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Type and frequency of mutations in the *LRRK2* gene in familial and sporadic Parkinson's disease*

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There is increasing evidence of genetic contribution to the pathogenesis of Parkinson's disease. The identification of mutations in the *leucine-rich repeat kinase 2* (*LRRK2*) gene has shed new light on genetic variations responsible for autosomal dominantly inherited Parkinson's disease. In this analysis, the most comprehensive published so far, we screened a second large series comprising 53 families with Parkinson's disease for mutations in the *LRRK2* gene by direct sequencing to further determine the frequency of the mutation and evaluate the clinical phenotype to establish a genotype/phenotype relation. For comparison, all novel and known mutations were investigated in a cohort of 337 patients with apparently sporadic Parkinson's disease and a cohort of 1200 control subjects using an ABI 7900 Allelic Detection system. We identified 7 more families with LRRK2 variations in the 53 families with Parkinson's disease. Four of these are novel amino acid substitutions (R793M, Q930R, S1096C, S1228T). Because of incomplete penetrance and possible phenocopies pathogenic relevance of the Q930R and S1096C mutations as well as for the previously described A3342G splice site mutation could not be established with certainty. The so far most common mutation (G2019) was not detected in our large cohort. Late onset and typical L-dopa responsive parkinsonian features were generally observed, often accompanied by impairment of executive functions and high interference values in neuropsychological testing, as well as sleeping disturbances but rare hallucinations. There were no abnormalities in electrophysiological investigations. Distinct intrafamily and interfamily differences could be observed, including the clinical presentation of diffuse Lewy body disease in one patient. The frequent finding of cerebral atrophy on MRI and less substantia nigra hyperechogenicity compared with idiopathic Parkinson's disease on transcranial ultrasound needs to be confirmed in further studies. Together with the findings obtained in 46 families in our first study, LRRK2 mutations, therefore, account for 13% of apparently autosomal dominant families in our population with varying but still generally typical clinical presentation of Parkinson's disease.

Keywords: *leucine-rich repeat kinase 2* (*LRRK2*) gene; PARK 8; frequency of mutations; genotype/phenotype relation

Abbreviation: LRRK2 = leucine-rich repeat kinase 2

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In more than 10% of patients with Parkinson's disease one or more relatives are also affected by this disorder (Elbaz *et al.*, 1999). However, genetic causes are only very rarely found. A recent breakthrough has been achieved by linkage of families with autosomal dominant Parkinson's disease to the PARK8 region located on chromosome 12p11.2-q13 (Funayama *et al.*, 2002; Zimprich *et al.*, 2004*b*; Paisan-Ruiz *et al.*, 2005) and the cloning of the mutant gene *LRRK2*

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(*leucine-rich repeat kinase 2*) (Paisan-Ruiz *et al.*, 2004; Zimprich *et al.*, 2004*a*). The gene encodes for a multifunctional protein, belonging to the Ras in complex protein (ROCO) family. Families linked to the PARK 8 region have autosomal dominant, generally late onset, dopa responsive parkinsonism (Hasegawa and Kowa 1997; Zimprich *et al.*, 2004*b*), with PET findings typical for idiopathic Parkinson's disease (Hernandez *et al.*, 2005). However, a closer look at the first five mutations described by our group showed a wide variety in phenotype and pleomorphic pathology (Wszolek *et al.*, 2004; Zimprich *et al.*, 2004*a*). Until now, seven different LRRK2 mutations have been published (Paisan-Ruiz *et al.*, 2004; Zimprich *et al.*, 2004*a*). So far, the most common mutation is the G2019S mutation, which accounts for 2–5% of the autosomal dominantly inherited cases with Parkinson's disease, depending on the population investigated (Deng *et al.*, 2005; Di Fonzo *et al.*, 2005; Nichols *et al.*, 2005; Toft *et al.*, 2005). Mean age of disease onset in the families described so far is 59–63 years. This late onset and the variable clinical phenotype may conceal the familial nature of the disorder. It is, therefore, not astonishing that this mutation has also been found in 1–2% of the sporadic Parkinson cases (Paisan-Ruiz *et al.*, 2004; Aasly *et al.*, 2005; Gilks *et al.*, 2005; Kachergus *et al.*, 2005). *LRRK2* contains 51 exons. Therefore, identification of single mutations responsible for the majority of patients with Parkinson's disease would be very helpful. However, before a mutation can be suggested for use in mutational screenings in Parkinson's disease its predominance needs to be confirmed in other cohorts.

In this analysis, the most comprehensive published so far, we screened another large cohort of families, most with an apparently autosomal dominant mode of inheritance, and searched for the identified variants in a large population of sporadic Parkinson's disease cases to

(i) further determine the frequency of mutations in patients with autosomal dominant inheritance and sporadic Parkinson's disease
(ii) identify or confirm possible major mutations
(iii) evaluate the clinical phenotype of patients affected by these variant mutations aiming to establish a genotype/phenotype relation.

Subjects and methods

Subjects

DNA of 53 index patients from families with Parkinson's disease compatible with an autosomal dominant mode of inheritance or with a mode of inheritance that could not be assigned to a typical Mendelian trait, as well as two affected sib pairs were analysed for mutations in the *LRRK2* gene. Clinical diagnosis was based on published criteria (Hughes *et al.*, 1992) and severity of the disease was rated according to the Unified Parkinson's Disease Rating Scale (UPDRS) (Fahn *et al.*, 1987) and Hoehn and Yahr staging. In one family (family E) typical parkinsonian features were only found in two members: in one Lewy bodies were pathologically confirmed in the substantia nigra (III-7-), the second affected family member with Parkinson's disease (III-12) remains alive. All other affected family members presented primarily with postural tremor. Moreover, all novel and known mutations were investigated in a cohort of 337 patients with apparently sporadic Parkinson's disease (204 male, 133 female, mean age 53 $\pm$ 13 years) and a cohort of 1200 subjects without any extrapyramidal disorders matched for age $\pm$5 years and sex. Allele frequency of the polymorphism N551K; 1653C>G was investigated in 888 of these control subjects.

DNA of patients with familial and sporadic Parkinson's disease was obtained from our gene bank, while DNA of control subjects comprised the Kora cohort obtained from the National Research Center of Environment and Health/Munich, Germany. All patients and controls had given informed consent to mutational screenings, which was approved by the local ethical committee.

