C9orf72 repeat expansions are a rare genetic cause of parkinsonism

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*See Acknowledgements for the French Parkinson’s Disease Genetics (PDG) Study Group.

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The recently identified C9orf72 gene accounts for a large proportion of amyotrophic lateral sclerosis and frontotemporal lobar degenerations. As several forms of these disorders are associated with parkinsonism, we hypothesized that some patients with Parkinson’s disease or other forms of parkinsonism might carry pathogenic C9orf72 expansions. Therefore, we looked for C9orf72 repeat expansions in 1446 unrelated parkinsonian patients consisting of 1225 patients clinically diagnosed with Parkinson’s disease, 123 with progressive supranuclear palsy, 21 with corticobasal degeneration syndrome, 43 with Lewy body dementia and 25 with multiple system atrophy-parkinsonism. Of the 1446 parkinsonian patients, five carried C9orf72 expansions: three patients with typical Parkinson’s disease, one with corticobasal degeneration syndrome and another with progressive supranuclear palsy. This study shows that (i) although rare, C9orf72 repeat expansions may be associated with clinically typical Parkinson’s disease and also with other parkinsonism; (ii) in several patients, parkinsonism was levodopa-responsive and remained pure, without associated dementia, for >10 years and (iii) interestingly, all C9orf72 repeat expansion carriers had positive family histories of parkinsonism, degenerative dementias or amyotrophic lateral sclerosis. This study also provides the tools for identifying parkinsonian patients with C9orf72 expansions, with important consequences for genetic counselling.
Introduction

Parkinson's disease is common in the ageing population, ranking second in prevalence after Alzheimer's disease. The prevalence of this condition is age dependent, affecting >1% of persons over the age of 65 years, but this rate is increased to 4–5% by the age of 85 years (De Lau and Breteler, 2006). Parkinson's disease is a clinical motor syndrome with variable combinations of akinesia, rigidity, rest tremor and postural instability, linked to a characteristic pattern of neurodegeneration of dopaminergic neurons, particularly in the substantia nigra pars compacta and the presence of intraneuronal cytoplasmic inclusions, known as Lewy bodies, in surviving dopaminergic neurons. In addition, cognitive dysfunction is frequent in Parkinson's disease (Barton, 2012) and also in atypical forms of parkinsonism, such as Lewy body dementia, progressive supranuclear palsy (PSP) and corticobasal degeneration syndrome. A GGGGCC repeat expansion in C9orf72 was recently reported to be the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degenerations (FTLDs) (DeJesus-Hernandez et al., 2011; Renton et al., 2011). As the clinical and neuropathological features of ALS, neurodegenerative dementias and parkinsonism overlap, we looked for C9orf72 repeat expansions in 1446 unrelated parkinsonian patients.

Subjects and methods

Subjects

The study included 1446 unrelated patients: 1225 were clinically diagnosed with Parkinson’s disease, 123 with PSP, 21 with corticobasal degeneration syndrome, 43 with Lewy body dementia and 25 with multiple system atrophy-parkinsonism (Table 1).

Table 1 Demographic and clinical characteristics of patients with parkinsonism and C9orf72 expansion carriers

