Clinical and molecular genetic study of movement disorders

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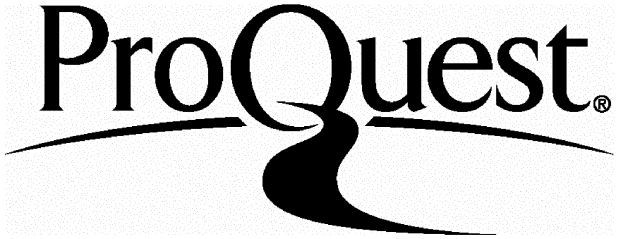

ABSTRACT

This thesis describes a molecular genetic study of three groups of inherited movement disorders: primary torsion dystonia (PTD), paroxysmal dyskinesias and autosomal recessive juvenile parkinsonism (ARJP). The principal methodology employed in the study of these disorders was genetic linkage analysis. Sets of highly polymorphic microsatellite markers were used to map the subchromosomal location of the disorders as the first step in a positional cloning or positional candidate strategy for disease gene identification.

Primary Torsion Dystonia

The prevalence of the DYT1 gene in the British population was analysed and genotype phenotype correlations were drawn. Moreover, a detailed haplotype analysis in Ashkenazi Jewish and non-Jewish British dystonic patients carrying the GAG deletion in the DYT1 gene and linkage disequilibrium analysis demonstrated a limited number of founder mutations in the United Kingdom.

Three large PTD families were ascertained, and a detailed description of clinical features obtained. These families were analysed for linkage between the disease and all PTD loci (DYT1, DYT6 and DYT7). Exclusion of linkage with the known PTD loci in these three families indicated the existence of at least one more genetic locus for PTD.

In family PTD01 a genome-wide search was performed, and a novel PTD locus (DYT13) was mapped to a 22 cM region on the short arm of chromosome 1. In families PTD02 and PTD03 linkage with this novel locus was also excluded, further supporting the wide genetic heterogeneity of primary torsion dystonia.

Paroxysmal dyskinesias

Three large families with paroxysmal kinesigenic dyskinesia were ascertained and clinical details were collected. A genome-wide analysis on the largest family mapped the disease on the pericentromeric region of chromosome 16, nearby but clearly separated from the only PKD locus so far identified and from the ICCA locus (another paroxysmal movement disorder associated with benign infantile convulsions). These data demonstrate the existence of a second PKD locus and are strongly suggestive of a cluster of genes for paroxysmal dyskinesias on human chromosome 16.

One large British family with paroxysmal exercise-induced dyskinesia and migraine was tested for linkage with microsatellite markers spanning paroxysmal dyskinesia and hemiplegic migraine loci (PNKD on chromosome 2q, PKD and ICCA on chromosome 16, FHM on chromosome 19p). Exclusion of linkage with all tested loci indicates the existence of a novel PED locus in this and possibly in other families.

Autosomal recessive juvenile parkinsonism

A large consanguineous family from Italy was ascertained and detailed clinical analyses were performed. A novel locus for autosomal recessive juvenile parkinsonism (PARK6) was identified by means of homozygosity mapping on the short arm of chromosome 1, in a 12.5 cM region. Linkage with PARK6 was further confirmed in other eight European families, allowing preliminary genotype-phenotype correlates and the refinement of the linked interval to 7.4 cM (4.2 Mb on the physical map).

These findings are discussed and future directions of study for identification of the disease genes involved are suggested.

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ABBREVIATIONS

A	adenine
AJ	Ashkenazi Jewish
APS	ammonium persulphate
ARJP	autosomal recessive juvenile parkinsonism
Ala	alanine
bp	base pair
C	cytosine
cDNA	complementary DNA
CEPH	Centre d'Études du Polimorphisme Humaine
CHLC	Cooperative Human Linkage Centre
cM	centimorgan
CNS	central nervous system
CSE	paroxysmal choreoathetosis and spasticity
CT	computerised tomography scan
dNTP	deoxynucleotide triphosphate
DAQ	dopamine-quinone
DNA	deoxyribonucleic acid
DRD	dopa-responsive dystonia
DYT1 – 13	dystonia gene symbols 1 - 13
DZ	dizygotic
EDTA	ethylene diamine tetraacetate
EEG	electroencephalogram
EKD2	episodic kinesigenic dyskinesia type 2 locus
EMG	electromyography
EST	expressed sequence tag
FPD1	familial paroxysmal dyskinesia type 1 locus
HGMP	Human Genome Mapping Project
ION	Institute of Neurology, London
G	guanine
GCH1	GTP cyclohydrolase gene
GDB	The Genome Database
GSPD	Genetic Susceptibility in Parkinson's Disease (Consortium for)

HGMP	Human Genome Mapping Project
5-HT	5-hydroxytryptamine
http	hypertext transmission protocol
IBD	identity by descent
IBS	identity by state
ICCA	infantile convulsions and choreoathetosis syndrome
kb	kilobase
L	likelihood
LEDD	levodopa equivalent daily dose
LOD	logarithm of odds ratio
Lys	lysine
Mb	megabase
mM	millimolar
MMSE	Mini Mental State Examination
MR	magnetic resonance scan
MZ	monozygotic
NCBI	National Centre for Biotechnology Information
NHNN	National Hospital for Neurology and Neurosurgery, London
nm	nanometer
OD	optical density
OMIM	On-line Mendelian Inheritance in Man database
PARK1-8	Parkinson's disease gene symbols 1 - 8
PCR	polymerase chain reaction
PED	paroxysmal exercise-induced dystonia
PD	Parkinson's disease
PDC	paroxysmal dystonic choreoathetosis
PKD	paroxysmal kinesigenic dyskinesia
PNKD	paroxysmal non kinesigenic dyskinesia
Pro	proline
PTD	primary torsion dystonia
RFLP	restriction fragment length polymorphism
SCL4A3	anion exchanger subtype, gene symbol
SD	standard deviation
SNP	single nucleotide polymorphism
T	thymine

TBE	tris-borate EDTA solution
TE	tris EDTA solution
TEMED	tetramethylethylenediamine
Thr	treonine
UCSC	University of California Santa Cruz
UPDRS	unified Parkinson's disease rating scale
VNTR	variable number tandem repeats
w	map distance
wt	wild type
WD	Wilson's disease
Z	LOD score

INTERNET SITES

- National Centre for Biotechnology Information (NCBI): http://www.ncbi.nlm.nih.gov/
- University of California Santa Cruz's (UCSC) Human Genome Working Draft: http://www.genome.ucsc.edu/
- Online Mendelian Inheritance in Men (OMIM): http://www3.ncbi.nlm.nig.gov/Omim/
- Marshfield Centre for Medical Genetics (genetic maps): http://www.marshfieldclinic.org
- The Genetic Location Database (LDB): http://www.cedar.genetics.soton.ac.uk/public_html/ldb.html
- UK Human Genome Mapping Project (HGMP): http://www.hgmp.mrc.ac.uk
- The Genome Database (GDB): http://gdbwww.gdb.org/
- Ensembl project: http://www.ensembl.org
- The Sanger Centre: http://www.sanger.ac.uk

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CHAPTER 1 - INTRODUCTION

The discovery of human genes and of functional mutations within these genes leading to diseases is of fundamental importance to genetics and medicine. The ability of researchers to isolate and clone the genes underpinning human diseases is the central tenet of the doctrine of 'the new genetics' (Comings, 1979). It has led to insights into the underlying biological mechanisms of disease, the development of molecular diagnostic and predictive testing, and may ultimately enable the development of rational forms of treatment, including, it is hoped, gene therapy. It is no exaggeration to assert that 'the new genetics' has revolutionised both genetics and medicine (Collins, 1992).

The human genome contains approximately 3×10^9 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). Identification of a point mutation in a gene, with no prior knowledge about its function, may be compared to searching for a needle in a haystack. Technological and methodological advances over the last two decades, however, have paved the way for the molecular characterisation of many disease genes. The availability of increasingly dense and polymorphic genetic maps of the human genome, in tandem with advances in the analysis of the markers comprised in these maps, have made possible an exponential increase in the numbers of disease genes isolated. In 1992 some 13 disease genes had been positionally cloned; by late 1995 more than 60 had been cloned based on their position, and more than 500 genes had been mapped to specific chromosomal locations (Collins, 1995; Lander and Kruglyak, 1995).

The announcement of the complete sequencing of the human genome in February 2001 has in many ways changed the approaches to the identification of disease genes. This draft sequence, independently generated by a public (Human Genome Mapping Project) and a private (Celera Genomics) effort, provides the researcher with the first comprehensive integration of diverse genomic resources, mostly accessible through free on-line databases. The ultimate goal of the Human Genome Mapping Project, still under completion, is to produce a single continuous sequence for all human chromosomes and to delineate the position of all genes. The working draft sequence described by the International Human Genome Sequencing Consortium was constructed by melting together sequence segments derived from over 20000 large-

insert clones. All of the results of this analysis are available on a web site maintained by the University of California at Santa Cruz (http://genome.ucsc.edu). Over the next few years, draft quality sequence will be steadily replaced by more accurate data. The National Centre for Biotechnology Information (NCBI – http://www.ncbi.nlm.nih.gov/) has developed a system for rapidly regenerating the genomic sequence and gene annotation, as sequences of the underlying clones are revised. Other databases apply a variety of approaches to large-scale annotation of genes and other features. One such database is Ensembl, a joint project of the European Bioinformatics Institute (EBI) and the Sanger Centre (http://www.ensembl.org; http://www.sanger.ac.uk).

Among the 25000-35000 estimated human genes, more than 10000 of them have been catalogued in the "Online Mendelian Inheritance in Men" (OMIM – http://www3.ncbi.nlm.nih.gov/Omim/), which documents all inherited human diseases and their causal gene mutations. The mapping of ESTs, gene predictions, STSs and SNPs onto the draft sequence can enable identification of alternative splicing, orthologues, paralogues, map positions and coding sequence variations (Wolfsberg et al, 2001). However, the Human Genome Mapping Project is far from completed, and a large number of genes have not been characterised yet. This chapter reviews the strategies available for gene identification, with particular emphasis on the technique of genetic linkage analysis, which forms the basis of much of the work described in this thesis.

Strategies for gene identification

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 maps. The theoretical background to these techniques will only be described in brief.

Functional cloning

Functional cloning makes use of fundamental information about the function of a disease gene, such as the presumed biochemical defect, to identify the gene without reference to its chromosomal map position. In most cases this approach relies on demonstration of an abnormal or deficient protein and its subsequent purification.

Amino acid sequencing of the protein allows complementary oligonucleotides to be synthesised and used to probe cDNA libraries. Once a cDNA fragment containing the gene of interest is identified, this may be used to screen genomic DNA to isolate the disease gene. An alternative approach makes use of a monoclonal antibody raised against the purified protein to screen expression vectors transfected with cDNA fragments, one of which may express the disease-associated protein. Functional complementation of mutant yeast strains with defined biochemical defects by human DNA fragments is also used as a means of gene identification.

Functional cloning was the first method of gene identification to be developed and has been employed to identify many disease genes including the genes for phenylketonuria (Robson et al, 1982) and factor VIII (Gitschier et al, 1984). However, 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.

Positional cloning

Since the early 1980s the strategy of positional cloning has emerged to identify genes for diseases where little or nothing is known about the nature of the disease gene product. 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 to a specific subchromosomal region, followed by successive narrowing of this candidate interval, which eventually results in the location of the gene itself. The initial localisation of the gene is usually achieved using the technique of genetic linkage analysis, described below. Alternatively, this information may be derived by other means, such as loss of heterozygosity screening in the case of genes involved in oncogenesis, or more commonly, the identification of cytogenetic abnormalities, such as chromosomal deletions or translocations, which have been used to pinpoint disease genes with great accuracy. The first success of the positional cloning technique was the identification of the gene for chronic granulomatous disease on the X chromosome in 1986 (Royer Pokora et al, 1986). Many of the first disease genes to be mapped were on the X chromosome because it was possible to localise the gene to this chromosome on the basis of an X-linked inheritance pattern, and because cytogenetically visible deletions and translocations involving the X chromosome frequently result in a clinical phenotype in males due to the haploid status of the X

chromosome. A substantial proportion of positional cloning successes have in fact relied on fortuitous identification of chromosomal rearrangements in individual patients. Despite the difficulties, the positional cloning approach has resulted in numerous successes, although the more efficient positional candidate approach (discussed below) has become the prevailing method of gene cloning.

In the absence of visible cytogenetic rearrangements to locate a gene, 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 (see section "genetic linkage analysis" later in this chapter). 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. It should be appreciated that genetic and physical distances are not linearly related, but that 1 cM corresponds to approximately 1 million base pairs (Mb) of DNA. A region of this size may contain a substantial number of genes (Fields et al, 1994). Cloning within a candidate region using the techniques of physical mapping is an expensive and time-consuming endeavour. Thus, reduction of the size of the genetic region to which a gene is mapped at the outset of a positional cloning project is of paramount importance.

The genetic and physical genome maps have been partially united to provide integrated maps containing a selection of microsatellites and other sequence tagged sites such as expressed sequence tags (ESTs -which are fragments of cDNA sequence most of which have been mapped to radiation hybrid or other physical maps) ordered relative to one another (Cox and Myers, 1996; Schuler et al, 1996). Nevertheless, the transition from knowledge of the genetic map position of a gene to its exact physical position is a laborious process. Characterisation of the gene for Huntington's disease, for example, followed some ten years after initial linkage was established (Gusella et al, 1983; Huntington's disease collaborative research group, 1993). An understanding of the techniques used for physical mapping is necessary to appreciate the scale of the undertaking required in moving from genetic map position to final gene identification.

The principle of physical mapping for the purposes of positional cloning rests upon the construction of an ordered series of overlapping, cloned DNA fragments spanning the candidate region - known as a contig. Genes within the contig, any of which may be disease gene candidates, are then systematically identified and screened for disease-associated mutations. A variety of cloning vectors are available for this purpose, each of which typically carries a different sized insert. These include yeast artificial chromosomes (YACs), the bacteriophage P1 and bacterial artificial chromosomes (BACs). The choice of vector depends upon the contig resolution required, as well as a number of technical factors. A hierarchy of contigs of increasing resolution is often constructed to cover large areas. Initial clones from the area of interest may be identified by screening DNA libraries for clones containing the microsatellite markers close to which the gene has been mapped. The contig may then be extended by creating probes from the end sequences of clones and using these to identify overlapping fragments (a process called chromosome walking), or by 'fingerprinting' of random clones to identify overlapping sequences. Whichever technique is used, the construction of a contig is highly labour-intensive despite increasing automation of the process. Nowadays it is often possible to bypass this stage by making use of pre-existing contigs of the area, produced as part of the Human Genome Project strategy for genome sequencing and available through public databases.

Assembly of the contig is followed by identification of coding sequences of genes from the clones, a process termed transcript mapping. A wide variety of methods have been developed for transcript mapping, including ingenious techniques such as exon trapping (Church et al, 1994) which makes use of splice sites flanking exons to express cloned exons in cell culture, as well as more straightforward approaches such as simple insert sequencing. Transcripts identified in the contig are often fragments of genes which may subsequently be fully characterised. Analysis of tissue expression patterns and putative function of novel genes as well as mutation analysis in patients are required to verify whether any gene identified is the cause of the disease studied.

Positional cloning has long been considered a standard means of gene identification. The sequencing of the whole human genome, although not completed yet, is making available on the databases a huge mass of information about the genomic structure of a region identified by linkage analysis. Powerful bioinformatic tools are being developed to analyse the large mass of data coming from the Human Genome Mapping Project. Several genes have already been identified and characterised, other genes

have been predicted and annotated on the basis of the presence of putative exons, of similarities with other genes in different species and of the predicted function (identification of specific structural domains in the predicted protein product). For this reason, the positional candidate approach (see below) is overtaking the positional cloning approach, and is currently considered the most efficient way of identifying disease genes.

The candidate gene approach

As the Human Genome Project nears completion, the base pair sequence of an increasing proportion of the human genome is becoming available. As a result, an increasing number of human genes are being identified and placed on the gene map (NCBI map viewer at: http://www.ncbi.nlm.nih.gov; UCSC map viewer at http://www.genome.ucsc.edu). The difficulties of a pure positional cloning approach may sometimes be circumvented by directly testing previously characterised genes which are considered to be putative disease-causing genes. Genes may be considered candidates either by virtue of their chromosomal position in the vicinity of the disease locus, or because the function of the gene is such that it may be envisaged to play a part in the pathophysiology of the disease, or preferably both. The most frequently used is the positional candidate approach, which is dependant upon knowledge of the genetic map position of the disease locus and of the genes previously assigned to this region. Some appreciation of the molecular pathology of the disorder being studied is also required. This strategy is much more efficient than pure positional cloning and has already resulted in numerous successes (Collins, 1995). The positional candidate approach looks likely to completely supersede positional cloning as the human gene map is completed.

If no suitable candidate genes are known to exist in the vicinity of a disease locus, syntenic chromosomal regions of model organisms such as the mouse may be searched for previously mapped candidate genes. Genes identified in this way are particularly convincing candidates if they are known to be associated with a mouse phenotype having features in common with the human disease. Such a situation was encountered in the case of Charcot-Marie-Tooth disease type 1A (CMT1A) which was known to be associated with duplications on chromosome 17p, syntenic to mouse chromosome 11. Subsequently, mutations in the peripheral myelin protein 22 gene

(PMP22) in the *Trembler* mouse (a model of CMT1A) led to identification of PMP22 mutations and duplications in CMT1A patients (Scherer and Chance, 1995).

When a predicted pathophysiological link between a disease and a known gene is sufficiently convincing, it is possible to screen the gene directly for mutations in patients, without first performing linkage analysis to map the disease locus. This approach has been termed the position-independent candidate gene approach.

Mutation analysis of candidate genes

Regardless of the approach employed to identify putative disease genes, disease-causing mutations must be demonstrated in patients to confirm the identity of a disease gene. The most widely applied method of mutation screening is by genomic DNA sequencing of the candidate gene. Differences in sequence between patients and controls may represent pathogenic mutations. Alternatively, sequence variants may be simple neutral polymorphisms of no pathological significance. Distinguishing between these two alternatives is essential. Features suggesting a pathogenic mutation include: large-scale deletion or rearrangements involving a gene, stop codon or frameshift mutations, non-synonymous changes (altering the amino acid specified by a codon), non-conservative amino acid substitutions (substitution of one amino acid by another with different chemical properties) and changes in amino acid residues highly conserved throughout evolution (suggesting an important role in protein function). In addition, putative mutations should segregate with the disease within a family and other mutations in the same gene may be identifiable in unrelated families; in some instances a single mutation may be associated with disease in unrelated families and even in different species, strengthening the case for a pathogenic role. The final requirement when studying dominantly inherited disorders is that putative disease-causing mutations should be absent in ethnically-matched controls. By convention, any sequence variant present in 1% or more of the population is termed a polymorphism, but rare variants present at lower frequency may not be identified in controls if insufficient numbers are screened. The ultimate distinction between pathogenic and neutral variants however can only be made by demonstrating changes in protein function consistent with models of disease pathogenesis. This might include demonstration of changes in the oxygen dissociation curve of haemoglobin due to globin gene mutations (Weatherall, 1985), or patch clamp recording from single channels, for example, in hyperkalaemic periodic paralysis where genes encoding

sodium channel subunits carry mutations (Hudson et al, 1995). Relatively recent advances have permitted the creation of transgenic animal models of human disease by introduction of mutation-carrying human genes and subsequent functional rescue by reinsertion of the wild-type gene. Studies of this type, performed to study the *Clock* gene for example (Antoch et al, 1997), provide unambiguous evidence of the pathogenic role of mutations.

Genetic linkage analysis

Genetic linkage analysis is the first essential 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, and its principles will be described in detail in the following paragraphs.

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, and 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 two loci will create recombinants. Therefore, sets of alleles on the same small chromosomal segment tend to be transmitted as a block thorough 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. The proportion of offspring in which two parental alleles are separated by recombination is the recombination fraction (θ). The recombination fraction varies from 0 (for adjacent loci) to 0.5 (for distant loci) and may serve as a stochastic 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 θ are equivalent to the actual map distance (w) between loci, and thus recombination fractions are additive over small distances. The simplest case relating θ 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 θ values into actual map distance. Some commonly used mapping functions are those of Haldane and Kosambi (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. It is the physical position of loci on the DNA molecule and the distance between loci measured in base pairs of DNA that is of greatest interest to researchers attempting to clone genes. The relationship between genetic and physical distance, however, is not constant but depends on the recombination rate which in turn varies quite considerably both between chromosomal regions and between males and females. However, if a total genetic map length of approximately 3,000 cM in man is assumed, this corresponds on average to 1 Mb of DNA per 1 cM of genetic distance in the 3×10^9 bp human genome.

For the purpose of localisation of disease genes, linkage analysis between two loci - the disease gene locus and anonymous DNA marker loci - is performed to determine whether the two co-segregate and are therefore in close physical proximity. Since the sub-chromosomal location of markers is known, the position of linked genes within the framework of the genetic map may be deduced. There are a number of prerequisites for linkage mapping of diseases. It is self-evident that the aetiology of the disease must have a genetic component; predominantly non-genetic diseases, such as vertically transmissible infectious diseases, may be mistaken for genetic disorders. Ideally, the disease should be monogenic (i.e. caused by a single disease gene) and the mode of inheritance established by segregation analysis. If these criteria are not met, for example in the case of polygenic diseases caused by the interaction of multiple disease-susceptibility genes with one another and with environmental factors, model-free methods of analysis, such as the affected sibling pair method, may be employed (Ott, 1996). However, a full discussion of non-parametric methods of linkage analysis is beyond the scope of this thesis. Clinical ascertainment of pedigrees in which the disease studied is segregating is of fundamental importance, and may, to a large extent, determine the success of a linkage study. 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 disorders. 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. Finally, linkage testing can fail as a consequence of insufficiently informative markers, of markers that are not enough close to the disease locus (and thereby having a high rate of recombination between the marker and disease gene).

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). 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 superseded in linkage projects by variable number tandem repeat markers (VNTRs) such as microsatellites.

A microsatellite is a genomic sequence in noncoding DNA consisting of a 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 therefore extremely useful as genetic markers, particularly since they are abundant (CA dinucleotide repeats occur on average every 18-28 kb - Stallings et al, 1991), dispersed throughout the genome, and may be easily typed by PCR (Weber and May, 1989; Silver, 1992). 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. Amplification of a short segment of DNA using PCR primers situated in non-repetitive DNA surrounding a microsatellite, produces multiple copies of a DNA fragment which may be sized by electrophoresis on a high resolution polyacrylamide gel, thus distinguishing between alleles with differing repeat numbers. 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. 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 (Mansfield et al, 1994; Schwengel et al, 1994). Systematic identification of microsatellites and careful ordering of these relative to one another in the genome by observation of recombinations in large pedigrees has enabled comprehensive genetic marker maps to be constructed for humans and several model organisms. The first phase of the Human Genome Project, completed in 1996, was the construction of a comprehensive high resolution genetic map of the human genome based on over 5,000 microsatellites with an average spacing of 1.6 cM and an average heterozygosity of 70% (Dib et al, 1996). The advent of such maps, along with the

technology for large-scale, rapid typing of markers, has made systematic disease linkage analysis possible, and provided a framework which is a prerequisite for construction of physical maps of the human genome and ultimately for complete genomic sequencing.

The most common type of human genetic variation is the single nucleotide polymorphism (SNP), a variation between two alternative bases occurring at a single position. SNPs are highly abundant, occurring on average at least once per kb (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. Although SNP maps are still at an early stage of development they have the potential to supersede microsatellite markers for mapping. Genotyping large numbers of microsatellite markers for linkage analysis is both costly and time-consuming. Unlike microsatellites which require gel-based methods to distinguish between alleles on the basis of length, SNPs are more amenable to automated genotyping using new technologies such as hybridisation to high density DNA probe arrays known as 'DNA chips' (Goffeau, 1997; Wang et al, 1998). 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 labelled, momentarily placed on a chip to hybridise, 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 individually less informative than microsatellites, the potential for rapid high throughput genotyping, and the predicted availability of SNP maps with a marker density much greater than 1 per cM, are likely to give SNPs significant advantages over current genetic markers (Jordan and Collins, 1996; Kruglyak, 1997).

Testing for linkage - 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. Although a LOD score at the Z = 3 threshold indicates an odds ratio of 1000:1 in favour of the data being explained by the hypothesis of linkage, it should be appreciated that this corresponds to a conventional probability of linkage at the p = 0.05 level of significance. This can be explained by the prior probability of linkage between any two randomly chosen loci (the probability that they are close enough for linkage to be detected) which is estimated to be in the order of only 0.02.

The odds ratio favouring linkage is defined as the likelihood that the two loci are linked at a specified recombination (θ) versus the likelihood that they are not linked (θ = 0.5). LOD scores are calculated by computer programs over a range of values of θ, facilitating the maximum probability of recombination (and hence genetic distance) between the two loci to be calculated. 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.

The direct method of linkage analysis depends upon observing and counting recombinants and nonrecombinants to directly calculate θ (Ott, 1991). In human genetics, however, the information needed to unambiguously identify the position of crossovers is often lacking, due to unknown phase and incomplete penetrance, for example, and an indirect statistical method is generally used. The most frequently used method is the likelihood method, which calculates the likelihood (L) that the observed genotypes could have arisen from two linked loci at a given value of θ, against the likelihood of observing the same genotypes for unlinked genes (L(0.5) - the null hypothesis of independent assortment). When the ratio of $L(\theta)/L(0.5)$ is greater than 1, it indicates that linkage is more likely than non-linkage to explain the observed genotypes. This odds ratio is usually expressed as a decimal logarithm, called a LOD score (for Log of ODds ratio), in order that scores from different families may be summed (Ott, 1991):

$$Z(\theta) = \log_{10}[L(\theta)/L(0.5)],$$

where Z is the LOD score at a given value of θ. The LOD score is calculated for various values of θ using computer programs such as MLINK (Lathrop and Lalouel, 1984 and 1988; Cottingham et al, 1993; Schaffer et al, 1994; Terwilliger and Ott, 1994) to obtain the value of θ associated with the highest LOD score. This provides an estimate of the genetic distance between the two loci studied, along with a measure of the weight of

the data in favour of the hypothesis of linkage (Ott, 1991). Calculation of LOD scores requires specification of the mode of inheritance, the penetrance of the disease gene and estimation of the disease gene frequency.

Pairwise and multipoint linkage analysis

Linkage analysis between two loci is a powerful technique for the localisation of disease genes. The technique depends upon pairwise linkage analysis between a disease locus and each of a large number of genetic markers. In practice genome-wide searches for linkage usually involve genotyping family members from one or more affected pedigrees, using a panel of microsatellite markers spaced evenly throughout the genome. Panels of microsatellite markers designed specifically for this purpose are available. These consist of markers selected on the basis of high heterozygosity and reliable amplification using the polymerase chain reaction. The density of markers screened in a genome-wide search for linkage will depend on the estimated maximum LOD score of the pedigrees studied. Once genetic linkage between a marker and the disease is identified, additional fine mapping may be undertaken, using closely spaced markers in the area of interest. This allows informative flanking recombinations between markers and disease to be identified by reconstruction of chromosomal haplotypes in family members. A disease gene can then be assigned to the genetic interval between the two closest flanking recombinations identified.

Pairwise analysis may be complemented by multilocus (or multipoint) linkage mapping, wherein three or more loci may be considered simultaneously. This technique is particularly useful for determination of probable marker order for a series of linked loci when constructing genetic maps, and is also helpful for exclusion mapping of disease loci to fully exclude a disease locus from the vicinity of a set of markers. Multipoint analysis between closely spaced loci yields more information than serial pairwise analysis and may therefore be helpful in a region where pairwise scores are equivocal. 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. Several computer programs 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.

Problems in linkage analysis

Genetic complexity may occur for one or more of the following reasons: a) the disease may be aetiologically heterogeneous, with only a subset due to genes conferring high risk; b) the disease may involve many different genetic loci that act together to cause disease; c) 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 some of 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. Also in some mendelian diseases, especially those with autosomal dominant inheritance, linkage analysis can be impaired by several critical factors, such as incomplete penetrance, variable phenotypic expression, genetic heterogeneity, phenocopies. Large families and accurate diagnostic criteria are needed in order to overcome these problems. Moreover, careful approaches such as "affecteds only linkage analysis" may be employed. In this approach, 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 often been used during the course of this thesis.

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. The identification of alleles IBD in consanguineous families is particularly useful to map rare autosomal recessive disorders through a homozygosity mapping approach (Lander and Botstein, 1987).

Linkage disequilibrium

While linkage refers to the relationship between two loci, the term linkage disequilibrium is used to describe the non-random association of alleles at linked loci. When a new mutation creating a disease allele is first introduced into a population, it occurs on a chromosome with a unique combination of surrounding alleles (a haplotype). During subsequent generations the original characteristic haplotype will be altered by random recombination, but the closer an allele to the disease allele, the greater the likelihood that these alleles will co-segregate through subsequent meioses. It follows that diseases which have originated relatively recently in a population (in generational terms) will be associated with a unique surrounding haplotype inherited from the founder. The more recent the common disease origin, the greater the genetic distance over which patients will share a common haplotype. These considerations make analysis of linkage disequilibrium a useful tool for high resolution genetic mapping, since the technique provides indirect information on a far greater number of recombinations than may be observed in any pedigree-based linkage study. In practice it is possible to map genes at a resolution of less than 1 cM by the identification of markers that are in strong linkage disequilibrium with the disease allele (Xiong and Guo, 1997).

Application of molecular genetics to neurological diseases

A large proportion of the human genes are expressed in the human central nervous system. It is perhaps not surprising then, that of the approximately 10,000 mendelian disorders of humans listed on the OMIM database, a significant proportion have a partly or wholly neurological phenotype. For these reasons, and because the pathophysiology of neurological disorders may sometimes be difficult to elucidate due to the complexity and inaccessibility of brain tissue, molecular genetic techniques have been applied extensively to the study of neurological disorders (Harding, 1993a; Baraitser, 1997). This approach has met with considerable success - a review published

in 1997 listed 96 disorders predominantly affecting muscle, peripheral nerve or spinal cord for which the causative genes have been identified or mapped (Kaplan and Fontaine, 1997). The OMIM database is continuously updated to reflect the daily progresses in the field of genetically inherited disorders.

Identification of neurological disease genes is of importance for several reasons. Perhaps most importantly, characterisation of a disease gene often provides biological insights into the function of that gene in health and disease. Greater understanding of pathophysiology as a result of cloning studies is essential to pave the way for the development of rational forms of therapy. It is also hoped that study of relatively uncommon monogenic variants of diseases such as Alzheimer's disease, Parkinson's Disease and amyotrophic lateral sclerosis will help to clarify the molecular pathogenesis of the far more common sporadic forms of these diseases. However, although many new disease-causing genes have been identified in recent years, understanding of the processes linking genotype to final disease phenotype is beginning to emerge only relatively slowly.

Cloning of disease genes has provided clinicians with the means for both diagnostic testing and predictive testing of at-risk individuals and foetuses. These advances have simplified genetic counselling and increased its accuracy, thus directly benefiting patients (MacMillan and Harper, 1994; Bird and Bennett, 1995). The nosology and classification of neurological diseases has also been clarified by characterisation of diseases at the molecular level. This is exemplified by genotype-phenotype studies of the hereditary ataxias, 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, a phenomenon recently designated as "splitting and lumping" of inherited diseases (Rosenberg, 1995). Future classifications of these disorders will thus be based on genetic locus as well as phenotype.

Many of the recent advances in the field of neurogenetics have been based on the elucidation of single gene disorders. Although these are important, many of the commoner neurological disorders are not caused by single genes but are thought to result from the interaction of environmental factors with multiple susceptibility genes. Diseases such as Parkinson's disease, multiple sclerosis, stroke, epilepsy and Alzheimer's disease, are all believed to have a genetic component. Studies based on use of non-parametric linkage methods and analysis of candidate genes in these

conditions are recommended and can yield better results (Bell and Lathrop, 1996; Günel and Lifton, 1996; Wood, 1997).

Application of molecular genetics to the study of movement disorders

Movement disorders are neurological syndromes in which there is either an excess of movement (hyperkinesias) or a paucity of voluntary or automatic movements, unrelated to weakness or spasticity (hypokinesias). The involuntary movements which comprise the hyperkinesias may be classified into five main categories: tremor, chorea, myoclonus, tics and dystonia. Parkinson's disease and a number of other akinetic-rigid syndromes with parkinsonian features constitute the hypokinesias (Marsden, 1996a).

Involuntary movements may occur as part of the clinical phenotype of numerous genetically determined neurological disorders. In many such disorders the involuntary movements are accompanied by other neurological or non-neurological features, producing a distinct clinical syndrome or disease. However, several movement disorders may occur in isolation, without associated abnormalities on clinical examination or routine investigation. Some such disorders appear to have a genetic basis, and a molecular genetic approach has begun to identify some of the genes responsible for these disorders. Study of a large American kindred of Czech ancestry and Icelandic kindred with essential tremor have resulted in identification of two genetic loci for tremor on chromosomes 2p22-p25 and 3q13 (Higgins et al, 1997; Gulcher et al, 1997). The molecular genetic basis of the dystonias, paroxysmal dyskinesias and Parkinson's Disease have likewise been the subject of intensive investigation and are discussed in chapters 3, 4 and 5 respectively.

Objectives of this thesis

The author has attempted to apply molecular genetic techniques to the study of families with a number of inherited movement disorders.

Mutation analysis of the DYT1 gene was performed in individuals with primary torsion dystonia, and a detailed haplotype study was performed to detect linkage disequilibrium. Families with dominantly inherited forms of primary torsion dystonia, with a variety of paroxysmal dyskinesia and with autosomal recessive juvenile parkinsonism were studied. An introduction to each disorder is given in the individual chapters. Kindred were first followed up and clinically ascertained, and DNA collected

for analysis. Exclusion mapping at the known loci was performed in three families with primary torsion dystonia and in three families with paroxysmal movement disorders.

Genome wide searches were performed in three families with primary torsion dystonia, paroxysmal kinesigenic dyskinesia and autosomal recessive juvenile parkinsonism respectively. These studies were undertaken with the aim of genetic mapping of the responsible disease genes as a first step in the process of gene characterisation, as described previously in this chapter.

CHAPTER 2 – MATERIALS AND METHODS

Outline of chapter

This chapter describes the materials used and experimental methodology employed in this study. The first part concerns the assessment and collection of affected family members and their unaffected relatives. In most cases, subjects were personally examined and videotaped using a standard protocol. The methodological stages of linkage analysis are described including extraction of DNA from blood, generation of DNA fragments using the polymerase chain reaction, separation of fragments by polyacrylamide gel electrophoresis, scoring of genotypes and computational linkage analysis. Buffers and solutions used are described at the end of the chapter. The clinical features of the families and patients studied are given in the relevant results chapters.

Diagnostic criteria and ascertainment of patients and families

Primary Torsion Dystonia

The dystonias are a clinically and genetically heterogeneous group of movement disorders characterised by sustained involuntary muscle contractions causing twisting movements and abnormal postures (Fahn et al, 1987a). The aetiological classification of dystonias has recently been revised with subdivision into four categories (Fahn et al, 1997):

1. Primary torsion dystonia (PTD), where the phenotype is of dystonia alone, which may be accompanied by tremor.
2. Dystonia-plus syndromes, where the dystonia may be accompanied by other neurological features such as parkinsonism (Dopa-responsive dystonia) or myoclonus (myoclonic dystonia).
3. Secondary dystonias, resulting from environmental factors (such as birth trauma, anoxia, stroke and so on).
4. Heredodegenerative diseases, where dystonia is part of a more complex clinical neurodegenerative phenotype, such as Huntington's disease and Wilson's disease.

Patients with primary torsion dystonia are clinically sub-classified according to two important parameters: a) age of onset (useful because it represents the best prognostic indicator as to whether there will be spread to other body parts); b) distribution (which can be considered a partial indicator of the severity of dystonia).

Three families with primary torsion dystonia have been ascertained during this project. The author has directly examined two families (PTD01 and PTD02), together with Dr AR Bentivoglio (Catholic University, Rome); videotapes were reviewed by Prof A Albanese (Catholic University, Rome). The third family (PTD03) has been on site examined by Dr A Münchau and revised by Dr K Bhatia (Institute of Neurology, London). Videotapes of family PTD01 were also revised by Prof CD Marsden and Prof NW Wood (Institute of Neurology, London).

Participating family members older than 18 years signed an informed consent. Minors were not included in the study for ethical reasons. All the examined subjects provided detailed information on familial and personal medical history and checked a detailed questionnaire of common motor problems observed in movement disorders. The neurological evaluation included specific tasks for dystonia, tremor or other movement disorders. Blink rate was assessed in all subjects according to published guidelines and compared to values expected (Bentivoglio et al, 1997a). Each subject was videotaped during the assessment. On-site examiners established a diagnosis of definite or probable dystonia and identified the body segments involved. A senior neurologist reviewed the videotapes blinded on the presumptive diagnosis and on the family history. A diagnosis of definite dystonia was made where unequivocal sustained muscle contraction resulting in twisting or repetitive movements was witnessed unanimously by the three examiners (Fahn et al, 1987a). A diagnosis of probable dystonia was made where a rapid jerky dystonic tremor was observed, when clinical features were subtle, or when not all examiners agreed on a diagnosis of definite dystonia. A diagnosis of definite or probably dystonia was made in deceased family members on the basis of history obtained from family members interviewed. Causes of secondary dystonia were excluded with appropriate investigations.

A definite diagnosis of blepharospasm was established when all the examiners observed at least two prolonged dystonic spasms of the orbicularis oculi muscle (or of any other peri-ocular muscles) during the whole duration of videotape (about 10 minutes). Subjects were considered probably affected by blepharospasm when their blink rate was increased as compared to the blink rate of the normal population

(Bentivoglio et al, 1997a) or when overactivity of the eyelids, but no prolonged spasms, were observed.

Definite cervical dystonia was characterised by slow dystonic movements or abnormal neck postures associated to jerky movements of the head. When just jerky movements or abnormal postures were observed, the diagnosis was considered probable.

A definite diagnosis of upper limb dystonia was established when slow dystonic movements or definitely abnormal postures were present, with or without irregular or asymmetric tremor. Subjects with jerky, irregular, asymmetric tremor evident at the nose-finger manoeuvre or mildly abnormal postures were considered probably affected. Scoliosis, mirroring or clumsiness of the limbs were annotated, but were not considered diagnostic for dystonia.

For the retrospective DYT1 study, patients were selected from the dystonia database of the movement disorder clinic and the neurogenetics clinic at the National Hospital for Neurology and Neurosurgery (NHNN), London and from previous clinical and genetic studies of familial PTD (Fletcher et al, 1990a; Warner et al, 1993). Inclusion criteria were 1) generalised, multifocal, segmental or focal dystonia as defined by published criteria (Fahn et al, 1987a); 2) a clinical course compatible with PTD with no features to suggest secondary dystonia or other dystonic states such as Dopa-responsive dystonia or paroxysmal dyskinesias (see Chapter 3).

Paroxysmal dyskinesias

Four families were ascertained in this study. Three families (PKD01, PKD02 and PKD03) had paroxysmal kinesigenic dyskinesia (PKD), while the fourth family (PED01) had paroxysmal exercise-induced dyskinesia (PED).

Family PKD01 is Indian; twenty-six family members were directly examined and videotaped by Dr GM Wali (KLE Society Hospital, Belguam, Karnataka, India) and diagnoses were discussed with Dr K Bhatia. Family PKD02 is a three-generation Caucasian English family, ascertained through Dr TT Warner (Royal Free Hospital, London), who personally examined all available family members. Family PKD03 is also a three-generation Caucasian English family. Dr PR Jarman (National Hospital for Neurology and Neurosurgery, London) examined and collected blood by all available family members. All examined subjects gave informed consent. A detailed history was obtained from each family member. Specific attention was paid to associated paroxysmal disorders such as epilepsy, migraine, and paroxysmal ataxia. Physical and

neurological examinations were performed in all affected members and attempts were made to provoke paroxysms with sudden movements, running and hyperventilation. Patients were designated as affected at the time of interview based on the observation or on the history of typical attacks of PKD (paroxysms of dystonia, chorea, ballistic or athetoid movements induced by a sudden change in position, usually manifesting in childhood or early adolescents, lasting from a few seconds to a few minutes and occurring up to 100 times daily, without loss of consciousness).

Family PED01 is a Caucasian English family ascertained by Dr A Münchau (Institute of Neurology, London). All available family members were examined on site and videotaped by Dr A Münchau; videotaped were revised by Dr K Bhatia. Physical and neurological examinations were performed in all affected members and attempts were made to provoke paroxysms with prolonged exercise. As for PKD, diagnosis was made based on the observation or on the history of typical attacks of PED (attacks of dystonia coming after 10-15 minutes of continuous exercise and disappearing shortly after ceasing the exercise).

Parkinson's Disease and Autosomal recessive juvenile parkinsonism

The European Consortium for Genetic Susceptibility in Parkinson's Disease established clear diagnostic criteria to be followed by all participating centres, in order to carefully define PD consistently across the EU partnership, to prevent any confusion from allied disorders such as multiple system atrophy and progressive supranuclear palsy, 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. They are similar to those previously proposed (Koller, 1992) but more rigorously exclude allied conditions.

Table 2.1 – diagnostic criteria for PD

Bradykinesia, rigidity, rest tremor, asymmetry of signs at onset	3 out of 4
Improvement at some stage with L-dopa therapy	over 50%
No supranuclear ophtalmoplegia	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 (6 months before onset)	

All the examined subjects provided detailed information on the following: birth, growth and development, education, use of well-water, medications, drug misuse, exposure to toxins or chemicals (particularly regarding organophosphorous compounds and other substances used in agriculture), previous illnesses, previous hospitalisations, any neurological or psychiatric disease, head or body injuries. The levodopa equivalent daily dose (LEDD) was calculated in order to have comparable data on drug therapy. All participating family members had a complete on-site neurological examination that included the UPDRS motor scale in the *on* condition (and, whenever possible, in the *off* condition), the mini-mental state examination (MMSE), and tasks aimed to disclose dystonia or paroxysmal dyskinesias (Folstein et al, 1983; Fahn and Elton, 1987b). Each subject was videotaped during the assessment. Exclusion criteria were supplemented by investigative data, including imaging and 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). Autosomal recessive juvenile parkinsonism (ARJP) was defined when a diagnosis of Parkinson's disease was associated with onset before 45 years of age (in at least one family member) and family history compatible with an autosomal recessive mode of inheritance. In this project, all cases of Parkinson's disease or autosomal recessive juvenile parkinsonism have been selected following these criteria.

Three ARJP families were ascertained through the Movement Disorder Unit of the Catholic University (Rome). The author personally examined, with Dr AR Bentivoglio, the Marsala kindred (a large consanguineous Sicilian family) and a second consanguineous family from Central Italy. The other family had been previously examined and videotaped, and DNA from available family members was already stored in the Movement Disorder blood and DNA bank based in the Movement Disorder Unit of the Catholic University (Rome). Videotapes from all patients were revised by Prof A Albanese, blinded to the previously assigned diagnoses.

For families collected by the European Consortium, 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, London) 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.

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. Thirty mls 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). Index subjects were only included if they fulfilled rigorous diagnostic criteria for clinically definite PD. Once an index subject was examined and found to fulfil 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 definite, probable and possible PD. Participating clinicians 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. All clinical information was stored in a standard database format. All core information on DNA samples and family trees was stored in the central databases in both London and Paris.

DNA extraction

DNA was extracted from venous blood samples (collected in EDTA) using the Nucleon II system (Scotlab). To extract leucocytes, 10 mls of blood was added to 40 mls of reagent A (to lyse erythrocytes), 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 leucocytes). The supernatant was discarded, 2 ml of reagent B added (to lyse leucocytes) and the pellet gently resuspended. 500 μl of 5 M sodium perchlorate was then added (to deproteinise the mixture) and the mixture shaken for 10 minutes at room temperature and for 15 minutes in a 65^{0}C water bath. DNA was extracted by the addition of 2 ml of chloroform (at -20^{0}C) 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 at 2,000 rpm. The aqueous DNA-containing phase was taken and DNA precipitated by the addition of two equal volumes of absolute ethanol, the mixture was then gently inverted. DNA was then hooked out into a tube containing 0.5 - 1 ml of sterile 1 x TE.

Measurement of DNA concentration and dilution of DNA

DNA concentration was estimated by measurement of optical density (OD) using a spectrophotometer (Cecil, model CE202). OD measurements were performed at a wavelength of 260 nm using quartz cuvettes. Measurements were calibrated using distilled water. DNA dilutions of 5 μl of DNA in 1 ml of distilled water (1 in 200) were used for OD measurements. For linkage analysis using fluorescently tagged primers and microtitre plates, DNA was diluted to a concentration of 10 ng/μl using 1x TE, and stored in covered deep-well titre plates (Beckman) at 4^{0}C when in frequent use, and at -20^{0}C when used infrequently. Concentrations of all other DNA samples were adjusted to approximately 50 - 100 ng/μl.

The polymerase chain reaction (PCR)

The polymerase chain reaction is a method for the selective enzymatic synthesis of a specific DNA sequence using two oligonucleotide primers hybridising to opposite strands of DNA flanking the target sequence. Repeated cycles of DNA denaturation, primer annealing and extension of the annealed primers by the thermostable DNA

polymerase derived from *Thermus aquaticus* (*Taq*), results in the selective amplification of the target DNA fragment. The extension products of each cycle are used as templates in subsequent cycles, resulting in exponential accumulation of target DNA. The requirements of PCR are as follows: DNA template, oligonucleotide primers, deoxynucleoside triphosphates (dNTPs), *Taq* DNA polymerase, a magnesium-containing buffer for the enzyme and a thermal cycler. Both primers and dNTPs must be present in excess. The method of visualising and sizing the final PCR product used in this study was the labelling of one primer from each pair with a fluorescent dye detectable by laser using an automated DNA sequencer.

Fluorescence-based PCR

PCR reactions using fluorescently-labelled primers were usually carried out in final reaction volumes of 20 μl in 96-well microtitre plates (Micro Test III, Falcon). The reaction mixture consisted of: 0.2 mM each of dATP, dCTP, dGTP and dTTP (2 μl of 10 x dNTP solution, Promega); 2 μl of GeneAmp 10 x magnesium-free PCR Buffer II (Perkin Elmer); 1.5 mM $MgCl_2$ (1.2 μl of 25 mM $MgCl_2$ solution, Perkin-Elmer); 10 ng of each primer; autoclaved and filtered distilled water to make up reaction mixture volume to 15 μl; 0.5 units of DNA polymerase added last (AmpliTaq Gold™ 5units/μl, Perkin-Elmer). The reaction mixture was prepared at room temperature and aliquoted into microtitre plate wells using an eight channel pipette (Scotlab). Fifty ng (5 μl) of template DNA was then added to each well. Microtitre plates were then centrifuged at 1000 rpm for 30 seconds (Beckman GS-6R centrifuge).

