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Glucocerebrosidase mutations in clinical and pathologically proven Parkinson's disease

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Mutations in the glucocerebrosidase gene (*GBA*) are associated with Gaucher's disease, the most common lysosomal storage disorder. Parkinsonism is an established feature of Gaucher's disease and an increased frequency of mutations in *GBA* has been reported in several different ethnic series with sporadic Parkinson's disease. In this study, we evaluated the frequency of *GBA* mutations in British patients affected by Parkinson's disease. We utilized the DNA of 790 patients and 257 controls, matched for age and ethnicity, to screen for mutations within the *GBA* gene. Clinical data on all identified *GBA* mutation carriers was reviewed and analysed. Additionally, in all cases where brain material was available, a neuropathological evaluation was performed and compared to sporadic Parkinson's disease without *GBA* mutations. The frequency of *GBA* mutations among the British patients (33/790 = 4.18%) was significantly higher (*P* = 0.01; odds ratio = 3.7; 95% confidence interval = 1.12–12.14) when compared to the control group (3/257 = 1.17%). Fourteen different *GBA* mutations were identified, including three previously undescribed mutations, K7E, D443N and G193E. Pathological examination revealed widespread and abundant α-synuclein pathology in all 17 *GBA* mutation carriers, which were graded as Braak stage of 5–6, and had McKeith's limbic or diffuse neocortical Lewy body-type pathology. Diffuse neocortical Lewy body-type pathology tended to occur more frequently in the group with *GBA* mutations compared to matched Parkinson's disease controls. Clinical features comprised an early onset of the disease, the presence of hallucinations in 45% (14/31) and symptoms of cognitive decline or dementia in 48% (15/31) of patients. This study demonstrates that *GBA* mutations are found in British subjects at a higher frequency than any other known Parkinson's disease gene. This is the largest study to date on a non-Jewish patient sample with a detailed genotype/phenotype/pathological analyses which strengthens the hypothesis that *GBA* mutations represent a significant risk factor for the development of Parkinson's disease and suggest that to date, this is the most common genetic factor identified for the disease.

Keywords: Parkinson's disease; GBA; Gaucher's disease; neuropathology

Abbreviations: AC = amygdaloid complex; BFB = basal forebrain; DMV = dorsal motor nucleus of vagus; GBA = glucocerebrosidase; HRC = human random control; LC = locus ceruleus; NBM = nucleus basalis of Meynert; SN = substantia nigra

Introduction

Gaucher's disease is the most common lysosomal storage disorder and results from the deficiency of the lysosomal enzyme glucocerebrosidase (GBA). It is caused by mutations in the gene coding for GBA and follows an autosomal recessive mode of inheritance. GBA deficiency leads to the accumulation of its substrate, glucosylceramide, within the lysosomes of a variety of cell types, including neurons and macrophages (Beutler and Grabowski, 2001). Clinically, Gaucher's disease is highly variable and the spectrum of disease correlates, at least in part, with residual enzyme activity (Cox and Schofield, 1997). In its most severe, infantile-onset form which has traditionally been termed type 2 disease, there is glucosylceramide accumulation in a variety of cell types, including neurones, which leads to rapidly fatal neurodegenerative disease (Cox and Schofield, 1997). In contrast, in late-onset, type 1 disease, there is enough residual enzyme activity to prevent storage in all cell types except macrophages which are exposed to an exceptionally high glycosphingolipid load due to their role in phagocytosis of effete blood cells. These lipid laden macrophages, termed Gaucher cells, infiltrate the liver, spleen and bone marrow, and patients can present with organomegaly, hypersplenism and, in its most severe form, bone infarction (Cox and Schofield, 1997). Recently, it has become clear that these subtypes are part of a spectrum of disease. In particular, there appears to be a greater range of neurological involvement than previously recognized and a variety of neurological deficits have been described in patients who have what has classically been thought of as type 1, non-neuronopathic disease (Capablo *et al.*, 2008; Sidransky, 2004).

The human *GBA* gene (MIM# 606463) is located on chromosome 1q21 in a gene rich area. *GBA* comprises 11 exons and 10 introns, spanning 7.6 kb of sequence. A non-processed pseudogene (*GBAP*) which shares 96% exonic sequence homology is located 16 kb downstream of the functional *GBA* gene (Horowitz *et al.*, 1989). The presence of this highly homologous pseudogene along with another six genes at the locus increases the occurrence of chromosomal rearrangements and misalignments in this region. These processes provide an explanation for the high number of complex recombinant alleles that have been detected in Gaucher's disease (Hruska *et al.*, 2008).

Although the *GBA* genotype plays a role in determining the type of Gaucher's disease, there is still enormous clinical variation between patients who have the same genotype, including twins, and genotype–phenotype correlations are difficult to make (Lachmann *et al.*, 2004; Sidransky, 2004). Surveys in the Ashkenazi Jewish population suggest that up to 60% of patients homozygous for the common N370S mutation may never present clinically (Beutler *et al.* 1993a). There is consensus, however, that heterozygosity for this relatively 'mild' allele protects the individual from neuronopathic involvement. In contrast, homozygosity for the L444P allele is invariably associated with brain involvement (Grabowski, 1997).

Parkinson's disease is a progressive, neurodegenerative condition, which is characterized primarily by the four cardinal motor symptoms: resting tremor, bradykinesia, rigidity and postural instability. Non-motor features include cognitive impairment, hallucinations, autonomic dysfunction and sleep disorders. Parkinsonism is one of the neurological symptoms described in Gaucher's disease and affected individuals exhibit classical symptoms, including tremor, rigidity and bradykinesia (Neudorfer *et al.*, 1996; Machaczka *et al.*, 1999; Bembi *et al.*, 2003). A relatively common finding is the early age of disease onset (AoO ⩽ 50 years) of parkinsonian symptoms in Gaucher's disease and the presence of cognitive symptoms, such as dementia (Tayebi *et al.*, 2003). Pathological evaluation of brains from Gaucher patients revealed Parkinsonian like features, including α-synuclein immunoreactive cortical and brainstem-type Lewy bodies (Wong *et al.*, 2004). An increased frequency of parkinsonism was noted amongst otherwise healthy relatives of Gaucher patients (Goker-Alpan *et al.*, 2004). Further analysis of these Gaucher relatives revealed a possible association between heterozygous changes in *GBA* and Parkinsonism. These findings led to the hypothesis that even heterozygous mutations in *GBA* might constitute a genetic risk factor for the development of Parkinsonism.

