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Clinical and Pathological Characteristics of Patients with Leucine-Rich Repeat Kinase-2 Mutations

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Abstract

Mutations in *LRRK2* are the single most common known cause of Parkinson's disease (PD). Two new PD patients with *LRRK2* mutation were identified from a cohort with extensive post-mortem assessment. One of these patients harbors the R793M mutation and presented with the typical clinical and pathological features of PD. A novel L1165P mutation was identified in a second patient. This patient had the classical and pathological features of PD, but additionally developed severe neuropsychological symptoms and dementia associated with abundant neurofibrillary tangles in the hippocampal formation; features consistent with a secondary diagnosis of tangle-predominant dementia. α -Synuclein-containing pathological inclusions in these patients also were highly phosphorylated at Ser-129, similar to other patients with idiopathic PD. These two PD patients also were characterized by the presence of occasional cytoplasmic TDP-43 inclusions in the temporal cortex, a finding that was not observed in three other patients with the G2019S mutation in *LRRK2*. These findings extend the clinical and pathological features that may be associated with *LRRK2* mutations.

Keywords

Parkinson's disease/Parkinsonism; Genetics; Parkinson's disease with dementia; Leucine-rich repeat kinase-2 (*Lrrk2*)

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease in the developing world and is characterized by bradykinesia, resting tremor, cogwheel rigidity and postural instability.^{1,2} These major clinical features of PD are associated with the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc).^{3,4} In addition, postmortem analysis of the majority of clinically diagnosed PD patients reveals intracytoplasmic inclusions known as Lewy bodies (LBs) and Lewy neurites (LNs) in some of the remaining dopaminergic neurons. LBs and LNs are formed as the result of the aberrant aggregation of the presynaptic protein α -synuclein (α -syn). These inclusions can also accumulate in other brain regions in PD as well as other related neurological disorders.

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5-7 The presence of these inclusions is a criterion used to differentiate PD from other disorders associated with parkinsonism.^{5,8}

Autosomal dominant mutations in the gene for leucine-rich repeat kinase-2 (*LRRK2*; also known as *PARK8*) have been identified in a significant percentage of late-onset PD cases.⁹⁻¹⁰ Five missense mutations with definite pathogenicity and many other missense mutations that are potentially pathogenic or may act as risk factors, have been reported.¹¹ G2019S, the most common mutation, is reportedly responsible for 0.6-1.6 % of sporadic¹²⁻¹⁴ and 2-8 % of familial cases of PD.¹²⁻¹⁸ In some specific ethnicities such as North African Arabs and Ashkenazi Jews, the G2019S mutation is linked to a much higher percentage (22-41%) of patients diagnosed with PD.¹⁹⁻²¹ In addition, the penetrance for this mutation increases with age from 15-17% at the age of 50 years to 32-100% at the age of 80 years depending on the reported cohort.^{14,19,22}

LRRK2 is a large 2527-amino acid protein with several distinct domains: Leucine-rich repeat (1010-1287), GTPase (1335-1504), COR (1517-1843), kinase (1875-2132) and WD40 repeat (2231-2276) (Fig. 1A).⁹ The understanding of the biological function of *LRRK2* is still rudimentary. The majority of patients with *LRRK2* mutations present with classical PD with LBs,^{13, 23-25} but some have parkinsonism without Lewy pathology,^{23, 26, 27} and diverse clinical and pathological findings have also been reported in others.^{9, 28, 29} Hence, the clinical and pathological features of two patients, one patient with the R793M mutation and another with a novel L1165P mutation in the Leucine-rich domain, are described.

MATERIALS AND METHODS

Antibodies

Murine anti- α -syn monoclonal antibodies LB 509, Syn 514 and Syn 211 were previously described.³⁰⁻³² SNL-4 is a purified rabbit polyclonal antibody raised against a peptide corresponding to amino acid residues 2-12 in α -syn.³¹ pSer129 is a novel mouse monoclonal antibody raised against phospho-peptide CAYEMPpSEEGYQ conjugated to maleimide-activated keyhole limpet haemocyanin (KLH) and this antibody specifically recognizes α -syn phosphorylated at Ser 129.³³ Antibody 17026 is a rabbit antiserum raised against full-length recombinant *tau* that detects all isoforms of *tau*. AP7099b is an affinity purified rabbit antibody to *LRRK2* raised against the peptide RVEKLHLSHNKLKEIPPEIG (Abgent, San Diego, CA).²³ Anti-TAR-DNA-binding protein 43 (TDP-43) rabbit polyclonal antibody was purchased from ProteinTech Group (Chicago, IL).

