3]	[2 3]	2 2	[2 3]	2 2	[2 3]	2 2	[2 3]
D6S1550	2 2	2 4	ND	2 2	2 2	ND	[3 4]	3 3
D6S1035	7 3	3 5	3 2	4 5	[2 4]	[2 5]	5 4	6 4

NOTE.—Data are as defined in Note to table 2.

eight patients with the Arg275Trp mutation compared with the control population (68% and 9%, respectively) ( $P < .0001$ ). It is possible that allele 2 is carried by all the chromosomes carrying the Arg275Trp mutation.

## Discussion

To discriminate between single founder effects and independent recurrent events, DHs in the *parkin* region were established for intragenic and tightly flanking markers in 48 families with repeatedly observed mutations in the *parkin* gene.

The disease-associated alleles and haplotypes in families with the same exonic rearrangement were discordant in most of the families, even for markers located close to the rearrangements. Furthermore, genotypes of marker D6S1599 revealed at least two distinct break-points for exon 2 deletions in four unrelated families. These results suggest that these recurrent mutations originated independently, although we cannot exclude a very ancient founder effect associated with recombinations or mutations involving tightly linked markers. The hypothesis of independent recurrent events was recently confirmed by molecular analysis of the break-points of the *parkin* gene in patients with some of the deletions analyzed here (Asakawa et al. 2000). These deletions were classified into 18 types. However, one type of exon 4 deletion was commonly found among six families. This may reflect the case of our patient IT05, who is homozygous, without known consanguinity, for an exon 4 deletion, as well as for three markers flanking the deletion. Thus, even if de novo mutations occur regularly, a given exon deletion that is transmitted to subsequent generations becomes a new founder for this deletion.

Unequal inter- or intrachromosomal crossovers that

result from the misalignment of two homologous flanking sequences may account for the existence of deletions and for duplications of the same regions of the *parkin* gene, as has been reported elsewhere in several genes, such as the steroid sulfatase gene (Yen et al. 1990) or the  $\alpha$ -globin locus (Nicholls et al. 1987). Unequal crossovers, which result in duplications and deletions of a 1.5-Mb region of chromosome 17p11.2, give rise, respectively, to Charcot-Marie-Tooth Type 1A (CMT1A) (Lupski et al. 1991) and hereditary neuropathy with liability to pressure palsies (HNPP) (Chance et al. 1993). Excisions of an intrachromatid loop, resulting exclusively in deletions, also occur in this region of 17p11.2 (Kiyosawa et al. 1995; Lopes et al. 1998; Lopes et al. 1999). If both these mechanisms also cause exon rearrangements in the *parkin* gene, this would explain why deletions appear to be more frequent than duplications (29 vs. 5 in our series) in the *parkin* gene.

Transposable elements (TEs), such as Alu elements, are also involved in recombination. Almost 0.5% of known genetic disorders result from TE insertions or TE-mediated recombination (Deragon and Capy 2000; Deininger and Batzer 1999). TEs are relatively abundant in the genome (~1 Alu/6 kb). The density of Alu elements in the first 130 kb of intron 2 of the *parkin* gene, which is involved in the deletion of exons 2 and 3, reaches 1 Alu/2.5 kb. This may explain the frequency of deletions of exons 2, 3, and 3-4, duplications of exon 3, and triplication of exon 2.

Unlike the exon rearrangements, point mutations in the patients studied may be accounted for by a limited number of founders. Strong linkage disequilibrium was observed between the c.255delA mutation in exon 2 and allele 7 of marker D6S1599 in intron 2 (table 5) and between the Arg275Trp mutation in exon 7 and allele 4 of marker D6S305 in intron 7 (table 7). These

Table 8

Linkage Disequilibrium for Three-Point Mutations Found Two or More Times in the *parkin* Gene

Mutation	Locus	Allele	P <sub>D</sub> <sup>a</sup>	P <sub>N</sub> <sup>b</sup>	χ <sup>2</sup>	P	P <sub>corrected</sub> <sup>c</sup>	D <sup>d</sup>	δ <sup>d</sup>
c.255delA	D6S1599	7	5/7	0/124	73.65	<.0001	<.001	.75	.75
c.202-203delAG	D6S1599	2	4/5	53/124	1.40	>.01	>.1	ND	ND
Arg275Trp	D6S305	4	5/5	39/140	8.71	<.003	<.03	.24	1

<sup>a</sup> The frequency of carrier chromosomes is calculated by  $P_D = (n_{\text{patient}}/N)$ , where  $N$  is the number of haplotypes in which the allele segregating with the mutation could be determined.

