DJ-1 mutations are a rare cause of recessively inherited early onset parkinsonism mediated by loss of protein function

P J Lockhart, S Lincoln, M Hulihan, J Kachergus, K Wilkes, G Bisceglia, D C Mash, M J Farrer


Parkinson's disease (PD; OMIM #168600) is a common neurodegenerative disorder characterised by bradykinesia, resting tremor, muscle rigidity, and postural instability. The pathological features include loss of dopaminergic neurones, in particular within the substantia nigra pars compacta, and eosinophilic, cytoplasmic inclusions termed Lewy bodies. Although rare, familial forms of parkinsonism provide a powerful tool to determine the molecular pathways perturbed in idiopathic PD.

Three loci have been associated with autosomal recessive early onset parkinsonism (EO-PD): parkin (PARK2), the as yet unidentified PARK6, and DJ-1 (PARK7). Loss of Parkin function is the predominant genetic cause of EO-PD in Japanese, Northern European, North American, and North African populations. Homozygous and compound heterozygous parkin mutations account for approximately 49% of familial and 19% of sporadic EO-PD (with onset prior to 45 years). Although the gene has yet to be identified, linkage analysis of chromosome 1p36 interval suggests that PARK6 mutations may also account for numerous families with EO-PD across Europe. Most recently, mutations in DJ-1 (PARK7) were identified in consanguineous Dutch and Italian kindreds affected with EO-PD. In the Dutch kindred, a 14 kb genomic deletion removed the promoter region and the first five exons (Δ1–5). In the Italian kindred, a highly conserved amino acid (leucine) was altered to proline (497 bp T→C; L166P). This substitution was predicted to disrupt protein folding and was demonstrated to affect cellular localisation. Both mutations showed complete segregation with disease in homozygous individuals, while heterozygous carriers were unaffected, suggesting that loss of function of DJ-1 is pathogenic. To date, DJ-1 Δ1–5 and 497 bp T→C (L166P) mutations have only been reported in the original Dutch and Italian families and in ethnically matched control individuals. There have been no reports on the frequency of DJ-1 variants in EO-PD generally, or in samples from other populations.

DJ-1 appears to have several functions within the cell. Originally recognised as a c-myc interacting protein, DJ-1 has been identified as an infertility related protein, associated with RNA stabilisation and shown to convert to a more acidic form under conditions of oxidative stress. Currently, the mechanism by which loss of DJ-1 causes parkinsonism is unclear.

With this background, we performed a comprehensive mutation analysis of DJ-1 in a cohort of 49 EO-PD probands, white subjects derived from North America and representing several European ethnicities. One sequence alteration was identified in one patient, a heterozygous 293 bp G→A transition, resulting in an R98Q change to the protein sequence. Subsequently, the functional properties of DJ-1 wild type, Q98, and P166 proteins were assessed.

Key points

- Mutations in DJ-1/PARK7 were recently identified as a cause of early onset parkinsonism (EO-PD). To identify known and novel DJ-1 mutations and assess their frequency, we performed a comprehensive analysis of DJ-1 in 49 EO-PD patients previously excluded for mutations in the parkin gene.
- There were no alterations to DJ-1 in 48 of the 49 cases studied. Mutations in DJ-1 appear to be a rare cause of recessively inherited EO-PD, accounting for <1% of cases with onset prior to 50 years.
- A novel G→A transition resulted in a R98Q heterozygous substitution in one affected individual; however, no other sequence alterations or exon deletion/duplication(s) were detected. The transition was identified in six of 258 ethnically matched controls. This alteration probably represents a rare polymorphism.
- Functional analysis of the DJ-1 P166 variant demonstrated a reduced steady state protein level that could be rescued by proteasomal inhibition. Loss of DJ-1 protein is sufficient to explain disease pathogenesis for both Δ1–5 and P166 mutations when recessively inherited in EO-PD.