Molecular genetic analysis of *LRRK2*

Genetic analysis of *LRRK2* was performed in a large cohort of neurodegenerative disease clinical and autopsy cases, including 98 cases (78 autopsied) with PD or dementia with LBs (DLB) as previously described.³⁴ Single nucleotide polymorphism (SNP) genotyping using TaqMan chemistry-based allelic discrimination assay with "Assay by Design" (Applied Biosystems, Foster City, CA) probes on an Applied Biosystems 7900 was performed for the *LRRK2* mutations: G2019S, I2020T, M1869T, R793M, and Y1699C. Appropriate positive and negative controls were used and data was analyzed using Sequence Detection System 2.2.1 software (Applied Biosystems) as described.³⁵ In the PD and LB autopsy cases, bi-directional DNA sequencing of a 251 bp product containing exon 25 was used to evaluate for the presence of the I1122V mutation which also allowed for the identification of a novel c.3494T>C, p.L1165P (Fig. 1B) variant within the exon 25 region as described.³⁴ All cases with *LRRK2* mutations were confirmed by bidirectional DNA sequencing using standard methods on a CEQ8000 (Beckman Coulter). To evaluate the novel exon 25 mutation c.

3494T>C, p.L1165P, a TaqMan “Assays by Design” allele discrimination assay was developed and performed on 366 control samples. The control samples were obtained from the following sources: 276 controls from the Coriell Institute (Neurologically Normal Caucasian control panels, Camden, NJ), 48 clinical controls (mean age 76) from the Alzheimer Disease Center at the University of Pennsylvania, and 42 brain autopsy samples (mean age 69) with normal pathology from the University of Pennsylvania Center for Neurodegenerative Disease brain bank. All research activities were approved by the University of Pennsylvania Institutional Review Board and all participants gave informed consent.

Immunohistochemistry and immunofluorescence

The harvesting, fixation and further processing of the tissue specimens were conducted as previously described.³⁶ Briefly, tissue blocks were removed at autopsy and fixed by immersion in 70% ethanol with 150 mM/L NaCl or 10% buffered formalin for 24-36 hr. Samples were dehydrated through a series of graded ethanols to xylene at room temperature and infiltrated with paraffin at 60°C as previously described.³⁶ Tissue blocks were then cut into multiple, near serial 6 µm sections for immunohistochemical staining. Immunohistochemistry was carried out using the avidin-biotin complex (ABC) detection system (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine as described previously with some modifications.³⁶ Briefly, sections were deparaffinized and sequentially rehydrated using 100-70% ethanol followed by water. Some sections were pretreated with 88% formic acid to enhance antigen detection. Endogenous peroxidases were quenched with 5% hydrogen peroxide in methanol for 30 min and sections were blocked in 0.1 M Tris with 2% fetal bovine serum (Tris/FBS) for 5 min. All antibodies were diluted in Tris/FBS. Primary antibodies were incubated overnight at 4°C. After washing, sections were sequentially incubated with biotinylated secondary antibodies for 1 hr and ABC complex for 1 hr. Bound antibody complexes were visualized by incubating sections in solution containing 100 mM Tris, pH 7.6, 0.1% Triton X-100, 1.4 mM DAB, 10 mM imidazole, and 8.8 mM hydrogen peroxide. Tissue sections were lightly counterstained with hematoxylin.

For immunofluorescence, tissue sections were re-hydrated and incubated with primary antibodies as described above. After washing, anti-mouse or anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 and 594 secondary were applied (Molecular Probes, Eugene, OR). Following washing and post-fixation with formalin, sections were cover-slipped with Vectashield with 4'-6-diamidino-2-phenylindole mounting medium (Vector Laboratories, Burlingame, CA).