<sup>b</sup> The frequency of control chromosomes is calculated by  $P_N = (n_{\text{control}}/N)$ , where  $N$  is the number of control chromosomes used.

<sup>c</sup> P value was corrected by a factor 10 (correction of Bonferroni), because the same control population was used to test allelic frequencies of the 10 microsatellites.

<sup>d</sup> ND = not determined.

associations may reflect the transmission of a single ancestral mutation or may be caused by recurrent mutations on a predisposing haplotype, as shown in spinocerebellar ataxia 7 (Stevanin et al. 1999). Interestingly, alleles at markers D6S1579 and D6S305, which cover a 0.3-cM interval that includes D6S1599, were not conserved in patients with the c.255delA mutation in exon 2. These observations indicate that multiple recombinations and/or mutations have modified the alleles that segregate with the closely flanking markers, suggesting that this mutation can be attributed to very ancient founder effects. The geographic diversity of the patients carrying the same allele also supports this hypothesis: patients with the c.255delA mutation come from France, Spain, and Portugal (tables 5-7).

In contrast, the Arg275Trp mutation may be more recent, because a common ancestral haplotype between markers D6S1579 and D6S1550 is suspected. Patients IT15 and IT63 shared a haplotype between markers D6S1579 and D6S1550 (not proved for markers AFMa155td9 and AFMb281wf1, because the phase was not determined). Furthermore, patients UK2329 and S96 (who were from Ireland and France, respectively) and probably patients S92 and UK4823 (who were from France and the United Kingdom) also shared part of this haplotype between marker D6S411 and marker AFMb281wf1. Although these associations were not statistically significant for the majority of the markers, associations between the Arg275Trp mutation and the markers D6S305 and AFMb281wf1 were significant ( $P < .001$ ). The ancestral founder haplotype could thus have been 2-2-4-3-1-2-2 for markers D6S1579 to D6S1550 before a recombination separated haplotype 2-2 (marker D6S1579 and D6S1599) from haplotype 4-3-1-2-2 (markers D6S305 to D6S1550). Because the suspected ancestral haplotype was observed in two Italian patients, the mutation may have arisen in Italy.

Allele 2 of marker D6S1599 was present in the six index patients with the c.202-203delAG mutation in

exon 2 and on four of the five chromosomes associated with the mutation. The association was not statistically significant, because allele 2 is also very frequent in the control population. Because of the absence of other known markers in the introns flanking this mutation, we cannot distinguish between a single founder effect on a chromosome carrying allele 2 or recurrent mutations on chromosomes that carry this allele by chance.

It is also difficult to draw conclusions concerning the three other point mutations analyzed in this study (Lys211Asn, c.321-322insGT and Gly430Asp). No allelic associations were observed. It is difficult, however, either to demonstrate or to exclude a founder effect because of (1) the small number of index patients, (2) the lack of polymorphic markers in flanking introns (except for mutation c.321-322insGT in exon 3), and (3) the absence of relatives who, if available, would enable us to determine the phase of transmission.

The release of sequence data for the *parkin* region of chromosome 6, which is expected in the near future, should allow us to clarify some of the patients in this study. The sequencing of a contig of BACs that contain the *parkin* gene is in progress, and some of the data are already available. It will therefore be possible to define new microsatellite markers in each intron of the *parkin* gene and to detect ancient founder effects. In addition, single-nucleotide polymorphism (SNP) databanks, which help in the reconstruction of haplotypes, are also being created. Only one SNP in the *parkin* gene, localized in intron 8, has been identified to date. The known SNPs in the coding region of the *parkin* gene (Abbas et al. 1999) were not informative for the present study.

In conclusion, a wide variety of mutations in the *parkin* gene have been found repeatedly in patients from many European populations. Interestingly, the majority of exon rearrangements seem to result from distinct mutational events, whereas at least two point mutations may have arisen from a small number of founders. Therefore, the mechanisms underlying these two groups

of mutations appear to be different. According to this hypothesis, the frequency of exon rearrangements would be expected, in the absence of selection, to increase with the passage of time because of new mutational events, whereas the frequency of point mutations would be expected to remain stable, because new mutations would be rare.