PCR reactions were performed using a Perkin Elmer 9700 thermal cycler. Reaction mixes were first heated to 95^0C for 11 minutes to activate the AmpliTaq Gold™; subsequent cycling conditions were:

94^0C (denaturation) 30 seconds

48 - 56^0C (annealing) 30 seconds

72^0C (extension) 30 seconds

repeated for 28 - 33 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. For microsatellite markers from the Linkage Mapping Set ABI PRISM LD-10 (see below), used for genome-wide searches, 2 couples of primers from the same

panel were multiplexed together in the same PCR reaction, to save time and consumables. PCR conditions for these markers have been already optimised by the manufacturers, and all markers in the set can be PCR-amplified under the same conditions. Reaction mixes were first heated to 95^0C for 11 minutes to activate the AmpliTaq Gold™; subsequent cycling conditions were:

94^0C (denaturation) 30 seconds

60^0C (annealing) 15 seconds

72^0C (extension) 15 seconds

repeated for 20 cycles, then:

94^0C (denaturation) 30 seconds

55^0C (annealing) 15 seconds

72^0C (extension) 15 seconds

repeated for 15 cycles.

As PCR is a sensitive technique capable of amplifying very small quantities of template, 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.

Oligonucleotide primers for microsatellite markers

For genome-wide linkage searches, a set of fluorescently labelled primers designed for this purpose were used in all families studied. This marker set (ABI PRISM Linkage Mapping Set LD-10) contains 400 individual markers spanning all the autosomes and the X chromosome with 10 cM average resolution. The markers are organized into 28 panels containing 10 to 20 fluorescent dye-labelled primer pairs (6-FAM, NED or HEX) that generate PCR products that can be pooled (combined) and detected in a single gel lane or capillary injection. Many of the primers have been redesigned to ensure efficient PCR amplification and to produce products over a broad molecular weight range. Forward and reverse primers are combined and supplied in a tube at 10 µM concentration (5 µM of each primer) in 10mM Tris-HCl, 1 mM EDTA, pH 8.0.

Additional microsatellite markers used for high resolution genetic mapping and exclusion mapping were analysed using custom-made fluorescently labelled primers. Primers were manufactured by Perkin-Elmer with a 5′ 6-FAM, HEX or TET (or NED) dye

on one of each primer pair. Markers used were dinucleotide (CA)n 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 di-, tri- and tetranucleotide repeats from the Cooperative Human Linkage Centre (CHLC) and the Marshfield Centre for Medical Genetics Human Genetic Map (Sheffield et al, 1995; http://www.marshfieldclinic.org). In all cases, markers with the highest heterozygosity were selected for use in order to maximise informativeness.

Agarose gel electrophoresis

To check for the presence of a PCR product of the desired size and quantity, 5 μl aliquots of reaction mix from four randomly selected wells (as well as the negative control) were visualised by electrophoresis on ethidium bromide stained agarose gels. Five μl of reaction mix was added to 2 μl of agarose gel loading buffer (6x buffer consists of 40% w/v sucrose, 0.25% bromophenol blue) and electrophoresed at 50 V through a 3.2% agarose minigel (Flowgen Instruments Ltd) for 30 - 60 minutes. A 100-bp size standard (Gibco) (1 μl) was run alongside the PCR products to enable estimation of their size. Ethidium bromide staining of the agarose gel (1 mg / ml, Sigma) permitted direct visualisation of DNA products using transillumination with ultraviolet light.

Polyacrylamide gel electrophoresis

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 travelled through the gel (smaller fragments migrate further than larger ones).

Polyacrylamide gel electrophoresis of fluorescently labelled PCR products

PCR products produced using fluorescently 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, labelled with the fluorescent dye TAMRA or ROX, is run in each lane to allow accurate sizing of PCR fragments. This method of DNA sizing has the great advantage over radioactive methods that markers of non-overlapping size and dye composition may be multiplexed in each lane so 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.

Polyacrylamide gel preparation

Thirty-six cm well-to-read glass plates were used with the 377 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 μl) and freshly prepared 10% ammonium persulphate solution (APS) (Sigma) (250 μl) 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 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 50^0C. Samples were then loaded. For preparation of acrylamide gel mix and of TBE buffer, see paragraph "Buffers and solutions" at the end of this chapter.

Pooling of PCR products for loading

Up to 20 non-overlapping microsatellite markers, amplified from a single DNA sample, were run simultaneously in each lane (multiplexed). PCR products from each DNA sample were first pooled according to the dye they contained as follows: FAM - 4 μl; TET - 10 μl (or NED - 10 μ); HEX - 10 μl. These volumes were adjusted according to the yield of the PCR as determined on agarose gel electrophoresis. Pooling was

performed in microtitre plates using an eight channel pipette. 2.5 μl 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 μl of deionised formamide, 20 μl of loading buffer (blue dextran, 50mg/ml, EDTA 25 mM, Perkin Elmer) and 24 μl of Genescan 350-TAMRA or Genescan 400-HD ROX size standard (Applied Biosystems). The final mix of pooled product and loading mix was denatured at 95^0C for 2 minutes in a Hybaid thermal cycler and then placed immediately onto ice before loading.

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 1.0 μl of final mix using a P2 pipette (Gilson) and Sorenson MiniFlex 0.2 mm 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 48 or 64 samples could be run in adjacent lanes.

Data analysis for fluorescently labelled PCR products

Initial data processing

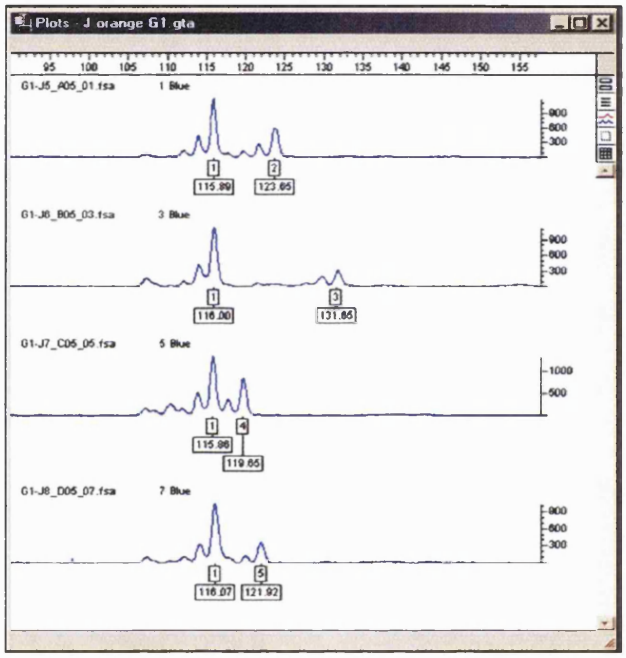
Data collected during the electrophoresis run were analysed automatically using the Genescan software in order to size DNA fragments separated by electrophoresis. Automatic lane tracking was checked using the gel image, and adjusted lane by lane where necessary. To ensure accurate sizing, the automatic 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.

Genotyping using the Genotyper programme

Fragment size data collected using the Genescan software were then analysed using the Genotyper software (Applied Biosystems) as described in the manual. The Genotyper software 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

electropherograms to ensure that alleles are correctly labelled. Although time-consuming, this step is extremely important as labelling 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 software is illustrated in figure 2.1.

Figure 2.1 – allele identification using the Genotyper software



Computational linkage analyses

The technique of genetic linkage analysis was used extensively throughout this study. The principles of linkage analysis are described in detail in chapter 1. Briefly, linkage analysis refers to the mapping of a disease gene to a sub-chromosomal location. The technique makes use of polymorphic microsatellite DNA markers to identify a chromosomal region segregating with the disease in one or more families. Markers are amplified from genomic DNA of family members using the polymerase chain reaction (PCR) and separated using polyacrylamide gel electrophoresis. Family members may be genotyped for each marker according to the number of tandem repeats present (which are determined by the length of the PCR fragments). Segregation of a marker

allele with the disease phenotype in a family suggests linkage between the microsatellite marker and disease gene.

Linkage analysis methods

Power simulation studies were performed using the SLINK software (Ott, 1989; Weeks et al, 1990). This simulation calculated the maximum and the average LOD score that can be obtained by performing linkage analysis in a given family. These simulated LOD score values reflect the power of the family to detect linkage and are therefore useful to decide whether or not to embark in a genome-wide search effort. Pairwise linkage analyses were performed using the FASTLINK 3.0P version of the MLINK program. For autosomal dominant families, the disease allele frequency was set at 0.0001, while for autosomal recessive families this value was set at 0.001 and loops in the pedigree were broken by using the automated loop breaking procedure described by Becker and co-workers (1998). Disease penetrance varied according to the disorder studied and these are given in the individual results chapters. Marker allele frequencies were assumed to be equal. Multipoint linkage analyses were performed using the SIMWALK2 program (Sobel and Lange, 1996). All linkage programs were remotely accessed through the Human Genome Mapping Project web site (http://www.hgmp.mrc.ac.uk). A conservative 'affecteds-only' methodology was used for the exclusion studies and for the first screening during each genome-wide search, in order to avoid biases resulting from inclusion of possibly affected individuals or incorrect estimation of penetrance or age of onset. In a powerful enough family, linkage analysis with affected individuals only allows the exclusion of a large part of the genome (70-80%). The regions surrounding markers showing possible linkage with the disease (pairwise LOD score > 1) and all regions surrounding non-informative markers were always saturated with more markers and all available family members were genotyped in order to allow haplotype construction.

Homozygosity mapping

The "Marsala kindred" is a consanguineous family with autosomal recessive parkinsonism where a genealogic search allowed tracing the origin of the family to a common ancestor couple seven generations back. Given the autosomal recessive pattern of inheritance, we expected homozygosity at the level of the disease locus in affected individuals due to identity by descent from a common progenitor. A conservative 'affecteds-only' homozygosity mapping methodology was therefore used

to perform the genome-wide search in this family, and other family members were genotyped in a second stage, only for those markers suggestive of linkage with the disease (homozygosity of the same allele in all affected individuals) and their flanking markers. Haplotype construction was then manually performed, to discriminate between false positive markers and markers truly in linkage with the disease.

Calculation of linkage disequilibrium

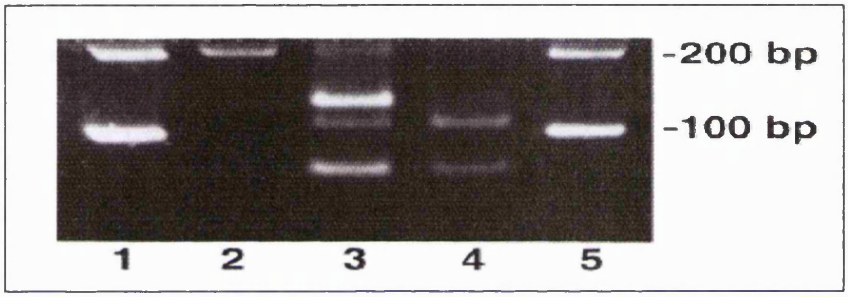
This was performed in Ashkenazi Jewish (AJ) and non-Jewish British dystonic patients carrying the GAG deletion in the DYT1 gene and allowed the identification of a limited number of DYT1 founder mutations in the United Kingdom. For comparison of allele frequencies, we obtained a total of 250 control chromosomes for the non-Jewish population, and 108 control chromosomes for the AJ group. In the non-Jewish population, full haplotype comparison between disease and control chromosomes was performed using 100 phased chromosomes from 25 unrelated Huntington's disease families as controls. Statistical comparison of single allele frequencies between disease and control chromosomes was based on a chi-square (χ^2) test for a 2x2 table, with alleles classified into two groups, one for the associated allele and all others combined into a single group. The same statistical method was used to compare haplotype frequencies between disease and control population. To assess the degree of linkage disequilibrium the author employed the parameter $\delta = (P_D - P_N)/(1 - P_N)$, where P_D is the frequency of the associated allele on disease chromosomes and P_N the frequency of the same allele on normal chromosomes (Risch et al, 1995). The value of δ represents the estimate of the proportion of disease chromosomes bearing the original associated allele at a certain locus. When δ is $\leq$ zero, the frequency of the presumed associated allele in the disease-bearing chromosomes is $\leq$ to that observed in controls; conversely, the more positive the value of δ (max: 1), the stronger the linkage disequilibrium between the disease and that particular allele.

DYT1 analysis

To detect the GAG deletion in the DYT1 gene, PCR was performed in a total volume of 20 μl using 100 ng of genomic DNA, 7 pmol of each primer, 200 μM dGTP, dCTP, dTTP and dATP, 1.5 mM $MgCl_2$, 10% Dimethylsulfoxid and 0.5 U of AmpliTaq Gold polymerase (Perkin Elmer). The primers used are those described by Ozelius and colleagues (1997). PCR conditions were as follows: one cycle at 94°C for 11 min,

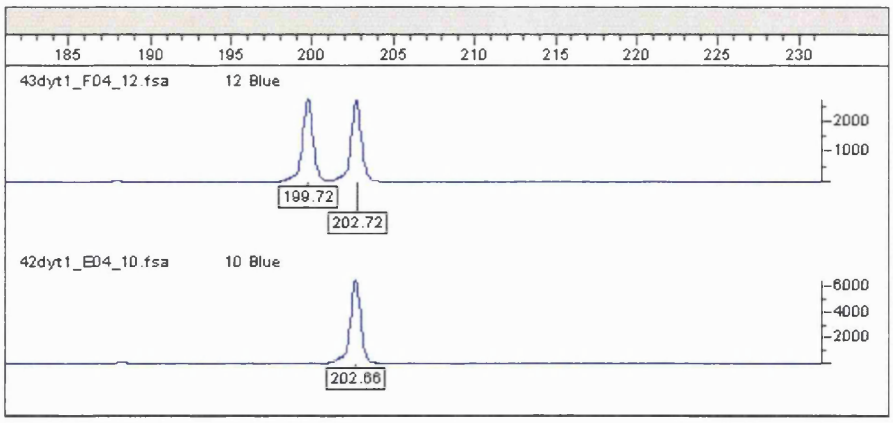
followed by 35 cycles of 94° for 30 s, 49°C for 30 s and 72°C for 30 s. Ten μl of each PCR product were digested with 4 U of the restriction endonuclease BseRI (New England Biolabs), loaded on a 4% intermediate melting temperature agarose gel (Promega) and electrophoresed at 50 Volts for 75 min. The 202-bp normal PCR product (figure 2.2, lane 2) contains two sites recognised by the restriction endonuclease BseRI; after digestion, two fragments of 120 and 72 bp are generated (figure 2.2, lane 4). Affected individuals have a novel 130-bp fragment resulting from abolition of a BseRI site by the GAG deletion (figure 2.2, lane 3). Lanes 1 and 5 contain 100bp DNA size standard (New England Biolabs).

Figure 2.2 – detection of DYT1 GAG deletion by BseRI digestion



Alternatively, we developed a much faster method of detecting the GAG deletion, using fluorescently labelled primers. The forward primer was tagged with a fluorescent dye (usually 6-FAM). The PCR reaction was carried out as described above. One μl of the PCR product was directly mixed with an equal volume of loading mix and 10 μl of Genescan 400-HD ROX size standard (Applied Biosystems). The final mix was denatured at 95^0C for 2 minutes in a Hybaid thermal cycler and then loaded in a 377 DNA fragment analyser (see paragraph "Polyacrylamide gel electrophoresis" above in this chapter). A positive and a negative control were always amplified and run together with the samples. In normal individuals, the 2 fragments amplified by PCR will be identical in size, and will be detected as a 202-bp peak with Genotyper (figure 2.3, lane 2). In patients carrying the GAG deletion in the DYT1 gene, one of the 2 fragments will be deleted and therefore 3-bp shorter. The Genotyper analysis will show 2 distinct peaks, of 199 and 202 bp respectively (figure 2.3, lane 1). This method is much faster and reliable of the restriction enzyme digestion, and avoids possible sources of mistakes due to partial digestion of the restriction enzyme or bad resolution of bands on the agarose gel.

Figure 2.3 – detection of DYT1 GAG deletion by using Genotyper software



Buffers and solutions

Unless stated otherwise, buffers and solutions were prepared using distilled water.

Chemicals were supplied by BDH unless otherwise stated.

10 x TBE (1 L)

- Tris (Sigma) 121.1 g
- Boric acid 61.8 g (anhydrous)
- EDTA (Sigma) 7.4 g

10 x TE (10mM Tris/1mM EDTA) (1 L)

- Tris 1.21 g
- EDTA 0.37 g

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

- Urea (Fison's) 18 g
- 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

Filter TBE through Whatman filter system (0.45 μm filter). Stir remaining constituents on a magnetic stirrer until dissolved. Filter onto TBE.

CHAPTER 3 – PRIMARY TORSION DYSTONIA

Outline of chapter

This chapter describes: a) a clinical-genetic study on the prevalence of DYT1-related dystonia in the UK and genotype-phenotype correlates; b) a linkage disequilibrium study on DYT1 founder mutations in the Ashkenazi Jewish and non-Jewish British population; c) linkage studies in three families with non-DYT1 primary torsion dystonia. In one of these families (PTD01) a novel PTD locus (DYT13) was assigned to the short arm of chromosome 1. In the other two families, linkage with all known PTD loci (DYT1, DYT6, DYT7 and DYT13) was excluded, suggesting the existence of at least one other PTD gene still unmapped.

The chapter begins with a review of the clinical features of the dystonias and summarises the current understanding of the molecular genetic basis of dystonia. The clinical features of patients carrying the DYT1 mutation and of the three PTD families are described together with the methods employed. Exclusion mapping of dystonia in all families at known PTD loci and the results of the genome search in family PTD01 are presented. The significance of these results are discussed in the context of current understanding of the molecular genetics of PTD, and future directions of study suggested.

An introduction to Primary Torsion Dystonia

Dystonia is defined as a syndrome of abnormal involuntary movements characterised by sustained muscle contractions, frequently causing twisting and repetitive movements, or abnormal postures (Fahn et al, 1987a). In the absence of an identified cause or other neurological abnormalities, this syndrome has been termed idiopathic torsion dystonia. However, with the mapping of several genes for idiopathic torsion dystonia, the term primary torsion dystonia (PTD) is now preferred (Fahn et al, 1997). The term secondary (or symptomatic) dystonia used to denote dystonia occurring in the setting of another neurological disease, for example a degenerative disorder of the CNS or a structural brain lesion, or as a result of environmental exposure to drugs such as tardive dystonia caused by dopamine receptor antagonists. It has been suggested that the term secondary dystonia should be reserved for dystonia resulting from environmental exposure or local CNS lesions (Fahn et al, 1987a). Fahn, Bressman and

Marsden (1997) proposed that the classification of dystonia be expanded to classify degenerative disorders causing dystonia (such as Wilson's disease or Huntington's disease) under the heading of 'heredodegenerative dystonia'. These authors also suggested that presumed neurochemical disorders, with neurological features in addition to dystonia (such as dopa-responsive dystonia), be classified as 'dystonia-plus syndromes' (see paragraph "Diagnostic criteria" in chapter 2). In this chapter, the primary dystonias will be sub-divided according to the evolving molecular genetic classification.

Primary torsion dystonia is not a rare disorder. A study of the prevalence of dystonia in Rochester, Minnesota, using the records of the Mayo Clinic, reported a prevalence in this community of 3.4 per 100,000 for generalised dystonia, and 29.4 per 100,000 for focal dystonias (Nutt et al, 1988). Although the relatively small sample population in this study necessitated wide confidence intervals (0.4-12.4 per 100,000 for generalised dystonia; 17.2-47.9 per 100,000 for focal dystonias) the results are broadly in line with figures reported from the UK: 1.6 per 100,000 for generalised dystonia, and 12 per 100,000 for focal dystonias (Duffey et al, 1997). The Epidemiological Survey of Dystonia (ESD) was started in May 1993 with the aim of identifying all dystonia cases in the population of North East England. According to this study, in 2001 the total number of primary and secondary dystonia cases within this geographical region was over 1100 people, proving that dystonia is the third most prevalent movement disorder after Parkinson's disease and benign essential tremor. One of the most interesting results was the identification of a positive family history (i.e. a proven member of the family with a form of dystonia) in 28.7% of all cases, increasing the present known incidence of familial dystonia (Butler, 2002).

Because focal dystonia is often mild and asymptomatic, case ascertainment may be incomplete, which means that the figures for focal dystonia could be underestimated. The prevalence of dystonia in the Ashkenazi Jewish population has long been recognised to be particularly high. Early estimates suggested a prevalence of up to 1 per 38,000 among Jews in the USA and indicated that the prevalence among Jews may be up to five times higher than among non-Jews (Zeman and Dyken, 1967; Eldridge, 1970; Zilber et al, 1984).

Clinical features and inheritance of dystonia

Dystonia may involve almost any muscle group, with a correspondingly wide range of clinical manifestations, encompassing equinovarus posturing of the foot with toe walking, hyperpronation of the arm, lordosis, scoliosis and torticollis. Writer's cramp, spasmodic dysphonia, blepharospasm, and oromandibular dystonia (the last two occurring together as Meige's syndrome), all fall within the rubric of dystonia. Dystonic spasms are often initially intermittent, appearing or intensifying with voluntary movement (which may lead to an erroneous diagnosis of hysteria), but dystonic postures may eventually become fixed. Electrophysiologically, dystonia is characterised by co-contraction of antagonist muscles and overflow of activity into extraneous muscles.

PTD is clinically heterogeneous, and may be classified according to age at onset (early onset: < 26 years of age), body part first affected and distribution. Focal dystonia is characterised by one body segment involved (i.e. blepharospasm, torticollis, spasmodic dysphonia). Segmental dystonia is diagnosed when at least two adjacent body parts are involved (i.e. Meige's syndrome, with upped and lower face muscles involved, or dystonia involving the neck and one upper limb). Generalised dystonia is defined by the involvement of one lower limb plus at least another body segment, while multifocal dystonia is characterised by involvement of at least two non adjacent body parts. Severity is largely determined by age at onset (Marsden and Harrison, 1974). The age at onset distribution of dystonia is bimodal with peaks at 9 and 45 years. There are clear clinical differences between patients with early-onset and late-onset PTD. Early-onset PTD usually begins in a limb, particularly the leg, and frequently progresses to generalised dystonia. Muscles of the head and neck are frequently spared. In contrast, late-onset PTD typically begins in the neck or head, for example with torticollis, and tends to remain focal in distribution. Segmental dystonia is of intermediate severity and may arise at any age (Marsden and Harrison, 1974; Greene et al, 1995).

Early, limb-onset PTD is particularly prevalent amongst the Ashkenazi Jewish population where inheritance is autosomal dominant with approximately 30% penetrance (Bressman et al, 1989). In the UK, generalised, multifocal and segmental dystonia are estimated to be dominantly inherited in 85% of cases, with approximately 40% penetrance (Fletcher et al, 1990a and 1990b). The remaining 15% of cases are thought to be non-genetic phenocopies.

The genetic contribution to adult-onset focal dystonia is less clear. Most cases are apparently sporadic, but careful examination of first degree relatives of patients with focal dystonia reveal that between 8% and 25% have an affected, often asymptomatic, relative (Waddy et al, 1991; Bressman et al, 1995, Leube et al, 1997a). These family studies suggest the existence of one or more autosomal dominant genes with low penetrance. A small number of large families exist in which focal or segmental dystonia is inherited as a dominant trait with relatively high penetrance (Uitti and Maraganore, 1993; Bressman et al, 1996; Leube et al, 1996; Brancati et al, 2002). Moreover, a recent case-control study on a British population showed a significant association between the dopamine receptor D5 (DRD5) and the susceptibility to develop adult onset focal dystonia (cervical dystonia and blepharospasm) (Placzek et al, 2001; Misbahuddin et al, 2002). The role of DRD5 in modulating the susceptibility to develop adult-onset cervical dystonia has been confirmed in an independent set of Italian patients (Brancati et al, in press). It seems likely, therefore, that non-genetic causes or interaction between minor genes and environment account for a large number of cases of sporadic adult-onset focal dystonia (Defazio et al, 1998).

The molecular genetic basis of dystonia

Over the last decade there have been considerable advances in the understanding of the genetics of dystonia. These have been driven by the need to take a positional cloning approach to understanding pathophysiology, as anatomical and neurochemical studies in PTD are generally unrevealing. A molecular genetic classification of the dystonias is now evolving, which complements the more traditional clinical classification, and allows phenotype-genotype correlations to be made. The Human Genome Organisation Nomenclature Committee have assigned the gene symbol 'DYT' to the dystonias, and there are currently thirteen loci: DYT1-13 (Ozelius et al, 1989 and 1997b; Gimenez-Roldan et al, 1988; Wilhelmsen et al, 1991; Parker, 1985; Ichinose et al, 1994; Almasy et al, 1997a; Leube et al, 1996; Fink et al, 1996; Auburger et al, 1996; Tomita et al, 1999; Klein et al, 1999a; Zimprich et al, 2001; Kramer et al, 1999; Valente et al, 2001) (table 3.1).

Table 3.1 – the DYT classification

Symbol	Phenotypic description	Gene	Locus
DYT1	early limb-onset generalised dystonia	TOR1A	9q34
DYT2	autosomal recessive dystonia in the Gypsy population	?	?
DYT3	X-linked dystonia / parkinsonism (Lubag syndrome)	?	Xq13.1
DYT4	whispering dystonia with Wilson's disease	?	?
DYT5	autosomal dominant Dopa-responsive dystonia	GCH1	14q
DYT6	"mixed" PTD phenotype	?	8
DYT7	adult onset pure focal dystonia	?	18p
DYT8	paroxysmal non kinesigenic dyskinesia (PNKD or PDC)	?	2q
DYT9	PNKD and spasticity (CSE)	?	1p
DYT10	paroxysmal kinesigenic dyskinesia (PKD or PKC)	?	16
DYT11	myoclonic dystonia myoclonic dystonia	D2 receptor ε-sarcoglycan	11q23 7q21-q31
DYT12	rapid-onset dystonia-parkinsonism	?	19q13
DYT13	PTD juvenile-adult onset, cranial-cervical and upper limb	?	1p36

Contrary to established practice, some clinically or genetically distinct dystonia syndromes have been ascribed a DYT designation leading to confusion. In fact, this classification includes some dystonia-plus syndromes, such as X-linked dystonia-parkinsonism (Lubag syndrome, DYT3), Dopa-responsive dystonia (DYT5) and myoclonus dystonia (DYT11), some diseases which are usually classified separately, such as the paroxysmal dyskinesias (DYT8, DYT9 and DYT10), and even some families with dystonic syndromes where no genetic loci have been identified (DYT2 and DYT4). Only four out of thirteen DYT symbols actually represent a distinct form of PTD (DYT1, DYT6, DYT7 and DYT13). Other dystonic syndromes, such as autosomal recessive Dopa-responsive dystonia, have not received a DYT symbol.

Each of the four PTD loci is associated with a characteristic, but sometimes overlapping, spectrum of phenotypic manifestations. The DYT1 locus is of considerable clinical importance, while the contribution of the other three loci has not yet been fully evaluated. A brief description of the three already known PTD loci (DYT1, DYT6 and DYT7) is reported in the following paragraphs, while the mapping of the fourth PTD locus (DYT13) is part of the work of this thesis and will be described in more detail later in this chapter. A brief description of the DYT5 locus, responsible for autosomal

dominant Dopa-responsive dystonia (DRD), is also reported, because a small number of DRD patients without identified mutations in the GCHI gene were tested for the DYT1 deletion. These results (all negative) are not presented in this thesis, but contributed to one of the papers that have arisen from this thesis (page 253, n. 1).

DYT1

Linkage studies in Jewish and non-Jewish families with PTD led to the identification of the DYT1 locus on chromosome 9q34 (Ozelius et al, 1989; Kramer et al, 1990 and 1994). Strong linkage disequilibrium has been demonstrated between DYT1 and a 9q34 haplotype in both familial and sporadic Jewish cases, indicating a founder mutation estimated to have occurred among the Ashkenazim of Eastern Europe about 350-380 years ago (Ozelius et al, 1992; Risch et al, 1995). The DYT1 gene is enriched in this population as a result of genetic drift, with a gene frequency estimated to be as high as 1/2000 (Risch et al, 1995). Very recently the DYT1 gene was cloned and a unique 3-bp (GAG) deletion identified in all chromosome 9q34-linked families, regardless of ethnic background and surrounding haplotype (Ozelius et al, 1997b). The deletion results in loss of one of a pair of glutamic acid residues in a novel protein named TorsinA. DYT1 mRNA and wild type (wt) TorsinA are expressed at very high levels in the dopaminergic cells of the substantia nigra pars compacta, a particularly interesting finding given the existing evidence implicating dopaminergic dysfunction in the pathogenesis of dystonia (Augood et al, 1998; Shashidharan et al, 2000). Also the mutant TorsinA is expressed in the brain of patients with dystonia and the 3-bp deletion in the DYT1 gene, with the same distribution shown by the wt-TorsinA (Walker et al, 2001). Immunohistochemical in vitro studies on cultured neuronal cells in which wt- or mutant TorsinA is over-expressed have shown that wt-TorsinA co-localised within specific markers of the endoplasmic reticulum in the cell body, dendrites and neurites, sparing the nucleus. Mutant TorsinA shows the same cellular distribution but somehow disrupts the endoplasmic reticulum structure forming large intracytoplasmic inclusions and dramatically altering the cell morphology (Hewett et al, 2000). Other expression studies have shown that TorsinA co-precipitates with α-synuclein within Lewy Bodies in the brain of patients with idiopathic Parkinson's Disease, suggesting a possible chaperone role within the endoplasmic reticulum of the cell (Shashidharan et al, 2000; Sharma et al, 2001). TorsinA is part of a family of proteins (Torsins and Torsin-related proteins) with ATP-binding and ATP-ase activity, with some similarity to the family of heat-shock proteins (Ozelius et al, 1997b).

Although its function in the nervous system and role in the pathogenesis of dystonia are not yet understood, it is likely that TorsinA is part of the Superfamily of proteins AAA (ATPase with a variety of cellular activities), which are cellular chaperones and play a crucial role within the endoplasmic reticulum by assisting other target proteins to undergo conformational changes. The AAA proteins are involved in several important cellular activities, such as protein degradation, solubilization of aggregates, vesicle biogenesis and fusion, vesicle and organelle transport, mitochondrial function, organelle biogenesis, signalling and gene transcription, cytoskeletal dynamics, protein translocation within the cell (Breakefield et al, 2001; Vale, 2000). Further studies and the identification of TorsinA substrates are needed to demonstrate its role within the cell, especially within the dopaminergic cells of the substantia nigra pars compacta.

The common 3-bp deletion appears to have arisen independently in different ethnic groups (Ozelius et al, 1997b and 1998; Klein et al, 1998a), and to date only another mutation (a 18-bp deletion) has been identified in the gene, in one patient with early onset dystonia with myoclonic features (Leung et al, 2001). This novel mutation has never been confirmed in a very large cohort of PTD patients; moreover, a mutation in the ε-sarcoglycan gene, responsible for myoclonus-dystonia, has been detected in the same patient carrying the DYT1 18-bp deletion (Klein et al, 2002; Doheny et al, 2002). These data support the hypothesis that this deletion could represent a rare polymorphism, irrelevant or with a minor role in determining the PTD phenotype. For this reason, in this thesis the term "DYT1 mutation" will refer to the 3-bp (GAG) deletion only. A small proportion of patients from families too small for linkage analysis, but with the typical DYT1 phenotype, do not carry the deletion (Ozelius et al, 1997b). It is not yet clear if these patients have novel mutations in the DYT1 gene, or whether other genes underlie dystonia in such cases. Recurrence of the same disease-associated mutation suggests either that the GAG deletion occurs at a mutational 'hotspot', or alternatively that only a single variation in the protein can give rise to the dystonia phenotype. The DYT1 mutation produces a relatively homogeneous clinical phenotype in both Jews and non-Jews. Typically onset is early (below 28 years of age), usually in a limb, and there is spread to at least one other limb but rarely to cranial-cervical muscles (Bressman et al, 1994a; Ozelius et al, 1997b). Patients with dystonia who carry the Jewish chromosome 9q34 haplotype ($DYT1^{AJ}$), have a mean age at onset of 12.5 years, and 70% eventually develop generalised or multifocal dystonia. However, a small proportion of patients with $DYT1^{AJ}$ have a less severe phenotype, with later onset (up to the age of 44), and little or no progression of symptoms after

long follow-up (Bressman et al, 1994a). In Europe, DYT1 mutations have been estimated to account for approximately 55% of families with generalised or segmental dystonia, although the confidence intervals on which this figure is based are wide (Warner et al, 1993). Mutation analysis of DYT1 in UK patients is one of the objectives of this thesis and will be reported in detailed in the following paragraphs of this chapter together with a comparison between UK data and other similar studies worldwide.

It has previously been suggested that DYT1 mutations may be a common cause of late-onset focal dystonia. However, there is mounting evidence that early and late-onset PTD are genetically, as well as clinically, distinct. Amongst the Ashkenazim, $DYT1^{AJ}$ is rarely found in patients with adult-onset or purely focal dystonia (Bressman et al, 1994a; Gasser et al, 1996), and exclusion of the DYT1 locus in large non-Jewish families with dominantly inherited late-onset focal dystonia also supports this view (Bressman et al, 1996; Brancati et al, 2002). In the study of Ozelius et al (1997b), none of the probands from 76 families with focal or segmental dystonia affecting cranial-cervical muscles were found to have the 3-bp deletion in the DYT1 gene. However, DYT1 mutations may occasionally result in a mixed dystonia phenotype with involvement of both cranial-cervical and limb muscles. The GAG deletion has been found in approximately 10% of probands from families with a mixed dystonia phenotype (Ozelius et al, 1997b).

DRD (formerly DYT5)

Dopa-responsive dystonia (DRD) occupies a unique place amongst the dystonias as the condition may be very effectively treated, and its molecular pathogenesis is better understood than that of any other dystonia. Segawa (Segawa et al, 1971 and 1976) provided the first detailed clinical description of DRD, and described the distinctive clinical phenotype and the dramatic, sustained, therapeutic response to low doses of L-dopa. Patients typically present in childhood with dystonia involving the lower limb which progresses to become generalised unless treated. Diurnal variation of symptoms with improvement of dystonia after sleep is a characteristic feature of DRD. It is now recognised that the DRD phenotype may encompass a number of atypical presentations, including parkinsonism, spastic paraplegia and a presentation mimicking athetoid cerebral palsy (Nygaard et al, 1988; Bandmann et al, 1996a). Most cases are inherited as an autosomal dominant trait with reduced penetrance, and females outnumber males by approximately three to one. Linkage between the DRD locus and

markers on chromosome 14q (Nygaard et al, 1993) led to the identification of heterozygote mutations in GCH1 in several cases of typical DRD (Ichinose et al, 1994). GCH1 enzyme activity in affected individuals was shown to be reduced to 2 - 20% of normal levels (Ichinose et al, 1994). The enzyme GCH1 catalyses the initial and rate-limiting step of tetrahydrobiopterin synthesis. Tetrahydrobiopterin is an essential co-factor for three aromatic amino acid mono-oxygenases, tyrosine hydroxylase, tryptophan hydroxylase and phenylalanine hydroxylase, as well as for nitric oxide synthase. Tyrosine hydroxylase is in turn the rate-limiting enzyme in the dopamine synthesis pathway. Dopamine deficiency in the CNS is thought to be central to the development of dystonia, as L-dopa therapy alleviates dystonia in DRD and treatment with dopamine receptor antagonists (such as antiemetics and neuroleptics) may cause both acute and tardive dystonia (Williams, 1995). Several different mutations have been detected in different families with autosomal dominant DRD, and most are private, thus making the mutation screening of the GCH1 gene a hard, expensive and time-consuming task (Hirano et al, 1995; Ichinose et al, 1995a and b; Bandmann et al, 1996a; Furukawa et al, 1996; Furukawa and Kish, 1999; Ichinose et al, 2001; Bandmann and Wood, 2002; Muller et al, 2002).

Rare autosomal recessive inheritance of a DRD phenotype has also been described in patients with homozygous mutations in other genes coding for enzymes in the dopamine synthesis, or tetrahydrobiopterin synthesis pathways. Ludecke and co-workers reported two brothers with homozygous missense mutations in the tyrosine hydroxylase gene associated with reduced activity of the enzyme (Ludecke et al, 1995; Knappskog et al, 1995). Hanihara and colleagues (1997) reported two sisters from a consanguineous marriage with an atypical form of DRD characterised by severe dopa-responsive dystonia with diurnal variation, mental retardation, and seizures. Both sisters had homozygous mutations in the gene coding for the enzyme 6-pyruvoyl-tetrahydropterin synthase, which catalyses the second step in tetrahydrobiopterin synthesis (Corner, 1952). Dystonia in a paternal uncle indicating autosomal dominant inheritance with reduced penetrance, as well as the clinical description, including prominent diurnal variation in symptoms, suggest that these patients may have had DRD. Subsequently Nygaard et al (1991) provided an update on one of the siblings, whose response to low dose benzhexol had been maintained for 30 years, and described another patient with a similar response. Both had been switched to L-dopa with good results, supporting the belief that these patients had DRD. Several other cases of generalised dystonia with marked response to low-dose anticholinergic

therapy have been described and have led to suggestions that DRD underlies many such cases.

DYT6

A locus for a mixed dystonia phenotype has been mapped to a 40 cM interval, spanning the peri-centromeric region of chromosome 8, in two German-American Mennonite families (Almasy et al, 1997a). Although some family members have a phenotype similar to the DYT1 phenotype, others have only segmental or focal cranial-cervical dystonia after long follow-up. Spread to cranial-cervical muscles occurs in most patients and is the major cause of disability in many. The wide age-at-onset distribution (2-69 years), and the overall tendency to involve limb and cranial-cervical muscles equally, are claimed as distinguishing features of the DYT6 phenotype. Both families share an identical haplotype over the 40 cM candidate interval, which suggests a common founder mutation despite the absence of known relationship between these families.

DYT7

Only one locus for late-onset, purely focal dystonia has been identified to date. Leube et al (1996) described a German kindred with purely focal dystonia showing autosomal dominant inheritance and reported linkage to markers spanning an approximately 30 cM interval at the telomere of chromosome 18p. Dystonia in this kindred was largely confined to the cervical muscles resulting in torticollis in most individuals. Similarly affected, apparently sporadic cases of focal dystonia in Northern Germany were found to share a common haplotype over a 6 cM region of 18p (Auburger et al, 1997; Leube et al, 1997b), suggesting that dominant inheritance at low penetrance of a founder mutation in the DYT7 gene may be an important cause of focal PTD among a relatively isolated population of Northwest Germany. However, these findings have not been confirmed in another study of focal dystonia in Northern Germany and must therefore be interpreted with caution (Klein et al, 1998b). The contribution of this locus to focal dystonia in other populations remains to be determined. The existence of a gene for focal dystonia on chromosome 18p is supported by the finding that patients with cytogenetic abnormalities of the terminal short arm of chromosome 18 (deletions or balanced translocations) have focal or segmental dystonia as part of a broader syndrome including mental retardation, dysmorphic appearance and short stature (Klein et al, 1999d).

Aims of the study

The aims of the present study were: a) to evaluate the role of the DYT1 GAG deletion in the British PTD population and to analyse the presence of founder mutations in the British PTD patients carrying the DYT1 mutation; b) to perform a linkage analysis with the known PTD loci in 3 PTD families; c) to identify other PTD loci by means of genome-wide searches.

What is the role of DYT1 in British patients with primary torsion dystonia?

In order to evaluate the role of the DYT1 3-bp deletion in British PTD patients, and to clarify the relationship between genotype and phenotype in dystonia, we studied 150 patients with PTD for the presence of the GAG deletion in the DYT1 gene. We also analysed the haplotypes surrounding DYT1 in 9 AJ and 15 non-Jewish British patients carrying the GAG deletion in the DYT1 gene, to search for linkage disequilibrium and for the presence of founder mutations in the Jewish and non-Jewish British population.

What is the role of known PTD loci (DYT1, DYT6 and DYT7) in PTD families?

Until the DYT6 and DYT7 genes are cloned, evaluation of the role of these loci in PTD will depend upon allelic association studies in patients with apparently sporadic PTD – such as that of Leube and co-workers (1997b) – and linkage analysis in suitable families. However, large families with non-DYT1 PTD are comparatively rare. Only seven such families had been reported at the time of this project (Parker, 1985; Uitti and Maraganore, 1993; Bressman et al, 1994b; Holmgren et al, 1995; Bressman et al, 1996; Bentivoglio et al, 1997b, Jarman et al, 1999), excluding the three families in which the DYT6 and DYT7 loci were mapped. One of these families (PTD01, Bentivoglio et al, 1997b) was available for further study; furthermore the author ascertained and clinically characterised two additional families (PTD02 and PTD03). All had juvenile or adult-onset dystonia with a predominantly cranio-cervical or a mixed PTD phenotype. In order to determine whether any of these families were linked to known loci, linkage analysis was performed using microsatellite markers spanning the DYT1, DYT6 and DYT7 loci. In family PTD01, linkage with the DYT1 locus had been previously excluded by means of linkage analysis and these results are therefore not presented in this thesis.

Genetic mapping of the DYT13 locus

A simulation linkage study in family PTD01 indicated that this family was informative to perform a genome-wide search. This allowed mapping a novel dystonia locus, which we termed DYT13, on the short arm of chromosome 1. Linkage with DYT13 was then tested in families PTD02 and PTD03.

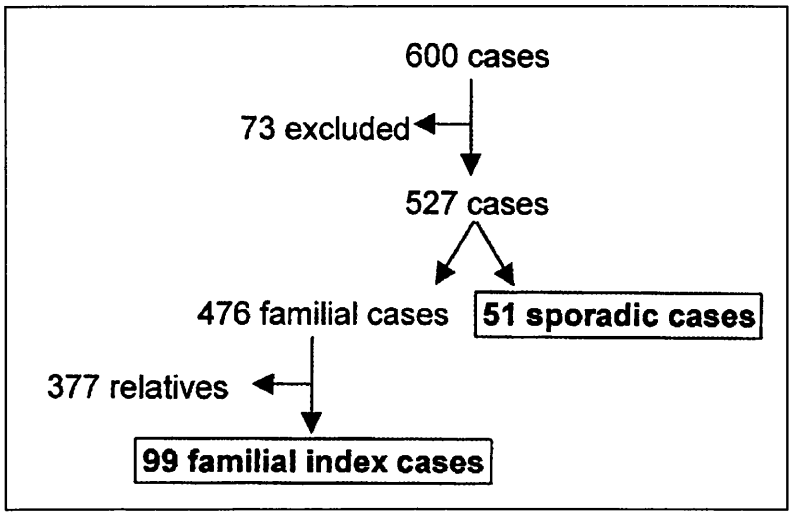
PTD patients

Patients selected for the DYT1 study

Patients were selected from the dystonia database of the movement disorder clinic and the neurogenetic clinic at the NHNN, London and from previous clinical and genetic studies of familial PTD (Fletcher et al, 1990a; Warner et al, 1993). Inclusion criteria were 1) generalised, multifocal, segmental or focal dystonia as defined by published criteria (Fahn et al, 1987a); 2) a clinical course compatible with PTD with no features to suggest secondary dystonia or other dystonic states such as Dopa-responsive dystonia or paroxysmal dystonias.

A total of 600 cases were initially ascertained, and the available hospital notes were examined. Seventy-three cases were excluded because of patient refusal, unavailability of clinical notes, uncertain diagnosis or probable secondary dystonia. In cases of familial PTD (476 patients in 99 families), only index cases were included. A total of 150 patients were included in the study (99 familial index cases and 51 sporadic cases) (figure 3.1).

Figure 3.1 – strategy for identification of PTD patients for the DYT1 study



All patients had been examined by a neurologist from the Movement Disorder Group (NHNN) and had blood sampled with informed consent; at the time of the study the majority of patients were under follow up at either the movement disorder clinic, the botulinum toxin clinic or the genetic clinic of the same hospital. Nationality, ethnicity and Jewish ancestry for each patient were obtained through clinical records or by phoning the patient directly. All patients were British except two Italian, three French and one Asian subject; eight were of Ashkenazi Jewish ancestry, one was Sephardic Jewish and two patients had one parent of Ashkenazi Jewish origin.

At the time of the DYT1 linkage disequilibrium study, more patients with the DYT1 mutation had been ascertained at the genetic lab of the NHNN. Twenty-four PTD patients carrying the GAG deletion were selected on the basis of the availability of at least one parent for the genetic study. Parents had already been consented and blood sampled previously, or alternatively they were contacted and agreed to participate in the study. For Ashkenazi Jewish DYT1 patients, fifty-four unrelated Ashkenazi Jews were included in the study as controls; these healthy individuals had been previously recruited from the Jewish community of London. For non-Jewish DYT1 patients, twenty-five unrelated, non-Jewish families (two parents and one child) selected from the Huntington's disease database at the NHNN were used as a control population. Additionally, this sample was supplemented with another 75 unrelated individuals from the neurofibromatosis database and the neuromuscular diseases database at the NHNN.

PTD families

Three families have been ascertained for this study. The clinical features of family PTD01 have been described in detail previously (Bentivoglio et al, 1997b). None of the three families have any known Ashkenazi Jewish heritage.

Family PTD01

Family PTD01 is an Italian family, first described by Bentivoglio and co-workers (1997b). The pedigree is illustrated in figure 3.2. To avoid presenting this pedigree twice and for clarity in result presentation, this figure has been placed in the "Results" section of this chapter (page 94). The family was examined for the first time in 1994 by Dr AR Bentivoglio and Dr E Cassetta. At that time, among the forty-five family members examined, eight individuals received a diagnosis of definite dystonia, six

received a diagnosis of probable dystonia and three deceased family members had definite dystonia by history. The age at onset in definitely affected subjects ranged from 5 to 40 years. The phenotype was characterised by focal or segmental dystonia with onset either in the cranial-cervical region or in the upper limbs, mild course and occasional generalisation. Symptoms began in the cranial-cervical region in six of the definitely affected cases, and in the arms in the remaining two. After a mean disease duration of 41 years (range 5-66 years) symptoms have remained focal in one patient, and progressed to involve the cranial-cervical muscles, the arms or the trunk in the others. Of the patients who showed progression, dystonia was segmental in four patients, and mild-generalised in two at the time of examination. Among the probable cases, progression of symptoms was less evident, with a focal or segmental distribution of dystonia in all cases. Of the fourteen living definitely and probably affected family members, twelve had prominent involvement of cranial-cervical muscles on examination.

All probably affected and unaffected family members agreed to be re-evaluated in March 2000 by Dr AR Bentivoglio and the author of this thesis. At that time, three more individuals (III:11, III:20 and IV:9) had developed a definite dystonia. Individuals III:11 and IV:9 had been diagnosed as unaffected in 1994. On the latest examination, individual III:11 presented with dystonic tremor and posturing of the neck. Individual IV:9 presented with marked irregular tremor and bilateral dystonic posturing of the upper limbs, and writer's cramp. Individual III:20 had received a diagnosis of probable dystonia in 1994. On the latest examination, she had dystonic posturing of the right arm while writing, and dystonic jerks and posturing of the neck partially controlled by a sensory trick. In these three subjects, aged 65, 41 and 58 respectively, the age at onset could not be accurately defined, as dystonia was very mild at onset and worsened slowly over time. The patients or relatives did not take special notice of the symptoms and could not be precise as to date of onset. The remaining five individuals who were diagnosed as probably affected in 1994 did not present any evolution over six years. They still had minor clinical signs (jerks of neck or of the arm or mild tremor), but no spasmodic movements or postures were evident, no directional or task-activated movements, no sensory tricks. These people have been considered in this study as "undetermined phenotype" and not included in the linkage analysis. The inheritance of PTD was autosomal dominant, with affected individuals spanning three consecutive generations and male-to-male transmission. A summary of

the clinical presentation of dystonia in definitely affected family members is given in table 3.2.