Materials and Methods

Patients

All subjects were evaluated by a neurologist specialising in movement disorders and met the criteria for PD. Age of onset of PD symptoms was prior to 50 years (mean (SD) 38.6 (8.1) years; male:female = 32:9, unknown gender = 8; n = 49), and two (possible PD, n = 33) or three (probable PD, n = 15) of the four cardinal signs of PD were noted. Patients displayed no atypical features or evidence of secondary parkinsonism caused by other neurological disease or drugs/toxins, except for one, where the patient was diagnosed with resting tremor/parkinsonism but was included in this study because of the very early age of onset (18 years). All patients were white, of diverse North American and/or Northern European ethnicity. Twenty four probands were defined as sporadic PD, while 22 probands had a family history of PD, here defined as having one or more first degree relatives with PD, and three probands

Abbreviations: EO-PD, early onset parkinsonism; ORF, open reading frame; PD, Parkinson’s disease
had an unknown family history. Three cases had a family history compatible with recessive inheritance, seven were suggestive of dominant transmission, and the remainder could not be assessed due to lack of pedigree information. All probands were screened for parkin mutations by gene dosage and direct sequencing as described previously and found to be negative.

Genetic analysis
Informed consent was obtained under an institutional review board approved protocol prior to the study. A 10–20 ml venous blood sample was drawn, and immortalised lymphoblastoid cells were generated by Epstein-Barr virus transformation using standard procedures. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Northern blot was performed essentially as described previously. The probe corresponded to the 3′UTR region of the pcDNA3.1 vector and was specific for plasmid derived transcripts. Genomic DNA was extracted using standard techniques, and semi-quantitative multiplex PCR assays were developed to detect exon rearrangements (deletions and duplications). Hex tagged, fluorescently labelled forward primers for rearrangements (deletions and duplications). Hex tagged, fluorescently labelled forward primers for rearrangements (deletions and duplications). Hex tagged, fluorescently labelled forward primers for rearrangements (deletions and duplications). Hex tagged, fluorescently labelled forward primers for rearrangements (deletions and duplications).

DJ-1 constructs, cell culture, and Western blotting
Full-length cDNA for DJ-1 was amplified as above and cloned into the mammalian expression vector pcDNA3.1-V5HIS/TOPO (Invitrogen). The resulting recombinant protein encoded an in-frame V5 epitope at the C terminus. The R98Q and L166P constructs were generated with the Transformer® mutagenesis kit (BD Biosciences Clontech, Palo Alto, CA, USA). All constructs were fully sequenced to confirm that the appropriate alterations were generated. HEK293 cells were cultured in OptiMEM supplemented with 10% fetal bovine serum (Invitrogen). Lactacystin and MG-132 (a specific inhibitor of the proteasome beta subunit and a reversible, transition state inhibitor of the proteasome respectively), used at the concentrations indicated in the text, were purchased from Calbiochem (EMD Biosciences, San Diego, CA, USA). Transient transfections were performed with FuGENE6 reagent (Roche Molecular Biology, Indianapolis, IN, USA) according to the manufacturer’s protocols. Western blotting was performed essentially as described previously. Primary antibodies were monoclonal anti-V5 (Invitrogen), monoclonal anti-β-arrestin (Chemicon International, CA, USA), and monoclonal anti-β-actin (Sigma, St Louis, MO, USA). Antibody binding was revealed using peroxidase conjugated secondary antibodies (Jackson ImmunoResearch Labs, West Grove, PA, USA) and enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturers’ protocols. Quantitation was performed using an Alpha Innotech gel documentation system (Alpha Innotech Corp, San Leandro, CA, USA).

RESULTS
Mutation screening
A comprehensive gene dosage analysis of all 7 DJ-1 exons did not detect any alteration in exon copy number, indicative of duplication and/or deletion, in any patient sample. All samples were analysed independently at least twice. For exons 1<sup>50</sup> through 5, haploid genomic controls were processed in parallel to confirm sensitivity and accurate quantitation of exon dosage. For all exons where a haploid control sample was available, we could accurately detect ~50% reduction in peak area, indicative of exon deletion (fig 1A).

For all patient samples, PCR amplification of the DJ-1 cDNA resulted in two products of 819 and 761 bp respectively. The different product sizes arise via alternate splicing of a different size were observed for any subject, suggesting there were no mutations affecting consensus splice motifs. Similarly, no sequence alterations were detected in the untranslated or coding portion of the DJ-1 cDNA in 48 of the patients examined. Taken with the gene dosage results, this study suggests that alterations in DJ-1 are a rare cause of EO-PD in the white population.