Mutational screening

Genomic DNA was isolated from peripheral blood using standard protocols. Mutational screening in patients of families with autosomal dominant Parkinson's disease was performed for all exons and exon–intron boundaries of the *LRRK2* gene by direct sequencing of both strands using the BigDye Terminator Cycle sequencing kit (Applied Biosystems) with the same primers (Table 1) and under the same condition as described previously (Zimprich *et al.*, 2004*a*).

Mutational screening in patients with sporadic Parkinson's disease and control subjects was performed using an ABI 7900 Allelic Detection system. As previously described genotyping was performed on a MALDI-TOF mass-spectrometer (Sequenom Mass Array system) using the homogeneous mass-extension process for producing primer extension products (Tang *et al.*, 1999; Zimprich *et al.*, 2004*a*).

In families with identical mutations haplotype analysis of the *LRRK2* region was performed. Haplotypes were constructed using five fluorescent-labelled microsatellite markers, two flanking and three intragenic (Table 2). DNA fragments containing the polymorphic marker sequences were amplified by polymerase chain reaction (PCR). Fluorescently labelled PCR products were analysed on an ABI 3100 automated sequencer with a fluorescence detection system.

DNA extraction from brain tissue

In the large family with only two patients with the clinical picture of Parkinson's disease and many others affected by symptoms resembling essential tremor (Family E, Danish-American), blood for DNA extraction was only available of one patient with the clinical diagnosis of Parkinson's disease (III-12). To disclose a possible association of an *LRRK2* mutation and clinical features of essential tremor, DNA was extracted from paraffin-embedded brain tissue of one other family member with the parkinsonian phenotype (III-7), who died in 1993 (Wszolek *et al.*, 1996) and had first degree relatives presenting only with essential tremor but not the clinical picture of Parkinson's disease.

Deparaffinization was performed using xylene and ethanol followed by a proteinase K digestion. The probe was then purified using phenol/chloroform extraction and finally precipitated with LiCl and ethanol.

Clinical investigations

The index patients of families with mutations in the *LRRK2* gene were invited for a genetic consultation and clinical and neuroimaging investigations under an approved protocol. After informed

Table 1 Primers for sequence analysis

Exon	Length	Primer-sequences, forward/reverse
1	284	CCTGCCGGTTCCCTGAG/TTTGCAAATGTAAGGAGGGG
2	203	GGGGTGCTGTGGATTGTG/GCCAGTAAAACGTCTCCTTCC
3	259	TTGAAGAGATTCATGTTTGACTGAG/CAAGGGAAGGTTGTCATGTG
4	330	ACTACAGGGAATTAAATACAATGAGAG/CAAGCTACCCTAATCCTGATCTTC
5	322	CCATGGGTCTACAAACCATTC/TGTGTTCTACTTTTCCCAGTATAAGC
6	399	GAAGGGCTGCTTCACAGAAA/GGGTTGAGCATCCACAAGTT
7	330	CATTATGCTGCCATCTATTTACAG/ATGCTACTTATCCATTATTATGCAAC
8	462	TTGCTCAATCACTTCCATTG/CACAGGACAGAAAAGGCATTG
9–10	601	AAGCACACCTCATTCTGTTGG/CCTGAAGGTGATGAGATCAAAATAC
11	468	CTCTTGTAAGTGGAGGTGGC/CACATAGAAGTCCGGAAAAATATAC
12	296	ATGCTTTCCTGTAAATTTGGAC/AAATATTGATATTCTACCTGGCCC
13	348	ATATTGGTTCTGCCCTCCTG/CAGAAAGATTTACATTTAGTCGACAG
14	506	CAGCATCTTAAAGCACAGCC/AAAAAGCGCCCCTAGTTCTC
15–16	686	TACAATGCCTGGCACAGAAC/TGCTCCAAAAGTGAAATTCG
17–18	597	GATAAATACTTTAAAGCACCAACCC/GATACACAATGGCAGGGCTC
19	428	GAAGTTTGATTTGCCAGTCTCC/TCAAACTGGCATGAATAACCAC
20	358	GCAATACGTAAGAACTTTGGTCC/GGTCAGGTTTTTGTCTTGGG
21	411	AAGTGAAAAACCAACATGGC/ACATCAGGGAAATCCCTACC
22	245	TTCCCATAATTATAAATACCATTAACC/ACCAAACACTGCATTCTGCC
23	436	AGCCTGATTGCTAGGAGGTG/GGGGGACTTATCACCCAGTG
24	356	GCTAGACTTAAGTTCCTCAGATGG/TCAGCATATTTAGGCAACCC
25	348	TCCTCTTTGATGCTGTTCTTTG/TGCCACTTTTAAATCCACAAC
26	233	CACTATTGGTAGCTGTTCTTATTTTTG/AAGGTTCTGTTCCAGCTAATGTG
27	565	GGTGGTTCAACTTCAGGCTC/AATGGAAATTAAATTAAGTGACACATC
28	300	CTTCCTTCCCACCAACAGG/TGTCCATCAAAGTCACAGAGAG
29	496	TTTTACCAAACATTATCAACTACCC/TCCCTGTTCCAAACAAATGG
30	289	GGATTCTTGCCTGTCGTTTG/ACTGAAGCAATTGTTTGCCC
31	332	AGCAGGCCCAGTTTGAAAG/GACATTTCTAGGCAGTTGAGAATC
32	387	CTGAATTTGCCAACCATTTG/GAACCGTATGGATATTCTCTCAAC
33	196	AAAGCCCCTTGATATTTGTTC/ATGCTTTGACCATAACCCCC
34	487	TGGTTGCTAGAGAAATTAGGTACTG/AATGATACATGTCAGTAGGAGGTTTAC
35	291	AGGTTGGGTGTTTTGTGAGG/ATGCCATCTCCCTAATTTCTC
36–37	900	TGGATCTTAATGTGCAGGGG/CAGCATTCAAACCCTCAAATC
38	467	GGATTGGTTAGAAAGGGAGGG/GATTATGTGCAAAACAAATTCCAG
39	394	TTCAATGAAACAAGTAGGTCAGG/CACAAAACTTTCTACTCCACAACG
40	376	CATGTTCAGCCTGTTGATGC/GGCACAGTGTTACTGGGAAG
41	331	GCACAGAATTTTTGATGCTTG/GAGGTCAGTGGTTATCCATCC
42	290	TATGAGCCCTGATGTTGGTC/CAATTAAATAAAAATGAAGCTGCTG
43	361	TTTCTTTGCAATGTCTGGACC/TTGTGCCTGGGATGGTG
44	471	TTGTGCCTGGGATGGTG/CAAAACACATTTGCTCTTGAATAC
45	362	CCTTTATTCTGTACAAGTTCTAGTTGC/TGAACAATCTTTGCTGATGC
46	233	AAAGTGGAGGAGAACATTAAGGC/AATCCCATAAGAGGGGTGTG
47	364	TTTGAAAGCACAGATTTTATGGAG/AAGATCTTCCTTATGAATTATCAACAG
48	474	TCAATTCAGAATGGTTAGGGAAG/GAAAAGATGGTGCTGAGAAGC
49	450	TGCATAATGGTGGTGGTGTC/TGTGACCCTCCAAGACCATC
50	239	TTCAGTTCCAAGGTATTTGTGTC/TGTTACCATCATTCACATCATTG
51	871	TTTGAACAGTGTTTGAAAAAGC/CATTTCGATGCAATCTAAGAAGG