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## Appendix

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## Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Centre d'Etude du Polymorphisme Humain, <http://www.cephb.fr> (for the control DNA sample)

DNA Databank of Japan, <http://www.ddbj.nig.ac.jp> (for the

cDNA sequence of the *parkin* gene [accession number AB009973])

Genome Database, <http://www.gdb.org> (for primer sequences)  
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (for early-onset parkinsonism [MIM 600116])

Sanger Center, <http://www.sanger.ac.uk> (for the clone 292F10 and RP1-45F6 sequences [accession numbers 2760544 and 5924005, respectively])

Whitehead Institute for Biomedical Research, <http://www.genome.wi.mit.edu> (for integrated map of chromosome 6)

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# Parkinson's Disease Is Not Associated with the Combined $\alpha$ -Synuclein/Apolipoprotein E Susceptibility Genotype

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A recent study showed significant association of sporadic Parkinson's disease with a polymorphism within the  $\alpha$ -synuclein gene and closely linked DNA markers on chromosome 4q and the *APOE*  $\epsilon 4$  allele. A combined  $\alpha$ -synuclein/*APOE*- $\epsilon 4$  genotype increased the relative risk of developing Parkinson's disease 12-fold. We failed to confirm this association in a much larger sample of histopathologically proven cases of Parkinson's disease and controls.

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Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer disease (AD), affecting 2% of the population over 65 years of age.<sup>1</sup> It is characterized by an akinetic rigid syndrome with bradykinesia, rigidity, and tremor.

The segregation of four disease-causing loci in rare autosomal dominant PD kindreds confirms not only that a genetic component exists, but that PD is genetically heterogeneous.<sup>2</sup> Sporadic PD is probably a complex trait which is non-Mendelian and multifactorial in aetiology owing to the interaction of one or more sus-

ceptibility genes together with hitherto undefined variable environmental influences. Association studies test whether alleles of a genetic marker (polymorphism) occur at different frequencies in cases compared with controls. If a true association emerges, either the polymorphism itself is the susceptibility locus or it is in linkage disequilibrium with the susceptibility locus.<sup>3</sup> In either case it should enable disease mapping and the identification of people at risk of developing disease.

Several functionally relevant associations in sporadic PD have been reported.<sup>2</sup> Krüger and colleagues recently investigated polymorphisms in two genes that are involved in neurodegeneration: the  $\alpha$ -synuclein (*SNCA*) and the apolipoprotein E (*APOE*) genes.<sup>4</sup> *SNCA* is mutated in a small subset of autosomal dominant PD kindreds,<sup>5,6</sup> and the  $\alpha$ -synuclein protein is a major fibrillar component of Lewy bodies (LBs).<sup>7</sup> The overlaps in clinicopathology of PD and AD may imply overlaps of susceptibility to age modulation of the disease by the *APOE*- $\epsilon 4$  allele. Krüger and colleagues identified a significant allelic association with PD of an *SNCA* polymorphism (NACP-Rep), closely linked DNA markers D4S1647 and D4S1628, and the *APOE*- $\epsilon 4$  allele, in up to 163 clinical PD cases in a German population. A combined *APOE*- $\epsilon 4$ /NACP-Rep genotype increased susceptibility to PD by 12-fold.<sup>4</sup>

We genotyped the same NACP-Rep polymorphism, adjacent chromosome 4q markers, and *APOE* in a much larger, mostly histopathologically proven PD population (305 cases) and a larger number of closely matched control samples (330 cases). Our PD samples also included a cohort of familial cases (FPD) and a larger number of younger onset cases (YPD). We also investigated a novel polymorphism of the intron 4 region of *SNCA* (IN4). In our analysis, susceptibility to PD was observed neither with any of the above markers nor with the combined NACP-Rep/*APOE*- $\epsilon 4$  genotype.