A single alteration in the open reading frame (ORF) of one proband was detected, a heterozygous 293 bp G→A transition (numbering based on position 1 being A of the initiating start codon), resulting in a R98Q substitution in the protein sequence. Semi-quantitative analysis of cDNA demonstrated

<p>| Table 1 Primer sets and pools used for DJ-1 gene dosage analysis |
|-----------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DJ-1 E1 F</td>
<td>5'-AGCTCTCCAGCTGAGAAAATCC</td>
<td>302</td>
</tr>
<tr>
<td>DJ-1 E1 R</td>
<td>5'-GACACGTCAGCAGACCC</td>
<td>297</td>
</tr>
<tr>
<td>DJ-1 E2 F</td>
<td>5'-CTCCTGCTCGAATAAGCTG</td>
<td>297</td>
</tr>
<tr>
<td>DJ-1 E2 R</td>
<td>5'-GCCAAGCAGTTAACAGCG</td>
<td>297</td>
</tr>
<tr>
<td>DJ-1 E3 F</td>
<td>5'-TAAAGAAGTCTGACTTGTGAT</td>
<td>297</td>
</tr>
<tr>
<td>DJ-1 E3 R</td>
<td>5'-ACGCGACCCACCACTTAC</td>
<td>297</td>
</tr>
<tr>
<td>DJ-1 E4 F</td>
<td>5'-GGCCCTCTGCTCCCTGGCA</td>
<td>297</td>
</tr>
<tr>
<td>DJ-1 E4 R</td>
<td>5'-TCACAGCCTCCTCCCAA</td>
<td>297</td>
</tr>
<tr>
<td>DJ-1 E5 F</td>
<td>5'-GTGATGTTTGGTAGTTGAC</td>
<td>297</td>
</tr>
<tr>
<td>DJ-1 E5 R</td>
<td>5'-ATCTGTTAATGACCACTT</td>
<td>297</td>
</tr>
<tr>
<td>DJ-1 E6 F</td>
<td>5'-TCTCAAGAATTITTTACCT</td>
<td>297</td>
</tr>
<tr>
<td>DJ-1 E6 R</td>
<td>5'-GAGGTGGTGGAAAAAGATCG</td>
<td>297</td>
</tr>
<tr>
<td>DJ-1 E7 F</td>
<td>5'-CCTCTCTGCTGCACTAG</td>
<td>297</td>
</tr>
<tr>
<td>DJ-1 E7 R</td>
<td>5'-GGACCTTCTGAAATGGGTGAC</td>
<td>297</td>
</tr>
<tr>
<td>Control F</td>
<td>5'-AGCTTCTCCTGAAATGGGTGAC</td>
<td>297</td>
</tr>
<tr>
<td>Control R</td>
<td>5'-CCTCTCCTGAAATGGGTGAC</td>
<td>297</td>
</tr>
</tbody>
</table>

Primers were pooled in sets of two DJ-1 exons and the control. The sets and primer concentrations were as follows. Set 1: exon 4 (0.6 μmol/l), exon 2 (0.8 μmol/l), and control (8 μmol/l); set 2: exon 1 (0.8 μmol/l), exon 6 (0.6 μmol/l), and control (0.8 μmol/l); set 3: exon 5 (0.8 μmol/l), exon 7 (0.8 μmol/l), and control (0.8 μmol/l); and set 4: exon 3 (1.2 μmol/l) and control (0.6 μmol/l).
that each allele was equally expressed. To comprehensively search for a second DJ-1 sequence alteration in this patient, we amplified and sequenced each exon from genomic DNA (for primer sequences, see table 1). No other sequence alterations were detected in the coding sequence or in a minimum of 50 bp flanking each intron. The 293 bp G→A transition was subsequently identified in 6 of 258 ethnically matched controls as a heterozygous alteration.