Given are the exons as well as the length of primers and the sequences of each forward/reverse primer. See GenBank accession no. AY792511.

Table 2 Primer sequences for haplotype analysis, consisting of two flanking and three intragenic markers

Location	Forward	Reverse
D12s2194	F_GAGGACTATGATTGCCATGG	R_AGGGCATACAAAATGTCCCT
D12s1048	F_GGTCTGCTTAGGTCCCTTTT	R_AAGGAACCAAGGAGTGGAAG
Intragenic_1 (Intron 5)	F_TTCAGATGTTTGGGGCAAGT	R_CATGAAGACTGTGAATGGTTTG
Intragenic_2 (Intron 20)	F_CCAGACAGAAGTCTGAAGGACA	R_TCCAAAACAGACAAGAGGTTGA
Intragenic_3 (Intron 29)	F_ ATGAAGCCTTGGCTCTTCAA	R_TCCCAATTCAAAATTTTAGTGC

consent was given we performed a thorough neurological examination and tested olfactory function using sniffing sticks (Daum *et al.*, 2000). A neuropsychological test battery sensitive for dementia, concentration, planning, as well as intelligence was chosen (Table 5; Owen *et al.*, 1992, 1995). To evaluate mood and sensitivity, patients were asked to complete the Beck Depression Inventory (BDI) and the Parkinson's Disease Quality of Life Questionnaire (PDQ-39) (Jenkinson *et al.*, 1997).

Electrophysiological investigations comprised neurography of the right tibial and sural nerve, and electromyography of the quadriceps to discern subclinical changes in motor unit potentials and possible abnormal spontaneous activity. Moreover, magnet evoked potentials were performed in all patients without contraindications.

Neuroimaging

Structural neuroimaging comprised transcranial sonography (TCS) and MRI.

For TCS a phased-array ultrasound system equipped with a 2.5 MHz transducer with an axial resolution of ~0.7 mm and a lateral resolution of ~3 mm (Elegra; Siemens, Erlangen, Germany) was used. The examination was performed through a preauricular