## Patients and Methods

The study population consisted of 305 unrelated PD cases (Table 1). Pathological confirmation was obtained for 170 samples from the U.K. Parkinson's disease Society (PDS) Brain Research Centre and the Institute of Psychiatry Brain Bank, London, U.K. All showed severe depletion of pigmented neurons in the substantia nigra and locus coeruleus and the presence of LBs. There were no glial cytoplasmic inclusions or additional pathology to account for parkinsonism. The remaining 135 samples fulfilled PDS Brain Bank criteria for the diagnosis of PD.<sup>8</sup> Of the clinical subjects 66 were FPD (having a sibling and/or an affected parent with PD). All subjects gave informed consent. The project was approved by the Joint Ethics Committee of the National Hospital for Neurology and Neurosurgery (NHNN). The 305 samples were also subdivided into YPD [mean onset age  $44 \pm 6$  years standard deviations (SD);  $n = 89$ ] and late-

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Table 1. Characteristics of PD Cases and Controls

Characteristics	PD Cases	Controls
Number	305	330
Age (mean years) ( $\pm$ SD)	61 (6)	63 (7)
Male (%)	55	53
Familial/nonfamilial	66/239	NA
YPD/LPD	89/216	NA
Histopathological cases	170	175
Clinical cases	135	155

PD = Parkinson's disease; SD = standard deviation; YPD/LPD = young onset PD/late-onset PD.

onset PD (LPD) (mean onset age  $63 \pm 7$  years SD;  $n = 216$ ).

Controls were selected to closely match age and sex of the PD population. We used 330 control samples of which 175 were from brain tissue with no abnormal histopathology, from the MRC Neurochemical Pathology Unit, Newcastle, U.K., and 155 clinical cases with no parkinsonism from the NHNN (52 cases of hereditary sensory motor neuropathy and 103 cases of spinocerebellar ataxia).

We extracted DNA from brain tissue and blood by use of standard methods.

#### $\alpha$ -Synuclein Promoter Polymorphism

The allele status of the NACP-Rep dinucleotide marker in the promoter region of *SNCA*<sup>9</sup> was determined by polymerase chain reaction (PCR) with the flanking primers: Rep1: (5' GCAATAGGAGTAGACAAAACGGATCG 3') and Rep2: (5' CTACATGACTGCGCCCAAGATTTAA 3'). PCR amplification was performed as previously described.<sup>4</sup> Amplimers were analysed on a Perkin-Elmer ABI 377 automated sequencer equipped with Genescan (v 3.1) software. Alleles were sized and assigned with Genotyper (v 2.5). Three alleles—Allele 0 (257 bp), Allele 1 (259 bp), and Allele 2 (261 bp)—as first described by Xia and coworkers<sup>9</sup> were identified (redesignated 3, 2, and 1, respectively, in Ref 4).

#### Markers of Chromosome 4q21-23 Region

Tetranucleotide (D4S1628, D4S1647) and dinucleotide [D4S1578 (used instead of D4S2422), D4S423, D4S2460] repeat markers were genotyped as described (GDB accession numbers 686793, 691159, 245064, 62948, and 424508, respectively). Alleles were sized and assigned as described for NACP-Rep D4S2460: 8 alleles (175–189 bp); D4S423: 12 alleles (100–122 bp); D4S1578: 10 alleles (210–32 bp); D4S1628: 4 alleles (146–158 bp) (corresponding to 149–161 bp, respectively, in Ref 4); and D4S1647: 6 alleles (132–152 bp, identical to those in Ref 4).

#### *SNCA* Intron 4 Polymorphism

Allele status for this TC-rich polymorphism approximately 0.9 kb downstream from the Exon 4/Intron 4 boundary (Dr E. Masliah, UCSD, personal communication) was determined by PCR using the flanking primers intron 4 Polymorphism (IN4) IN4F: 5' ATTCTTCTCACCTCTGGTATTC3' and IN4R: 5' TTAAAGGTGAATAACACTTTGGC 3'. Amplification was performed in the presence of 5% di-

methylsulphoxide with identical PCR conditions used for NACP-Rep. Four alleles were identified: 265, 266, 288, and 366 bp.

#### Apolipoprotein E Genotyping

Apolipoprotein E genotyping was performed as previously described.<sup>10</sup>

#### Statistical Analysis

Allele frequencies rather than genotype frequencies were analyzed to improve power. However, this method assumes Hardy-Weinberg equilibrium (HWE). The HWE assumption was tested for each marker using an exchangeable kappa statistic. The allelic association test is a  $\chi^2$  test on the  $m \times 2$  table where  $m$  is the number of alleles. When  $m$  is large for highly polymorphic markers the table may be sparse. This may invalidate the asymptotic sampling distribution of the Pearson  $\chi^2$  statistic. The table was therefore analyzed using Monte-Carlo methods as described.<sup>11</sup> Linkage disequilibrium was tested between pairs of markers using an expectation-maximization algorithm to resolve phase.<sup>12</sup>