Subsequent *GBA* genotyping studies on various cohorts of Parkinson's disease patients showed an increased frequency of *GBA* mutations (Supplementary Table S4). Whilst most studies focused on the more common pathogenic mutations in *GBA*, such as N370S and L444P (Aharon-Peretz *et al.*, 2004; Clark *et al.*, 2005; Sato *et al.*, 2005; Mata *et al.*, 2008; Gan-Or *et al.*, 2008), some smaller studies also performed a complete sequencing screen of the entire *GBA* gene (Lwin *et al.*, 2004; Goker-Alpan *et al.*, 2006; Ziegler *et al.*, 2007; Bras *et al.*, 2007; Clark *et al.*, 2007; Spitz *et al.*, 2008). Carrier frequencies for *GBA* mutations differed between 10% and 31% in the Ashkenazi Jewish Parkinson's disease population, and 2.9% and 12% in Parkinson's disease cohorts of non-Ashkenazi-Jewish origin, such as North American (with European background), Taiwanese, and Italian. The lowest carrier frequency was reported to be 2.3% in Norwegian Parkinson's disease patients as compared to 1.7% in controls (Toft *et al.*, 2006).

In this study, we explored the association between mutations in *GBA* and Parkinson's disease by performing a sequencing screen of 790 British patients with the disease and 257 age and ethnicity-matched controls. Our study examined all *GBA* exons and flanking intronic sequences where possible and, to our knowledge, represents the largest study to date on a non-Ashkenazi-Jewish Parkinson's disease patient sample in which an extensive review of clinical data on all *GBA* mutation carriers and a pathological evaluation of *GBA* carriers was performed. The aim of this study was to more accurately define the role of *GBA* mutations in a non-Ashkenazi-Jewish population and to provide detailed phenotype and neuropathological correlation data to clarify the clinical parameters.

Methods

Parkinson's disease patient cohort and control groups

Genomic DNA samples from 790 patients diagnosed with Parkinson's disease were screened for mutations in the *GBA* gene. A total of

380 cases had been diagnosed with pathologically proven Parkinson's disease and were procured from the Queen Square Brain Bank at the Institute of Neurology, UCL (346 cases) or from the Parkinson's Disease Society Tissue Bank, Imperial College London, UK (34 cases). 410 cases were from a series collected by the Department of Molecular Neuroscience at the Institute of Neurology, UCL. All subjects met the UK Brain Bank Clinical Criteria for Parkinson's disease (Hughes *et al.*, 1992). The mean age of disease onset was 58.7 ± 12.3 years. The male-to-female ratio in this series was $3.5:1$. Among the 790 Parkinson's disease samples, 83 were associated with familial Parkinsonism, whereas 707 samples were diagnosed with sporadic Parkinson's disease and showed no pattern of Mendelian inheritance. Familial Parkinson's disease was defined as showing a positive family history compatible with the diagnosis of Parkinsonism in at least one first or second degree relative.

Control DNA was procured from three different sources. The first control DNA set, control group 1, consisted of 115 samples of the European Collection of Cell Cultures (ECACC), the Human Random Control (HRC) panels 1 and 2. DNA was extracted from lymphoblastoid cell lines generated from peripheral blood lymphocytes of healthy donors. The male-to-female-ratio in this set was 1:1 and the mean age at donation was 38 years. Another subset of 73 DNA controls, control group 2, was extracted from brain tissue which was derived from the Newcastle Brain Tissue Resource. The male-to-female-ratio in this control group was approximately $3:2$ and the mean age at death was 57 years. The control DNAs of group 1 and 2 were kindly provided by Dr Rohan de Silva, Reta Lila Weston Institute, UCL. A last group of 69 DNA control samples, control group 3, derived from brain samples or blood samples, was provided by the Department of Molecular Neuroscience at the Institute of Neurology. The mean age at death or at sample collection was 72 years. No information was available about the gender distribution in this last control group. All three control groups were of UK Caucasian origin and no individual reported an Ashkenazi Jewish background.

The DNA samples were obtained according to ethical guidelines and written consent was given by all donor individuals.

Molecular genetic analysis of GBA mutations

For amplification of the *GBA* gene, three different PCR reactions were performed as described previously (Stone *et al.*, 2000). In order to avoid amplification of the pseudogene, primer sequences were designed to DNA regions exclusively found within the *GBA* gene. Three distinct fragments were amplified spanning all exonic and most intronic sequences of *GBA*. As an internal control, the size of the PCR products resulting from amplification of the pseudogene for these three fragments was calculated and confirmed as being of an alternative size to those amplified from *GBA*. Different PCR conditions were set up to optimize the annealing temperature and extension time for each fragment. For a detailed description of the PCR conditions used in this study please see Supplementary Table S1. The following reagents were used for the PCR in a total reaction volume of $15\,\mu l$: $7.5\,\mu l$ fast start PCR master mix (Roche), $1\,\mu l$ of $10\,\mu M$ forward primer, $4.5\,\mu l$ deionised water and $1\,\mu l$ genomic DNA template $(50\,ng/\mu l)$. All PCR products were run on a 1% agarose gel with ethidium bromide and size checked to rule out amplification of the *GBA* pseudogene.

