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Absence of *C9ORF72* expanded or intermediate repeats in autopsy confirmed Parkinson Disease

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Abstract

Background—We have reported that intermediate repeat lengths of the *C9ORF72* repeat are a risk factor for Parkinson Disease (PD) in a clinically- diagnosed dataset. As 10-25% of clinically diagnosed PD have different diagnoses upon autopsy, we hypothesized this may reflect phenotypic heterogeneity or concomitant pathology of other neurodegenerative disorders.

Methods—We screened 488 autopsy-confirmed PD cases for the expansion haplotype tag, rs3849942T. In 196 identified haplotype carriers, the *C9ORF72* repeat was genotyped using the repeat-primed PCR assay.

Results—No larger (intermediate or expanded) repeats were found in these autopsy-confirmed PD samples. This absence of larger repeats is significantly different from the frequency in clinically-diagnosed datasets ($p=0.002$).

Conclusions—Our results suggest that expanded or intermediate *C9ORF72* repeats in clinically-diagnosed PD or Parkinsonism might be an indication of heterogeneity in clinically-diagnosed PD cases. Further studies are needed to elucidate the potential contribution of the *C9ORF72* repeat to autopsy-confirmed PD.

Keywords

autopsy confirmed; Parkinson Disease; *C9ORF72* repeat; parkinsonism

Introduction

Parkinson disease (PD) is a neurodegenerative movement disorder that affects approximately 4-5% of the population at 85 years and older (1). Diagnosis of PD requires at least two out of the three cardinal symptoms of bradykinesia, rigidity and tremor, and is often accompanied by postural instability. Parkinson-plus syndromes such as progressive supranuclear palsy (PSP), multiple system atrophy (MSA) and corticobasal syndrome (CBS) share symptoms with PD of akinetic rigidity, though each supplemented with disease specific symptoms (e.g. PSP; supranuclear ophthalmoplegia / MSA; dysautonomia / CBS; dystonia). In addition, several other neurodegenerative disorders display symptom overlap with PD, such as amyotrophic lateral sclerosis (ALS), frontotemporal dementia with Parkinsonism (FTDP), dementia with Lewy bodies (DLB) and Alzheimer disease (AD). Thus it is not surprising that up to 25% of clinically-diagnosed PD cases have one of these other disorders identified by neuropathologic evaluation (2). This is true even when the patients are examined by an experienced movement disorder specialist.

In the last two years, carriers of large (> 30 repeats) or intermediate (20-30) expansions of a six base pair repeat in the *C9ORF72* gene have been reported to cause different neurodegenerative disorders. These longer repeats (expanded or intermediate) were initially reported in ALS (20-40%; (3, 4, 19)) and FTD families (10-25%, (3, 4, 20)) but are also observed in other neurodegenerative disorders, albeit at much lower frequency (AD; 0.5-1% out of ~5000 reported, PSP; ~1.5% out of ~200 reported, CBS; ~2% out of ~35 reported) (5-15). Further, our recent report (16) evaluated intermediate repeat lengths (20-30 copies) in two independent clinically-diagnosed PD datasets, without known family history of other neurodegenerative disorders, and provided evidence for association between these intermediate repeat lengths and increased risk for clinical PD.

Given the known heterogeneity of the neuropathologic diagnoses associated with clinically-diagnosed PD case series (2), we hypothesized that the presence of intermediate and expanded *C9ORF72* repeats in clinically-diagnosed PD patients reflects this neuropathological heterogeneity. To test this hypothesis, we set out to genotype a large group of autopsy-confirmed PD cases, effectively filtering out other Parkinsonian syndromes.

Material and Methods

Sample selection

A total of 488 individuals with PD were included after evaluation with strict clinical and pathological criteria. All had an antemortem clinical diagnosis of PD, moderate to severe neuronal loss in the substantia nigra and presence of Lewy bodies in the substantia nigra or other areas in the brain upon autopsy. Individuals were excluded if any of the following existed: a prominent dementia syndrome within one year of diagnosis (17), competing pathologic features (e.g., PSP rather than PD), or Braak neurofibrillary tangle stage greater than IV. As ascertainment for most samples was through the initial autopsy, no information on age-at-onset or family history was available on most individuals. None of the 488 individuals overlap with the previously reported clinically-diagnosed PD dataset (16). However, samples with over 20 repeat copies from the previously reported dataset (16) were included as positive controls.