<table>
<thead>
<tr>
<th>Parkinsonian disorders</th>
<th>PD</th>
<th>LBD</th>
<th>PSP</th>
<th>CBDS</th>
<th>MSA-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, n</td>
<td>1235a</td>
<td>43</td>
<td>123</td>
<td>21</td>
<td>25</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>734/501</td>
<td>31/12</td>
<td>65/58</td>
<td>12/9</td>
<td>16/9</td>
</tr>
<tr>
<td>Age at disease onset, years (range)</td>
<td>48.3 ± 12.5</td>
<td>63.6 ± 11.9</td>
<td>64.1 ± 8.2</td>
<td>64.2 ± 8.8</td>
<td>56.3 ± 13.2</td>
</tr>
<tr>
<td>Age at examination, years (range)</td>
<td>56.8 ± 13.1</td>
<td>71.1 ± 10.0</td>
<td>67.8 ± 9.8</td>
<td>67.8 ± 8.5</td>
<td>63.7 ± 14.7</td>
</tr>
<tr>
<td>Dementia (%)</td>
<td>10</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Patients with family histories of other neurodegenerative disordersb, n (%)</td>
<td>47 (4)</td>
<td>11 (26)</td>
<td>15 (12)</td>
<td>7 (33)</td>
<td>17 (68)</td>
</tr>
<tr>
<td>C9orf72 expansion carriers, n</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Expansion carriers with family histories of other neurodegenerative disordersb (%)</td>
<td>6</td>
<td>0</td>
<td>7</td>
<td>14</td>
<td>0</td>
</tr>
</tbody>
</table>

a Forty per cent of the patients had family histories of typical Parkinson's disease.
b Other neurodegenerative disorders include parkinsonism, degenerative dementias or ALS.

CBDS = corticobasal degeneration syndrome; LBD = Lewy body dementia; MSA-P = multiple system atrophy-parkinsonism; PD = Parkinson's disease.
histories of parkinsonism and excluded for the LRRK2 G2019S mutation, were examined at age 64.9 ± 8.5 years (range 51–89 years). Local ethics committees approved the study, and written informed consent was obtained from all participants.

Methods

Genomic DNA from all participants was obtained from venous blood using standard protocols. The GGGGCC repeat expansions in the first intron of C9orf72 were searched for, using the repeat primed PCR adapted from Renton et al. (2011). In short, 80 ng of genomic DNA was mixed with 14 μl FastStart PCR Master-Mix (Roche Diagnostics), 0.18 mM 7-Deaza-2’-deoxy-guanosine-5’-triphosphate (Roche Diagnostics), Q solution (Qiagen Inc.), 7% dimethyl sulphoxide, 0.9 mM MgCl2, 0.7 mM reverse primer consisting of four GGGGCC repeats and an anchor tail (TACGCATCCAGTTGAGACGGGGGCGGGGGCCGGGG), 1.4 μM 6FAM-fluorescent forward primer located 280 bp 3’ to the repeat sequence (AGTCGCTAGGCGAAAGC) and 1.4 μM anchor primer corresponding to the anchor tail of the reverse primer (TACGCATCCAGTTGAGAC). A touchdown PCR cycling protocol was used, in which the initial annealing temperature was lowered from 70 to 56 °C in 2 °C increments with a 3-min extension in each cycle (Renton et al., 2011). Fragment-length analysis was performed on an ABI 3730 genetic analyser (Applied Biosystems), and data were analysed using Peak Scanner software version v1.0 (Applied Biosystems).

To determine the accurate sizing of the non-expanded allele and score all alleles in heterozygous or homozygous state, we performed a classical FAM-fluorescent labelled PCR assay, using previously described primers (Renton et al., 2011). To determine the mean number of C9orf72 hexanucleotide repeats in non-mutation carriers, we summed the number of repeats on both non-expanded alleles.

To confirm the size of the largest expanded alleles, we performed a Southern blot analysis of DNA from the putative C9orf72 expansion carriers and also from patients with an intermediate length hexanucleotide repeat-containing allele for whom DNA was available. Briefly, 10 μg of genomic DNA extracted from blood was digested with MboI restriction endonuclease (New England Biolabs), and the digested samples were separated on a 0.8% agarose gel in 1 x Tris/Borate/ Ethylenediamine Tetraacetic Acid buffer at 1 V/cm overnight. DNA was alkali-transferred onto a GeneScreen Plus® filter (NEN Life Sciences Products Inc.) according to the manufacturer’s protocol. A double-labelled probe (NEBlot® kit, New England Biolabs, Alpha 32P deoxyadenosine triphosphate and Alpha 32P deoxycytidine 5’-triphosphate 3000 ci/mmol, PerkinElmer) corresponding to chromosome 9 position 27, 573, 698–27, 573, 938 in the hg19 assembly was hybridized to blots in modified Church and Gilbert hybridization buffer (0.5 M NaH 2PO4/Na2HPO4, pH 6.8, 7% sodium dodecyl sulfate, 1 mM EDTA, 1% bovine serum albumin) at 65°C overnight (Church and Gilbert, 1984). Blot membranes were then washed twice at room temperature and twice at 65°C in 40 mM NaH2PO4/Na2HPO4,
Results