Table 3.2 – phenotype of family PTD01

Subject	onset		latest examination	
(sex)	age	site	age	PTD distribution
III:2 (F)	5	cranial-cervical	76	upper face, larynx, neck, upper limbs (segmental)
III:6 (M)	10	cervical	67	upper face, neck, upper limbs (segmental)
III:10 (F)	26	cranial-cervical	68	upper face, larynx, pharynx, neck, upper limbs (segmental)
III:11 (F)	unknown	cervical	65	neck, right upper limb (segmental)
III:14 (F)	6	upper limbs	61	upper face, larynx, neck, trunk, limbs (generalised)
III:16 (F)	5	cervical	59	upper and lower face, neck, trunk (segmental)
III:18 (M)	20	cervical	56	lower face, neck, limbs (generalised)
III:20 (F)	unknown	cervical	58	neck, right upper limb (segmental)
IV:1 (M)	40	right upper limb	45	right upper limb (focal)
IV:3 (M)	14	cranial-cervical	32	lower face, larynx, neck (segmental)
IV:9 (M)	unknown	upper limbs	41	upper limbs (segmental)

Detailed clinical description of definitely affected family members

Subject III:6 (segmental dystonia). This 67 year-old man is the index case. His personal history was uneventful until the age of 10, when he had cervical and head trauma as a consequence of a fall from a six-meters high (the patient reports the head trauma as "severe", but he was not hospitalised). At the age of 15 he presented abnormal movements of the head, described as rapid (clonic) rotational movements and lateral head tilt. The clinical picture did not change until 1973 (when he was aged 46), apparently related to a high fever (over 42°C) of unknown origin. After the fever, cervical dystonia exacerbated without spreading to other body regions. Several years later, he developed right arm dystonia; it started with task-induced dystonic

movement, rapidly progressing to dystonic posture at rest. Several oral therapies, also in combination, failed to induce any clinical improvement; so, at age of 54, he underwent surgical resection of right sterno-cleido-mastoidal muscle without clinical benefit. During evaluation, he presented with segmental dystonia, involving the cranio-cervical segment and the right upper limb. Dystonic symptoms consisted in involuntary jerky movements of the head and of the right shoulder, inducing a tremulous right rotational torticollis, pain and rigidity of the neck and hyperlordosis. He had abnormal posture of the right wrist and dystonic movements of both arms (more severe in the right side), and right foot mirroring. Because of upper limbs dystonia, the patient was clumsy in performing tasks such as writing, drawing or handling objects.

Subject III:2 (segmental dystonia). This 76-year woman suffered from fever-related seizures during infancy. Dystonic symptoms in the upper body (cranial-cervical and upper limbs) are reported since the age of 5. She had a head trauma and right arm fracture at age of 18. On last examination the patient presented with segmental dystonia involving cranial, cervical, upper limbs and larynx (adductor dysphonia). The patient and her relatives reported that she was fully symptomatic since presentation and that progression was in terms of severity of symptoms but no spreading to other body regions was observed. A pregnancy ended with caesarean delivery and a surgery for kidney stones were not reported to influence dystonia. The progression was slow, as she was able to work until young adulthood. Several combinations of oral drugs and botulinum toxin treatment did not improve substantially dystonic symptoms. At last visit, the patient presented cranial-cervical dystonia moderate in severity, and severe upper limb dystonia, which incapacitated her to use both upper limbs: the patient was unable to write and needed help in tasks of daily living requiring fine hand and finger movements.

Subject III:10 (segmental dystonia). This 68-year woman had uneventful personal history until the age of 20, when she was cured with penicillin for rheumatic fever. As a child, she was stuttering. She had two pregnancies and one abortion. She reported the onset of jerky movements of the head when aged 26, after the first pregnancy. Later, dystonia progressed to involve pharyngo-laryngeal muscles; as a consequence the patient had troubles in swallowing and reported hypophonia and voice tremor.

At last visit the patient presented with a cranial, cervical, larynx and upper limb dystonia. Dystonic movements of the neck consisted in backward jerks and gross

irregular head tremor; she presented also tremor and dystonic posture of the hands, but she was still able to write with a trembling but understandable writing.

Subject III:11 (segmental dystonia). This 65-year old housekeeper had an uneventful personal history. She was unaware of any involuntary movement. When examined in 1995 at the age of 60, she presented slight involuntary movements of the head and tremor of both hands during the finger-to-nose manoeuvre, but no dystonic movements or posture were evident. When re-evaluated in 2000 she presented torticollis and left hand dystonia, and was diagnosed as definitely affected by segmental dystonia. The symptoms did not impair any task of daily living.

Subject III:14 (generalised dystonia). This 61-year old woman reported phobic symptoms which had started in young age. She developed hand dystonia at the age of 6; since almost the beginning of the disease, she had a shaking tremor involving all the body. A mild occipital trauma occurred at age of 7. She had three pregnancies; after the first one at the age of 26, she noted that the severity of dystonia had progressed. At the moment of evaluation she presented with spasmodic dysphonia, dystonic posture of the head and strong head tremor, dystonic posture of shoulder and arms (more evident in the right arm), and dystonic tremor of legs. Upper limbs dystonia impaired moderately activities of daily living such as feeding, writing and handling small utensils.

Subject III:16 (segmental dystonia). This 59 year old woman reported a nervous breakdown in her thirties. At the moment of evaluation she presented with facial involuntary movements (more evident when speaking), rigidity and pain of the neck due to dystonic posture (right laterocollis), dystonic posture of both arms and involuntary movements of the fingers (evident when walking) and writer's cramp. She had also pronounced scoliosis with hypertrophic paravertebral muscles and hyperlordosis, with an abnormal truncal posture when walking. Several years ago, she was treated with increasing doses of levodopa (up to 200 mg q.i.d.) without any clinical improvement. She had three pregnancies (one ended with abortion) which are not reported to influence dystonia.

Subject III:18 (generalised dystonia). This 56 year old man reported the onset of a mild cervical dystonia at the age of 20. Dystonia did not progress for three decades, and only when he was in his fifties, he experienced a mild exacerbation of symptoms, consisting in dystonic posture of right hand and involuntary movements of both hands and head. At the moment of evaluation he presented with scoliosis, tremulous

dysphonia, dystonic tremor of both hands, involuntary movements of the lips and eyes. The subject also reported early morning painful contracture of both legs and rapid jerky movements of the neck; this latter symptom sometimes woke him up during the night. Symptoms moderately affected writing and activities of daily living, this man being a farm worker employed in a full time heavy job.

Subject III:20 (segmental dystonia). This 58-year old woman had been diagnosed as probably affected in 1994. On the second evaluation in 2000 she was diagnosed as definitely affected, presenting with dystonic posturing of the right arm while writing, and dystonic jerks and posturing of the neck partially controlled by a sensory trick. Her writing was, however, only moderately clumsy and all words were legible.

Subject IV:1 (focal dystonia). This 45-year old employee had normal development and an uneventful personal history until the age of 40, when he noticed painful and distressing writing, a change in the way of holding the pen when writing and a different hand writing. At the moment of evaluation he presented with a writer's cramp, which did not impair excessively his handwriting. He also presented an abnormally high blinking rate, reported since he was an adolescent. In the five years after onset, dystonia symptoms were reported as steady.

Subject IV:3 (segmental dystonia). This 32-year old man had pre-term delivery with forcipes and normal psycho-motor developmental steps. Since the age of 14 he presented mild dystonic symptoms, exacerbated from stress. Symptoms at onset are described as stuttering with tic-like movements of facial muscles. During the first evaluation, in 1994, he presented with rapid tic-like movements in left upper and lower facial muscles, frequent irregular blinking, mild dysphagia with occasional chocking (when drinking), voice tremor, stuttering and hypophonia, dystonic movements of the neck, and scoliosis. During writing, he presented abnormal posturing of the hands and jerky myoclonic-like movements of the neck, pulling the head backwards. When re-evaluated, six years later, we did not observe any progression of dystonia.

Subject IV:9 (segmental dystonia). He is a 41-year old man, son of III:14. This subject was unaffected in 1994. When re-evaluated, six years later, he presented with marked irregular tremor and bilateral dystonic posturing of upper limbs and writer's cramp. During writing, he had some tic-like movement of the face.

Family PTD02

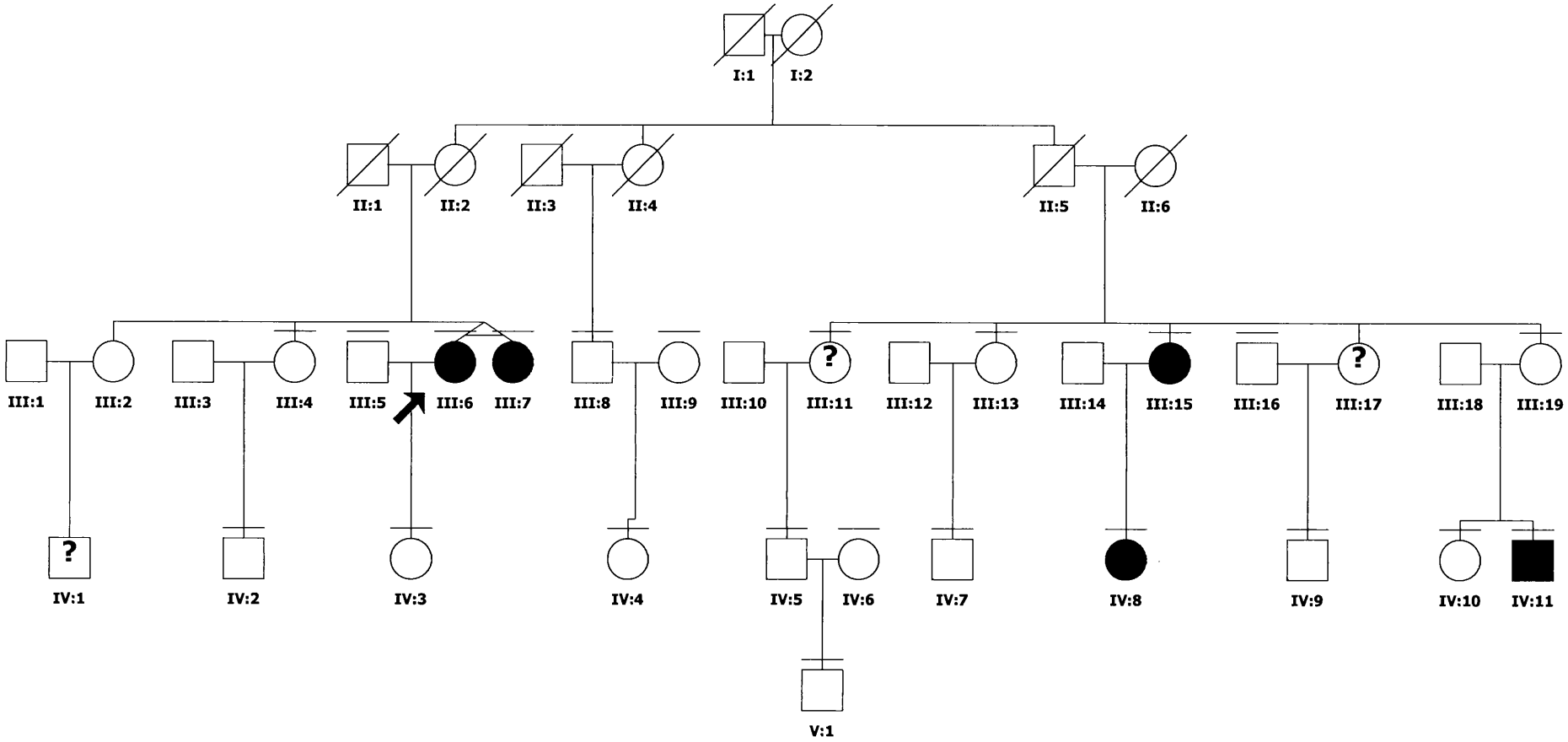
Forty-three subjects, including thirty-six family members and seven spouses, were personally examined by the author of this thesis, together with Dr AR Bentivoglio and Dr E Cassetta (Catholic University, Rome). Twenty subjects belonged to the third generation and twenty-three to the fourth. Among the thirty-six family members studied, eight had a diagnosis of dystonia (either definite or probable) and twenty-eight were unaffected. All the definitely or probably affected members belonged to the third or the fourth generation. The family pedigree indicated an autosomal dominant pattern of inheritance, with reduced penetrance (figure 3.3). Table 3.3 summarises the clinical data.

Table 3.3 – phenotype of family PTD02

Subject	onset		latest examination	
(sex)	age	site	age	PTD distribution
III:6 (F)	65	cervical	69	face, larynx, neck, trunk, upper limbs (segmental)
III:7 (F)	66	cranial	69	upper and lower face, neck, (segmental)
III:15 (F)	unknown	lower face	74	upper and lower face (segmental)
IV:8 (F)	3rd decade	upper face	54	upper face (focal)
IV:11 (M)	40	writer's cramp	43	writer's cramp (focal)
III:11 (F)*	unknown	upper face	80	upper and lower face, neck, (segmental)
III:17 (F)*	unknown	upper face	56	upper face (focal)
IV:1 (F)*	unknown	upper face	60	upper face (focal)

*probable cases

Figure 3.3 – pedigree of family PTD02



Detailed clinical description of affected family members

Five subjects (four women and one man) received a diagnosis of definite PTD. Two patients (III:6 and III:7) were identical twin sisters, the remaining two women were first-degree relatives (subject III:15 and her daughter IV:8). The mean age (± SD) at the time of study was 61.8 ± 12.9 years (range: 43-74). The age at disease onset (clearly identified only for cases III:6, III:7 and IV:2) was 65, 66 and 40 years respectively. The interval between disease onset and the first clinical examination was four years in one case, three years for the two other cases. Dystonia started in the cranial district in three subjects, in the cervical region in one, in one upper limb in one. Progression to other body regions was observed only in two subjects. At the time of examination, the clinical presentation of dystonia was segmental in three patients and focal in two (one with cranial dystonia, one with writer's cramp).

Subject III:6 (segmental dystonia). This 69-year-old woman is the index case of the family. She was born after an uncomplicated full-term twin-pregnancy; no problems occurred during delivery. At the age of 65, she noted the onset of retrocollis. Dystonia rapidly progressed to cranial muscles, to the platysma, larynx, trunk, and eventually to the upper limbs within one year from onset. On examination, blepharospasm, spasmodic dysphonia, facial, cervical, upper limb and trunk dystonia were the prominent signs. Blink rate was higher than expected: 84 blinks per minute during conversation, 80 at rest and 6 while reading. Video-laryngoscopic evaluation confirmed the diagnosis of laryngeal dystonia. She had no pyramidal, cerebellar or sensory signs, and the intellect was normal. No clinical features of secondary dystonia, paroxysmal dystonia or alcohol-responsive myoclonic dystonia were observed. Brain CT and MR imaging were unremarkable. Biochemical routine for secondary dystonia was negative. The patient received a treatment with levodopa/carbidopa (250/25 mg q.i.d.) for eight weeks without clinical improvement. Acute ingestion of alcohol (30 ml) failed to ameliorate dystonia. She was treated with different combinations of psychoactive drugs, myorelaxants and anticholinergics in association with botulinum toxin, which improved blepharospasm and cervical dystonia. At variance with her twin sister, this woman used to carry heavy weights on the head for many years before the onset of cervical dystonia.

Subject III:7 (segmental dystonia). She is the identical twin sister of the index case. Early development was normal. At the age of 66, she first had involuntary movements of the peri-oral muscles. Later, dystonia progressed to other cranial muscles and to the

neck. Brain CT scan and biochemical laboratory tests were unremarkable. There were neither identifiable causes of secondary dystonia nor any precipitating factor. At the time of first examination she had blepharospasm and cranial dystonia, involuntary contractions of the platysma, a slight bilateral tremor of the upper limbs and a tremulous voice. Blink rate was 80 during conversation, 54 at rest and 24 while reading.

Subject III:15 (segmental dystonia). This 74-year-old woman denied any symptom. Her relatives had noticed abnormal eye movements during the last few years. On examination, involuntary movements of the lower eyelids and occasional spasmodic contractures of the orbicularis oculi were observed. Voice tremor and "yes-yes" tremor of the head were also evident. Prolonged spasms occurred during conversation; blink rate was within the normal range: 19 during conversation, 22 at rest, 2 while reading. Still, at variance with the normal pattern, blink rate at rest was higher than that during conversation (Bentivoglio et al, 1997a).

Subject IV:8 (focal dystonia). At the time of clinical evaluation, this woman was 54 and unaware of suffering from dystonia. She reported increased blink rate as a consequence of emotional states and ocular discomfort when exposed to bright light. She could not reckon when this trouble started; other family members reported that her blinking was exaggerated since she was a young girl. On examination, she had blepharospasm, involuntary movements of the lips and jaw, and a mild bilateral postural tremor of the upper limbs. Blink rate was slightly higher than normal during conversation (60 blinks per minute) and while reading (11), but normal at rest (26).

Subject IV:11 (focal dystonia). This 43-year-old right-handed bank employee was born after an uncomplicated pregnancy and a normal delivery. Personal history was uneventful. Since adolescence, he presented bilateral hand tremor and eye discomfort for bright light. He had suffered with difficulties in writing for the last three years. Approximately one year before examination, he had to stop writing with his right hand and to start typing, because of the increasing pain and the non-intelligibility of handwriting. For this reason he required medical intervention. At neurological examination, abnormalities during handwriting were observed: writing with the right hand was effortful, forearm muscles were abnormally tense and contracted although the quality of handwriting did not worsen progressively; handgrip was abnormal. Blink rate was normal: 32 during conversation, 19 at rest and 6 while reading. The patient's father, unrelated to family PTD02, was reportedly affected by bilateral hand tremor

and presented in his late adulthood abnormalities in his handwriting suggestive of writer's cramp. An evaluation of the paternal family of this patient revealed familial dystonia (one case of definite and two of probable upper limb dystonia).

Three women received a diagnosis of probable dystonia. Their mean age at the time of the study was 65.3 ± 12.9 years (range 56-80). Age at disease onset could not be ascertained.

Subjects III:11 (segmental dystonia). This 80-year-old woman denied any symptom. In 1985 she received a diagnosis of depression and was treated with nortriptiline. On observation, over-activity of the lower eyelid was evident. Blink rate was 27 during conversation, 3 at rest; it was impossible to evaluate blink rate while reading due to a severe visual impairment. A mild laterocollis and occasional lip and jaw protrusion were observed. Contra-lateral mirror movements occurred bilaterally when performing repetitive movements of the lower limbs.

Subject III:17 (focal dystonia). This 56-year-old patient reported frequent and intermittent involuntary movements of the lips and tongue, rigidity of the neck, clumsiness in drawing and eye discomfort (sensation of dry eyes). On examination, frequent eye blinking, involuntary lip protrusion, some involuntary movements of upper and lower facial muscles and of the platysma were evident. Blink rate was normal: 60 during conversation, 72 at rest and 14 while reading.

Subject IV:1 (focal dystonia). This 60-year-old woman reported eye discomfort when exposed to bright light, and head tremor. On examination, her blink rate was higher than expected: 46 during conversation, 78 at rest and 6 while reading. The relative blink rate pattern was also abnormal. A mild head tremor and clumsy movements of the left lower limb were observed.

Family PTD03

Figure 3.4 shows the pedigree of the family and table 3.4 the clinical features of the affected family members. Seventeen family members were personally examined and videotaped by Dr A Münchau and videos were reviewed by Dr K Bhatia. Five members had definite dystonia and one had probable dystonia (III:2). Three members of the family were considered affected by history: two were deceased (II:4, I:1), while the third one is a young girl (IV:15) whose parents did not agree to a clinical examination. Relatives reported that she had excessive eye blinking. No other family member was reported to be affected.

Figure 3.4 – pedigree of family PTD03

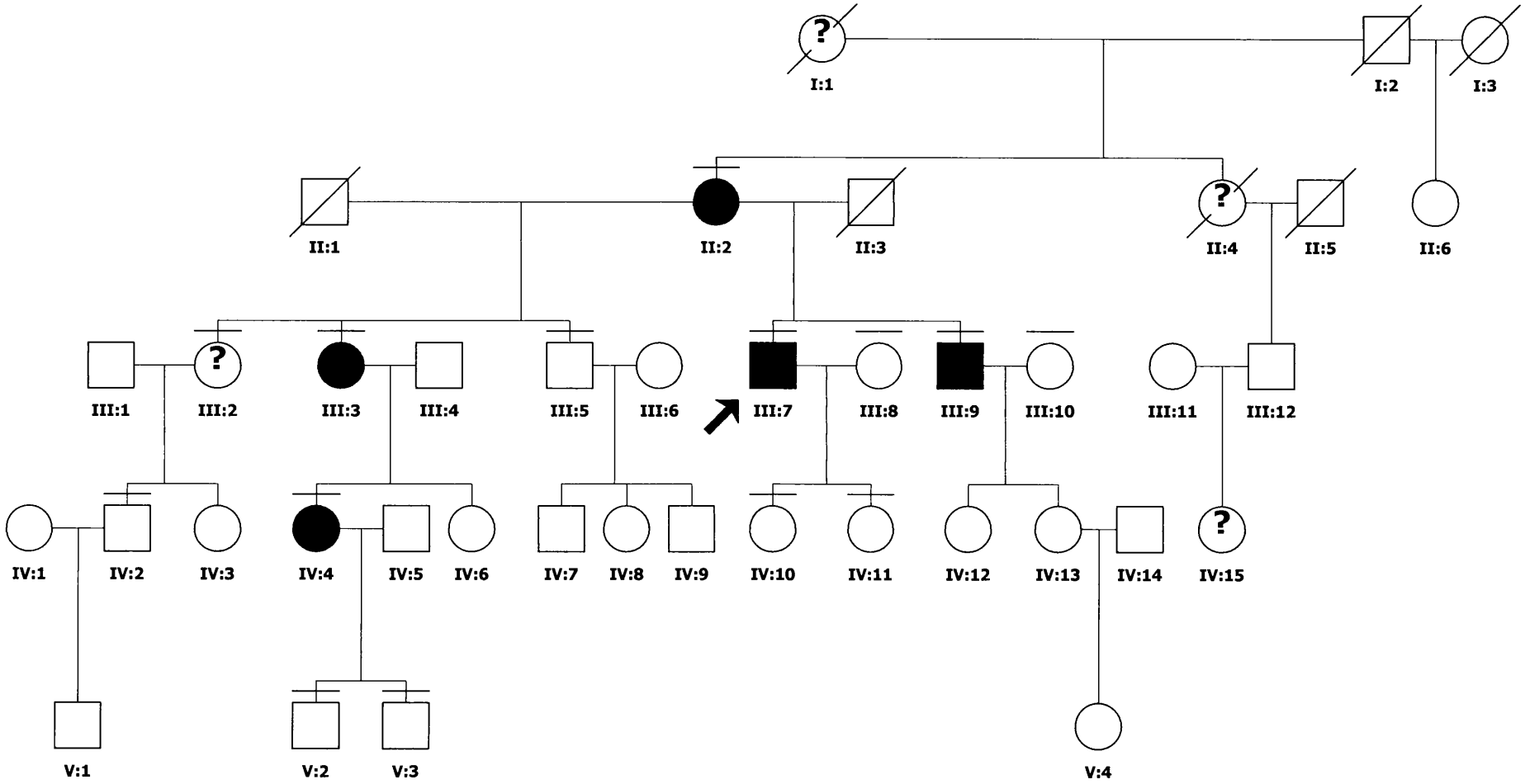


Table 3.4 – phenotype of family PTD03

Subject	onset		latest examination	
(sex)	age	site	age	PTD distribution
III:7 (M)	40	cervical	55	upper and lower face, larynx, neck , upper limb (segmental)
III:9 (M)	30	larynx	57	upper and lower face, larynx neck (segmental)
III:3 (F)	30	cervical	45	neck, larynx, upper limb (segmental)
IV:4 (F)	19	cervical	24	neck, upper limb (segmental)
II:2 (F)	27	cervical	85	neck, larynx, upper limb (segmental)
III:2 (F)*	unknown	cervical	47	neck, upper limb (segmental)
II:4 (F)*	unknown	cervical	deceased	neck (focal)
I:1 (F)*	unknown	cervical	deceased	neck (focal)
IV:15 (F)*	unknown	upper face	15	upper face (focal)

* cases with probable dystonia or affected by history

The transmission pattern appeared to be autosomal dominant with reduced penetrance. Mean age of onset in affected subjects was 29 years (range: 19-40 years). The average duration of dystonia (from onset until examination) was 24 years with a range of 4-58 years. All affected members had jerky cervical dystonia. Two of them also had blepharospasm, four had voice tremor and one had writing tremor. Dystonic posturing was found in four subjects and mild postural tremor in the arms in two.

The index case (III:7) was a 55-year old man who developed a head shake and started having difficulties with singing at the age of 40. Alcohol slightly improved the head tremor. On examination at the age of 55 he had a mild jerky torticollis to the right which improved when he was touching his chin with the index finger. He also had mild blepharospasm, a voice tremor and bilateral postural arm tremor. When he attempted to write his right hand adopted dystonic postures and he developed a severe tremor so that writing became impossible. He was receiving Botulinum toxin injections, which improved the stiffness of his neck muscles but had no effect on jerky head movements.

DNA analysis

DYT1 study

For the DYT1 study, the GAG deletion in the DYT1 gene was looked for in 150 PTD patients. For the haplotype study, six microsatellite loci from chromosome 9q34 were genotyped in twenty-four DYT1 mutation carriers (nine Ashkenazi Jews and fifteen non-Jewish patients) and thirty-five healthy parents. The six markers were also typed in a control population formed by fifty-four healthy unrelated Ashkenazi Jews, twenty-five unrelated non-Jewish families (two parents and one child) selected from the Huntington's disease database at the NHNN and seventy-five other unrelated individuals from the neurofibromatosis database and the neuromuscular diseases database at the NHNN. The six loci were ordered as follows: *cen* - D9S62B - *170Kb* - D9S2160 - *<40Kb* - D9S2161 - *150Kb* - D9S63 - *130Kb* - D9S2162 - *>40Kb* – ASS - *tel*. In selected subsets of AJ and non-Jewish patients (groups A, B, D and E – see Results section) the additional locus D9S2163 was typed. This locus is situated in between D9S2162 and ASS. Distances between adjacent markers are those reported by Ozelius and colleagues (1997a). PCR analysis of the microsatellite markers was carried out on genomic DNA as described in chapter 2. To allow comparison between our results in the British AJ population and the data previously reported in the North American Jewry, the 7 microsatellite loci were also typed in a North American AJ known to carry the $DYT1^{AJ}$ and assigned allele numbers accordingly. The other alleles for each locus have been numbered arbitrarily.

Linkage analysis of known PTD loci in 3 families

For family PTD01, DNA from 47 family members was available for study. For families PTD02 and PTD03, DNA was available from 43 and 17 individuals respectively.

DYT1 locus

The DYT1 mutation (3-bp deletion) was analysed in 2 affected members from each family. Additionally, the following microsatellite markers spanning the DYT1 region were typed in families PTD02 and PTD03, to ensure that dystonia in these families was not due to mutations in the DYT1 gene other than the 3-bp deletion:

cen - D9S2160 - *<40Kb* - D9S2161 - *150Kb* - D9S63 - *130Kb* - D9S2162 - *tel*.

In family PTD01, linkage of dystonia with the DYT1 region had already been excluded (Bentivoglio et al, 1997b).

DYT6 and DYT7 loci

The candidate regions containing the DYT6 and DYT7 loci are large (30-40 cM each). To fully exclude either region for each family required a marker density which varied with the size of the family and the number of recombinations with affected individuals in each region. For the DYT6 region, the following microsatellite markers were analysed:

Family PTD01: *cen* - D8S1791 - *6.6cM* - D8S538 - *2.2cM* - D8S509 - *7.9cM* - D8S285 - *4.9cM* - D8S507 - *4.0cM* - D8S260 - *3.4cM* - D8S1797 - *7.0cM* - D8S543 - *5.8cM* - D8S279 - *4.3cM* - D8S286 - *tel*

Family PTD02: *cen* - D8S1791 - *8.3cM* - D8S285 - *8.2cM* - D8S260 - *11.4cM* - D8S279 - *3.3cM* - D8S286 - *tel*

Family PTD03: *cen* - D8S1791 - *4.3cM* - D8S601 - *2.1cM* - D8S509 - *1.6cM* - D8S166 - *2.8cM* - D8S374 - *1.6cM* - D8S507 - *2.5cM* - D8S1113 - *4.9cM* - D8S1797 - *4.7cM* - D8S543 - *4.0cM* - D8S279 - *3.1cM* - D8S286 - *tel*

For the DYT7 region, the following microsatellite markers were analysed:

Family PTD01: *cen* - D18S59 - *12.9cM* - D18S1140 - *2.0cM* - D18S818 - *1.2cM* - D18S1368 - *4.0cM* - D18S1098 - *1.2cM* - D18S481 - *1.3cM* - D18S54 - *1.0cM* - D18S52 - *12.8cM* - D18S62 - *0cM* - D18S452 - *7.3cM* - D18S1163 - *3.4cM* - d18S843 - *tel*

Family PTD02: *cen* - D18S54 - *10cM* - D18S62 - *0cM* - D18S452 - *14.7cM* - D18S464 - *2.3cM* - D18S1153 - *1.6cM* - D18S1150 - *tel*

Family PTD03: *cen* - D18S1140 - *2.0cM* - D18S1105 - *2.7cM* - D18S1098 - *1.4cM* - D18S481 - *1.4cM* - D18S54 - *10.0cM* - D18S452 - *5.3cM* - D18S1163 - *4.0cM* - D18S843 – *tel*.

Microsatellite markers were from the Généthon and CHLC maps. All map distances are according to the Marshfield Clinic genetic map.

In each family, pairwise LOD scores were calculated for dystonia and markers in the candidate regions using the FASTLINK 3.0P version of the MLINK linkage program. An autosomal dominant model of inheritance was used with a disease gene frequency of 0.0001. In the first analysis, only definitely affected family members were classified as

'affected', while unaffected and probably affected family members were classified as phenotype 'unknown'. This more conservative, 'affecteds only', approach is useful to avoid biases from the inclusion of possibly affected individuals or incorrect estimation of penetrance or age of onset. Repeating all linkage calculations considering healthy family members as "unaffected" and setting a reduced penetrance (0.40) did not result in a significant difference in LOD score values. Obligate gene carriers were considered to be definitely affected for the purposes of linkage analysis. The affected identical twins in family PTD02 were considered as a single person for the purposes of linkage.

Genome-wide search in family PTD01

Four hundred and twenty-three markers were analysed in family PTD01. The first screening was done typing 380 markers from the ABI PRISM Linkage Mapping Set LD-10 in the 11 definitely affected individuals. Pairwise LOD scores were calculated using the FASTLINK 3.0P version of the MLINK linkage program, using an autosomal dominant model of inheritance with a disease allele frequency of 0.0001. Regions surrounding non-informative markers or markers showing possible evidence of linkage (LOD score > 1) were saturated with more microsatellite markers (selected from genetic maps and obtained ordering custom-made fluorescently labelled primer pairs). Unaffected individuals were also typed in order to manually construct haplotypes. For these additional markers calculations were performed twice: once using an "affecteds only" approach, and again including unaffected family members in the analysis and setting a reduced penetrance value (0.40).

Results

Analysis of the DYT1 mutation and genotype-phenotype correlation

Phenotypes of mutation-positive and mutation-negative patients are summarised in table 3.5. The correlates between the clinical subtypes, the presence or absence of a family history and the results of mutation analysis are shown in table 3.6.

Table 3.5 – phenotypes of DYT1-positive and DYT1-negative patients

Phenotype	DYT1+	DYT1 -	Total
generalised dystonia			
- limb onset without spread to cranial-cervical muscles (typical DYT1 phenotype)	15 (60%)	10 (40%)	25
- limb onset spreading to cranial-cervical muscles	4 (29%)	10 (71%)	14
- neck onset spreading to limbs	0	3 (100%)	3
segmental dystonia			
- cranial-cervical onset	0	37 (100%)	37
- arm onset	2 (18%)	9 (82%)	11
focal dystonia	1 (2%)	59 (98%)	60

Table 3.6 – PTD family history in DYT1-positive and DYT1-negative patients

Phenotype	Familial	Sporadic	Total
DYT1 + (n=22)			
- generalised dystonia	17 (89%)	2 (11%)	19
- segmental dystonia	1 (50%)	1 (50%)	2
- focal dystonia	1 (100%)	0	1
DYT1 – (n=128)			
- generalised dystonia	21 (91%)	2 (9%)	23
- segmental dystonia	35 (76%)	11 (24%)	46
- focal dystonia	24 (41%)	35 (59%)	59

Mutation positive cases

Twenty-two patients were found to have the GAG deletion in the DYT1 gene. All but three of these (88%) had at least one relative affected by PTD. Four were Ashkenazi Jewish, and one had a single parent of Jewish origin. The onset of dystonia was before the age of 28 in all mutation positive patients (mean ± SD: 9.9 ± 4.3 yrs; range: 2-21 yrs).

Fifteen patients presented with the typical DYT1 phenotype, i.e. early leg-onset dystonia spreading to other limbs but not to cranial-cervical muscles.

Four patients presented a mildly atypical phenotype. In three of them dystonia started in a leg and spread to the upper limbs eventually involving cranial-cervical muscles; in

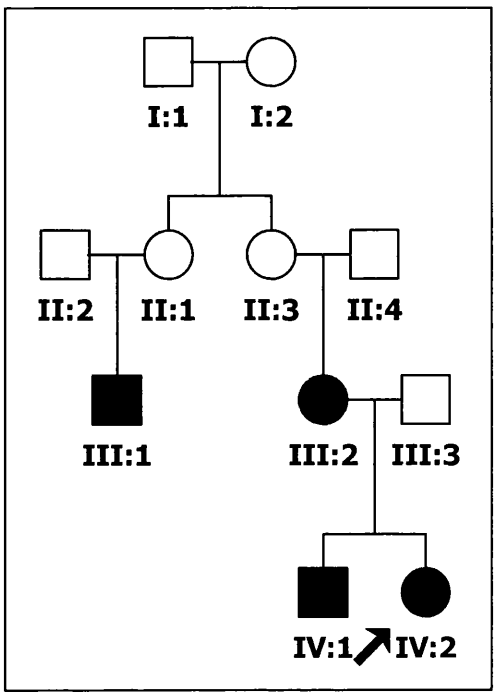
the fourth patient dystonia started in the right arm and spread to the other arm, to the legs and the cervical muscles. Two affected relatives of this patient (a maternal uncle and a cousin) had a milder phenotype with focal dystonia of the hand and neck respectively; both of them carried the GAG deletion.

Three mutation positive patients had a clearly atypical phenotype and merit more detailed description:

Patient 1: this patient had one parent of Ashkenazi Jewish origin. He presented at the age of 10 with segmental dystonia in the right arm, which spread up to cranial-cervical muscles but not to the other limbs. He had no affected relatives.

Patient 2: This individual (IV:2) is the index case of a non-Jewish kindred (family W) whose pedigree is shown in figure 3.5. She is a 28 year old female who presented at the age of 21 with writer's cramp affecting her right arm. Age 23 she developed dystonic posturing of that arm, but no further spread of dystonia has occurred. Her mother (III:2) also has atypical features for DYT1, with onset of cervical dystonia age 50. Age 62 this spread to involve spasms in her back (segmental axial dystonia). The brother of the index case (IV:1) developed dystonia of the left foot age 9, which spread to involve both legs and arms, the trunk and larynx. A maternal cousin (III:1), however, has typical limb onset generalised dystonia, which started in the right leg age 11 and spread to involve both lower limbs, the trunk and the right arm. All affected individuals in this family carry the GAG deletion.

Figure 3.5 – pedigree of family "W"



Patient 3: This 35 year old man, of Ashkenazi Jewish descent, developed dystonia of his right and then left arm at the age of 10 years. He worsened for a year or two after onset and then had no further progression. When he was seen, he had segmental dystonia affecting his arms alone, with no involvement of his legs or cranial-cervical region. His father also developed dystonia of his arms when he was 8 years old. On follow-up at 50 years of age he still only had segmental dystonia of his arms and has had no further progression since.

Mutation negative cases

One hundred and twenty-eight patients were negative for the GAG deletion in the DYT1 gene. Forty-eight patients (38%) were sporadic cases, while eighty (62%) had at least one relative affected by PTD. Four were Ashkenazi Jewish, one was Sephardic Jewish and one had a single parent of Ashkenazi Jewish origin. The age at onset varied widely (mean ± SD: 35.0 ± 17.7 yrs; range: 2 -74 yrs).
One hundred and five of these patients (82%) had focal (fifty-nine cases) or segmental (forty-six cases) dystonia. The age at onset in this group was below 28 years in twenty-six patients and above 28 years in seventy-nine patients (mean ± SD: 38.3 ± 25.1 yrs; range: 4-74 yrs).

Twenty-three of the mutation negative patients (18%) presented with generalised dystonia: in thirteen of them, dystonia involved the limbs and the cranial-cervical muscles, while ten patients presented with early, limb-onset dystonia without cranial-cervical involvement (typical DYT1 phenotype). The age at onset in this group was below 28 years in all but two patients (mean ± SD: 13.1 ± 8.1 yrs; range: 3-33 yrs).

Haplotype analysis at the DYT1 locus

Ashkenazi Jewish population

All the nine AJ disease bearing chromosomes analysed shared partially or completely the North American AJ haplotype associated with the $DYT1^{AJ}$ mutation (i.e. 8-4-5-16-4-12). The frequency of each haplotype-associated allele was markedly in excess on DYT1 chromosomes versus 108 control chromosomes. Results of $\chi 2$ tests and values of the linkage disequilibrium parameter δ are given in table 3.7.

Table 3.7 – linkage disequilibrium in DYT1 Ashkenazi Jewish patients

locus	allele	χ^2	p	δ
D9S62B	8	26.1	< 0.001	0.87
D9S2160	4	27.7	< 0.001	1
D9S2161	5	13.4	< 0.001	0.84
D9S63	16	49.3	< 0.001	1
D9S2162	4	26.4	< 0.001	1
ASS	12	40.9	< 0.001	0.88

Full haplotypes for 9 AJ disease-bearing chromosomes for the six microsatellite loci are shown in table 3.8.

Table 3.8 – full haplotypes at the DYT1 locus in Ashkenazi Jewish patients

	locus						N.		
group	D9S62B	D9S2160	D9S2161	D9S63	D9S2162	ASS	Def.	Prob.	Tot.
A	**8**	**4**	**5**	**16**	**4**	**12**	6	1	7
B	12	**4**	**5**	**16**	**4**	**12**	1	0	1
C	**8**	**4**	4	**16**	**4**	2	0	1	1

Def.: phased disease-bearing chromosomes determined by inheritance. Prob.: phased disease-bearing chromosomes not determined by inheritance. Bold numbers denote the haplotype-associated alleles.

On the centromeric side, only one chromosome showed an historic recombination event (i.e. not occurring in the generation under study) between the loci D9S62B and D9S2160 (group B). This individual was homozygous for the allele 4 at the D9S2160 locus; in this case the recombination event may have been centromeric or telomeric to D9S2160. Another chromosome (group C) showed an historic recombination on the telomeric side, between loci D9S2162 and ASS. This chromosome had also a different allele (i.e. 4 instead of 5) at D9S2161. However, the DYT1 associated haplotype was conserved both on the proximal side (loci D9S62B and D9S2160) and on the distal side (loci D9S63 and D9S2162). It is possible that the different allele at D9S2161 was due to a slippage event, as a double recombination around that locus would be highly unlikely. The locus D9S2163 was typed in groups A and B: all chromosomes in these groups carried the same allele (allele 8 - see Discussion, page 99).

Non-Jewish population

Allele frequencies for the six microsatellite loci (D9S62B, D9S2160, D9S2161, D9S63, D9S2162 and ASS) for the non-Jewish population are given in table 3.9. Comparison of allele frequencies between disease-bearing and control chromosomes showed a statistically significant excess of allele 5 at D9S62B, of allele 4 at D9S2161, of allele 6 at D9S63 and of allele 4 at D9S2162. The locus D9S2160 also revealed a high frequency of allele 5, but the difference was not significant as this allele was also frequent in control chromosomes. The locus ASS displayed association with two different alleles, 6 and 12. Results of χ^2 tests and values of δ are given in table 3.10.

Table 3.9 – allele frequencies at the DYT1 locus (non-Jewish population)

locus	D9S62B		D9S2160		D9S2161		D9S63		D9S2162		ASS	
	D	C	D	C	D	C	D	C	D	C	D	C
allele												
-2		0.020										
1	0.067	0.076		0.004		0.168	0.267	0.252	0.067	0.040		
2		0.108	0.133	0.140		0.168		0.108		0.064	0.133	0.328
3		0.004		0.004	0.133	0.212		0.016	0.333	0.368		0.004
4		0.064	0.200	0.204	0.733	0.180		0.072	0.600	0.228		0.100
5	0.667	0.188	0.667	0.444	0.067	0.240	0.067	0.068		0.104		0.004
6		0.036		0.160	0.067	0.028	0.533	0.128		0.124	0.333	0.104
7		0.004		0.024		0.004				0.012		0.004
8	0.067	0.120		0.016						0.060		0.116
9		0.004		0.004				0.032			0.133	0.088
10	0.067	0.216						0.020			0.067	0.048
11	0.067	0.068						0.016			0.067	0.116
12	0.067	0.004						0.004			0.267	0.088
13		0.036										
14		0.012										
15		0.008										
16		0.012					0.133	0.156				
17		0.008										
18		0.004						0.092				
19		0.004						0.036				
20		0.004										

D: disease chromosomes; C: control chromosomes

Table 3.10 – linkage disequilibrium in DYT1 non Jewish patients

locus	allele	χ^2	p	δ
D9S62B	5	19.2	< 0.001	0.59
D9S2160	5	2.8	n.s.	0.40
D9S2161	4	26.0	< 0.001	0.68
D9S63	6	18.1	< 0.001	0.47
D9S2162	4	10.5	= 0.001	0.48
ASS	12	5.1	n.s.	0.20
	6	7.2	< 0.01	0.26

Full haplotypes for 15 non-Jewish disease-bearing chromosomes are shown in table 3.11.

Table 3.11 – full haplotypes at the DYT1 locus in non Jewish patients

	locus						N.		
group	D9S62B	D9S2160	D9S2161	D9S63	D9S2162	ASS	Def.	Prob.	Tot.
D	8	4	5	16	4	12	1	0	1
E	**5**	**5**	**4**	**6**	**4**	**12**	3	0	3
F	**5**	**5**	**4**	**6**	**4**	**6**	3	1	4
G	**5**	**5**	**4**	**6**	**4**	10	1	0	1
H	**5**	**5**	**4**	5	1	11	1	0	1
I	**5**	2	**4**	16	3	2	1	0	1
J	10	5	**3**	**1**	**3**	2	1	0	1
K	11	2	**3**	**1**	**3**	6	1	0	1
L	1	4	6	**1**	**3**	9	1	0	1
M	12	4	4	**1**	**3**	9	1	0	1
N	4	5	5	2	4	8	2	0	2

One of the non-Jewish disease chromosome showed the full AJ haplotype (group D). Eight disease chromosomes (groups E, F, and G) presented the same combination of alleles (5-5-4-6-4) at the five proximal loci; at the ASS locus, three of them had allele 12, four had allele 6, and one had allele 10, probably representing historic recombinations between loci D9S2162 and ASS. One chromosome (group H) shared only the proximal part of this haplotype (5-5-4), suggesting an historical crossover

between D9S2161 and D9S63. A single chromosome (group I) carried only allele 5 at D9S62B and allele 4 at D9S2161, but not the other associated alleles; this could result from a slippage event at D9S2160 and a recombination event distal to D9S2161, but the possibility that this chromosome carried the two associated alleles purely by chance could not be excluded. The full haplotype 5-5-4-6-4 with either allele 6 or 12 at ASS locus was not observed in any of the 100 phased control chromosomes. Only three control chromosomes carried different portions of the haplotype, i.e. x-5-4-6-x-x, x-x-x-6-4-6 and x-x-x-6-4-12 respectively (where x represents any other non-associated allele at that locus).

Two disease-bearing chromosomes (groups J and K) presented with a different, more restricted haplotype, characterised by the alleles 3-1-3 at loci D9S2161, D9S63 and D9S2162 respectively. Another two chromosomes (groups L and M) shared only the alleles 1-3 at D9S63 and D9S2162, which could be accounted for by a recombination event distal to D9S2161 and proximal to D9S63. Only four out of 100 control chromosomes carried the same combination of alleles (i.e. x-x-3-1-3-x).

The marker locus D9S2163 was typed only in groups D and E. The chromosome in group D shared the same allele as the AJ chromosomes in groups A and B (allele 8), while all the chromosomes in group E shared the allele 1 (see Discussion, page 99).

Two American patients of British ancestry (group N) carried a completely different distinctive haplotype. However, the small (n=2) number of American British patients and the lack of control chromosomes did not allow any statistical comparison.

Genotype-phenotype correlation

No significant differences in phenotype expression were noted in patients with different haplotypes. All patients had onset of dystonia before 28 years. Among the AJ patients, seven presented with a typical DYT1 phenotype (limb onset dystonia without spread to cranial-cervical muscles). Two patients (both in group A) showed an atypical phenotype, with dystonia beginning in the arm and spreading to cranial-cervical muscles and to the other arm respectively.

Among the non-Jewish patients, ten (in groups D, E, F, H, I, L and M) presented with a typical DYT1 phenotype, while four (in groups F, J and K) had limb onset dystonia with further spread to the cranial-cervical region. Only one patient (group G) showed an atypical phenotype characterised by writer's cramp and posturing of the right arm only, but the typical DYT1 phenotype was observed among her affected relatives.