RESULTS

Genetic analysis of LRRK2

Among the 98 sporadic and familial PD patients screened, 4 (~4%) were identified with the G2019S mutation (3 heterozygous and 1 homozygous). Of these, the clinical and pathological findings for three cases have been previously reported,²³ while the fourth was a living patient with onset of PD at age 44, a strong family history of PD, and a homozygous G2019S mutation. Additionally, the p.R793M (c.2378 G>T) mutation was identified in 1 PD case (described further below as Patient D). The I2020T, M1869T, and Y1699C mutations were not identified. DNA sequence analysis of exon 25 did not identify any I1122V mutations; however, the novel missense mutation in exon 25 (c.3494T>C, p.L1165P) (Fig. 1B) was identified in one PD case (described further below as Patient E). The L1165P mutation was absent from 366 controls tested using TaqMan SNP analysis. Thus, overall, 5

cases with LRRK2 mutations were found in the cohort of autopsies PD and DLB patients (5/78, 6.4%).

Clinical and pathological findings in cases harboring the R793M and L1165P mutations in LRRK2

Patient D—Patient D is a woman that first manifested unexplained falls at the age of 77. Within one year, clear evidence of parkinsonism had developed, and she was started on carbidopa/levodopa therapy for relief of rest tremor, rigidity and general bradykinesia. She responded well to treatment in the early years of her illness, but she developed slowly progressive disability and died 15 years after onset at 92.

At autopsy, the SN and the locus coeruleus (LC) were severely depigmented, but other brain regions were unremarkable. Detailed histological analysis revealed abundant LBs, LNs and α -syn immunoreactive spheroids in the SNpc (Fig. 2A, B), other brain stem nuclei, the hippocampus and the amygdala (Fig. 2C), but these inclusions were only sparsely present in the neocortex (Fig. 2D) with a distribution characteristic of PD.⁵⁻⁷ Neurofibrillary tangles (NFTs) and tau-positive dystrophic neurites were moderately abundant in the hippocampal formation (Fig. 2E), but rare in other brain regions. Senile plaques (SPs) were rare in all brain regions. No inclusions were detected with antibodies specific to LRRK2.

Patient E—This man developed the first signs of parkinsonism at age 47, including resting tremor, stooped posture and shuffling gait, but no sensory impairments. He was treated with carbidopa/levodopa therapy and responded well. At the age of 56, he developed visual and auditory hallucinations that only partly improved following withdrawal of levodopa therapy. His neurological condition gradually deteriorated with the emergence of apathy, delusions, confusion, memory impairments and severe depression. He died at the age of 81.

Postmortem examination revealed extensive loss of pigmented neurons in the SNpc and LC without neuronal loss in other brain regions. Numerous LBs, LNs and α -syn immunoreactive spheroids were found in the SNpc (Fig. 2F). α -Syn inclusions were frequent in the amygdala and in the hippocampal formation (Fig. 2G) and sparse in the neocortex. NFTs were abundant in the hippocampal formation (Fig. 2H, I) and the amygdala. Tau-containing inclusions were rare in other brain regions. SPs were infrequent in all regions of the brain. No inclusions were detected with antibodies specific to LRRK2. The clinical and pathological features of this patient are consistent with a primary diagnosis of PD and a secondary diagnosis of tangle predominant senile dementia.

Phosphorylation of Ser129 in α -Syn in the Pathological Inclusions of Patients with LRRK2 Mutations

Since the multiple functional domains and *in-vitro* kinase activity of LRRK2 has made LRRK2 a prime candidate for regulating signal transduction pathways, and α -syn is phosphorylated at Ser129 in pathological inclusions,³⁷ we therefore analyzed the phosphorylation state of α -syn in patients with LRRK2 mutations. Using an anti-phospho-Ser129 α -syn specific antibody (pSyn 129), the vast majority of α -syn pathological inclusions in the brains of both patients D and E were immuno-positive for phosphorylated Ser129 α -syn (Fig. 3). Similar phosphorylation of α -syn was also observed in 2 other previously described patients²³ carrying the G2019S mutation (data not shown).

TDP-43 cytoplasmic inclusions in patients with LRRK2 mutations

“Tau-negative, α -syn negative, ubiquitin-only” positive inclusions have been reported in some patients with LRRK2 mutations.⁹⁻²⁹ Recently, a patient with frontotemporal lobar degeneration with ubiquitinated neuronal inclusions has been reported to carry the G2019S

mutation³⁷. Since TDP-43 has recently been identified as a major component of ubiquitin inclusions in frontotemporal lobar degeneration³⁹ and a subset of PD patients have TDP-43-containing inclusions⁴⁰, the presence of TDP-43-positive inclusions in patients with *LRRK2* mutations was ascertained. Occasional cytoplasmic TDP-43 inclusions were observed in both patient D and E, but only in the temporal cortex (Fig. 4). No similar cytoplasmic TDP-43 inclusions were observed in the brains of the three previously reported PD patients carrying the G2019S *LRRK2* mutation,²³ or an additional five sporadic PD patients analyzed (data not shown).