Cycle sequencing was performed for each exon and the flanking intronic sequences using the Dye Terminator Sequencing Kit (Applied Biosystems) and run on an ABI 3700xl genetic analyzer

(Applied Biosystems). Initially, predesigned sequencing primers were used as described previously (Stone *et al.*, 2000). However, for some exons sequencing with the mentioned primers did not result in a sequencing read over the entire exon. Therefore, an alternative set of sequencing primers was designed (Supplementary Table S2). All identified mutations were confirmed by re-amplification of the individual patient DNA and sequenced both in the forward and the reverse direction. Sequence chromatograms were analysed using the Sequencher software (Genecodes) and a cDNA reference sequence for *GBA* was taken from GenBank (NM_001005749). All exons and the flanking intronic regions were analysed when clean, complete sequence reads were obtained. This approach allowed us to take into account all successful sequencing reads for each exon rather than excluding data when complete *GBA* gene reads could not be obtained for individual patients. The overall number of mutations found was then used to calculate the carrier frequencies. To evaluate the degree of conservation of amino acids which were altered due to novel missense mutations, an online version of the ClustalW2 software was used. Protein sequences for glucosylceramidase were obtained from the UniProt (www.uniprot.org) and Ensembl (www.ensembl.org/index.html) database. The amino acid sequences of eight different species were compared: *Homo sapiens* (human), *Pan troglodytes* (chimpanzee), *Pongo abelii* (sumatran orangutan), *Sus scrofa* (pig), *Bos Taurus* (cow), *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Drosophila melanogaster* (fruit fly), *Caenorhabditis elegans* (worm) and *Danio rerio* (zebrafish).

Clinical data analyses

Clinical notes of all *GBA* mutation carriers were reviewed independently by two experienced neurologists. The data was analysed with the main focus on age of disease onset, age of death (in the case of brain derived samples), sex, levodopa (L-Dopa) responsiveness, motor symptoms and non-motor symptoms, especially the presence of cognitive impairment, visual hallucinations and depression. The following criteria were applied for the assessment of L-Dopa responsiveness: a reported improvement of at least 30% after first introduction of L-Dopa was regarded as being a positive response. The degree of improvement was based on the clinical impression documented by the treating clinician, with specific changes in formal rating scales of Parkinsonism, such as the Unified Parkinson's Disease Rating Scale. Visual hallucinations which were considered as side effects of L-Dopa or dopamine agonist therapy and which resolved after changing medications were not counted. Similarly, hallucinations which occurred in the context of febrile illnesses and delirium were not taken into account.

Neuropathological assessment

Seventeen Parkinson's disease brains from *GBA* mutation carriers and from 16 sporadic Parkinson's disease control brains without *GBA* mutations matched for age at onset, disease duration and gender had been fixed in 10% buffered formalin and dissected according to the standardized protocol used in the Queen Square Brain Bank for Neurological Disorders. Brain samples from selected regions were embedded in paraffin, cut into $8\,\mu m$ thick tissue sections and were deparaffinized and rehydrated according to established procedures. For α-synuclein (α-syn) immunohistochemistry, the sections were autoclaved in citrate buffer for 10 min and pre-treated with 98% formic acid at room temperature for 15 min. Following epitope unmasking, a monoclonal antibody to human α-synuclein$_{1-140}$ (Novocastra, Newcastle upon Tyne, UK) was applied at a dilution of $1:1000$ and

incubated overnight at $+4^{\circ}$C. For detection, the Histostain SP kit (Zymed, San Francisco, CA, USA) was used with Romulin AEC chromogen (Biocare Medical, Walnut Creek, CA, USA). Finally, the expression of α-syn was assessed in ten brain regions: (i) medulla with dorsal motor nucleus of vagus (DMV); (ii) pons with locus ceruleus (LC); (iii) midbrain with substantia nigra (SN); (iv) basal forebrain (BFB) including the nucleus basalis of Meynert (NBM) and amygdaloid complex (AC); (v) posterior hippocampus including the CA2 subregion at the level of the lateral geniculate body; (vi) entorhinal cortex; (vii) medial temporal gyrus; (viii) anterior cingulate gyrus; (ix) anterior frontal cortex; and (x) inferior parietal cortex. The selection of regions was based on the currently used staging and grading systems for Lewy body disorders (Braak *et al*., 2003; McKeith *et al*., 2005).

Statistical analysis

Genotype frequencies in Parkinson's disease patients and controls were compared using Fisher's exact test, statistical significance was considered to be $P < 0.05$ using a one-tailed test. To determine the odds ratio (OR) and the 95% confidence interval (95% CI) an online calculator was used (DJR Hutchon Calculator; http://www.hutchon.net/ConfidOR.htm). The statistical differences in Braak staging and McKeith grading were estimated by Fisher's exact test. The differences in Lewy body scores between *GBA* carriers and sporadic Parkinson's disease controls were estimated using the non-parametric Mann–Whitney U-test. For the statistical analyses, SPSS (version 14.0) for Windows (SPSS Inc., Chicago, IL, USA) was used.

Results

Mutations in GBA

In this study, a total number of 33 mutations were found in 790 screened Parkinson's disease patient samples (4.18%) as compared to three sequence changes in 257 controls (1.17%) (Table 1). The frequency of *GBA* mutations detected in the patients is statistically significantly higher than the frequency observed in age and ethnicity matched controls ($P = 0.01$; OR = 3.7; 95% CI = 1.12–12.14). Due to technical difficulties, clear sequencing reads could not be obtained for all exons. We therefore decided to analyse each exon and the flanking intronic region separately in an exon-by-exon approach. This approach allowed us to determine the maximum number of mutations for all successfully sequenced exons of *GBA* in our 790 patients. The sequencing changes included 30 missense mutations, one deletion and two complex alleles resulting from recombination events with the *GBA* pseudogene (*GBAP*). Out of the 33 mutations observed in Parkinson's disease patients, 11 individuals were found to be heterozygous for L444P (Carrier frequency 1.39%) a mutation which in homozygous carriers is unequivocally associated with the neuronopathic type 3 of Gaucher's disease. In addition, eight heterozygous carriers of the N370S allele could be identified (Carrier frequency 1.01%). In patients of non-Ashkenazi-Jewish origin, these two mutations (N370S and L444P) represent the