Exclusion mapping of DYT1, DYT6 and DYT7 loci in 3 PTD families

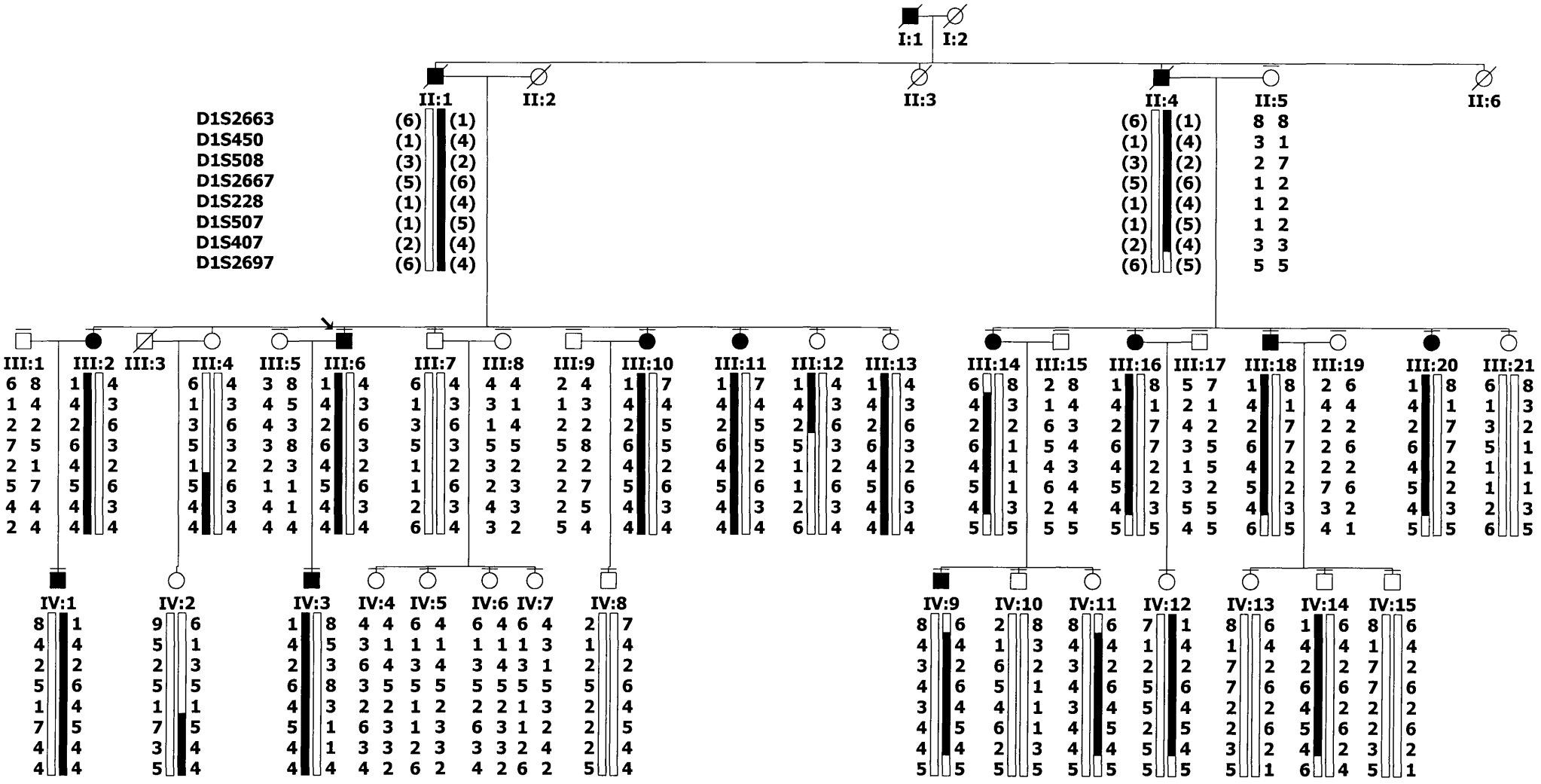
The results of pairwise LOD scores between dystonia and markers at the DYT1 (chromosome 9), DYT6 (chromosome 8) and DYT7 (chromosome 18) loci are presented in appendix 1. These results show no evidence of linkage between dystonia and any locus in any of the three families studied. Recombinations are present between dystonia and all informative markers in each family. In family PTD03, few markers produced small positive LOD scores in the DYT6 and DYT7 candidate regions (D8S1113: $Z_{max} = 0.89$ at $\theta = 0$; D18S1105: $Z_{max} = 0.52$ at $\theta = 0$). However, haplotype construction allowed exclusion of both the DYT6 and the DYT7 intervals.

Mapping of the DYT13 locus in family PTD01

Four hundred microsatellite markers covering all autosomes were analysed in the eleven affected members of the family. Two-point LOD scores are presented in appendix 2. All markers generated negative or non-significant LOD scores at all tested recombination fractions ($\theta = 0.0 - 0.5$), except five markers on chromosomes 1, 4, 10, 12 and 15, which generated maximum LOD scores between 1.0 and 2.3. The regions surrounding these five loci and all regions surrounding non-informative markers were then saturated with closely spaced microsatellite markers, all available individuals were genotyped and haplotypes were constructed. The negative LOD scores obtained and the detection of different haplotypes carried by the affected individuals in the family allowed excluding all the autosomes except a region on the short arm of chromosome 1. All markers spanning this candidate interval produced positive LOD scores, with a maximum LOD score of 3.44 ($\theta = 0.0$) between the disease and marker D1S2667 (appendix 3a).

Linkage calculations assuming different penetrance values (0.20 – 0.80) or using affected individuals only did not result in a significant change in pairwise LOD scores. All affected individuals in the family shared a common haplotype between D1S2663 and D1S2697 (figure 3.2), allowing the identification of a 22cM interval containing a novel PTD gene (DYT13). The upper extent of the region is determined by recombinations detected in subjects III:14 and IV:9 between markers D1S2663 and D1S450. The lower extent of the region is defined in individual II:4 and his descendants between D1S407 and D1S2697.

Figure 3.2 – pedigree of family PTD01 and haplotypes spanning the DYT13 locus



Families PTD02 and PTD03 were tested for linkage with this novel locus. The following microsatellite markers were analysed: *tel* – D1S2663 – *1.8cM* – D1S450 – *2.0cM* – D1S508 – *4.0cM* – D1S2667 – *4.9cM* – D1S228 – *4.4cM* – D1S507 – *0.8cM* – D1S407 – *2.5cM* – D1S2697 – *cen.*

Haplotype construction and linkage analysis allowed excluding linkage to the DYT13 locus in both families. Two-point LOD score values for families PTD02 and PTD03 are shown in appendices 3b and 3c respectively.

Discussion

The role of DYT1 in primary torsion dystonia in the UK

The work presented here provides evidence that the GAG deletion in the DYT1 gene is responsible for the majority of cases of early, limb-onset dystonia in both Jewish and non-Jewish British families. These results are consistent with the findings of Ozelius and colleagues (1997b) in North American PTD families, and support the contention that a single mutation is responsible for the majority of cases with typical early-onset dystonia, regardless of the ethnic background and the ancestral origin of the patients. The clinical subgroups used in this work differ slightly from those used by Ozelius and colleagues (1997b). The author decided to use a simple clinical classification, i.e. generalised, segmental and focal dystonia groups, rather than the broad categories "typical", "atypical" and "uncertain". The term "typical DYT1 phenotype" was maintained only for the selected group characterised by early limb-onset generalised dystonia without spread to the cranial-cervical muscles. These criteria allow a simpler and more direct comparison between genotype and phenotype in dystonia. Furthermore, applying Ozelius's classification system to the present data, the percentage of DYT1 positive cases with "typical or probable DYT1 phenotype" is 61%, thus very similar to the figure obtained using the new classification. In the first screening on 150 patients, the DYT1 mutation was found in 60% (15/25) of patients with typical DYT1 phenotype, the majority of whom were from small non-Jewish families of unknown linkage. Only three of these were Ashkenazi Jewish and all carried the $DYT1^{AJ}$ haplotype. In the USA, 74% of individuals with this phenotype carried the deletion. This may reflect the fact that the European population sampled in this study was more heterogeneous than the population of North America, or contained a smaller number of Ashkenazi patients. It should be noted that the majority of the British cases

studied here were recruited through an epidemiological study and therefore may well represent a more accurate estimate of the true proportion of DYT1 positive cases. This study also confirms the data reported by Almasy and colleagues, who found significant differences in age at onset and site at onset of PTD among different ethnic groups (Almasy et al, 1997b). The high proportion of European PTD patients with typical DYT1 phenotype who do not carry the mutation (40%) may represent either allelic or locus heterogeneity in dystonia.

In a recent paper, Leung and co-workers (2001) have identified a novel 18-bp deletion (966_983del) in one patient with early onset dystonia and myoclonic features. This deletion was not detected in a cohort of over 1800 samples from dystonic individuals, thus its role in determining early-onset dystonia remains unclear. The 18 base pairs deleted are enclosed in the same DNA fragment amplified to detect the GAG deletion (primers 6419 and h48) (Ozelius et al, 1997b; Leung et al, 2001) and therefore both deletions can be identified with the same test. Therefore, the 18-bp deletion can also be excluded in the British cohort of patients tested in this study. Recently, a mutation in the ε-sarcoglycan gene (SGCE), responsible for myoclonus-dystonia, was identified in the same patient harbouring the 18-bp deletion in the DYT1 gene (Klein et al, 2002; Doheny et al, 2002). Although the authors hypothesise a possible functional role of both mutations in determining the myoclonus-dystonia phenotype, it is well known that SGCE mutations alone are sufficient to induce the clinical manifestations of myoclonus-dystonia (Zimprich et al, 2001). It is therefore likely that the 18-bp deletion plays only a minor, if any, part in determining the phenotype and its role in primary torsion dystonia remains to be evaluated.

In two recent studies, the whole coding region of the DYT1 gene was sequenced in several patients with typical early limb-onset dystonia not carrying the GAG deletion, and no mutations were identified (Ozelius et al, 1999; Tuffery-Giraud et al, 2001). It is likely that at least another, still unidentified gene is responsible for early limb-onset primary torsion dystonia, and that the GAG deletion is the only mutation in the DYT1 gene leading to the dystonia phenotype.

The present study broadens the phenotype associated with the GAG deletion in the DYT1 gene. This mutation was found in four patients affected by generalised dystonia with cranial-cervical involvement, two patients with segmental dystonia and one patient with focal right arm dystonia. The available affected relatives of these patients (five subjects) were subsequently tested: one presented with a typical DYT1

phenotype and another with the DYT1 phenotype plus laryngeal involvement. The other three subjects had a milder clinical picture (focal dystonia of the arm or neck, segmental cranial-cervical dystonia). All these five subjects carried the DYT1 mutation. Family W demonstrates that there is phenotypic variation within a single kindred. Whether this represents variable expression of the GAG mutation or the effect of modifying genes or environmental factors is unclear.

For the majority of cases of focal dystonia, however, the GAG deletion does not appear to be the pathogenic mutation. Only one family with autosomal dominant writer's cramp due to the DYT1 mutation has been reported so far (Gasser et al, 1998a). Ozelius and co-workers (1997b) found no deletion carriers among patients with focal or segmental cranial-cervical dystonia, and several studies failed in detecting the DYT1 mutation in patients with adult-onset focal dystonia (Kamm et al, 2000; Friedman et al, 2000; Sessa et al, 2001). This may be due to the fact that other genetic loci are involved or that the cases are not genetic. It is interesting to note that Bressman and colleagues reported several cases of Ashkenazi Jews with segmental or focal dystonia carrying the Ashkenazi DYT1 haplotype (Bressman et al, 1994a), implying they should have the GAG deletion. However, the molecular analysis of the DYT1 gene in these patients has not been reported so far.

Several studies have been published on the incidence of the DYT1 mutation in cohorts of dystonic patients of different nationalities (Klein et al, 1999b; Slominsky et al, 1999; Leube et al, 1999; Kamm et al, 1999; Lebre et al, 1999; Brassat et al, 2000; Bressman et al, 2000; Tuffery-Giraud et al, 2001, Matsumoto et al, 2001; Hjermind et al, 2002).

A clinical comparison among these studies is made difficult by the different criteria used to sub-classify patients, and this also explains the high variability of percentages of patients carrying the DYT1 mutation. Most studies have been performed on small groups of patients and this makes the obtained figures unreliable for comparison purposes. However, a few considerations can be made. If we look at patients with generalised dystonia, the percentage of DYT1-positive cases ranges from 28% to 76%. The highest proportion of DYT1 positive patients is in the study by Klein an co-workers (1999b), which includes only patients with limb-onset dystonia, without specifying the cranial-cervical involvement. The percentage obtained in the present study is 47%, but it raises to 60% if we consider patients with limb-onset and without cranial-cervical involvement (typical DYT1 phenotype). Also, it can be noted that the highest percentages of DYT1-positive patients are found in studies including Ashkenazi Jewish

patients. The low figure (31%) in the study by Ozelius and colleagues (1997b) is likely explained by the fact that the group also includes patients with early-onset writer's cramp. If we consider only patients with generalised dystonia without cranial-cervical involvement, the figure raises up to 74%; AJ patients are also included in this study. Figures from European studies not including AJ patients are more variable, but the percentage of non-Jewish European patients with early-onset generalised dystonia carrying the GAG deletion seems to be lower, between 30 and 50%. Further studies are needed to identify the genetic cause of early-onset dystonia in DYT1-negative patients.

A common haplotype in the chromosomal region surrounding the DYT1 gene has been described in the North American AJ population prior to the cloning of the DYT1 gene (Risch et al, 1995). This haplotype was present in 94% of unrelated AJ individuals with early-onset dystonia, suggesting that most early-onset PTD cases in the North American Ashkenazim were due to a unique founder mutation in the DYT1 gene ($DYT1^{AJ}$), and that the DYT1 gene was located in a 1.8-cM region between the two polymorphic loci D9S62A and ASS. A more detailed analysis of the region surrounding DYT1 allowed localisation of the DYT1 gene to a 150 Kb region distal to D9S2161 and proximal to D9S63 (Ozelius et al, 1997a). The subsequent identification of the DYT1 gene by positional cloning and the finding of a single 3-bp deletion in the gene responsible for all cases of 9q34-linked dystonia allowed the selection of a more homogeneous disease population (i.e. mutation carriers only) for the study reported here, thus increasing the reliability of the population genetic data.

The finding that the same haplotype is shared by North American and European Ashkenazi Jews is interesting. Risch and colleagues (1995) identified Russia and Poland as the country of origin in Europe of many of the oldest carriers in their AJ families. They postulated that the first appearance of the $DYT1^{AJ}$ mutation was in Lithuania or Byelorussia about 350-380 years ago, at the time of the brutal Cossack massacre, which dramatically decimated the East European Jewry. The migration process that took place afterwards was directed not only across the ocean, with the creation of the American Jewry, but also towards Central and Western European countries, such as Italy, Germany, France and England (Ankori, 1979). This explains the finding of a unique haplotype shared by North American and European Ashkenazi Jews, and the postulated recent origin of the mutation. Part of the $DYT1^{AJ}$ associated haplotype was already detected in the affected members from three small AJ British families (Warner

et al, 1993). In the present study, all British AJ patients shared the full $DYT1^{AJ}$ haplotype, with the exception of two recombination events at the centromeric or telomeric end of the haplotype, and a possible slippage event at D9S2161. This is probably due to the fact that our AJ population was more homogeneous than the North American, as all the AJ patients were carriers of the GAG deletion in the DYT1 gene. The North American AJ population was selected prior to the identification of the DYT1 gene, on the basis of the clinical phenotype and probably included a few non-DYT1 dystonia patients. One of our non-Jewish patients carried the full AJ haplotype. The patient was unaware of Jewish ancestry in her family, but the simple explanation of a remote AJ ancestor whose memory has been lost in the family history cannot be ruled out.

The religious and cultural strength that has always bonded Jewish people together and the habit of marriages in small communities can easily explain the fact that a single mutation is responsible for the majority of cases of DYT1-dystonia, and the high frequency of this disease among the Ashkenazim. On the contrary, the finding of a single or few mutations responsible for most cases of early-onset PTD in the British population would be intuitively less likely. Interestingly, 9 out of 14 unrelated British patients (groups E, F, G, H – see Results) shared completely or in part a common haplotype, different from the $DYT1^{AJ}$ haplotype. It seems likely that haplotype "I" derives from the same ancestral chromosome. Allele frequency comparisons in patients and controls, and values of the parameter δ supported linkage disequilibrium between the associated alleles and the disease, although the results were not as significant as those in the AJ group.

The two distal alleles in three British disease-bearing chromosomes (group E: allele 4 at D9S2162 and allele 12 at ASS) were the same as the ones in the DYT1 AJ population (groups A and B). This suggested the intriguing hypothesis that a unique mutation predating that seen in the AJ population might have been responsible for early-onset dystonia both in AJ and in a subset of non-Jewish patients. To address this matter, an additional microsatellite marker (D9S2163), located in between D9S2162 and ASS, was typed in groups A, B, D and E. The finding of two different alleles in the two subsets of patients (allele 8 in the AJ chromosomes and allele 1 in group E) ruled out this hypothesis, the most likely explanation being that the alleles 4 at D9S2162 and 12 at ASS are shared by the two groups purely by chance. The chromosome in group D carried allele 8, and is therefore identical to the AJ haplotype. Observed

recombination events were consistent with the localisation of DYT1 between D9S63 and D9S2161 as reported by Ozelius and co-workers (1997a). Four other British disease-bearing chromosomes (groups J to M) appeared to derive from a different ancestral haplotype, characterised by the alleles x-x-3-1-3-x at the D9S2161, D9S63 and D9S2161 loci. Two of these chromosomes only carried the distal part of this haplotype (x-x-x-1-3-x), and could have arisen by recombination consistent with the known position of DYT1. However, these data need to be supported with the inclusion of more patients and with additional typing of more polymorphic loci, as the hypothesis of a casual occurrence of the same restricted haplotype in four patients cannot be excluded.

The same GAG deletion in the DYT1 gene has arisen independently a few times in past history. Ozelius and colleagues (1997b) found several different haplotypes in their non-Jewish patients, and even two different patterns of alleles in three new polymorphisms located very closely (within 5 Kb) to the GAG deletion. Several *de novo* GAG deletions in the DYT1 gene have been described in non-Jewish patients with early-onset dystonia (Klein et al, 1998a; Hjermind et al, 2002). This is also supported by the finding of a different haplotype in two American patients of British descent. The interesting question is: how often does this recurrent deletion arise in the DYT1 gene? The data presented here suggest that most cases of British patients with PTD due to the DYT1 deletion can be accounted for by a limited number of founder mutations. Only two distinct haplotypes were identified in British PTD patients with the deletion. Moreover, the author was not able to identify any *de novo* GAG deletion in the sporadic cases. The finding of a restricted haplotype shared by four non-Jewish patients (groups J to M) would suggest a founder mutation dating well before the $DYT1^{AJ}$. Further studies with an increased number of patients are needed to enlighten this matter.

Linkage analysis in the three PTD families

Three large unrelated families with non-DYT1 dystonia were studied for evidence of linkage to the DYT6 or DYT7 genetic loci. These three families had important phenotypic similarities to both the DYT6 and DYT7 kindred. Considering all three families and the DYT6 and DYT7 families together, there are features in common between all families, including a juvenile or adult onset of symptoms in most individuals, and prominent involvement of cranial-cervical muscles (20 out of 22 or 91% in the original DYT6 and DYT7 kindred, and 22 out of 24 or 92% in the three PTD

families). These clinical features are in contrast to the childhood onset and cranial-cervical sparing seen in DYT1 dystonia. The data presented exclude linkage of dystonia to either DYT6 or DYT7 locus in all families.

DYT13, a novel PTD locus

As part of the work of this thesis, a fourth locus for primary torsion dystonia, DYT13, was identified on the short arm of chromosome 1 in family PTD01, a non-Jewish family from central Italy. The phenotype is characterised by prominent involvement of the cranial-cervical region and the upper limbs; age of onset is variable; progression is mild and disease course is relatively benign with occasional tendency to generalisation. All the affected individuals, including those with generalised dystonia, were able to accomplish common domestic chores and to perform daily living activities.

Nineteen individuals shared partially or completely the chromosome 1p haplotype segregating with the disease; eleven of them (58%) were affected by dystonia. This value of penetrance is slightly higher than the penetrance usually attributed to primary dystonia genes (30-40%); however it is worth noticing than not all the unaffected members of the family were available for clinical examination and genotyping, and so the exact value of penetrance for the DYT13 gene remains to be defined.

The clinical picture is noticeably different from the DYT1 phenotype, where dystonia presents generally in a limb, rarely affects the cranial-cervical region, and has a higher tendency to generalise, producing a much more disabling disease. The DYT6-associated phenotype is characterised by a wider distribution of body regions involved at onset and in the course of the disease, which has the tendency to be more severe and to generalise more frequently. The phenotype in family PTD01 is also different from that described for the DYT7 gene, which is characterised by adult-onset pure focal cervical dystonia without tendency to spread to other body regions.

In several PTD families reported so far linkage to the known PTD loci has been excluded; in some of these families the phenotype shares relevant clinical features with DYT13-linked dystonia. In two large non-Jewish families reported in 1996 by Bressman and co-workers (one previously described by Uitti and Maraganore in 1993), the affected members presented with early or adult-onset dystonia confined to cervical and brachial region. Two other PTD families, of Swedish and Italian origin (Holmgren et al, 1995; the latter is family PTD02), had a similar phenotypic presentation: variable age at onset (spanning from the second to the fifth decade), cranial-cervical prominent

involvement, and upper limb tremor or occasional generalisation. Families whose phenotype is remarkably different from DYT13-linked dystonia have also been reported. A family observed by Parker had a variable phenotypic presentation, characterised by prominent laryngeal involvement, torticollis, and infrequent generalisation; Wilson's disease also occurred in the same family. The underlying dystonia gene in this family was named DYT4, but its chromosomal location has not been established (Parker, 1985). An Italian family from South Tyrol displayed an unusually variable phenotype: most affected members had cervical or upper limb dystonia with onset in adulthood, although some patients suffered from typical early-onset generalised dystonia (Klein et al, 1998c). Some of these families may link to the DYT13 locus, as many of them were characterised by variable age of onset (juvenile or adult) and prominent cranial-cervical involvement.

A large number of genes map within the 22cM candidate interval identified in family PTD01, but none of them represents an obvious candidate for dystonia. The most interesting gene mapping to the region is a gene coding for a member of the heat shock protein family, called cvHsp. This protein is mainly expressed in cardiovascular tissues, but a low expression has been also detected in specific areas of the brain, i.e. putamen, caudatus, substantia nigra and amygdala (Krief et al, 1999). This gene represents an interesting candidate because TorsinA, the DYT1 gene product, is a protein with high similarities to heat shock proteins. Other genes map within the linked region and bear a possible role in neurological diseases: SCNN1D, an amiloride-sensitive non-voltage gated sodium channel, isoform delta, expressed in brain and other tissues, is putatively involved in neurodegeneration; EPHA2, a tyrosine kinase receptor expressed in projecting neurones and their target fields, is involved in short-range, contact-mediated, axonal guidance; KCNA2B codes for the $\alpha 2$ subunit of a voltage gated "shaker" potassium channel; and DVL1, a widely expressed homologue of a Drosophila gene, is putatively involved in neural and heart development.

Family PTD02

This Italian family presented with a very homogeneous phenotype, characterised by cranio-cervical dystonia. Clinical presentation was so remarkably homogeneous that the observation of subject IV:11 with upper limb dystonia prompted us to study his paternal family, which was indeed affected by familial writer's cramp. The clinical

presentation of dystonia in subject IV:11 actually suggests that he may have inherited writer's cramp from his father.

The structure of the family was poorly informative. Out of five definitely affected subjects, two were monozygotic twins, thus sharing the same DNA information, and one possibly inherited writer's cramp from the unrelated parent. Linkage analysis was therefore carried out on three definitely affected and three probably affected subjects.

The GAG deletion in the DYT1 gene was not detected in the index case, the most severely affected family member, and linkage analysis with markers spanning the DYT1 interval gave negative results. Also, the clinical presentation of dystonia in this family differed noticeably from the typical DYT1 phenotype.

Pairwise linkage analysis showed negative LOD-scores throughout the DYT6, DYT7 and DYT13 regions, either considering the subjects with probable dystonia as unknown phenotype or as affected. Also, the affected members of the family did not share the same haplotype for DYT6, DYT7 and DYT13 areas. These results indicate that dystonia in this family is not linked to any of the known PTD loci.

Families with cervical dystonia have already been described. The family described here resembles quite clearly the German family linked to DYT7, in which six out of the seven definitely affected members presented with adult onset focal cervical dystonia (Leube et al, 1996). Conversely, family PTD02 appears phenotypically different from the two German Mennonite families linked to the DYT6 gene.

The onset and progression of dystonia were different in the index case and in her monozygotic twin. The index case, which was the only subject in the family who used to carry heavy weights on her head, had a clinical picture noticeably worse than that of the remaining affected subjects. It appears plausible that this chronic environmental factor, lasting for years and directly affecting the cervical region, could be responsible for repeated strain and a more severe phenotypic expression of dystonia.

Family PTD03

Only few families have been described with four or more affected relatives presenting predominant cranial-cervical dystonia without significant spread (Uitti et al, 1993; Leube et al, 1996; Bressman et al, 1996), the first report dating back to 1896 (Thompson, 1896).

In family PTD03 at least five members in three generations presented with predominantly cranio-cervical dystonia. Over an average period of 24 years spread of dystonia to other body regions was limited with mild dystonic posture in one arm in three and writer's cramp in one patient. The phenotype and age of onset was noticeably different from the typical DYT1 phenotype, the GAG deletion in the DYT1 gene was not detected in the proband and linkage with the DYT1 region was excluded in the family. Similarly, linkage to DYT1 was also excluded in two of the four families with predominant cranio-cervical dystonia reported in the literature (Leube et al, 1996; Bressman et al, 1996), thus arguing against the previously held assumption that focal or segmental dystonias represent a forme fruste of generalised PTD.

The mean age of onset (29 years) in family PTD03 was similar to the families reported by Bressman et al (30.9 years) and Uitti and Maraganore (35 years). Apart from jerky cranial-cervical dystonia, four family members also had voice tremor, two had postural tremor in the arms and one had writing tremor. Tremor was sometimes severe and caused significant disability. Tremor was reported only in two out of seven and three out of five affected members in the family reported by Bressman and by Uitti and Maraganore respectively, and was usually mild. It is well known that PTD patients may exhibit tremor as their only clinical abnormality and isolated head tremor may occur as a manifestation of cervical dystonia (Fletcher et al, 1990a; Rivest and Marsden, 1990). The tremor in this family did not appear to be essential tremor using the criteria defined by Bain et al (1994). Head movements were rather jerky and voice tremor was present either in the absence of postural arm tremor, or was more severe than the arm tremor if this was present. Additionally, jerky head movements were ameliorated by a geste in four patients, which is more in favour of dystonia than essential tremor.

Phenotypically there were differences between family PTD03 and the DYT7 family (Leube et al, 1996). Firstly, anticipation, reported in the DYT7 family, was not found in family PTD03. Secondly, in the DYT7 family tremor was found in only one out of seven affected relatives. The phenotype in family PTD03 was also different from the two Mennonite families linked to the DYT6 locus (Almasy et al, 1997a). The age at onset in these families was earlier (mean 18,9 years) but widely variable. Dystonia often started in the cranio-cervical region but progressed to involve at least one arm and one leg in seven cases and became generalised in three. Tremor was not a feature. LOD scores and haplotype construction excluded linkage between the disease and the DYT6, DYT7 and DYT13 loci in family PTD03.

Future directions of study

The findings presented here indicate the existence of at least one other as yet unmapped gene for PTD. Thus, the phenotypes of adult-onset focal dystonia and mixed phenotype dystonia are genetically heterogeneous, and may result from mutations of as yet unidentified gene(s) as well as from DYT6, DYT7 and DYT13 mutations. Such a finding is in contrast to the apparent genetic homogeneity of the DYT1 phenotype of early, limb-onset dystonia, the great majority of which is accounted for by mutations of TorsinA (Bressman et al, 1994; Ozelius et al, 1997b). The contribution of the DYT6, DYT7 and DYT13 loci to families and singleton patients with focal and mixed phenotype dystonia remains to be fully evaluated. The presence of allelic association at D18S1098 in patients with torticollis in a rural region of Northwest Germany suggests that this locus is an important cause of dystonia, at least in this community. The Mennonite community in which the DYT6 locus was described is known to be genetically isolated, and it may be that this locus is not of widespread importance outside the Mennonite community.

Large families with non-DYT1 dystonia such as those described in this chapter are rare and provide a unique opportunity to identify unmapped PTD genes. Identification of these genes will depend on initial linkage mapping using genome-wide searches, followed by a concerted physical, or positional candidate, mapping strategy. Also, the identification of PTD families in linkage with the DYT13 locus will allow a refinement of the linkage region, easing the identification of candidate genes to be tested for functional mutations.

Allelic association studies at D18S1098 in patients with torticollis would be of interest to determine whether a founder mutation common to the British and German populations contributes to this common form of dystonia. However, definitive mutation analysis of these genes in patients with dystonia will have to await the identification of the genes themselves.

Attempts to evaluate the importance of environmental factors in the causation of dystonia, particularly the interaction of environmental factors with the 'disease-susceptibility' genotype will also be of importance in the future.

CHAPTER 4 – PAROXYSMAL DYSKINESIAS

Outline of chapter

This chapter describes a clinical and genetic linkage study undertaken in three families with paroxysmal kinesigenic dyskinesia (PKD) and in one family with paroxysmal exercise-induced dyskinesia (PED). The ascertainment of the families and the clinical features of affected family members are described. A genome-wide search for genetic linkage using microsatellite markers was performed in family PKD01. Haplotypes were constructed for an area segregating with the PKD phenotype in order to identify flanking recombinations. This work allowed the identification of a novel PKD locus (named EKD2) on the pericentromeric region of chromosome 16, close to the first PKD locus identified in a Japanese family (Tomita et al, 1999). The significance of the results presented in this chapter are discussed and future directions of study suggested.

An introduction to paroxysmal dyskinesias

The paroxysmal dyskinesias are an unusual group of hyperkinetic movement disorders characterised by paroxysmal attacks of a mixed movement disorder, encompassing elements of dystonia, chorea and ballism in varying proportions (Fahn, 1994). Patients are entirely normal between attacks and clear consciousness is preserved throughout attacks. The current classification of these disorders divides them into three groups according to the duration and precipitation of attacks: paroxysmal non kinesigenic dyskinesia (PNKD, previously known as paroxysmal dystonic choreoathetosis or PDC), paroxysmal kinesigenic dyskinesia (PKD, previously known as paroxysmal kinesigenic choreoathetosis or PKC) and paroxysmal exercise-induced dyskinesia (PED) (Lance, 1977; Demirkiran and Jankovic, 1995; Marsden, 1996b).

Paroxysmal kinesigenic dyskinesia (PKD)

PKD is the commonest of the three forms of paroxysmal dyskinesia, first described by Kertesz in 1967. The episodes of hyperkinetic movements are initiated by a sudden movement or change in movement velocity. The attacks frequently manifest as dystonia or choreodystonia induced by a sudden change in position, classically from a sitting to standing position; however startle, hyperventilation and continuous exercise

can also trigger them (Houser et al, 1999). Even changes in velocity (from slow walking to walking more quickly) can initiate an episode. Many patients report variable "aura like" sensations preceding an attack. The onset is in childhood or early adulthood but can range from 6 months to 40 years, and attack frequency often decreases in adult life (Fahn, 1994). The attacks usually last from seconds to 1-2 minutes, occasionally up to 5-7 minutes. Attacks commonly involve the hemibody, in some almost always on the same side or alternating sides. However, bilateral attacks have been reported. Speech can be affected due to dystonic spasms of the face or jaw, but consciousness is never lost. Patients with PKD may have dozen of attacks per day (up to 100) and respond dramatically to low doses of carbamazepine or phenytoin. After an attack there is usually a short refractory period before another attack can be triggered. Some patients may abort an attack by stopping moving or warming up slowly. Some PKD patients or their relatives have a history of afebrile infantile convulsions, usually with a favourable outcome (Hudgins and Corbin, 1966; Sadamatsu et al, 1999). Although there are reports of symptomatic PKD (Berger et al, 1984; Camac et al, 1990), the majority of cases of PKD are idiopathic, often with a family history of autosomal dominant inheritance. In a review of 26 idiopathic PKD cases by Houser et al (1999), there was a notable predominance of males (23 males, 3 females). Twenty-six percent of patients had other affected family members, with an autosomal dominant inheritance pattern in most.

Paroxysmal non kinesigenic dyskinesia (PNKD)

PNKD is characterised by spontaneous attacks which tend to be more dystonic than those seen in PKD. Attacks are frequently precipitated by alcohol, caffeine, stress or fatigue. Patients with PNKD have longer (10 minutes to 6 hours) and less frequent attacks as compared to PKD, with long attack-free intervals. Patients may have only 1-3 attacks in a day, and may have months of attack-free intervals. As in PKD, many patients report an "aura" like sensation (not necessary in the affected limb) just prior to the onset of the attack. This may take the form of paresthesias, tension in the limbs or an undefined feeling which the patients recognise as the onset of another paroxysm. Speech may be affected during an attack, but consciousness is always preserved. Most of the cases have their onset in childhood or early teens, and the attacks tend to diminish with age. As in PKD, more males than females are affected (1,4:1) (Fahn, 1994). The initial PNKD cases reported were familial with autosomal dominant pattern of inheritance (Mount and Reback, 1940; Richards and Barnett,

1968; Tibbles and Barnes, 1980; Walker, 1981). Subsequently, sporadic cases were reported (Bressman et al, 1988). Generally PNKD cases have no detectable abnormalities between attacks, although there has been one report of patient with PNKD who had also some interictal dystonia (Bressman et al, 1988). There has also been a family with PNKD and additional myokimia (Byrne et al, 1991). EEG and brain imaging in the idiopathic cases are normal. Pathological examination at autopsy in two cases revealed no abnormalities (Lance, 1977). PNKD is more difficult to treat than PKD. Patients do not benefit from antiepileptic drugs like carbamazepine, although some patients may respond to levodopa or clonazepam (Fink et al, 1997; Jarman et al, 2000). Although most cases of PNKD are idiopathic, there are many different conditions which can be associated. As with PKD, the most commonly reported association is with multiple sclerosis (Berger et al, 1984; Sethi et al, 1992). However, closer inspection often reveals that the attacks associated with multiple sclerosis are very brief (seconds to minutes) and often painful, thus typical of tonic spasms and not the long duration attacks of classical PNKD (Berger et al, 1984). Also cases with vascular lesions involving the basal ganglia (particularly the thalamus), encephalitis and head trauma had been associated with PNKD (Lee and Marsden, 1994; Bressman et al, 1988; Drake et al, 1986).

Paroxysmal exercise-induced dyskinesia (PED)

Lance (1977) described members of a family over three generations who developed attacks of dystonia lasting 5-30 minutes following prolonged exercise. Subsequently, a second family and fifteen sporadic cases with PED have been reported (Plant et al, 1984; Nardocci et al, 1989; Wali, 1992; Demirkiran and Jankovic, 1995; Bhatia et al, 1997). PED differs from both PKD and PNKD in the following respects: the phenomenology of attacks is different (dystonia is the only abnormal movement in most cases of PED, ballism and chorea are usually absent), precipitation is always by prolonged exercise rather than initiation of movement, alcohol, caffeine, and finally, the duration of attacks is intermediate between that of PKD and PNKD. The attacks usually appear in the body part involved in the exercise, most commonly the legs after prolonged walking or running. The dystonic episodes usually cease in 10-15 minutes after stopping the exercise. Anticonvulsants are generally not useful treatment, but one case showed some benefit with acetazolamide (Bhatia et al, 1997). PED is a rare disorder and only a few families have been described mostly with an autosomal dominant pattern of inheritance (Bhatia et al, 1997). There has been debate as to

whether familial PED is a forme fruste of PNKD (Lance, 1977; Kurlan et al, 1987). As a part of this thesis, we have analysed a family with autosomal dominant PED and have excluded linkage with the PNKD locus and other known paroxysmal dyskinesia loci, thus supporting the conclusion that PED is a distinct entity within the group of paroxysmal dyskinesias. No PED loci have been identified so far, and the number of affected individuals in the family studied here is too small to allow a genome-wide search to identify the locus.

Other complex paroxysmal dyskinesias

The ICCA syndrome

Until recently an association of PKD with epilepsy in affected patients or other family members was not recognised. However, two papers have now described families with infantile convulsions and later onset of episodes of paroxysmal choreoathetosis (called "Infantile Convulsions and ChoreoAthetosis – ICCA syndrome) (Szepetowski et al, 1997; Lee et al, 1998). In both these reports, attacks of paroxysmal choreoathetosis in the affected members were very brief, frequent and induced by sudden movements and therefore similar to PKD.

The RE-PED-WC syndrome

Recently, a family with an apparent recessive disorder characterised by rolandic epilepsy, episodes of exercise-induced dystonia and writers cramp (RE-PED-WC syndrome) affecting three members of the same generation has been described (Guerrini et al, 1999). This is the only report of this syndrome so far.

The CSE syndrome

Auburger et al (1996) reported a large German family in whom affected members had dyskinetic attacks (choreodystonic) induced by alcohol, fatigue and exercise, and thus similar to typical PNKD. However, in addition these patients also had perioral paresthesias, double vision, headache and generalised myoclonic jerks often culminating in a seizure with unconsciousness. Some affected members also had marked spastic paraplegia. This syndrome, named paroxysmal choreoathetosis and spasticity syndrome, is therefore clearly different from typical PNKD.

Pathophysiology of paroxysmal dyskinesias

Since the outset there has been much controversy regarding the pathophysiology of these disorders. The debate so far has been whether these conditions are a form of reflex epilepsy or a basal ganglia disorder (Lishman et al, 1962; Stevens, 1966). With regard to PKD for example, arguments suggesting epilepsy include the paroxysmal non-progressive remitting character of the disorder and the excellent response to anticonvulsants (Kinast et al, 1980). A sub-cortical focus is suggested by the absence of seizure discharges on EEG in the majority of cases and the lack of an associated alteration of consciousness, or of amnesia. That the supplementary motor area may be the onset focus has been shown by depth electrode recordings in one patient during an episode (Lombroso, 1995). Others have suggested that these are basal ganglia disorders in view of the clinical characteristics of the involuntary movements, the absence of EEG abnormalities during attacks, and the occurrence of similar attacks caused by symptomatic lesions affecting the basal ganglia. In support of the extrapyramidal theory are abnormalities on specialised electrophysiological studies of contingent negative variation (Franssen et al, 1983), reciprocal inhibition (Lee et al, 1999) and the Bereitschaftspotential (Houser et al, 1999) in patients with PKD suggesting a possible functional disturbance of the prefrontal basal ganglia circuit.

However, as discussed below, it is clear that the paroxysmal dyskinesias have similarities to other episodic disorders of the nervous system, many of which are now known to be due to disorders of ion channels (Browne et al, 1994; Brunt and van Weerden, 1990; Griggs and Nutt, 1995), and therefore may have a common pathophysiology.

Similarities between paroxysmal dyskinesias and other episodic disorders

The paroxysmal dyskinesias have many features in common with other episodic nervous system disorders, many of which are known to be due to mutations of genes regulating ion channels, i.e. channelopathies (Griggs and Nutt, 1995; Hanna et al, 1998). Clinically the episodic ataxias, paroxysmal dyskinesias, periodic paralysis and other episodic disorders including migraine and epilepsy syndromes share the common feature of episodic attacks on a normal interictal background (Ptácek, 1997; Bhatia et al, 1999 and 2000). Many of these disorders have similar precipitating factors like stress, fatigue and diet. There is also an overlap for many of these disorders with regard to drug treatment. Carbamazepine, for example, prevents epileptic seizures and

is also very effective in patients with PKD. Acetazolamide is helpful not only in patients with periodic paralysis but also for myotonia, episodic ataxia and some paroxysmal dyskinesias. There are also reports of families with multiple episodic disorders, for example paroxysmal dyskinesia in a family with episodic ataxia and association of episodic problems like migraine and epilepsy in families with paroxysmal dyskinesias (Szepetowski et al, 1997). These similarities suggest a common pathophysiological process. It is interesting to observe the similarities between PKD and episodic ataxia type 1 (EA1), which is caused by mutations of the potassium channel KCNA1 gene (Browne et al, 1994). Both conditions begin early in life, and both tend to abate in adulthood. Like PKD, episodes of ataxia in patients with EA1 can be provoked by kinesigenic stimuli, and tend to be brief and frequent (Brunt and Van Weerden, 1990). Although EA1 typically responds to acetazolamide, anticonvulsants may reduce EA1 attacks in some patients and also help the interictal myokimia seen in this disorder (Griggs et al, 1978; Brunt and Van Weerden, 1990).

The relationship of paroxysmal dystonias, in particular PKD, to epilepsy has been a matter of considerable debate. The paroxysmal nature of attacks of involuntary movements bears clear similarities to epilepsy, although the dystonic and choreoathetoid nature of the movements is unlike the tonic or clonic movements usually associated with generalised or focal motor seizures. Electroencephalogram (EEG) recordings between attacks in patients with PKD or PNKD have shown no consistent abnormality but there have been a number of reports of paroxysmal epileptiform EEG discharges during attacks of PKD (Perez-Borja et al, 1967; Kato and Araki, 1969; Hirata et al, 1991). Hirata and co-workers argued that the origin of the abnormalities they observed (a generalised 5-Hz discharge with prolonged spike duration) suggested a subcortical origin of discharge. The excellent therapeutic response of PKD to anticonvulsants, and the coexistence of PKD and epilepsy in some patients (Hudgins and Corbin, 1966) support the contention that PKD may be a form of epilepsy.

It is therefore suspected that the familial paroxysmal dyskinesias may also be due to defects in genes regulating ion channels. The section below reports on the advances in genetics of these disorders so far.

The genetics of paroxysmal dyskinesias

Paroxysmal non kinesigenic dyskinesia (PNKD)

Fink and co-workers performed a genome-wide search in a large American kindred of Polish descent, with 28 affected members, and mapped PNKD to chromosome 2q33-q35 (Fink et al, 1996). In a five-generation Italian family with 20 affected members, Fouad and co-workers also showed tight linkage between PNKD and microsatellite markers on distal 2q (2q31-q36) (Fouad et al, 1996). The smallest region of overlap of the candidate intervals identified by the two groups placed the PNKD locus in a 6 cM interval. In a six-generation British family, Jarman and colleagues confirmed linkage to distal chromosome 2q and narrowed the candidate region to a 4 cM interval (Jarman et al, 1997). Linkage to the same genetic location, designated FPD1 (familial paroxysmal dyskinesia type 1) was further confirmed by Hofele et al (1997) in a German family originally described by Przuntek and Monninger (1983) as classical of Mount and Reback type of PNKD. Also linked to the same locus are two other typical PNKD families, one North American of German descend (Raskind et al, 1998) and a Japanese family (Matsuo et al, 1999a). This suggests that classical familial PNKD is indeed genetically homogeneous. With regard to the identification of candidate genes within the linked interval, SLC4A3 – a ion channel gene – represented a suitable candidate. This gene is the third member of a family of anion exchangers and encodes a membrane-bound protein which functions as a chloride/bicarbonate exchanger and an alkali extruder. It plays a role in the regulation of intracellular pH, intracellular chloride concentration and maintenance of cell volume (Kopito, 1990). The protein isoform is widely expressed throughout the brain, namely in the deep pontine grey matter, the substantia nigra and the caudal medulla (Alper, 1991). Jarman and colleagues (1997) analysed polymorphic tandem repeat sequences within the gene, and found no recombinations between PNKD and the intragenic polymorphisms. Linkage of PNKD with the SLC4A3 gene was also confirmed in the two other families described by Raskind et al (1998) and by Matsuo et al (1999a) respectively. However, the direct sequencing of the whole coding region of the gene failed to reveal functional mutations (Matsuo et al, 1999b). Although large rearrangements (deletions or duplications) within the gene cannot be excluded by direct sequencing and should be searched for in PNKD families, further genes need to be selected as candidates on the basis of the function and expression pattern, and evaluated for mutations.

The CSE syndrome

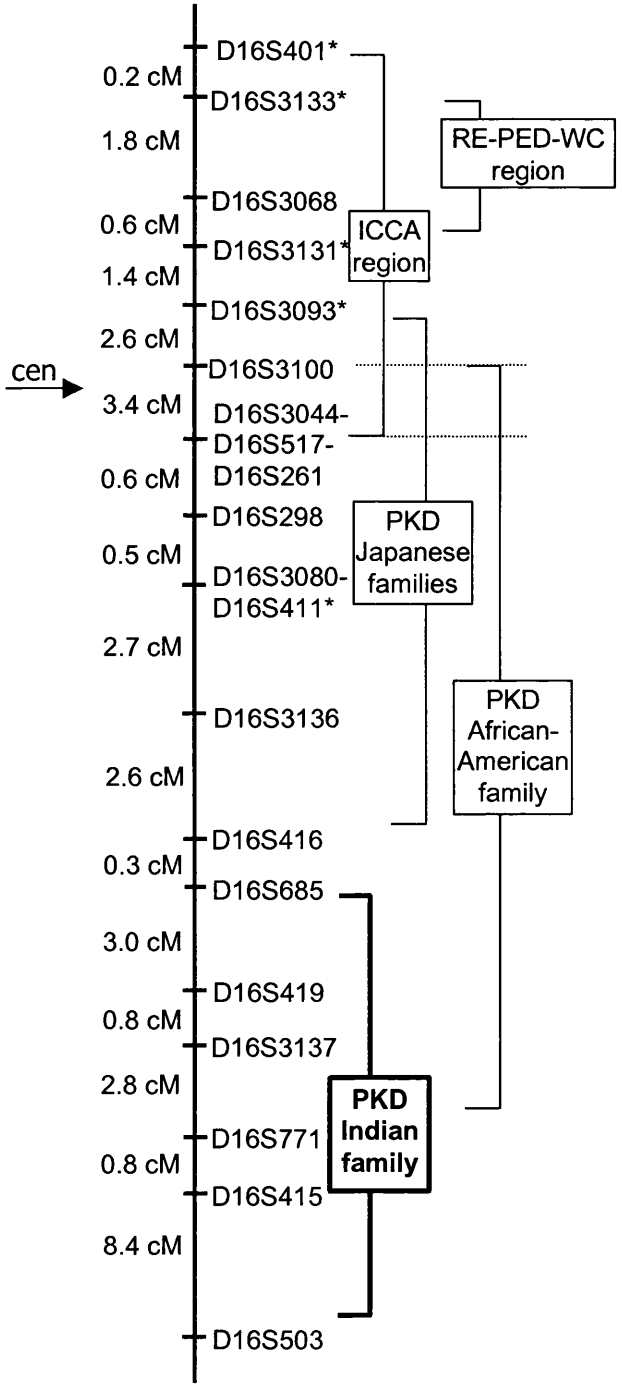
This syndrome (paroxysmal choreoathetosis and spasticity) was described only once in a German family by Auburger and colleagues (1996). Linkage analysis of the entire genome in this family placed the disease locus in a 12-cM interval on chromosome 1p21. There is a cluster of several potassium channel genes mapping to this region but further investigations are needed to determine the gene responsible for this disease.

PKD syndrome, ICCA syndrome and RE-PED-WC syndrome

It is necessary to consider the above three entities together, given the linkage of all three to the pericentromeric region of human chromosome 16. Szepetowski and colleagues (1997) described four French families with the ICCA syndrome and linked this to the pericentromeric region of chromosome 16. Critical recombinants narrowed the region of interest to a 10 cM interval around the centromere. Linkage to the same locus was further confirmed in a Chinese family with a similar syndrome (Lee et al, 1998), supporting genetic homogeneity for this condition. The clinical characteristic of some of the paroxysmal dyskinetic episodes in families with the ICCA syndrome were very similar to those described for PKD in terms of their brevity, frequency and onset by kinesigenic stimulus in some episodes. It was therefore not surprising that in one report of eight Japanese families (Tomita et al, 1999) and in a subsequent report of a three-generation African-American kindred (Bennett et al, 2000) with typical PKD attacks, the disease locus was mapped by genome-wide searches to the pericentromeric region of chromosome 16. The PKD region in the Japanese families spans 12.4 cM and overlaps the ICCA region by 6 cM (figure 4.1). As there was an increased prevalence of afebrile infantile convulsions in the Japanese families with PKD, it has been postulated the one gene might be responsible for both PKD and ICCA (Tomita et al, 1999). The PKD interval identified in the African-American family in which individuals have PKD alone (and no infantile seizures) spans 16.7 cM and overlaps by 3.4 cM with the ICCA region and by 9.8 cM with the PKD region identified in the Japanese families. The three regions overlap by 3.4 cM between microsatellite markers D16S3100 and D16S517 (figure 4.1). Making the situation more interesting (and perhaps complicated) is the family with autosomal recessive rolandic epilepsy, paroxysmal exercise-induced dyskinesia and writer's cramp (RE-PED-WC syndrome) which has also been linked to chromosome 16 within the ICCA region, but outside the 3.4 cM overlap between ICCA and PKD. Thus RE-PED-WC might be allelic to ICCA but

it is probably not allelic to PKD. Furthermore, epilepsy is the most striking feature of both the ICCA and RE-PED-WC syndromes and some of the ICCA attacks were induced by exercise, which perhaps suggests a common underlying gene for these two conditions which may be different from that giving rise to PKD.