A total of 1446 patients with parkinsonism, including 1225 clinically diagnosed with Parkinson's disease and 464 healthy control subjects, were successfully genotyped with both assays to study the C9orf72 repeat in all patients: a repeat-primed PCR to identify expansion carriers and a classical fluorescent PCR to allow the accurate sizing of the non-expanded allele.

Five of the 1446 patients with parkinsonism showed the typical saw tooth tail pattern detected by repeat-primed PCR analysis (Renton et al., 2011), characteristic of C9orf72 repeat expansions in the pathogenic size (\( \geq 560 \) repeat units) (Fig. 1A) and a single peak and potentially unamplifiable repeat expansions detected by fluorescent fragment-length PCR analysis (Fig. 1B, Table 2). The presence of these large expanded alleles was further confirmed on Southern blots (Fig. 1C), which showed additional expanded alleles 412 kb in size in the five patients with parkinsonism.

Although we confirmed the presence of repeat expansions by Southern blot, significant repeat size heterogeneity resulting in a smear on the blot complicated accurate sizing of the repeats in the five patients and the positive control subject (patient with FTLD with a C9orf72 expansion).

In the subgroup of 1225 patients with Parkinson's disease, there were three carriers of C9orf72 repeat expansions (0.2%, 95% confidence intervals 0.09–0.7) who had isolated parkinsonian symptoms at onset. Ages at onset ranged from 29 to 64 years. In the first patient (Patient PD1), parkinsonism began at age 48 with bilateral rest tremor and akinesia. At age 54, she developed axial rigidity and a severe gait disorder. She showed cognitive deficits (Mini-Mental State Examination score 25/30) at age 56 and died at age 59. The second patient (Patient PD2) developed a levodopa-responsive akinetic rigid syndrome and asymmetric lower limb dystonia at age 64. She developed dysplasia, visual hallucinations and delusions under dopamine agonists. At age 69, she had no effect on her autonomy in daily life (Mini-Mental State Examination 26/30; Frontal Assessment Battery 15/18). The third patient (Patient PD3) developed left hemi-parkinsonism at age 29 with upper limb rest tremor, ataxia, rigidity and dystonia (UPDRS Part III motor ‘ON/OFF’ score = Unified Parkinson’s Disease Rating Scale rated ON/OFF treatment with levodopa at evaluation time).