Table 1 Mutations in GBA

Allele name[a]	cDNA[b]	Protein[c]	Exon	PD Subjects		Controls	
				n	Carrier frequency[d] (%)	*N*	Carrier frequency[e]
L444P	c.1448T>C	p.Leu483Pro	10	11	1.39	0	0%
D443N[f]	c.1444G>A	p.Asp482Asn	10	1	0.13	0	0%
R463C	c.1504C>T	p.Arg502Cys	10	3	0.38	0	0%
Rec*Nci*I	c.1448T>C	p.Leu483Pro	10	2	0.25	0	0%
	c.1483G>C	p.Ala495Pro					
	c.1497G>C	p.Val499Val					
RecA456P	c.1448T>C	p.Leu483Pro	10	1	0.13	0	0%
	c.1483G>C	p.Ala495Pro					
N370S	c.1226A>G	p.Asn409Ser	9	8	1.01	1	0.39%
D409H	c.1342G>C	p.Asp448His	9	1	0.13	0	0%
D380A	c.1256A>C	p.Asp419Ala	9	1	0.13	0	0%
c.1263-1317 del55	c.1263-1317 del55	p.N421PfsX4	9	1	0.13	0	0%
R257Q	c.887G>A	p.Arg296Gln	7	1	0.13	1	0.39%
G193E[f]	c.695G>A	p.Gly232Glu	6	1	0.13	0	0%
R131C	c.508C>T	p.Arg170Cys	5	1	0.13	0	0%
K7E[f]	c.136A>G	p.Lys46Glu	3	1	0.13	0	0%
V458L[f,g]	c.1489G>T	p.Val497Leu	10	0	0	1	0.39%

a Allele names follow the common nomenclature and apply to the processed protein, not including the 39-residue signal peptide.
b cDNA sequence numbering starts with the adenine of the first translated ATG start codon (GenBank reference sequence NM_001005749).
c Protein names are based on the primary translation product and include the 39-residue signal peptide.
d Carrier frequency of GBA mutations among Parkinson's disease patients where the percentage of each mutation is shown in regard to the total number of screened Parkinson's disease patients (total $n = 700$).
e Carrier frequency of GBA mutations among Parkinson's disease patients where the percentage of each mutation is shown in regard to the total number of screened controls (total $n = 257$).
f Previously undescribed mutation.
g Change only found in the control group.

most frequent changes in *GBA*. Three individuals were carriers of the complex alleles Rec*Nci*I (Carrier frequency 0.25%) and RecA456P (Carrier frequency 0.13%), respectively. These alleles include the non-synonymous changes L444P and A495P and are reported to result from a recombination between *GBA* and *GBAP* (Latham *et al*., 1990; Hatton *et al*., 1997). Therefore, whilst these three individuals carry the L444P mutations, they have not been counted as L444P exclusive carriers. The third most common change in sequence was R463C (carrier frequency 0.38%). All allele names used in this report follow the common nomenclature and refer to the processed protein, not including the 39-residue signal peptide.

One individual carried a 55 bp deletion in exon 9 (c.1263–1317 del55). This 55 bp deletion, along with additional DNA base changes, is present in exon 9 of the pseudogene. Therefore, the presence of this deletion suggests that a gene conversion or another recombination event between the functional gene and the pseudogene must have occurred. In support of this, no other DNA alterations normally present within the pseudogene sequence were identified in exon 9 in this individual confirming that the presence of this deletion was not due to an accidental amplificiation of the pseudogene. The c.1263–317 del55 results in a non-functional gene and has been associated with severe clinical manifestations of Gaucher's disease (Beutler *et al*., 1993*b*). Two more previously undescribed point mutations were found in exons 10 and 6 – D443N and G193E (carrier frequencies 0.13%, respectively), both resulting in amino acid changes (p.Asp482Asn and p.Gly232Glu). Whilst most of the mutations identified were clustered in the region spanning exons 9 and 10, we discovered another novel change in exon 3 resulting in the amino acid change p.Lys46Glu (K7E) (carrier frequency 0.13%). An interspecies comparison of the amino acids affected by these novel mutations revealed that K7E and D443N are highly conserved in most mammalian species, but not in rat, zebrafish, *C. elegans* and *D. melanogaster* (data not shown). Interestingly, G193E is conserved in all species screened except for zebrafish, indicating that this amino acid is particularly well conserved during evolution. These findings suggest that the three novel *GBA* mutations not only cause an alteration in the amino acid sequence but also are likely to be pathogenic mutations. However, the precise functional effects of these novel mutations remain to be investigated. In the control groups, three individuals (1.2%) were heterozygous for the following changes: N370S (control group 1), R257Q (control group 2), and a previously unpublished alteration V458L (control group 1).

Clinical data

Of the 33 *GBA* mutation carriers within the patient group, four (12%) have been diagnosed with familial Parkinsonism as compared to 29 patients (88%) with sporadic Parkinson's disease (Table 2). Therefore, the prevalence of *GBA* mutations in British patients diagnosed with sporadic Parkinson's disease can be estimated at ∼3.7% (29/790). No clinical data was available for patients 27 and 31, and therefore these individuals were not included in the clinical data analyses. The mean ± ST age of onset (AoO) of all *GBA* mutation carriers in the Parkinson's disease