Figure 4.1 – genetic map of the pericentromeric region of chromosome 16



cen: centromere

Aims of the study

The aims of this part of the study were: a) to characterise clinically and genetically three families with autosomal dominant paroxysmal kinesigenic dyskinesia; b) to identify novel PKD loci by means of genome-wide searches; c) to clinically and genetically characterise a British family with autosomal dominant paroxysmal exercise-induced dyskinesia and migraine.

PKD and PED families

Family PKD01

Family PKD01 is an Indian family. The proband was identified at the KLE Society's Hospital and Medical Research Centre in Belgaum, Karnataka, India and subsequent family members were traced to the surrounding area. Seventeen patients with PKD were identified in this pedigree (figure 4.2). To avoid presenting this pedigree twice and for clarity in result presentation, this figure has been placed in the "Results" section of this chapter (page 130). Eleven were male and six were female. The mode of inheritance was autosomal dominant and the male-to-female ratio was 1.8:1. The age of onset of attacks varied from 7 to 13 years with an average age of onset of 9.6 years. Eight of the affected had choreic episodes whereas the other nine had dystonic attacks. The involuntary movements involved the limbs predominantly and the face was involved to a lesser extent, which led to mild dysarthria. None of the attacks were associated with loss or impairment of consciousness. Six patients experienced sensory aura in the form of creeping paresthesias involving the same part of the body. Laterality of the attacks was variable, only two members had strictly one-sided attacks, nine individuals had episodes which were unilateral but alternated between sides and five others had both alternating episodes and bilateral episodes. The duration of episodes was 5 seconds to 2 minutes and the frequency of attacks was 1-20/day. The severity of attacks was variable, some had episodes severe enough to make them nearly fall down while others had episodes which were barely noticed by their friends and relatives. Attacks were precipitated by sudden movement in all cases, and four patients could precipitate attacks by hyperventilation. An additional two patients could provoke attacks by "mental tension" while cold precipitated attacks in two

others. There was good therapeutic response in the four patients who tried anticonvulsants. Withdrawal of the drugs led to reappearance of the episodes within 24-48 hours. Spontaneous remission occurred in nine of the seventeen affected family members and the average age of remission was 23.3 years. To date, there has been no relapse of the remission in any of the family members.

Five family members suffered from seizures (two of these individuals also have attacks of PKD). These individuals are marked with a plus symbol in figure 4.2. Individual II:5 had PKD and had three episodes of generalized tonic-clonic seizures from the age of 10 to 25, and was treated with phenytoin for two years. Individual III:14 had had four episodes of generalized tonic clonic seizures between the age of 14 and 15 years and has had no seizures since. Individual IV:8 does not have PKD but had onset of generalised tonic clonic seizures from the age of 11 years with a frequency of 1-2 seizures per year. His seizures are well controlled on carbamazepine 600mg/day. Individual IV:10 does not have PKD but had onset of generalized tonic clonic seizures from the age of 16 years with a frequency of attacks of 1-3/year. His seizures are also successfully controlled on 600 mg/day of carbamazepine. Individual IV:9 had a single seizure at the age of 6 years. His EEG was normal, he did not receive medication and does not suffer from PKD.

EEG recordings were obtained from five family members with PKD. Only one person (III:21) had an abnormal EEG, demonstrating interictal generalized low voltage spike discharges. Interestingly this person did not suffer from seizures but did suffer from PKD. CT scans of the head were performed on three of these patients and were normal; unfortunately a scan was not obtained on individual III:21. It is important to stress than none of these individuals suffered from infantile convulsions. The family does not have the ICCA syndrome. The predominant condition is PKD and the seizures are not infantile but develop in the preadolescent/adolescent period. Table 4.1 shows a summary of the clinical details.

Table 4.1 – clinical details of affected members in family PKD01

Subject (sex)	Age	Age at onset	Age at remission	Nature	Duration of episodes	Attacks per day	Trigger factors	Sensory aura	Associated disorders	EEG	CT scan	Response to therapy
II: 2 (F)	dead	9 yrs	25 yrs	dystonic	1 min	6 to 8	SM	no	---	---	---	no therapy
II: 5 (M)	dead	10 yrs	25 yrs	dystonic	30 sec	10 to 11	SM	no	epilepsy	---	---	Phenytoin
II : 6 (M)	dead	8 yrs	25 yrs	dystonic	30 sec	5 to 6	SM	no	---	---	---	no therapy
III: 3 (M)	58	9 yrs	22 yrs	dystonic	30 sec	6 to 7	SM	yes	---	---	---	no therapy
III: 5 (M)	52	9 yrs	20 yrs	dystonic	1 min	4 to 4	SM	no	---	---	---	no therapy
III: 7 (M)	48	10 yrs	25 yrs	dystonic	1 min	6 to 7	SM	no	---	---	---	no therapy
III: 10 (F)	45	10 yrs	21 yrs	dystonic	30 sec	2 to 3	SM	yes	---	---	---	no therapy
III: 13 (M)	40	9 yrs	28 yrs	choreic	30 sec	3 to 4	SM	no	---	---		no therapy
III: 14 (M)	36	9 yrs	---	dystonic	1 to 2 min	15 to 20	SM, HV	yes	epilepsy	normal	normal	Phenytoin
III: 15 (M)	40	10 yrs	---	choreic	30 sec	2 to 3	SM, HV	yes	---	---	---	no therapy
III: 21 (F)	23	13 yrs	---	choreic	5 sec	1 to 2	SM, MT	no	---	abnormal	---	no therapy
IV: 11 (M)	18	9 yrs	---	choreic	1 min	10 to 12	SM, cold , HV	yes	---	normal	normal	Phenytoin
IV: 13 (F)	20	9 yrs	---	choreic	20 sec	2 to 3	SM	yes	---	---	---	no therapy
IV: 18 (F)	32	11 yrs	---	dystonic	1 min	15 to 20	SM, MT	no	---	normal	normal	Phenobarb
IV: 19 (M)	7	7 yrs	---	choreic	10 sec	1 to 2	SM, cold	no	---	normal	---	no therapy
IV: 20 (F)	11	10 yrs	---	choreic	15 sec	1 to 2	SM, cold, HV	no	---	---	---	no therapy

SM: sudden movement; HV: hyperventilation; MT: mental tension

Family PKD02

This family is a Caucasian English family. The proband was identified at the Royal Free Hospital, London, UK. It is a three-generation family, and four family members are affected with PKD (figure 4.3). To avoid presenting this pedigree twice and for clarity in result presentation, this figure has been placed in the "Results" section of this chapter (page 132). Inheritance of PKD was autosomal dominant and the sex ratio in the affected individuals was 1:1. The age of onset of attack ranged from 6 to 13 years. Attacks were clearly provoked by sudden movement in three family members (I:1, II:4 and II:8) and by exercise in two (I:1 and III:3). The nature of the movement was dystonic in all members and the duration was seconds in all cases. The attacks were never associated with an alteration in the level of consciousness. There were no other paroxysmal conditions such as epilepsy or migraine, identified in this family. Table 4.2 presents a summary of the clinical findings.

Family PKD03

Family PKD03 is also a Caucasian English family. The proband was identified at the York General Hospital, York, UK. It is a three generation family in which family members suffered from either PKD or epilepsy (figure 4.4). To avoid presenting this pedigree twice and for clarity in result presentation, this figure has been placed in the "Results" section of this chapter (page 133). Four individuals in this family had PKD alone, three were female and one was male. The oldest affected member of this family (I:2), was not directly examined but was reported to have had onset of intermittent choreic movements at age 12 which resolved spontaneously when the patient was 19 years of age. The mode of inheritance in this family was autosomal dominant. The age of onset ranged from 9 to 12 years. In all cases the attacks were choreic in nature and lasted less than a minute in duration. The attacks were provoked by sudden movements in all cases and also by stress in individual II:2. These subjects did not suffer from any other paroxysmal condition such as seizures and their attacks responded well to carbamazepine 200mg twice a day. Table 4.3 presents the clinical details of the affected members.

Additionally, there were four family members who suffered from seizures but not PKD. Subject II:5 had infantile convulsions with onset of multiple generalized tonic clonic seizures at the age of 6 months. These subsequently developed into partial seizures that were associated with an alteration in the level of consciousness but were never

associated with falls or motor movements. These attacks were never treated and resolved spontaneously at the age of 15 years.

Subject III:4 also suffered from seizures since the age of 9. He had two seizure types, generalized tonic clonic and partial seizures. The partial seizure consisted of an expressive aphasia and an alteration in the level of consciousness associated with hand stiffening and flexion of both arms. The episodes typically lasted two minutes in duration and the individual was post-ictal following the attack. The attacks were not preceded by an aura. This person had approximately one seizure per month pre-treatment but responded well to carbamazepine 200 mg twice a day.

Subject III:5 had onset of non febrile generalized tonic clonic seizures at age 14 months. The patient had a total of six attacks before being put on phenytoin. The patient was treated with phenytoin for six months and then medications were discontinued. There have been no further seizures.

Subject III:7 was born prematurely at 32 weeks gestation and suffered an intra-ventricular haemorrhage. At the age of 6 months this patient had a non-febrile generalized convulsion and was put on carbamazepine for two years. Medications have been dismissed for one year now and there have been no further seizures.

The clinical features in family PKD03 shared similarities with both ICCA syndrome and family PKD01. In both cases family members suffered from PKD or epilepsy. In the ICCA syndrome, seizures start in infancy. There were three individuals (II:5, III:5, III:7) in family PKD03 who suffered from generalized infantile seizures. Subject III:7 however was born premature and suffered an intra-ventricular haemorrhage, and therefore his seizures may be secondary to this. One family member (III:4) experienced seizures in adolescence and not in infancy. This particular clinical feature is similar to that seen in family PKD01, the Indian family.

Table 4.2 – clinical details of affected members in family PKD02

Subject (sex)	Age	Age at onset	Age at remission	Nature	Duration of episodes	Attacks per day	Trigger factors	Sensory aura	Associated disorders	EEG	CT scan	Response to therapy
I:1 (M)	68	10	30	dystonic	< 20 sec	< 5	SM, exercise	no	none	---	---	no therapy
II:4 (F)	38	11	31	dystonic	< 10 sec	3-4	SM, fatigue	no	none	---	---	no therapy
II :8 (F)	31	13	28	dystonic	5-10 sec	2	SM	no	none	---	---	no therapy
III:3 (M)	8	6	---	dystonic	10 sec	2-3	Exercise	no	none	---	---	CBZ

Table 4.3 – clinical details of members affected by PKD in family PKD03

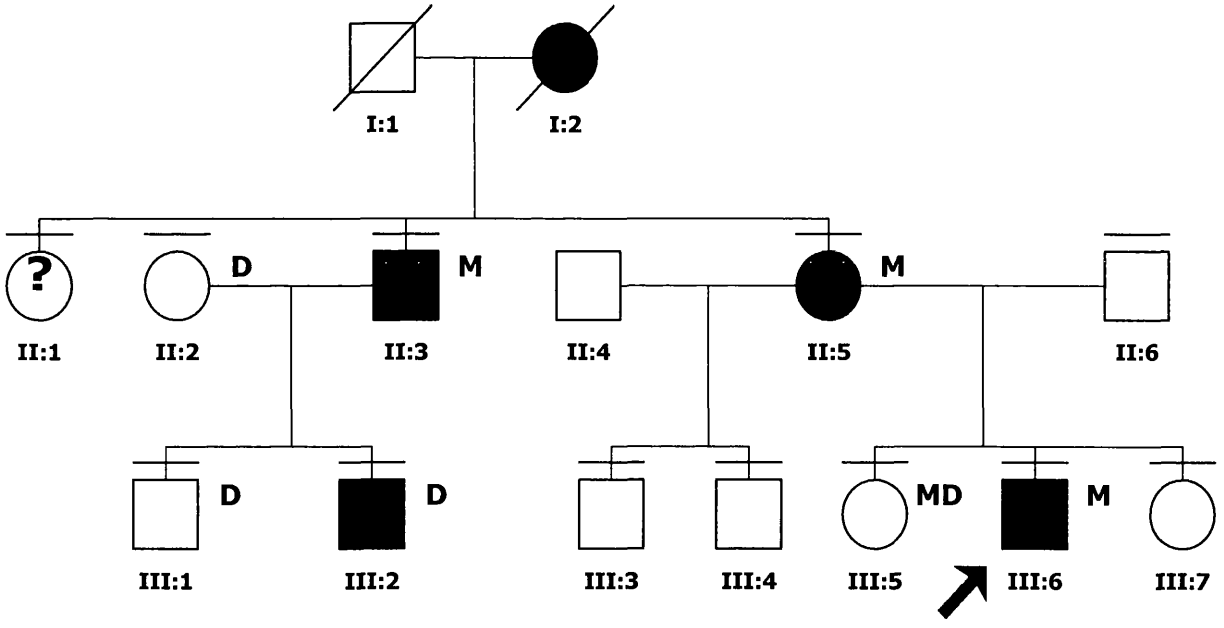
Subject (sex)	Age	Age at onset	Age at remission	Nature	Duration of episodes	Attacks per day	Trigger factors	Sensory aura	Associated disorders	EEG	CT scan	Response to therapy
I:2	73	12	19	choreic	---	---	---	---	---	---	---	---
II:2	50	11	---	choreic	< 1 min	up to 100, less when older	SM, stress	---	none	---	---	CBZ
II:7	40	8	---	choreic	< 1 min	up to 50	SM, stress	yes	none	---	---	CBZ
III:1	25	9	---	choreic	10-60 sec	up to 50	SM	---	none	---	---	CBZ

SM: sudden movement; CBZ: carbamazepine

Family PED01

Of fourteen members examined, four were definitely and one probably affected by PED; three of them had migraine also. One member who is deceased was reportedly affected by history. The transmission pattern in this family appeared to be autosomal dominant with reduced penetrance. Of the four definitely affected members, interictal examination was normal in one, showed postural tremor in the arms in two patients and dystonic posturing and chorea in one patient. The remaining nine examined members of the family were not affected by PED, but one had dystonic posturing and mild chorea in the arms and one had dystonic posturing and position specific arm tremor. Additionally, one spouse had mild dystonia and chorea. Table 4.4 summarises the abnormal clinical findings in the examined family members. Mean age of onset in subjects affected by PED was 12 (range: 9-15 years). Male-to-female ratio was 3:1. Figure 4.5 shows the pedigree of the family. Case reports of affected family members are reported below.

Figure 4.5 – pedigree of family PED01



Black symbols: subjects affected by PED; D: choreodystonic movements; M: migraine

Table 4.4 – clinical details of affected members in family PED01

Subject (sex)	Age	Age at onset	Prodromal symptoms	Features during PED attacks	Other symptoms	Trigger factors	Attack duration	Recovery time	Interictal clinical examination
II:3 (M)	41	15	tingling thighs	dystonia of legs, hand stiffness, slurred speech	migraine	long walk, cycling, writing	10-15 min	40 min	postural arm tremor
II:5 (F)	44	11	legs feeling like jelly	dystonia of legs, jaw and hand cramps, slurred speech	migraine	long walk, chewing gum	up to 30 min	30 min	normal
III:2 (M)	12	9	none	leg jerks, loss of balance, hand spasms	---	long walk, writing	15 min	30 min	choreodystonic movements of arms
III:6 (M)	25	12	tingling thighs	dystonia of legs, tightening of hands, pectoralis spasms, slurred speech	migraine, leg cramps	long walk, using tools, talking, chewing gum	10-15 min	30 min	postural arm tremor
I:2 (F)	decease	12	none	leg spasms	---	long walk	---	---	---
II:2 (F)*	33	---	---	no PED	arm tremor	---	---	---	dystonia and tremor of arms
III:1 (M)*	7	---	---	no PED	---	---	---	---	mild choreodystonic movements of arms
III:5 (F)*	19	---	---	no PED	migraine	---	---	---	mild dystonia and postural tremor of arms
II:1 (F)**	35	18	none	diffuse spasms, hand cramps	seizures	---	hours	---	normal

* family members with dystonia, chorea or tremor, but no PED; **possibly affected by PED

Family members definitely affected by PED

Subject II:3. He is the 41-year-old maternal uncle of the index case. He developed symptoms at the age of 15. Typically after walking for one mile (or cycling for several miles) his leg muscles gradually become very stiff and twist. His feet and legs turn inwards and he becomes unable to walk. He looses control over his legs and has to sit down to prevent falling. He has to rest for at least 10 to 15 minutes before the spasms subside and he is able to walk again but then usually gets another attack after walking for 10-20 yards. To prevent getting another attack after short exercise he has to rest for at least 35-40 min. Before an attack he has tingling in his thighs, his heart rate is increased and he feels panicky and sometimes has numbness around his mouth. During a typical attack his speech sometimes becomes slurry. He has not noticed any other precipitating events apart from exercise and feels neither cold nor heat has an influence on his symptoms. Frequency of attacks solely depends on physical activity. On average he has 3-4 attacks a weeks. On several occasions he developed similar stiffness and cramps in his right hand after writing for a long time. Very often he has painful muscle spasms between his shoulder blades and in the lower spine without radiation to the legs. These spasms can be brought on by walking, bending and sometimes by sudden movements. Occasionally when yawning his legs start to quiver uncontrollably for several seconds. Intermittently he suffers from double vision with images side-to-side. This could happen at any time and is not provoked by exercise or fatigue. Once or twice a week he has right-sided severe pulsating headache that usually lasts for 6 hours and is accompanied by sensitivity to light. He thinks that when having these headaches he is more likely to get attacks of muscle stiffness. He also describes faecal urgency. Before having the attacks when he was a child he was prone to develop painful muscle spasms in his calf muscles at night. At the age of 7 there was one episode when he woke up in the morning and couldn't get up, as his legs were floppy. This lasted for 35 minutes. He has never tried any medication. On neurological examination there was a mild, jerky postural tremor in the arms. When he was imitating a typical attack, the posture of his legs and arms appeared dystonic.

Subject II:5. This is a 44 year-old lady, mother of the index case. Her symptoms started at the age of 11. When walking for more than half a mile her legs start feeling like jelly before beginning to twist involuntarily. She then becomes unable to walk and has to sit down or else she would fall. Cramps last for up to 30 minutes. She has to rest for at least 30 minutes before she is able to walk again without immediately getting another attack. Usually she feels very exhausted after an attack. She also

occasionally gets muscle spasms in her hands after carrying a heavy grocery bag or in her right hand after writing for a while. She has also had cramps in her abdominal muscles after exercise. When chewing gum for some time her tongue starts to feels heavy and her speech becomes slurred. She feels she is more prone to get either of these attacks in hot weather or when she is upset. During pregnancy or during her periods she did not notice any change of the attacks. She tried Phenobarbital without success. She is now avoiding any physical exercise and rarely has an attack but used to have attacks 2-3 times a week. Once a week she has left-sided pounding headache that usually starts in the morning and lasts for a few hours. She is then sensitive to light and noise. Neurological examination was normal.

Subject III:2. He is a 12-year-old boy, cousin of the index case. At the age of 9 he had his first episode of involuntary movements in his legs after exercise. After walking for several hours he suddenly lost control over his legs which started to "jerk". His whole body was then swaying, he lost his balance and was forced to sit down. He had to rest for 30 min before he was able to walk normally. So far he has had three attacks. The last attack was brought on after playing in the garden with other children for about 15 minutes. For several days before this event he had been very inactive. He is also getting painful spasms in his right hand when writing for an hour. Neither he nor his mother had noticed precipitating events other than exercise. On neurological examination he had mild dystonic posturing and chorea in his arms.

Subject III:6 (index case). He is 25 years old. His symptoms started at the age of 12. He develops stiffness and spasms in his leg muscles after walking for one hour. His legs tend to "turn in", his toes "curl in" and he has to sit down. Spasms in his leg muscles last 10-15 minutes. He has to rest for at least 30 min before being able to walk a reasonable distance. If he gets up too soon he is able to walk only for several yards before developing spasms in his legs again. His hands sometimes "tighten up" when he is using tools for some time, e.g. holding a paintbrush. When he is talking for a long time or chewing gum his speech becomes slurred. On one occasion he also developed spasms in his pectoralis muscles after lifting of barrels. He has several attacks a week. After an attack he usually feels light-headed and exhausted. His heartbeat tends to slow down. Other than exertion there are no precipitating events. Before an attack he might get tingling and mild aching in his thigh muscles. Sometimes he has cramping in his calf muscles at night. Now and again, usually after some exercise he has pounding bi-frontal headache, feels irritable and light-headed. This

headache lasts only for 15 minutes to half an hour. He tries to go to sleep and usually feels fine after a short nap. Apart from mild jerky postural tremor in the outstretched arms, neurological examination was normal. EMG, nerve conduction studies and muscle biopsy were normal, as well as routine laboratory investigations including CK, lactate, liver enzymes and renal function. Autoantibodies including ANA, ENA, ASMA, striated muscle antibodies, thyroid and anti-voltage gated potassium channels antibodies were negative. Ischaemic exercise test showed a normal response.

Family members probably affected with PED

Subject II:1. This maternal aunt of the index case started having generalised tonic-clonic seizures at the age of 18. These were successfully treated with Phenytoin. Several years later she was also diagnosed as having frontal lobe seizures with attacks of bizarre behaviour, confusion and hallucinations. Apart from that she also mentioned intermittent episodes of muscle spasms starting in one arm or one leg with spread to other body regions. These attacks could happen at any time at rest or after short exercise. She also complained of painful cramps in her hands after writing for a long time, occasional hoarseness and numbness of her whole body. Descriptions of her symptoms were rather vague. Neurological examination was normal.

Family member affected by history

Subject I:2. This is the maternal grandmother of the index case who died at the age of 71. She had developed spasms in her legs after walking for a mile at the age of 12. She mentioned to her daughter (mother of the index case) that the severity of her attacks improved as she got older. Other details of her symptoms are not known.

Family members affected with dystonia, chorea or tremor

Subject II:2. This is the 33-year-old ex-wife of II:3 and maternal aunt in law of the index case. She mentioned having tremor in both arms since the age of 17. On examination there was bilateral dystonic posturing of her arms and hands and mild to moderate irregular postural and kinetic tremor in her hands, more marked on the left.

Subject III:1. This 7-year-old cousin of the proband did not complain of any symptoms. On examination he showed dystonic posturing of his arms and abnormal involuntary jerky movements involving his arms and shoulder muscles particularly when holding out his arms. There was some stimulus sensitivity. They were interpreted as chorea.

Subject III:5. This is the 19-year-old sister of the index patient. She complained of occasional left-sided headache, lasting for several hours and responding well to Paracetamol. On examination she had a dystonic posture of both arms when holding out the arms and position specific tremor of the left arm on supination.

DNA analysis

Genome-wide search in family PKD01

DNA was extracted from leukocytes as described in the "methods" section. A simulation study performed with the program SLINK confirmed that family PKD01 was suitable for a genome-wide search (maximum expected LOD score: 3.98 at $\theta = 0.00$). Prior to the reported linkage of PKD to the pericentromeric region of chromosome 16 (Tomita et al, 1999), the author analysed 350 highly polymorphic fluorescent microsatellite markers spanning the 22 autosomes with an average distance of 10 cM (Linkage Mapping Set version 2, PE Applied Biosystems). Microsatellite markers were amplified from genomic DNA using the PCR technique, and electrophoresed on a denaturing acrylamide gel using a 377 DNA Sequencer. DNA fragment size analysis was performed semi-automatically using the Genescan and Genotyper software, to determine genotypes (see chapter 2).

Two-point LOD scores were generated using the FASTLINK version of MLINK using an assumption of autosomal dominant inheritance, age-related reduced penetrance (0.8 for individual older than 14 years and 0.5 for individuals 14 years old or younger), a gene frequency of 0.0001, and equal allele frequencies for each marker. The results of our genome search mapped the PKD locus to the pericentromeric region of chromosome 16. Additional informative microsatellite markers were analysed to refine this localisation. The position of these markers with respect to PKD and ICCA is shown in figure 4.1. Haplotypes were constructed to detect recombination events, assigning phase based on the minimum number of recombinants. Marker order and genetic distances were based on framework markers of the latest Genetic Location Database chromosome 16 consensus map and published recombinants (Szepetowski et al, 1997; Tomita et al, 1999; Bennett et al, 2000).

Testing for linkage to known loci (families PKD02, PKD03, PED01)

The number of affected individuals in each of these families was too small to allow the identification of a novel locus through a genome-wide search. However, these families were tested for linkage to the loci known to be responsible for other paroxysmal dyskinesias or episodic disorder.

Families PKD02 and PKD03

Families PKD02 and PKD03 were tested for linkage to the pericentromeric region of chromosome 16, with markers covering both the ICCA, the RE-PED-WC and the PKD intervals: D16S3133 – *1.8cM* – D16S3068 – *0.6cM* – D16S3131 – *1.4cM* – D16S3093 – *6.0cM* – D16S261 – *0cM* – D16S3044 – *0.6cM* – D16S298 – *3.2cM* – D16S3136 – *2.9cM* – D16S685 – *7.4cM* – D16S415 – *8.4cM* – D16S503.

DNA was extracted from whole blood as described in the Methods section. Fluorescent PCR products were analysed by electrophoresis on an acrylamide gel using a 377 DNA sequencer and Genescan software. Genotypes were assigned using the Genotyper program. LOD scores were calculated as for family PKD01. An additional analysis of family PKD03 calculated LOD scores based on a phenotype which included PKD +/- infantile convulsions. Haplotypes were assigned manually, assuming the smallest number of recombination events.

Family PED01

To perform haplotype analysis in this family, the following microsatellite markers spanning the critical regions on chromosome 2 (Paroxysmal Non Kinesigenic Dyskinesia), 19 (Familial Hemiplegic Migraine / Episodic Ataxia 2) (Joutel et al, 1993) and 16 (Infantile Convulsions and ChoreoAthetosis / Paroxysmal Kinesigenic Dyskinesia) were analysed:

PNKD: *cen* – D2S164 – *1.1cM* – D2S295 – *0.2cM* – D2S173 – *0.5cM* – D2S2250 – *2.1cM* – D2S2359 – *2.1cM* – D2S377 – *tel*

FHM/EA2: *cen* – D19S432 – *3.9cM* – D19S899 – *0.6cM* – D19S1150 – *0.4cM* – D19S199 – *tel*

ICCA/PKD: D16S401 – *0.2cM* – D16S3133 – *1.8cM* – D16S3068 – *0.6cM* – D16S3131 – *1.4cM* – D16S3093 – *6.6cM* – D16S298 – *6.1cM* – D16S685 – *7.4cM* – D16S415.

Map distances were taken from the Genetic Location Database.

For the PNKD locus, the markers D2S164, D2S295 and D2S377 are located outside the linked area, while the other three markers span the 4 cM linked region. The gene responsible for FHM/EA2 has been identified and sequenced (calcium channel CACNL1A4) (Ophoff et al, 1996). The microsatellite marker D19S1150 is located within the gene, while D19S899 and D19S199 are placed at very short distance on either side of the gene. For the ICCA/PKD loci, the flanking markers D16S401 and D16S415 are just outside the region containing the three loci. Microsatellite markers were amplified from genomic DNA using the polymerase chain reaction and analysed as previously described. Haplotypes were reconstructed in all subjects using all the typed markers spanning the three regions.

The sequencing of the FHM gene was performed by Dr. Louise Eunson, co-author of the paper which came out of this project. For this reason, the sequencing methods are briefly reported here and not in detail in chapter 2. Three sets of oligonucleotide primers were used to amplify the four exons containing the known FHM mutations (exons 4, 16, 17 and 36). The primers employed were tagged with M13 tails to facilitate subsequent DNA sequencing. Conditions for the polymerase chain reaction were as follows: an initial denaturing step of 94°C for 4 minutes followed by 30 cycles of the following: 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds. A final extension step of 72°C for 10 minutes was used. The products were cleaned using Qiagen Qiaquick PCR purification kits and both strands were sequenced using a Dye Primer Taq cycle sequencing kit (Applied Biosystems). The sequence data were analysed using Sequencing Analysis and AutoAssembler software (Applied Biosystems).

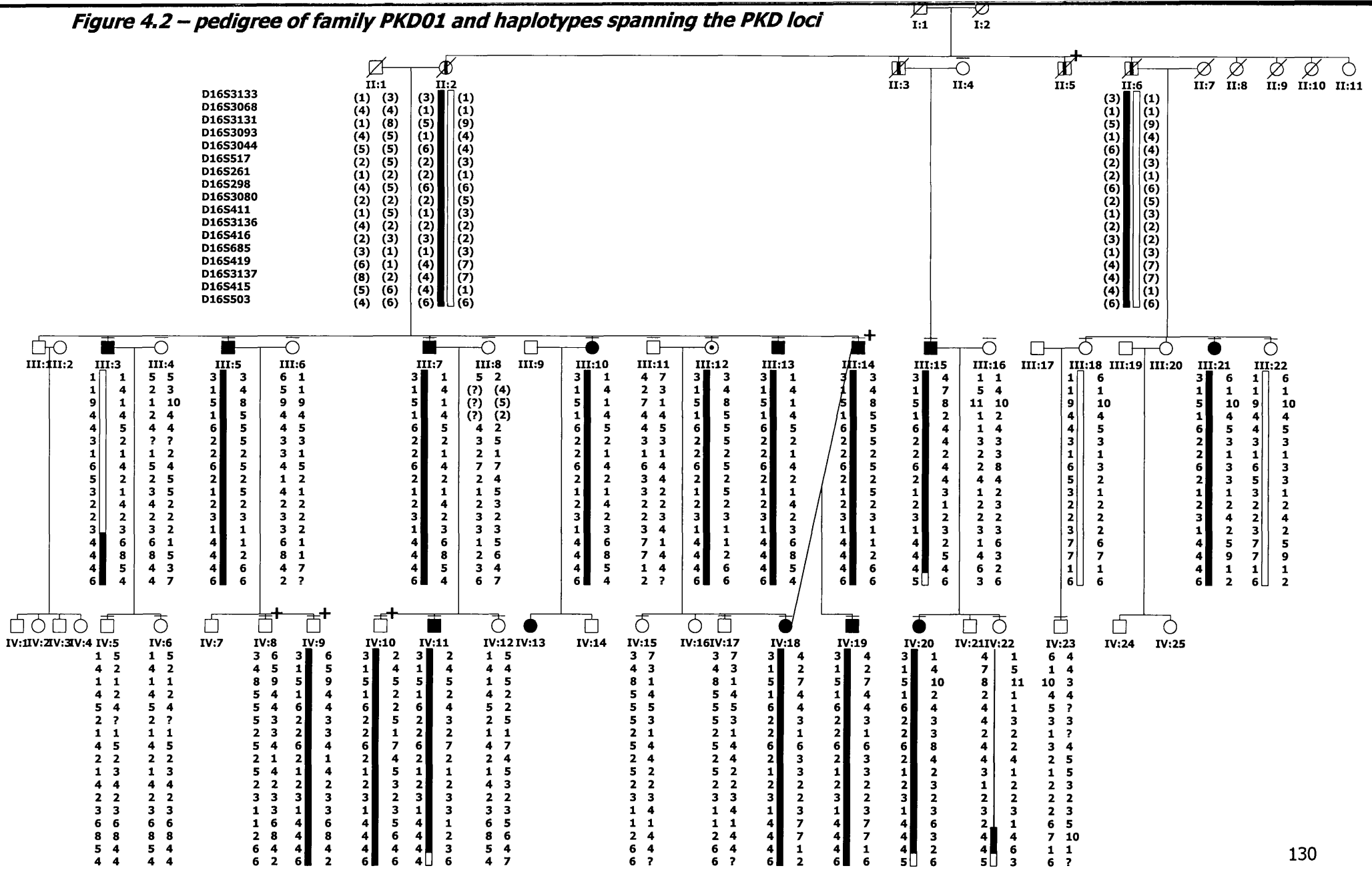
Results

Genome-wide linkage analysis in family PKD01

Pairwise LOD scores between PKD and marker loci on the centromeric region of chromosome 16 are given in appendix 4a. Positive results were obtained over the pericentromeric region of chromosome 16, with LOD score values > 3 at $\theta = 0.00$ for markers D16S419, D16S3137 and D16S415, with a maximum LOD score at D16S419 ($Z = 3.66$, $\theta = 0.00$). These markers are located telomeric to the PKD region identified in the Japanese families (Tomita et al, 1999). All affected individuals in the family shared a common haplotype between D16S685 and D16S503 (figure 4.2). The lower extent of the region is determined by recombinations detected in subjects III:15 and

IV:11 between markers D16S415 and D16S503. The upper extent of the region is defined in individual III:3 between D16S685 and D16S419. This recombination was more difficult to map, as both parents of III:3 are dead and the only unaffected brother (III:1) was unavailable for examination. The full parental haplotype segregating with the disease (black bar) was shared by most affected family members. Construction of the unaffected chromosome of the affected mother (II:2) was made possible by considering the haplotypes of individuals III:18 and III:22. These two individuals are the unaffected sisters of III:21, who is affected and carries the same haplotype as the other affected individuals in the family. III:18 and III:22 carry the same haplotype as III:3 for markers D16S3133 to D16S685, but a different haplotype for markers D16S419 to D16S503. It is likely that they inherited the haplotype not segregating with the disease (figure 4.2 - white bar) from their affected father (II:6), brother of II:2 and presumably haploidentical for this region. It is therefore likely that individual III:3 is recombinant between D16S685 and D16419. Clinical assessment, blood sampling and marker analysis have been carried out twice for this individual to avoid possible errors. The disease haplotype did not segregate with epilepsy in the family, suggesting that generalised juvenile-onset epilepsy is not part of the PKD phenotype in this family. These results assign a second PKD locus to a 15.8 cM region between markers D16S685 and D16S503, which is distinct from both the ICCA locus and the PKD locus described in Japanese families (Szepetowski et al, 1997; Tomita et al, 1999).

Figure 4.2 – pedigree of family PKD01 and haplotypes spanning the PKD loci



Linkage analysis in families PKD02 and PKD03

In family PKD02, significantly negative LOD scores were obtained across the ICCA and PKD regions (appendix 4b). Construction of the haplotypes for markers D16S3133, D16S3068, D16S3131, D16S3093, D16S3044, D16S261, D16S298, D16S3136, D16S685 and D16S415 excluded linkage with all chromosome 16 loci in this family (figure 4.3).

For family PKD03, haplotypes for markers D16S3133, D16S3068, D16S298, D16S3136, D16S415 and D16S503 are shown in figure 4.4. LOD scores were not significant in this family for the PKD phenotype alone (appendix 4c). Including infantile convulsions as part of the affected phenotype, linkage analysis generated positive LOD scores across the whole region (appendix 4d). Haplotype construction showed that the haplotype 5-3-2-2-2-2 co-segregated both with the PKD and the infantile onset seizures phenotypes. Conversely, this haplotype did not segregate in the individual who had onset of seizures in the adolescent period (III:4). The 5-3-2-2-2-2 haplotype was also identified in one unaffected individual (III:2). These data are consistent with the disease locus in this family being one of those in the pericentromeric region of chromosome 16, but the absence of recombinations in affected individuals across the whole region does not allow a precise localisation.

Figure 4.3 – pedigree of family PKD02 and haplotypes spanning the PKD loci

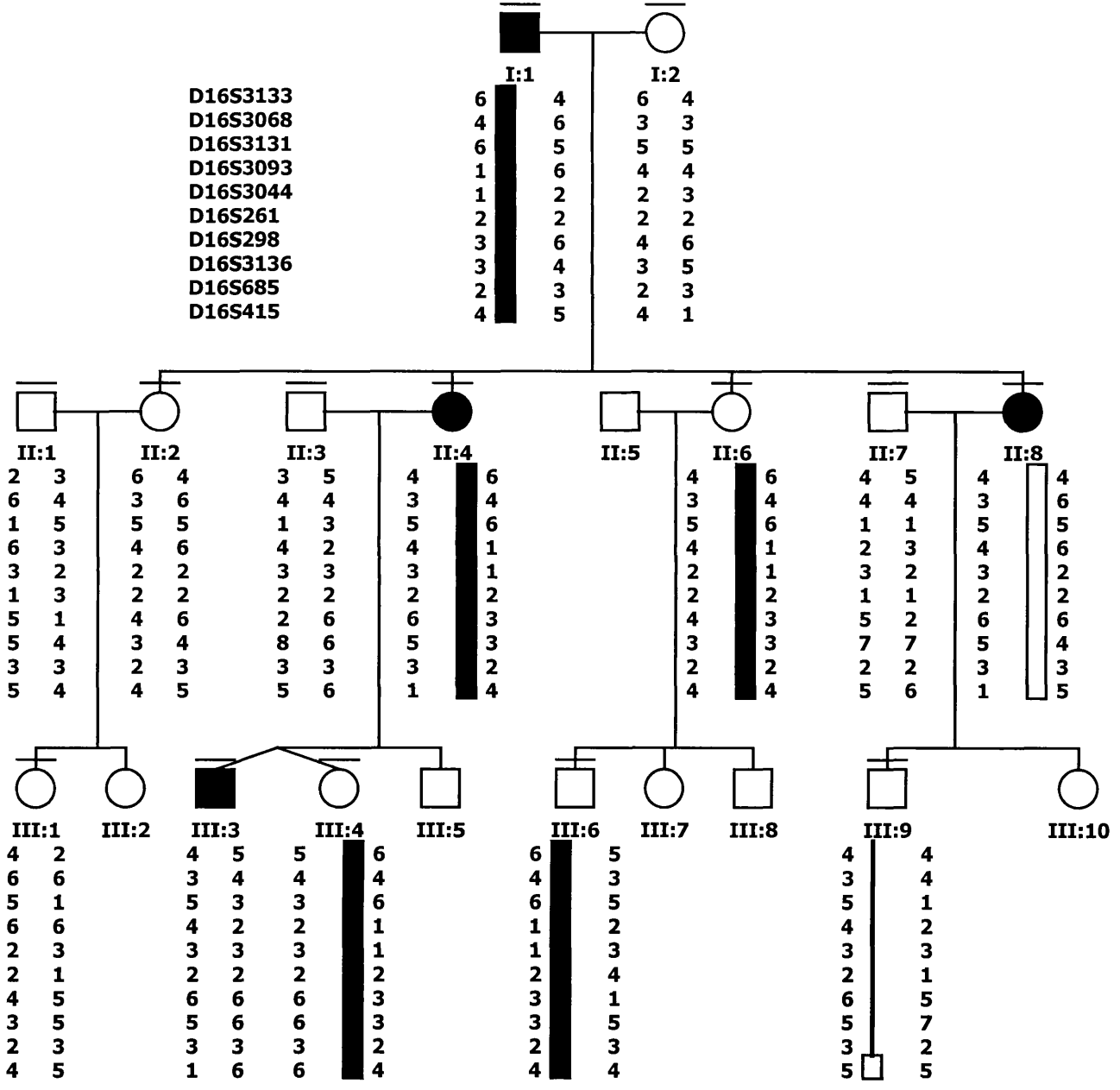
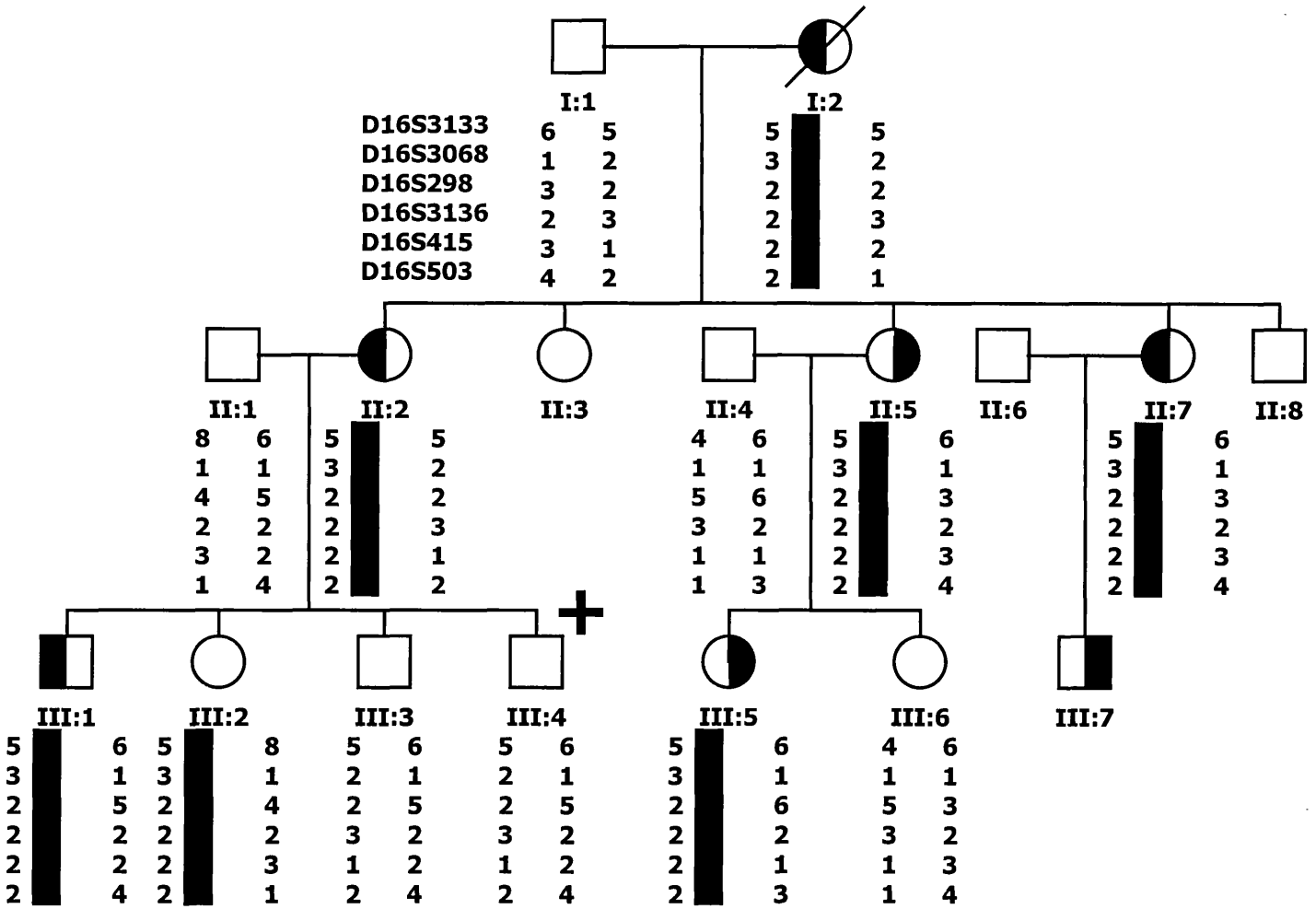


Figure 4.4 – pedigree of family PKD03 and haplotypes spanning the PKD loci



Symbols shaded on the left: PKD; symbols shaded on the right: infantile-onset seizures; plus symbol: adolescent-onset seizures

Linkage analysis in family PED01

The haplotype reconstruction for PNKD, FHM/EA2 and ICCA/PKD areas failed to show a common haplotype shared by all the four definitely affected members. Moreover, linkage analysis between the disease and all tested markers generated negative LOD scores at all recombination fractions, hence allowing us to exclude linkage between the disease and all the tested loci (appendix 5). Direct DNA sequencing of the exons 4, 16, 17 and 36 of the FHM/EA2 gene did not reveal the previously described mutations R192Q, T666M, V714A and I1811L, nor any other mutation.

Discussion

PKD families

This study identified three PKD families. Clinical and genetic data from these three families report evidence of both clinical and genetic heterogeneity in PKD. In all three families the mode of inheritance was autosomal dominant but the degree of penetrance differed. While there was incomplete penetrance in family PKD01 (75%), the absence of an asymptomatic obligate gene carrier in families PKD02 and PKD03 suggests complete penetrance. It should be noted however that these families were small. Other studies, which have reported incomplete penetrance in familial PKD, have identified that the disease was transmitted through an unaffected female (Sadamatsu et al, 1999; Nagamitsu et al, 1999). This was also found in family PKD01. Surprisingly, the male preponderance typically reported in this disease (Fahn, 1994; Houser et al, 1999; Nagamitsu et al, 1999) was only born out in one of the families. In family PKD01 the male:female ratio was 1.8:1. In family PKD02 the ratio was 1:1 and in family PKD03 the male:female ratio was reversed with a female preponderance (ratio of 1:3). The overall male:female ratio was 1.3:1. Although the age of onset of attacks was similar in all three families (ranging from 6 to 13 years), the nature of the attacks themselves not only varied between families but also within families. Within a family the attacks can be choreic or dystonic, unilateral or bilateral, mild or disabling. Although sudden movements induced attacks in all cases, within families there maybe other precipitating factors such as cold, hyperventilation or exercise. Clearly there are multiple factors at play even within families, which determine the phenotype.

One PKD locus has been mapped to a 12.4 cM interval on the pericentromeric region of chromosome 16, between D16S3093 and D16S416 (Tomita et al, 1999). A subsequent

study confirmed linkage of PKD to a partially overlapping 16.7 cM interval between markers D16S3100 and D16S771 in an African-American family (Bennett et al, 2000). There is a common overlap of 3.4 cM between these 2 regions and the ICCA locus (Szepetowski et al, 1997). Initially it appeared that PKD (and ICCA) were genetically homogeneous, as families from wide geographic regions (France, China, Japan, and America) all mapped to the pericentromeric region of chromosome 16 (Szepetowski et al, 1997; Lee et al, 1998; Tomita et al, 1999; Bennett et al, 2000; Swoboda et al, 2000).

The genome search performed on family PKD01 (before the first PKD locus had been published) allowed the localization of PKD in this family to chromosome 16q13-q22.1, to a region which is close to, but distinct from, the PKD locus identified in the eight Japanese families (Tomita et al, 1999). This locus has been assigned the symbol EKD2. The localisation of PKD in the African-American family (Bennett et al, 2000) showed a region of overlap with both these regions. The African-American PKD locus may therefore be allelic with either the Japanese or the Indian PKD locus or represent a third gene (figure 4.1). The results obtained in family PKD01 demonstrated genetic heterogeneity of PKD, making it unlikely that one gene in the pericentromeric region of human chromosome 16 could give rise to both PKD and ICCA. Another paroxysmal disorder, characterised by autosomal recessive rolandic epilepsy with paroxysmal exercise-induced dystonia and writer's cramp (RE-PED-WC), has been mapped to the same region as the ICCA locus (flanking markers: D16S3133 and D16S3131) (Guerrini et al, 1999). In both syndromes epilepsy is the most striking feature, suggesting that the underlying gene/s for these two conditions are different from those which give rise to PKD.

The mapping of a second PKD locus lying in close proximity of the first one raises the possibility that a family of genes in the pericentromeric region of chromosome 16 are responsible for multiple paroxysmal disorders (PKD, ICCA, RE-PED-WC). The identification of several duplicated regions and frequent chromosomal rearrangements in the pericentromeric region of human chromosome 16 is in favour of this hypothesis. (Loftus et al, 1999). Clusters of genes causing similar but distinct phenotypes, which map in close proximity have previously been reported, e.g. two hereditary non-chromaffin paraganglioma loci on the long arm of chromosome 11 (Heutink et al, 1992; Mariman et al, 1993), two loci for familial benign hypercalcemia on the pericentromeric region of chromosome 19 (Heath et al, 1993; Lloyd et al, 1999), and

several epilepsy genes causing phenotypically different epilepsy syndromes on the long arm of human chromosome 8 (Steinlein et al, 1995; Zara et al, 1995; Wallace et al, 1996; Fong et al, 1998; Mikami et al, 1999).