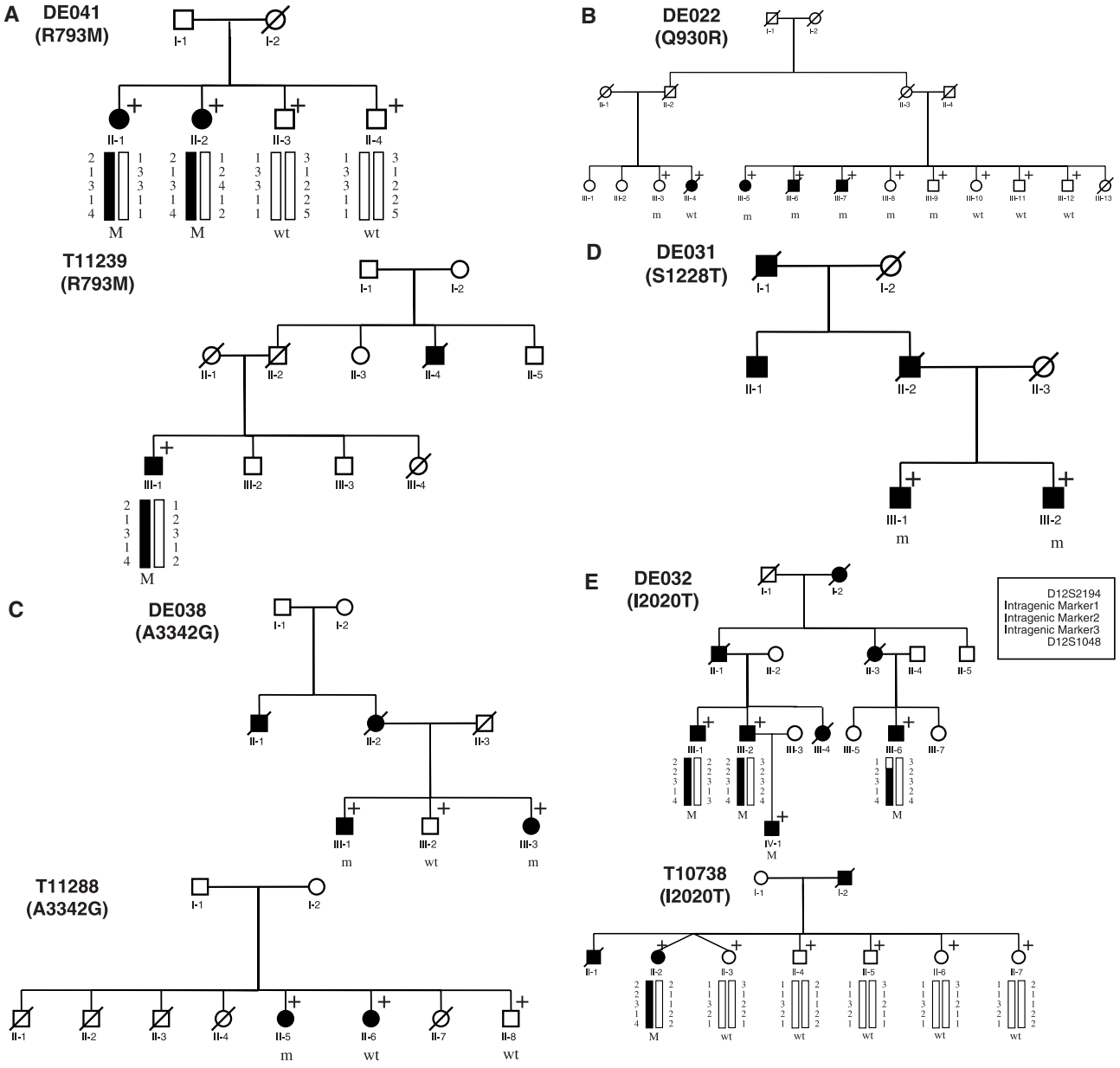

Fig. 1 (**A**–**F**) Pedigree structures of families with *LRRK2* mutation. Except family DE038, which is shown to demonstrate co-segregation in the first family investigated (Zimprich *et al.*, 2004*a*, *b*) and DE032 (**E**), which is shown to demonstrate the same haplotypes in the two families affected by the I2020T mutation, all pedigrees display novel families. Closed symbols denote family members with the clinical presentation of Parkinson's disease, '+' denotes a genotyped individual, with 'M' for mutation carriers and 'wt' for wild-type *LRRK2*. The shaded symbols in **F** denote family members with the clinical presentation of tremor. To protect confidentiality the genotypes of some unaffected family members are not shown. Moreover, the gender of individuals in the youngest generation of family E is disguised.

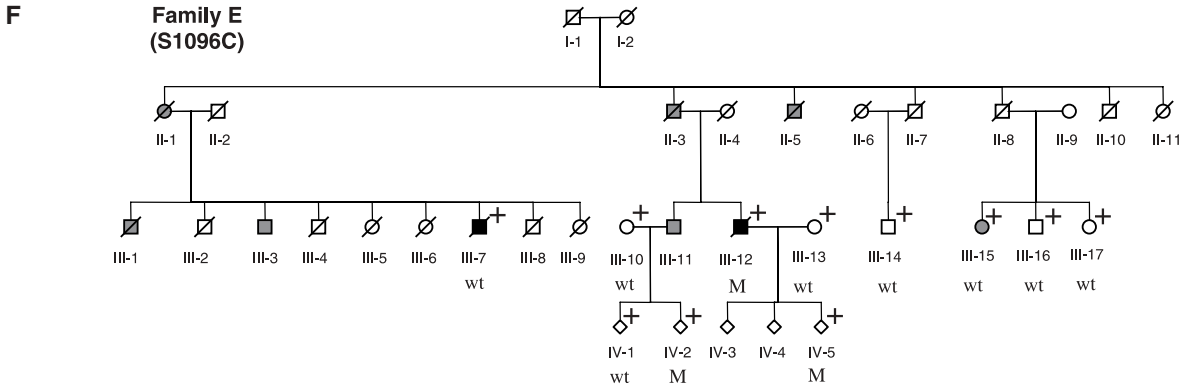

Fig. 1 continued.

acoustic bone window with a penetration depth of 16 cm and a dynamic range of 45 dB as described previously (Berg *et al.*, 1999). The substantia nigra was identified within the butterfly-shaped structure of the mesencephalic brainstem as clearly as possible, scanning from both temporal bone windows, then the area of hyperechogenic signals in the substantia nigra region was encircled and measured (Berg *et al.*, 1999, 2001). An area of substantia nigra hyperechogenicity $\leq$0.19 cm^2 was classified as normal, an area >0.19 and $\leq$0.24 cm^2 as moderately and an area of >0.24 cm^2 as markedly hyperechogenic (Berg *et al.*, 1999).

MRI was performed on a Magnetom Avanto 1.5 Tesla, Siemens AG, Germany, Erlangen using a modern 12 channel head coil for parallel imaging. Among others, imaging protocol consisted of standard strongly T_2-weighted turbo spin echo images in three orthogonal orientations. These images were used to perform an evaluation concerning brain atrophy, iron deposition and microangiopathic changes by a senior neuroradiologist.

Results

Mutational screening

Screening the entire coding region of the *LRRK2* gene of one index patient each from 53 families in addition to the 46 described initially by our group (Zimprich *et al.*, 2004*a*), we identified 7 novel families with amino acid substitutions (Fig. 1). Four of these are novel missense mutations: R793M; 2378G>T in family DE041 and family T11239, Q930R; 2789A>G in family DE022; S1096C; 3287C>G in family E and S1228T; 3683G>C in family DE031. The missense mutation R793M was also found in one patient with late onset sporadic Parkinson's disease presenting with rigidity and resting tremor (Table 4) and one female control person, aged 40 years with no extrapyramdial symptoms.

The so far most common amino acid substitution G2019S; 6055G>A was only found in one patient with sporadic Parkinson's disease, who presented at an age of 43 years with rigidity, resting and intermittent postural tremor (Table 4). Moreover we identified one additional patient with the already described splice site mutation 3342A>G (family T11288) and one more family with the described I2020T mutation (family T10738) (Zimprich *et al.*, 2004*a*).

Table 3 Frequency of the novel mutations

Variant	Exon	PD families (n = 53)	Idiopathic PD (n = 337)	Controls (n = 1200)
R793M; 2378G>T	19	2	1	1
Q930R; 2789A>G	21	1	0	0
S1096C; 3287C>G	24	1	0	0
A3342G	24	1	0	0
S1228T; 3683G>C	27	1	0	0
G2019S; 6055G>A	41	0	1	0
I2020T; 6059T>C	41	1	0	0

Mutational screening was performed in 53 families with Parkinson's disease additional to the 34 families of our first study, 337 patients with sporadic Parkinson's disease and 1200 matched controls.

Except for the R793M mutation, which was found in one control person, none of the mutations were found in the control group (Table 3). There was no significant difference in the minor allele frequency of the known N551K; 1653C>G polymorphism between patients with sporadic Parkinson's disease (6.5%) and control subjects (7.3%).

Haplotype analysis

Haplotype analysis revealed common haplotypes for the two novel families affected by the R793M mutation as well as for family T10758 and family DE032 affected by the I2020T mutation, indicating common founders for these mutations (Fig. 1A and E). Although members of family DE032 and T10758 were not aware of common ancestors, they originate from the same geographical area (Baden Wuerttemberg, Southern Germany). Family T11239 and DE041 were recruited from more distinct geographical areas (Baden Württemberg family T11239 and Hesse family DE041). Members of these families were also not aware of common ancestors. For the A3342G splice site mutation no common haplotype was found in the affected families (DE038 and T11288).

Clinical findings

Extensive clinical and neuroimaging examination revealed the features listed in Table 4.

