

**ELECTRONIC LETTER**

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

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**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 immunolysed 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). Hextagged, fluorescently labelled forward primers for DJ-1 exons (table 1) were optimised in pooled sets of three primer pairs for multiplexing with an internal control. An initial 95˚C denaturation (5 minutes) was followed by 23 cycles of 95˚C (30 seconds), 53˚C (45 seconds), and 68˚C (2.5 minutes), with a final extension 68˚C (5 minutes). PCR products were purified and diluted to give peak heights in the 1000–3000 scalar range, ensuring accurate assessment of peak area on an ABI 3100 analyser using Genotyper™ software.

The entire DJ-1 ORF was amplified by RT-PCR using SuperScript II reverse transcriptase (Invitrogen) and a 60–50˚C touchdown protocol over 35 cycles. The forward primer corresponded to base pairs 23–40 of the Genbank sequence AF021819 (5’-TGTGACTTCCATACTTCG-3’) and the reverse primer to base pairs 764–783 (5’-TGTGACTTCCATCTTCG-3’). Products were analysed for size alterations by fluorescently labelled forward primers for this alteration, a 294 bp fragment was amplified from genomic DNA (forward primer, 5’-GTGAGTGATTGGTTAGTG-3’ and reverse primer 5’-ATCTCTGAAATGACACCAACTT-3’). Products were analysed for size alterations by digestion with MspI 1 resulted in two fragments of 194 and 100 bp) if the 293 bp G→A transition was present. To assay that the appropriate alterations were generated, the BigDye Terminator Reagent mix (version 1.1) and an ABI3100 automated sequencer. Heterozygote base calls and sequence alignment were performed with Sequencher™ software (Gene Codes Corp., Ann Arbor, MI, USA).

The 293 bp G→A transition disrupts an MspI site. To assay for this alteration, a 294 bp fragment was amplified from genomic DNA (forward primer, 5’-TGCGTTCACTTTCAGCC-3’) and the reverse primer 5’-TGCGTTCACTTTCAGCC-3’) using a BigDye Terminator Reagent mix (version 1.1) and an ABI3100 automated sequencer. Heterozygote base calls and sequence alignment were performed with Sequencher™ software (Gene Codes Corp., Ann Arbor, MI, 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 1A–B through to 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 on the non-coding exon 1A, as reported previously. No PCR products 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

### Table 1

**Primer sets and pools used for DJ-1 gene dosage analysis**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DJ-1 E1 F</td>
<td>[Hex] 5’-GTGACTTCCATACTTCG-3’</td>
<td>302</td>
</tr>
<tr>
<td>DJ-1 E1 R</td>
<td>5’-GACACCGTCGACCC-3’</td>
<td></td>
</tr>
<tr>
<td>DJ-1 E2 F</td>
<td>[Hex] 5’-CTCTGCTCCCTGATAAAC-3’</td>
<td>392</td>
</tr>
<tr>
<td>DJ-1 E2 R</td>
<td>5’-GCCAACAGTATAACAGCC-3’</td>
<td></td>
</tr>
<tr>
<td>DJ-1 E3 F</td>
<td>[Hex] 5’-TAAAGACAGTGACTGATT-3’</td>
<td>388</td>
</tr>
<tr>
<td>DJ-1 E3 R</td>
<td>5’-CATCCGACCCACCCCTAC-3’</td>
<td></td>
</tr>
<tr>
<td>DJ-1 E4 F</td>
<td>[Hex] 5’-GCGTACCTTGCTACCTCC-3’</td>
<td>297</td>
</tr>
<tr>
<td>DJ-1 E4 R</td>
<td>5’-TCAAGCACCCCTGACCAA-3’</td>
<td></td>
</tr>
<tr>
<td>DJ-1 E5 F</td>
<td>5’-GTGAGTGATTGGTTAGTG-3’</td>
<td>293</td>
</tr>
<tr>
<td>DJ-1 E5 R</td>
<td>5’-ATCTCTGAAATGACACCA-3’</td>
<td></td>
</tr>
<tr>
<td>DJ-1 E6 F</td>
<td>5’-TCAAGAACATTITTTTCAC-3’</td>
<td>367</td>
</tr>
<tr>
<td>DJ-1 E6 R</td>
<td>5’-GAGCGTGAGGAGAAGAAT-3’</td>
<td></td>
</tr>
<tr>
<td>DJ-1 E7 F</td>
<td>5’-CGTCTTCTCGACTA-3’</td>
<td>360</td>
</tr>
<tr>
<td>DJ-1 E7 R</td>
<td>5’-TGGACTCCTACCTCCTCC-3’</td>
<td></td>
</tr>
<tr>
<td>Control F</td>
<td>5’-AGTCTCTGGTAATGGGGAT-3’</td>
<td>328</td>
</tr>
<tr>
<td>Control R</td>
<td>5’-CCCTCCTCGACCAAGTG-3’</td>
<td></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 (0.8 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 and transfected HEK293 cells. The cells were harvested, split into two pellets and analysed by Northern and Western blotting. The Northern blot demonstrated that significant levels of DJ-1 mRNA generated by the plasmid was being produced for each DJ-1 construct (fig 2), suggesting that efficient transfection had occurred. However, the steady state levels of the P166 protein were significantly lower than the wild type or Q98 forms (fig 3), and required extended exposure times or increased protein loading to be visualised. Similar results were obtained when transfected cells were examined by indirect immunofluorescence for plasmid derived DJ-1 protein. Wild type and Q98 DJ-1 demonstrated robust levels of expression and displayed diffuse cytoplasmic and nuclear immunoreactivity, whereas P166 DJ-1 levels were significantly reduced (data not shown).

To confirm these observations were the result of proteasome inhibition, we repeated the experiment using a second inhibitor (MG-132) and GFP, an in vivo indicator of proteasomal inhibition. HEK293 cells were transiently transfected with the GFP 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 GFP, confirming effective proteasome inhibition. Similarly, steady state levels of V5-P166 recombinant protein were significantly elevated (fig 5A). Quantitation 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

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 (representative results shown in fig 4). To confirm that these observations were the result of proteasome inhibition, we repeated the experiment using a second inhibitor (MG-132) and GFP, an in vivo indicator of proteasomal inhibition. HEK293 cells were transiently transfected with the GFP 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 GFP, confirming effective proteasome inhibition. Similarly, steady state levels of V5-P166 recombinant protein were significantly elevated (fig 5A). Quantitation 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
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. 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. 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 α-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. 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. 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. 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 α-helix, and proline can disrupt helix formation. 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. 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, 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. Additionally, not all proteins that are degraded by the proteasome are ubiquitylated. 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 A1–5 and P166 mutations, when recessively inherited in EO-PD. Although mitochondrial relocation of DJ-1 P166 has been noted in COS7 cells, our work suggests it is a minor observation in HEK293 cells compared with the dramatic decrease in steady state protein levels. These differences may be the result of alternate cell models or be an artefact of cell culture conditions. Examination of the amount and localization 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, if PARK7 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 play a part of a molecular pathway perturbed in idiopathic PD. Furthermore, genetic insights into the creation of cellular and animal models will ultimately provide a rational basis for novel drug design.

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