Genetic Susceptibility to Parkinson's Disease

B.S. Harhangi

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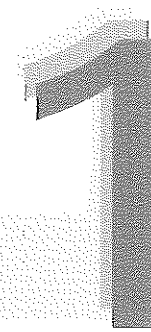

Introduction

PARALYSIS AGITANS HAS existed in the world since ancient times. Its first clear description is found in the old Indian medical system Ayurveda described from 4500 to 1000 BC under the name Kampavata.[1] Herbal preparations like mucuna pruriens, commonly known as velvet bean or cowitch and containing levodopa, were used in the treatment of Kampavata.[2] At present this entity is labeled Parkinson's disease (PD) which has been described by James Parkinson in his monograph "Shaking Palsy" in 1817.[3] He defined PD as "*Involuntary tremulous motion, with lessened muscular power, in parts, not in action and even when supported; with propensity to bend the trunk forward, and to pass from a walking to a running pace; the senses and intellects being uninjured.*" Nowadays PD is known as a common neurodegenerative disorder characterized by the clinical combination of tremor, bradykinesia, rigidity and postural disturbances. Additional features in PD are dementia, dysautonomia, dystonic cramps, postural abnormalities and asymmetry of signs at onset.

Neuropathologically, PD is characterized by a selective degeneration of the dopaminergic neurons in the pars compacta of the substantia nigra, resulting in dopamine deficiency in striatal projection areas of these neurons. The presence of neuronal inclusions, known as Lewy bodies, in the surviving dopaminergic neurons are generally considered as the pathologic hallmark of PD, albeit not totally specific.[4]

In the elderly, PD is a major disabling disorder putting a high claim on health care budgets. The prevalence of PD varies from 0.6% for those aged 65-69 years to 4.3% for those aged 85 years and over.[5] The overall prevalence is 1.6% for individuals aged 65 years or older.[5] Since the number of elderly individuals of the population is increasing and individuals tend to live longer, we may expect an increase in the prevalence of PD, which could have major social and financial implications in our current health care system. The etiology of PD is still largely unknown and treatment is symptomatic with only temporary results. However, currently it is generally accepted that PD is a genetically complex disease in which genetic factor(s), environmental factor(s) or a combination of both may determine the risk of PD. For many decades several investigators reported clustering of PD in families.[6,7] Studies on familial aggregation in PD with an autosomal dominant inheritance pattern have clearly indicated that genetic factors play a significant role in PD.[8] Familial linkage studies and candidate gene studies have been carried out to investigate the role of genetic factors and the pattern of inheritance in PD but the results are controversial.[6,7] The most convincing evidence that PD can be inherited as a single gene defect comes from families with PD with an autosomal dominant inheritance pattern [9-11] or with an autosomal recessive inheritance pattern.[12,13] At least two other loci have been found in relation with PD.[14,15] These observations provide important evidence that PD is a genetically heterogeneous disorder.

The objective of the work presented in this thesis was to study genetic susceptibility to PD. We did this in two different studies, the Rotterdam Study, which is a prospective population based cohort study on frequency, etiology, and prognosis of chronic diseases, and a large European collaborative sib pair study on PD. We used different methods.

Firstly, as presented in chapter 2, we analyzed whether there was familial aggregation of PD. We did this by assesing the risk of PD in the Rotterdam Study as a function of a positive family history of PD or dementia.

Secondly, we used genetic association studies, presented in chapter 3, to study candidate genes in PD. In chapter 3.1 and 3.2 we report the association between candidate genes, N-acetyltransferase-2 (NAT-2) Cytochrome P450 (CYP2D6) and PD, within the Rotterdam Study. The risk of PD with or without dementia as a function of the APOE genotype in the Rotterdam Study is presented in chapter 3.3.

Finally, linkage studies and genetic association studies in affected sib pairs, were used to study genetic susceptibility to PD (chapter 4). In chapter 4.1 we present the study design and linkage results on chromosmes 2p and 4 in Parkinson's Disease in a large study of European affected sib pairs. Within the framework of this European study we also evaluated the importance of the reported mutations in the UCH-L1 gene (chapter 4.2) and the mutations in the Parkin gene (chapter 4.3).

In chapter 5 the findings presented in this thesis are discussed in general together with issues relating to study design. Some mechanisms that may potentially underlie PD are considered and finally, we discuss clinical, social and scientific impact of our and other genetic studies in PD.

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2 Aggregation study

FAMILY HISTORY AND THE RISK OF PARKINSON'S DISEASE IN A PROSPECTIVE POPULATION-BASED STUDY

Abstract

Previous studies on familial aggregation of Parkinson's disease (PD) were mostly hospital-based case-control studies or pertained to younger individuals. We prospectively determined the risk of PD according to family history of PD in a population-based cohort of 6341 elderly. After 5.3 year follow-up we identified 57 PD-patients. Persons with at least one first degree relative with PD had a 1.5-time higher risk, and persons with at least two first degree relatives had a five-time higher risk of PD. Our study suggests that hereditary factors contribute to the occurrence of PD in the elderly.

INTRODUCTION

Several studies reported familial aggregation of Parkinson's disease (PD). However, most were case-control studies,[1-6] mostly based on selected hospital populations in which the information had been collected retrospectively,[1-4] or pertained to younger subjects.[2-4] Recently a large twin study on PD suggested that genetic factors are only important when the onset of disease is at or before age 50 years.[7] Because of these considerations, it remains a question to what extent a positive family history of PD increases the risk of PD among elderly, unselected people from a general population. We prospectively assessed the risk of PD as a function of family history of PD or dementia in the Rotterdam Study.

METHODS

The study was part of the Rotterdam Study, an ongoing prospective population-based cohort study on frequency, etiology and prognosis of chronic diseases in the elderly.[8]

Baseline examinations were in 1990-1993; follow-up examinations took place in 1993-1994 and 1997-1999. In addition, the cohort is continuously being monitored for major outcomes of interest through computer linkages with general practitioners' medical records and pharmacies automated medical record systems. Until the end of December 1999, the proportion lost to follow up was less than 1 percent.

Case ascertainment and diagnosis of PD

The cohort examined at baseline for symptoms of parkinsonism exists of 6969 participants and has been described extensively elsewhere.[9] Screening and diagnostic procedures were the same throughout the entire study. Briefly, all participants were interviewed about previous diagnosis of PD or anti-parkinsonian drug use and screened for symptoms of parkinsonism by study physicians at the research center. All participants who either used anti-parkinsonian drugs, reported that they had PD or had at least one possible cardinal sign of parkinsonism (resting tremor, bradykinesia, rigidity, and postural disturbances) at the screening examination, got a structural diagnostic work-up by a neurologist.[9] PD was diagnosed if at least two out of four cardinal signs and no other apparent cause of parkinsonism were present. For individuals who could not be re-examined in person for parkinsonism at follow-up, we obtained information through our surveillance system and checked their medical records.

Study population

The study population at risk for incident PD consisted of all persons who had a neurologic screening examination at base-line, were free of parkinsonism and dementia at that time and of whom base-line data on family history were available (n=6,341).

Data collection on family history

Data on family history for various diseases including PD and dementia among first degree relatives were obtained as part of the base-line data collection in the Rotterdam Study. All participants were asked to indicate for each family member whether that family member was affected by a specific disease. Formal pedigrees were constructed using data on year of birth and if applicable year of death of their parents, siblings, and children.

Data analysis

The follow up period ended either at the second screening examination, at age at onset of PD, parkinsonism or dementia, or at death. We used Cox proportional hazard models to estimate age and gender adjusted relative risks (RRs) with the corresponding 95% confidence intervals (95%CI) for a positive family history of PD. In addition, we calculated the risk of PD according to a positive family history for dementia.

RESULTS

The characteristics of the study population at risk for PD are listed in Table 1. In our study population 64.8 % were ever smokers. After a total follow up time of 33,887 personyears with a mean follow-up duration of 5.3 year we had identified 57 cases with PD. The personyears of follow-up, incidence rates and adjusted RRs are listed in Table 2. Persons with at least one relative with PD had a 1.5-time higher risk of PD whereas persons with at least two relatives had a five-time higher risk of PD, albeit not significant at the level of 5%. Adjusting for ever smoking status did not alter the point-estimates. The risk of PD was similar for persons with or without a positive family history of dementia.

Table 1
Baseline characteristics of the study population at risk of incident Parkinson's disease (PD).

Number of persons at risk of PD	6,341	
Mean age in years (± SD*)	68.5	(8.6)
Number of women (%)	3,731	(58.8%)
Number of persons with at least one relative with PD (%)	313	(4.9%)
Number of persons with at least two relatives with PD (%)	24	(0.4%)
Number of persons with at least one relative with dementia (%)	1497	(23.6%)

* SD = standard deviation

Table 2
The incidence of Parkinson's disease (PD) in persons with a family history of PD, and in those without such history with corresponding adjusted relative risk (RR) with 95 % confidence interval (95%CI) of PD.

Baseline family history	Persons at risk of PD*	Incident PD cases*	Person-years	Incidence rate**	RR (95%CI)
Persons without a relative with PD	6,028	53	32,181	1.7	1.0 (ref)
Persons with at least one relative with PD	313	4	1,706	2.3	1.5 (0.5-4.1)
Persons with at least two relatives with PD	24	1	110	9.0	5.1 (0.7-37.1)

*Numbers of individuals; ** Incidence rate in cases per 1,000 personyears; RRs adjusted for age and sex.

Discussion

This is the first prospective population based study that investigated the risk of PD among elderly subjects according to history of PD in first degree relatives. We found that individuals with first degree relatives with PD in particular with at least two affected first degree relatives had a higher risk to develop PD than persons without such a family history. This suggests that genetic factors do play a role in sporadic late-onset PD.

Our results did not reach statistical significance at the conventional 5% level, which asks for a cautious interpretation of our findings. There are a number of reasons why we may have failed to find a statistically significant effect. First, the power in our study was limited. Although the present prospective population-based study is the largest in its kind, the number of incident cases was still relatively small. Furthermore, only a small proportion of all participants reported a positive family history of PD among first degree relatives. Second, we had to rely on self-reported family history of participants. Due to the size and the age distribution of this cohort, it was not feasible to verify the family history of each participant. This probably may have introduced some misclassification of disease status of family members. Since all participants were free of PD or dementia at baseline and were, like the interviewers, not aware of their future disease status, this exposure misclassification was probably similar in those who developed PD and those who did not. Yet, it will have attenuated any existing effect. Third, since we only assessed family history at baseline and did not update the disease status of relatives

of our cohort members during follow-up, we will have misclassified the relatives who developed PD after our initial inquiry. Assuming that there is indeed familial aggregation of PD, this will have resulted in a more severe underestimation of the proportion with a positive family history of PD among those who actually developed PD, and hence in an underestimation of the relative risk. These considerations suggest that, if anything, we underestimated the relation between family history and the risk of dementia in this elderly population.

Our results seem at odds with a recently published twin study that suggested that heredity is not a major etiologic component in PD beginning after age 50 years.[7] However, that study was cross-sectional and an average of almost 10 years elapsed before the second twin was diagnosed with PD.[7] More concordant pairs may be identified if the twins are followed over time. Moreover, with increasing age at onset in the first affected twin, the probability increases that the second twin will not survive long enough to develop PD. The study of heredity of PD in twins with late-onset PD is difficult, and negative results should be interpreted cautiously. Evidence that genetic factors may play a role in familial PD with an onset after age 50 years came from a recent report on Parkin mutations in Europe.

In conclusion, our findings suggest that genetic factors contribute to the occurrence of sporadic PD in the elderly.

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3

Genetic association studies in the Rotterdam Study

3.1

N-ACETYLTRANSFERASE-2 POLYMORPHISM IN PARKINSON'S DISEASE

THE ROTTERDAM STUDY

Abstract

The N-acetyltransferase-2 (NAT-2) gene has been associated with Parkinson's disease (PD). The genotype associated with slow acetylation has been reported to be increased in PD patients. Three mutant alleles M1, M2, and M3 of the NAT-2 gene were investigated in 80 patients with idiopathic PD and 161 age matched randomly selected controls from a prospective population-based cohort study. The allelic frequencies and genotype distributions in cases were very similar to those found in controls. In controls the frequency of the wild type allele increased significantly with age suggesting that the mutant alleles are associated with an increased risk of mortality. These findings suggest that NAT-2 polymorphism is not a major genetic determinant of idiopathic Parkinson's disease, but may be a determinant of mortality in the general population.

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder among elderly people.[1,2] The etiology of PD still remains largely unknown but most likely results from interaction between genetic and environmental factors.[3-5] Several functionally relevant polymorphisms in xenobiotic metabolism have been studied in relation to patients with PD, but with no consistent results.[6,7] Explanations for these inconsistencies include different methodologies, different diagnostic criteria, poor selection of control groups and small sample sizes. Recently, an association of slow acetylators for the NAT-2 gene with PD was reported.[8] The NAT-2 gene, which maps to chromosome 8p22[9,10], is associated with speed of acetylation of certain drugs and xenobiotics.[9-11] Slow acetylators are homozygous for any of the mutant alleles and may be more susceptible to low-level environmental exposure to neurotoxins.[8] The finding of an increased frequency of subjects homozygous for the NAT-2 gene mutations is compatible with the view that

PD patients may be less capable to handle certain endogenous or exogenous toxins.[12] However, the findings on the NAT-2 gene were not confirmed.[13] The aim of this study was to investigate the possible association of the NAT-2 gene polymorphism in idiopathic PD.

METHODS

The study formed part of the Rotterdam Study, a prospective population-based cohort study on the frequency, etiology, and prognosis of chronic diseases. The cohort exists of 7,983 independently living or institutionalised inhabitants from a suburb of Rotterdam, the Netherlands, who are aged 55 years or older. The Rotterdam Study started in June 1990 and has been described extensively elsewhere.[14] Informed consent was obtained from each participant and the study was approved by the Medical Ethics Committee of Erasmus University Rotterdam. Participants were screened at baseline (1990-1993) and at follow-up (1993-1994) for symptoms of parkinsonism by study physicians. All screenpositives had a diagnostic investigation by a neurologist. PD was diagnosed in persons with at least two out of four cardinal signs (resting tremor, bradykinesia, rigidity, and postural disturbances) and no other apparent cause of parkinsonism.[2] In the Rotterdam Study 97 prevalent and 35 incident PD patients were identified until 1994. Blood samples for DNA extraction and genotyping were available for 80 patients (68 prevalent PD patients and 12 incident PD patients, mean age 77.3 (8.3), range 57.5-99.2, 27 males and 53 females). For each case we randomly selected two age matched (± 5 years) controls (mean age 76.8 (8.3), range 57.9-98.8, 63 males and 98 females) from the same study population who did not have PD and of whom baseline data regarding smoking history as well as blood samples were available. A polymerase chain reaction (PCR) was conducted using specific PCR primers for the NAT-2 gene.[15] The amplification products were digested using restriction enzymes, separated on an agarose gel and visualised with ultra violet light. We investigated the genotype of the NAT-2 gene using restriction enzymes *Kpn*I, *Taq*I and *BamH*I for mapping the three mutant alleles M1, M2, M3 and the wild type allele of the NAT-2 gene.[8] Genotyping was performed on coded samples without knowledge of the clinical diagnosis of the subjects. The mutations M1, M2, and M3 account for most of the slow acetylators in Caucasian patients.[16] Slow acetylators were defined as carrying any two of the mutant alleles M1, M2, and M3. Allele frequencies were determined by counting alleles and calculating sample proportions. Allele frequencies and genotype frequencies were compared using Chi-square statistics.

Table 1
Age stratified and overall frequencies of acetylator genotypes in PD patients and controls from the Rotterdam Study.

genotype	55-64		65-74		≥75		overall	
	control (n=14)	case (n=7)	control (n=46)	case (n=22)	control (n=101)	case (n=51)	control (n=161)	case (n=80)
WT/WT	0	0	0.11	0.14	0.04	0.06	0.06	0.07
WT/Mx	0.29	0.86	0.30	0.27	0.49	0.37	0.42	0.39
Mx/Mx	0.71	0.14*	0.59	0.59	0.47	0.57	0.53	0.54

WT = wild type, Mx = any mutant allele M1, M2, or M3. *significant lower proportion of slow acetylator genotype as compared to cases (OR = 0.067; 95%CI 0.06-0.75; Fisher Exact 2-sided P = 0.024).

Results

Genotype distributions were in Hardy-Weinberg equilibrium. Frequencies of mutations in cases and controls and stratified on age are listed in the table. Overall, the mutation frequencies were very similarly distributed among cases and controls.
The proportion of slow acetylators in the youngest age category, 55-64 years, was significantly lower in patients compared to controls. An interesting observation was that the frequency of the wild type allele in controls increased significantly with age ($P_{trend}<0.001$).

Discussion

Our results do not confirm the association between the slow acetylator genotype for the NAT-2 gene and PD as was found previously.[8] Sample size calculations with a 5% significance level showed that we had a power of 90% in our study to detect differences in genotype frequencies at an odds ratio level of 2.5 for slow acetylators as was found in the study of Bandmann et al.[8] This latter study was based on pathologically proven PD, whereas we used clinical diagnostic criteria to assess PD. One might consider that we misclassified some patients in our study and that this has biased our estimates. It is highly unlikely however that the resulting bias, if any, would be big enough to explain the discrepant findings. Moreover, the overall frequencies of slow acetylators in cases and controls found in our study are similar to that found in other studies.[13,17]

There are several other possible explanations for the discrepancy between our findings and those of Bandmann et al.[8] Firstly, they reported a significant association between the NAT-2 gene polymorphism and familial PD, but not with sporadic PD. However, the frequency of the slow acetylator genotype in sporadic PD in their initial analysis was significantly higher as compared to controls (odds ratio=2.45; p=0.003) and after correction for multiple comparison the point estimate of the association remained the same but only became borderline significant (odds ratio=2.45; p=0.06). Secondly, the association between the NAT-2 gene and PD may not be due to a causal relationship but rather to the NAT-2 gene being in linkage disequilibrium with a neighbouring gene that is involved in the etiology of PD. This explanation is in line with the finding of a lower proportion of slow acetylators in the early-onset group in the present study whereas Bandmann et al.[8] found a higher proportion of slow acetylators in PD patients as compared to controls. Thirdly, their controls came from a heterogeneous population submitted to the UK Parkinson's Disease Brain Bank and the brain bank at

the institute of Psychiatry, London, UK and were not matched for ethnic background. This could explain the relatively low frequency of slow acetylators found in their control population as compared to our study and other studies[13,17], and may have introduced some bias. Fourthly, we found that the frequency of the wild type allele in controls increased significantly with age suggesting that the mutant alleles are associated with an increased risk of mortality, possibly because of the increased risk of cancer for mutation carriers.[7,18] This suggests that bias may be introduced if cases and controls are not matched for age which was the case in the series of Bandmann et al.[8] where the mean age of the control population (77.1 years) was almost nine years higher than the mean age from patients selected from the familial cases (68.4 years). A final explanation could be that slow acetylators are not only more susceptible to neurotoxins which are inactivated by an acetylation reaction[8], but are simultaneously less susceptible to potential neurotoxins which are activated by acetylation. This dual activation-detoxification of the NAT-2 gene polymorphism makes the interpretation of any association debatable.[19]

The findings of this population-based study suggest that NAT-2 gene polymorphism is not a major genetic determinant of idiopathic PD, but may be a determinant of mortality in the general population.