Table 4 Clinical features

Family/patient and sporadic mutation carriers	T11239	DE041/ II-1//II-2	Sporadic	DE022/ III-5//III-6// III-7	Fam. E	T11288/ II-5	DE031/III-1//III-2	Sporadic	T10738/II-2
Mutation	R793M	R793M	R793M	Q930R	S1096C	3342A>G	S1228T	G2019S	I2020T
Age at onset (years)	42 Uncle: 79	55//70	71	68//58//47	63	77	49//49 father: 62 grandmother	43	57 father: 59 68
Disease duration	25∼	14∼//10∼	1	8∼//19//30	15	4∼	8∼//12∼	1	3∼
Initial symptom	B/RT	PT//RT	RT	B,RT//B// B,RT	RT	B	RT//B, RT	RT	B,RT
Response to L-dopa	+	+//+	+	+//+//+	+	+	+//+	+	+
Bradykinesia	+	+//+	+	+//+//+	+	+	+//+	+	+
Rigidity	+	+//+	+	+//+//+	+	+	+//+	+	+
Resting tremor	+	−//+	+	+//+//+	+	−	+//+	+	+
Postural instability	+	+//+	−	−//+//+	+	+	−//−	−	−
Sleeping disturbances	+	+//+	+	+//+//+	−	+	−//+	+	+
Long-term complications	+	+//+	−	+//+//+	−	−	+//+	−	−
Hallucinations	+	−//−	−	−//−//+	−	+	−//−	−	−
UPDRS (on)	31	41//38#	20	26//34//58#	25#	51	27//56	16	44
Additional findings	Tongue dystonia	−	−		−	Dementia L-dopa hypersensitivity		−	
Sniffing test	2/8	nd	nd	nd	nd*	7/8	5/8//5/8	nd	7/8
Electro-neurography	na	nd	nd	nd	nd	na	na//na	nd	na
Electro-myography	na	nd	nd	nd	nd	na	na//na	nd	na
Magnet evoked potentials	na	nd	nd	nd	nd	na	na//na	nd	na
TCS (r;l)	0.21; 0.24	nd	nd	nd	nd	0.24; 0.19	0,23;0,24// 0,22;0,24	nd	0.16; 0.17
MRI	normal	nd	nd	nd	nd	global atrophy, microangiopathy	slightly increased atrophy in both, slight micro-angio-pathie in III-2	nd	Slight fronto-temporally enhanced atrophy

Clinical and neuroimaging features of affected members of the families and the two sporadic patients with *LRRK2* mutations of our second cohort. Not all subjects could be investigated with the same methods, which is indicated using nd (not done). No change in comparison with normal is indicated using na (no alteration). ∼, ongoing at the time of examination; B, bradykinesia; R, rigidity; RT, resting tremor; UPDRS, unified Parkinson's disease rating scale. Patients were examined at the time blood was taken when they were on medication. While disease duration and all symptoms listed refer to the time the manuscript was written, blood was taken 3–5 years earlier in patients marked with #, and UPDRS-scores were obtained at that time. For brief evaluation of olfaction a sniffing test consisting of 8 different odours (/8) was used. No sniff test but University of Pennsylvania Smell Identification (UPSIT) test was performed on the patient of family E, who was normosomic with score of 35/40.

All patients investigated had typical signs of Parkinson's disease. However, features differed between members within the same family affected by the same mutation as well as between different families with the same mutation. Moreover, penetrance was found to vary for different mutations.

Common findings in patients with *LRRK2* mutations

All mutation carriers with clinically apparent Parkinson's disease had the typical parkinsonian features including bradykinesia, tremor and rigidity. Moreover, all patients experienced substantial relief of symptoms after application of L-dopa, although therapy was complicated in one patient (T11288 II-5) by hallucinations. Estimation of olfactory function by application of eight sniffing sticks revealed a moderate to severe loss of identification capacity in three of five subjects. Postural instability was only found late in the disease course. Hallucinations were seldom reported and only occurred after long disease duration or associated with dementia, whereas sleep disturbances were reported by 85%, comprising one or several of the following: difficulty falling asleep, frequent wake up and very early wake up in the morning without going back to sleep (Table 4).

Intrafamily differences in clinical presentation

R793M: Two sisters are affected with a difference of age of onset of 15 years. While at disease onset II-1 had only slight postural tremor on the right side, the initial symptom of II-2 was resting tremor on the left side. An equivalent type of

Parkinson's disease developed in II-2 while II-1 showed no resting tremor at all but an akinetic-rigid type of Parkinson's disease (Fig. 1A).

Q930R: Span of age of onset was 21 years among the three members of the same generation affected. Only brother III-7 developed severe dementia and hallucinations after >20 years of disease duration (Fig. 1B).

3342A>G: While sister II-7 of family T11288 presented with typical parkinsonian features, the clinical picture of early severe dementia, hypersensitivity to dopaminergic hallucinations and daytime sleepiness with fluctuation of vigilance resembled diffuse Lewy body disease in II-5. However, mutational analysis revealed the wild-type allele in II-7. A phenocopy for the more typical Parkinson's disease presentation must, therefore, be postulated, while the atypical diffuse Lewy body disease-type was indeed associated with the 3342A>G splice site mutation. However, the fact that this variation co-segregated with the mutation in the previously described family DE038 (Zimprich *et al.*, 2004*a*) argues for a mutation rather than a benign polymorphism.

Interfamily differences in clinical presentation for the same mutation

R793M: While in III-3 of family T11239 speaking was almost impossible because of severe tongue dyskinesia, the affected sisters of family 41 did not show any atypical signs except of postural tremor in II-1 (Fig. 1A).

3342A>G: In family T11288 both sisters and in the previously reported family DE038 father and III-1 were severely affected by the disease. III-3, however, did not show any parkinsonian symptoms except of minimal resting tremor of the right thumb for >15 years (Fig. 1C).

Age of onset

Mean age of onset in the novel families was 59 ± 13 years. However, age of onset differed between members of the same family. In offspring of mutation carriers of the three novel families, in whom clear data of ancestors were available (Table 4) the diagnosis of Parkinson's disease was established earlier and also investigation of an additional family member in family DE032 (described in Zimprich *et al.*, 2004*a*) revealed an earlier diagnosis (41 years), while mean age of onset was 54 (48–59 years) in generation I–III (Fig. 1E).