In addition to the gene loci on chromosome 16, the findings in family PKD02 demonstrate the presence of at least one other PKD locus elsewhere in the genome.

As a number of other paroxysmal disorders including some epilepsies are due to mutations in ion channels (Steinlein et al, 1997; Hanna et al, 1998; Bievert et al, 1998; Charlier et al, 1998; Singh et al, 1998) it would be reasonable to expect that PKD is also due to a mutation in a gene controlling ion channel function. However, ion channel genes have not yet been identified in this region. A cluster of genes encoding for solute carriers, comprising sodium/chloride and potassium/chloride co-transporters, neurotransmitter transporters and sodium/hydrogen exchangers map to chromosome 16. Most members of this cluster lie in the pericentromeric region of chromosome 16, within the ICCA and PKD intervals. Also, several members of small inducible cytokine subfamilies and a cluster of several metallothionein genes map to the region.

The mapping of the EKD2 locus has used framework markers of the Genetic Location Database consensus map for intermarker distances and relative position. Markers D16S401, D16S3133, D16S3131, D16S3093 and D16S411 were localised according to previously observed recombinants. (Szepetowski et al, 1997; Tomita et al, 1999). When this study was performed (1999-2000), the available genetic and physical maps were not so accurate, especially for critical regions such as the pericentromeric region of chromosome 16. The completion of the Human Genome Mapping Project, and the free availability on the Internet of up-to-date Human Genome Databases allows now a more precise positioning of microsatellite markers, single nucleotide polymorphisms and genes on the chromosome physical map. A recent re-evaluation of the position of all typed markers on the most recent chromosome 16 physical map (UCSC Human Working Draft, release November 2002) confirmed the existence of two distinct PKD loci on chromosome 16, although the relative position of a few markers has changed.

The presence and type of epilepsy varied between the three PKD families identified. Family PKD02 is a family with pure PKD with no associated epilepsy. Family PKD01, the Indian family, has PKD and preadolescent/adolescent onset of seizures. In this family the disease haplotype did not segregate with epilepsy, supporting the hypothesis that adolescent onset epilepsy is not part of the PKD phenotype. Swoboda et al (2000) also observed adolescent onset seizures in their PKD families, however the individuals with

seizures did not have PKD. Studies in family PKD03 are also consistent with this hypothesis. Although family PKD03 is too small to generate significant LOD scores, the same haplotype segregated in all individuals affected by PKD and also in subjects who had infantile benign convulsions, but was not present in the only individual with adolescent-onset seizures. These data are consistent with the disease locus in family PKD03 being on chromosome 16 and suggest that infantile convulsions may be part of the PKD phenotype, while adolescent onset seizures are not.

Recognition of the full clinical spectrum of PKD and developments in linkage of ICCA syndrome and PKD to chromosome 16 are bringing us closer to identifying the genes responsible for these conditions and will hopefully shed some light on the relationship between PKD and epilepsy and the underlying pathophysiology.

Family PED01

Attacks in all affected family members fulfil criteria of PED as outlined by Demirkiran and Jankovic (1995) and Bhatia and co-workers (1997). The clinical presentation of the affected members was remarkably stereotyped. The age of onset varied little (9-15 years), the duration of an attack was always between 15 and 30 minutes and attacks were consistently precipitated by walking. All members had attacks involving the legs, which were mainly dystonic. However, depending on the exercise attacks could also occur in other body regions, e.g. in the hand after writing for a long time in all affected members, in the jaw after chewing or in the pectoralis muscles after heavy lifting. Generalization did not occur. Speech was slurred during an attack in three cases and these patients also reported prodromi, mainly sensory symptoms in their legs. Between the attacks two members had a postural tremor in the arms and one had dystonic posturing and chorea in the arms, a feature that has also been mentioned by Nardocci and colleagues (1989). Interestingly, another two family members unaffected by PED also had mild dystonia in their arms (two also had mild chorea and one had tremor in the arms). However, it has to be pointed out that at least the two cousins of the index case (cases III:1 and III:2) might have inherited their symptoms from their mother (case II:2 and spouse of II:3) who had dystonia in her arms, and a relation to PED can therefore not definitely be claimed.

While following the same pattern of paroxysmal dyskinesias after exertion, the families with PED reported by Lance (1977), Plant and co-workers (1984) and Kluge and co-workers (1998) also presented each with a characteristic phenotype. In the three

members reported by Lance, spasms and cramps were almost exclusively confined to the legs, lasted for 5-30 minutes and could be precipitated by walking or other strenuous exercise. Attacks in the cases described by Plant and colleagues (1984) involved legs, arm and trunk and apart from exercise could also be induced by passive movements and vibration. In the two family members reported by Kluge and colleagues (1998) a tendency of the attacks which usually involved the legs and sometimes the arms after prolonged manual work to generalize was pointed out. In a comprehensive review of PED, Bhatia and co-workers (1997) stressed that in sporadic cases attacks are mostly dystonic and most commonly involve the legs but it was also outlined that considerable differences in the clinical presentation between patients exist. Therefore it can be concluded that PED is heterogeneous in its presentation with little intrafamilial variation in familial cases.

In addition to paroxysmal dyskinesias, three of the affected members in our family also had migraine without aura. The association of PED and migraine has only been reported once before in a girl also affected with learning impairment, ataxia and epilepsy (case 5 in Bhatia et al, 1997 and Neville et al, 1998). Linkage of the disease with the FHM/EA2 locus was excluded in our family by haplotype reconstruction and linkage analysis. Moreover, direct sequencing of the four exons of the FHM/EA2 gene previously shown to harbour mutations failed to reveal any mutation in the index case.

The occurrence of different paroxysmal disorders in the same family is intriguing and raises the question of a similar underlying cause. As a number of paroxysmal neurological disorders are known to be caused by mutations in ion channels, it appears likely that the defect underlying PED is also a channelopathy, and it is possible that novel mutations in an already identified gene might be responsible for the PED phenotype. Recently, a novel autosomal recessive syndrome characterised by PED plus rolandic epilepsy and writer's cramp (RE-PED-WC) has been linked to chromosome 16 (Guerrini et al, 1999). The haplotype construction with markers spanning this region (contained within the ICCA region) showed that the affected members in family PED01 did not share a common haplotype, thus excluding linkage of the disease with this novel locus.

In familial episodic ataxia with interictal myokimia (EA type 1), which has been mapped to chromosome 12p13 and is caused by a missense point mutation in the potassium channel gene KCNA1 (Browne et al, 1994), attacks of involuntary movements can be precipitated by sudden movements or after prolonged exercise (van Dyke et al, 1975;

Gancher and Nutt, 1986; Brunt and van Weerden, 1990). Also during episodes of ataxia some patients exhibit dystonic posturing (van Dyke et al, 1975). On electromyography, however, myokimia was not found in the index case, which makes the diagnosis of EA1 in this family very unlikely.

Although clinically the family under discussion differed from PNKD, there appears to be some overlap between PNKD and PED. In the family with PNKD reported by Schloesser and colleagues (1996), two affected female members described exercise as a precipitant of attacks that were longer than usual attacks of PED and more typical of PNKD. Prolonged exertion induced PNKD attacks in three of five members with PNKD reported by Kurlan and co-workers (1987). Linkage to the PNKD locus on chromosome 2 was therefore analysed in family PED01. Haplotype construction and linkage analysis allowed exclusion of linkage between the disease and this locus. At the moment, no PED loci have been identified. Further work is required to characterise this family genetically, although this family is too small for genome-wide purposes. A mutation in an as yet unidentified ion channel gene is likely to be the underlying cause of the paroxysms observed in family PED01.

Future directions of study

The findings presented here indicate the existence of a cluster of genes giving rise to paroxysmal disorders on the pericentromeric region of human chromosome 16; moreover, linkage with the entire chromosome 16 interval was excluded in family PKD02, supporting the genetic heterogeneity of this condition and the existence of at least one other as yet unmapped PKD gene. The contribution of the identified loci to families and singleton patients with PKD and the genotype-phenotype correlations remain to be evaluated. However, the coexistence of different paroxysmal disorders lying in close proximity on human chromosome 16 is particularly intriguing. Infantile benign seizures and PKD can coexist in the same family and individuals with infantile benign seizures might develop PKD. Conversely, other types of epilepsy do not seem to be associated with the PKD phenotype.

Large PKD families such as those described in this chapter are rare and provide a unique opportunity to identify unmapped genes. The identification of candidate genes requires additional PKD families to refine the linked intervals and monitoring new sequence information generated by the Human Genome Mapping Project.

CHAPTER 5 – AUTOSOMAL RECESSIVE JUVENILE PARKINSONISM

Outline of chapter

A genetic basis for Parkinson's Disease (PD) and Autosomal Recessive Juvenile Parkinsonism (ARJP) has been now clearly established. Several loci have been identified and four genes have been implicated in the pathogenesis of familial PD and ARJP. This represents a significant progress in the understanding of the disease, considering the rarity of large families, low heritability and genetic heterogeneity. Characterisation of the gene products encoded by these PD genes in the future should help elucidate the molecular mechanisms of neurodegeneration in these disorders 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.

The recent advances on pathogenetic mechanisms underlying autosomal dominant PD and ARJP will be briefly covered here, illustrating how the existence of genetic factors in PD has now become firmly established. The genetic work described in this chapter relates to the identification of a novel locus for ARJP and therefore the emphasis will be on this form of parkinsonism.

An introduction to Parkinson's Disease and Autosomal Recessive Juvenile Parkinsonism

Idiopathic (or sporadic) PD is one of the most common neurodegenerative disorders, with a clear age-dependent prevalence of 1 to 2 percent after the age of 65 years (De Rijk et al, 1997). The phenotype of idiopathic PD is characterised by resting tremor, rigidity and bradykinesia, which responds well to levodopa treatment. The pathological hallmarks of PD are the presence of Lewy bodies and massive loss of dopaminergic neurons in the pars compacta of the substantia nigra. Although there are symptomatic treatments, none modifies disease progression. 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. Up until 1996 the role of genetic factors in PD remained controversial, and though 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 (Polymeropoulos et al, 1996). The subsequent finding of a mutation in the α-synuclein gene (SNCA) in this family (Polymeropoulos et al, 1997) led to intensive global research into the genetics of PD, and a genetic contribution to the aetiology of PD is now well established. In families with autosomal dominant PD, one gene, α-synuclein has been proven and a second, ubiquitin carboxy-terminal hydrolase L1 (Leroy et al, 1998a) has been implicated in disease causation; moreover, three other genetic loci (PARK3 on chromosome 2p13, PARK4 on chromosome 4p14-16.3 and PARK8 on chromosome 12p11-q13) have been mapped (Gasser et al, 1998b; Farrer et al, 1999; Funayama et al, 2002). However, autosomal dominant forms are rare and seem to account only for a small number of families.

Autosomal recessive juvenile parkinsonism (ARJP) is a distinct clinical and genetic entity within familial PD, characterised by typical PD features associated with early age at onset (< 40 years), slow progression of the disease, sustained response to levodopa with early levodopa-induced complications (fluctuations and dyskinesias) that are often severe, hyperreflexia, sleep benefit and mild dystonia, mainly in the feet (Yamamura et al, 1973; Ishikawa and Tsuji, 1996). Pathological studies demonstrated a highly selective degeneration of dopaminergic neurons in the substantia nigra pars compacta and the locus coeruleus, and in most cases the absence of Lewy bodies (Takahashi et al, 1994). A gene for ARJP (Parkin) has been identified on human chromosome 6q25.2-27 (Kitada et al, 1998). The Parkin gene codes for a protein with ubiquitin-ligase activity, absent in the brain of patients with 6q-linked ARJP (Shimura et al, 1999 and 2000). The Parkin gene plays an important role in determining ARJP, as a wide range of independently occurring mutations has been detected in sporadic and autosomal recessive cases with early-onset parkinsonism (Hattori et al, 1998a and 1998b; Leroy et al, 1998b; Lücking et al, 1998; Abbas et al, 1999; Nisipeanu et al, 1999; Klein et al, 2000a; Lücking et al, 2000; Bonifati et al, 2001; Lücking et al, 2001; Periquet et al, 2001). As part of the work of this thesis, the author has mapped a second locus for ARJP (PARK6). A third locus (PARK7) has been subsequently identified in a Dutch ARJP

family (van Dujin et al, 2001), and the responsible gene (DJ-1) has been very recently cloned (Bonifati et al, 2003). Only two consanguineous families with DJ-1 mutations have been described so far, but a larger number of ARJP families and sporadic cases need to be tested in order to assess the relevance of DJ-1 in determining early-onset parkinsonism.

As briefly outlined above, the identification of loci and genes responsible for autosomal dominant PD and ARJP represents the most promising line of research to gain understanding into the pathogenetic mechanism of PD, and will be described in detail in the following paragraphs of this chapter. Other three lines of evidence have emerged in favour of a genetic predisposition to PD, and will be briefly summarised below: epidemiological surveys, twin data and candidate gene studies.

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 (Lazzarini et al, 1994; Payami et al, 1994; De Michele et al, 1996; Marder et al, 1996; Payami et al, 2002). 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; Payami et al, 2002). 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).

Twin studies

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 four of the MZ pairs were concordant from this sub-set, as compared to only two of twelve 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). 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). A recent study used 18F-dopa and positron emission tomography to evaluate dopaminergic function in twin pairs at baseline clinically discordant for PD. The concordance for subclinical dopaminergic dysfunction was found to be significantly higher in monozygotic than in dizygotic twins, suggesting a substantial role for inheritance in sporadic PD (Piccini et al, 1999). 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.

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 proteasomal degradation in PD. Therefore research has largely focused on association studies in candidate genes which might logically be involved in PD, such as the genes involved in dopamine synthesis and metabolism, cellular detoxification, oxidative stress and mitochondrial function, or genes mutated in familial Parkinson's disease. A huge number of such studies has been published over the last 10 years, of which the most significant are referenced here (Kurth et al, 1993; Tsuneoka et al, 1993; Hotamisligil et al, 1994; Plante-Bordeneuve et al, 1994a and b; Agundez et al, 1995; Bandmann et al, 1995a; Higuchi et al, 1995; Ho et al, 1995; Bandmann et al 1997a and b; Plante-Bordeneuve et al, 1997; Agundez et al, 1998; Riedl et al, 1998; Wood, 1998; Bon et al, 1999; Ho et al, 1999; Kruger et al, 1999; Maraganore et al, 1999; Mellick et al, 1999; Nicholl et al, 1999; Sabbagh et al, 1999; Satoh and Kuroda, 1999; Bandmann et al, 2000; Klein et al, 2000b; Mellick et al, 2000; Pastor et al, 2000; Zhang et al, 2000; Khan et al, 2001; Eerola et al, 2002; Skipper and Farrer, 2002; Momose et al, 2002; Ischiropoulos and Beckman, 2003; Gasser, 2003). The role of candidate genes in determining susceptibility for sporadic Parkinson's disease has been confirmed only for a limited number of genes, while for others only isolated reports are available. Not all the association studies can be considered reliable; a recent review by Tan and coworkers analysed the validity and variability of such association studies (Tan et al, 2000).

Seven candidate genes for proteins involved in cell protection were originally studied by genetic linkage in eight 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. The other three candidates examined were tyrosine hydroxylase (TH), brain-derived neurotrophic factor (BDNF) and amyloid precursor protein (APP). 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).

Another interesting approach was aimed at investigating possible candidate genes by direct DNA sequencing, particularly because most PD kindreds are too small for linkage analysis. In one study, sequencing of the SOD1 gene in 23 index cases of familial PD failed to reveal any mutations in the coding region (Bandmann et al, 1995b). 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 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 familial and sporadic cases of PD has so far failed to find any abnormal sequences (Bandmann et al, 1996b). Recently, two mutations in the NR4A2 gene were identified in ten out of 107 individuals with familial PD, but not in any individuals with sporadic PD or in unaffected controls. This gene encodes a member of nuclear receptor superfamily and has an essential role for the differentiation of dopaminergic neurons. The clinical picture in the 10 patients harbouring mutations in this gene was not distinguishable from typical PD. Both identified mutations resulted in a marked decrease of NR4A2 mRNA levels in transfected cell lines and in lymphocytes of affected individuals (Le et al, 2003).

However, the role of this gene in a larger cohort of familial and sporadic PD patients needs further evaluation.

Autosomal Dominant PD

Although several pedigrees had previously been described with parkinsonian features (Bell and Clark, 1926; Allan, 1937; Spellman, 1962), often there was no pathological data, and only more recently an increasing number of well-documented multi-generational parkinsonian kindreds have been reported with evidence of autosomal dominant inheritance and variable penetrance. However, only a few kindreds have been described where the clinical-pathological features are indistinguishable from the sporadic form of the disease with a late age of onset, good levodopa 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 (Muenter et al, 1998), or atypical parkinsonian features such as apathy, hypoventilation and scattered Lewy bodies (Perry et al, 1975 and 1990).

The recent identification of mendelian PD families 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 α-synuclein and Parkin in Lewy bodies – suggests that this is likely (Spillantini et al, 1997).

PARK1 (chromosome 4q21-q23) – alpha-synuclein

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 sixty affected members in four 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 SNCA gene, causing an alanine to threonine substitution (Ala53Thr) in the α-synuclein protein (Polymeropoulos et al, 1997).

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 ±13 years. In this family, the penetrance of the gene was estimated to be 85%. This mutation was also found in a few other apparently unrelated Greek kindreds. 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 (Polymeropoulos et al, 1997; Athanassiadou et al, 1999; Papadimitriou 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.

Mutation analysis of all five 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 the gene, causing the Ala30Pro substitution in the resulting protein. 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 (Krüger 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 families collected by the EU consortium (GSPD) in order to detect the numerical importance of the locus (Gasser et al, 1997). No mutations were detected in a large number of EU sibling pairs and a series of autosomal dominant PD families (Bennett and Nicholl, 1998; Farrer et al, 1998; Vaughan et al, 1998a and b).

The Synuclein family of proteins

Three distinct synucleins in human brain, α-, β- and γ-synuclein, have 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 that α-synuclein shares 95% sequence homology with rat synuclein (Maroteaux and Scheller, 1991; Campion et al, 1995), which is also expressed in the brain associated with synaptosomal membranes in neurons. A fragment of α-synuclein forms the non-β amyloid component (NAC) of amyloid plaques in Alzheimer's disease (Ueda et al, 1993) and secondary structure modelling predicted that this peptide has a strong tendency to form β–structures consistent with its association with amyloid. Campion and colleagues (1995) mapped the α-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's disease were sequenced, but no mutations were found (Higuchi et al, 1998). However, three 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 and co-workers (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 α-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 α–synuclein in the zebra finch, may play a role in song learning, but the function of mammalian synucleins remains unclear. Alpha-synuclein binds to phospholipids present in the cell membrane and can inhibit phopholipase D2, which plays a role in lipid membrane turnover (McLean et al, 2000). One hypothesis proposed that α–synuclein is involved in the turnover of pre-synaptic membranes and synaptic signalling, processes important in learning and memory (Clayton and George, 1998 and 1999). No mutations in β- or γ- synuclein have been found in PD subjects to date (Lavedan et al, 1998; Lincoln et al, 1999a).

Alpha Synuclein, the Lewy body and neurodegenerative disease

Despite the rarity of SNCA mutations in familial PD, the protein product, α–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 α–synuclein in PD and related disorders such as diffuse Lewy body disease and dementia with Lewy bodies has since been recognized, as α-synuclein protein is a major fibrillar component of Lewy bodies and Lewy neurites found in PD, Lewy Body Dementia and some cases of Alzheimer's disease (Spillantini et al, 1997) and the glial cell inclusion bodies (GCIs) of Multiple System Atrophy (Tu et al, 1998). These disorders have therefore been called "synucleinopathies" (Heintz and Zoghbi, 1997; Duda et al, 2000). Lewy bodies are roughly spherical structures comprising radially arrayed intraneuronal aggregations of at least twenty 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 (Lowe et al, 1988; Spillantini et al, 1997). Indeed, immunostaining for α–synuclein has now become diagnostic for Lewy bodies. Functional in vitro studies with wild type and mutant α-synuclein over-expressed in cultured cells have demonstrated that mutant Ala53Thr α-synuclein has a higher tendency to assume a β-sheet secondary structure and to form intracellular aggregates highly similar to the filaments contained within Lewy Bodies, while Ala30Pro α-synuclein is less efficient in binding synaptic vesicles (Conway et al, 1998; Giasson et al, 1999a). Mutant α-synuclein has also been shown to induce mitochondrial depolarisation, to facilitate the activation of the apoptotic cascade and to reduce proteasomal activity (El-Agnaf et al, 1998; Bennett et al, 1999; Narhi et al, 1999; Conway et al, 2000; Ancolio et al, 2000). The interaction of mutant α-synuclein with membrane phospholipids and brain vesicles is disrupted and there is increase susceptibility to dopamine toxicity (Jensen et al, 1998; Perrin et al, 2000; Tabrizi et al, 2000). The mechanism leading to α-synuclein aggregation and the selectivity of the damage to dopaminergic neurons of the substantia nigra have not yet been completely understood. The transformation of α-synuclein from a soluble state to mass aggregated fibrils involves an increase in β-sheet content and progressive oligomerisation. Transient small units of oligomers of β-folded proteins are known as protofibrils (Giasson et al, 1999a). Dopamine metabolism produces high quantities of free radicals and facilitates heavy metals deposition within neurons. Free radicals have been shown to facilitate the aggregation of human α-synuclein in vitro (Giasson et al, 1999b; Hashimoto et al, 1999). Moreover, recent work has demonstrated that α-

synuclein and cytosolic dopamine interact to form adducts that stabilize protofibrils, presumably toxic intermediates of fibril formation. Catecholamines are readily oxidized in the presence of iron to highly reactive metabolites, such as DA-quinone (DAQ), that covalently binds proteins. Conway and coworkers have recently isolated DAQ-α-synuclein adducts and have demonstrated that these adducts inhibit the conversion of protofibrils to fibrils, causing accumulation of α-synuclein protofibrils. In other words DAQ may actually inhibit Lewy bodies formation by binding α-synuclein, and have a neurotoxic effect on the dopaminergic neuron. A number of cellular mechanisms keep dopamine and its metabolites from associating with α-synuclein and causing toxicity. Several of these focus on maintaining cellular DAQ levels below a certain threshold, such as uptake into synaptic vesicles, monoamino-oxidase activity, glutathione. If, despite these mechanisms, DAQ remains and succeeds in binding α-synuclein, the ubiquitin-proteasome pathway (of which Parkin is an important element – see later in this chapter) can degrade the damaged proteins. In case this mechanism is also insufficient, fibrillation and Lewy bodies formation might be a last resort for removing DAQ-damaged α-synuclein. If this is true, the Lewy body can be seen as a desperate attempt by the neuron to save itself by sequestering toxic protofibrils. The role of cytosolic catecholamines in protofibril stabilization could explain why most PD cell death occurs in dopaminergic neurons of the substantia nigra and locus coeruleus (Conway et al, 2001; Sulzer, 2001).

Several transgenic mice and Drosophila models have been generated, which express the wild-type or mutated α-synuclein. Transgenic mice over-expressing human α-synuclein demonstrate a number of features of PD, including loss of nigrostriatal dopaminergic terminals in the striatum, development of α-synuclein and ubiquitin-positive cytoplasmic inclusions, and motor impairments (Abeliovich et al, 2000; Masliah et al, 2000). The inclusions observed in these animals lacked fibrillar organization, which is characteristic of the Lewy bodies observed in PD. Some inclusions were also present in the nucleus, a feature not seen in PD. Dopaminergic and behavioural defects were only observed in the high expressing line of transgenic mice, suggesting that a critical threshold of α-synuclein may be required for these defects. Other transgenic mice had α-synuclein-positive inclusions and motor deficit, but there was no evidence of nigrostriatal dopaminergic degeneration. In these mice, neurons of the brainstem and motor neurons were most vulnerable. Mice expressing mutated forms of α-synuclein developed a severe and complex motor impairment leading to paralysis and death. These animals developed age-dependent intracytoplasmic neuronal α-

synuclein inclusions paralleling disease onset, and the inclusions were similar to the human counterpart. Immunoelectron microscopy revealed that the inclusions contained 10-16 nm wide fibrils similar to human pathologic inclusions (van der Putten et al, 2000; Giasson et al, 2002). Another interesting PD animal model is the Drosophila expressing normal and mutated α-synuclein. These flies demonstrate many features of PD including dopaminergic cell loss, filamentous intraneuronal inclusions and motor defects (Feany and Bender, 2000). Because of the well characterised Drosophila genetics and the short lifespan of flies, this model offers a valuable opportunity to uncover novel proteins involved in PD pathogenesis, for example suppressor genes that prevent dopaminergic degeneration or susceptibility genes that exacerbate the effects of α-synuclein expression.

Animal models based on the transgenic expression of wild-type and mutated α-synuclein provide an important opportunity to study the involvement of α-synuclein in PD pathogenesis. However, there are some limitations to the use of transgenic α-synuclein models. Not all transgenic mouse models demonstrate key features of PD, such as nigrostriatal dopaminergic degeneration. Furthermore, it should be kept in mind that one of the mutations associated with PD in humans is the wild type sequence in mice (Trojanowski and Lee, 1999). Despite these limitations, transgenic α-synuclein mice provide an excellent model system for studying the formation of α-synuclein-positive protein aggregates. This issue warrants detailed investigation since protein aggregation is associated with a number of neurodegenerative disorders. Additionally, these mice provide accurate models for examining the interplay between genetic mutations and environmental exposures in the aetiology of PD (Dawson, 2000).

PARK5 (chromosome 4p14) – Ubiquitin C-terminal hydrolase 1

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 hydrolyse 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 tumours (Doran et al, 1983; Solano et al,

2000), and is selectively present in ubiquitinated inclusion bodies characteristic of human neurodegenerative disease (Lowe et al, 1990). 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 et al, 1990). 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* hybridisation, the assignment was regionalised to 4p14. Interestingly, an Ile93Met missense mutation in the 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, 1998a). The reduced enzymatic activity may impair the ubiquitin-proteasome degradation machinery through at least two mechanisms: a) the reduced availability of free ubiquitin monomers can lead to a deficient ubiquitination of substrate proteins which cannot therefore be degraded efficiently through the proteasome; b) the free polyubiquitin chains can compete with ubiquitinated substrates to enter the proteasome, therefore leading to a reduced degradation rate of abnormal or toxic proteins. Also, in the gracile axonal dystrophy (gad) mouse, an autosomal recessive mutant showing motor ataxia, "dying back" type axonal degenerations and formation of spheroid bodies in nerve terminals, the pathogenetic mutation is an in-frame deletion including exons 7 and 8 of UCH-L1. These mice show accumulation of amyloid β-protein and ubiquitin-positive deposits along the sensory and motor nervous system (Saigoh et al, 1999). These data support the hypothesis that altered function of the ubiquitin system directly causes neurodegeneration. However, a mutation screening of a large cohort of PD families from the EU GSPD Consortium failed to show any mutation within the UCH-L1 gene (Harhangi et al, 1999; Lincoln et al, 1999b; Wintermeyer et al, 2000). Therefore, the role of this gene in determining autosomal dominant PD is still unclear and needs to be confirmed in other PD families.

PARK3 (chromosome 2p13)

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, 1998b). Dementia was prominent in addition to parkinsonism in two of the kindreds. Autopsy data from three 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 40 cM

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 seven 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 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, 1999c). The disease gene at this locus has yet to be identified, and sequencing of several biologically plausible candidate genes in the area (TGF-α, sideroflexin 5 and others) did not reveal any mutations (West et al, 2001; Lockhart et al, 2002). Further parkinsonian kindreds which show linkage to 2p13 have yet to be reported. The studies on PARK3 highlight 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 and 2p13 (Gasser et al, 1997).

PARK4 (chromosome 4p15)

Farrer and colleagues identified an 8.7 cM 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, 1999). This kindred had previously been reported in detail (Muenter et al, 1998). Affected members of the family share many features with typical 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 (Gwinn-Hardy et al, 2000). 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 typical PD 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, 1999). More recently identified members of this kindred have allowed reanalysis at this locus such that the multipoint LOD score is now above 3.0 (Dr M. Farrer, personal communication). Identification of additional families linked to 4p15-linked families would provide statistical support for the Iowan haplotype and help refine the linked region and select candidate genes.

PARK8 (chromosome 12p11-q13)

This locus has been recently mapped by means of a genome-wide search in a large Japanese family (the Sagamihara family) with autosomal dominant parkinsonism. The clinical description of the family had already been published in detail (Hasegawa and Kowa, 1997). The onset the disease is earlier than idiopathic PD (mean ± SD = 51 ± 6 years) and neuropathological examination in four cases revealed a pure nigral degeneration and absence of Lewy bodies; however, the clearly autosomal dominant transmission distinguishes this family from families with ARJP (see below). The highest LOD score (Z = 4.32) was obtained at marker D12S345. The observation of recombination events in affected individuals placed the disease locus in a genetic interval of 13.6 cM on the pericentromeric region of chromosome 12 (Funayama et al, 2002). The detection of the disease haplotype also in eight unaffected family members suggests a low penetrance of the underlying gene. Several candidate genes have been localised within the linked interval, but the causative gene has not been identified yet.

Autosomal Recessive Juvenile Parkinsonism

PARK2 (chromosome 6q25-q27) – Parkin

Autosomal recessive juvenile parkinsonism (ARJP) is one of the monogenic forms of Parkinson's disease initially described in Japan. ARJP 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 levodopa induced dyskinesias. The clinical features of 17 patients from 12 Japanese families with ARJP 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 along with classic parkinsonism.

Symptoms of tremor, rigidity, and bradykinesia were mild. Patients responded well to levodopa therapy, but levodopa-induced dyskinesias and wearing-off phenomena occurred frequently. A distinguishing feature of the phenotype was sleep benefit, with reduction of parkinsonian symptoms after awakening. ARJP is pathologically characterised by highly selective degeneration of dopaminergic neurons in the pars compacta of the substantia nigra and the absence of Lewy bodies (Takahashi et al, 1994).

By linkage analysis using a diallelic polymorphism of the manganese superoxide dismutase gene (SOD2), segregation of the disease was found at the SOD2 locus (Matsumine et al, 1997). By extending the linkage analysis to thirteen ARJP families, they obtained 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. This locus was termed PARK2. Linkage analysis was then performed on further families to narrow the region prior to positional cloning to identify the responsible gene.

Non-Japanese PARK2 families were first demonstrated in Europe, the United States and the Middle East. Homozygous deletions in three ARJP families greatly reduced the initial 17 cM candidate interval (Matsumine et al, 1998; Tassin et al, 1998; Jones et al, 1998). Linkage of ARJP to 6q25.2-q27 was described in one Algerian and ten 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, two of the three cardinal signs of PD (akinesia, rigidity, and tremor) were found. Marked improvement with levodopa treatment occurred in all except 2 untreated secondary cases found in family studies. Age at onset was less than 40 years for at least one affected sib. Linkage to 6q25.2-q27 was also found in a group of fifteen families from four 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 ARJP locus to a 6.9 cM region and excluded SOD2 (Jones et al, 1998).

The gene responsible for ARJP (Parkin) was isolated by positional cloning techniques in a Japanese patient with deletion of 6q, including the closely linked marker D6S305 (Kitada et al, 1998). 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 ARJP patients from three unrelated families had a deletion affecting exon 4 alone (Kitada et al, 1998). Alternative splicing of these exons produces different Parkin transcripts in different tissues (Sunada et al, 1998). The Parkin protein in brains of control individuals is widely expressed, mainly in the pigmented neurons of the substantia nigra and locus coeruleus. Neuropathological studies in brains of patients with sporadic late onset PD have shown that Parkin is a major component of the Lewy bodies, where it is detected in association with α-synuclein, ubiquitin and several other proteins. Conversely, the protein is absent in brains of patients with ARJP and mutations in the Parkin gene (Hayashi et al, 2000). The Parkin protein is composed of 465 amino acids with a moderate similarity to ubiquitin at the amino terminus and two RING-finger motifs at the carboxy-terminus (Kitada et al, 1998) flanking an IBR (In Between Ring) region. These domains are typical of proteins which act as ubiquitin-ligase. Functional in vitro studies have indeed demonstrated that Parkin acts as a ubiquitin-ligase (E3), and represents a crucial protein in the ubiquitin-proteasome degradation pathway (Shimura et al, 2000). This pathway is of fundamental importance for cell survival as it is deputed to degrade abnormal misfolded proteins, which tend to precipitate within the endoplasmic reticulum leading to cell toxicity (endoplasmic reticulum stress). In order to be degraded by the proteasome, abnormal proteins need to be tagged with a polyubiquitin chain, which is the signal recognised by the proteasome to internalise and degrade proteins. The adding of a polyubiquitin chain to a protein is obtained by the concurrent action of three different enzymes: a) the ubiquitin-activating enzyme (E1); b) the ubiquitin-conjugating enzyme (E2); c) the ubiquitin-ligase (E3). Proteins tagged with a polyubiquitin chain are incorporated within the 26S proteasome and degraded. Free polyubiquitin chains are therefore degraded by ubiquitin-hydrolase enzymes (such as UBH-L1 – see paragraph "Autosomal dominant PD) and ubiquitin molecules can be recycled. Each cell has its own ubiquitin-proteasome degradation machinery, and the specificity of substrates recognised by this system is given by the E3 enzyme. The correct functioning of the ubiquitin-proteasome machinery is crucial, and each step of the process is regulated within the cell by several specific factors. An increase of misfolded proteins is recognised by the cell and triggers a signal to the nucleus, enhancing the transcription of genes involved in the ubiquitin pathway. The mechanism of selective neural cell death still remains to be clarified. The absence of Parkin protein in brains of patients with ARJP demonstrates a main disruption of the ubiquitin-proteasome machinery; the consequent accumulation of abnormal proteins

which cannot be ubiquitinated and degraded can lead to endoplasmic reticulum stress and selective neuronal degeneration. This can also justify the absence of Lewy bodies, which are mainly composed of aggregates of ubiquitinated proteins (Nussbaum, 1998; Giasson and Lee, 2001). However, Lewy bodies are not always absent in the brain of patients with parkin mutations, and a kindred has been reported with typical Lewy body pathology in the brain of patients with early onset parkinsonism and parkin mutations (Chen et al, 2000; Farrer et al, 2001).

A number of functional studies have been undertaken to identify the substrates of Parkin. Several proteins have been identified (a form of glycosilated α-synuclein, synphilin-1, three proteins of unknown function called CDCrel-1, PAEL-receptor and CHIP) but these studies have not been replicated so far and their full relevance remains to be established (Zhang et al, 2000; Chung et al, 2001; Imai et al, 2001; Shimura et al, 2001; Imai et al, 2002). However, the role of parkin in the ubiquitination of synphilin-1 is particularly intriguing. It has been demonstrated that synphilin-1 is a component of Lewy bodies and interact with α-synuclein promoting the formation of protein aggregates (Engelender et al, 1999; Wakabayashi et al, 2000). Co-expression studies of parkin, α-synuclein and synphilin-1 within cultured cells resulted in the formation of Lewy body-like ubiquitin-positive cytosolic inclusions (Chung et al, 2001). Thus, parkin may be important for the formation of Lewy bodies and it can be said that parkin and α-synuclein are linked in a common pathogenetic mechanism through their interaction with synphilin-1.

Detailed mutational analyses have now clearly shown that Parkin is numerically far more important than other genes so far described in PD. Several different mutations (comprising homozygous and compound heterozygous missense mutations, exon deletions and multiplications) have been detected in ARJP patients worldwide (Hattori et al, 1998a and 1998b; Leroy et al, 1998b; Lücking et al, 1998; Abbas et al, 1999; Nisipeanu et al, 1999; Klein et al, 2000a; Lücking et al, 2000; Maruyama et al, 2000; Bonifati et al, 2001; Hedrich et al, 2001; Lücking et al, 2001; Periquet et al, 2001; Ujike et al, 2001; van de Warrenburg et al, 2001; West et al, 2002). The systematic screening of ARJP patients has also evidenced pseudo-dominant inheritance in some families, with more than two mutations segregating in some affected individuals (Hedrich et al, 2001; West et al, 2002).

The largest European study on ARJP families (mainly affected sib-pairs with age of onset < 45 years in at least one affected sib) and sporadic early-onset PD cases,

carried on by the EU GSPD Consortium, found mutations in the Parkin gene in about half of the familial cases (37 out of 73) but only in 18 out of 100 sporadic patients, most of them with a very early onset (below 20 years of age) (Lücking et al, 2000). Phenotype-genotype correlations in Parkin-positive families demonstrated that the typical ARJP features, such as dystonia and hyperreflexia, were more frequent in Parkin-positive than in Parkin-negative patients. However, none of these features could specifically distinguish between the two groups. The age of onset in the Parkin-positive group was on average lower than in the Parkin-negative group, but mutations were identified in patients with age of onset up to 64 years and clinical features difficult to distinguish from PD, making genotype-phenotype correlation uncertain.

^{18}F-Dopa and ^{11}C-raclopride PET studies in patients with ARJP and parkin mutations showed a profound decrease of ^{18}F-Dopa uptake, both in the putamen and in the caudate nucleus, and of ^{11}C-raclopride striatal binding index. These data evidence that parkinsonism in Parkin-positive patients is associated with presynaptic dopaminergic dysfunction (similar to idiopathic PD but involving dopaminergic projections to both caudate and putamen), along with alterations at the postsynaptic D2 receptor level. A longitudinal PET study of idiopathic PD and parkin-positive ARJP patients, repeated after 5-10 years, demonstrated that disease progression is much slower in parkin patients than in idiopathic PD. In asymptomatic carriers of one parkin mutation, a significant striatal dopaminergic dysfunction is also present, but with no progression over a 7-years period (Broussolle et al, 2000; Hilker et al, 2001; Khan et al, 2002; Wu et al, 2002).

PARK7 (chromosome 1p36)

The project described in this chapter concerns the mapping of the second locus for autosomal recessive juvenile parkinsonism (PARK6) in an Italian family, followed by confirmation of linkage in other European ARJP families and genotype-phenotype correlates. More recently, a third ARJP locus (PARK7) has been placed by homozygosity mapping to the short arm of chromosome 1, 25 cM telomeric to PARK6, in a large consanguineous family originating from a genetically isolated community in the southwestern region of the Netherlands (van Duijn et al, 2001). Four individuals in the same generation received a diagnosis of ARJP. The age of onset ranged from 27 to 40 years; none of the patients exhibited atypical features or signs of involvement of additional neurological systems. The parkinsonian symptoms responded well to therapy

with levodopa or dopamine agonists in two patients, the other two had a very mild phenotype and did not require any antiparkinsonian treatment at the time of latest examination. Off-dystonia, levodopa- induced dyskinesias and fluctuations were observed. Tendon reflexes in lower limbs were brisk in three of the patients, while Babinski sign was always absent. Two of the patients showed neurotic signs, and one had suffered from psychotic episodes. The disease progression was slow. A brain PET study performed in two patients with dopamine-transporter tracer showed severe abnormalities consistent with presynaptic dysfunction of nigrostriatal dopaminergic systems. The high rate of consanguinity and the identification of a common ancestor in the family strongly suggested an autosomal recessive mode of inheritance. The PARK7 locus spans a 16 cM region on chromosome 1p36 and is clearly distinct from PARK6. Linkage analysis in the Dutch family with markers spanning the PARK6 interval produced negative LOD scores throughout the region. Among four autosomal recessive early-onset families analysed, two supported linkage to PARK7, one with conclusive evidence.

Very recently, the underlying gene has been identified through a systematic screening of all transcripts present in the PARK7 region, using an RT-PCR approach. The cDNA corresponding to the whole DJ-1 gene open reading frame could not be amplified in one patient from the original Dutch family, while it was normally amplified from a control sample. Amplification of the $1^{A/B}$ to 5 exons from genomic DNA yielded no product in the same patient, whereas the two more centromeric exons 6 and 7 were normally amplified. This deletion involved only the DJ-1 and showed complete co-segregation with the disease allele in the Dutch family, whereas it was absent in a very large control sample from the general Dutch population and from the genetic isolate where the family came from. Mutation screening of the DJ-1 gene in the Italian consanguineous family positively linked to PARK7 revealed a missense mutation (T497C), resulting in the substitution of a highly conserved leucine at position 166 by a proline. This change showed complete co-segregation with the disease allele in the Italian family and was absent in 320 control chromosomes from the general Italian population. No other mutation were identified in the other small families consistent for linkage to PARK7 (Bonifati et al, 2003).

The DJ-1 gene contains 8 exons, of which the first two (exons 1^{A} and 1^{B}) are non-coding and alternatively spliced in DJ-1 mRNA. One major transcript of about 1Kb contains a 570-bp ORF encoding a protein of 189 aminoacids ubiquitously expressed in

body tissues and brain areas, including those more affected in PD. DJ-1 is a member of the ThiJ/PfpI family, which includes ThiJ, a protein involved in thiamine biosynthesis in prokaryotes; PfpI and other bacterial proteases; araC and other bacterial transcription factors; and the glutamine amidotransferases family. Transfection of wild type DJ1 in COS and PC12 cells resulted in diffuse cytoplasmic and nuclear DJ-1 immunoreactivity, while transfection of the mutant form showed a similar uniform nuclear staining, while the cytoplasmic staining appeared mostly co-localised with mitochondria. These data suggest that the loss of cytoplasmic activity of DJ-1 is pathogenic, or that the nuclear activity is affected by the mutation even if the protein retains its translocation capability. The absence of DJ1 protein (as in the Dutch family) or its functional disruption (as in the Italian family) leads to early onset autosomal recessive parkinsonism (Bonifati et al, 2003).

The function of DJ-1 is still unknown. It has been shown that DJ-1 binds to PIAS proteins, a family of SUMO-1 ligases that modulate the activity of various transcription factors. Human DJ-1 was first identified as an oncogene, and later as a regulatory subunit of an RNA-binding protein (RBP) and as a hydroperoxide-responsive protein, which is converted into a variant in response to oxidative stimuli, suggesting a possible function as an antioxidant protein. It is possible that DJ-1 participates in the oxidative stress response by buffering cytosolic redox changes and by modulating gene expression at transcriptional and post-transcriptional levels (Bonifati et al, 2003).

Aims of the study

The aims of this part of the study were: a) to map a novel locus in a large consanguineous ARJP family from Sicily; b) to assess its relevance in determining the ARJP phenotype in Europe; c) to draw genotype-phenotype correlations.

Patients

The "Marsala" kindred

Clinical ascertainment of the family

A large family from Marsala (Sicily, Italy) comprising one hundred and twenty-two members was investigated. The author, together with Dr AR Bentivoglio, personally examined thirty-five subjects, including thirty family members and five spouses. After

obtaining informed consent, venous blood samples were taken from twenty-seven members and five spouses for DNA analysis. All the examined subjects provided detailed information on the following: birth, growth and development, education, use of well-water, medications, drug misuse, exposure to toxins or chemicals (particularly regarding organophosphorous compounds and other substances used in agriculture), previous illnesses, previous hospitalisations, any neurological or psychiatric disease, head or body injuries. The levodopa equivalent daily dose was calculated in order to have comparable data on drug therapy. All participating family members had a complete on-site neurological examination that included the UPDRS motor scale in the *on* condition (and, whenever possible, in the *off* condition), the mini-mental state examination and tasks aimed to disclose dystonia or paroxysmal dyskinesias. Each subject was videotaped during the assessment. The diagnosis of parkinsonian syndrome was made when at least two of the following were present: resting tremor (4-6 Hz), bradykinesia or akinesia (with fatiguing), rigidity. A simplified family tree is shown in figure 5.1. To avoid presenting this pedigree twice and for clarity in result presentation, this figure has been placed in the "Results" section of this chapter (page 165).

The analysis of genealogic records from the local church and town hall permitted to trace the origin of the family to a common ancestral couple seven generations back (XVIII century). All affected individuals descended, both on the maternal and the paternal side, from subjects I:1 and I:2, who lived in the late XIX century. Their descendants remained in the same region and frequently had consanguineous marriages in subsequent generations. The high rate of inbreeding and the occurrence of affected men and women within one generation suggest an autosomal recessive mode of inheritance. All family members were farmers, who denied the use of pesticides. Four subjects received a diagnosis of early-onset parkinsonism. Their mean age at disease onset was 40.8 years (± 7.2); at the time of the study their average disease duration was 16.5 years (± 6.4) and they were aged 59.8 years (± 13.5). The clinical features of the four affected individuals are illustrated in table 5.2.

Table 5.1 – phenotype of the "Marsala" kindred

Affected family members (sex)	VI:7 (M)	VI:9 (M)	VI:17 (M)	VI:23 (F)
Age at onset	45	48	32	38
Asymmetry at onset	Yes	No	Yes	Yes
Disease duration (years)	29	20	20	7
Levodopa-induced dyskinesias	Moderate	Moderate	Severe	Mild
Phenotype at examination:				
Resting tremor	+	±	+	+
Bradykinesia	+	+	+	+
Rigidity	+	+	+	+
Postural instability	+	+	+	-
Hoehn & Yahr stage (on-state)	II.5	II.5	III	II
Daily medication:				
Levodopa	300 mg	625 mg	800 mg	500 mg
Pergolide	4 mg		3 mg	

The European ARJP families

Twenty-eight families with early-onset parkinsonism were studied. Twenty-five had been collected by the EU GSPD Consortium, while three other families were selected from the Movement Disorder Database of the Catholic University in Rome. All families were negative for mutations in the Parkin gene. All patients fulfilled the UK Brain Bank criteria, and in addition the following criteria for ARJP were met: i) inheritance compatible with autosomal recessive transmission; ii) age of onset of 45 years or younger in at least one affected member in the family. Out of twenty-eight families, eight were British, six were Dutch, six were Italian, five were German and three were French. There were at least two affected sibs in each nuclear family. Blood samples were available from sixty-two affected individuals and forty-eight clinically unaffected first-degree relatives.

DNA analysis

Homozygosity mapping in the Marsala kindred

Linkage to PARK2 had been previously excluded by genotyping four markers spanning the PARK2 region and by mutation analysis of the Parkin gene in the proband. A simulation study performed with the program SLINK revealed a maximum expected

LOD score of 4.2 at recombination fraction (θ) = 0.00. The family was then considered suitable to perform a genome-wide homozygosity mapping (Lander and Botstein, 1987). In the first screen, only the four definitely affected individuals were genotyped. The author analysed 400 highly polymorphic fluorescent microsatellite markers spanning the 22 autosomes (as described in chapter 2). Two-point LOD scores were generated using the FASTLINK version of the MLINK program using an assumption of equal male-female recombination rate, autosomal recessive inheritance, a gene frequency of 0.001 and equal allele frequencies for each marker. Loops were broken by using the automated loop breaking procedure described by Becker and coworkers (1998). Marker order and genetic distances were based on the Marshfield chromosome 1 genetic map and on the NCBI chromosome 1 physical map.

Linkage analysis in other ARJP families

All affected individuals from the twenty-eight families were genotyped for eleven microsatellite markers spanning the PARK6 region on chromosome 1p35-p36, as identified in the Marsala kindred (see "Results" below). Haplotypes were manually constructed. In families where the affected individuals shared identical haplotypes within the PARK6 region, all available unaffected family members were genotyped and haplotypes were phased based on the minimum number of recombinants. Pairwise LOD scores were obtained as for the Marsala kindred. Cumulative multipoint LOD scores were generated by use of the SIMWALK2 program. Pairwise and multipoint LOD scores were calculated in all families using affected individuals only. In the eight families consistent for linkage with PARK6, LOD scores were recalculated adding the available unaffected family members.