group was 52.7 ± 11.3 years. Twelve patients (38.71%) had an AoO ⩽ 50 years which represents the cut-off value for early-onset of the disease. The mean AoO of the 790 Parkinson's disease patients in this study was 58.7 ± 12.3 years which is statistically significantly higher than in the *GBA* mutant group (*t*-test for equality of means: *t* = 2.658; *P* = 0.008). Comparing these results to previous studies our findings confirm that mutations in *GBA* are associated with an early onset of the disease. The male-to-female ratio of *GBA* carriers within the Parkinson's disease group was 5.2 (26 male : 5 female) which is considerably higher than the overall male-to-female ratio of 3.5 in the total study group (Pearson's chi-squared test: 5.12; *P* = 0.024). 28 out of 31 (90.32%) Parkinson's disease patients who carried a *GBA* mutation were initially responsive to L-Dopa treatment. Patients 28 and 29 did not respond to L-Dopa therapy and patient 8 showed a minimal response to L-Dopa. Notably, patient 9 was initially responsive but became unresponsive to L-Dopa treatment over the course of 5 years. Fifteen out of the 31 (48.39%) Parkinson's disease patients with *GBA* mutations developed symptoms of cognitive decline during the course of the disease. Patients 1, 13, 16, 21, 22, 23, 24, 25, 26 and 30 were diagnosed with Parkinson's disease and dementia or probable dementia, whereas patients 4, 7, 10, 12 and 28 had not been formally given the diagnosis of dementia but showed clear symptoms of cognitive degeneration (e.g. memory loss, cognitive slowing, confusion). None of these fifteen cases had a reported onset of cognitive symptoms prior to or within the first year after diagnosis of Parkinson's disease, thus no patient fulfilled the formal criteria for dementia with Lewy bodies. Information about the mean disease duration was available for 12 of these cognitively impaired Parkinson's disease patients and was 11.7 ± 5 years. Interestingly, 40% (6/15) of the patients with cognitive symptoms and/or dementia had an AoO ⩽ 50 years. In this study, we also evaluated the presence of visual hallucinations in Parkinson's disease patients with *GBA* mutations. Visual hallucinations (VH) were present in 45.16% (14/31) of patients with Parkinson's disease, of which 44.86% (6/14) had an AoO ⩽ 50 years. None of the GBA mutation carriers had an occurrence of VH prior to or concurrent with the onset of Parkinson's disease motor symptoms. The minimum interval to developing VH was 42 months and the average interval was 125 months after motor symptom onset. To conclude, we can summarize that the clinical features of Parkinson's disease patients with *GBA* mutations comprise an early age of disease onset (AoO ⩽ 50 years) and a good responsiveness to L-Dopa treatment. Symptoms of cognitive decline and/or dementia were a common finding and non-treatment associated hallucinations were present frequently in almost 45% of the cases.

Neuropathological data

All pathologically examined cases with *GBA* mutations (*n* = 17) showed morphological changes, which were within the spectrum of classical (idiopathic) Parkinson's disease and were not considered to represent an atypical form of the disease. Braak staging, which is used to establish the topographical extent of α-syn-immunopositive inclusions (Lewy bodies and neurites), revealed that, in addition to involvement of subcortical structures, cortical

Table 2 Clinical features of Parkinson's disease patients with GBA mutations

Patient No.	Genotype	Clinical diagnosis	Sex	Age at onset (years)	Age of death (years)	L-Dopa responsiveness	Family history of Parkinson's disease	First symptom	Cognitive symptoms	Other non-motor symptoms
1	L444P/wt	Familial PD	Male	34	NA	Yes	Yes	Tremor	Dementia, Confusion, Impaired memory	Frontal lobe dysfunction, Hallucinations
2	K7E/wt[a]	Familial PD	Male	48	NA	yes	yes	Decreased dexterity and stiffness in the left hand	None	
3	L444P/wt	Familial PD	Female	43	NA	Yes	Yes	Stiffness in the left arm, unilateral tremor on the left	None	
4	N370S/wt	Idiopathic PD	Male	57	NA	Yes	No	Bradykinesia	Moderate global cognitive deterioration, memory loss	
5	N370S/wt	Idiopathic PD	Male	54	NA	Yes	No	Stiffness in the shoulder	None	Anxiety with panic attacks
6	L444P/wt	Early onset PD	Male	41	NA	Yes	No	Bradykinesia (?)	None	
7	N370S/wt	Idiopathic PD	Female	53	NA	Yes	No	Rigidity in the neck, Bradykinesia	Confusion, Impaired memory	Faint episodes with falls due to postural drops in blood pressure
8	N370S/wt	Idiopathic PD	Male	57	NA	Minimal response	No	Resting tremor, Loss of dexterity in the left hand	None	REM sleep behavioural disorder, hallucinations anxiety attacks
9	D380A/wt	Idiopathic PD	Male	56	NA	Yes (but poor response over the course of 5y)	No	Unilateral tremor on the left	None	REM sleep behavioural disorder
10	D443N/wt[a]	Early onset PD	Male	43	NA	yes	No	Unilateral tremor on the right	Frontal executive dysfunction with cognitive slowing	REM sleep behavioural disorder, visual perceptive disturbances
11	c.1263-1317 del	Early onset PD	Male	26	NA	Yes	No	Tremor on the right, Bradykinesia on the left	None	
12	G193E/wt[a]	Idiopathic PD[b]	Male	49	62	Yes	No	Back pain	Yes	Hallucinations
13	R131C/wt	Idiopathic PD[b]	Male	56	65	Yes	No	Bradykinesia, Lack of energy, Bradyphrenia	Dementia	Hallucinations, Depression
14	N370S/wt	Idiopathic PD[b]	Male	69	79	Yes	No	Subarachnoid hemorrhage	None	
15	L444P/wt	Idiopathic PD[b]	Female	50	67	Yes	No	Lethargy	None	Hallucinations, Depression

(continued)