Penetrance

Combining findings of the actual study and our first examination we found a clear autosomal dominant mode of inheritance in at least one affected family for the splice site mutation of exon 24, and for the missense mutations of exon 25, exon 27, exon 31 and exon 41. No strong genetic pattern was found in families affected by missense mutations in exon 19, 21 and 24.

Exon 19, R793M: In family T11239 only the uncle of the index patient was affected, while the father who died at the age of 68 did no show any extrapyramidal sign during lifetime. In family DE041 two sisters showed typical signs of Parkinson's disease during lifetime, while none of the parents who died both at the age of 74 showed any parkinsonian signs (Fig. 1A).

Exon 21, Q930R: Of nine sisters and brothers in family DE022 three were affected by the mutation and had clinical signs of Parkinson's disease, while one other sister and brother, also mutation carriers, are not affected by parkinsonian symptoms at an age of >70 years. Neither the mother (II-3), who died at the age of 90 years, nor her brother (II-2), who died at 75 years of age, showed any parkinsonian features during lifetime. The cousin of the affected members of the family (III-4) displayed signs for typical Parkinson's disease but was not carrier for the Q930R mutation, indicating sporadic Parkinson's disease in this family member. However, her sister (III-3) was found to have the mutation. Having already reached the age of 77, she has no clinical signs allowing the diagnosis of Parkinson's disease. Both II-3 and II-2 must have been mutation carriers. The fact that none of them and also III-3 have not shown any parkinsonian features during lifetime argues for incomplete penetrance of this mutation.

Exon 24, S1096C: In this large family with an additional tremor phenotpye (1F) only III-12 was a mutation carrier and affected by Parkinson's disease. One child of his brother, who showed only features of essential tremor but no parkinsonian symptoms until death at the age of 66 years, is also a mutation carrier, indicating incomplete penetrance for this mutation as well.

Phenocopies and simultaneous occurrence of tremor

One family member with typical Parkinson's disease of DE022 associated with the Q930R mutation and one of the sisters of family T11288 (A3342G splice site mutation of the other sister), again with typical parkinsonian features, had wild-type alleles, arguing for idiopathic Parkinson's disease in these cases.

In family E an autosomal dominant inheritance of tremor is evident (Fig. 1F). Two family members (III-7 and III-11) showed typical parkinsonian features during lifetime, in III-12 the S1096C mutation was detected. As there was no blood available of III-7, DNA extracted from brain tissue was investigated. However, in this patient the C3287G mutation could not be detected, indicating a different cause for Parkinson's disease. Of one of the siblings with a tremor phenotype (III-15, presenting with postural and vocal tremor) also only wild-type alleles could be identified. The only family member with a tremor phenotype carrying the S1096C mutation must have been the brother of III-12, as one of his children is also mutation carrier. However, as the mutation could not be detected in III-15 incomplete penetrance of parkinsonian symptoms in a subject also affected by tremor

Table 5 Neuropsychological assessment

Family/patient	T11239	T11288/II-5	DE031/III-1//	DE031/III-2	T10738/II-2
Mutation	R793M	3342A>G		S1228T	I2020T
LPS-K/SPM (IQ)	/ 90↓	75↓↓ /	111↑ /	117↑ /	120↑ /
Tower of London (PR)	–	21↓	6↓↓	27↓	72↑
FWIT (*T*-value INT)	–	(63)	37	41	41
CERAD 1 (verbal fluency)	–	6	20∼	31↑	36↑
CERAD 2 (Boston naming test)	–	12	14∼	15∼	14∼
CERAD 3 (MMSE)	21	24	30∼	30∼	29∼
CERAD 4 (word list memory)	–	10	19∼	22∼	29↑
CERAD 5 (constructional praxis)	–	7	10∼	10∼	11∼
CERAD 6.1 (word list recall)	–	1	7∼	7∼	10↑
CERAD 7.1 (word list recognition)	–	10	9	10	10
CERAD 8 (recall of constructional praxis)	–	2	10∼	13↑	12↑
D2-test (concentration)	–	–	38	46	46
BDI	23↑	11	3	9	7.5
PDQ-39 (PDSI)	40.4	25.6	8.8	18.6	23.6

Tests applied for intelligence (short version of the common German intelligence test Leistungsprüfsystem' LPS-K, Rawen's standard progressive matrices—SPM), executive function (Tower of London), interference (German version of the Stroop test—FWIT), dementia (Consortium to establish a registry for Alzheimer's disease—CERAD 1–8; MMSE, mini mental state examination); concentration (D2) as well as mood (Beck's depression inventory—BDI) and quality of life (Parkinson's Disease Questionnaire—PDQ-39). ↓, Performance below; ∼, average; ↑, above mean ± standard variation of matched healthy controls.

is more likely than an association of tremor with the mutation in this family.

Neuropsychological findings

Of the five patients examined in a thorough neuropsychological investigation, three were able to complete the whole test battery. In all three intelligence was above average of a matched control group in the short version of the common German intelligence test 'Leistungsprüfsystem' (LPS-K), suggesting that subtle neuropsychological deficits may well be compensated. Still, two of the three showed deficits in executive functions (Tower of London) and all had high interference scores in the German version of the Stroop test (FWIT) indicating incapacity to blind overstimulation (Table 5). This pattern is in accordance with neuropsychological deficits in idiopathic Parkinson's disease. The two others investigated were graded as demented. In patient III-3 of family T11239 mini mental state examination (MMSE) was 21. Additionally, severe tongue dystonia prevented accomplishing the test of the consortium to establish a regristry for Alzheimer's disease (CERAD). In Patient II-5 of family T11288 with 3342A>G splice site mutation exhaustibility and dementia thwarted completion of neuropsychological testing.

TCS and MRI findings

Transcranial sonography: Moderate hyperechogenicity, at least on one side, was found in all but one patient with *LRRK2* mutations. Interestingly, none of the patients displayed marked substantia nigra hyperechogenicity. MRI showed mild to marked atrophy in three of the four patients investigated (Table 4). The patient with the diffuse Lewy body disease phenotype had additionally some evidence for microangiopathy.

Discussion

Screening the entire coding region of the *LRRK2* gene in a cohort of 53 apparently unrelated families, we identified seven more families with amino acid substitutions or one splice site mutation. In our previous study, we reported mutations in the *LRRK2* gene in 6 out of 46 families (34 with features consistent with autosomal dominant Parkinson's disease and 12 affected sib pairs). Mutations in the *LRRK2* gene, therefore, account for 13% of familial Parkinson's disease in our total cohort. Three families were recruited in North America with ancestors from Denmark, Northern Germany and England (Denson and Wszolek 1995). The other families originated from Southern or Middle Germany.