Results

Identification of a novel ARJP locus (PARK6) in the Marsala kindred

Given the autosomal recessive pattern of inheritance, we expected homozygosity at the level of the disease locus in affected individuals due to identity by descent from a common progenitor. Out of four hundred microsatellite markers, the four definitely affected family members were homozygous for the same allele at seven marker loci, located on chromosomes 1, 8, 10, 14 and 19. The regions surrounding these seven loci and all regions surrounding non-informative markers were saturated with closely spaced microsatellite markers; all available family members were genotyped and

haplotypes were constructed. A combination of negative LOD scores and the detection of different haplotypes carried by the affected individuals allowed exclusion of all the autosomes except a region on the short arm of chromosome 1. All markers spanning this candidate interval produced positive LOD scores, with a maximum LOD score of 4.01 (θ = 0.00) between the disease and marker D1S199 (appendix 6a).

Calculation of pairwise LOD scores assuming reduced penetrance values and under the assumption "affected individuals only" did not result in a significant change. All affected individuals in the family shared a region of homozygosity between D1S199 and D1S2885 (figure 5.1), allowing the identification of a 12.5 cM interval containing a novel gene for autosomal recessive parkinsonism (PARK6). The upper and lower extents of the region are determined by recombinations detected in subjects VI:7, VI:9 and VI:17 between markers D1S483 and D1S199 (telomeric end) and between markers D1S2885 and D1S247 (centromeric end) respectively.

Linkage analysis in the European ARJP families

Linkage to PARK6 was first tested in the sixty-two affected members of the twenty-eight families. The eleven microsatellite markers spanning the PARK6 region were ordered as follows: *tel* – D1S483 – *0cM* – D1S199 – *3.2cM* – D1S2732 – *0cM* – D1S2828 – *0cM* – D1S478 – *0.6cM* – D1S2702 – *3.6cM* – D1S2734 – *0cM* – D1S2698 – *1.6cM* – D1S2674 – *0.8cM* – D1S2885 – *2.7cM* – D1S247 – *cen*. Eight families generated positive LOD scores at all recombination fractions, and haplotype construction showed identical haplotypes shared by the affected members of each family. The other families generated negative LOD scores at all recombination fractions and affected individuals had different haplotypes across the entire 12.5 cM region; these were therefore excluded from further analysis. In the eight families consistent with linkage to PARK6, the eleven markers were genotyped in all available unaffected family members. Figure 5.2 shows simplified pedigrees of these families and haplotypes for the markers spanning the PARK6 locus. Linkage analysis including unaffected family members gave a cumulative maximum pairwise LOD score of 5.39 for marker D1S478. Pairwise LOD scores for all markers covering the PARK6 interval are given in appendix 6b.

Figure 5.1 – pedigree of the Marsala kindred and haplotypes spanning the PARK6 locus

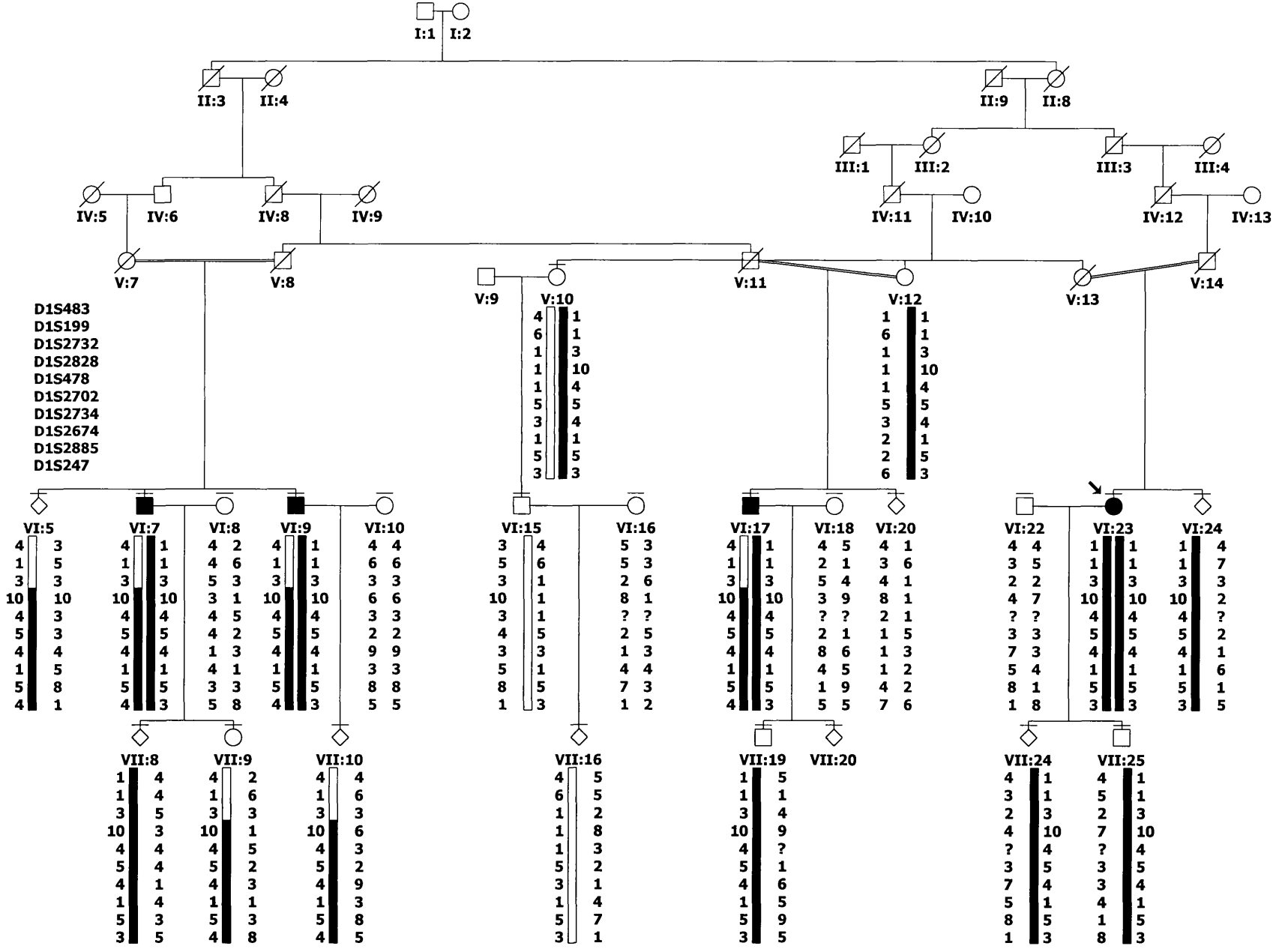
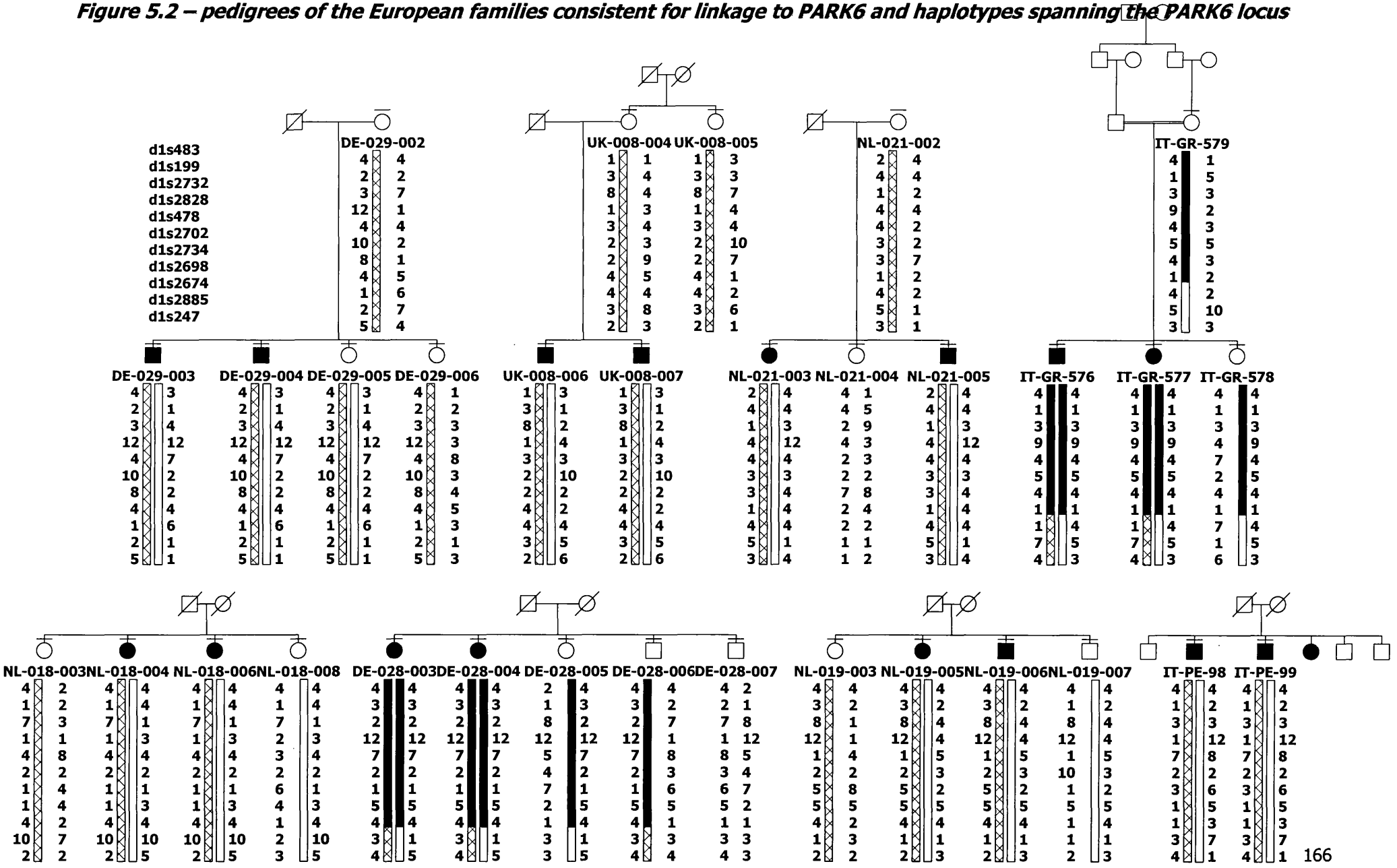
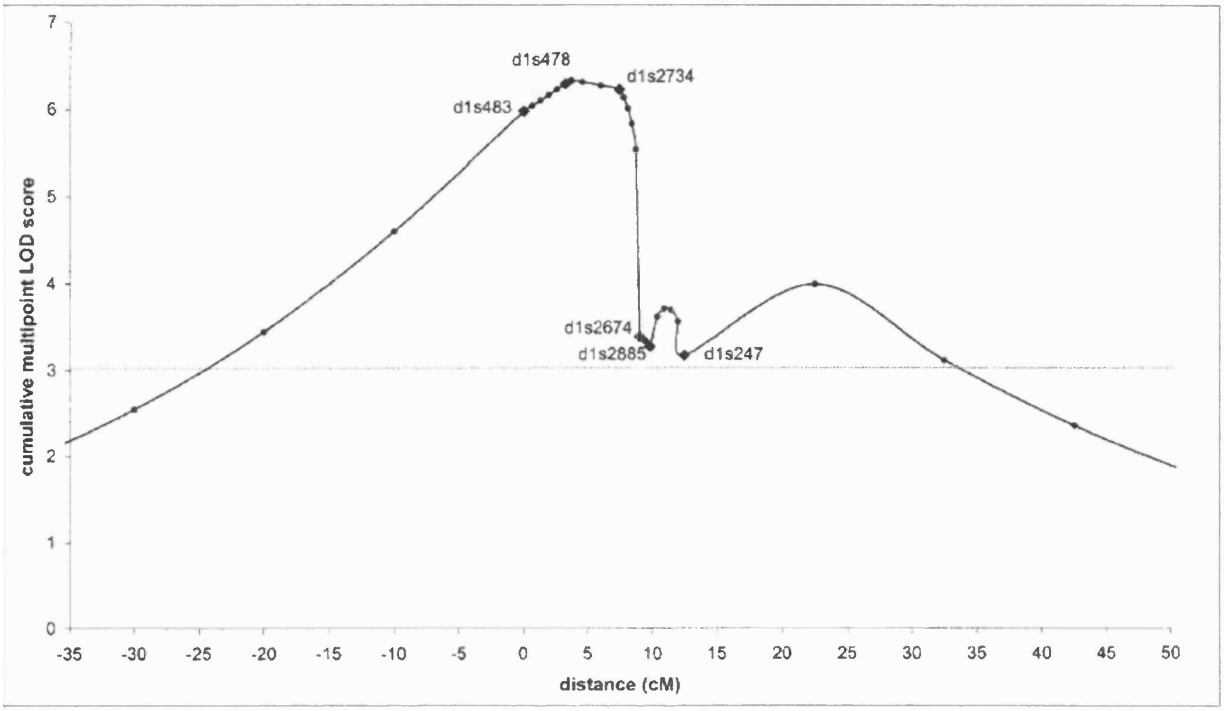


Figure 5.2 – pedigrees of the European families consistent for linkage to PARK6 and haplotypes spanning the PARK6 locus



The maximum cumulative multipoint LOD score obtained on the eight families using six markers (D1S483 – D1S478 –D1S2734 – D1S2674 – D1S2885 – D1S247) was 6.29 at locus D1S478 (figure 5.3).

Figure 5.3 – cumulative multipoint linkage analysis at the PARK6 locus in 8 European families



No recombination events were detected at the telomeric end of the region, which is still defined by the flanking marker D1S483 by a recombination between markers D1S483 and D1S199 identified in the Marsala kindred. Families DE-028 and IT-GR showed homozygosity across the entire region, except for markers D1S2885 and D1S247 (family DE-028) and for markers D1S2674, D1S2885 and D1S247 (family IT-GR). This was of great interest especially in the latter family (IT-GR), where the parents of the two affected siblings were first-degree cousins, indicating homozygosity by descent in the affected individuals. The lack of homozygosity at D1S2674 and at more centromeric markers in this family suggested a recombination event between this marker and D1S2698. This was confirmed by the negative LOD scores obtained for family IT-GR only at loci D1S2674, D1S2885 and D1S247 (at $\theta = 0.00$: –1.10, –1.11 and –1.40 respectively) and allowed the candidate interval to be reduced to a 9 cM region between flanking markers D1S483 and D1S2674. Each family showed a

different haplotype, excluding a common founder effect and suggesting independent mutational events (figure 5.2).

Genotype-phenotype correlations in the European ARJP families

Eight of twenty-eight families tested were consistent with linkage to PARK6. Of these families, three were Dutch, two were German, two were from Italy and one was from the UK. Consanguinity between parents of affected sibs was detected only in one family from central Italy (IT-GR). Affected individuals were observed in only one generation in all families. Mean age of onset was 42.1 ± 9.4 years in the sixteen affected individuals, and at least one of the two affected sibs had an onset before age of 45 years. However, four affected individuals (from family UK-008 and from the three Dutch families) had an age of onset above 45 years, with the latest onset at 68 years in individual NL-018-004. Clinical features were indistinguishable from Parkin-positive ARJP especially of non-Japanese origin, being characterised in all patients by a mild to moderate parkinsonian syndrome with good response to levodopa and slow progression (mean disease duration = 12 ± 6.9 years). Levodopa-induced dyskinesias occurred in eight out of sixteen patients (50%). Dystonia at onset and sleep benefit, mainly reported in Japanese ARJP subjects, were not detected in any patient. Hyperreflexia was observed only in two of the sixteen patients.

Twenty of twenty-eight families did not show evidence of linkage to the PARK6 locus. The mean age of onset in the forty-six affected individuals was 46.4 ± 9.1 years. Eighteen of these individuals from sixteen families had an age of onset above 45 years. This figure is slightly higher compared to that in PARK6-linked families. Clinical presentation, progression of disease, response to levodopa treatment and occurrence of distinct features, including levodopa-induced dyskinesias and hyperreflexia, were similar to those in PARK6-linked families, preventing any attempt to sub-classify the two groups of patients on clinical ground alone. These families were not tested for linkage to PARK7, as this locus had not been identified when the work of this thesis was performed.

Discussion

As a part of this thesis, the author has used homozygosity mapping in a large family from Sicily (the Marsala kindred) to identify a second locus for autosomal recessive early onset parkinsonism, named PARK6, on the short arm of chromosome 1. The

phenotype in this family was characterized by early onset parkinsonism, with slow progression, sustained response to levodopa and frequent levodopa-induced fluctuations and dyskinesias. Other signs often reported in AR-JP, such as dystonia at onset and sleep benefit, were absent. The clinical picture in the Marsala family overlaps with that described in Parkin-positive families of non-Japanese origin (Abbas et al, 1999). Lücking and coworkers (2000) screened seventy-three families with age of onset $\leq$ 45 years in at least one affected member, and one hundred sporadic cases with onset $\leq$ 45 years. They found Parkin mutations in about half of the families, but only in eighteen sporadic cases, mostly (14/18) with age of onset $\leq$ 20 years. The typical AR-JP features, such as dystonia, hyperreflexia, sustained response to levodopa and early levodopa-induced dyskinesias, were more frequent in the Parkin-positive patients than in the Parkin-negative group; however, these signs were less frequent than in previous reports and none could specifically distinguish between the two groups. The age of onset in the Parkin-positive group was on average lower than in the Parkin-negative group, but mutations were identified in patients with age of onset well above 45 years (up to 64 years) with clinical features difficult to distinguish from sporadic PD (Lücking et al, 2000; Klein et al, 2000a).

The identification of several autosomal recessive families without Parkin mutations and clinically indistinguishable from the Parkin-positive families strongly suggested the existence of at least one other, possibly similar gene responsible for autosomal recessive parkinsonism. The identification of a novel locus for autosomal recessive early onset parkinsonism in a family with a similar phenotype to that reported for Parkin represented a crucial step toward the identification of another gene, which could account for at least a subset of the Parkin-negative cases. The disease in the Marsala kindred was more characteristic of sporadic, typical Lewy body PD, as it had a later age of onset than Parkin-positive patients and fewer atypical features. Still, the absence of autoptic confirmation did not allow drawing a definite diagnosis of PD. The term "early onset parkinsonism" is preferred in the case of families, such as the present one, when information on the occurrence of Lewy bodies and on the pattern of neuronal loss is missing.

Linkage with the PARK6 locus was then tested in twenty-eight European ARJP families and eight of them, from four different European countries, were consistent with linkage to PARK6. No common haplotype could be detected in these families, suggesting independent mutational events. These results indicated that PARK6 is not restricted to

Italy, thus confirming the role of this locus in determining the parkinsonian phenotype in European Parkin-negative cases. The PARK6-associated phenotype does not resemble the typical ARJP presentation, as it is often seen in Japanese patients. The clinical presentation is more similar to the phenotype described in Parkin-positive non-Japanese ARJP patients, with a broader clinical spectrum and range of ages at onset. Four of sixteen affected individuals had an age of onset above 45 years, with the highest onset at 68 years. In these late-onset cases, clinical features are indistinguishable from idiopathic PD. Thus, PARK6 could also be responsible for a subset of cases diagnosed as idiopathic PD. However, no pathological data on PARK6-positive patients are available at present. It is still unclear whether PARK6 shares the same neuropathological features as Parkin (a more selective neuron loss than idiopathic PD and absence of Lewy bodies) or idiopathic PD (with presence of Lewy bodies), or represents a distinct disorder within familial parkinsonisms.

Parental consanguinity was detected only in one (IT-GR) of eight families. The two affected sibs in this family were homozygous across the entire linked region except for the three centromeric markers (figure 5.2). This finding strongly suggested a recombination between markers D1S2698 and D1S2674 and allowed the refinement of the candidate interval to 9.0 cM between markers D1S483 and D1S2674. Homozygosity was also detected in another family from Germany (DE-028), without any obvious consanguinity. However, parents of the two affected siblings were originating from a restricted geographic area and the existence of a common ancestor whose memory has been lost in the family could not be ruled out.

The identification of an unaffected individual (DE-029-005) sharing the same haplotypes with the two affected sibs could be explained assuming a reduced penetrance of the underlying gene. However, this individual was 54 years old at the time of examination and might not have developed the disease yet.

The 9.0 cM PARK6 region on chromosome 1, which is equivalent to approximately 9 Mb on physical maps, contains a large number of genes (about 100), either predicted or already known, but none of them represents a striking candidate for parkinsonism. Two interesting genes mapping to the region are coding for the serotonin receptors subtype 1D (HTR1D) and 6 (HTR6). In fact, it has been shown that serotonin receptors play an important role in serotonin-mediated striatal dopamine release and that serotoninergic-mediated mechanisms are damaged in the parkinsonian brain (Volpi et

al, 1997; Sershen et al, 2000). The genes have been cloned and fully characterized, and the exon-intron junction structure of both genes are completely available.

Another interesting gene mapping within the region is FLJ23277 (previously named FLJ11328). This gene has a complex genomic structure. It is composed by 27 exons and at least 9 different mRNAs can be generated by alternative splicing (NCBI Evidence Viewer). Analysis of the open reading frames showed that the different mRNA forms may code for different peptides. Two main groups of mRNAs can be recognized, the first transcribed by the initial fourteen exons and the second lacking the first nine exons. The existence of other unrecognised mRNA forms cannot be ruled out, in particular very long transcripts encompassing both the 5' exons (comprising the ubiquitin hydrolase domain on exon 9) and the distal gene portions. Evidence from ESTs comprised within the gene structure demonstrates that FLJ23277 transcripts are expressed in tissues from the central nervous system, although more specific expression studies on different brain regions have not been performed yet.

Future directions of the study

The whole coding region of the three candidate genes selected will be completely sequenced. The already established collaboration with several Italian and European movement disorder groups will allow in the future to test a larger group of ARJP Parkin-negative families for linkage to PARK6, in order to further refine the linked interval. This will ease the identification of candidate genes, to be directly sequenced in search of pathogenic mutations. Moreover, the identification of other PARK6-linked families will allow a more accurate definition of the PARK6 phenotype and will improve genetic counselling and therapeutic approaches to patients. MRI in a patient from the Marsala kindred was completely negative. Functional studies, such as brain SPECT and PET, are therefore needed to better understand the pathogenetic mechanism acting in the brain of patients with PARK6-parkinsonism and to evaluate abnormalities in the metabolism of dopamine and other neurotransmitters. Autoptic studies of the PARK6 brain, whenever available, will be of great help in understanding the pathogenetic mechanism of the disease, and in helping distinguishing this form of parkinsonism from idiopathic PD and from Parkin-related ARJP.

CHAPTER 6 – CONCLUSIONS AND FUTURE DIRECTIONS OF STUDY

General conclusions

Over the last decade there have been considerable advances in molecular genetics, which have resulted in the discovery of genes responsible for numerous monogenic diseases. Rapid advances in technology and genetic resources, such as increasingly detailed genetic and physical maps, have made possible the identification of novel genes and disease-associated mutations. The aims of this study were to apply the strategy of linkage analysis to locate the genes for two dominantly inherited movement disorders (primary torsion dystonia and paroxysmal kinesigenic dyskinesia) and one recessively inherited disorder (early onset parkinsonism), and to perform genotype-phenotype correlations in families with these diseases. In addition, a mutation screening and linkage disequilibrium study of the DYT1 gene was performed. 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.

Primary torsion dystonia

The findings presented in this thesis indicate the existence of at least one other as yet unmapped gene for PTD. The contribution of the DYT6, DYT7 and DYT13 loci to PTD families and singleton PTD cases remains to be evaluated. Large families with non-DYT1 dystonia are rare but provide a very useful resource to refine the genetic location of the already known PTD loci or to identify novel PTD loci. Only one PTD gene – DYT1 – has been identified so far. The identification of other PTD genes will depend on initial linkage mapping using genome-wide searches, the refinement of candidate regions in linked families, followed by a positional candidate mapping strategy using the available information from the Human Genome Mapping Project. Association studies in sporadic adult-onset PTD using selected polymorphisms in candidate genes will help elucidate the genetic basis of PTD and the molecular pathways, still largely unknown, involved in the pathogenesis of this movement disorder.

Paroxysmal dyskinesias

The results presented here indicate the existence of a cluster of genes giving rise to paroxysmal disorders on the pericentromeric region of human chromosome 16, and demonstrate the existence of at least one other as yet unmapped PKD gene. As for autosomal dominant PTD, the contribution of the identified loci to families and singleton patients with PKD and the genotype-phenotype correlations remain to be fully evaluated. However, the coexistence of different paroxysmal disorders in close proximity on human chromosome 16 is particularly intriguing. A re-evaluation of the available human genome databases will help select candidate genes on the basis of their function (i.e. ion channel genes) and expression pattern.

Autosomal recessive juvenile parkinsonism

The identification of genetic forms of PD in the last few years has dramatically transformed our understanding of the pathogenesis of Parkinson's disease and parkinsonism. The two genes so far discovered in autosomal dominant PD families, α-synuclein (PARK1) and UCH-L1 (PARK5), other dominant loci PARK3, PARK4 and PARK8, and the identification of two autosomal recessive genes, Parkin (PARK2) and DJ1 (PARK7), emphasize the importance of molecular genetics in the aetiology of PD. Genetic studies on 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. In this view, the identification of the PARK6 gene will be of great importance to improve our knowledge on the molecular mechanisms leading to dopaminergic cell death in PD and ARJP.

The identification of other PARK6-linked families will allow a more accurate definition of the PARK6 phenotype and will help refine the linked interval, facilitating the identification of candidate genes to be sequenced for functional mutations. Candidate genes will be chosen on the basis of their expression in brain and their function, in the light of the recent knowledge on the pathogenetic pathways leading to cell death in PD and ARJP. RT-PCR approaches will be used to search for homozygous deletions across the region. Autoptic studies of the PARK6 brain, whenever available, will be of great help in understanding the pathogenetic mechanism of the disease, and in helping distinguish this form of parkinsonism from idiopathic PD and from other subtypes of ARJP.

Future directions of study

Concurrent work to refine the genetic mapping of these disorders in additional families, using linkage analysis or linkage disequilibrium, may narrow the candidate regions for the novel loci identified. Similarly, it may be possible to map new loci for these movement disorders, although success will be determined by the availability of suitable families. Study of the pathophysiology of movement disorders using physiological, pharmacological and functional imaging techniques will complement genetic analysis, and may improve selection of candidate genes as understanding of these disorders increases.

Molecular genetics holds great promise for the future in the fields of diagnosis, understanding of pathophysiology, and ultimately for therapeutic advances in treating human disease. The present era of disease gene discovery is not an end in itself but a new beginning for medicine.

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APPENDICES

APPENDIX 1 – Results of two-point linkage analysis between dystonia and DYT1, DYT6 and DYT7 loci in families PTD01, PTD02 and PTD03

Two-point LOD scores for microsatellite markers spanning the DYT1, DYT6 and DYT7 loci for families PTD01 (appendix 1a), PTD02 (appendix 1b) and PTD03 (appendix 1c) are presented here. Parameters used for linkage analysis are given in chapter 3. Marker order and genetic distances are taken from the Marshfield Clinic genetic maps at: http://www.marshmed.org/genetics/.

Appendix 1a – Family PTD01

Linkage to the DYT1 locus had been previously excluded (Bentivoglio et al, 1997) and therefore it is not reported in this thesis.

DYT6

		LOD scores at θ =						
Markers	*Intermarker distance*	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D8S1791		-5.84	-4.05	-2.99	-2.07	-0.98	-0.42	-0.13
	(6.6 cM)							
D8S538		-2.31	-2.01	-1.70	-1.35	-0.64	-0.26	-0.07
	(2.2 cM)							
D8S509		-2.59	-0.95	-0.33	-0.12	0.02	0.06	0.05
	(7.9 cM)							
D8S285		-4.82	-2.63	-1.87	-1.21	-0.41	-0.07	-0.04
	(4.9 cM)							
D8S507		-2.28	-0.97	-0.38	-0.18	-0.04	0.01	0.06
	(4.0 cM)							
D8S260		-5.28	-3.35	-1.89	-1.23	-0.60	-0.31	-0.13
	(3.4 cM)							
D8S1797		-2.81	-2.66	-1.61	-0.92	-0.34	-0.11	-0.02
	(7.0 cM)							
D8S543		-2.20	-1.87	-0.80	-0.32	0.04	0.13	0.10
	(5.8 cM)							
D8S279		-6.25	-3.53	-1.81	-1.07	-0.45	-0.19	-0.07
	(4.3 cM)							
D8S286		-5.50	-2.20	-0.91	-0.43	-0.07	0.05	0.05

DYT7

Markers	*Intermarker distance*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D18S59		-4.93	-3.18	-1.94	-1.29	-0.51	-0.16	-0.01
	(12.9 cM)							
D18S1140		0.24	0.22	0.16	0.09	-0.03	-0.08	0.00
	(3.2 cM)							
D18S1368		-2.10	-0.53	0.05	0.21	0.23	0.16	0.08
	(4.0 cM)							
D18S1098		-2.20	-1.81	-1.25	-0.71	-0.19	-0.08	-0.05
	(1.2 cM)							
D18S481		-0.12	-0.12	-0.11	-0.09	-0.06	-0.03	-0.01
	(1.3 cM)							
D18S54		-1.70	-1.31	-0.61	-0.23	0.00	0.00	-0.04
	(1.0 cM)							
D18S52		-4.65	-2.95	-1.73	-1.16	-0.48	-0.17	-0.03
	(12.8 cM)							
D18S62		-0.07	-0.08	-0.10	-0.11	-0.09	-0.05	-0.02
	(0.0 cM)							
D18S452		-8.09	-4.00	-2.63	-1.82	-0.78	-0.25	-0.03
	(7.3 cM)							
D18S1163		-0.04	-0.05	-0.05	-0.05	-0.04	-0.02	-0.01
	(3.4 cM)							
D18S843		-1.73	-1.38	-0.73	-0.37	-0.07	0.04	0.05

Appendix 1b – Family PTD02

DYT1

Markers	*Intermarker distance*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D9S2160		-2.85	-2.57	-1.53	-0.99	-0.49	-0.24	-0.08
	(40 Kb)							
D9S2161		-2.44	-0.94	-0.36	-0.20	-0.15	-0.15	-0.09
	(150 Kb)							
D9S63		-2.78	-2.42	-1.32	-0.79	-0.33	-0.12	-0.03
	(130 Kb)							
D9S2162		-2.01	-1.94	-1.07	-0.59	-0.18	-0.03	0.02

DYT6

Markers	*Intermarker distance*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D8S1791		-3.62	-2.60	-1.48	-0.96	-0.47	-0.15	-0.03
	(8.3 cM)							
D8S285		-3.22	-1.10	-0.44	-0.20	-0.03	0.01	0.01
	(8.2 cM)							
D8S260		-3.21	-1.23	-0.58	-0.33	-0.13	-0.04	0.00
	(11.4 cM)							
D8S279		-4.16	-2.23	-1.18	-0.70	-0.29	-0.11	-0.02
	(3.3 cM)							
D8S286		-3.40	-1.50	-0.82	-0.53	-0.26	-0.11	-0.03

DYT7

Markers	*Intermarker distance*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D18S54		-3.43	-1.56	-0.80	-0.46	-0.15	-0.03	0.01
	(10.0 cM)							
D18S62		-3.53	-1.54	-0.80	-0.47	-0.18	-0.06	-0.02
	(0.0 cM)							
D18S452		-3.69	-1.47	-0.76	-0.45	-0.17	-0.05	-0.01
	(14.7 cM)							
D18S464		-0.28	-0.27	-0.24	-0.19	-0.11	-0.05	-0.01
	(2.3 cM)							
D18S1153		-0.73	-0.65	-0.40	-0.20	-0.11	-0.06	0.00
	(1.6 cM)							
D18S1150		-3.21	-1.38	-0.67	-0.32	-0.14	-0.04	-0.02

Appendix 1c – Family PTD03

DYT1

Markers	*Intermarker distance*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D9S2160		-4.05	-3-66	-2.23	-1.46	-0.73	-0.34	-0.12
	(40 Kb)							
D9S2161		-3.03	-1.99	-1.02	-0.32	-0.04	0.12	0.04
	(150 Kb)							
D9S63		-3.15	-2.63	-1.43	-0.88	-0.39	-0.15	-0.04
	(130 Kb)							
D9S2162		-2.80	-1.10	-0.46	-0.23	-0.06	-0.01	0.00

DYT6

Markers	*Intermarker distance*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D8S1791		-4.13	-2.08	-1.24	-0.82	-0.39	-0.17	-0.04
	(4.3 cM)							
D8S601		-0.15	-0.05	-0.05	-0.04	-0.03	-0.01	-0.01
	(2.1 cM)							
D8S509		0.04	0.04	0.04	0.03	0.02	0.01	0.00
	(1.6 cM)							
D8S166		-3.10	-1.34	-0.69	-0.44	-0.24	-0.15	-0.09
	(2.8 cM)							
D8S374		-3.21	-1.37	-0.71	-0.46	-0.26	-0.16	-0.09
	(1.6 cM)							
D8S507		0.09	0.09	0.07	0.06	0.03	0.01	0.00
	(2.5 cM)							
D8S1113		0.89	0.87	0.80	0.71	0.51	0.29	0.09
	(4.9 cM)							
D8S1797		-2.66	-0.87	-0.24	-0.03	0.08	0.05	-0.02
	(4.7 cM)							
D8S543		0.14	0.13	0.11	0.09	0.05	0.02	0.01
	(4.0 cM)							
D8S279		-2.71	-0.91	-0.28	-0.06	0.06	0.04	-0.02
	(3.1 cM)							
D8S286		-1.28	-1.25	-1.03	-0.76	-0.41	-0.22	-0.09

DYT7

Markers	*Intermarker distance*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D18S1140		-0.41	-0.39	-0.32	-0.26	-0.18	-0.12	-0.06
	(2.0 cM)							
D18S1105		0.52	0.51	0.47	0.42	0.29	0.16	0.05
	(2.7 cM)							
D18S1098		-0.15	-0.12	-0.10	-0.07	-0.05	-0.03	-0.02
	(1.4 cM)							
D18S481		-3.18	-1.19	-0.52	-0.27	-0.08	-0.04	-0.04
	(1.4 cM)							
D18S54		-0.50	-0.48	-0.42	-0.35	-0.24	-0.15	-0.07
	(10.0 cM)							
D18S452		-3.37	-1.37	-0.69	-0.41	-0.19	-0.10	-0.07
	(5.3 cM)							
D18S1163		0.10	0.08	0.07	0.06	0.04	0.03	0.02
	(4.0 cM)							
D18S843		-3.68	-1.58	-0.88	-0.58	-0.27	-0.11	-0.02

APPENDIX 2 – Results of two-point linkage analysis from the genome-wide scan in family PTD01

Two-point LOD scores for 423 microsatellite markers analysed in family PTD01 (see chapter 3). These were analysed in a genome-wide search which allowed mapping the DYT13 locus. Markers are arranged in chromosome order and are ordered from p-ter to q-ter.

Chromosome 1

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D1S468	*4*	-7,19	-2,75	-1,39	-0,84	-0,37	-0,18	-0,08
D1S214	*14*	-3,46	-0,81	-0,19	0,01	0,09	0,06	0,01
D1S2663	*15*	-5.94	1.24	1.76	1.81	1.56	1.10	0.51
D1S450	*17*	2.52	2.49	2.36	2.17	1.71	1.15	0.50
D1S244	*20*	0,56	0,54	0,47	0,40	0,26	0,14	0,06
D1S508	*21*	2.81	2.76	2.56	2.29	1.70	1.06	0.40
D1S2667	*25*	3.44	3.39	3.17	2.88	2.23	1.49	0.67
D1S2740	*29*	0,27	0,26	0,22	0,18	0,11	0,05	0,01
D1S228	*30*	3.32	3.27	3.06	2.79	2.17	1.46	0.66
D1S507	*33*	3.03	3.00	2.83	2.60	2.05	1.39	0.63
D1S407	*34*	3.05	3.00	2.80	2.54	1.96	1.30	0.57
D1S2697	*47*	-∞	-2,56	-1,22	-0,69	-0,25	-0,07	0,00
D1S2644	*43*	-6,97	-2,05	-0,76	-0,29	0,03	0,08	0,04
D1S199	*52*	-∞	-2,54	-1,18	-0,64	-0,20	-0,04	0,00
D1S234	*55*	-8,44	-3,05	-1,47	-0,81	-0,27	-0,09	-0,03
D1S255	*65*	-∞	-2,01	-0,60	-0,18	0,04	0,03	-0,02
D1S2797	*76*	-3,61	-2,71	-1,41	-0,86	-0,37	-0,15	-0,04
D1S2890	*86*	-∞	-4,13	-2,13	-1,29	-0,53	-0,18	-0,02

D1S230	*95*	-∞	-4,17	-2,05	-1,16	-0,39	-0,08	0,02
D1S2841	*106*	-∞	-3,42	-2,01	-0,92	-0,29	-0,05	0,03
D1S207	*114*	-∞	-5,36	-2,75	-2,01	-0,71	-0,27	-0,06
D1S2868	*126*	-∞	-3,33	-2,01	-1,18	-0,55	-0,24	-0,08
D1S206	*134*	-∞	-4,71	-2,59	-2,01	-0,83	-0,41	-0,17
D1S2726	*144*	-0,10	-0,09	-0,05	-0,01	0,01	0,01	0,01
D1S252	*150*	-10,25	-4,17	-2,14	-1,32	-0,62	-0,31	-0,15
D1S498	*156*	-∞	-3,77	-2,01	-1,02	-0,40	-0,16	-0,07
D1S484	*170*	-∞	-0,97	-0,30	-0,05	0,10	0,09	0,02
D1S2878	*178*	-∞	-5,74	-3,52	-2,50	-1,42	-0,79	-0,35
D1S196	*182*	-3,70	-1,36	-0,71	-0,46	-0,27	-0,18	-0,12
D1S218	*192*	-∞	-3,92	-2,52	-2,01	-1,07	-0,61	-0,28
D1S238	*203*	-∞	-5,41	-3,48	-2,50	-1,39	-0,72	-0,29
D1S413	*212*	-3,88	-3,24	-2,00	-1,39	-0,78	-0,41	-0,16
D1S249	*221*	-∞	-3,72	-2,01	-0,96	-0,33	-0,07	0,02
D1S425	*231*	-∞	-4,21	-2,16	-1,32	-0,57	-0,24	-0,07
D1S23	*240*	-∞	-2,03	-0,73	-0,24	0,11	0,18	0,13
D1S2800	*252*	-∞	-4,16	-2,12	-1,30	-0,56	-0,22	-0,06
D1S2785	*266*	-10,55	-5,02	-2,82	-2,01	-0,87	-0,38	-0,12
D1S2842	*273*	-7,26	-3,43	-2,00	-1,20	-0,53	-0,21	-0,05
D1S2836	*285*	-3,31	-1,31	-0,65	-0,39	-0,18	-0,09	-0,04

Chromosome 2

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D2S319	*7*	-7,12	-4,01	-2,29	-1,47	-0,71	-0,32	-0,11
D2S281	*14*	-10,70	-4,58	-2,51	-2,01	-0,79	-0,36	-0,12
D2S2211	*16*	-7,44	-2,70	-1,37	-0,84	-0,40	-0,19	-0,07

D2S131	*31*	-∞	-5,43	-2,72	-2,01	-0,65	-0,22	-0,04
D2S305	*39*	-4,21	-2,01	-0,94	-0,63	-0,33	-0,18	-0,08
D2S165	*47*	-∞	-5,04	-2,85	-2,01	-0,95	-0,45	-0,16
D2S177	*59*	-∞	-4,85	-2,28	-1,24	-0,39	-0,06	0,04
D2S391	*70*	-7,36	-3,21	-1,42	-0,70	-0,14	0,05	0,07
D2S134	*84*	-∞	-2,48	-1,22	-0,16	-0,08	-0,04	0,05
D2S286	*94*	-∞	-2,01	-0,26	0,15	0,34	0,28	0,14
D2S139	*101*	-∞	-3,31	-1,97	-0,88	-0,25	-0,09	0,00
D2S2333	*103*	-2,29	-2,01	-0,98	-0,52	-0,19	-0,09	-0,04
D2S2216	*111*	-∞	0,21	0,77	0,88	0,78	0,51	0,20
D2S160	*123*	-∞	-4,49	-2,43	-1,12	-0,24	-0,09	-0,04
D2S347	*132*	-∞	-2,05	-0,75	-0,27	0,05	0,09	0,04
D2S114	*143*	-∞	-5,73	-2,97	-2,01	-0,79	-0,30	-0,07
D2S142	*161*	-∞	-2,89	-1,38	-0,73	-0,19	-0,01	0,02
D2S326	*177*	-5,69	-2,16	-1,01	-0,15	0,12	0,22	0,07
D2S152	*188*	-2,24	-0,03	0,47	0,52	0,35	0,14	0,03
D2S72	*199*	-∞	-2,01	-0,49	-0,05	0,20	0,17	0,06
D2S157	*206*	-∞	-3,89	-2,00	-1,22	-0,55	-0,22	-0,05
D2S126	*221*	-∞	-3,21	-2,01	-0,85	-0,28	-0,07	-0,01
D2S159	*229*	-9,76	-1,33	-0,10	0,30	0,47	0,39	0,21
D2S396	*233*	-∞	-3,79	-2,01	-1,01	-0,36	-0,13	-0,04
D2S206	*241*	-∞	-5,66	-2,95	-2,01	-0,83	-0,35	-0,10
D2S338	*250*	-∞	-4,40	-2,37	-1,52	-0,74	-0,34	-0,12
D2S125	*260*	-10,30	-5,35	-3,12	-2,02	-0,99	-0,47	-0,16
D2S2330	*169*	-6,06	-2,36	-1,07	-0,59	-0,21	-0,06	-0,01
D2S335	*176*	-5,67	-2,12	-1,01	-0,60	0,34	0,24	0,08
D2S117	*194*	-7,13	-2,44	-1,13	-0,63	-0,24	-0,11	-0,05

Chromosome 3

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D3S1297	*2*	-∞	-4,43	-2,14	-1,18	-0,37	-0,08	0,01
D3S1304	*15*	-6,34	-3,68	-1,96	-0,88	-0,24	0,07	0,04
D3S1263	*29*	-∞	-4,28	-2,01	-0,89	-0,12	0,15	0,09
D3S2338	*37*	-7,68	-2,01	-0,55	-0,10	0,19	0,23	0,14
D3S1266	*49*	-3,31	-2,01	-1,23	-0,78	-0,25	-0,12	-0,05
D3S1277	*59*	-7,09	-2,63	-1,28	-0,75	-0,30	-0,11	-0,03
D3S1578	*71*	-∞	-2,95	-2,01	-1,01	-0,50	-0,24	-0,08
D3S1289	*72*	-∞	-3,88	-2,01	-1,09	-0,43	-0,15	-0,03
D3S1300	*80*	-∞	0,08	0,64	0,76	0,66	0,41	0,14
D3S3571	*91*	-∞	-2,00	-0,71	-0,27	0,00	0,02	0,00
D3S1285	*92*	-∞	-2,04	-0,76	-0,32	-0,04	-0,01	-0,01
D3S1566	*99*	-∞	-3,79	-2,01	-1,13	-0,48	-0,19	-0,05
D3S3681	*110*	-3,23	-1,23	-0,58	-0,33	-0,14	-0,06	-0,02
D3S1271	*119*	-∞	-4,04	-2,00	-1,19	-0,50	-0,21	-0,07
D3S1278	*130*	-∞	-5,48	-2,93	-2,01	-0,83	-0,35	-0,10
D3S1303	*141*	-∞	-5,75	-3,27	-2,15	-1,07	-0,50	-0,18
D3S1292	*153*	-7,63	-4,35	-2,30	-1,44	-0,63	-0,24	-0,05
D3S1569	*166*	-3,19	-2,04	-0,78	-0,31	0,02	0,09	0,07
D3S1279	*175*	-∞	-4,70	-2,59	-2,01	-0,84	-0,39	-0,13
D3S1614	*185*	-∞	-5,34	-2,83	-2,01	-0,74	-0,27	-0,05
D3S1282	*192*	-∞	-6,00	-3,23	-2,06	-0,97	-0,42	-0,12
D3S1565	*197*	-∞	-2,76	-1,47	-1,00	-0,57	-0,31	-0,12
D3S1262	*210*	-∞	-4,32	-2,24	-1,37	-0,56	-0,19	-0,03
D3S1580	*219*	-∞	-3,82	-2,01	-1,04	-0,36	-0,09	0,01
D3S1265	*232*	-∞	-2,83	-1,41	-0,80	-0,25	-0,01	0,05

Chromosome 4

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D4S412	*5*	-3,71	-2,01	-1,23	-0,35	-0,05	0,04	0,06
D4S2935	*14*	0,03	0,03	0,03	0,03	0,01	0,00	0,00
D4S403	*26*	-6,11	-3,01	-1,87	-0,66	-0,18	-0,11	-0,04
D4S419	*33,4*	-∞	-4,48	-2,01	-1,03	-0,36	-0,05	-0,01
D4S418	*44*	-∞	-4,52	-2,44	-2,01	-0,67	-0,28	-0,09
D4S405	*57*	-∞	-2,40	-1,10	-0,61	-0,23	-0,07	0,00
D4S1592	*69*	-7,19	-4,33	-2,01	-0,89	-0,36	-0,12	-0,07
D4S398	*73*	-∞	-2,01	-0,71	-0,37	-0,07	0,02	0,03
D4S392	*79*	1,00	0,98	0,91	0,81	0,61	0,40	0,20
D4S2964	*88*	-∞	-4,54	-2,14	-1,13	-0,32	-0,03	0,04
D4S231	*98*	-∞	-3,60	-2,01	-0,82	-0,19	0,03	0,07
D4S1572	*108*	-6,89	-2,91	-1,42	-0,78	-0,23	-0,02	0,04
D4S406	*117*	-∞	-2,65	-1,24	-0,66	-0,17	0,01	0,05
D4S430	*126*	-∞	-5,54	-3,26	-1,86	-0,78	-0,24	-0,06
D4S1575	*132*	0,53	0,52	0,48	0,43	0,33	0,22	0,11
D4S1565	*143*	-∞	-2,55	-1,49	-0,97	-0,46	-0,21	-0,03
D4S424	*144*	-∞	-2,22	-0,93	-0,47	-0,15	-0,06	-0,03
D4S413	*158*	-∞	-3,08	-2,01	-1,03	-0,45	-0,18	-0,04
D4S1539	*176*	-6,73	-3,15	-1,68	-0,95	-0,38	-0,11	-0,02
D4S415	*181*	-∞	-3,37	-2,01	-1,13	-0,48	-0,17	-0,04
D4S408	*195*	-∞	-6,15	-3,36	-1,98	-0,45	-0,09	0,04
D4S426	*207*	-∞	-4,89	-2,78	-2,01	-1,00	-0,54	-0,24