#### Results

##### *NACP-Rep, Intron 4 Polymorphism, and Markers of Chromosome 4q21-22*

In this population  $\chi^2$  analysis confirms the complete absence of allelic association between any markers of the chromosome 4q21-23 and PD (Table 2). In particular, we failed to replicate the independent association of disease with alleles of NACP-Rep allele 2 (261 bp; designated allele 1 in Ref 4), D4S1628 (158 bp), or D4S1647 (140 bp) previously observed<sup>4</sup> despite the comparable allelic distributions in U.K. and German control samples. In addition, we found no association of IN4 (see Tables 2 and 3) and NACP-Rep Allele 2 and with the YPD ( $\chi^2 = 1.04$ ;  $p = 0.677$ ) and FPD ( $\chi^2 = 0.341$ ;  $p = 0.84$ ) subgroups.

Linkage disequilibrium was not detected between pairs of Chromosome 4 markers, NACP-Rep, or IN4.

##### APOE

In a total of 305 PD samples and 330 age- and sex-matched controls, we found no difference in the allele

Table 2. Comparison of Allele Frequencies between PD Cases and Controls

Marker	Number of Alleles	Full Table $\chi^2$	Empirical $p$ Value
D4S2460	8	3.72	0.89
D4S423	12	13.3	0.365
D4S1578	10	15.55	0.09
D4S1628	4	3.47	0.39
D4S1647	6	3.97	0.615
NACP-Rep	3	5.10	0.26
IN4	4	1.00	0.8
APOE	3	1.10	0.505

Table 3. Allele Frequencies of NACP, D4S1628, and D4S1647 in Control and Parkinson's Disease (PD) Cases

	257 bp (allele 0)		259 bp (allele 1)		261 bp (allele 2)	
NACP-Rep	257 bp (allele 0)		259 bp (allele 1)		261 bp (allele 2)	
Controls	0.261		0.655		0.084	
PD cases	0.251		0.694		0.055	
IN4	265 bp	266 bp	288 bp	366 bp		
Controls	0.490	0.198	0.069	0.242		
PD cases	0.474	0.187	0.077	0.262		
D4S1628	146 bp	150 bp	154 bp	158 bp		
Controls	0.059	0.328	0.510	0.102		
PD cases	0.046	0.286	0.547	0.123		
D4S1647	132 bp	136 bp	140 bp	144 bp	148 bp	152 bp
Controls	0.164	0.028	0.180	0.312	0.231	0.085
PD cases	0.194	0.028	0.181	0.332	0.185	0.080

distribution of *APOE* ( $\chi^2 = 1.09$ ; empirical  $p = 0.05$ ). The *APOE*- $\epsilon 4$  allele did not have a significant association with either the YPD group ( $\chi^2 = 4.21$ ; empirical  $p = 0.14$ ) or FPD group ( $\chi^2 = 0.357$ ;  $p = 0.77$ ).

#### Combined $\alpha$ -Synuclein/Apolipoprotein E Genotype

It is notable that we were unable to replicate the finding of a 12-fold increased risk of PD with a combined *APOE*- $\epsilon 4$ /NACP-Rep Allele 2 genotype ( $p = 0.96$ ).

#### Discussion

Population-based genetic association studies can be a powerful method of identifying disease susceptibility loci.<sup>3</sup> However, this requires associations to be confirmed by larger study numbers with stringent standardization of control samples. In our sample of 305 PD cases and 330 controls, we did not observe an increased susceptibility to disease due to the NACP-Rep Allele 2, D4S1628 (158 bp), D4S1647 (140 bp), and *APOE*- $\epsilon 4$  or the combined NACP-Rep Allele 2/*APOE*- $\epsilon 4$  genotype. This is in contrast to the finding by Krüger and coworkers.<sup>4</sup> We failed to detect an association with additional markers D4S2460 and D4S423 that lie closer to and flank *SNCA*. The order of markers used in this study is cen-D4S2460-NACP Rep/IN4-D4S423-D4S2380-D4S1578-D4S1628-D4S1647-tel (www.ncbi.nlm.nih.gov, Stanford Radiation Hybrid Map, ftp://cedar.genetics.soton.ac.uk), and data indicate that D4S1647 and D4S1628 lie up to 8 MB distal to *SNCA*.