Table 2 Continued

Patient No.	Genotype	Clinical diagnosis	Sex	Age at onset (years)	Age of death (years)	L-Dopa responsiveness	Family history of Parkinson's disease	First symptom	Cognitive symptoms	Other non-motor symptoms
16	RecA456P	Idiopathic PD[b]	Male	48	57	Yes	No	Shuffling gait	Dementia	Hallucinations, Depression
17	R463C/wt	Idiopathic PD[b]	Female	61	78	Yes	No	Pain in the left shoulder, lower back pain	None	Hallucinations, Depression
18	R463C/wt	Idiopathic PD[b]	Male	35	51	Yes	No	Tremor in the left hand	None	Hallucinations, Depression
19	N370S/wt	Idiopathic PD[b]	Male	79	91	Yes	No	Tremor, Bradykinesia	None	
20	N370S/wt	Familial PD[b]	Male	64	85	Yes	Yes	Stiffness in the left side	None	Hallucinations, Depression
21	L444P/wt	Idiopathic PD[b]	Male	60	75	Yes	No	Slowing down	Dementia	Hallucinations, Depression
22	R463C/wt	Idiopathic PD[b]	Male	61	68	Yes	No		Dementia	Hallucinations, Depression
23	L444P/wt	Idiopathic PD[b]	Male	57	75	Yes	No		Dementia	Hallucinations
24	D409H/wt	Idiopathic PD[b]	Male	44	62	Yes	No	Tremor	Dementia, Confusion	Hallucinations
25	R257Q/wt	Idiopathic PD[b]	Male	58	66	Yes	No		Dementia	
26	L444P/wt	Idiopathic PD[b]	Male	58	65	Yes	No	Akinesia, Rigidity	Dementia	
27	RecNciI	Early onset PD[b]	Female				No			
28	RecNciI	MSA[b]	Female	58	64	no	No	Unusual gait and difficulty writing	Cognitive impairment	Hallucinations, Depression
29	L444P/wt	MSA[b]	Male	52	59	no	No		None	Urinary urgency, Nocturia
30	L444P/wt	Early onset PD[b]	Male	37	56	Yes	No	Dragging of the left foot	Probably demented, Confusion	Depression
31	L444P/wt			68		Yes	No			
32	N370S/wt	Idiopathic PD[b]	Male	68	82	Yes	No	Impaired walking, Tremor	None	Depression
33	L444P/wt	Idiopathic PD[b]	Male	59	61		No	Unilateral tremor with autonomic bulbar involvement	None	Depression

PD, Parkinson's Disease; MSA, Multiple System Atrophy; wt, wild-type; NA, not applicable.
a previously undescribed mutations.
b Initial clinical diagnosis for pathologically proven Parkinson's disease cases.

areas were also affected by α-syn-immunoreactive inclusions corresponding to Braak stages 5–6 in all 17 patients (Table 3). There was no statistically significant difference in Braak stages between the GBA carriers and sporadic Parkinson's disease controls (P = 0.537, Fisher's exact test). However, 13 of the 17 GBA carriers (76%) and 6 of the 16 Parkinson's disease controls (38%) fulfilled the McKeith criteria for diffuse neocortical Lewy body pathology. This shows a positive trend for a higher McKeith grade among the GBA mutation carriers, as the difference between the two groups just reached statistical significance (P = 0.049, Fisher's exact test). Lewy body scores generated by the McKeith protocol were used to give an indicative of the overall cortical burden and did not differ between the two groups; GBA carriers 7.3 ± 3.0 (mean ± ST), Parkinson's disease controls 6.3 ± 2.8 (P > 0.5, Mann–Whitney U-test). For a detailed description of the sporadic Parkinson's disease controls, please see Supplementary Table S3.

Discussion

The frequency of GBA mutations found in the British Parkinson's disease population is clearly a striking result as it represents the highest frequency of mutations of a single gene related to the development of the idiopathic disease in this population. Although former studies on the same series of British patients showed that other genes such as PTEN induced putative kinase 1 (PINK1), leucine-rich repeat kinase 2 (LRRK2) and DJ-1 may also play a role in the sporadic form of the disease (Abou-Sleiman et al., 2003, 2006; Gilks et al., 2005) mutations in GBA have the highest prevalence with ~3.7% of all sporadic Parkinson's disease cases being affected (Fig. 1). Several studies have evaluated the frequency of GBA mutations among Parkinson's disease patients and show similar results (Supplementary Table S4). However, the majority of previous studies specifically screened the GBA gene for previously reported common mutations and did not attempt sequencing analysis of the complete gene. Whilst we were unable to obtain a complete gene sequencing read for all 790 of our patients, our compiled data from all clear exon and intronic sequence reads represents the largest sequencing study of GBA mutations in Parkinson's disease patients to date. However, one needs to take into consideration the fact that our data is based on a subset of patients who were referred to a specialized university clinic or who have donated their brains to our brain bank for research. We acknowledge the limitations of the retrospective nature of our data and the selection bias that is expected in this series. Nevertheless, the pathological evaluation of the GBA mutant cases revealed that the observed morphological features were typical for sporadic Parkinson's disease, suggesting that a proportion of classical sporadic Parkinson's disease might indeed be caused by mutations in the GBA gene.

The mutant GBA gene frequency in the general population in the UK has been estimated at ~0.0016 in contrast to 0.034 in the Ashkenazi-Jewish population (Benson and Fensom, 1985; Zimran et al., 1991). Studies on control subjects from other non-Ashkenazi-Jewish populations have found very different frequencies ranging from 0.004 in a North American cohort

(with a European ethnic background) to 0.017 in a Norwegian control sample (Toft et al., 2006; Mata et al., 2008). Thus, the observed frequency of 0.012 in our British control group is representative for a European population, and provides the best estimate for the British population to date. Regarding the clinical data on the Parkinson's disease patient group with GBA mutations, it can be summarized that, in general, our findings confirm previously published results, stating that GBA mutation carriers frequently have an earlier age of disease onset (AoO ⩽ 50 years), show a good response to L-Dopa treatment, and have an increased likelihood to present with symptoms of cognitive decline and dementia. In addition to that, we looked at the occurrence of other non-motor features such as visual hallucinations, which have been associated with Parkinson's disease.