Chromosome 5

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D5S1981	*2*	-∞	-2,01	-0,40	0,03	0,27	0,24	0,12
D5S406	*12*	-∞	-3,86	-2,01	-1,07	-0,41	-0,14	-0,02
D5S630	*20*	-6,88	-2,95	-2,01	-1,00	-0,45	-0,18	-0,05
D5S416	*29*	-4,10	-1,06	-0,41	-0,17	0,00	0,03	0,02
D5S268	*33*	-∞	-3,05	-2,01	-1,07	-0,46	-0,16	-0,02
D5S419	*40*	-∞	-2,94	-1,41	-0,77	-0,25	-0,06	0,00
D5S426	*52*	-∞	-3,40	-1,46	-0,75	-0,22	-0,04	0,02
D5S418	*59*	-∞	-3,50	-2,01	-0,85	-0,27	-0,05	0,02
D5S407	*65*	-∞	-3,44	-1,48	-0,75	-0,20	-0,04	0,00
D5S647	*74*	-∞	-2,40	-1,10	-0,61	-0,24	-0,09	-0,02
D5S424	*82*	-10,30	-3,80	-2,01	-1,04	-0,41	-0,17	-0,06
D5S641	*92*	-3,69	-0,99	-0,29	-0,02	0,14	0,13	0,06
D5S428	*96*	-∞	-3,35	-2,01	-0,76	-0,22	-0,03	-0,02
D5S409	*109*	0,29	0,29	0,27	0,24	0,19	0,13	0,07
D5S421	*122*	-∞	-4,21	-2,98	-1,78	-0,66	-0,46	-0,17
D5S471	*130*	-0,07	-0,07	-0,06	-0,06	-0,04	-0,03	-0,01
D5S210	*147*	0,82	0,82	0,78	0,71	0,51	0,27	0,07
D5S410	*156*	-∞	-2,01	-1,17	-0,83	-0,48	-0,27	-0,12
D5S422	*164*	-∞	-5,95	-3,17	-2,01	-0,93	-0,40	-0,11
D5S400	*175*	-∞	-5,72	-3,37	-2,27	-1,14	-0,53	-0,18
D5S408	*195*	-∞	-3,68	-2,01	-0,89	-0,11	0,03	0,01
D5S644	*105*	-∞	-4,12	-1,89	-0,98	-0,37	-0,11	0,04

Chromosome 6

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D6S1574	*9*	-∞	-4,56	-2,89	-1,21	-0,34	-0,11	-0,03
D6S470	*18*	0,71	0,70	0,63	0,56	0,40	0,25	0,12
D6S289	*29*	-∞	-3,69	-1,89	-0,78	-0,21	-0,06	-0,02
D6S260	*30*	-∞	-0,44	0,22	0,44	0,51	0,37	0,15
D6S276	*43*	-∞	-4,46	-2,38	-1,21	-0,30	0,03	0,06
D6S273	*45*	0,19	0,19	0,19	0,17	0,12	0,06	0,02
D6S291	*50*	-∞	-2,81	-1,47	-0,94	-0,47	-0,23	-0,08
D6S1610	*55*	-∞	-2,05	-0,76	-0,30	0,01	0,07	0,05
D6S257	*80*	-∞	-3,92	-2,00	-1,07	-0,36	-0,06	0,03
D6S286	*90*	-∞	-3,97	-2,00	-1,15	-0,46	-0,15	-0,02
D6S462	*99*	-∞	-1,03	-0,38	-0,14	0,01	0,03	0,01
D6S434	*109*	-11,19	-4,03	-2,01	-1,20	-0,49	-0,19	-0,05
D6S287	*122*	-∞	-2,66	-1,34	-0,82	-0,36	-0,13	-0,01
D6S262	*130*	-∞	2,46	-1,12	-0,59	-0,15	0,00	0,03
D6S292	*137*	-∞	-3,62	-2,01	-0,90	-0,31	-0,09	-0,01
D6S308	*145*	-∞	-2,87	-1,48	-0,91	-0,38	-0,14	-0,03
D6S290	*154*	-∞	-3,49	-1,49	-0,71	-0,11	0,06	0,05
D6S305	*166*	-∞	-3,69	-2,01	-0,90	-0,28	-0,08	-0,03
D6S264	*179*	-∞	-3,67	-2,01	-0,87	-0,26	-0,07	-0,02
D6S446	*189*	-6,85	-4,01	-2,03	-1,22	-0,52	-0,21	-0,06
D6S281	*190*	0,48	0,47	0,43	0,38	0,28	0,17	0,08

Chromosome 7

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D7S531	*5*	-∞	-2,92	-2,00	-0,96	-0,44	-0,23	-0,13
D7S517	*8*	-2,92	-0,38	0,21	0,36	0,36	0,23	0,07
D7S513	*18*	-∞	-4,32	-2,36	-1,28	-0,49	-0,19	-0,02
D7S493	*34*	-∞	-4,04	-2,01	-1,19	-0,47	-0,15	-0,01
D7S629	*37*	0,73	0,71	0,63	0,52	0,30	0,10	0,01
D7S516	*42*	-3,68	-1,08	-0,44	-0,21	-0,04	0,01	0,01
D7S484	*53*	-∞	-5,38	-2,43	-1,16	-0,33	-0,07	-0,05
D7S510	*60*	-3,25	-0,78	-0,19	-0,01	0,06	0,03	0,00
D7S519	*69*	-∞	-5,16	-2,48	-1,43	-0,54	-0,17	-0,01
D7S502	*79*	-∞	-2,00	-0,66	-0,21	0,06	0,08	0,02
D7S669	*90*	-∞	-2,01	-0,61	-0,17	0,08	0,09	0,05
D7S524	*97*	-∞	-2,66	-1,35	-0,86	-0,43	-0,20	-0,06
D7S527	*108*	-∞	-3,79	-2,01	-1,12	-0,38	-0,01	0,05
D7S515	*112*	0,50	0,48	0,44	0,38	0,26	0,15	0,07
D7S486	*124*	-∞	-1,06	-0,42	-0,18	-0,01	0,03	0,03
cftr	*131*	-∞	-5,63	-2,87	-2,01	-0,72	-0,27	-0,06
D7S640	*138*	-∞	-3,90	-2,00	-1,10	-0,42	-0,13	-0,01
D7S684	*147*	-∞	-5,58	-2,82	-2,01	-0,67	-0,22	-0,03
D7S661	*155*	-∞	-5,46	-3,05	-2,00	-0,88	-0,37	-0,11
D7S483	*165*	-∞	-2,45	-1,09	-0,55	-0,13	-0,01	-0,02
D7S550	*178*	-∞	-2,71	-1,35	-0,80	-0,33	-0,12	-0,03
D7S2465	*180*	-3,02	-0,68	-0,08	0,10	0,15	0,08	0,02

Chromosome 8

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D8S504	*0*	-∞	-3,21	-2,01	-1,15	-0,54	-0,26	-0,13
D8S277	*8*	-∞	-4,82	-2,75	-2,01	-0,95	-0,47	-0,18
D8S550	*21*	-∞	-4,95	-2,65	-2,01	-0,73	-0,31	-0,10
D8S552	*27*	-∞	-4,78	-2,69	-2,01	-0,89	-0,42	-0,16
D8S261	*37*	-4,67	-2,01	-0,98	-0,70	-0,39	-0,20	-0,07
D8S1771	*50*	-4,48	-2,60	-1,27	-0,75	-0,31	-0,12	-0,03
D8S283	*60*	-∞	-2,60	-1,26	-0,73	-0,29	-0,10	-0,02
D8S285	*71*	-5,21	-3,01	-1,78	-0,77	-0,33	-0,12	-0,02
D8S286	*94*	-∞	-3,37	-2,01	-1,25	-0,62	-0,31	-0,12
D8S273	*102*	-∞	-4,75	-2,65	-2,01	-0,89	-0,44	-0,17
D8S257	*111*	-∞	-2,54	-1,19	-0,66	-0,22	-0,05	0,01
D8S556	*117*	-∞	-2,38	-1,05	-0,55	-0,16	-0,04	-0,01
D8S1784	*118*	-∞	-2,09	-0,80	-0,33	-0,01	0,05	0,03
D8S198	*128*	-∞	0,72	1,24	1,30	1,10	0,72	0,28
D8S514	*130*	1,52	1,49	1,34	1,15	0,76	0,39	0,10
D8S284	*143*	-∞	0,62	1,14	1,22	1,03	0,69	0,28
D8S272	*154*	-∞	0,21	0,78	0,91	0,83	0,57	0,23
D8S258	*41*	-7,53	-2,59	-1,26	-0,74	-0,30	-0,11	-0,03
D8S538	*67*	-2.44	-1,87	-1,15	-0,35	-0.15	-0.15	-0.09
D8S509	*69*	-2.85	-2.57	-1.53	-0.99	-0.49	-0.24	-0.08
D8S1791	*73*	-5.48	-3.06	-2,14	-1.3	-0.79	-0.45	-0.19
D8S507	*75*	-5.61	-2.67	-1.4	-0.93	-0.55	-0.34	-0.15
D8S260	*79*	-∞	-4,28	-2,19	-1,31	-0,53	-0,18	-0,05
D8S1797	*83*	-5.68	-3.03	-1.65	-1.02	-0.44	-0.17	-0.04
D8S543	*87*	-5.38	-2.01	-0.75	-0.31	-0.02	0,00	0,00

D8S279	*91*	-2.78	-2.42	-1.32	-0.79	-0.33	-0.12	-0.03

Chromosome 9

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D9S288	*10*	-∞	-5,44	-3,09	-2,03	-1,01	-0,48	-0,17
D9S286	*18*	-∞	-3,27	-2,01	-1,30	-0,69	-0,33	-0,11
D9S144	*25*	-∞	-5,78	-3,38	-2,29	-1,23	-0,65	-0,27
D9S156	*32*	-7,00	-2,88	-1,50	-0,93	-0,42	-0,19	-0,07
D9S171	*43*	-6,80	-2,68	-1,34	-0,80	-0,36	-0,17	-0,07
D9S147e	*48*	-6,54	-2,48	-1,15	-0,64	-0,23	-0,10	-0,06
D9S43	*55*	-∞	-4,02	-2,21	-0,98	-0,24	-0,11	-0,06
D9S15	*67*	-3,21	-1,01	-0,37	-0,14	0,00	0,02	0,00
D9S175	*71*	-6,10	-2,66	-1,34	-0,81	-0,36	-0,17	-0,07
D9S167	*83*	-10,15	-4,33	-2,28	-1,45	-0,70	-0,36	-0,17
D9S283	*95*	-∞	-1,05	-0,41	-0,19	-0,06	-0,04	-0,03
D9S176	*105*	-∞	-3,38	-2,00	-1,29	-0,67	-0,34	-0,13
D9S1677	*117*	-5,53	-2,89	-1,12	-0,48	-0,15	-0,12	-0,07
D9S1776	*123*	0,94	0,91	0,80	0,66	0,41	0,20	0,04
D9S1682	*132*	-3,47	-1,02	-0,39	-0,19	-0,08	-0,06	-0,04
D9S159	*142*	0,84	0,81	0,68	0,52	0,24	0,05	-0,02
D9S164	*148*	-3,01	-1,22	-0,61	-0,42	-0,34	-0,34	-0,22
D9S1826	*160*	-3,27	-2,02	-1,15	-0,38	-0,08	-0,08	-0,07

Chromosome 10

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D10S249	*2*	0,34	0,33	0,30	0,25	0,17	0,10	0,04
D10S591	*13*	-5,56	-3,38	-2,01	-1,16	-0,46	-0,16	-0,06
D10S189	*19*	-∞	0,16	0,73	0,85	0,76	0,51	0,19
D10S547	*29*	-7,54	-2,69	-1,35	-0,82	-0,34	-0,13	-0,03
D10S191	*38*	-∞	-2,82	-1,35	-0,74	-0,22	-0,03	0,02
D10S1653	*41*	1,77	1,74	1,61	1,44	1,07	0,67	0,25
D10S197	*52*	-∞	-3,35	-1,89	-0,88	-0,34	-0,11	0,11
D10S208	*61*	-2,18	-0,20	0,37	0,50	0,46	0,29	0,10
D10S220	*70*	-∞	-4,15	-2,21	-1,02	-0,56	-0,12	-0,03
D10S1652	*81*	-2,16	-0,18	0,40	0,55	0,52	0,34	0,12
D10S537	*91*	-∞	0,31	0,89	1,01	0,91	0,61	0,24
D10S1730	*99*	-∞	0,14	0,72	0,85	0,77	0,51	0,21
D10S201	*102*	1,10	1,07	0,97	0,83	0,57	0,32	0,11
D10S1686	*105*	-2,72	0,21	0,78	0,90	0,80	0,54	0,23
D10S583	*115*	-∞	0,07	0,68	0,85	0,82	0,58	0,26
D10S185	*116*	-∞	0,33	0,88	0,99	0,87	0,59	0,26
D10S192	*124*	0,72	0,71	0,67	0,60	0,47	0,32	0,16
D10S1731	*134*	-∞	-0,18	0,44	0,64	0,66	0,48	0,22
D10S1693	*137*	-∞	-2,20	-0,89	-0,40	-0,04	0,05	0,05
D10S190	*139*	0,96	0,94	0,86	0,77	0,57	0,37	0,18
D10S587	*147*	-∞	-3,27	-2,01	-1,27	-0,67	-0,34	-0,14
D10S1723	*148*	-∞	-5,05	-2,84	-2,01	-0,81	-0,30	-0,05
D10S217	*158*	-∞	-3,14	-1,98	-0,67	-0,14	0,03	0,02
D10S1770	*168*	-∞	-5,56	-3,31	-2,28	-1,22	-0,63	-0,24
D10S212	*171*	-∞	-2,29	-1,39	-0,86	-0,35	-0,12	-0,02

D10S1647	*88*	-∞	-0,57	0,06	0,27	0,33	0,23	0,07
D10S1744	*107*	-∞	-0,01	0,58	0,73	0,68	0,46	0,18

Chromosome 11

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D11S922	*3*	-∞	-2,09	-0,81	-0,36	-0,06	0,00	0,00
D11S1338	*13*	-7,18	-3,22	-2,01	-1,22	-0,61	-0,27	-0,08
D11S899	*23*	-∞	-4,13	-2,63	-2,01	-0,91	-0,42	-0,14
D11S904	*33*	-∞	-2,13	-1,45	-1,14	-0,73	-0,39	-0,14
D11S907	*43*	-∞	-3,69	-2,01	-0,95	-0,36	-0,13	-0,03
D11S903	*55*	-∞	-2,75	-1,39	-0,84	-0,36	-0,16	-0,07
D11S905	*52*	0,59	0,58	0,52	0,45	0,29	0,13	0,01
D11S987	*67*	-∞	-4,49	-2,87	-1,76	-0,96	-0,27	-0,13
D11S901	*85*	-∞	-1,38	-0,71	-0,45	-0,27	-0,19	-0,11
D11S4175	*91*	-7,24	-3,91	-2,46	-2,01	-1,00	-0,53	-0,22
D11S35	*97*	-∞	-4,08	-2,05	-1,24	-0,53	-0,21	-0,05
D11S927	*104*	-∞	-3,56	-1,57	-0,80	-0,18	0,04	0,08
D11S908	*109*	-6,84	-2,81	-1,49	-0,96	-0,48	-0,21	-0,06
D11S925	*118*	-∞	-6,54	-3,65	-2,38	-1,13	-0,49	-0,15
D11S910	*140*	-∞	-2,49	-1,16	-0,65	-0,25	-0,09	-0,02
D11S968	*148*	-∞	-2,01	-0,59	-0,16	0,07	0,06	0,01
D11S4151	*128*	-6,61	-3,60	-2,27	-0,93	-0,31	-0,13	-0,03
D11S1320	*142*	-∞	-4,43	-2,39	-1,56	-0,81	-0,43	-0,18

Chromosome 12

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D12S352	*0*	-9,11	-5,26	-3,38	-1,89	-0,68	-0,21	-0,06
D12S99	*12*	-∞	-2,17	-0,81	-0,28	0,10	0,16	0,10
D12S77	*20*	-∞	-2,20	-0,85	-0,33	0,05	0,13	0,09
D12S358	*26*	1,16	1,12	0,96	0,76	0,39	0,12	-0,01
D12S364	*31*	-∞	-2,00	-0,66	-0,19	0,12	0,16	0,09
D12S62	*33*	-∞	-2,17	-0,81	-0,28	0,11	0,17	0,11
D12S1617	*44*	-3,22	-2,25	-1,18	-0,47	-0,07	0,05	0,06
D12S87	*52*	0,04	0,04	0,03	0,02	0,00	-0,01	-0,01
D12S368	*66*	-∞	-5,64	-2,90	-2,01	-0,79	-0,33	-0,11
D12S83	*75*	-4,45	-2,01	-0,79	-0,56	-0,34	-0,21	-0,10
D12S43	*80*	-∞	-4,06	-2,02	-1,21	-0,51	-0,22	-0,09
D12S92	*83*	-∞	-5,30	-3,15	-2,18	-1,18	-0,61	-0,25
D12S95	*95*	-4,60	-2,01	-0,97	-0,78	-0,67	-0,54	-0,29
D12S351	*96*	-∞	-3,63	-2,26	-2,01	-1,03	-0,59	-0,27
D12S346	*104*	-3,30	-1,09	-0,46	-0,25	-0,16	-0,17	-0,13
D12S78	*112*	-∞	-3,45	-2,13	-2,00	-1,04	-0,61	-0,27
D12S366	*133*	-∞	-3,14	-2,01	-1,12	-0,54	-0,25	-0,08
D12S342	*144*	-∞	-4,42	-2,89	-1,88	-1,02	-0,32	-0,12
D12S324	*147*	-∞	-2,01	-1,02	-0,72	-0,38	-0,17	-0,05
D12S97	*161*	-∞	-3,75	-2,01	-0,96	-0,30	-0,04	0,04
D12S345	*53*	-∞	-2,28	-1,62	-0,87	-0,31	-0,08	-0,02
D12S85	*61*	-7,51	-4,12	-2,21	-1,11	-0,03	0,03	0,04
D12S1659	*156*	-∞	-5,17	-2,72	-1,68	-0,74	-0,30	-0,08
D12S1723	*165*	0,81	0,79	0,73	0,65	0,49	0,32	0,16

Chromosome 13

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D13S192	*5*	-∞	-3,58	-2,01	-0,78	-0,16	0,03	0,03
D13S120	*15*	-∞	-2,69	-1,34	-0,81	-0,36	-0,17	-0,08
D13S171	*25*	-7,02	-2,60	-1,26	-0,73	-0,28	-0,10	-0,02
D13S263	*38*	-10,32	-4,71	-2,65	-2,01	-0,94	-0,49	-0,20
D13S153	*45*	-∞	-2,81	-1,40	-0,80	-0,29	-0,10	-0,04
D13S279	*54*	-∞	-3,26	-2,01	-1,14	-0,51	-0,20	-0,05
D13S170	*64*	-5,67	-2,62	-2,01	-1,31	-0,71	-0,38	-0,19
D13S265	*69*	-3,08	-1,09	-0,45	-0,21	-0,04	0,01	0,01
D13S159	*79*	-3,16	-1,18	-0,57	-0,37	-0,22	-0,13	-0,05
D13S158	*85*	-∞	-5,70	-3,08	-2,00	-0,89	-0,37	-0,10
D13S1265	*99*	-∞	-3,32	-1,98	-0,66	-0,28	-0,09	-0,02
D13S285	*110*	-∞	-3,88	-2,01	-1,10	-0,44	-0,16	0,03

Chromosome 14

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D14S72	*9*	-∞	-2,67	-1,25	-0,67	-0,20	-0,04	0,00
D14S50	*13*	-∞	-3,19	-2,01	-1,19	-0,57	-0,23	-0,05
D14S80	*26*	-∞	-0,42	0,17	0,33	0,34	0,23	0,08
D14S49	*36*	-∞	-4,08	-2,03	-1,20	-0,47	-0,16	-0,03
D14S70	*40*	-∞	-2,01	-0,95	-0,63	-0,31	-0,15	-0,06
D14S75	*44*	-∞	-3,86	-2,01	-1,01	-0,34	-0,09	-0,01
D14S276	*56*	-7,70	-3,09	-2,01	-1,19	-0,64	-0,32	-0,11
D14S63	*69*	-∞	-3,86	-2,01	-1,01	-0,34	-0,09	-0,01
D14S258	*76*	-3,94	-2,01	-1,06	-0,79	-0,45	-0,23	-0,09

D14S74	*87*	-∞	-2,77	-1,41	-0,86	-0,37	-0,15	-0,03
D14S68	*95*	-∞	-4,67	-2,43	-1,44	-0,53	-0,13	0,01
D14S280	*105*	-4,04	-1,36	-0,73	-0,48	-0,25	-0,11	-0,03
D14S51	*115*	-∞	-3,70	-2,01	-0,74	-0,08	0,10	0,08
D14S267	*122*	-∞	-3,11	-1,15	-0,42	0,09	0,18	0,11
D14S292	*134*	-6,99	-2,14	-0,84	-0,38	-0,08	-0,03	-0,04
D14S261	*6*	-∞	-3,42	-1,47	-0,74	-0,17	0,01	0,03
D14S283	*14*	-7,73	-4,19	-2,14	-1,29	-0,54	-0,20	-0,04
D14S275	*28*	-2,47	-1,33	-0,97	-0,09	0,03	0,05	0,04
D14S65	*117*	-2,84	-1,91	-0,69	-0,24	0,04	0,09	0,05
D14S985	*127*	-∞	-4,25	-2,23	-1,40	-0,66	-0,30	-0,10

Chromosome 15

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D15S128	*6*	-∞	-2,14	-0,82	-0,33	0,02	0,10	0,07
D15S1002	*15*	-∞	-2,01	-0,60	-0,16	0,12	0,15	0,09
D15S165	*20*	-∞	-4,25	-2,12	-1,24	-0,48	-0,17	-0,04
D15S118	*32*	-4,21	-1,17	-0,48	-0,21	-0,02	0,02	0,01
D15S994	*40*	-∞	-2,00	-0,70	-0,23	0,07	0,11	0,05
D15S117	*51*	-∞	-3,71	-2,01	-0,85	-0,19	0,03	0,05
D15S153	*62*	-∞	-2,79	-1,48	-0,97	-0,51	-0,24	-0,07
D15S114	*72*	-3,72	-0,69	-0,07	0,15	0,29	0,25	0,14
D15S205	*79*	-2,37	0,48	1,00	1,05	0,83	0,49	0,20
D15S979	*83*	-2,98	0,04	0,62	0,76	0,69	0,45	0,17
D15S127	*87*	2,28	2,25	2,12	2,01	1,50	0,99	0,42
D15S130	*100*	-5,87	-3,22	-2,01	-1,35	-0,66	-0,27	-0,07
D15S120	*112*	-∞	-2,84	-1,47	-0,92	-0,41	-0,18	-0,05

D15S1007	*26*	-12,19	-4,12	-2,63	-1,15	-0,32	0,05	0,08
D15S1012	*36*	-3,55	-2,02	-1,12	-0,48	-0,17	-0,06	-0,03
D15S131	*71*	-2,41	-2,11	-1,03	-0,36	-0,01	0,06	0,03

Chromosome 16

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D16S423	*10*	-∞	-4,30	-2,22	-1,36	-0,58	-0,24	-0,07
D16S407	*18*	-∞	-3,84	-2,01	-1,01	-0,33	-0,06	0,03
D16S405	*28*	-∞	-0,88	-0,28	-0,09	0,01	0,02	0,01
D16S287	*36*	0,20	0,20	0,18	0,16	0,11	0,06	0,02
D16S420	*44*	-∞	-2,42	-1,12	-0,64	-0,27	-0,11	-0,03
D16S261	*58*	-∞	-1,24	-0,58	-0,34	-0,14	-0,06	-0,02
D16S411	*60*	-∞	-1,24	0,01	0,41	0,58	0,45	0,21
D16S415	*67*	-∞	-2,32	-1,00	-0,49	-0,12	-0,02	-0,01
D16S320	*75*	-∞	-2,93	-1,55	-0,99	-0,49	-0,26	-0,12
D16S503	*83*	-∞	-2,99	-2,01	-1,05	-0,54	-0,27	-0,11
D16S515	*92*	-4,76	-2,01	-1,03	-0,73	-0,43	-0,24	-0,11
D16S516	*100*	-∞	-3,28	-2,00	-1,30	-0,71	-0,36	-0,13
D16S289	*105*	-∞	-0,04	0,53	0,66	0,57	0,31	0,06
D16S422	*111*	-∞	-2,21	-0,88	-0,39	-0,06	-0,03	-0,08
D16S520	*125*	-3,89	-2,29	-1,52	-1,14	-0,71	-0,43	-0,21
D16S404	*18*	0,24	0,24	0,23	0,21	0,16	0,11	0,06
D16S3075	*24*	-∞	-3,87	-1,86	-1,06	-0,40	-0,12	-0,01
D16S3103	*32*	-∞	-2,01	-1,12	-0,31	0,03	0,26	0,14
D16S3046	*41*	-∞	-4,43	-2,11	-1,02	-0,25	0,12	0,07
D16S3068	*48*	-∞	-3,15	-1,88	-0,68	-0,23	0,24	0,11
D16S3091	*111*	-10,08	-3,82	-2,21	-1,04	-0,41	-0,18	-0,08

D16S3112	*74*	-∞	-2,11	-0,82	-0,36	-0,07	-0,03	-0,04

Chromosome 17

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D17S513	*8*	0,68	0,67	0,61	0,53	0,37	0,23	0,10
D17S786	*17*	-∞	-3,31	-2,01	-1,03	-0,31	-0,01	0,07
D17S799	*32*	-∞	-2,63	-0,72	-0,05	0,36	0,38	0,24
D17S1857	*43*	0,39	0,38	0,34	0,29	0,19	0,11	0,05
D17S798	*53*	-∞	-2,01	-1,14	-0,80	-0,45	-0,26	-0,12
D17S250	*62*	-∞	-3,59	-2,00	-1,14	-0,49	-0,20	-0,06
D17S934	*64*	-∞	-3,88	-2,01	-0,85	-0,15	0,08	0,10
D17S787	*75*	-∞	-1,19	-0,52	-0,27	-0,07	-0,01	0,00
D17S807	*86*	-∞	-4,24	-2,19	-1,34	-0,59	-0,23	-0,05
D17S789	*89*	-∞	-2,01	-0,69	-0,20	0,14	0,18	0,11
D17S949	*97*	-10,72	-5,46	-2,86	-2,01	-0,73	-0,26	-0,05
D17S939	*105*	-∞	-4,09	-2,04	-1,20	-0,47	-0,15	-0,02
D17S784	*117*	-∞	-2,00	-0,61	-0,14	0,17	0,21	0,13
D17S928	*126*	-3,54	-2,75	-1,48	-0,93	-0,44	-0,18	-0,05
D17S849	*1*	-6,91	-3,35	-1,81	-1,10	-0,45	-0,16	-0,03
D17S831	*6*	-10,34	-4,65	-2,58	-1,69	-0,83	-0,37	-0,12
D17S938	*15*	-∞	-2,01	-1,03	-0,73	-0,41	-0,22	-0,08
D17S1852	*22*	0,49	0,49	0,45	0,41	0,31	0,21	0,11
D17S921	*36*	0,11	0,11	0,09	0,08	0,04	0,01	0,00

Chromosome 18

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D18S59	*0*	-∞	-2,67	-1,29	-0,74	-0,27	-0,08	0,00
D18S52	*9*	-∞	-2,11	-0,81	-0,33	0,00	0,06	0,05
D18S62	*19*	-∞	-2,64	-1,29	-0,76	-0,29	-0,09	-0,01
D18S464	*31*	0,62	0,60	0,55	0,48	0,34	0,21	0,10
D18S53	*41*	-∞	-1,28	-0,63	-0,37	-0,15	-0,05	0,00
D18S71	*44*	-∞	-1,00	-0,36	-0,13	0,03	0,06	0,04
D18S478	*53*	-∞	-1,16	-0,52	-0,28	-0,10	-0,02	0,01
D18S57	*63*	-∞	-0,66	-0,03	0,17	0,23	0,14	0,02
D18S474	*71*	0,34	0,34	0,31	0,27	0,19	0,12	0,05
D18S64	*85*	-∞	-2,84	-1,47	-0,90	-0,40	-0,16	-0,04
D18S68	*96*	-∞	-3,54	-2,11	-1,47	-0,79	-0,40	-0,15
D18S61	*105*	-∞	-3,70	-2,13	-1,41	-0,70	-0,33	-0,11
D18S1161	*114*	-∞	-2,71	-1,40	-0,87	-0,39	-0,16	-0,04
D18S70	*126*	-∞	-5,05	-2,43	-1,37	-0,50	-0,15	-0,03
D18S818	*2,8*	-5.48	-3.06	-2,01	-1.3	-0.79	-0.45	-0.19
D18S54	*8,3*	-2.44	-0.94	-0.36	-0.2	-0.15	-0.15	-0.09
D18S452	*18,8*	-5.61	-2.67	-1.4	-0.93	-0.55	-0.34	-0.15
D18S1163	*24,1*	-5.48	-2.61	-1.28	-0.73	-0.27	-0.09	-0.02
D18S843	*28,1*	-2.38	-0.86	-0.27	-0.08	0,00	0,00	0,00
D18S363	*72*	0,59	0,58	0,55	0,49	0,34	0,18	0,05

Chromosome 19

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D19S209	*11*	-∞	-3,25	-1,29	-0,55	0,02	0,18	0,15

D19S884	*26*	-7,71	-2,37	-1,04	-0,52	-0,12	0,01	0,03
D19S221	*36*	-∞	-2,56	-1,04	-0,42	0,04	0,14	0,09
D19S226	*42*	-2,48	-0,50	0,08	0,25	0,29	0,21	0,09
D19S225	*56*	-∞	-4,39	-2,31	-1,43	-0,64	-0,26	-0,07
D19S220	*62*	-3,76	-2,01	-0,73	-0,41	-0,15	-0,07	-0,05
D19S902	*72*	-7,75	-2,12	-0,81	-0,32	0,02	0,07	0,02
D19S572	*89*	-∞	0,09	0,66	0,78	0,69	0,44	0,17
D19S418	*92*	0,55	0,53	0,47	0,39	0,23	0,11	0,03
D19S210	*100*	-3,01	-2,01	-0,53	-0,07	0,20	0,21	0,13

Chromosome 20

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D20S117	*3*	-10,14	-2,00	-0,66	-0,18	0,15	0,19	0,11
D20S889	*11*	-11,04	-2,50	-1,18	-0,68	-0,28	-0,12	-0,04
D20S115	*21*	-7,34	-2,64	-1,28	-0,72	-0,24	-0,05	0,02
D20S186	*32*	-∞	-2,03	-0,74	-0,27	0,04	0,07	0,02
D20S112	*39*	-∞	-3,52	-2,00	-0,77	-0,17	0,03	0,07
D20S200	*50*	-∞	-4,38	-2,21	-1,30	-0,51	-0,18	-0,04
D20S106	*51*	-∞	-3,55	-2,09	-1,44	-0,75	-0,37	-0,13
D20S107	*56*	-∞	-3,21	-1,26	-0,54	-0,03	0,08	0,05
D20S178	*66*	-∞	-3,29	-1,34	-0,61	-0,06	0,11	0,10
D20S196	*75*	-∞	-2,75	-1,37	-0,79	-0,25	-0,02	0,05
D20S120	*83*	-∞	-2,88	-1,51	-0,95	-0,43	-0,16	-0,02
D20S100	*85*	-∞	-4,33	-2,31	-1,50	-0,74	-0,34	-0,11
D20S173	*98*	0,50	0,48	0,44	0,38	0,26	0,15	0,06
D20S171	*95*	-∞	-4,21	-2,20	-1,40	-0,70	-0,35	-0,14
D20S93	*100*	-∞	-4,43	-2,36	-1,50	-0,68	-0,28	-0,07

Chromosome 21

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D21S258	*5*	-∞	-2,45	-1,10	-0,56	-0,12	0,04	0,07
D21S1256	*10*	-∞	-2,34	-1,03	-0,54	-0,16	-0,03	0,01
D21S1914	*19*	-∞	-3,66	-2,04	-1,30	-0,62	-0,32	-0,17
D21S265	*21*	-∞	-3,21	-1,80	-1,18	-0,59	-0,28	-0,10
D21S263	*27*	-∞	-3,79	-1,78	-0,99	-0,35	-0,12	-0,07
D21S65	*34*	-∞	-2,30	-1,00	-0,54	-0,26	-0,20	-0,12
D21S1252	*36*	-∞	-1,60	-0,35	0,07	0,29	0,25	0,13
D21S167	*38*	-3,30	-0,28	0,32	0,48	0,47	0,31	0,11
D21S270	*39*	-∞	-2,33	-0,99	-0,47	-0,05	0,04	0,00
D21S268	*41*	-∞	-3,01	-1,04	-0,31	0,19	0,26	0,14
D21S266	*46*	-∞	-3,02	-1,09	-0,40	0,07	0,15	0,09

Chromosome 22

Markers	*Distance from top of chromosome (cM)*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D22S420	*4*	-3,69	-0,94	-0,31	-0,09	0,04	0,06	0,04
D22S539	*14*	-4,28	-2,57	-1,24	-0,71	-0,26	-0,06	0,01
D22S315	*21*	-10,98	-3,86	-2,01	-1,07	-0,42	-0,15	-0,03
D22S280	*31*	-∞	-3,24	-1,28	-0,55	-0,02	0,11	0,08
D22S283	*39*	-∞	-5,10	-2,42	-1,37	-0,50	-0,16	-0,03
D22S274	*51*	-7,31	-2,55	-1,09	-0,50	-0,07	0,02	0,01

APPENDIX 3 – Results of two-point linkage analysis between dystonia and DYT13 locus in families PTD01, PTD02 and PTD03

Two-point LOD scores for microsatellite markers spanning the novel DYT13 locus for families PTD01 (appendix 3a), PTD02 (appendix 3b) and PTD03 (appendix 3c) are presented here. Parameters used for linkage analysis are given in chapter 3. Marker order and genetic distances are taken from the Marshfield Clinic genetic maps at: http://www.marshmed.org/genetics/.

Appendix 3a – Family PTD01

		LOD scores at θ =						
Markers	*Intermarker distance*	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D1S2663		-5.94	1.24	1.76	1.81	1.56	1.10	0.51
	(1.8 cM)							
D1S450		2.52	2.49	2.36	2.17	1.71	1.15	0.50
	(2.0 cM)							
D1S508		2.81	2.76	2.56	2.29	1.70	1.06	0.40
	(4.0 cM)							
D1S2667		3.44	3.39	3.17	2.88	2.23	1.49	0.67
	(4.9 cM)							
D1S228		3.32	3.27	3.06	2.79	2.17	1.46	0.66
	(4.4 cM)							
D1S507		3.03	3.00	2.83	2.60	2.05	1.39	0.63
	(0.8 cM)							
D1S407		3.05	3.00	2.80	2.54	1.96	1.30	0.57
	(2.5 cM)							
D1S2697		-6.97	-2.05	-0.76	-0.29	0.03	0.08	0.04

Appendix 3b – Family PTD02

		LOD scores at θ =						
Markers	*Intermarker distance*	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D1S2663		-3.40	-1.48	-0.70	-0.44	-0.12	-0.02	0.01
	(1.8 cM)							
D1S450		-3.89	-1.66	-0.89	-0.50	-0.27	-0.13	-0.02
	(2.0 cM)							
D1S508		-2.58	-2.21	-1.12	-0.59	-0.28	-0.13	-0.02
	(4.0 cM)							
D1S2667		-3.60	-1.74	-0.98	-0.63	-0.33	-0.15	-0.02
	(4.9 cM)							
D1S228		-0.68	-0.60	-0.46	-0.24	-0.09	-0.03	0.01
	(4.4 cM)							

D1S507		-2.72	-1.95	-1.10	-0.72	-0.38	-0.09	-0.01
	(0.8 cM)							
D1S407		-3.21	-1.23	-0.58	-0.33	-0.13	-0.04	0.00
	(2.5 cM)							
D1S2697		-3.27	-1.15	-0.49	-0.23	-0.07	-0.01	0.01

Appendix 3c – Family PTD03

		LOD scores at θ =						
Markers	*Intermarker distance*	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D1S2663		-3.37	-1.37	-0.69	-0.41	-0.19	-0.10	-0.07
	(1.8 cM)							
D1S450		-4.05	-3-66	-2.23	-1.46	-0.73	-0.34	-0.12
	(2.0 cM)							
D1S508		-3.10	-1.34	-0.69	-0.44	-0.24	-0.15	-0.09
	(4.0 cM)							
D1S2667		-3.68	-1.58	-0.88	-0.58	-0.27	-0.11	-0.02
	(4.9 cM)							
D1S228		-2.66	-0.87	-0.24	-0.03	0.08	0.05	-0.02
	(4.4 cM)							
D1S507		-0.50	-0.48	-0.42	-0.35	-0.24	-0.15	-0.07
	(0.8 cM)							
D1S407		-3.18	-1.19	-0.52	-0.27	-0.08	-0.04	-0.04
	(2.5 cM)							
D1S2697		-2.71	-0.91	-0.28	-0.06	0.06	0.04	-0.02

APPENDIX 4 – Results of two-point linkage analysis between PKD and chromosome 16 markers in families PKD01, PKD02 and PKD03

Two-point LOD scores for microsatellite markers covering the pericentromeric region of chromosome 16 for families PKD01 (appendix 4a), PKD02 (appendix 4b) and PKD03 (appendix 4c) are presented here. Parameters used for linkage analysis are given in chapter 4. Marker order and genetic distances were based on framework markers of the latest Genetic Location Database chromosome 16 consensus map (G-map, updated November 1999) and published recombinants (Szepetowski et al, 1997; Tomita et al, 1999; Bennett et al, 2000).

Appendix 4a – Family PKD01

		LOD scores at θ =						
Markers	*Intermarker distance*	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D16S3133		- ∞	0.69	1.22	1.28	1.06	0.67	0.25
	(1.8 cM)							
D16S3068		2.34	2.30	2.14	1.92	1.44	0.95	0.47
	(0.6 cM)							
D16S3131		- ∞	2.85	3.28	3.20	2.62	1.79	0.82
	(1.4 cM)							
D16S3093		- ∞	1.73	2.22	2.22	1.84	1.24	0.52
	(6.0 cM)							
D16S3044		-2.16	2.43	2.86	2.79	2.24	1.46	0.61
	(0.0 cM)							
D16S517		- ∞	2.07	2.50	2.44	1.96	1.28	0.53
	(0.0 cM)							
D16S261		- ∞	0.51	1.01	1.06	0.84	0.48	0.14
	(0.6 cM)							
D16S298		2.05	2.03	1.91	1.73	1.33	0.90	0.45
	(0.5 cM)							
D16S3080		0.46	1.40	1.81	1.79	1.42	0.88	0.32
	(0.0 cM)							
D16S411		- ∞	2.18	2.59	2.50	1.96	1.25	0.49
	(2.7 cM)							
D16S3136		1.24	1.21	1.11	0.98	0.71	0.44	0.21
	(2.6 cM)							
D16S416		- ∞	0.59	1.07	1.10	0.83	0.45	0.12
	(0.3 cM)							
D16S685		- ∞	0.38	0.86	0.89	0.64	0.32	0.07
	(3.0 cM)							
D16S419		3.66	3.59	3.31	2.93	2.10	1.26	0.53
	(0.8 cM)							
D16S3137		3.64	3.58	3.31	2.94	2.12	1.27	0.53
	(3.6 cM)							
D16S415		3.27	3.21	2.95	2.61	1.84	1.08	0.44

	(8.4 cM)							
D16S503		-5.88	-2.60	-1.28	-0.76	-0.35	-0.17	-0.06

Appendix 4b – Family PKD02

		LOD scores at θ =						
Markers	*Intermarker distance*	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D16S3133		0.51	0.50	0.43	0.35	0.20	0.08	0.02
	(1.8 cM)							
D16S3068		-5.44	-4.12	-2.46	-1.65	-0.84	-0.40	-0.14
	(0.6 cM)							
D16S3131		-5.24	-4.15	-2.57	-1.78	-0.96	-0.49	-0.19
	(1.4 cM)							
D16S3093		-5.44	-4.28	-2.53	-1.68	-0.85	-0.41	-0.14
	(6.0 cM)							
D16S3044		-5.40	-4.04	-2.42	-1.63	-0.83	-0.39	-0.14
	(0.0 cM)							
D16S261		0.00	0.00	0.00	0.00	0.00	0.00	0.00
	(0.6 cM)							
D16S298		-5.17	-4.08	-2.53	-1.76	-0.95	-0.49	-0.19
	(3.2 cM)							
D16S3136		-5.17	-3.81	-2.28	-1.55	-0.79	-0.38	-0.13
	(2.9 cM)							
D16S685		0.82	0.80	0.72	0.60	0.38	0.17	0.04
	(7.4 cM)							
D16S415		-5.21	-4.52	-2.96	-2.11	-1.18	-0.63	-0.25

Appendix 4c – Family PKD03 (PKD only)

		LOD scores at θ =						
Markers	*Intermarker distance*	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D16S3133		0.10	0.10	0.09	0.08	0.06	0.04	0.02
	(1.8 cM)							
D16S3068		0.67	0.66	0.63	0.58	0.45	0.31	0.16
	(8.6 cM)							
D16S298		0.10	0.10	0.09	0.08	0.06	0.04	0.02
	(3.2 cM)							
D16S3136		0.14	0.14	0.14	0.12	0.07	0.03	0.00
	(10.3 cM)							
D16S415		0.36	0.37	0.38	0.37	0.34	0.26	0.15
	(8.4 cM)							
D16S503		0.40	0.39	0.34	0.28	0.17	0.09	0.03

Appendix 4d – Family PKD03 (PKD and infantile convulsions)

Markers	*Intermarker distance*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D16S3133		0.52	0.52	0.48	0.44	0.35	0.25	0.13
	(1.8 cM)							
D16S3068		1.39	1.38	1.31	1.20	0.94	0.64	0.31
	(8.6 cM)							
D16S298		0.52	0.52	0.48	0.44	0.35	0.25	0.13
	(3.2 cM)							
D16S3136		0.57	0.56	0.52	0.46	0.31	0.14	0.03
	(10.3 cM)							
D16S415		0.79	0.79	0.77	0.74	0.62	0.47	0.26
	(8.4 cM)							
D16S503		1.12	1.10	1.02	0.91	0.67	0.42	0.18

APPENDIX 5 – Results of two-point linkage analysis between PED and PNKD, FHM/EA2 and ICCA/PKD loci in family PED01

PNKD locus (chromosome 2q)

Markers	*Intermarker distance*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D2S164		-8.25	-2.69	-1.34	-0.81	-0.35	-0.15	-0.05
	(1.1cM)							
D2S295		0.30	0.29	0.26	0.21	0.13	0.06	0.02
	(0.2cM)							
D2S173		-3.96	-1.22	-0.57	-0.33	-0.14	-0.07	-0.04
	(0.5cM)							
D2S2250		-4.08	-1.51	-0.83	-0.54	-0.28	-0.14	-0.05
	(2.1cM)							
D2S2359		-4.38	-1.47	-0.77	-0.48	-0.20	-0.07	-0.01
	(2.1cM)							
D2S371		-3.86	-1.18	-0.52	-0.26	-0.07	-0.01	0.00

FHM/EA2 locus (chromosome 19p)

Markers	*Intermarker distance*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D19S432		-3.86	-1.16	-0.50	-0.25	-0.07	-0.01	0.00
	(3.9cM)							
D19S899		-3.89	-1.09	-0.44	-0.21	-0.04	0.00	0.00
	(0.6cM)							
D19S1150		0.12	0.12	0.10	0.08	0.05	0.02	0.01
	(0.4cM)							
D19S199		-4.05	-1.30	-0.62	-0.36	-0.14	-0.05	-0.01

ICCA and PKD loci (chromosome 16)

Markers	*Intermarker distance*	LOD scores at θ = 0.0	0.01	0.05	0.1	0.2	0.3	0.4
D16S401		-3.90	-1.62	-0.91	-0.60	-0.29	-0.12	-0.03
	(0.2 cM)							
D16S3133		-3.88	-1.58	-0.88	-0.57	-0.27	-0.12	-0.03
	(1.8 cM)							
D16S3068		-3.88	-1.58	-0.88	-0.57	-0.27	-0.12	-0.03
	(0.6 cM)							
D16S3131		-3.80	-1.51	-0.82	-0.53	-0.25	-0.11	-0.03
	(1.4 cM)							
D16S3093		-0.06	-0.06	-0.05	-0.05	-0.04	-0.03	-0.01
	(6.6 cM)							
D16S685		-0.18	-0.17	-0.15	-0.13	-0.09	-0.05	-0.01
	(6.1 cM)							
D16S298		-3.88	-1.58	-0.88	-0.57	-0.27	-0.12	-0.03

APPENDIX 6 – Results of two-point linkage analysis between parkinsonism and the novel PARK6 locus in ARJP families

Two-point LOD scores for microsatellite markers spanning the novel PARK6 locus for the "Marsala" kindred (appendix 6a), and cumulative two-point LOD scores for European ARJP families (appendix 6b) are presented here. Parameters used for linkage analysis are given in chapter 5. Marker order and genetic distances are taken from the Marshfield Clinic genetic maps at: http://www.marshmed.org/genetics/, and from the NCBI chromosome 1 physical map at: http://www.ncbi.nlm.nih.gov.

Appendix 6a – The "Marsala" kindred

		LOD scores at θ =						
Markers	*Intermarker distance*	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D1S483		0.18	0.53	0.83	0.83	0.59	0.31	0.10
	(0.0 cM)							
D1S199		4.01	3.92	3.55	3.08	2.14	1.24	0.48
	(3.2 cM)							
D1S2732		2.65	2.60	2.39	2.10	1.46	0.83	0.32
	(0.0 cM)							
D1S2828		3.49	3.42	3.12	2.72	1.89	1.08	0.41
	(0.0 cM)							
D1S478		3.45	3.36	3.03	2.61	1.78	1.00	0.38
	(0.6 cM)							
D1S2702		3.36	3.16	2.84	2.44	1.65	0.93	0.36
	(3.6 cM)							
D1S2734		3.62	3.55	3.26	2.87	2.03	1.19	0.46
	(1.6 cM)							
D1S2674		3.29	3.21	2.87	2.44	1.60	0.87	0.32
	(0.8 cM)							
D1S2885		3.94	3.85	3.49	3.03	2.11	1.23	0.49
	(2.7 cM)							
D1S247		1.01	1.35	1.59	1.48	1.03	0.58	0.19

Appendix 6b – European ARJP families (cumulative LOD scores)

		LOD scores at θ =						
Markers	*Intermarker distance*	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D1S483		2.78	2.72	2.43	2.07	1.33	0.67	0.20
	(0.0 cM)							
D1S199		4.43	4.31	3.83	3.29	2.13	1.09	0.35
	(3.2 cM)							

D1S2732		3.85	3.78	3.43	2.94	1.91	0.96	0.28
	(0.0 cM)							
D1S2828		4.96	4.85	4.36	3.72	2.41	1.24	0.39
	(0.0 cM)							
D1S478		5.39	5.25	4.68	3.96	2.54	1.30	0.40
	(0.6 cM)							
D1S2702		4.45	4.35	3.90	3.32	2.14	1.09	0.33
	(3.6 cM)							
D1S2734		5.17	5.05	4.54	3.86	2.46	1.23	0.36
	(0.0 cM)							
D1S2698		3.55	3.46	3.10	2.62	1.67	0.84	0.25
	(1.6 cM)							
D1S2674		2.89	3.40	3.48	3.08	2.00	0.97	0.25
	(0.8 cM)							
D1S2885		1.69	2.22	2.41	2.17	1.41	0.68	0.18
	(2.7 cM)							
D1S247		2.33	2.86	3.01	2.70	1.78	0.87	0.23

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