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3.2

CYP2D6 POLYMORPHISM IN PARKINSON'S DISEASE

THE ROTTERDAM STUDY

Abstract

*The CYP2D6 polymorphism has been studied extensively in association with Parkinson's Disease (PD), with no consistent results. Several explanations, like differences in study design or bias in the selection of the control population have been offered for these inconsistent results. We designed a case-control study nested within a prospective population based cohort study in which cases and controls were sampled from the same source population. To assess the significance of the CYP2D6 gene in PD, we investigated two mutant alleles, CYP2D6*3 and CYP2D6*4, associated with poor metabolism and the wild type allele in 80 patients with PD and 156 matched controls, frequency matched on age and gender. No differences between cases and controls were found for the poor metabolizer genotype. However, we found that in contrast to earlier reports, the CYP2D6*4 mutant allele frequency was lower in cases as compared to controls, albeit not statistically significant. Our result supports the hypothesis that the CYP2D6 gene is not a major gene responsible for PD.*

INTRODUCTION

Parkinson's disease (PD) is a common disabling disorder among elderly characterized by a progressive neuronal degeneration in the nigrostriatal system.[1] Clinically, PD is characterized by the presence of resting tremor, bradykinesia, rigidity and postural disturbances. Several environmental and genetic factors have been suggested to play a role in the etiology of PD. Of all candidate genes studied thusfar, the CYP2D6 gene has been studied most extensively. It has been suggested that poor metabolizers of debrisoquine have an increased risk of PD.[2,3] After the initial report of Smith and colleagues in 1992[3], several studies regarding the association between PD and the CYP2D6 gene have been published with no consistent result.[4] A meta-analysis of the association between the CYP2D6 gene and PD showed a slightly increased, borderline significant,

risk of PD in poor metabolizers.[4] Exclusion of the only statistically significant study[3] increased the P-value of the meta-analysis however to 0.5. Despite all effort put in studying the association of the CYP2D6 gene with PD, the hypothesis that poor metabolizers are at greater risk of PD could neither be supported nor refuted.[5] Different diagnostic criteria across studies, different study designs, small sample sizes and in particular poor control selection may have played a role in these inconsistent results.

To avoid several epidemiological pitfalls in studying the CYP2D6 gene in PD, we designed a case-control study nested within a population based cohort study in which cases and controls were sampled from the same source population, to investigate the significance of the CYP2D6 gene in PD.

METHODS

This study was nested within the Rotterdam Study, a prospective population-based cohort study on the frequency, etiology, and prognosis of neurologic, cardiovascular, locomotor and ophthalmologic diseases. The Rotterdam Study started in June 1990 and has been described extensively elsewhere.[6] The cohort examined for symptoms of PD exists of 6,969 independently living or institutionalized inhabitants from a suburb of Rotterdam, the Netherlands, who are aged 55 years or older. Informed consent was obtained from each participant and the study was approved by the Medical Ethics Committee of Erasmus Medical Center Rotterdam. Participants were screened at baseline (1990-1993) and at follow-up (1993-1994) for symptoms of parkinsonism by study physicians. All screenpositives got a diagnostic work-up by a neurologist. PD was diagnosed in persons with at least two out of four cardinal signs (resting tremor, bradykinesia, rigidity, and postural disturbances) and no other apparent cause of parkinsonism.[7] In the Rotterdam Study 97 prevalent and 35 incident PD patients were identified until 1994. Blood samples for DNA extraction and genotyping were available for 80 patients (68 prevalent PD patients and 12 incident PD patients, mean age 77.3 (8.3), range 57.6-99.2, 29 males and 51 females) and 156 randomly selected controls, frequency matched on age (in 5 years age categories, mean age 76.6 (8.3), range 57.9-98.8) and gender from the same study population who did not have PD nor parkinsonism and of whom baseline data regarding smoking history as well as blood samples were available.

We investigated the CYP2D6 functional wild type allele (CYP2D6wt) and two defective mutant alleles classified as CYP2D6*3 and CYP2D6*4[8] in association with PD. These two mutant alleles compromise approximately 90% of the poor metabolizers among Caucasians in Europe.[9] Genotyping was performed similarly to a previously re-

ported study[3] on coded samples without knowledge of the clinical diagnosis of the participants. For the evaluation of the genotype distribution, the CYP2D6 alleles were classified according to functional activity as wild type (wt) and mutant (mut). Individual genotypes were characterized as either homozygous for the wild type (wt/wt), heterozygous (mut/wt) or homozygous mutant (mut/mut). Allele frequencies were determined by counting alleles and calculating sample proportions. Allele frequencies and genotype distributions were compared using Chi-square statistics. Logistic regression analysis methods were used to calculate odds ratios (ORs) with 95% confidence intervals (95%CI) for the association between the heterozygous and homozygous mutant genotypes and PD as compared to controls. The homozygous wt genotype was used as the reference category. In the analysis we adjusted for age, sex and ever smoking.

RESULTS

Genotype distributions in controls were in Hardy-Weinberg equilibrium. The distribution of the CYP2D6 genotypes and alleles in cases and controls are listed in Table 1. No significant differences were found for the allele frequencies between cases and controls. However we observed that the frequency of the CYP2D6*4 allele was unexpectedly lower (OR = 0.72; 95%CI 0.45-1.19) in cases as compared to controls, albeit

Table 1
Distribution of CYP2D6 genotypes and alleles in Parkinson's disease (PD) and controls.

	Controls		PD-cases	
genotypes	n	frequency	n	frequency
wt/wt	90	0.58	57	0.71
wt/*3	48	0.31	15	0.19
*3/*3	11	0.07	5	0.06
wt/*4	7	0.04	0	0
*3/*4	0	0	3	0.04
Total	156	1	80	1
alleles				
wt	235	0.753	129	0.806
CYP2D6*3	7	0.022	3	0.019
CYP2D6*4	70	0.225	28	0.175
Total	312	1.00	160	1.00

wt = wild type allele; *3 = CYP2D6*3 (basepair deletion); *4 = CYP2D6*4 (G-A transition)

Table 2
Genotype distributions of the CYP2D6 gene in PD cases and controls and the association between CYP2D6 genotypes and PD as compared to controls.

	Controls	Cases	OR (95%CI) adjusted for:	
Genotype	frequency (n=156)	frequency (n=80)	age & sex	age, sex & smoking
wt/wt	0.58	0.71	ref.	ref.
wt/mut	0.35	0.19	0.43 (0.22-0.83)	0.37 (0.18-0.76)
mut/mut	0.07	0.10	1.18 (0.44-3.15)	1.28 (0.47-3.43)

$\chi^2=6.99$; p = 0.03; df=2
n = number of study participants; ref. = reference category
wt = wild type; mut = mutant alleles CYP2D6*3 = basepair deletion; CYP2D6*4 = G-A transition

not statistically significant. No significant differences between cases and controls were found for the CYP2D6*3 allele (Fisher's exact test two tailed, p=1.0). The distribution of the pooled heterozygous or homozygous mutant genotypes and corresponding ORs with 95%CI for the association between the heterozygous or homozygous mutant genotypes in PD as compared to controls are presented in Table 2. Among PD patients there were 57.6 percent ever smokers and among controls 50.8 percent.

Overall, we observed significant differences in the genotype distribution between cases and controls ($\chi^2=6.99$; p=0.03; df=2). This occurred merely due to a lower frequency of the heterozygous genotype in cases as compared to controls. The frequency of the heterozygous genotype was significantly lower (p=0.01) among patients with PD as compared to controls whereas the homozygous mutant genotype was slightly increased in the cases, however not significantly.

Discussion

Within this population based cohort study we found a significantly lower frequency of the heterozygous genotype among patients with PD as compared to controls from the same source population. We did not observe a significant difference in the frequency of the homozygous mutant genotype between cases and controls. The frequency of the CYP2D6*4 allele was lower among patients with PD as compared to the control population. These results were contrary to the initial report of Smith and colleagues[3] and also in contrast to a recently published meta-analysis on the CYP2D6 genotype and

PD.[4] The CYP2D6*4 association with PD in our study was not consistent with a genetic causal mechanism because the decrease in frequency was found for the heterozygotes but not for the homozygotes.

The strength of the present study is the use of a population based cohort with systematic data collection and classification of PD. All participants were examined in person and diagnosis of PD was made by neurologists. Controls were, in contrast to most other studies of the CYP2D6 gene in PD[3,4], randomly selected from the same source population where the cases came from and frequency matched on age and gender. This minimizes the probability of bias due to poor control selection. A limitation is the number of cases which is relatively small and hence yields little power.

How should we interpret our findings and the discrepancies across studies? Firstly, since so many studies between PD and the CYP2D6 gene have been published without consistent results and our results also do not provide evidence for an association between the CYP2D6 gene and PD, it is most likely that this gene does not affect the susceptibility to PD. A second explanation for the inconsistent results could be that PD is a genetically heterogeneous disorder. In a genetic complex trait like PD it could be possible that more than one genetic factor may determine the risk of PD. This complicates the reproducibility of a candidate gene association study, like the CYP2D6 gene in PD, in different populations with different genetic background and ethnic origin. Thirdly, difficulties reproducing candidate gene studies may result from interaction with environmental factors. Smoking, for example, has been inversely associated with PD and poor metabolizers may smoke less.[10] This, in part, could explain the conflicting findings due to confounding by smoking, since most studies[4] did not adjust for smoking in their analysis. However, adjusting for smoking in our analysis did not alter our results. Other factors that might interact with the CYP2D6 gene in causing PD include exogenous neurotoxins like MPTP[11] or pesticides[12] or endogenous isoquinolines.[13] Hitherto, there is no clear evidence that these interactions may cause PD. Finally, the CYP2D6 gene may be in linkage disequilibrium with another susceptibility locus in the same region. Up to present there is one report that tested the hypothesis of linkage disequilibrium and provided evidence that the CYP2D6 gene may be in linkage disequilibrium with another, yet unidentified locus responsible for susceptibility to PD.[14] Our results do not support the hypothesis of linkage disequilibrium since we would expect a lower proportion of both the heterozygous genotype and homozygous mutant genotype in cases as compared to controls which was not the case.

Although we cannot fully exclude that the CYP2D6 gene may have an effect in a subgroup of PD patients through interaction with exogenous or endogenous toxins, we can conclude that the CYP2D6 gene is not a major gene in PD in the elderly.

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3.3

APOE AND THE RISK OF PARKINSON'S DISEASE WITH OR WITHOUT DEMENTIA IN A POPULATION-BASED STUDY

Abstract

The objective of this study was to investigate the association between apolipoprotein-E genotype (APOE) and Parkinson's disease (PD) with or without dementia. The study formed part of the Rotterdam Study, a prospective population based cohort study on the frequency, etiology, and prognosis of chronic diseases. The cohort examined for PD exists of 6969 independently living or institutionalized inhabitants from a suburb of Rotterdam, the Netherlands, who are aged 55 years or older. All participants were screened at baseline (1990-1993) and at follow-up (1993-1994) for symptoms of parkinsonism by study physicians and screenpositives got a diagnostic work-up by a neurologist. APOE genotyping was available for 107 PD patients (26 with and 81 without dementia) and 4805 non-parkinsonian controls. The presence of at least one ε2-allele significantly increased the risk of PD (OR = 1.7; 95%CI:1.0-2.8). When we looked separately for demented and non-demented PD patients as compared to non-parkinsonian controls, APOE appeared not associated with PD without dementia, but both the ε2-allele and the ε4-allele increased the risk of PD with dementia (OR = 5.6; 95%CI:2.0-15.2 and OR = 3.6; 95%CI:1.3-9.9 respectively). The risk of dementia for ε4-allele carriers was not significantly different for persons with or without PD. However, the ε2-allele strongly increased the risk of dementia in patients with PD (interaction p < 0.007).

We conclude that in the elderly the APOE ε2-allele increases the risk of PD and in particular the risk of PD with dementia.

INTRODUCTION

It has been suggested that Parkinson's disease (PD) and Alzheimer's disease (AD) share clinical and neuropathologic features.[1-3] It is known that PD patients frequently develop dementia, and AD patients frequently develop parkinsonism. Both are age-related disorders characterized by intraneuronal inclusion bodies.[4]

The apolipoprotein ε4-allele has been associated with both AD[5] and Lewy Body disease (LBD).[6,7] Reports on APOE genotype and PD are not consistent. Some studies

reported an association between the apolipoprotein ε4-allele and PD or PD with dementia whereas others did not.[8,9] In the majority of these studies little attention has been paid to the ε2-allele and PD. However, in most of these studies, but not all,[10] the ε2-allele frequency was higher in PD patients,[8, 11-13] or in PD patients with dementia,[14,15] albeit not statistically significant. Recently a significant association of the ε2-allele with sporadic PD has been reported, however no subdivision was made between demented and non-demented PD patients.[16] We studied differences in the APOE genotype distribution among PD patients with and without dementia as compared to non-parkinsonian controls from the Rotterdam Study.

METHODS

Study population

The study formed part of the Rotterdam Study, a prospective population based cohort study on frequency, etiology and prognosis of neurologic, cardiovascular, locomotor and ophthalmologic diseases as has been described previously.[17] Briefly, all inhabitants of a suburb of Rotterdam, aged 55 years and over, including those living in the homes for the elderly, were invited to participate. A total of 7983 (response rate 78 percent) individuals agreed to participate and of these, 6969 (68%) individuals participated in the screening of parkinsonism at baseline (1990-1993) as described extensively elsewhere.[18] Informed consent was obtained from each participant and the study was approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam, The Netherlands.

Case ascertainment and diagnosis of PD

All 6969 participants were interviewed about previous diagnosis of PD or anti-parkinsonian drug use and screened for symptoms of parkinsonism by study physicians at the research center. All individuals who either used anti-parkinsonian drugs, reported that they had PD or had at least one possible cardinal sign of parkinsonism (resting tremor, bradykinesia, rigidity, and postural disturbances) at the screening examination, got a structural diagnostic work-up by a neurologist. PD was diagnosed in persons with at least two out of four cardinal signs and no other apparent cause of parkinsonism.[18] At baseline of the 129 individuals with parkinsonism 98 had PD. The causes of parkinsonism were: parkinsonism associated with dementia (9); drug induced parkinsonism

(3); parkinsonism related to vascular disease (1); multi system atrophy (2); progressive supranuclear palsy (1) and other parkinsonism (15) which included parkinsonism and dementia with no clear time relationship between dementia and parkinsonism or more than one possible cause as well as those subjects in whom all causes of PD could be excluded but who had not shown any progression over more than 15 years in the course of disease and who did not respond to antiparkinsonian drugs.[18] At follow-up (1993-1994), 6840 participants, screened for symptoms of parkinsonism at baseline, were at risk to develop parkinsonism. Participants who were demented at baseline, were considered at risk for parkinsonism but not for PD. Follow-up information was available on 6778 (99%) individuals either through completely re-examination at the research center or through our surveillance system that continuously monitors the total cohort for interval cases of parkinsonism. Through this surveillance system that consists of computer linkages with general practitioners and pharmacies automated medical record systems, we were notified of incident cases of parkinsonism, including PD, and had access to their medical records. A total of 5,310 participants were completely re-examined similarly to the two-phase design used at baseline. Of those who could not be re-examined, 449 had died, 558 refused screening examinations and in 461 the screening examination was incomplete. In the follow-up period, 62 individuals with parkinsonism were identified of whom 35 had PD, three of whom with dementia. The other causes of parkinsonism were very similar to those at baseline: parkinsonism with dementia (8 of whom 6 were already demented at baseline); drug induced parkinsonism (1); multi system atrophy (2) progressive supra nuclear palsy (1) and other parkinsonism (15) which included parkinsonism and dementia with no clear time relationship between dementia and parkinsonism (7), subjects with more than one possible cause, and subjects in whom all other causes of parkinsonism could be excluded but who did not respond to anti-parkinsonian drugs (8). Neuroimaging was only performed in subjects in which the cause of parkinsonism was not obvious from physical examination alone (n=20, 35%).

In total 133 individuals were identified with PD of whom 107 patients had the APOE genotype. Of these 107 patients 26 were diagnosed as PD with dementia and 81 as PD without dementia. From the 26 PD patients with dementia, in nine individuals the diagnosis was obtained from general practitioners in which eight were confirmed by a neurologist not affiliated to our institute. From the 81 PD patients without dementia, in 12 individuals the diagnosis was obtained from the general practitioner in which 10 were confirmed by a neurologist. All other PD patients were seen in person and the diagnosis was made by the neurologists affiliated to our department. In total six of the

61 PD patients with APOE genotyping who were not demented at baseline developed dementia at follow up.

Diagnosis of dementia

A three-phase design was used, both at baseline and at follow-up, to diagnose dementia according to the American Psychiatric Association's criteria (DSM-III-R)[19] and has been described elsewhere.[20,21] Briefly, all participants were screened with a cognitive test; those who screened positive underwent further neuropsychological testing and those suspected for dementia were examined in detail and, if possible, had a Magnetic Resonance Imaging brain scan made. Of subjects who could not be examined in person, diagnosis was obtained from the general practitioner and medical records through our surveillance system. For the diagnosis PD with dementia, the onset of PD had to clearly precede the onset of dementia.