DISCUSSION

The identification and characterization of patients with mutations in *LRRK2* is pivotal in understanding the effects of aberrations of the various domains of this protein. Herein, we describe the clinical and pathological findings of two patients with missense substitutions in *LRRK2* identified from a cohort of patients with pathologically confirmed PD and DLB. One subject (patient D) was found to harbor the R793M amino acid substitution and presented with the typical clinical and pathological features of PD. This variant has been previously reported in three patients with PD (2 familial and 1 sporadic), one patient with primary progressive aphasia, and four 40-61 year old control individuals (4 out of a total of 2065, 0.2%).^{34,35,41,42} The presence in a small percentage of unaffected individuals contests the pathogenicity of the R793M substitution; however, since these individuals are still relatively young (40-61 year old) it is possible that they may yet manifest disease with age. Like the G2019S mutation,^{14,19,22} the R793M substitution may be associated with reduced, age-dependent penetrance, or it may confer increased risk for expression of clinical disease, similar to the G2385R mutation.^{43,44} The nature of the R793M substitution, a disruption of two highly conserved basic residues within a stretch of hydrophobic residues (Fig. 1D), further suggests potential pathogenic properties. Irrespective of the pathogenicity or risk association, this is the first pathological characterization of a patient with the R793M variant. This patient demonstrated the classical clinical and pathological features of PD.

The second patient (patient E) was found to have a novel mutation L1165P in *LRRK2*. This patient presented with onset in middle age and had a long duration of illness (34 years). Although this patient had typical features of PD, he also became severely demented. The abundance of tau pathology in the hippocampal formation without SPs and concurrent dementia is consistent with a secondary diagnosis of tangle predominant senile dementia (also termed senile dementia with tangles). The relatively early onset of illness in this patient and the lack of this substitution in more than 366 control patients suggest that this mutation may be pathogenic. In addition, L1165 is highly conserved across multiple species (Fig. 1C) and the Leu to Pro substitution would be predicted to cause a dramatic structural change within the Leu-rich domain. However, since the function of this region in *LRRK2* is unknown, the overall effect of this mutation is unclear, and it is uncertain if the atypical pathological features of this patient are directly associated with this amino acid substitution. Although, it is notable that severe neuropsychiatric symptoms also have been described in a subset of patients with the G2019S and I2020T mutations.^{45,46}

Most patients with mutations in *LRRK2* present with clinical PD and have typical LB pathology, but a subset of patients have diverse pathological findings. These differences cannot solely be explained by the nature of specific mutations since significant differences can be observed between patients with the same mutation. For example, while 17 of the 20 autopsied PD patients reported with the G2019S mutation have shown classical nigral degeneration with nigral LBs^{13, 23-25}, three cases have presented pathological findings indicating concurrent Alzheimer disease.^{23,24} Two patients were shown to have nigral degeneration without Lewy pathology,^{23,26} and the third lacked Lewy pathology but

presented with a tauopathy with features suggestive of supranuclear palsy and early Alzheimer disease-type pathology.²⁸

Atypical clinical features associated with *LRRK2* mutation were first observed in the Canadian family A with the Y1699C mutation where amyotrophy was observed in addition to parkinsonism in some patients.⁹ The autopsies of two individuals from this kindred demonstrate SN degeneration, a paucity of α -syn positive LB and LN pathology, and the presence of “ubiquitin-positive” cytoplasmic and nuclear inclusions.⁹ On the other hand, individuals from the Lincolnshire kindred that carry the same mutation had clinical PD and LB brain pathology.⁴⁷ Diverse pathological findings have also been reported for the R1441C mutations carried within the Canadian family D. Autopsies of two of the documented cases present classical α -syn positive LB and LN pathology, but the third has tau pathology reminiscent of progressive supranuclear palsy. The fourth patient lacked Lewy pathology; however nonspecific loss of dopaminergic neurons with ubiquitin-positive inclusions were found.^{9, 29}