Table 2: Clinical characteristics of C9orf72 expansion carriers

<table>
<thead>
<tr>
<th>Patients</th>
<th>Diagnosis</th>
<th>Age at onset</th>
<th>DD at examination</th>
<th>DD at last visit</th>
<th>Symptoms at onset</th>
<th>Parkinsonism</th>
<th>UPDRS Part III ON/OFF (/108)</th>
<th>Hoehn and Yahr stage (/5)</th>
<th>Cognitive deficit (DD before cognitive deficit occurred)</th>
<th>MMSE (/30) FAB (/18) at examination</th>
<th>Gaze-palsy</th>
<th>Dystonia(^b)</th>
<th>Other symptoms</th>
<th>Family history of neuropsychiatric disorders</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD1</td>
<td>PD</td>
<td>48</td>
<td>6</td>
<td>11</td>
<td>P</td>
<td>R, A, T</td>
<td>7/20</td>
<td>4</td>
<td>+ (8)</td>
<td>25/na</td>
<td>–</td>
<td>–</td>
<td>Sleep disorders</td>
<td>Dementia, PD</td>
</tr>
<tr>
<td>PD2</td>
<td>PD</td>
<td>64</td>
<td>5</td>
<td>6</td>
<td>P and dystonia</td>
<td>R, A (left, LL &gt; UL)</td>
<td>13/na</td>
<td>3</td>
<td>+ (5)</td>
<td>26/15</td>
<td>–</td>
<td>LL</td>
<td>Depression, dementia</td>
<td>Depression, P</td>
</tr>
<tr>
<td>PD3</td>
<td>PD</td>
<td>29</td>
<td>6</td>
<td>12</td>
<td>P and dystonia</td>
<td>R, A (left, LL &gt; UL)</td>
<td>9/37</td>
<td>2</td>
<td>–</td>
<td>27/18</td>
<td>–</td>
<td>LL</td>
<td>Enhanced reflexes</td>
<td>Depression, dementia, P, ALS Dementia, PSP, P</td>
</tr>
<tr>
<td>PD4</td>
<td>PSP</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>+ (at onset)</td>
<td>na</td>
<td>–</td>
<td>UL</td>
<td>Enhanced reflexes</td>
<td>Dementia</td>
</tr>
<tr>
<td>PD5</td>
<td>CBDS</td>
<td>50</td>
<td>2</td>
<td>2</td>
<td>P, anarthria, ophal apraxia, dystonia</td>
<td>R, A (left, UL &gt; LL)</td>
<td>na</td>
<td>na</td>
<td>+ (at onset)</td>
<td>na</td>
<td>–</td>
<td>UL</td>
<td>Enhanced reflexes</td>
<td>Dementia</td>
</tr>
</tbody>
</table>

a UPDRS Part III motor ‘ON/OFF’ score = Unified Parkinson’s Disease Rating Scale rated ON/OFF treatment with levodopa at evaluation time.

b Before levodopa treatment.

A = akinesia; DD = disease duration; FAB = Frontal Assessment Battery; LL = lower limb; MMSE = Mini-Mental State Examination; na = not available; P = parkinsonism; PD = Parkinson’s disease; R = rigidity; T = rest tremor; UL = upper limb.
We also found a $C9orf72$ repeat expansion in a patient with PSP (Patient PD4) with parkinsonism, gaze-palsy and dementia (1/123, 0.8%, 95% CI 0.2–4.4), and in a patient with corticobasal degeneration syndrome (Patient PD5) with left akinetic rigid parkinsonism, upper limb dystonia, orofacial apraxia, anarthric speech and frontal lobe syndrome (1/21, 5%, 95% CI 1.1–22.8) (Table 2). In addition, two patients with PSP, 59 (Patient PD6) and 74 of years at onset (Patient PD7), had intermediate repeat lengths of 26 and 30, respectively (Fig. 1). Both patients presented typical features of isolated PSP, including parkinsonism, supranuclear gaze-palsy, postural instability early in the disease course, dysarthria and mild frontal dementia.

No expansions were found in patients with multiple system atrophy-parkinsonism or with Lewy body dementia or in control subjects. The average $C9orf72$ repeat number was $4.5 \pm 3.3$ (range 2–22) in the 445 control subjects.

Discussion

In a recent study, 35% of patients with $C9orf72$ expansions developed parkinsonism within the first 2 years after onset of frontotemporal dementia and ALS (Boeve et al., 2012). Using a stepwise protocol according to previously described methods, combining fluorescent fragment-length, repeat-primed PCR and Southern blot analyses (DeJesus-Hernandez et al., 2011), we have now identified disease-causing repeat expansions in a subset of patients from a large cohort with different forms of parkinsonism, extending the clinical phenotype associated with these expansions from degenerative dementias, including Alzheimer’s disease (Majounie et al., 2012a) and ALS to parkinsonism. Two patients had typical Parkinson’s disease for 5–8 years before cognitive deficits or dementias developed; one patient had no cognitive deficits 12 years after Parkinson’s disease onset. This study shows that (i) although rare, $C9orf72$ repeat expansions may be associated with clinically typical Parkinson’s disease and also with PSP and corticobasal degeneration syndrome; (ii) the parkinsonism could be levodopa-responsive and, as in Parkinson’s disease, remains pure, without associated cognitive deficits or dementias for >10 years and (iii) interestingly, all $C9orf72$ mutation carriers had positive family histories of parkinsonism, degenerative dementias or ALS. Among the patients with positive family histories, the proportion of patients with expansions...
in C9orf72 reached 6% (3/47, 95% CI 2.3–17.2) in Parkinson’s disease, 7% (1/15, 95% CI 1.6–30.2) in PSP and 14% (1/7, 95% CI 3.2–52.7) in corticobasal degeneration syndrome.