Apolipoprotein E genotyping

APOE genotyping was performed on coded DNA-samples of the total cohort as described previously,[22] without knowledge of the clinical diagnosis. In summary, polymerase chain reaction was conducted and the amplification products were digested with H*ha*I, separated on an agarose gel, stained with ethidium bromide and visualized with UV-light. The results were analyzed by three independent experts. The APOE genotyping was repeated in case of discrepancies.

Data analysis

We used multivariate logistic regression analysis to calculate the odds ratios (OR) with 95% confidence intervals (95%CI) to assess whether the presence of at least one ε4-allele (APOE*4) or at least one ε2-allele (APOE*2) was associated with PD, with or without dementia as compared to non parkinsonian controls from the Rotterdam Study. In this logistic model PD was the dependent variable whereas APOE genotype (with dummy indicating APOE*4, APOE*2 and APOE3E3) was the independent variable. The most frequent genotype APOE3E3 was used as the reference. Additionally we used multivariate logistic regression analysis to examine the risk of dementia in PD patients as compared to non-parkinsonian controls, stratified by APOE genotype. In this model dementia was the dependent variable whereas PD, APOE genotype and the multiplicative interaction between PD and APOE were used as independent variables.

To test whether our results were not merely due to misclassification of AD as PD with dementia, we conducted similar analyses for patients with dementia with parkinsonism from the same study population. The diagnosis dementia with parkinsonism was made for patients with at least two cardinal signs, with no other apparent cause of parkinsonism except dementia taking into account that the onset of dementia had to clearly precede the onset of parkinsonism or both were diagnosed at the same time. Moreover, to rule out the possibility of survivorship bias as an alternative explanation for our results, we used COX proportional hazard models to firstly determine prospectively if the presence of an ε2-allele or an ε4-allele increased the risk of PD and secondly to determine the risk of the development of dementia during longitudinal follow-up in PD patients who were not demented at baseline. In the first analysis PD was the dependent variable and the APOE genotype the independent variable, whereas in the second analysis dementia was the dependent variable and APOE genotype the independent variable. In all analyses we adjusted for age at examination at baseline and gender and we excluded subjects with the APOE2E4 genotype since this genotype may obscure differences between APOE*2 and APOE*4.

RESULTS

The characteristics of the study population and the APOE genotype distributions are summarized in Table 1. After exclusion of participants with the APOE2E4 genotype (three patients with PD and 132 non-parkinsonian participants), APOE genotype for the analyses was available for 104 PD patients (22 prevalent and three incident PD with dementia and 55 prevalent and 24 incident PD without dementia) and 4673 non-parkinsonian controls. All genotype distributions were in Hardy-Weinberg equilibrium. The crude and adjusted ORs with 95%CI for the associations between the APOE genotype with PD, with and without dementia are listed in Table 2. Persons with at least one ε2-allele had significantly more often PD (OR=1.7; 95%CI: 1.0-2.8). We found no association between the ε4-allele and PD (OR=1.0; 95%CI: 0.6-1.6). For non-demented PD patients as compared to non-parkinsonian subjects, no association was found for either the ε2-allele (OR=1.2; 95%CI: 0.7-2.2) or the ε4-allele (OR=0.7; 95%CI: 0.4-1.2). However, both the ε2-allele (OR=5.6; 95%CI: 2.2-15.2) and the ε4-allele (OR 3.6; 95%CI: 1.3-9.9) strongly increased the risk of PD with dementia. To assess whether these findings could be explained by misclassification of AD as PD with dementia, we performed similar analyses for 27 patients with dementia with parkinson-

Table 1
The characteristics and the distribution of the APOE genotype and alleles of the study population.

	All PD (n = 107)	PD without dementia (n = 81)	PD with dementia (n = 26)	Dementia with parkinsonism (n = 27)	Controls (n = 4805)
age (SD)	77.4 (8.0)	75.8 (7.8)	82.1 (6.7)	84.0 (6.0)	69.0 (8.6)
proportion men (%)	35.5	37.0	30.8	29.6	44.8
APOE genotype frequency % (n)					
apoe ε3/ε3	54.2 (58)†	62.9 (51)	26.9 (7)	37.0 (10)	57.9 (2783)
apoe ε2/ε2	0.9 (1)	0 (0)	3.8 (1)	3.7 (1)	0.8 (38)
apoe ε2/ε3	20.6 (22)	17.3 (14)	30.7 (8)	11.1 (3)	13.0 (625)
apoe ε2/ε4	2.8 (3)	2.5 (2)	3.8 (1)	0 (0)	2.7 (132)
apoe ε3/ε4	20.6 (22)	16.1 (13)	34.6 (9)	44.5 (12)	23.0 (1106)
apoe ε4/ε4	0.9 (1)	1.2 (1)	0 (0)	3.7 (1)	2.5 (121)
APOE allele frequency					
apoe *2	12.6 (27)‡	9.9 (16)	21.2 (11)	9.3 (5)	8.7 (833)
apoe *3	74.8 (160)	79.6 (129)	59.6 (31)	64.8 (35)	75.9 (7297)
apoe *4	12.6 (27)	10.5 (17)	19.2 (10)	25.9 (14)	15.4 (1480)

†numbers of subjects are given in parentheses.
‡numbers of alleles are given in parentheses.

Table 2
The association between the APOE genotype and Parkinson's disease (PD) with and without dementia and dementia with parkinsonism.

APOE genotype	All PD (n=104)	PD without dementia (n=79)	PD with dementia (n=25)	Dementia with parkinsonism (n=27)
APOE3E3	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
APOE*2				
crude	1.67 (1.01-2.72)	1.15 (0.63-2.09)	5.40 (2.00-14.54)	1.68 (0.53-5.37)
adjusted*	1.69 (1.03-2.80)	1.18 (0.65-2.17)	5.55 (2.02-15.22)	1.69 (0.52-5.55)
APOE*4				
crude	0.90 (0.55-1.46)	0.62 (0.34-1.13)	2.91(1.08-7.85)	2.95 (1.28-6.74)
adjusted*	0.98 (0.60-1.61)	0.67 (0.36-1.22)	3.60 (1.31-9.87)	4.10 (1.73-9.74)

*Values are odds ratios adjusted for age and gender with 95% confidence intervals in parentheses.

ism. We found a strong association for the ε4-allele (OR=4.1; 95%CI: 1.7-9.7) as expected, but no association for the ε2-allele (OR=1.7; 0.5-5.6) with dementia with parkinsonism (Table 2). When we restricted ourselves to incident cases of PD, we found results that were similar to those found for the entire group of PD (adjusted OR for at least one ε2-allele: OR=2.0; 95%CI: 0.8-5.2, for at least one ε4-allele: OR=1.5; 95%CI: 0.6-3.6). When we looked prospectively at the risk of dementia in PD patients not demented at baseline, we found that the presence of at least one ε2-allele strongly increased the risk of dementia (OR=13.5; 95%CI: 1.3-136.2); for presence of at least one ε4-allele we found no such relation (OR=1.5; 95%CI:0.6-3.7).

The ORs with 95%CI for the association between PD and dementia stratified on the APOE genotype are listed in Table 3. Overall, patients with PD had an almost three times higher risk of dementia as compared to non-parkinsonian participants. This appeared entirely due to the increased risk of dementia among PD patients with either an APOE*4 or APOE*2 genotype. The strength of the association between PD and dementia in the APOE*2 stratum as compared to the APOE*4 stratum or the APOE3E3 stratum, suggested multiplicative interaction between PD and the APOE*2 genotype. Adding a multiplicative interaction term of PD and the APOE genotype to the multivariate model, revealed a strong interaction between the APOE*2 genotype and PD ($p<0.007$).

Finally, we verified the well-established association of the ε4-allele with dementia in the control population (n=4763). As expected, the presence of at least one ε4-allele significantly increased the risk of dementia (OR=2.2; 95%CI: 1.7-2.9), whereas the ε2-allele was not associated with dementia (OR 0.8; 95%CI: 0.5-1.2).

Table 3
The risk of dementia in Parkinson's disease patients stratified by the APOE genotype.

APOE genotype	Overall (n=4777)	APOE3E3 (n=2841)	APOE*4 (n=1250)	APOE*2 (n=686)
Non-parkinsonian controls	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
PD cases				
crude	6.2 (4.1-9.3)	2.2 (1.0-4.9)	7.3 (3.1-17.2)	14.6 (5.8-36.5)
adjusted*	2.8 (1.8-4.5) (n=104)	0.9 (0.3-2.1) (n=58)	2.5 (0.9-6.8) (n=23)	7.7 (2.2-27.3) (n=23)

*Values are odds ratios adjusted for age and gender with 95% confidence intervals in parentheses.

Discussion

In this study among the elderly we found that carriers of the apolipoprotein ε2-allele had a significantly increased risk of PD, in particular of PD with dementia, and that presence of at least one ε2-allele multiplied the risk to develop dementia in PD patients. The apolipoprotein ε4-allele was not associated with PD overall, but, as expected, the ε4 allele increased the risk of dementia in PD patients in a very similar way as it increases the risk of dementia in the general population. No associations were found for the APOE genotype with PD without dementia.

One should consider whether our findings could result from bias, in particular misclassification, selection or survival bias. We consider misclassification of AD or LBD patients as PD patients with dementia unlikely, as we carefully restricted the diagnosis PD to those in whom the onset of parkinsonian signs had clearly preceded the cognitive changes in absence of other clinical features. Moreover, if misclassification would have occurred, it might, in part, account for the associations we found for the ε4-allele but not for the ε2-allele. Selection bias is a potential threat in association studies. In contrast to other studies,[8,9] our control population was both derived from the same source population as the PD patients and based on a general, not necessarily healthy, elderly population, including institutionalized persons. This minimized the possibility of selection bias in our study. Finally, since the ε2-allele has been reported to be a genetic factor for longevity,[23] one should consider that our findings might be a result of selective survival. In order to rule out bias due to selective survivorship we looked at the risk of dementia prospectively in non-demented PD patients and found that presence of the ε2-allele did increase the risk of the development of dementia in non-demented PD patients. Although the confidence interval of the point estimate was wide, mainly due to the small sample size, these results confirm the overall findings. Moreover, we studied survival of carriers of the ε2-allele in the Rotterdam Study, but found no association between APOE*2 and survival (unpublished data). Hitherto, only one study reported an association between the ε2-allele and PD, however without distinguishing between PD patients with or without dementia.[16] Nevertheless, when we reviewed the studies that investigated APOE genotype in relation to PD, we found that in most of them the frequency of the ε2-allele in PD patients,[8, 11-13] or in PD patients with dementia[14,15] was actually increased, albeit not statistically significant. These other observations corroborate our view that the association that we found for the ε2-allele with PD with dementia is true. However, the population screened in this survey was aged 55 years and over and therefore our findings may not generalize to younger patients with PD.

It is as yet unclear what might be the underlying pathologic mechanism. Whereas the ε4-allele occurs at an increased frequency in AD,[24] and β-amyloid plaques and neuro-fibrillary tangles (NFT's) are increased in the brains of individuals with the ε4-allele as compared to those who lack the ε-4 allele,[25,26] the ε2-allele does not increase the risk of AD. If anything, it has been suggested to play a protective role in AD and senile plaque formation.[24,27] Interestingly however, recently a high frequency of the ε2-allele in a subset of NFT-predominant senile dementia has been reported.[28] The authors hypothesized that this type of dementia was distinct from AD.[28] Moreover, the ε2-allele has been associated with Argyrophilic Grain Disease,[29] and an association of the ε2-allele with cerebral amyloid angiopathy has been reported.[30]

The strong association of APOE*2 with PD with dementia and not with PD without dementia or dementia with parkinsonism and the observation that the ε2-allele strongly increases the risk of dementia in non-demented PD patients suggests that PD with and without dementia have, at least partly, a different pathogenesis. Further research to elucidate a possible specific role of the ε2-allele in neurodegeneration is required.

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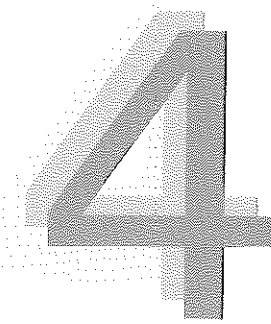

The European sib pair study

4.1

STUDY DESIGN FOR A GENOME-WIDE SCREEN FOR SUSCEPTIBILITY GENES IN PARKINSON'S DISEASE IN EUROPE AND LINKAGE RESULTS ON CHROMOSOME 2P AND 4

Abstract

In the last few years evidence increased that genetic factors influence the occurrence of Parkinson's disease (PD). We established a European Consortium on Genetic Susceptibility in PD (GSPD) with the objective to study other, not yet identified, susceptibility genes in affected sib pairs with PD. We present here the overall design of the study and results from genetic analyses on three chromosomal regions that are of interest in PD (2p, 4p, 4q).

The GSPD-study started in 1995 with the recruitment of families with at least two affected siblings with PD and was conducted in five European countries. The diagnosis PD was made if proven pathologically or if three out of the four cardinal signs (bradykinesia, rigidity, resting tremor, asymmetry of signs at onset), at least a 30 % improvement with levodopa and no exclusion criteria were present. All the affected family members were personally examined, videotaped and blood samples were taken using a standardised protocol. In addition blood was collected from parents and unaffected siblings. Up to the end of 1999 a total of 194 affected sib pairs from 176 families was identified. For the current analyses regarding chromosome 2p, 4q and 4p we genotyped 125 highly informative families with PD using 12 DNA-markers.

We observed a nominal p-value lower than 0.05 for the pairwise lod score on D2S1394, suggesting the presence of a susceptibility region. Country-specific lod scores suggested possible linkage to the PARK3 locus for German sib pairs.

We have successfully established a European Consortium and recruited a large number of affected sib pairs with PD for a genome wide search for susceptibility genes in PD. Our initial analyses did not reveal significant susceptibility regions on Chr 2p, 4p and 4q.

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder among the elderly.[1,2] In Europe the overall age-standardised prevalence for PD in subjects 55 years or older

is 1.6 per 100, with an increasing frequency up to 4.3% in those aged 85 years and over.[2] Despite intensive efforts, the cause of PD remains still largely unknown and treatment is symptomatic with only temporary results. However, there is increasing evidence that genetic factors play an important role in the etiology of PD.

For many decades several investigators reported family clustering of PD.[3,4] Familial linkage studies and candidate gene studies have been carried out to investigate the role of genetic factors and the pattern of inheritance in PD, but the results are controversial.[3,4] The first evidence that PD can be inherited as a single gene defect came when a mutation in the α-synuclein gene was identified that caused PD in the Contursi kindred, a large family with PD with an autosomal dominant inheritance pattern.[5] Since then, another mutation in α-synuclein,[6] a mutation in UCH-L1[7] and two loci (2p13[8] and 4p14-16.3[9]) have been found in relation to autosomal dominantly inherited PD. Furthermore, a wide variety of mutations in the Parkin gene responsible for autosomal recessive juvenile parkinsonism (AR-JP) have been reported.[10,11] The PD genes reported so far provide convincing evidence that PD is a genetically heterogeneous disorder, yet they account for only a minority of familial cases of PD.[12-15] It is likely that several other yet not identified genes are responsible for genetic susceptibility to PD. We therefore started a European collaborative study on affected siblings to further investigate genetic factors in PD. The affected sib pair (ASP) method does not need an assumption about the mode of inheritance and is therefore an appropriate strategy for mapping genes of a complex disease trait such as PD.[16-18] The European Consortium on Genetic Susceptibility to Parkinson's Disease (GSPD), consisting of five clinical partners and four lab partners, was established in 1995 and aimed to recruit ASPs and large families with PD for a genome-wide search for susceptibility genes in PD.

The first analysis was conducted on chromosomal regions that were of potential interest, because of earlier findings related to PD with loci on 4q21-q23 (α-synuclein gene),[5] 4p14 (UCH-L1 gene),[7] 4p14-16.3 (susceptibility locus)[9] and 2p13 (susceptibility locus).[8] We report here the overall design of the GSPD study as well as the results of the first genetic analyses on chromosome 2p, 4p and 4q.

METHODS

Families

The GSPD-study, which is still ongoing, started in 1995 with the recruitment of families with PD and was conducted in France, Germany, Italy, the Netherlands and the

United Kingdom. Different established links and communication networks were used to identify families with at least two affected sibs with PD. In France, a network of senior clinicians from 15 neurological departments of university hospitals was established to identify families with PD. In Germany families were ascertained through university departments of Neurology and the German PD lay organisation. In Italy families were identified through a case control study of PD in Naples, studies of familial PD in Rome, and a collaborative network of neurologists interested in PD from the Italian Neurological Society, the Italian League against PD and two patients organisations. In the Netherlands all neurologists were requested to notify affected families to the study centre in Rotterdam. In the UK all neurologists were requested to notify families with PD to the centre in London. Moreover, the PD society (a lay organisation), the PD research group (a network of clinicians interested in PD research) and the British Neurological Surveillance Unit were asked to identify families.

Ascertainment and diagnostic procedures.