In this paper we describe four novel mutations (R793M, Q930R, S1096C and S1228T). Therefore, together with the seven published mutations, until now, ten missense mutations and one splice site mutation have been described. The *LRRK2* gene consists of 51 exons comprising five conserved domains: (i) a leucine-rich repeat, (ii) a Ras in complex (ROC) domain indicating the affiliation of the protein to the Ras/GTPase superfamily, (iii) a C-terminal domain of ROC (COR), (iv) a tyrosine kinase catalytic domain and (v) a WD domain. Nine mutations are all within these conserved domains (Fig. 2). The R793M and the Q930R mutations, however, are located in exons 19 and 21, respectively, which are part of the ancyrin repeat region (amino acid 678–806) that seems to take part in protein–protein interactions.

In contrast to previous reports, the so far most common mutation (G2019S; 6055G>A) was not detected in any of the families investigated but only in 1 out of 337 patients with

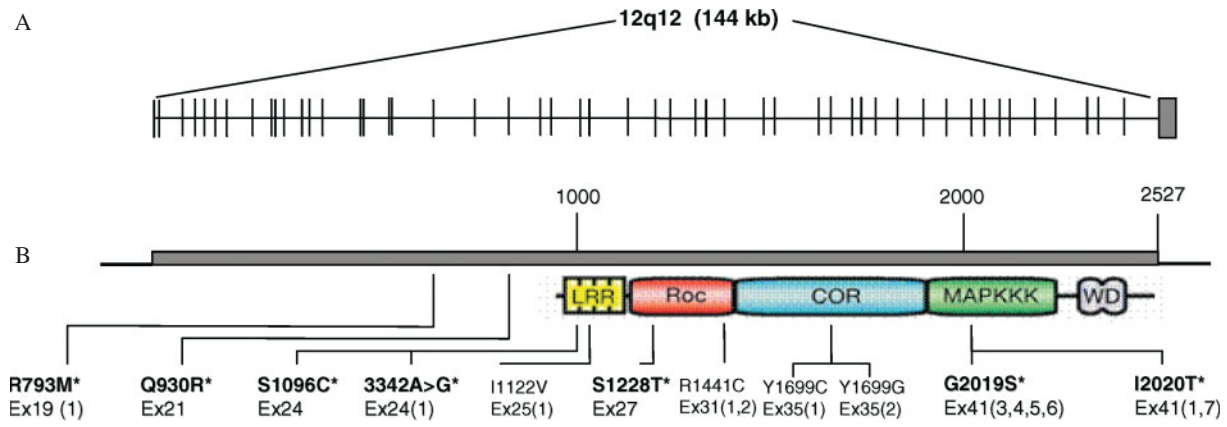

Fig. 2 (**A**) Exon positions. (**B**) Schematic diagram of *LRRK2* domains and positions of mutations. Bold asterisks, mutations found in this study; in parentheses, reference of mutations found previously. (1) Zimprich *et al.* (2004*a*); (2) Paisan-Ruiz *et al.* (2004); (3) Di Fonzo *et al.* (2005); (4) Nichols *et al.* (2005); (5) Gilks *et al.* (2005); (6) Toft *et al.* (2005); (7) Funayama *et al.* (2005).

sporadic Parkinson's disease. We, therefore, conclude that predominance of this mutation (Gilks *et al.*, 2005; Toft *et al.*, 2005) cannot be established for all populations. We additionally found the novel R793M in one sporadic patient in our cohort comprising 337 patients with sporadic Parkinson's disease. Therefore, all known *LRRK2* mutations investigated account for only 0.6% of sporadic Parkinson's disease cases in our population.

In three families the specific variation did not co-segregate with one family member each: In family DE022 (Q930R) only three of the four family members affected by the disease were mutation carriers (Fig. 1B), in family E in fact only one of the two family members with a Parkinson's disease phenotype was a carrier of the S1096C mutation (Fig. 1F), and the splice site mutation co-segregating with Parkinson's disease in one previously investigated family (DE038) was only found in one of the clinically affected sisters (T112888) (Fig. 1C). As none of these variations was found in any of the 1200 controls investigated and the splice site variation affected two distinct Parkinson's disease families it is likely that they are causative for the disease, although incomplete penetrance at least in family DE022 could indicate that additional factors may contribute to manifestation of the disease in affected subjects. We, therefore, suggest phenocopies in these three families, as the high prevalence of Parkinson's disease in the population makes it well possible that other causes of Parkinson's disease occur in a family affected by *LRRK2* mutations. Disease phenocopy is not uncommon in Parkinson's disease. It has been described in the original α-synuclein A53T kindred (Polymeropoulos *et al.*, 1997), in family D with the *LRRK2* R1441C mutation (Zimprich *et al.*, 2004*a*, *b*) as well as in a family with the *LRRK2* G2019S mutation (Hernandez *et al.*, 2005). However, association of these mutations with the disease in other families with autosomal dominant Parkinson's disease would be helpful and examination of greater cohorts of controls and functional analyses are mandatory to prove the pathogenic relevance of these three mutations.

Three of our mutations affect at least two families. For two of these (R793M and I2020T) haplotype analysis revealed a common haplotype indicating a common founder. None of the families was aware of a possible relation to the respective family although the two families harbouring the I2020T mutation lived in the same geographic region. The same mutation has also been described in the Japanese family, who served as the basis for the original defining of the *PARK8* locus (Funayama *et al.*, 2005). It, therefore, needs to be established, whether this family shares the same haplotype indicating either a common founder or an association with a frequently occurring haplotype.

The R793M mutation, detected in two distinct families with the same haplotype, was also found in one patient with sporadic Parkinson's disease and one control person. Because of technical problems in assessing this CG rich exon call rate of the population screened was low (~50% in three different tries). Therefore, it may well be, that this mutation is more frequent in patients with apparently sporadic Parkinson's disease. Also, the possibility of a polymorphism needs to be taken into account, if this variation was detected in more controls. On the other hand, as the control person carrying this variation was fairly young, development of Parkinson's disease later in life is still possible. Common founders are also suggested for other families affected by mutations in the *LRRK2* gene (Kachergus *et al.*, 2005; Lesage *et al.*, 2005; Mata *et al.*, 2005).