Symptoms of cognitive decline are a common feature in parkinsonism. In a systematic review of prevalence studies which looked at dementia in the disease, a proportion of ~24–31% has been suggested (Aarsland et al., 2005). In our Parkinson's disease patient group of GBA mutation carriers, 48% had been given the diagnosis Parkinson's disease with dementia or showed clear symptoms of cognitive decline. Moreover, 40% of the patients with cognitive symptoms had an age of onset ⩽ 50 years. Therefore, we hypothesize that mutations in GBA might increase the risk of developing dementia or cognitive impairment in individuals with an early disease onset. This finding might be of importance given that patients rarely show symptoms of cognitive decline at an age younger than 55 years. Hence, in future research it will be interesting to determine whether GBA mutations have an impact on the development of dementia in younger patients with an early disease onset.

The male-to-female ratio in our series was 3.5 : 1 which is comparable to the published range of 3:2 (Wooten et al., 2004). In studies on Parkinson's disease patients which carry a GBA mutation, the male-to-female ratio has been reported to be higher, ranging between 2 : 1 to 5 : 2 (Toft et al., 2006; Gan-Or et al., 2008; Mata et al., 2008). In the present study, male GBA mutation carriers were by far more frequently affected than women (26 male : 5 female; Pearson's chi-squared test: 5.12; P = 0.024). Thus, the ratio observed in GBA mutation carriers suggests that male individuals who have a mutated GBA gene are more susceptible to develop Parkinson's disease than female mutation carriers.

Overall, the initial response to L-Dopa treatment was good to very good. This finding is in accordance with results from previous research which described an excellent response to L-Dopa therapy in Parkinson's disease probands heterozygous for GBA mutations (Ziegler et al., 2007; Mata et al., 2008). However, one of the characteristics of Gaucher patients with parkinsonism is that their symptoms are mostly refractory to standard Parkinson therapy. Thus, it is possible that identical mutations in GBA result in different phenotypic traits of Parkinson's disease (e.g. good to no response to L-Dopa treatment) and that other genetic modifiers play a role in the susceptibility to the disease.

In our subset of Parkinson's disease patients with GBA mutations, non-treatment associated visual hallucinations were present in almost half of the cases. Visual hallucinations are a common feature in Parkinson's disease and have been estimated to occur in up to 50% of patients (Williams and Lees, 2005). As in idiopathic

Table 3 Distribution and the extent of α-synuclein positive inclusions

Patient No.	Genotype	Age of onset	Disease duration	DMV[a] LBs	DMV[a] neurites	LC[a] LBs	LC[a] neurites	SN[b] LBs	SN[b] neurites	AC	CA2	ERC	MTG	CG	FC	PC	Braak stage	McKeith grade	LB score
12	G193E/wt*	49	13	3	+++	3	++	3	+++	3	+	2	1	2	2	0	6	3	7
13	R131C/wt	56	9	3	+++	3	+++	3	+++	4	+	4	3	4	2	0	6	3	13
15	L444P/wt	50	17	1	+	NA	NA	1	–	3	–	1	1	2	1	0	6	2	5
16	RecA456P	48	9	1	–	3	+++	1	+	2	+	3	2	NA	3	0	6	3	NA
17	R463C/wt	61	17	2	+	3	+	3	+	3	+	2	1	2	1	0	6	2	6
18	R463C/wt	35	16	1	+	0	–	1	+	NA	+	1	0	2	0	0	5	2	3
19	N370S/wt	79	12	2	+	3	+++	1	–	NA	–	3	2	3	2	0	6	3	10
20	N370S/wt	64	21	3	+++	3	++	3	+++	4	+	4	3	3	2	0	6	3	12
21	L444P/wt	60	15	NA	NA	NA	NA	1	–	3	+	4	2	1	1	0	6	3	8
22	R463C/wt	61	7	2	++	2	+	2	++	4	+	3	1	2	0	0	5	3	6
23	L444P/wt	57	18	3	++	3	+++	2	+	3	+	3	3	3	3	0	6	3	12
24	D409H/wt	44	18	NA	NA	2	+	2	+	NA	+	1	1	2	0	0	5	2	4
28	RecNcil	58	6	2	+++	3	+++	2	++	4	+	2	1	2	0	0	5	3	5
29	L444P/wt	52	7	2	++	1	+	1	+	2	+	1	1	2	1	1	6	3	6
30	L444P/wt	37	19	2	++	3	++	2	++	4	NA	2	1	3	1	1	6	3	8
31	L444P/wt	NA	NA	NA	NA	3	++	1	+	4	+	1	1	2	1	1	6	3	6
32	N370S/wt	68	14	1	+	2	+	NA	NA	1	–	1	1	2	2	0	6	3	6

DMV, dorsal motor nucleus of vagus; LC, locus coeruleus; SN, substantia nigra; AC, amygdaloid complex; CA2, CA2 region of the hippocampus; ERC, entorhinal cortex; MTG, medial temporal gyrus; CG, cingulate gyrus; FC, frontal cortex; PC, parietal cortex; LBs, Lewy Bodies; N/A, data not available; *previously unpublished mutation in *GBA*

α-S-immunopositive inclusions in the brain stem were counted unilaterally and assessed according to an arbitrary grading system as follows [a]1 = 1–2 LBs, 2 = 2–10 LBs, 3= >10 LBs; [b]1 = <25 LBs, 2 = 25–50 LBs, 3 = >50 LBs

McKeith's grading was applied for regions AC, ERC, MTG, CG, FC and PC: 1 = Mild (sparse Lewy bodies or neurites), 2 = Moderate (more than one Lewy body in a high power field and sparse neurites), 3 = Severe (four or more Lewy bodies and scattered neurites in a low power field), 4 = Very severe (numerous Lewy bodies and numerous neurites)

Braak stage was assigned according to the topographic distribution of α-S-immunopositive inclusions. This was done by dichotomized assessment (present or not), disregarding any density of inclusions.