In all countries the affected family members were visited either at home or in a hospital for an interview, blood sampling and a clinical examination. This was done by a clinician with experience in the diagnosis of PD, using a standard protocol. All patients were videotaped according to a standard procedure. Any doubtful case was discussed with other reviewers experienced in the diagnosis of PD and a consensus agreed as to include or exclude the patients concerned. Patients and their affected relatives were included in the study if PD was proven pathologically or if they met the following rigorous diagnostic criteria for clinically definite PD: (1) at least three out of four cardinal signs (bradykinesia, rigidity, resting tremor, asymmetry of signs at onset), and (2) at least a 30 % improvement with levodopa therapy, and (3) no exclusion criteria. Exclusion criteria were: supranuclear palsy, pyramidal syndrome, cerebellar signs, dyspraxia, severe early loss of postural reflexes, prominent early urinary symptoms, significant postural hypotension, a mini mental state examination (MMSE) score of less then 24/30 within two years of onset, neuroleptic drug ingestion in the six months prior to onset, encephalitis or possible toxic exposure in the six months prior to onset. If the age of onset was younger then 40 years, Wilson's disease and other metabolic causes of parkinsonism had to be excluded too. From each participant, 30-ml venous blood was sampled for DNA extraction. In order to determine allele segregation in the families, we collected blood from parents and unaffected siblings, if available. If both parents were alive, we did not collect blood samples from unaffected siblings. A sib was defined as unaffected if he or she did not show clinical characteristics compatible with PD. If one

parent was alive, we collected blood samples from this parent and two unaffected siblings. If parents were not alive anymore, we collected blood from up to four unaffected siblings if available. All participants gave their informed consent according to the Declaration of Helsinki for examination, videotaping, bloodsampling and genotyping. Each partner obtained ethical approval for this project from his or her institutional ethical committee.

Datamanagement

Three databases were designed for accurate storage and transfer of data for appropriate data analyses. All clinical information is stored in a standard database format, centralised in Paris (AD), that will allow the study of phenotype/genotype correlations. All core information on DNA-samples and family trees is stored in the central database in London (NW). Results of the genotyping are stored in the genotype database in Paris (MM). This database is used to perform the linkage analyses and to control inter-lab consistency.

Genotyping

Four partners from Paris (AB), Munich (TG), Rotterdam (BA) and London (NW) are participating in the laboratory work. At each lab centre and the Italian clinical centre, DNA was extracted from venous blood samples using standard techniques and send to the repository in London. We analysed three regions on chromosome 2p, 4p and 4q in 125 highly informative families with ASPs using the markers D2S441, 2S2109, D2S1394, D4S2397, D4S391, D4S1609, D4S230, D4S405, D4S3350, D4S1647, D4S1578 and D4S2380 from the Génethon map for the genotyping. In all families DNA of parents or unaffected siblings was available to determine allele segregation. Techniques for semi-automated DNA amplification using the polymerase chain reaction and fluorescent oligonucleotides primers to analyse fragment sizes on an automated sequencer (ABI) were used for the genotyping.[19,20]

Data-analysis

Marker allele frequencies were set to their maximum likelihood values estimated in the data with the computer program VITESSE.[21] Pairwise and multipoint model free linkage analyses were conducted with the SIBPAIR[22] and the MLBGH[23,24] programs, respectively. These programs provide a likelihood-based test statistic for linkage which applies to the whole sibship of affected siblings. The pairwise statistic is equivalent to

the LOD score calculated under the assumption of a simple recessive disease model.[25] To test for linkage, the likelihoods over all families are maximised as a function of the rate of marker alleles IBD (y) among affected siblings, and the likelihood ratio test statistic is calculated against the null hypothesis of no linkage (y=0.5). The statistic follows a chi-square distribution with one degree of freedom (df) and can thus be expressed as a LOD score.

RESULTS

Up to the end of 1999, 246 families were recruited with familial PD. In total 43 families were excluded because one or both of the affected sibs did not (yet) fulfil our stringent criteria, mainly did not receive L-DOPA treatment. Another 27 families were excluded because they had mutations in the Parkin gene (unpublished data). A total of 176 ASP families fit the criteria for inclusion in the genome-wide screen. The description of families and country of origin are presented in Table 1. We have 167 families with two affected siblings (167 ASP) and 9 families with three affected siblings (27 ASPs), hence a total of 194 ASPs out of 361 patients with PD. The clinical characteristics of all patients included in the GSPD study thusfar are listed in Table 2.

At present only two point LOD scores were available. Multipoint LOD scores were not available due to uncertainties on marker location and order.

Table 1
Characteristics and origin of the sib pair families.

Origin	number of families with 2 affected sibs	number of families with 3 affected sibs	total number of families	total number of affected sib pairs
Germany	21	1	22	24
France	59	2	61	65
Italy	29	0	29	29
Netherlands	26	2	28	32
UK	32	4	36	44
Total	174	9	176	194

Table 2
Clinical characteristics of the 366 patients with definite Parkinson's disease in 198 sib pairs

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

UPDRS, Unified Parkinson's Disease Rating Scale; on, under treatment; off, without treatment; MMSE, Mini Mental State Examination

Table 3
Overall pairwise LOD scores.

Chr 2p		Chr. 4p		Chr 4q	
marker	LOD score	marker	LOD score	marker	LOD score
D2S441	0.09	D4S2397	0.06	D4S1647	0
D2S2109	0.13	D4S391	0.05	D4S1578	0.006
D2S1394	1.61	D4S1609	0.14	D4S2380	0
		D4S230	0.02		
		D4S405	0.04		
		D4S3350	0.012		

The genotyping of the regions 2p, 4p and 4q in 125 families revealed one marker on Chr 2p, D2S1394, with a pairwise lod score, LOD=1.61 (nominal p-value=0.003). For the other markers overall LOD scores were <0.14 (Table 3). When the data were analysed for specific countries, the pairwise linkage analysis showed p-values smaller than 5% for marker D4S405 in German families (LOD=1.22; p=0.01), marker D2S1394 in German families (LOD=0.71; p=0.04) and British families (LOD=0.91; p=0.02) and marker D4S1647 in Italian families (LOD=0.67; p=0.04). However, when these country specific results were adjusted for multiple comparisons (initial p-values adjusted by multiplying the p-values with the number of comparisons (n=6; one overall and five country specific comparisons)), no marker retained p-values smaller than 5%.

Discussion

Thusfar, the European Consortium on GSPD has identified 194 strictly diagnosed ASPs without known PD mutations. The initial screen on a highly informative subset of 125 families with ASPs regarding chromosomal regions 2p, 4p, and 4q resulted in one marker, with a p-value smaller than 5 %, providing weak evidence for linkage with Parkinson's disease. Although three markers produced initial p-values <0.05 in country specific analyses, the findings did not reach significance at the level 5% after correction for multiple comparisons.

The strengths of this ASP study include the systematic data collection with standardised documentation and videotaping of the affected individuals, the strict diagnostic criteria that were used and the international ongoing collaboration through which we have collected and will continue to collect a large number of families with PD. An issue is whether we could have misclassified disease. Although a definite diagnosis of PD is based on neuropathological findings, we consider it unlikely that our results are biased because of misclassification of PD. We used very strict criteria to define our cases, with response to L-DOPA included as a major criterion and a review of any doubtful case by other experts.

The ASP method is a very appropriate approach to map genes in a complex disease trait as PD because it does not need an assumption about the mode of inheritance. A drawback, however, is that the power is usually low, in particular for susceptibility loci with a low relative risk or low frequency of the risk allele. Because the mode of inheritance of PD is unknown we cannot exactly estimate the statistical power of our study.[26] Hence, absence of linkage in our data may not be interpreted as excluding linkage. At

present we focussed on recessive models, but shortly we will extend the analyses without an assumption about the mode of inheritance.

Although the country-specific results did not reach significance at the level of 5% after correction for multiple comparison, we did some observations that merit further discussion. First, the D4S405 marker, which is closely localised to the UCH-L1 gene, showed some weak evidence for linkage in German families. The mutation in the UCH-L1 gene that causes PD was originally detected in a German family.[7] However, we did not find that specific mutation or any other mutations in the coding regions of UCHL-1 in our families.[14,15] Next, the D4S1647 marker, closely localised to the α-synuclein (PARK1), showed some evidence for linkage in Italian families. The first mutation in the α-synuclein was also described in Italian families from the Contursi kindred. However, mutations in the exons of the α-synuclein in these families were not found.[12,13] Still, the D4S1647 marker remains interesting, since a polymorphism in the promoter region of the α-synuclein gene (NACP-Rep 1), as well of the closely linked markers D4S1647 and D4S1628marker have been associated with PD.[27] Moreover, linkage disequilibrium between NACP-Rep1 and the D4S1647 marker was identified in PD cases, which might include the presence of a susceptibility gene.[27]

The only marker for which we found increased lod score in the overall analysis, D2S1394, is localised near to the PARK3 locus.[8] In their initial description of this locus, Gasser et al. did not find a significant maximum two-point LOD score for D2S1394, yet the marker was part of the haplotype segregating with two of their families.[8] The fact that we found an initial nominal p-value of 0.04 in German families and that the initial locus was also detected in German families strengthens the role of the PARK3 locus as a susceptibility factor for PD.

We have successfully established a European Consortium and recruited a large number of affected sib pairs with PD for a genome wide search for susceptibility genes in PD. Our initial analyses did not reveal significant susceptibility regions on Chr 2p, 4p and 4q.

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4.2

THE ILE93MET MUTATION IN THE UBUQUITIN CARBOXY-TERMINAL-HYDROXYLASE-L1 GENE IS NOT OBSERVED IN EUROPEAN CASES WITH FAMILIAL PARKINSON'S DISEASE

Abstract

Recently an Ile93Met mutation in the ubiquitin-carboxy-terminal-hydrolase-L1 gene (UCH-L1) has been described in a German family with Parkinson's Disease (PD). The authors showed that this mutation is responsible for an impaired proteolytic activity of the UCH-L1 protein and may lead to an abnormal aggregation of proteins in the brain. In order to determine the importance of this or any other mutation in the coding region of the UCH-L1 gene in PD, we performed mutation analysis on Caucasian families with at least two affected sibs. We did not detect any mutations in the UCH-L1 gene, however, we cannot exclude mutations in the regulatory or intronic regions of the UCH-L1 gene since these regions were not sequenced.

We conclude that the UCH-L1 gene is not a major gene responsible for familial PD.

INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative disorders. The prevalence of PD in Europe in subjects aged 55 years and older is 1.6%.[1,2] PD is characterized by a progressive neuronal degeneration, which mainly affects the dopaminergic neurons in the nigrostriatal system but also other regions of the brain.[3] The presence of intraneuronal inclusions, known as Lewy bodies in the pars compacta of the substantia nigra, is generally considered as the pathologic hallmark of PD, albeit not totally specific.[3] The major clinical signs of the disease include resting tremor, bradykinesia, rigidity and postural disturbances with a good response to treatment with levodopa. The cause(s) of PD are still largely unknown. In the vast majority, PD occurs in a sporadic form. However, in the last few years there is increasing evidence that some forms of familial PD are genetically determined.[4-9] At present, it is generally accepted that PD is a genetically heterogeneous disorder. It is known that mutations in the α-

synuclein gene are responsible for a small minority of autosomal dominant inherited PD[7,8], whereas mutations in Parkin are responsible for some cases of autosomal recessive inherited parkinsonism.[4,6,9]. Recently, a missense mutation in the ubiquitin-carboxy-terminal-hydrolase-L1 (UCH-L1) gene encoding for the UCH-L1 protein has been reported in relation to PD.[10] The authors showed that this Ile93Met mutation is responsible for an, in vitro, partial loss of the catalytic thiol protease activity of the UCH-L1 protein, which may lead to aberrations in the proteolytic pathway and aggregation of proteins.[10] This missense mutation was found in two affected sibs of a German family in which two other members also were affected with PD. It was suggested that the disease appeared to be autosomal dominant with incomplete penetrance.[10] The gene for human UCH-L1 has been mapped to chromosome 4p14.[11] However, a large family with levodopa-responsive Lewy body parkinsonism, in which a chromosome 4p14 haplotype segregates with disease, has also been described. UCH-L1 was excluded as a candidate gene by both gene sequencing, for coding regions, and as a heterozygous coding varient (S18Y) in two affected individuals demonstrated expression of the gene from both chromosomes.[5] We present here the results of mutation analysis on the complete coding region of the UCH-L1 gene in families with PD, that were recruited as part of the study of the European Consortium on Genetic Susceptibility in Parkinson's disease.[12] All these families were previously tested for allele sharing on chromosome 4p (unpublished data).

METHODS

In this study, we only included European Caucasian families with PD. In all families, at least two affected sibs in each family were present. PD was diagnosed using the rigorous criteria of idiopathic PD according to a similar study design as has been described by Maraganore and co-workers.[13] All patients gave their informed consent according to the Declaration of Helsinki. As part of the ongoing total genome screen in families with PD, up to present, 96 affected families with PD were tested for allele sharing on chromosome 4p. The markers used to test for allele sharing on chromosome 4p are D4S230, D4S1609, D4S391, D4S2397, and D4S405, spanning 12.4cM telomeric to centromeric on the Genethon Map. Since the family described by Leroy and colleagues[10] showed an apparent autosomal dominant (AD) inheritance pattern, only those families, that showed an inheritance pattern compatible with AD transmission, were eligible for mutation analysis for the UCH-L1 gene. Mutation analysis was performed on the complete coding region of the UCH-L1 gene in one randomly selected sibling

Table 1
Characteristics and origin of the individuals sequenced for the UCH-L1 gene.

Origin	number of families	individuals sequenced	mean age of onset years (SD)	mean duration of illness years (SD)
French	4	4	57.0 (9.3)	12.8 (14.5)
German	1	1	51	18
Italian	4	4	56.5 (7.3)	7.5 (5.8)
Dutch	2	2	51 (12.7)	10.5 (6.4)

Table 2
Primer sequences and PCR product size of the UCH-L1 gene.

exon	primer	forward (5'-3')	reverse (3'-5')	product size (bp)
1+2	Ex 1+2	CTCCCCCTGCACAGGCCTCA	GTCCCTGCCAGCAGCCGGAA	353
3	Ex 3	CTCCTCCCAGGCTCGGGT	CTCAAGCTGGGGAGCGGC	307
4	Ex 4	TGCACTCTCATTCTGAGATG	GATGGGTGGCCTCTAAACCC	228
5+6	Ex 5+6	AGGTTGCTCAGCATGTTCAG	CAGTAGAAACACAGATGGC	364
7	Ex 7	CTTAGTGGGCTTAGAATAGG	AAGTGCCCTCATGAGAATAC	372
8	Ex 8	ATCTAGGCTAGGTAAGCACG	TGCTGGCTATACTGGAAGAG	271
9	Ex 9	GGAGCCTTTCCCTATGTGAC	ACCACATCCAAGGTCTTAAC	465

from those families in which two affected siblings shared a haplotype for all 5 markers. The characteristics and country of origin of the individuals tested for mutations in the UCH-L1 gene are listed in Table 1. DNA was extracted from blood using standard techniques. All exons of the UCH-L1 gene, including flanking intronic sequences, were amplified from genomic DNA using specific primers. The sequences of the primers are shown in Table 2. The PCR reactions and product sequencing were done similarly to the study of Lincoln and co-workers.[14]

RESULTS

In total, 29 out of the 96 families showed an inheritance pattern compatible with autosomal dominant transmission. Of these 29 families, DNA of 11 affected siblings was sequenced for mutations in the UCH-L1 gene because they shared a haplotype for all five markers. The overall mean age at onset of PD was 55.2 years ± 8.1 years with a

mean duration of illness of 10.9 years ± 9.4 years. All individuals were analyzed for mutations in any coding region of the UCH-L1 gene. First, we searched for the Ile93Met alteration at nucleotide position 277 as described previously.[10] We did not observe this mutation in the UCH-L1 gene in the 11 affected siblings that were sequenced. To further detect mutations in the UCH-L1 gene we systematically performed sequence analysis of exons 1 to 9. Subsequently we identified the C54A variant in exon 3 in one Dutch sibling and one Italian sibling. This creates a novel RsaI restriction site and is responsible for a serine to tyrosine substitution at codon 18 (S18Y) (Table 2). The allele frequency we found for this polymorphism was somewhat lower as compared to other studies.[5,14] This could easily be a chance finding since our sample size was relatively small. Alternatively the difference could be explained by the difference in genetic background with different allele frequencies.

Discussion

The lack of the Ile93Met mutation or any other mutations in the coding region of the UCH-L1 gene in our study population suggests that a mutation in the UCH-L1 gene is not a major cause of familial PD in Europe. However, we cannot exclude mutations in the regulatory or intronic regions, as these regions were not sequenced. Since the prevalence of the Ile93Met mutation is currently unknown and no mutations were found in 500 control individuals of different ethnic background[10], it could be hypothesized that the mutation found in the German family may be unique in this family. This explanation is supported by the fact that yet no other mutations in the UCH-L1 gene were found in the present study, which included German families. Although our results and very recently another report[14] do not support the involvement of the UCH-L1 gene in the pathogenesis of PD, the malfunctioning of the ubiquitin-dependent proteolytic system is known to be involved in the formation of intraneuronal inclusion bodies.[15-17] Like α-synuclein, UCH-L1 is a constituent of Lewy bodies. It is thought to play a role in the cleavage of polymeric ubiquitin into monomers and may as well hydrolyze C-terminal esters and amides of ubiquitin.[18] Lewy bodies are strongly ubiquitinated and immunoreactive with neuro-specific ubiquitin carboxy terminal hydrolase and some subunits of the 26S proteasome. The reports of mutations in the α-synuclein gene[7,8] and the observation that α-synuclein is a major component of Lewy bodies[19] has accelerated research in Lewy body formation and deposition. Alpha-synuclein antibodies have enabled novel, occult pathology to be described in both neurites in idiopathic PD and glia in multiple system atrophy (MSA) [19,20]. Recently, another gene-defect, ini-

tially described in Japan and associated with an autosomal recessive juvenile parkinsonism (AR-JP), has been mapped to chromosome 6q25.2-q27.[6] Mutations in this gene, named Parkin, have now also been reported in European and Algerian families.[4,9] The similarity of the Parkin protein[6] to the ubiquitin protein family suggests that the ubiquitin-dependent proteolytic system may play an important role in the pathogenesis of PD, but the lack of Lewy bodies in AR-JP suggests that not only Lewy body formation but also other factors involved in neurodegeneration like oxidative stress and specific neurotoxins may be involved in the pathogenesis of PD. At present, it is generally accepted that a genetic contribution in the etiology to PD is of major importance and therefore research should be focussed on other yet unidentified susceptibility genes in PD.