Mode of inheritance of *LRRK2* mutations is autosomal dominant. It has been suggested that penetrance of *LRRK2* mutations is age dependent (Di Fonzo *et al.*, 2005; Toft *et al.*, 2005) accounting for the reduced penetrance in some families. In our families reduced penetrance was only observed in mutations of exons 19 and 21 located before the highly conserved LRR domain. This might indicate that mutations in this region are less severe and have to be associated with other so far unknown factors for disease manifestation. From the splice site mutation of exon 24 onwards, penetrance was complete, although one splice mutation carrier (DE038, III-1) had only

slight resting tremor for several years, while his sister (III-3), mother and uncle were affected by severe Parkinson's disease.

In all families with definite documentation of age of onset an earlier recognition of first parkinsonian signs was observed in the younger generations. So far, there are no known pathomechanisms that allow the hypothesis of anticipation. Rather, a number of biases may account for this observation including a greater awareness of a possible affliction and a more thorough investigation in families in whom Parkinson's disease has already been diagnosed.

In accordance with our previous observation the clinical presentation of *LRRK2* mutation carriers varies within families and between families affected by the same mutation. In general the typical phenotype of Parkinson's disease with resting tremor, bradykinesia, rigidity and olfactory dysfunction can be observed. Interestingly, tremor, the main and naming feature of some of the initially described families (Paisan-Ruiz *et al.*, 2004), was neither the main initial nor the leading symptom in many of our patients with Parkinson's disease. Two patients did not report any resting tremor in their medical history. Rather, the typical pattern of different subtypes known from idiopathic Parkinson's disease could be observed. All patients reported a substantial relief of symptoms after application of dopaminergic treatment, which was hampered by hallucinations in only the one patient with diffuse Lewy body disease-phenotype.

In patients with *LRRK2* mutations, a frequent strongly afflicting symptom seems to be sleeping abnormality. Out of 13 patients, 11 (85%) reported suffering from difficulties of either falling asleep, staying asleep or both. According to several studies, sleeping disturbances occur in ~40–75% of Parkinson's disease patients (Lees *et al.*, 1988; Kumar *et al.*, 2002), but only the minority (~20%) report sleeping abnormalities as a problem (Lees *et al.*, 1988). In our study 85% stated that sleeping disturbances were indeed a problem. More detailed assessment on sleeping behaviour and pattern is needed to decide whether this symptom is more pronounced in *LRRK2* mutations carriers, possibly indicating an earlier involvement of the respective systems. Postural instability occurs late in the course of the disease. As also described by others (Paisan-Ruiz *et al.*, 2005) dementia is not a common finding in *LRRK2* associated Parkinson's disease and seems to occur rather late in the disease process. The same holds true for hallucinations in our patient cohort, occurring either late in the disease process or in combination with dementia. In this cohort, one patient presented with the typical clinical picture of diffuse Lewy body disease. Autopsy of one subject with dementia in our first cohort revealed diffuse Lewy body pathology in one family affected by the Y1699C mutation (Zimprich *et al.*, 2004*a*). Description of the same phenotype in another patient in this study affected by a different mutation favours the hypothesis that the clinical presentation of diffuse Lewy body disease may be caused by the same pathophysiological alterations as the clinical picture of Parkinson's disease. Obviously specific pathophysiological changes (in this case caused by mutations in the *LRRK2* gene) may lead to the clinical and histopathological entity of both Parkinson's disease and diffuse Lewy body disease. A similar observation has been made concerning α-synuclein multiplications (Singleton *et al.*, 2003; Farrer *et al.*, 2004).

In our first study, one patient showed mild signs of motor neuron disease (Zimprich *et al.*, 2004*a*). In this cohort, however, motor neuron symptoms were neither clinically nor electrophysiologically disclosed in any patient investigated.

Structural neuroimaging revealed slight to marked atrophy in three of four patients investigated. Disease duration was only 3–12 years in these patients and none of them was classified as demented (Table 4). This contrasts findings of idiopathic Parkinson's disease, where structural MRI is usually normal and atrophy only occurs with disease progression, usually associated with dementia. Comparison of larger patient samples and volumetry is necessary to prove, whether *LRRK2* mutations are indeed more often associated with brain atrophy. The patient with the clinical presentation of diffuse Lewy body disease had marked signs of microangiopathy, which may also be causative for an atypical parkinsonian syndrome. The clinical presentation with fluctuation of vigilance, good response to L-dopa hampered by hypersensitivity and dementia developing over a short period of time, however, makes the diagnosis of diffuse Lewy body disease more likely.

Transcranial sonography revealed substantia nigra hyperechogenicity—the typical sign for idiopathic Parkinson's disease, found in >90% of patients with Parkinson's disease (Berg *et al.*, 2001; Walter *et al.*, 2002)—on at least one side of *LRRK2* mutation carriers. Interestingly, substantia nigra hyperechogenicity was only moderate in all patients investigated, as opposed to sporadic Parkinson's disease, where it is marked in 73–79% of the patients (Berg *et al.*, 2001; Walter *et al.*, 2002). This highly characteristic finding is supposed to be associated with an increase in tissue iron content and possible alterations in iron binding, antedating the manifestation of disease onset (Berg *et al.*, 1999, 2002). An only moderate hyperechogenicity of the substantia nigra in *LRRK2* associated Parkinson's disease may argue for a different course of underlying pathomechanisms, which may finally lead to less iron accumulation in *LRRK2* associated than in idiopathic Parkinson's disease. Similarly, the slower disease progress, documented by less, although, typically located reduction of F-dopa uptake in PET examinations (Hernandez *et al.*, 2005) favours the hypothesis of a different course of the disease.

In conclusion, we could show in two consecutive studies that *LRRK2* mutations account for ~13% of apparently autosomal dominantly inherited Parkinson's disease and sib pairs in our population. Although the phenotype varies within and between families affected by the same mutations, it is very similar to the clinical presentation of idiopathic Parkinson's disease. The causal relation between disease

manifestation and variation is not equally clear for all variations described. In three families the specific variations did not co-segregate with one family member each affected by the disease. As none of these mutations was found in 1200 control persons and one variation was found in two distinct Parkinson's disease families phenocopies are likely. Still, the pathogenetic relevance of these variations needs to be proven.

Moreover, two patients with the clinical presentation of diffuse Lewy body disease should lead to the consideration of *LRRK2* mutations in families with the simultaneous occurrence of diffuse Lewy body disease and Parkinson's disease.

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