We also identified two patients with PSP with intermediate numbers of GGGGCC repeats, ranging from 26 to 30. Notably, a 29-hexanucleotide repeat allele was found in a patient from a Dutch family with C9FTLD/ALS (Simón-Sánchez et al., 2012), suggesting that intermediate alleles might be pathogenic in patients with PSP. Of note, the maximum size of the repeat in most control subjects was reported not to exceed 24 units (DeJesus-Hernandez et al., 2011; Gijselink et al., 2012; Renton et al., 2011; Rutherford et al., 2012; Sabatelli et al., 2012). In our study and that of Millecamps et al. (2012), repeat lengths ranged from 2 to 23 in the control group of French origin (n = 600). Although the repeat size in our patients was clearly larger than in ethnically matched control subjects and their ages at onset were in the range observed in typical patients with C9orf72 repeat expansions, we cannot completely exclude that the observed repeat expansions and the presence of typical Parkinson’s disease are unrelated (i.e. the patients have two different neurological diseases). A recent study in a large cohort of 781 Caucasian cases with Parkinson’s disease, one-third of which reported family histories of Parkinson’s disease, failed to identify any pathogenic hexanucleotide expansions in C9orf72, although a patient with typical Parkinson’s disease harboured a marginally 38-GGGGCC hexanucleotide repeat allele (Majounie et al., 2012b). Large multi-centric studies worldwide are needed to determine the frequency of C9orf72 mutations in idiopathic Parkinson’s disease, particularly when positive family histories of parkinsonism, degenerative dementias or ALS are present.

The present study, therefore, suggests that patients with Parkinson’s disease or other parkinsonian syndromes, particularly when a family history of other neurodegenerative disorders is present, should be screened for C9orf72 expansions. This provides the tools for identifying potential carriers with important consequences for genetic counselling.

Acknowledgements

The authors are grateful to the patients and their families. They thank Merle Ruberg for careful reading of the manuscript, the DNA and Cell Bank of the CRICM for sample preparation and Agnès Camuzat and Léna Guillot-Noel for technical assistance. The French Parkinson Disease Genetic Group members are Y. Agid, MD, PhD; M. Anheim, MD, PhD; A.-M. Bonnet, MD; M. Borg, MD; A. Brice, MD; E. Broussolle, MD, PhD; J.-C. Corvol, MD, PhD; P. Damier, MD, PhD; A. Destée, MD; A. Dürr, MD, PhD; F. Durif, MD, PhD; C. Duyckaerts, MD, PhD; S. Klebe, MD, PhD; P. Krack, MD; S. Lesage, PhD; E. Lohmann, MD; M. Martinez, PhD; P. Pollak, MD, PhD; O. Rascol, MD, PhD; F. Tison, MD, PhD; C. Tranchant, MD; M. Vérin, MD, PhD; F. Viallet, MD and M. Vidailhet, MD.

Funding

PSP-France Association (to I.L.) (R12116DD), France Parkinson (to A.B. and S.L.) (R12010DD), French Agency for Research (ANR) program (to A.B. and A.D.) (R08200DS/ANR-08-NEUR-004-01, R08109DS/ANR-08-MNP-012-02).

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