We conclude that the UCH-L1 gene is not a major gene responsible for familial PD.

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4.3

A WIDE VARIETY OF MUTATIONS IN THE *PARKIN* GENE IS RESPONSIBLE FOR AUTOSOMAL RECESSIVE PARKINSONISM IN EUROPE

Abstract

Autosomal recessive juvenile parkinsonism (AR-JP, PARK2, OMIM 602544), one of the monogenic forms of Parkinson's Disease (PD), was initially described in Japan. It is characterized by early onset (before age 40), marked response to levodopa treatment, and levodopa induced dyskinesias. The gene responsible for AR-JP was recently identified and designated parkin. *We have analyzed the 12 coding exons of the* parkin *gene in 35 mostly European families with early onset autosomal recessive parkinsonism. In one family, a homozygous deletion of exon 4 could be demonstrated. By direct sequencing of the exons in the index patients of the remaining 34 families, eight previously undescribed point mutations (homozygous or heterozygous) were detected in eight families that included 20 patients. The mutations segregated with the disease in the families and were not detected on 110 to 166 control chromosomes. Four mutations caused truncation of the parkin protein. Three were frameshifts (202-203delAG, 255delA, 321-322insGT) and one a nonsense mutation (Trp453Stop). The other four were missense mutations (Lys161Asn, Arg256Cys, Arg275Trp, Thr415Asn) that probably affect amino acids that are important for the function of the parkin protein, since they result in the same phenotype as truncating mutations or homozygous exon deletions. Mean age at onset was 38 ± 12 years, but onset up to age 58 was observed. Mutations in the* parkin *gene are therefore not invariably associated with early onset parkinsonism. In many patients, the phenotype is indistinguishable from that of idiopathic PD. This study has shown that a wide variety of different mutations in the* parkin *gene is a common cause of autosomal recessive parkinsonism in Europe, and that different types of point mutations seem to be more frequently responsible for the disease phenotype than are deletions.*

INTRODUCTION

Parkinson's Disease (PD) is a frequent neurodegenerative disorder with a prevalence close to 2% after age 65.[1] The major signs of the disease are tremor, rigidity and bradykinesia associated with a good response to treatment with levodopa. The disorder is

caused by a massive loss of dopaminergic neurons in the pars compacta of the substantia nigra. The etiology of the disease is still unknown, but the existence of genetic susceptibility factors is strongly suspected.[2] Several familial forms of PD with autosomal dominant transmission have been reported. Mutations in the α-synuclein gene on chromosome 4q21-q23 were described in several PD families with early onset and rapid disease progression.[3,4] However, α-synuclein is a minor locus, found only in a subset of families with dominant transmission.[5-7] A second susceptibility locus was localized on chromosome 2p13 in German families with autosomal dominant PD.[8] A parkinsonian syndrome with autosomal recessive transmission (AR-JP, PARK2, OMIM 602544) was first described in Japan.[9,10] AR-JP patients show the typical signs of PD, but they are associated with: i) early onset, typically before the age of 40; ii) dystonia at onset; iii) diurnal fluctuations; iv) slow disease progression; v) early and severe levodopa induced dyskinesias. In a few cases, neuropathological examination has shown a massive loss of dopaminergic neurons in the pars compacta of the substantia nigra, but the absence of Lewy bodies, the histopathological hallmark of PD.[9,11,12] Recently, the AR-JP locus, designated PARK2, was mapped to chromosome 6q25.2-27 in consanguineous Japanese families.[13] Subsequently, linkage analyses, our own[14] and others'[15], have demonstrated the existence of non-Japanese PARK2 families in Europe, the United States and the Middle East. Homozygous deletions of one or more microsatellite markers in 3 AR-JP families greatly reduced the initial 17 cM candidate interval.[14-16] Very recently, Kitada et al. identified a novel gene, designated *parkin*, in which homozygous deletions of either exon 4 or 3-7 were detected in four Japanese families with AR-JP.[17] The *parkin* gene has an estimated genomic size of 500 kb and consists of 12 coding exons with an open reading frame of 1395 bp. The corresponding protein, parkin, composed of 465 amino acids, shows moderate homology to ubiquitin at the amino terminus and contains a RING-finger motif at the carboxy terminus.[17] Subsequently, homozygous deletions of exon 3 were found in two European families and of exon 8-9 in one Algerian family.[18] To date, two point mutations in the parkin gene have been reported in two Turkish AR-JP families.[19]

In order to determine the frequency and the diversity of mutations in the *parkin* gene as a cause for the AR-JP phenotype in Europe, we amplified the 12 coding exons of the *parkin* gene in 35 families with autosomal recessive early onset parkinsonism and sequenced the exons in the patients that did not show homozygous exon deletions.

RESULTS

A new family with an exon 4 deletion

A homozygous deletion of exon 4 was detected in all 3 patients of an Italian family (IT-005), previously shown to be homozygous for four markers at the disease locus[14] (figure 1).

Point mutations in the *parkin* gene

In the index patients from the 34 families that did not show homozygous exon deletions, sequence analysis of all coding exons, including the exon intron boundaries, revealed 11 sequence variations in exons and 3 in introns (table 1). Eight of the exonic variations cosegregated with the disorder in the families (figures 2) and were not detected in 110 to 166 control chromosomes (table 1). They are therefore most probably causative mutations (figure 3).

Four mutations resulted in truncated proteins: 202-203delAG in exon 2 of families IT-020 and UK-086, 255delA in exon 2 of family FR-096, 321-322insGT in exon 3 of family FR-119, and 1459G>A (Trp453Stop) in exon 12 of family IT-006. These mutations were homozygous in patients, except for the 202-203delAG mutation in families IT-020 and UK-086.

Four missense mutations were observed: In family IT-020, a heterozygous 584A>T transversion (Lys161Asn) in exon 4 was detected in addition to the 202-203delAG mutation described above. Segregation analysis showed that the two mutations were located on different alleles, and that all patients were compound heterozygotes (figure 2). Two other heterozygous, non-conservative amino acid changes were observed in exon 7 of patients from families DE-012 and IT-015: 867C>T (Arg256Cys) and 924C>T (Arg275Trp), respectively. Finally, a homozygous 1345C>A transversion in exon 11 of all patients from family IT-014 resulted in a conservative (Thr415Asn) amino acid change.

In three of the families with heterozygous mutations (UK-086, DE-012,IT-015), no complementary heterozygous mutation could be detected by sequencing.

Polymorphisms

Three of the 11 exonic sequence variations were detected in control chromosomes, and were thus classified as polymorphisms (table 1). They resulted in two conservative and one non conservative amino acid change. A 601G>A transition (Ser167Asn) was het-

Table 1
Variants of the *parkin* gene

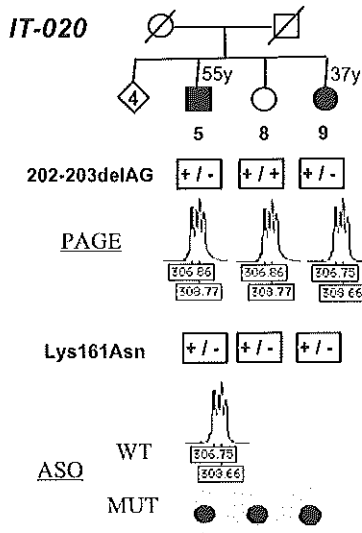
	nucleotide change	amino acid change/ position of the stop codon	type of mutation	technique of detection	expected fragment length in bp	number of control cases	frequency of the variant
exonic							
Ex2	***202-203delAG***	***Gln34/Stop37***	frameshift	PAGE	WT: 308 V: 306	55	0%
Ex2	***255delA***	***Asn52/Stop81***	frameshift	*Fok* I creation of site	WT: 278+30 V: 222+57+30	83	0%
Ex3	***321-322insGT***	***Trp74/Stop81***	frameshift	ASO		83	0%
Ex4	***584A>T***	***Lys161Asn***	missense	ASO		83	0%
Ex4	601G>A	Ser167Asn	missense	*Alw*N I loss of site	WT: 164+97 V: 261	83	1%
Ex7	***867C>T***	***Arg256Cys***	missense	ASO		83	0%
Ex7	***924C>T***	***Arg275Trp***	missense	*Sau*3A I loss of site	WT: 142+97 V: 239	83	0%
Ex10	1239G>C	Val380Leu	missense	ASO		45	16%
Ex11	1281G>A	Asp394Asn	missense	*Taq* I loss of site	WT: 221+84 V: 303	90	7%
Ex11	***1345C>A***	***Thr415Asn***	missense	ASO		83	0%
Ex12	***1459G>A***	***Trp453Stop***	nonsense	*Nla* IV loss of site	WT: 142+17+35+61 V: 159+35+61	83	0%
intronic							
Intron2	IVS2+25T>C			*Bst*N I creation of site	WT: 308 bp V: 264+44 bp	45	19%
Intron3	IVS3-20C>T			*Mnl* I loss of site	WT: 201+60 V: 261	45	10%
Intron7	IVS7-35A>G			*Mae* III creation of site	WT: 206 V: 159+47	36	27%

The nucleotide numbers are given according to the cDNA sequence published in the DNA Data Bank of Japan (DDBJ; accession number: AB009973); PAGE = polyacrylamide gel electrophoresis; ASO = allele specific oligonucleotide; causative mutations are given in bold-faced italics.

Figure 1
Homozygous exon 4 deletion in the patients of the Italian family IT-005. Bands (PCR products of the indicated exons) correspond to the numbered individuals. Black squares (men) and circles (women) represent affected individuals. Age at onset (in years) is given above the patient's symbol. Slashed symbols indicate deceased individuals. Ex = exon, i = inner, o = outer.

erozygous in only one of the two affected members of family FR-730 and on 1% of the control chromosomes (2/166). In addition, it was found in all sibs, including an unaffected, of family IT-020, in which the disease is already explained by the compound heterozygosity of two causative mutations (see above). A 1239G>C transversion (Val380Leu) in exon 10 was either heterozygous or homozygous in 11 families (IT-014, IT-020, IT-058, FR-017, FR029, FR-038, FR-096, FR-431,UK-006, UK-086, DE-022). It did not segregate with the disease and was found on 16% of control chromosomes. A 1281G>A transition (causing the non conservative Asp394Asn change) in exon 11 was heterozygous in the index patient of family UK-046 and heterozygous or homozygous on 7% of control chromosomes.

Variants in introns 2, 3 and 7 (IVS2+25T>C, IVS3-20C>T, IVS7-35A>G), were located at distance from the splice sites. These polymorphisms were detected on control chromosomes at a frequency of 19%, 10% and 27%, respectively (table 1). Finally, a homozygous 768C>T transition (Pro223Ser) was found in all individuals sequenced, suggesting that 768C was an error in the initial cDNA sequence.[17]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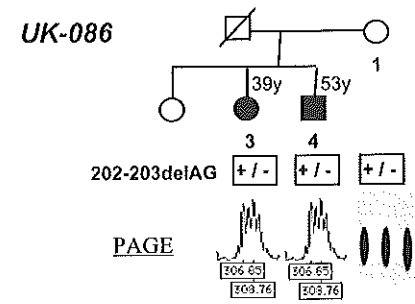
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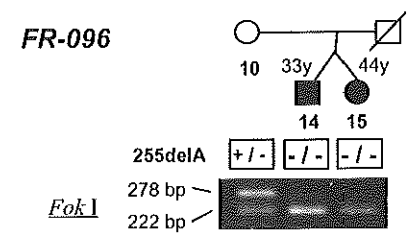
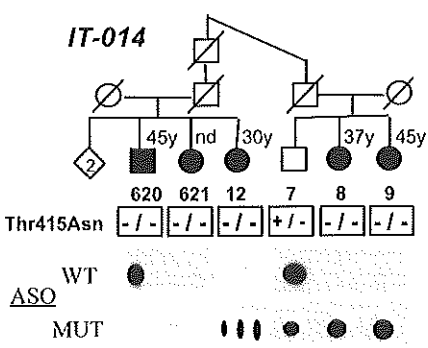
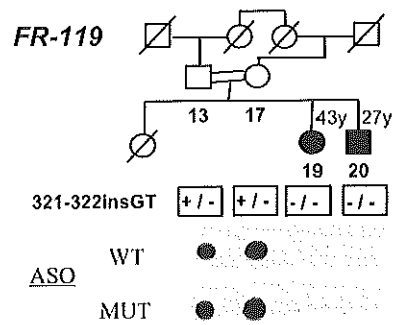
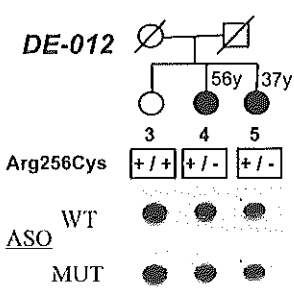
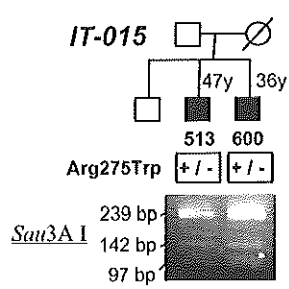
306.65
309.76


FR-096
10
33y
44y
14
15
255delA
+ / -
- / -
- / -
Fok I
278 bp
222 bp


IT-014
45y
nd
30y
37y
45y
2
620
621
12
7
8
9
Thr415Asn
- / -
- / -
- / -
+ / -
- / -
- / -
ASO
WT
MUT


FR-119
13
17
43y
27y
19
20
321-322insGT
+ / -
+ / -
- / -
- / -
ASO
WT
MUT


DE-012
56y
37y
3
4
5
Arg256Cys
+ / +
+ / -
+ / -
ASO
WT
MUT


IT-015
47y
36y
513
600
Arg275Trp
+ / -
+ / -
Sau3A I
239 bp
142 bp
97 bp

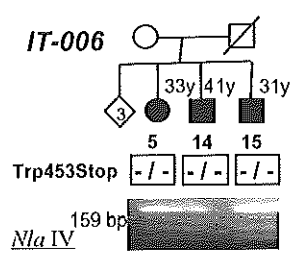

IT-006
33y
41y
31y
3
5
14
15
Trp453Stop
- / -
- / -
- / -
Nla IV
159 bp

Figure 2 (left)
Families with point mutations in the *parkin* gene. Complete cosegregation of the mutation with the disease is shown. Black squares (men) and circles (women) represent affected individuals with age at onset (in years) indicated above the patient's symbol. Slashed symbols indicate deceased individuals. The numbers of unaffected sibs not analyzed are given in diamonds. For each mutation the genotype of the family member is indicated (+/+ = homozygous wild type, +/- = heterozygous for the mutation, -/- homozygous for the mutation). Under each genotype, the detection data are shown. PAGE: electrophoregrams with allele sizes in bp. ASO: autoradiograms of mutated and wild type allele. Restriction assay: PCR products after digestion with the appropriate restriction enzyme. Fragment lengths in bp are indicated. Mut = mutated; nd = age at onset not determined, as patient is unaware of her clinical signs.

Functional domains of the parkin protein

According to the PROSITE program, the conservative amino acid change Thr415Asn is located within the consensus sequence for putative phosphorylation sites of cAMP- and cGMP-dependent protein kinases (KKTT) and protein kinase C (TTK). The truncating nonsense mutation Trp453Stop is located within a putative N-myristoylation site (GCEWNR).

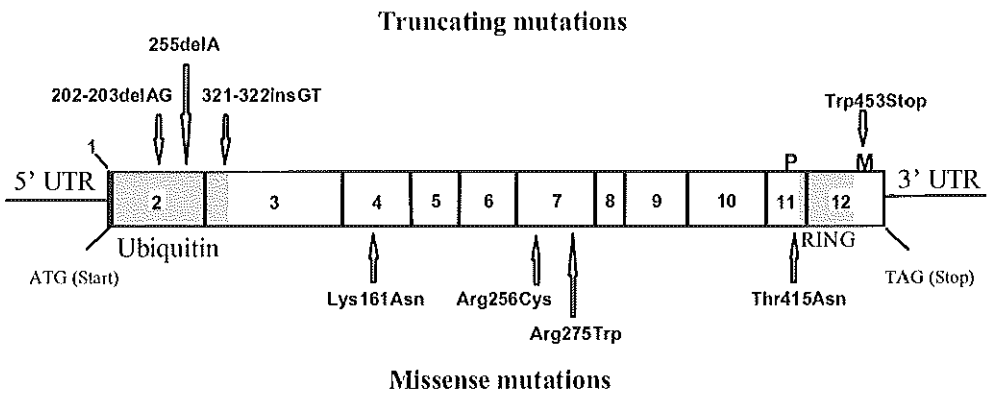


Figure 3
Point mutations in the *parkin* gene. The coding sequence of the gene with its 12 exons is represented as a bar. Exons are numbered 1 to 12. The eight causative point mutations are positioned according to their effect on the protein (truncating versus missense). The ubiquitin-like domain and the RING finger motif are hatched. For the mutations Thr415Asn and Trp453Stop the putative phosphorylation sites (P) and the N-myristoylation site (M), respectively, are indicated. UTR = untranslated region.

Table 2
Clinical characteristics of 31 patients from 12 Parkin families according to the type of mutation

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

Genotype/phenotype correlations

Point mutations were present in eight families, that included 20 patients (table 2). The patients of family IT-020 with a truncating mutation on one allele and a missense mutation on the other allele were classified in the missense mutation group, assuming that missense mutations may be less deleterious if they result in only partial loss of function of the parkin protein, thereby determining the severity of the illness. Accordingly, the patients of the families for whom only one heterozygous mutation could be detected were included among the truncating (UK-086) or missense mutations (DE-012, IT-015).

The 11 patients with homozygous exon deletions of the *parkin* gene (3 previously published families[18] and family IT-005), the 11 patients with missense mutations (4 families) and the 9 patients with truncating mutations (4 families), showed an overall phenotype of early onset parkinsonism (table 2). Mean age at onset was 38±12, ranging from 7 to 58. Parkinsonian rigidity (97%) and bradykinesia (97%) were almost always present, whereas tremor was observed in only 68%. These features were associated with dystonia at onset in 30% and with brisk reflexes in the lower limbs without a Babinski sign in 33%. After a mean disease duration of 16±8 years, dyskinesias and fluctuations were present in 71% and 50%, respectively. The effect of the mutations on age at onset, severity or frequency of signs did not differ significantly among the patients carrying different types of mutations.