Although α -syn inclusions are more frequently observed in patients with *LRRK2* mutations than aggregates comprised of other proteins, all types of proteinaceous inclusions may contribute to neuronal dysfunction. TDP-43 has been identified as a major component within ubiquitinated neuronal intranuclear inclusions of patients with frontotemporal lobar degeneration.³⁹ Recently, the G2019S mutation was identified in an individual with frontotemporal lobar degeneration with ubiquitinated neuronal intranuclear inclusions,³⁸ and these inclusions have recently been found to be comprised of TDP-43.³⁹ Within the current study, histological analysis revealed occasional cytoplasmic TDP-43 inclusions within the temporal cortex of patients D and E. This is the first report of a histological analysis for TDP-43-positive inclusions in patients harboring *LRRK2* variants. It would be interesting to determine whether the “ubiquitin-only” inclusions that have been previously reported in some patients with the Y1699C and R1441C mutations are comprised of TDP-43.

It can be surmised from the wide range of disease onset, incomplete penetrance, and diverse pathological findings associated with *LRRK2* mutations that these alterations may render neurons more vulnerable to other insults that can result in neuronal degeneration with or without protein aggregation. Further studies will be required to better understand the mechanisms of neurodegeneration associated with *LRRK2* mutations and the contribution of protein aggregation.

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Figure 1. Identification of Patients with *LRRK2* Mutations and Sequence Alignment of Amino Acid Surrounding the Mutations

(**A**) Schematic representation of the *LRRK2* protein with major domains. The locations of the R793M, L1165P, and the most common G2109S mutation are indicated. Abbreviations used: LRR, leucine-rich repeat like domain; ROC, Ras of complex; COR, C-terminal of Roc. (**B**) Portion of sequencing electropherogram showing the region of *LRRK2* exon 25 in which the heterozygous c.3494T>C, p.L1165P was identified in Patient E compared to normal sequence. The mutation is near the 3' end of exon 25. The exon/intron junction is indicated by the dashed line. (**C**) Cross-species alignment of the amino acid sequence surrounding residue L1165. (**D**) Cross-species alignment of the amino acid sequence surrounding residue R793.

Figure 2. Histological Characterization of Patients with *LRRK2* Mutations

(A, B) Staining for α -syn in the SNpc of patient D demonstrating a dopaminergic neuron with multiple LBs (A) or a typical spheroid (B). (C) Abundant LBs and LNs in the amygdala of patient D. (D) Cortical LBs in the cingulate cortex of patient D. (E) NFTs and tau-positive dystrophic neurites in the hippocampus of patient D. (F) A classical LB in a dopaminergic neurons in SNpc of patient E. (G) Abundant LBs and LNs in hippocampus of patient E. (H, I) NFTs and tau-positive neurites in the hippocampus and entorhinal cortex, respectively, of patient E. Tissue sections were stained with the following antibodies: anti- α -syn antibody Syn 514 (A, C, D, and G), anti- α -syn antibody Syn 211 (B), anti- α -syn antibody LB509 (F), and anti-tau antibody 17026 (E, H and I). Scale bar = 25 μ m in A, B, D, E, F, and 50 μ m in C, G, H, and I.

Figure 3. Double-labeling Immunofluorescence Analysis of α -Syn Phosphorylated at Ser129 in Pathological Inclusions of Patients with LRRK2 Mutations

Double-labeling immunofluorescence analysis demonstrating the presence of α -syn phosphorylated at Ser129 in the vast majority of α -syn inclusions in patients with LRRK2 mutations. Depicted are tissue sections from the SNpc from patient D (**A-C**) and the amygdala from patient E (**D-F**). Panels **A** and **D** show immunostaining with the rabbit anti- α -syn antibody SNL-4 (green) and panels **B** and **E** depicts staining with mouse antiphospho-Ser129 α -syn antibody pSyn 129 (red). The overlays are shown in **C** and **F**. Scale bar = 65 μ m.

Figure 4. TDP-43 Cytoplasmic Inclusions in Patients with LRRK2 Mutations

Staining with an antibody to TDP43 revealed the presence of TDP-43 cytoplasmic inclusions in the temporal cortex of patient D (A) and patient E (B, C). Aberrant cytoplasmic TDP-43 inclusions are depicted by arrows, and normal nuclear localization is indicated by arrowheads. Scale bar = 25 μ m.