Analysis of DJ-1 protein variants

The R98 amino acid is well conserved in evolution (fig 1B) and the R98Q substitution may have significant effects on protein structure and localisation, as reported for the L166P alteration. To test this possibility, we generated non-tagged and V5 tagged constructs encoding the wild type, P166, and Q98 forms of DJ-1. The R98 amino acid is indicated in bold. Accession numbers are those referenced in Bonifati et al.²

## Figure 1 Genetic analysis of DJ-1 in EO-PD patients. (A) A representative exon dosage chromatogram demonstrating detection of a heterozygous exon 1 and exon 5 deletions. Ratios are generated by dividing the area under the DJ-1 exon peak by the area under the control peak. A ratio half of that observed for wild type is indicative of a heterozygous exon deletion. (B) ClustalW alignment of DJ-1 proteins. The R98 amino acid is indicated in bold. Accession numbers are those referenced in Bonifati et al.²

V5-Q98 DJ-1 transfected cells for 14 hours with 5 μmol/l and 10 μmol/l lactacystin, a specific proteasomal inhibitor, did not significantly alter the levels of these two forms of recombinant DJ-1 protein. However, this treatment did result in significantly elevated levels of V5-P166 recombinant protein. To confirm that these observations were the result of proteasome inhibition, we repeated the experiment using a second inhibitor (MG-132) and GFP₆, an in vivo indicator of proteasomal inhibition.²⁴ HEK293 cells were transiently transfected with the GFP₆ reporter and V5-P166 DJ-1 constructs. Treatment with increasing concentrations of MG-132 resulted in a significant, dose dependent increase in the steady state levels of GFP₆, confirming effective proteasome inhibition. Similarly, steady state levels of V5-P166 recombinant protein were significantly elevated (fig 5A). Quantification of three independent experiments performed in triplicate demonstrated that steady state V5-P166 DJ-1 increased greater than two-fold following exposure to 5 μmol/l MG-132 for 14 hours (fig 5B). This corresponded to approximately 20% of the steady state level of the wild type and Q98 forms of DJ-1. The lower molecular weight bands consistently observed in the MG-132 treated V5-P166 DJ-1

## Figure 2 Northern blot analysis of HEK293 cells transfected with pcDNA3.1-DJ-1. HEK293 cells were transiently transfected with the indicated constructs and total RNA was isolated and analysed by Northern blotting. (A) The blot was probed with the 3' UTR sequence of the transgene. Strong expression of non-tagged and V5 tagged wild type DJ-1 (lanes 3 and 4), non-tagged and V5 tagged R98Q DJ-1 (lanes 5 and 6), and non-tagged and V5 tagged L166P DJ-1 (lanes 7 and 8) was observed. (B) The same blot was subsequently reprobed with the β-actin cDNA probe to demonstrate loading and integrity of the RNA. Approximate sizes in kilobase pairs are indicated.

Two major mechanisms of intracellular protein degradation are the lysosomal pathway and the ubiquitin proteasome system. Inhibition of the lysosome with 100 μmol/l chloroquine or 20 m mol/l ammonium chloride didn’t alter steady state levels of V5-wildtype, V5-Q98, or V5-P166 DJ-1 (data not shown). Similarly, treatment of V5-wildtype and
cells probably represent breakdown products of the full length protein.

**DISCUSSION**

Genetic approaches are rapidly refining the causes of early onset PD, a complex disorder with a multifactorial genetic aetiology.22 However, the frequency of DJ-1 (PARK7) mutations in parkinsonism has yet to be fully evaluated. Apart from the initial description of mutations in DJ-1 causing EO-PD, there have been no further reports examining different EO-PD cases or populations. Although pathogenic mutations were reported in two consanguineous European kindreds, analysis of nine additional EO-PD families and 22 sporadic EO-PD cases failed to identify additional DJ-1 mutations.6 These observations suggest that mutations in DJ-1 are not a common cause of EO-PD, analogous to the low frequency of autosomal dominant PD resulting from mutations in a-synuclein. However, in the prior report, EO-PD cases were not excluded for parkin mutations, nor was DJ-1 gene dosage analysis performed. Interestingly, DJ-1 maps to a fragile region of 1p36 analogous to the FRA6E/parkin locus, suggesting that DJ-1 may also be prone to deletion and loss of heterozygosity.20–21 In this study, we assessed all possible variants within the DJ-1 gene in a cohort of EO-PD cases, formerly excluded from having mutations within the parkin (PARK2) gene. Our methods included RT-PCR, direct sequencing of cDNA, and exon dosage methods. We did not detect any alterations in exon copy number, PCR product size, or cDNA sequence in 48 of the 49 patients examined. A single heterozygous alteration in the ORF of one proband was detected, which resulted in a R98Q substitution in the protein sequence. However, a second sequence alteration in the alternate allele was not identified, and the alteration was also detected in control samples, suggesting that it represents a rare polymorphism. Our results suggest that DJ-1 mutations are rare in EO-PD, as we also failed to identify any mutations in a cohort of 41 EO-PD cases of ethnic Chinese descent originating from Taiwan (manuscript in preparation).