Discussion

The initial report of Kitada et al. describing homozygous deletions of exon 4 or 3-7 in 4 Japanese families[17], our own observation of homozygous deletions of exon 3 or 8-9 in two European and one Algerian family[18], and the newly identified exon 4 deletion in an Italian family show that a variety of deletions in the *parkin* gene can cause autosomal recessive parkinsonism, with age at onset as late as 58, as observed in the Italian family IT-005 (figure 1). However, is was suspected that other types of mutations (e.g. point mutations) might be a more common cause of the disease.[18] The only point mutations reported to date were two homozygous single base pair substitutions in two consanguineous Turkish families, which resulted in a Thr240Arg missense mutation in exon 6 and an Ala311Stop nonsense mutation in exon 8. Until now, however, it could not be determined whether point mutations in the *parkin* gene account for a significant proportion of autosomal recessive early onset parkinsonism.

Taken together, our 3 families with previously reported exon deletions[18] and the 9 families with mutations detected in the present study demonstrate that mutations in the *parkin* gene are the cause of the disease in approximately 30% of the families with autosomal recessive parkinsonism analyzed (12/38), including 11/35 from Europe. Point mutations were detected in 2/3 of our families (8 of 12 families) and thus seem to be more frequent than homozygous exon deletions (4 of 12 families).

The fact that a second mutation could not be detected in families DE-012, IT-015 and UK-086 (which were heterozygous for only one mutation) suggests the presence of a complementary mutation that could not be detected by the techniques used in this study. This second mutation might be located in regions of the gene that were not sequenced (e.g. the promotor region) or might be present as a heterozygous exon deletion that can not be analyzed by simple PCR amplification. However, in order to estimate the frequency of compound heterozygotes resulting from the combination of a point mutation and an exon deletion or of two different exon deletions, we compared the frequencies of the homozygous exon deletions (n=4) and the homozygous point mutations (n=4) in 8 families with known or suspected consanguinity. The frequency for the two types of homozygous mutations was 50% each in this sample. The frequency of compound heterozygotes for each of the two combinations (deletion + point mutation or deletion + deletion) could therefore reach up to 25%. However, the small sample size precludes accurate estimation of these frequencies.

Only one of the point mutations, the 202-203delAG frameshift, was found in two families of different origins (Italy and UK), suggesting that the mutation occurred independently. Although the patients from these families shared two frequent alleles for the PARK2-markers D6S411 (allele 2 = 59%) and D6S1550 (allele 2 = 68%), the alleles for two other tightly linked markers, D6S305 and D6S1579, were different (data not shown), indicating that a recent common founder effect is unlikely.

All the point mutations are novel and show that a wide variety of different mutations in the *parkin* gene can account for the disease. The pathogenic role of the point mutations was shown by: i) cosegregation of the mutations with the disorder in the families and ii) their absence in a large number of control chromosomes (110 to 166 depending on the mutation). All the point mutations identified are likely to have major functional consequences. The four truncating mutations (202-203delAG, 255delA, 321-322insGT and Trp453Stop), which were detected in homozygous state in 3 of 5 families, clearly cause a loss of function of the parkin protein, compatible with the recessive mode of inheritance. Three of the missense mutations result in non conservative amino acid changes (Lys161Asn, Arg256Cys and Arg275Trp). In family IT-020, the 202-203delAG frameshift mutation on one allele, which already results in a loss of function,

is associated with an apparently deleterious Lys161Asn missense mutation on the other allele. The conservative Thr415Asn amino acid change, that involves neutral amino acids with different polar side chains, homozygous in all five patients of family IT-014, is located within two consensus sequences of different protein kinases (cAMP- and cGMP-dependent protein kinases and protein kinase C), and might alter post-translational modifications. In addition, codon 415 is located very close to the first cysteine of the RING-finger motif (codon 418) and could affect its structure.

The mutations, which had different effects on the parkin protein, were distributed over 6 exons, excluding a mutational hot spot. It is interesting to note that, in contrast to the reported Gln311Stop mutation[19], the truncating point mutations identified in this study correspond to the N- and C-terminal regions of the parkin protein (ubiquitin-like and RING-finger motif, respectively), whereas the missense mutations affect the more central regions of the protein, as does the Thr240Arg mutation.[19] The previously described ubiquitin-like and RING-finger domains were not affected by the missense mutations. The carboxy terminal region appears to be extremely important since a homozygous Trp453Stop nonsense mutation, which only removes the last 12 carboxy terminal residues, causes the disease, perhaps by altering a N-myristoylation site between codons 450-455, that prevents a necessary post-translational modification of the protein. The absence of significant clinical differences in this study among the patients with different types of mutations suggests that the modified amino acids are all of functional importance and that their replacement, like the truncating mutations, causes loss of function. The location of the mutations may therefore point to unknown functional domains.

The phenotype of the families with point mutations in the *parkin* gene and those with exon deletions covered a clinical spectrum that was broader than in the Japanese families originally described[17,19], and is often very close to that of idiopathic PD (table 2). Mean age at onset, 38, was that of early onset PD. In 13 patients, however, onset occurred after age 40. Dystonia or brisk reflexes were less frequent than previously reported.[10,19] Overall, the phenotypes of patients classified according to the effect of the mutations on the parkin protein were similar, although the earlier ages at onset, 7 to 18 years, were observed in the Algerian family with deletion of exons 8 and 9.[18] Similarly early onset was also observed in the Japanese family with deletion of exon 3-7 (17) as well as in the patient with the Gln311Stop mutation[19], raising the question of the functional consequences of exon deletions and truncating events in specific regions of the parkin protein, especially as onset within our patients group with truncating mutations was later (between 27 and 53 years).

Finally, three exonic variants (Ser167Asn, Val380Leu and Asp394Asn) were classified as polymorphisms, since they were detected at different frequencies (between 1 and 16 %) on control chromosomes. In some families, they did not segregate with the disease. Furthermore, they were found in a family in which the disease was explained by other mutations (the polymorphism Ser167Asn in family IT-020) or were homozygous in healthy controls (Val380Leu and Asp394Asn). They are therefore insufficient to cause the disease by themselves. They might, however, alter the function of the parkin protein and contribute to the pathogenesis of idiopathic Parkinson`s Disease. Association studies will help to clarify this question.

In conclusion, this study shows that point mutations in the *parkin* gene are a common cause of autosomal recessive parkinsonism in Europe and seem to be more frequent than the exon deletions so far described. Furthermore, the mutations in the *parkin* gene are associated with a wide range of ages at onset as well as of clinical signs and can result in familial parkinsonism clinically indistinguishable from idiopathic Parkinson's Disease. The mutations detected are diverse in their effects on the parkin protein as well as in their localization within the gene. The diversity of the mutations and the absence of a mutational hot spot will complicate molecular diagnosis, but the evident importance of the amino acids affected will help to analyze the function, still unknown, of the parkin protein.

MATERIALS AND METHODS

Patients and statistical analysis

Thirty-eight families were selected according to the following criteria: i) presence of parkinsonism with good response to levodopa (≥ 30% improvement) in at least 2 siblings, and absence of excluding criteria such as extensor plantar reflexes, ophthalmoplegia, early (after 2 years of disease evolution) dementia or autonomic failure; ii) onset ≤ 45 years in at least one of the siblings; iii) inheritance compatible with autosomal recessive transmission (several patients in a single generation with or without known consanguinity). The families originated from France (n=12), Italy (n=10), Germany (n=7), Great Britain (n=4), Algeria (n=1), Morocco (n=1), The Netherlands (n=1), Portugal (n=1), Vietnam (n=1). Four families from Algeria, France, Italy and Portugal were excluded from sequence analysis because they were found to carry homozygous deletions of either exons 3 or 8-9 (18) or exon 4 (family IT-005).

The patients and unaffected relatives were examined by us in one of the movement disorder clinics of the European Consortium on Genetic Susceptibility in Parkinson's Disease (GSPD) or the French Parkinson's Disease Genetics Study Group, according to a standardized protocol using the same inclusion and exclusion criteria. All patients were videotaped and the clinical data were centralized. The Kruskal-Wallis test was used for comparisons of means, and the χ^2 test for comparison of frequencies (Yates corrected). Blood samples were taken with informed consent from the patients and their first degree relatives.

PCR amplification and sequence analysis

The 12 coding exons of the *parkin* gene from 35 index patients were amplified from genomic DNA by PCR as described by Kitada et al.[17] In family IT-005, an additional pair of exonic primers was used for exon 3 (Ex3iFor (5'-AATTGTGACCTGGATCA-GC-3') and Ex3iRev (5'-CTGGACTTCCAGCTGGTGGTGAG-3')). For exons 4 and 7 only the intronic primer pairs were used. The same primers were used for the sequencing of the PCR products of the 12 exons on both strands using the "Big Dye Terminator Cycle Sequencing Ready Reaction" DNA Sequencing Kit (ABI PRISM), according to the manufacturer's recommendations, on an ABI 377 automated sequencer with the "Sequence Analysis 3.0" (ABI PRISM) software.

Each time a nucleotide variant was identified in an index case, its cosegregation with the disease was established in the patient's family, and its frequency was determined in 36 to 90 controls (with or without other neurological diseases). Three different techniques were used to detect mutations in the PCR products corresponding to the different exons: i) allele specific oligonucleotides (ASO) to detect the wild type and the variant sequence; ii) digestion with the appropriate restriction endonuclease, iii) polyacrylamide gel electrophoresis (table 1). Nucleotide positions were determined according to the cDNA sequence published in the DNA Data Bank of Japan (DDBJ; accession number: AB009973).

ASO

Ten microliter of the PCR product were blotted onto Hybond N^+ nylon membranes (Amersham) after denaturation at 95°C for 5 min and fixed in a microwave oven at 600W for 2 min. For exon 3, the primers Ex3iFor and Ex3iRev were used. The oligonucleotides (table 3), labeled with $dCTP^{32}$ using the Terminal Transferase Kit (Boehringer Mannheim), were hybridized to the membrane at 44°C overnight in a buffer consisting of 5X SSPE, 5X Denhardts and 0,1% SDS. The membranes were

Table 3
Oligonucleotides used for the ASO technique

location	nucleotide change	oligonucleotide sequence
Ex3	321-322insGT	WT: 5'-TGCAGAGACC--GTGGAGAAAA-3' V: 5'-GCAGAGACC**GT**GTGGAGAAA-3'
Ex4	584A>T	WT: 5'-GCCGGGAAA**A**CTCAGGGTA-3' V: 5'-GCCGGGAAA**T**CTCAGGGTA-3'
Ex7	867C>T	WT: 5'-TGCAACTCC**C**GCCACGTGA-3' V: 5'-TGCAACTCC**T**GCCACGTGA-3'
Ex10	1239G>C	WT: 5'-TGCAGTGCC**G**TATTTGAAG-3' V: 5'- TGCAGTGCC**C**TATTTGAAG-3'
Ex11	1345C>A	WT: 5'-AGAAAACCA**C**CAAGCCCTG-3' V: 5'-AGAAAACCA**A**CAAGCCCTG-3'

The nucleotide change in the oligonucleotides is represented in bold and underlined; WT = wild type, V = variant

washed twice for 30 min in 2X SSC at 59°C and exposed to MP film (Amersham) for 3-6 hours.

Restriction assay

Fifteen µl of PCR product were digested with restriction enzymes according to the manufacturer's recommendations. The expected fragment lengths are given in table 1.

Polyacrylamide gel electrophoresis

A 5'-fluorescent (Hex) forward primer (Kitada et al.[17]) was used to amplify exon 2. The presence of the 202-203delAG variant, resulting in a shorter PCR product (306 versus 308 bp), was established by fragment size measurement using an ABI 377 automated sequencer with the "Genescan 2.0.2" and "Genotyper 1.1.1" software (ABI PRISM).

Determination of additional functional domains in the parkin protein

The internet web site PROSITE (http://expasy.hcuge.ch/cgi-bin/scanprosite) was used to determine additional functional domains of the parkin protein.

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5

General discussion

IN THIS THESIS several studies concerning the genetic susceptibility to Parkinson's disease (PD) are described. We studied possible genetic causes of PD in two different settings, a population-based cohort study and an affected sib pair study, using different approaches, including aggregation, association, and linkage sib pair analysis. In this chapter, after briefly summarizing our main findings, we will discuss some of the general and specific methodological issues that pertained to these studies and we will discuss the definition of the outcome used in our studies. Furthermore, we will discuss how the identification of genetic factors for PD may further our understanding in the etiology of this disease. Finally, we will discuss possibilities of future epidemiological research regarding the etiology of PD.

BACKGROUND

Up to present several hypotheses have been postulated in the etiology of PD, however the causes of PD still largely remain unknown. Over the last two centuries prevailing thinking about the etiology of PD alternated continuously between genetic and environmental hypotheses. More than a century ago, Gowers[1] observed that 15% of patients with PD did have a positive family history, and thus emphasized the importance of inheritance in the etiology of PD. In the early 20th century a possible infectious etiology, became more popular. In the last decades several investigators reported familial aggregation of PD.[2-4] The hypothesis of environmental factors in PD was favored in the early nineteen eighties[5] and the genetic hypothesis regained strong attention in the nineteen nineties. The identifications of genetic defects in families with PD with an autosomal dominant inheritance pattern[6-8] or with an autosomal recessive inheritance pattern[9,10] and the reports of two other loci in families with PD[11,12] stressed the impor-

Table 1
Chromosomal location of genes and loci related to Parkinson's Disease (PD) or subtypes of PD with the specific mode of inheritance.

Gene	First report	Chromosomal location	Mode of inheritance
α-synuclein	Polymeropoulos et al. 1997	4q21-q23	Autosomal dominant
Parkin	Kitada et al 1997	6q25.2-q27	Autosomal recessive
UCH-L1	Leroy et al. 1998	4p14	Autosomal dominant
Unknown	Gasser et al. 1998	2p13	Autosomal dominant
Unknown	Farrer et al. 1999	4p14-16.3	Autosomal dominant

tance of genetic factors in the etiology of PD or subtypes of PD. The chromosomal location of these genes and loci together with the mode of inheritance are listed in Table 1. These observations provide convincing evidence that PD is genetically heterogeneous. Although genes like α-synuclein,[7,8] UCH-L1[6] and Parkin[9,10] can cause PD, they account for a small minority of patients with PD. The aim of the work presented in this thesis was to obtain more insight in the role of known and other yet unidentified genetic factors in the occurrence of PD.

MAIN FINDINGS

Familial aggregation study

We prospectively studied the risk of PD among participants from the Rotterdam Study with a family history of PD and compared it to those without such a history (chapter 2). We found that individuals with a positive family history of PD, in particular with at least two participants with PD, did have an increased risk of PD, suggesting that genetic factors may play a role in the cause of PD in the elderly.

Genetic association studies

We investigated the role of three candidate genes in PD within the Rotterdam Study (chapter 3). The first two candidate genes, N-acetyltransferase-2 (NAT-2) and cytochrome P-450 debrisoquine hydroxylase (CYP2D6), thought to be involved in the metabolism of neurotoxins, have been associated with PD.[13,14] In our study, both genes were not associated with PD suggesting that these genes are not likely to play an important role in the etiology of PD in the Rotterdam Study. Moreover, we studied the role of the APOE gene in PD and found that in the elderly the APOE ε2-allele increases the risk of PD and in particular the risk of PD with dementia.

The sib pair study

The design of the European sib pair study with first results from genetic analyses as well as the evaluation of the significance of mutations in two genes, UCH-L1 and Parkin, in these sib pair families are presented in chapter 4. Up to the end of 1999 a total of 246 families with at least two affected siblings were identified and all information has been stored in centralized databases. We performed genotyping on three interesting regions for PD on chromosome 2p, 4p and 4q and observed for one marker D2S1394,

a p-value<0.05 suggesting the presence of a susceptibility region. Mutations in the UCH-L1 gene in families with PD with an inheritance pattern compatible with autosomal dominant transmission were not found. Different mutations in the Parkin gene, responsible for an autosomal recessive juvenile parkinsonism (AR-JP) initially described in Japan,[9] are a common cause for early onset autosomal recessive parkinsonism in Europe. However, the phenotype of this subtype of PD in our study was indistinguishable from that of idiopathic PD.

METHODOLOGICAL CONSIDERATIONS

In this section we will discuss specific methodological issues that pertained to the three different approaches of studying genetic susceptibility to PD presented in this thesis.

Familial aggregation study

A familial aggregation study is generally the first step to provide evidence for inheritance of a disease. In familial aggregation studies the frequency of the same disease in relatives of a group of cases with the disease of interest are compared to the frequency of the disease in relatives of an appropriate control population. However, family members sharing environmental risk factors may explain familial aggregation. Therefore studies on familial aggregation should be interpreted cautiously. Although several studies have reported familial aggregation of PD, no prospective population based studies on familial aggregation of PD in the elderly have been published. These considerations were motive to study familial aggregation of PD in the Rotterdam Study.

Selection bias due to selective lost to follow-up is a major threat in prospective population based studies. Since lost to follow-up was less than one percent in the Rotterdam Study, it is unlikely that this type of bias has occurred. One might consider that if family history were related to early mortality, selection bias might have been introduced because the individual did not live long enough to develop PD. We did not find an association between family history and survival suggesting that selective mortality did not bias our results.

Information bias, due to misclassification of disease status of family members, is another threat in our aggregation study. A problem in a positive family history is that individuals also refer to other diseases like essential tremor or senile tremor and other parkinsonian disorders. On the other hand, elderly people are more likely to loose track of their relatives. Due to the large number of individuals it was not feasible to verify family history for each relative and therefore had to rely on self-reported family history.

Since this misclassification was most likely to be non-differential it will have attenuated the risk of PD.

Confounding may have occurred since several environmental risk factors that could mimic familial aggregation have been postulated to play a role in PD. However, apart from smoking no consistent results have been published.[15] Smoking has been reported to protect against PD.[15] It could be hypothesized that individuals from families with a positive history for PD may smoke less thus increasing their risk of PD. We did not find evidence for smoking being a confounder in familial aggregation of PD in our study.