There are several additional explanations for the discrepancy, including differences in population stratification, statistical artifact, small sample sizes, different phenotypes, poorly matched controls, and the biological credibility of the gene-disease association.<sup>3,13</sup>

It is known that up to 25% of clinical subjects with PD do not have characteristic histopathological lesions at autopsy.<sup>14</sup> In our study, the majority of PD cases were pathologically proven. Furthermore, possible differences in disequilibrium levels between populations from the United Kingdom and Germany may not be as significant.<sup>15,16</sup> We used a much larger sample size

and stringent statistical analysis. In addition, the power of both our study and that of Krüger and coworkers<sup>4</sup> would be lower owing to the low frequency of the NACP-Rep Allele 2 (261 bp) and *APOE*- $\epsilon 4$  in the population. However, it is surprising that we failed to detect any association whatsoever in light of the previously reported significantly increased risk.<sup>4</sup> Even larger controlled studies on the order of 1,000 cases with at least as many controls<sup>13</sup> and a dense linkage disequilibrium map of single-nucleotide polymorphisms stemming from the Human Genome Mapping Project<sup>17</sup> may be necessary to resolve this discrepancy definitively. Our results cast considerable doubt on the importance of the combined  $\alpha$ -synuclein/apolipoprotein E genotype as a risk factor in sporadic PD.

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nels and is highly expressed in the cerebellum. Using a pointing paradigm and infrared optoelectronic tracking system, we found subclinical hypermetria and other subtle cerebellar signs in the common forms of migraine. These were more pronounced in migraine with than without aura. Whether this reflects involvement of  $\text{Ca}^{2+}$  channel genes in the common types of migraine needs to be investigated by genetic analyses.

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Recent genetic findings suggest that one important pathogenetic aspect in migraine might be an alteration in central nervous system ion channels. Mutations in the chromosome 19p13 gene CACNA1A coding for the  $\alpha_1$  subunit of a neuronal P/Q- $\text{Ca}^{2+}$  channel can cause familial hemiplegic migraine (FHM), a rare subtype of migraine with aura (MA), and episodic ataxia Type 2.<sup>1</sup> In FHM, permanent cerebellar ataxia and cerebellar atrophy can be associated features.<sup>2</sup> P/Q-type  $\text{Ca}^{2+}$  channels are strongly expressed in the cerebellum.<sup>3</sup> Furthermore, subclinical abnormalities of neuromuscular transmission (depending on P/Q- $\text{Ca}^{2+}$  channels) were found in subgroups of MA patients<sup>4</sup> and in a CACNA1A mouse mutant.<sup>5</sup> The CACNA1A locus is linked to common forms of migraine<sup>6,7</sup> in which interictal finger dexterity was found to be impaired.<sup>8</sup>

On this background we searched for subclinical cerebellar signs in migraine patients using repetitive, free, and unrestrained reaching movements of the arm.

#### Participants and Methods

All subjects were right-handed: 15 healthy volunteers (HV) [8 females, age  $31 \pm 8$  years (mean  $\pm$  standard deviation [SD])] without personal or familial history of headache and 35 migraine patients from a specialized headache clinic without regular medication and with normal neurological examination. According to International Headache Society criteria,<sup>9</sup> 16 patients had migraine without aura (MO) and 19 MA with the following characteristics: 12 MO/10MA females, age  $36 \pm 9/28 \pm 14$  years, disease duration  $15 \pm 12/14 \pm 12$  years, attack frequency  $3 \pm 2/2 \pm 1$  per month. They were studied at least 3 days after and before an attack (checked by telephone interview). The study was approved by the local ethics committee; informed consent was obtained.

Movements were recorded at 100 Hz in three dimensions (3D) using an infrared optoelectronic-tracking system (ELITE™, BTS Milan, Italy) with a reflective marker attached to the tip of the index finger and another marker to the movement target.

Participants were seated with the target in the midsagittal plane on eye level (Fig 1). They were instructed to start with the right arm extended to the right, touch the target with high precision (but quickly) without any trunk movement, go back to the starting position, and repeat above movements, in a given pace over 15 seconds (one trial), to result in 8 to 10 movements. After one practice trial, trials

## Subclinical Cerebellar Impairment in the Common Types of Migraine: A Three-Dimensional Analysis of Reaching Movements

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Mutations in the CACNA1A gene can cause familial hemiplegic migraine (FHM) and/or cerebellar ataxia. CACNA1A codes for the  $\alpha_1$  subunit of P/Q- $\text{Ca}^{2+}$  chan-

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