Additionally, DJ-1 sequence alterations were detected in only one of 107 EO-PD patients in a separate study (Dr S Hague, personal communication).

Recessive inheritance of DJ-1 mutations appears to be a prerequisite for EO-PD, as at least five carriers of the DJ-1 Δ1–5 mutation and three carriers of the P166 substitution remain unaffected.26–28 However, as the disease phenotype in the Q98 carrier was of very early onset, we explored the functional properties of the variant protein.

Northern and Western blot analysis of wild type and Q98 DJ-1 transfected cells demonstrated robust levels of expression. In contrast, Northern blot analysis showed that significant levels of plasmid generated P166 DJ-1 transcript was being produced but that the P166 DJ-1 protein was practically undetectable. We hypothesised that the P166 alteration might destabilise the protein, increasing turnover of the mutant compared to wild type DJ-1. To further investigate the mechanism, we treated cells expressing wild type and variant DJ-1 with proteasomal inhibitors (lactacystin and MG-132) at concentrations previously shown to inhibit proteasomal function but not induce significant cellular toxicity.28–29 Treatment with proteasome inhibitors did not significantly affect the steady state level of wild type or Q98 DJ-1, but did significantly increase the steady state levels of both P166 DJ-1 and the in vivo GFP reporter protein. Our results suggest P166 DJ-1 is rapidly and efficiently degraded by the proteasome. The molecular basis for enhanced degradation of P166 DJ-1 by the proteasome is unknown. The 166 position of DJ-1 is predicted to form a conserved helix,6 and proline can disrupt helix formation.30 Recent crystallisation data support this hypothesis, demonstrating that the 166 position is important for helix formation and the generation of a hydrophobic core between three helices. The P166 alteration appears to prevent DJ-1 dimerisation and crystallisation.31 This may alter protein conformation allowing/enhancing ubiquitylation by a currently unknown E3 protein–ubiquitin ligase. While lysine 130 is proposed as a site for sumoylation,33 DJ-1 encodes several lysine residues that are potential targets for ubiquitylation. Interestingly, a
proline residue can function directly as a targeting determinate for E3 protein—ubiquitin ligases.14 Additionally, not all proteins that are degraded by the proteasome are ubiquitylated.15 Future studies will be required to distinguish between these possibilities.

In conclusion, loss of DJ-1 protein is sufficient to explain disease pathogenesis for both α1–5 and P166 mutations, when recessively inherited in EO-PD. Although mitochondrial relocalisation of DJ-1 P166 has been noted in COS7 cells,16 our work suggests it is a minor observation in HEK293 cell models or be an artefact of cell culture conditions. Examination of the amount and localisation of DJ-1 in patient samples derived from the Italian kindred could help to resolve these observations. Although further case-control studies of the 293 bp G→A transition identified here need to be performed, our results suggest the R98Q substitution represents a rare polymorphic change, not a pathogenic mutation. DJ-1 mutations are rare; there are few polymorphic or pathogenic coding variants in the gene in white populations. Hence, PARK5 is unlikely to explain EO-PD within individuals in which mutations in PARK2 have been excluded. Nevertheless, identifying the molecular components of parkinsonism in rare families is useful, as these proteins may be part of a molecular pathway perturbed in idiopathic PD. Furthermore, genetic insights allow the creation of cellular and animal models and will ultimately provide a rational basis for novel drug design.

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