Genetic association studies

Genetic association methods are conceptually straightforward methods to study genetically determined disease entities. In these kind of studies the frequencies of different genotypes and alleles of the candidate gene are compared between individuals with and without the outcome of interest. In reality, there is quite a number of difficulties. Firstly, an appropriate candidate gene has to be found. This may be a gene/protein playing a possible role in the pathogenesis of disease, or a gene that is associated with other disorders that share clinical and/or pathological features. However, usually there is hardly any solid ground to base the selection of candidate genes on. Secondly, the power is often very low. If the etiology of the disease is multifactorial, a true association might not be observed. Moreover, if there is a low increase in the risk, or the frequency of the risk allele is low, we may lack statistical power. In the Rotterdam Study we studied the role of two candidate genes (CYP2D6 and NAT-2) in 80 patients with PD. The lowest odds ratio (OR) at α=0.05 and β=0.80 that could be detected would be an OR>2, depending on allele frequency and number of controls. This would mean that we may have missed a true association if the increased risk of PD for the these genetic risk factors is lower than 2. Biological plausibility and initial reports showing an increased risk of PD with ORs>2 for both CYP2D6[14] and NAT-2[13] were motive to study these genes in PD in the Rotterdam Study. Consistent associations between APOE and other neurodegenerative diseases like Alzheimer's disease[16] were motive to perform a candidate gene association study between APOE and PD.

Selection bias is likely to occur in candidate gene association studies that are performed in a cross sectional case-control design, like in the studies of chapter 3. Since controls were chosen from the same source population from which the cases were derived and individuals from the cohort were examined in person, it is not likely that selection bias may have occurred due to an ill-defined sampling frame of controls. How-

ever, selection bias may have occurred due to the inclusion of prevalent and incident cases in our studies. Based on the assumption that the genetic determinants are not associated with duration of disease, this mixing should not lead to bias. However, if the genetic polymorphisms are associated with co-morbidity, which may alter disease duration due to selective survival or change in clinical course, this may lead to bias. The prevalent cases in the mixture may represent a subset of cases that have survived long enough to be enrolled in the study. This group may be different with respect to risk factors, and clinical course. We may partly, solve this problem by using left truncation methods if age at onset can be determined exactly. However, this is difficult with an insidious disease like PD. A large case-control study on incident cases, nested within a prospective cohort study design is probably the most accurate method to avoid this and other forms of bias in genetic association studies. In contrast to the NAT-2 and CYP2D6 genes, the APOE gene was studied also in incident cases. The results did confirm the overall findings suggesting that the association between APOE and PD is true.

Potential confounders in genetic association studies besides age and gender are racial and ethnic background. We did not adjust for racial and ethnic background in the Rotterdam Study. Most participants are Dutch Caucasian individuals living in this area for a very long time. However, if unmeasured potential confounders introduce differences in allele frequencies this may lead to spurious results.

The sib pair study

It is generally accepted that PD is a genetically complex disease trait in which the mode of inheritance is often not clear. The major advantage of the affected sib pair approach is that the definition of the mode of inheritance is not required. The major disadvantage of this method is the large number of families required. Another disadvantage we may have is that it is likely that parents and even sibs are not alive anymore due to the late onset of PD, which may make it impossible to assess parental haplotypes and handle the number of alleles identical by descent. The major problem to overcome in ASP studies is genetic heterogeneity. The proportion of ASPs that may be explained due to the same gene is determining the power of the ASP study. Strategies to increase homogeneity by studying a founder population may increase chances of success.

Successful applications of mapping genes for complex disease traits[17-19] in sib pairs as well as the availability of more sophisticated statistical methods motivated the establishment of a consortium to recruit affected sib pairs for a genome wide screen for susceptibility genes in sib pairs with PD. With the large set of sib pairs we also have the

opportunity to test the significance of published genetic defects responsible for PD or subtypes of PD.

DEFINITION OF OUTCOME

The generally accepted diagnostic criteria of PD in epidemiological studies[15,20] were used within the Rotterdam Study and have been presented in the introduction of this thesis. The diagnostic criteria in the sib pair study were even more rigorous, including neuropathological diagnosis of PD. However, even neuropathological diagnosis may bear some misclassification.[21] In our studies diagnosis of PD was made before the genotyping. This minimizes the probability of introducing misclassification which, if any, would be non-differential. To minimize misclassification in the Rotterdam Study it would be necessary to use more rigorous criteria. However, this would reduce the power of several association studies due to the decrease in the number of definite cases.[20] To minimize misclassification one might consider a follow-up strategy of the affected individuals in which diagnosis of PD may be rejected if initially unidentified causes may explain the parkinsonism. This will protect us against false positive diagnosis, preventing false negative results, but will lower the power of the study. Another advantage is that we will have the opportunity to monitor unaffected family members at risk of PD.

Our probability of success would be even higher with the inclusion of multiplex families, because we reduce the probability to include nongenetic phenocopies. However, such families are relatively rare and again we would reduce the power of the study.

Definition of the outcome is a difficult problem to overcome since heterogeneity is likely to found in a complex disease entity like PD. Whether all genes responsible for PD or parkinsonism represent a spectrum of one clinicopathologic entity or that they each reflect a different disease entity is not clear and thus subject of debate. From genotype-phenotype correlation studies we know that there are clinical differences between the responsible gene and corresponding disease status. However, in many patients, the phenotype is often indistinguishable from that of idiopathic PD.[22]

GENETIC DEFECTS AND PATHOGENETIC MECHANISMS

The identification of genetic defects responsible for disease may be important in understanding the pathogenesis of disease. The nature of mutations in genes associated with

PD has been unknown for a long time. The discovery of a mutation in the α-synuclein in the Contursi kindred in 1997[8] has stimulated research in the pathogenetic mechanisms of PD. The availability of α-synuclein antibodies has enabled novel, occult pathology to be described in both neurites in idiopathic PD and other parkinsonian disorders.[23,24] The presence of Lewy bodies in association with neuronal loss and depigmentation of the substantia nigra is considered the neuropathologic hallmark in PD, albeit not totally specific. Lewy bodies are also found in other neurodegenerative diseases like Lewy body dementia,[25] and Alzheimer's disease.[26] The α-synuclein protein appeared a major component of Lewy bodies,[23] which accelerated research in Lewy body formation and deposition. We know that the pathogenic α-synuclein gene mutations are responsible for a minority of familial PD.[27] Moreover, we know that the wild type α-synuclein is a major component of Lewy bodies in sporadic PD.[28,29] This suggests that the aggregation of the normal α-synuclein protein could be triggered by other mechanisms. For example, damage to the protein[30,31] or abnormal catabolism[32] may lead to an abnormal aggregation. Synuclein abnormalities are also found in other interesting parkinsonian disorders like multiple system atrophy (MSA)[23] and amyotrophic lateral sclerosis (ALS).[24] Moreover, it has been shown that axon pathology in PD and Lewy Body dementia hippocampus contains α-, β-, and γ-synuclein.[33] This provides even more evidence that synuclein pathology is playing a role in PD and other neurodegenerative diseases.

Another mutation, responsible for familial PD, was found in the UCH-L1 gene. Like the α-synuclein gene product, the UCH-L1 protein is a constituent of Lewy bodies.[34] UCH-L1 is thought to play a role in the cleavage of polymeric ubiquitin into monomers and may as well hydrolyze C-terminal esters and amides of ubiquitin.[35] The malfunctioning of the ubiquitin-dependent proteolytic system is known to be involved in the formation of intraneuronal inclusion bodies.[36,37] Recently it has been shown that the α-synuclein protein is degraded by the ubiquitin-proteasome proteolyic pathway.[32] This suggests that malfunctioning of this system may be involved in the etiology of PD.

Finally, the similarity of the Parkin protein[9] to the ubiquitin protein family strengthens the hypothesis that the ubiquitin-dependent proteolytic system may play a role in the pathogenesis of PD. However, the lack of Lewy bodies in AR-JP suggests that other factors in neurodegeneration like oxidative stress[31] and specific neurotoxins[38,39] may be involved in the pathogenesis of PD.

RECOMMENDATION FOR FUTURE ETIOLOGICAL RESEARCH

The genes or loci described so far in PD are responsible for only a minority of PD cases. We have to consider that the cause of PD is likely to be multifactorial, in which two or more genes together with environmental effects may lead to the phenotype(s) of PD during life. If there is some biologic plausibility for interaction, it might be worthwhile studying these kind of interactions. Since these possibilities are so diverse, any genetic association study, prone to interaction with other genetic or environmental factors, is subject of debate and results should be interpreted cautiously. To disentangle these interactions we first have to identify the genetic risk factors since these factors are usually stable during life. Once genetic risk factors are identified the study of interaction will become more feasible. Different methods can be used to identify other genetic risk factors in PD.

Major genes are more likely to be found in large families or in genetic isolates. Large families are rare and a major gene responsible for PD in that family may be specific and not generalizible to other families. However, we may obtain more insight in the pathogenetic mechanisms of PD.

The advantage of mapping genes in genetic isolates is that a limited number of genes with a limited number of mutations may be responsible for PD or subtypes of PD. This reduces the complexity of genetic heterogeneity and the probability of finding a gene responsible for PD will be higher.

Recently a twin study showed that heritability plays a role in early onset PD[40]. We therefore may increase our chances to find a gene, if we focus on early onset PD. However, the findings presented in this thesis suggest that genetic factors also play a role in the elderly.

As discussed previously the affected sib pair method is a good approach to identify susceptibility genes in PD. Since this method has been successful in other complex traits,[17-19] it will be worthwhile to collect as much as possible affected sib pairs. Up to present five European countries have participated in the collection of affected sib pairs. It is warranted to expand this project both on national and international level. With the increasing number of DNA-markers and rapid improvements in molecular genotyping, the 'fishing expedition', often used by epidemiologists in classical case-control studies will aid the identification of susceptibility genes in PD.

The role of the candidate gene association study in identifying genetic risk factors of a heterogeneous disease like PD is subject of debate. The study design is often susceptible to several forms of bias. This does not imply that these kind of studies are not important in the studying genetic factors in PD. When there is biological plausibility

and the sample size is large enough it can be extremely worthwhile performing these studies. It is important that cases and controls are sampled from the same source population in which there is no population admixture. The results should be reproduced in other independent datasets with similar study design.

Moreover in genetically complex diseases entities candidate gene association studies can play an important role in the fine mapping of regions once susceptibility regions have been found in linkage studies. Once novel genetic defects have been identified,

it will be important to assess its relevance in a general population. Existing datasets, on population level, like the Rotterdam Study, will be extremely worthwhile to test the significance of these identified genetic defects in PD.

Not only the identification of novel genes, but also the search for other genes/proteins that might be associated with the genes already known as PD-genes may be of interest and could be analyzed in the affected sib pair study or the Rotterdam Study. An excellent example is the structural similarity of the apolipoprotein-E to the α-synuclein and the increased susceptibility to sporadic PD by a certain combined α-synuclein/apolipoprotein-E genotype.[41] The availability of the sequence of the entire human genome (HUGO) by the year 2002 will be of major help in the search of candidate genes and genes with homology to the yet identified PD genes. Finally we may perform genotype-phenotype correlation studies. With the identification of other genes we may classify PD or subtypes of PD.

Current thinking is that the etiology of PD is multifactorial and that several susceptibility genes and environmental factors may be involved. The genes and loci described so far in PD are responsible for a minority of PD patients. It is likely that other genetic risk factors for PD may be discovered shortly. A challenge will then be to find out whether such genes act in isolation or in interaction with other genes or environmental risk factors in bringing out the disease. Studies on the function of the genes that have been identified will help in understanding the pathophysiology of PD but may also help direct future etiologic research. The better we understand the causes of PD, the closer we may get to the ultimate aim of prevention of this common disabling disorder.

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6 Summary

SUMMARY

6.1

PARKINSON'S DISEASE (PD) is a common neurodegenerative disorder among the elderly, and a major health problem due to its disabling character. Clinically, PD is characterized by resting tremor, bradykinesia, rigidity and postural disturbances. The cause of PD is still largely unknown and treatment is symptomatic with only temporary results. Studies on families with PD have clearly indicated that genetic factors may play a role in PD. Since 1997, mutations in three genes, α-synuclein, UCH-L1, and Parkin have been identified as causes of PD. Moreover two loci (2p13 and 4p14-16.3) have been found that are associated with PD. These observations provide convincing evidence that PD is a genetically heterogeneous disorder.

The objective of the work presented in this thesis was to study genetic susceptibility to PD. We did this in two different studies, the Rotterdam Study, which is a prospective population-based cohort study on frequency, etiology and prognosis of chronic diseases among 7983 individuals aged 55 years and over, in Ommoord a suburb of Rotterdam, and in a large European collaborative sib pair study on PD. We used different methods to study the significance of genetic factors in PD. Firstly, as presented in chapter 2, we analyzed whether there was familial aggregation of PD in the Rotterdam Study. Secondly, we performed genetic association studies to study candidate genes in PD (chapter 3). Finally, linkage studies and genetic association studies in affected sib pairs were used to study genetic susceptibility to PD (chapter 4). All studies were approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam and all participants gave their informed consent according to the Declaration of Helsinki.

In **chapter 2.1,** we describe the association between family history of PD and the risk of PD in the elderly population of the Rotterdam Study. To date, no prospective population based studies on familial aggregation of PD have been published. We found

that individuals with a first degree relative with PD had a 50% increased risk to develop PD as compared to those without such a family history. For subjects with at least two first degree relatives with PD the risk was more than fivefold increased. These observations suggest that genetic factors may play a role in the occurrence of PD in the elderly.

Next, we studied the association between three candidate genes and PD within the Rotterdam Study (chapter 3). The first two candidate genes, N-acetyltransferase-2 (NAT-2) and cytochrome P-450 debrisoquine hydroxylase (CYP2D6) are thought to be involved in the metabolism of neurotoxins and have been associated with PD. The third candidate gene, apolipoprotein-E (APOE), is as far as known, not involved in the metabolism of neurotoxins but has been associated with other neurodegenerative diseases including Alzheimer's disease and Lewy body disease.

In **chapter 3.1** we evaluated the role of the NAT-2 gene in PD. The genotype responsible for slow acetylation was previously reported to be increased in PD patients. Slow acetylators are homozygous for any of the mutant alleles and may be more susceptible to low-level environmental exposure to neurotoxins. The finding of an increased frequency of subjects homozygous for the NAT-2 gene mutations would have been compatible with the view that PD patients may be less capable to handle certain endogenous or exogenous toxins. We investigated three mutant alleles, M1, M2 and M3 and the wild type allele of the NAT-2 gene in 80 patients with idiopathic PD and 161 age matched randomly selected control subjects from the Rotterdam Study. The allelic frequencies and genotype distributions were very similar in cases and controls. Interestingly, in controls the frequency of the wild type allele increased significantly with age suggesting that the mutant alleles may be associated with an increased risk of mortality ($P_{trend}<0.001$). We concluded that the NAT-2 gene is not a major genetic determinant of PD, but may be a determinant of mortality in the general population.

In **chapter 3.2** the association between the CYP2D6 gene and PD is described. The CYP2D6 gene is known to be involved in the metabolism of the neurotoxin MPTP, which has been shown to induce parkinsonism in humans. The initial reports on the CYP2D6 gene and PD described that poor metabolizers homozygous for any mutant allele had an increased risk of PD. Later studies, in other populations, have tried to confirm the initial findings but with conflicting results. Several explanations, like differences in study design or bias in the selection of the control population have been offered for this. The Rotterdam Study provided the possibility to assess the significance of the CYP2D6 gene in PD in a case-control study in which cases and controls were sampled from the same source population. We investigated two mutant alleles, CYP2D6*3 and CYP2D6*4, both associated with poor metabolism, and the wild type allele in 80 patients with PD and 156 control subjects, frequency matched on age and

gender. No differences between cases and controls were found for the poor metabolizer genotype. Our results suggest that the CYP2D6 gene is not a major determinant of PD.

In **chapter 3.3** we report the association between the APOE genotype and PD. The apolipoprotein ε4-allele is associated with Alzheimer's disease and Lewy Body disease. Reports on APOE genotype and PD are not consistent. We therefore studied the risk of PD with or without dementia as a function of APOE genotype. APOE genotyping was available for 107 PD patients (26 with and 81 without dementia) and 4805 non-parkinsonian controls. Individuals with at least one ε2-allele were almost twice as likely to have PD as compared to individuals with the most frequent genotype APOE3E3. When we looked separately for demented and non-demented PD patients as compared to non-parkinsonian controls, APOE genotype appeared not associated with PD without dementia, but carriers of the ε4-allele and the ε2-allele had a four to five times increased risk of PD with dementia. For carriers of an ε4-allele the risk of dementia was similarly increased in non-PD and PD subjects, however, for carriers of an ε2-allele the increased risk of dementia was confined to PD patients. When we prospectively studied the risk of dementia in non-demented PD patients we found that ε2-allele carriers had a more than 10 times higher risk of dementia as compared to ε3ε3 subjects. These findings suggest that in the elderly the APOE ε2-allele increases the risk of PD and in particular the risk of PD with dementia.

Chapter 4 is devoted to the analysis of susceptibility genes in European affected sib pairs with PD. We established a European Consortium on Genetic Susceptibility to PD (GSPD) with the objective to study known and other, not yet identified, susceptibility genes in affected sib pairs with PD. In this chapter the design of the European sib pair study is presented as well as the evaluation of the significance of known mutations in two genes, UCH-L1 and Parkin, in these sib pairs.

In **chapter 4.1** we present the sib pair study design and the results from genetic analyses on three interesting chromosomal regions in PD. The GSPD-study started in 1995 with the recruitment of families with PD and was conducted in five European countries. Different established links and communication networks were used to identify families with at least two affected sibs with PD using rigorous diagnostic criteria. All the affected family members were personally examined, videotaped and blood samples were taken using a standardized protocol. In order to determine allele segregation in the families, we collected blood from parents and unaffected siblings if available. Up to the end of 1999 a total of 246 families with at least two affected siblings from five countries were identified and all information had been stored in centralized databases.