Lewy body score was calculated as sum of semiquantitative scores in 5 cortical areas (ERC, MTG, CG, FC, PC), maximum value 16.
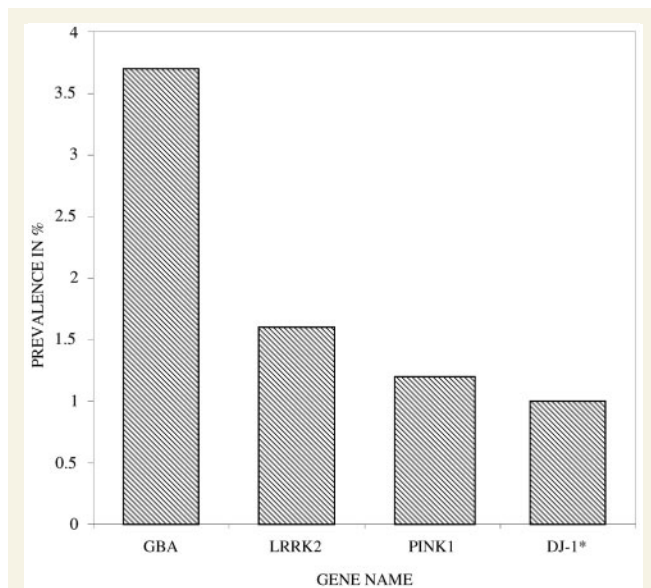
Figure 1 Comparison of single gene mutations in a series of British sporadic Parkinson's disease. Prevalence of single gene mutations in a British series of sporadic Parkinson's disease. The data refers to former studies on the same series of sporadic Parkinson's disease: *LRRK2*, *PINK1*, *DJ-1*, *GBA*, *The *DJ-1* study showed a prevalence of ~1% in young-onset Parkinson's disease patients, but not in late-onset sporadic Parkinson's disease

Parkinson's disease, the occurrence of visual hallucinations in patients with *GBA* mutations is likely to be the consequence of the extension of the Lewy body pathology in the temporal lobe (Harding *et al*., 2002).

These data implicating *GBA* mutants in Parkinson's disease pathogenesis strongly motivates an evaluation of potential pathways. There are two broad possibilities. First haploinsufficiency of *GBA* leads directly to an accumulation of glucosylceramide and a concomitant impairment of ceramide metabolism and thereby increases the risk of developing the disease. The second possibility is that a novel property of the mutant enzyme is contributing to the risk of developing parkinsonism.

If one considers the neuronopathic form of Gaucher's disease it seems unlikely that the association between mutant *GBA* alleles and parkinsonism relates solely to the catalytic activity of the mutant enzyme although it is possible that there will be a subtle dysregulation in ceramide metabolism. In heterozygote mutant carriers, the unaffected allele would likely provide adequate GBA activity to degrade most of the glucosylceramide entering the lysosome.

If there is a novel toxic function playing a role it is of note that most of the mutations described here are missense alleles which would be predicted to produce a protein product. A precise toxic function is unclear but for a number of these alleles, it has been demonstrated that the mutant enzyme produced is unstable and, instead of being targeted to the lysosome, is diverted by the quality control mechanisms of the cell to proteosomal degradation (Schmitz *et al*., 2005). Lewy bodies are seen in the brains of Gaucher patients who develop Parkinson's disease and a

particularly severe involvement of neuronal populations of the CA2-CA4 hippocampal subregions has been documented. Immunohistochemical studies have demonstrated that constitutive levels of GBA expression are high in these hippocampal subregions (Wong *et al*., 2004). Therefore it seems likely that the expression of high levels of the unstable mutant enzyme may play a role in the formation of Lewy bodies in Gaucher patients with parkinsonism.

In the cases presented in this study, neuropathological analysis (including Braak staging and grading using consensus criteria) demonstrated extensive Lewy body pathology in a pattern identical to that seen in sporadic Parkinson's disease controls matched for age at disease onset, disease duration and gender. Furthermore a larger proportion of the cases with *GBA* mutations tended to have neocortical Lewy body pathology than the sporadic cases, although investigation of larger cohorts is required to confirm this. The autophagy-lysosome pathway, including chaperone-mediated autophagy and macroautophagy is an important mechanism for the degradation of cellular α-synuclein (Vogiatzi *et al*., 2008). The findings of this study supports the hypothesis that mutant glucocerebrosidase may interfere with cellular pathways related to lysosomal degradation of cellular α-synuclein and that these mechanisms might also be fundamental in Lewy body formation in the sporadic form of the disease. Further research will be essential to establish whether neuronal cell death might be a consequence of a misfolded GBA enzyme or how alteration of the glucosylceramide/ceramide metabolism could contribute to the development of Parkinson's disease.

The results from this study and those from other reports in Parkinson's disease patients clearly establish that *GBA* mutations account for a significant minority of cases. The clinical and pathological data reported here emphasise that these cases are indistinguishable from what is normally considered as idiopathic Parkinson's disease. This has important implications for genetic counselling of such patients and indeed relatives of patients with Gaucher's disease. The classical scenario of autosomal recessive disease is that carriers are both unaffected and the recurrence risk to their offspring is incredibly low in the absence of a consanguineous relationship. These data and findings emerging from studies of proven autosomal recessive Parkinson's disease genes (e.g. *parkin*, *DJ-1* and *PINK1*) in which there is some, but controversial, evidence to support a role of heterozygous mutations have changed the terrain and suggest that carrying a single heterozygous mutation is associated with increased risk. To provide accurate information to patients and their families one really requires a reliable and accurate estimate of prevalence to be made. This will be difficult but will probably require international collaboration to achieve sufficient numbers of cases. Even then given the allelic variability which may in part influence penetrance means that accurate predictive risk counseling will be fraught. However it is obligatory for the clinicians who are making these genetic diagnoses that a discussion of these difficulties is conducted with the patients and their families. Our data reinforce the proposed association between *GBA* mutations and Parkinson's disease. While this link has been investigated in a number of genetic association studies in the last few years, the functional aspects

of how *GBA* mutations might impact on neuronal cell death in Parkinson's disease remains to be elucidated.

Supplementary material

Supplementary material is available at *BRAIN* online.

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