To address the possibility that the repeat length is variable between different tissues within the same individual, we used DNA extracted from the brain when available (85%), with blood DNA as the source in the remaining 15%. As the substantia nigra is degraded in PD, DNA from the frontal cortex was utilized in order to have sufficient material.

Genotyping

TagSNP rs3849942 genotyping—The T allele at SNP rs3849942 is found in 95 percent of all individuals with greater than eight *C9ORF72* repeats, and all individuals with greater than 20 repeats (16, 18). Thus this SNP was genotyped as a screening step, using a custom TaqMan® genotyping assay (Life Technologies, Applied Biosystems, USA), to identify an enriched pool of patients who were appropriate for full *C9ORF72* repeat typing.

C9ORF72 repeat-primed PCR—The primers developed by DeJesus-Hernandez et al (4) were used in the *C9ORF72* repeat-primed PCR assay. The PCR cycling program of Renton et al (3) was modified to achieve more robust results on a Veriti 96-well Fast Thermal Cycler (Life technologies, Applied Biosystems). A custom PCR cycling program was used (4min at 94°C; 50 cycles of 1min at 94°C, 1min at 64°C and 2min at 72°C; 10min at 72°C). Fragment length analysis was performed on an ABI 3730xl genetic analyzer (Life Technologies, Applied Biosystems), and analyzed using GeneMapper software (version 4.0, Life technologies, Applied Biosystems).

Statistical Methods

We defined the threshold for ‘larger’ repeat copies as over 20 copies. This value was chosen as it is the most commonly reported lower limit of ‘intermediate’ *C9ORF72* repeats (3, 4, 6, 12, 13, 16, 19-32). To test for significant difference in frequency between different datasets we conducted Fisher's exact tests using the *a priori* threshold of greater than 20 repeat copies (RCs). P-values of 0.05 or below were considered statistically significant.

Results

We identified 196 out of 488 cases (~40%) with the T allele at rs3849942, which tags the repeat expansion haplotype. These individuals, together with the positive controls, were then typed using the *C9ORF72* repeat-primed assay. All positive controls had repeats ≥ 20 repeats, but no carriers were detected with the intermediate (20-30 copies) or expanded (>30) repeat copy alleles (range of autopsy cases: 4-19 RCs). Similar to previous reports, approximately 92% of the T allele carriers carried over 8 repeat copies (Figure 1).

To determine whether the absence of intermediate repeat carriers in this group is significantly different from the frequency of repeats in individuals with clinically-diagnosed PD, we performed a Fisher's exact test using the previously defined threshold of 20 RCs. Comparing frequencies of intermediate repeat carriers between the previously reported clinically-diagnosed dataset (14 out of 889) and the present autopsy confirmed dataset revealed a significant difference between the two groups (one-tailed Fisher's exact test $p=0.002$). Alternatively, assuming a true carrier frequency at the lower bound of the 95% confidence interval in the clinically-diagnosed dataset (~0.8%), we had a chance of $> 98\%$ of seeing at least one carrier in 488 autopsy-confirmed individuals, indicating that we would have likely seen the intermediate repeat if it existed in the autopsy-confirmed PD cases.

Discussion

We recently described the intermediate sized *C9ORF72* repeats (20 to 30 repeats in size) as a risk factor for PD in two large clinically-diagnosed datasets (16). Only a small number of large expansions (>30 RC) have been found in PD, suggesting that repeats over 30 copies are not a common cause of PD (~0.2% out of ~3500 tested). However, additional intermediate repeat carriers (20 to 30 repeats) have been reported, totaling ~1% of both intermediate and expanded repeats in clinical PD cases (11-14, 28, 33-37).

The significant absence of intermediate or expanded repeats in our autopsy-confirmed dataset supports the hypothesis that the presence of intermediate and larger *C9ORF72* repeat expansions in clinically-diagnosed PD might arise from phenotypic heterogeneity. Xi et al recently reported nominal association with PD and the 10-repeat allele, which would not survive multiple testing thresholds.