We performed genotyping in 125 highly informative families with affected sib pairs on three interesting regions for PD on chromosome 2p and 4p and 4q using 12 DNA-markers. For one marker D2S1394, we observed a nominal p-value of 0.05 or lower suggesting the presence of a susceptibility region.

In **chapter 4.2** we present our assessment of mutations in the UCH-L1 gene in the sib pair study. A German group had reported a mutation in the UCH-L1 gene, responsible for PD with an inheritance pattern compatible with autosomal dominant transmission in a German sibling pair. The mutation in this gene is responsible for an impaired proteolytic activity of the UCH-L1 protein and may lead to an abnormal aggregation of proteins in the brain. In order to determine the importance of this or any other mutation in the coding region of the UCH-L1 gene in PD, we performed mutation analysis on the complete coding region of the UCH-L1 gene among 96 Caucasian affected sib pair families. We did not detect any mutations in the UCH-L1 gene, however, we could not exclude mutations in the regulatory or intronic regions of the UCH-L1 gene since these regions were not sequenced. We concluded that mutations in the UCH-L1 gene are a rare cause of familial PD.

In **chapter 4.3** we studied the Parkin gene, which was initially described in Japan where it was found to be responsible for autosomal recessive juvenile parkinsonism (AR-JP). Several mutations in this gene have been described since. We have analyzed the 12 coding exons of the Parkin gene in 35 families, mostly European, with early onset autosomal recessive parkinsonism. This study has shown that a wide range of different mutations in the Parkin gene are a common cause for early onset autosomal recessive parkinsonism in Europe, and that point mutations of various types are more frequently responsible for the disease phenotype than deletions are. Mean age at onset was 38±12 years, but onset up to age 58 was observed, showing that mutations in the Parkin gene were not invariably associated with early onset parkinsonism defined as an onset of younger than 45 years. In many patients, the phenotype was indistinguishable from that of idiopathic PD.

In **chapter 5.1** the findings presented in this thesis were placed in a broader perspective, as were issues relating to study design. We discussed some mechanisms that may potentially underly PD and reflected on the clinical and scientific impact of our and other genetic studies in PD.

Current thinking is that the etiology of PD is multifactorial and that several susceptibility genes and environmental factors may be involved. The genes and loci described so far in PD are responsible for a minority of PD patients. It is likely that other genetic risk factors for PD may be discovered shortly. A challenge will then be to find out whether such genes act in isolation or in interaction with other genes or environmental

risk factors in bringing out the disease. Studies on the function of the genes that have been identified will help in understanding the pathophysiology of PD but may also help direct future etiologic research. The better we understand the causes of PD, the closer we may get to the ultimate aim of prevention of this common disabling disorder.

SAMENVATTING

6.2

DE ZIEKTE VAN Parkinson is een frequent voorkomende neurodegeneratieve aandoening onder ouderen en vormt door haar invaliderende karakter een belangrijk gezondheidsprobleem. Klinisch wordt de ziekte van Parkinson gekenmerkt door rusttremor, bradykinesie, rigiditeit en gestoorde houdingsreflexen. De oorzaak van de ziekte van Parkinson is nog steeds grotendeels onbekend en de behandeling is symptomatisch met alleen tijdelijk resultaat.

Studies in families met de ziekte van Parkinson hebben aangetoond dat genetische factoren mogelijk een rol spelen bij de ziekte van Parkinson. Vanaf 1997 zijn in drie genen, α-synuclein, UCH-L1 en Parkin, mutaties geïdentificeerd die de ziekte van Parkinson veroorzaken. Daarnaast zijn twee loci (2p13 en 4p14-16.3) gevonden die geassocieerd zijn met de ziekte van Parkinson. Deze bevindingen leveren overtuigend bewijs dat de ziekte van Parkinson een genetisch heterogene aandoening is.

De doelstelling van het werk beschreven in dit proefschrift was het bestuderen van de genetische gevoeligheid bij de ziekte van Parkinson. We hebben dit gedaan in twee verschillende studies, het Erasmus Rotterdam Gezondheid en Ouderen (ERGO) onderzoek – in het Engels "the Rotterdam Study" – een prospectief bevolkingsonderzoek naar frequentie, oorzaak en prognose van chronische aandoeningen onder 7983 inwoners van 55 jaar en ouder in de wijk Ommoord in Rotterdam, en in een groot samenwerkend Europees sibparen-onderzoek (paren van broers en/of zusters met de ziekte van Parkinson). We hebben verschillende methoden gebruikt om de significantie van genetische factoren bij de ziekte van Parkinson te bestuderen. Ten eerste – zoals beschreven in hoofdstuk 2 – hebben we onderzocht of er familieaggregatie bestond bij de ziekte van Parkinson binnen de ERGO-studie. Ten tweede deden we genetisch associatieonderzoek om kandidaat-genen bij de ziekte van Parkinson te bestuderen

(hoofdstuk 3). Ten slotte hebben we gebruik gemaakt van linkage studies en genetisch associatieonderzoek om genetische gevoeligheid bij de ziekte van Parkinson te bestuderen in aangedane sibparen. Alle studies werden goedgekeurd door de Medisch Ethische Commissie van de Erasmus Universiteit en alle deelnemers hebben toestemming, gebaseerd op het verdrag van Helsinki, verleend voor het verrichten van het onderzoek.

In **hoofdstuk 2.1** beschrijven we het verband tussen familiegeschiedenis van de ziekte van Parkinson en de kans op de ziekte van Parkinson bij ouderen in het ERGO-onderzoek. Tot op heden is er geen prospectief bevolkingsonderzoek over familieaggregatie bij de ziekte van Parkinson gepubliceerd. We vonden dat personen met ten minste een eerstegraads familielid met de ziekte van Parkinson 50% meer kans hadden op het krijgen van de ziekte van Parkinson dan personen zonder familieleden met deze aandoening. Bij personen met ten minste twee eerstegraads familieleden met de ziekte van Parkinson was dit risico meer dan vijf keer verhoogd. Deze bevindingen geven aan dat erfelijke factoren mogelijk een rol spelen bij het ontstaan van de ziekte van Parkinson.

Vervolgens bestudeerden we de associatie tussen drie kandidaat-genen en de ziekte van Parkinson binnen de ERGO-studie (**hoofdstuk 3**). De eerste twee kandidaat-genen, N-acetyltransferase-2 (NAT-2) en het cytochroom P450 debrisoquine hydroxylase (CYP2D6), zijn mogelijk betrokken bij het metabolisme van neurotoxines en werden geassocieerd met de ziekte van Parkinson. Het derde kandidaat-gen, apolipoproteine-E (APOE), is voor zover bekend niet betrokken bij het metabolisme van neurotoxinen, maar werd geassocieerd met andere neurodegeneratieve aandoeningen, inclusief de ziekte van Alzheimer.

In **hoofdstuk 3.1** evalueerden we de rol van het NAT-2 gen bij de ziekte van Parkinson. Het genotype verantwoordelijk voor langzame acetylering werd in het verleden in een verhoogde frequentie gevonden bij patiënten met de ziekte van Parkinson. Langzame acetyleerders zijn homozygoot voor gemuteerde allelen en kunnen een verhoogde gevoeligheid vertonen voor expositie aan bepaalde neurotoxines. De bevinding van een verhoogde frequentie van het genotype homozygoot voor het gemuteerde allel zou verenigbaar zijn met de hypothese dat mensen met de ziekte van Parkinson slechter in staat zijn bepaalde exogene en endogene neurotoxines te verwerken. We hebben gekeken naar drie gemuteerde allelen M1, M2 en M3 en het normale, meest voorkomende allel van het NAT-2-gen in 80 patiënten met de ziekte van Parkinson en 161 aselecte controlepersonen uit het ERGO-onderzoek met dezelfde leeftijd. De verdeling van de allelen en genotype in patiënten was vergelijkbaar met die van de controlepersonen. Een interessante bevinding was dat de frequentie van het normale allel toenam met de leeftijd ($P_{trend}<0.001$) waardoor een associatie tussen gemuteerde allelen en verhoogd risico op sterfte wordt gesuggereerd. We concluderen dat het NAT-2-gen geen belang-

rijke determinant is bij de ziekte van Parkinson, maar mogelijk een determinant zou kunnen zijn van sterfte in de algemene populatie.

In **hoofdstuk 3.2** wordt het verband tussen het CYP2D6-gen en de ziekte van Parkinson beschreven. Van het CYP2D6-gen is bekend dat dit gen betrokken is bij het metabolisme van het neurotoxine MPTP, waarvan is aangetoond dat het parkinsonisme veroorzaakt. De initiële studie naar het CYP2D6-gen bij de ziekte van Parkinson beschreef dat slechte metaboliseerders, homozygoot voor gemuteerde allelen, een verhoogd risico hadden op de ziekte van Parkinson. In latere studies in andere populaties is geprobeerd de resultaten te reproduceren, echter zonder consistente resultaten. Hiervoor wordt een aantal verklaringen, zoals verschillen in studieopzet en vertekening van de associatie door selectie van de controlepersonen, gepostuleerd. De ERGO-studie verschafte de mogelijkheid deze associatie te bestuderen in een patiënt/controle-onderzoek waarin de patiënten en controlepersonen werden geselecteerd uit dezelfde onderzoekspopulatie. We hebben gekeken naar twee gemuteerde allelen en het normale, meest voorkomende allel, in 80 patiënten met de ziekte van Parkinson en 156 aselecte controlepersonen uit het ERGO-onderzoek met dezelfde leeftijdsverdeling als bij de patiënten. Er werden geen verschillen tussen patiënten en controlepersonen gevonden voor het genotype verantwoordelijk voor slechte metabolisering. Onze resultaten suggereren dat het CYP2D6-gen geen belangrijke determinant is bij de ziekte van Parkinson.

In **hoofdstuk 3.3** rapporteren we de associatie tussen APOE genotype en de ziekte van Parkinson. Het apolipoproteine ε4-allel is geassocieerd met de ziekte van Alzheimer en de Lewy body ziekte. Studies over het APOE gen bij de ziekte van Parkinson zijn niet consistent. Daarom bestudeerden wij de kans op de ziekte van Parkinson, met of zonder dementie, als functie van het APOE genotype. APOE gentypering was beschikbaar voor 107 patiënten met de ziekte van Parkinson (26 met en 81 zonder dementie) en 4805 controlepersonen zonder parkinsonisme. Personen met ten minste een ε2-allel hadden een bijna twee keer verhoogd risico op het krijgen van de ziekte van Parkinson vergeleken met personen met het meest frequente genotype APOE3E3. Indien we apart keken naar demente en niet demente patiënten met de ziekte van Parkinson vonden we geen associatie met de ziekte van Parkinson zonder dementie, maar dragers van zowel het ε2-allel als het ε4-allel hadden een vier- tot vijfvoudig verhoogd risico op de ziekte van Parkinson met dementie. Voor dragers van het ε4-allel was de kans op dementie niet significant verschillend voor personen met of zonder de ziekte van Parkinson, maar voor dragers van het ε2-allel was het risico van dementie alleen verhoogd bij patiënten met de ziekte van Parkinson. Indien we prospectief keken naar de kans op dementie bij niet demente patiënten met de ziekte van Parkinson, von-

den we dat dragers van het ε2-allel een meer dan tienvoudig verhoogd risico hadden op dementie vergeleken met het APOE3E3-genotype. Deze bevindingen suggereren dat het APOE-ε2-allel bij ouderen het risico voor de ziekte van Parkinson, in het bijzonder de ziekte van Parkinson met dementie, verhoogt.

Hoofdstuk 4 is gewijd aan de analyse van gevoeligheidsgenen in Europese sibparen met de ziekte van Parkinson. Wij richtten een Europees consortium aangaande de genetische susceptibiliteit voor de ziekte van Parkinson (GSPD) op, met als doelstelling bekende en onbekende, nog niet geïdentificeerde, gevoeligheidsgenen te bestuderen in aangedane sibparen. In dit hoofdstuk worden het ontwerp van deze studie alsmede de evaluatie van de significantie van bekende mutaties in twee genen, UCH-L1 en Parkin, in aangedane sibparen gepresenteerd.

In **hoofdstuk 4.1** beschrijven we de opzet van de sibparenstudie en presenteren we resultaten van drie interessante chromosomale loci bij de ziekte van Parkinson. De GSPD-studie startte in 1995 met de werving van families met de ziekte van Parkinson, uitgevoerd in vijf Europese landen. Verschillende links en communicatienetwerken werden gebruikt om families met ten minste twee aangedane broers en of zusters met de ziekte van Parkinson te identificeren met gebruikmaking van zeer strikte diagnostische criteria. Alle aangedane familieleden werden persoonlijk onderzocht, gefilmd en bij alle deelnemers werd bloed afgenomen volgens een gestandaardiseerd protocol. Om de allel segregatie vast te stellen werd, indien beschikbaar, bloed afgenomen bij ouders en niet aangedane broers en of zusters. Tot eind 1999 hebben we in totaal 246 families met ten minste twee aangedane broers en of zusters geïdentificeerd en alle gegevens zijn opgeslagen in centrale databestanden. In 125 zeer informatieve families met ten minste twee aangedane broers en/of zusters hebben we gezocht naar predisponerende gebieden met behulp van 12 markers op chromosoom 2p en 4p and 4q, gebruikmakend van twopoint en multipoint aangedane sibparen analyses. Voor één marker, D2S1394, vonden we een nominale p-waarde kleiner dan 0.05 waardoor de aanwezigheid van een genetisch suspect gebied wordt gesuggereerd.

In **hoofstuk 4.2** beschrijven we het onderzoek naar mutaties in het UCH-L1-gen in de sibparen. Een Duitse groep rapporteerde in een Duitse familie een mutatie in het UCH-L1-gen, verantwoordelijk voor de ziekte van Parkinson met een overervingspatroon compatibel met autosomaal dominante transmissie. De mutatie in dit gen is verantwoordelijk voor een verminderde proteolytische activiteit van het UCH-L1-eiwit, mogelijk leidend tot een abnormale aggregatie van eiwitten in de hersenen. Om de significantie van deze en/of andere mutaties in coderende gebieden van dit gen te bestuderen, hebben we mutaticanalyses uitgevoerd bij 96 Kaukasische sibparenfamilies. We hebben geen mutaties in het UCH-L1-gen gevonden, alhoewel we mutaties in de

niet coderende gebieden niet konden uitsluiten, daar deze gebieden niet werden geanalyseerd. We concluderen dat het UCH-L1-gen een zeldzame oorzaak is voor de familiaire ziekte van Parkinson.

In **hoofdstuk 4.3** presenteren we het Parkin gen, voor het eerst beschreven in Japan, waar het verantwoordelijk bleek te zijn voor autosomaal recessief juveniel parkinsonisme (AR-JP). We hebben de 12 coderende gebieden op dit Parkin gen onderzocht in 35 families, de meeste van Europese origine, met een op vroege leeftijd beginnend parkinsonisme. Deze studie laat zien dat een brede variatie aan mutaties in het Parkin gen een algemene oorzaak is van autosomaal recessief parkinsonisme in Europa en dat puntmutaties vaker verantwoordelijk blijken voor het fenotype van de ziekte dan deleties. De gemiddelde leeftijd waarop de ziekte begon was 38±12 jaar, maar een beginleeftijd van 58 jaar werd ook waargenomen. De ziekte lijkt hierdoor dus niet beperkt tot een vroege aanzet Bovendien bleek bij veel patiënten de ziekte niet te onderscheiden van de idiopatische ziekte van Parkinson.

In **hoofdstuk 5.1** worden de resultaten, beschreven in dit proefschrift, geplaatst in een breder perspectief, evenals opmerkingen aangaande studieopzet. We bespreken sommige mechanismen die mogelijk onderliggend zijn aan de ziekte van Parkinson, gereflecteerd aan klinische en wetenschappelijke impact van onze en andere studies aangaande de ziekte van Parkinson.

Het is algemeen aanvaard dat de oorzaak van de ziekte van Parkinson multifactorieel is, waarbij verschillende gevoeligheidsgenen en omgevingsfactoren mogelijk betrokken zijn. De tot dusver beschreven genen en loci zijn verantwoordelijk voor slechts een kleine groep van patiënten met de ziekte van Parkinson. Het is waarschijnlijk dat andere genetische risicofactoren binnenkort zullen worden ontdekt. Een uitdaging zal dan zijn om te onderzoeken of deze genen alleen of in interactie met andere genen en/of omgevingsfactoren leiden tot de ziekte. Studies naar de functie van deze geïdentificeerde genen zullen bijdragen aan het begrijpen van de pathofysiologie van de ziekte van Parkinson, maar ook toekomstig etiologisch onderzoek initiëren. Hoe beter we de oorzaak van de ziekte van Parkinson begrijpen, des te dichter we zullen komen bij het ultieme doel, de preventie van deze invaliderende aandoening.

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About the author

SANJAY HARHANGI WAS born on March 13, 1970 in Paramaribo, Surinam. In 1974 he and his family immigrated to the Netherlands. He graduated in 1989 from high school (*Atheneum B, 'Lorentz College', Arnhem*). In that same year he started his medical studies at the University of Nijmegen. During his studies he co-operated in a research project on acoustic neuromas at the department of Otorhinolaryngology and the department of Neurosurgery and participated for four months in a primary health care project in San Felix, Venezuela. In 1996, he obtained his medical degree, and worked for three months as a resident in the home for the elderly 'Bruggerbosch', Enschede. In the same year he started his research project, described in this thesis at the department of Epidemiology & Biostatistics (head: Prof.dr. A. Hofman) and the department of Clinical Genetics (head: Prof.dr. B.A. Oostra) of the Erasmus Medical Center Rotterdam. During his research he was trained as an epidemiologist at the Netherlands Institute of Health Sciences in Rotterdam (Msc in Clinical Epidemiology, 1998). From February 2000 he is working as a resident in Neurosurgery (head: Prof.dr. C.J.J. Avezaat).

He is married to Devina Asarfi. They have a daughter called Madhavi.