Interestingly, positive family histories for other clinical neurodegenerative disorders (including ALS and FTD) have been observed in some of the *C9ORF72* positive PD/parkinsonism families (11, 12, 33, 34), though this is not addressed in all reports. Besides phenotypic heterogeneity in the PD patients, these observations might also suggest possible concomitant diseases in these families. Patients with symptoms reminiscent of Parkinson-plus syndromes, dementia within one year of PD onset, or positive family history of FTD were excluded from the previously reported clinically-diagnosed dataset. Though not specifically ascertained in this dataset, identification of positive family history of ALS by the examiner sufficed for exclusion.

The hypothesis of concomitant pathologies seems to be supported by another report on *C9ORF72* in autopsy confirmed PD (34). The authors observed one carrier of the *C9ORF72*

expansion (out of 377 patients with Lewy body positive α -synucleinopathy). As they did not use any exclusion criteria, the neuropathologic evaluation in this individual also showed TDP-43 pathology with frontotemporal lobar degeneration features. In combination with the described family history for ALS, it suggests the clinically diagnosed PD patient may also have had subclinical FTD.

In addition, the hypothesis of an underlying concomitant pathology might also be relevant to the control individuals that were reported to carry the *C9ORF72* repeat expansion or intermediate length repeat copies (3, 4, 6, 19-26). Recently, clinical controls have been shown to present with some measure of “disease”-associated change upon autopsy (38), allowing for the possibility that the asymptomatic intermediate repeat copy carriers will still present with pathological indications of disease. This concept gets some support from our analyses in the clinically-diagnosed datasets (16), where we included only controls with an age-at-exam higher than 60 years. With this threshold we wanted to reduce the chance of including preclinical individuals. We in fact observed less controls with over 20RCs in this group than generally reported so far (<0.5% versus 0.5-1%) (3, 4, 6, 19-26).

In conclusion, we observed that expanded or intermediate *C9ORF72* repeats are not associated with stringently selected autopsy confirmed PD. Our findings underscore the clinical heterogeneity of PD, and support the hypothesis that the presence of *C9ORF72* repeats in PD patients may represent this heterogeneity rather than a direct contribution to PD itself.

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Author Roles

1) Research project:

a. Conception; KN, GWB, LW, ERM, WKS, JMV

b. Organization; KN, VI, JMV

c. Execution; KN, VI

2) Statistical Analysis:

a. Design; GWB, ERM

b. Execution; KN, ERM

c. Review and Critique; GWB, ERM, WKS

- 3) Manuscript:
- d. Writing of the first draft; KN, JMV
- e. Review and Critique; WKS, DWD, JQT, VMYL, DCM, MPF, TF, LSH, TJM, TMD, JMV

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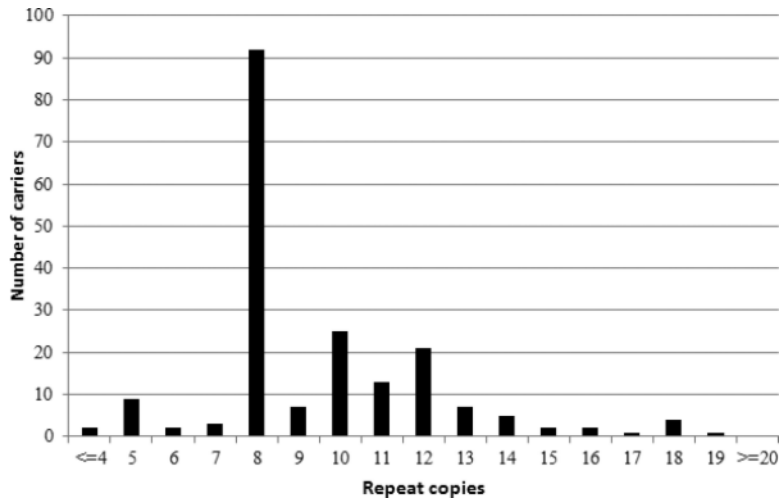


Figure 1. Histogram of autopsy confirmed PD dataset
 Histogram of the maximum number of *C9ORF72* repeat copies (X-axis) for 196 rs3849942 T positive, autopsy confirmed PD cases.