Absence of *C9ORF72* Expanded or Intermediate Repeats in Autopsy-Confirmed Parkinson's Disease

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ABSTRACT

Background: We have reported that intermediate repeat lengths of the *C9ORF72* repeat are a risk factor for Parkinson's disease (PD) in a clinically diagnosed data set. Because 10% to 25% of clinically diagnosed PD have different diagnoses upon autopsy, we hypothesized that this may reflect phenotypic heterogeneity or concomitant pathology of other neurodegenerative disorders.

Methods: We screened 488 autopsy-confirmed PD cases for expansion haplotype tag rs3849942T. In 196 identified haplotype carriers, the C9ORF72 repeat was genotyped using the repeat-primed polymerase chain reaction 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.

Key Words: autopsy confirmed; Parkinson's disease; *C9ORF72* repeat; parkinsonism

Parkinson's disease (PD) is a neurodegenerative movement disorder that affects approximately 4% to 5% of the population at 85 years and older.[1] Diagnosis of PD requires at least two of the three cardinal symptoms of bradykinesia, rigidity, and tremor and is often accompanied by postural instability. Parkinson-plus syndromes, such as PSP, MSA, and corticobasal syndrome (CBS), share symptoms with PD of akinetic rigidity, though each is supplemented with disease-specific symptoms (e.g., PSP: supranuclear ophtalmoplegia, MSA: dysautonomia and CBS: dystonia). In addition, several other neurodegenerative disorders display symptom overlap with PD, such as amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) with parkinsonism, dementia with Lewy bodies, and Alzheimer's 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

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evaluation.[2] This is true even when the patients are examined by an experienced movement disorder specialist.

In the last 2 years, carriers of large (>30 repeats) or intermediate (20-30) expansions of a 6-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]) and FTD families (10%-25%[3,4]), but are also observed in other neurodegenerative disorders, albeit at much lower frequency (AD; 0.5%-1% of ~5,000 reported, PSP; ~1.5% of ~200 reported, CBS; ~2% of ~35 reported).[5-15] Furthermore, our recent report[16] evaluated intermediate repeat lengths (20-30 copies) in 2 independent clinically diagnosed PD data sets, without known family history of other neurodegenerative disorders, and provided evidence for the 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.

Patients 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 (SN) and presence of Lewy bodies (LB) in the SN or other areas in the brain upon autopsy. Individuals were excluded if any of the following existed: a prominent dementia syndrome within 1 year of diagnosis[17]; competing pathologic features (e.g., PSP rather than PD); or Braak neurofibrillary tangle stage greater than IV. Because 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 data set.[16] However, samples with over 20 repeat copies from the previously reported data set[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%. Because the SN 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 single-nucleotide polymorphism (SNP) rs3849942 is found in 95% of all individuals with greater than 8 *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, Foster City, CA), to identify an enriched pool of patients who were appropriate for full *C9ORF72* repeat typing.

C9ORF72 Repeat-Primed Polymeras Chain Reaction

The primers developed by DeJesus-Hernandez et al.[3] were used in the *C9ORF72* repeat-primed polymerase chain reaction (PCR) assay. The PCR cycling program of Renton et al.[4] was modified to achieve more-robust results on a Veriti 96-well Fast Thermal Cycler (Life Technologies, Applied Biosytems). A custom PCR cycling program was used (4 minutes at 94°C; 50 cycles of 1 minute at 94°C, 1 minute at 64°C, and 2 minutes at 72°C; and 10 minutes 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 Biosytems).

Statistical Analysis

We defined the threshold for "larger" repeat copies as over 20 copies. This value was chosen because 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 data sets, 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 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 ≥20 repeats, but no carriers were detected with the intermediate (20-30 copies) or expanded (>30) RC alleles (range of autopsy cases: ≤4-19 RCs). Similar to previous reports, approximately 92% of the T-allele carriers carried over 8 RCs (Fig. 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.

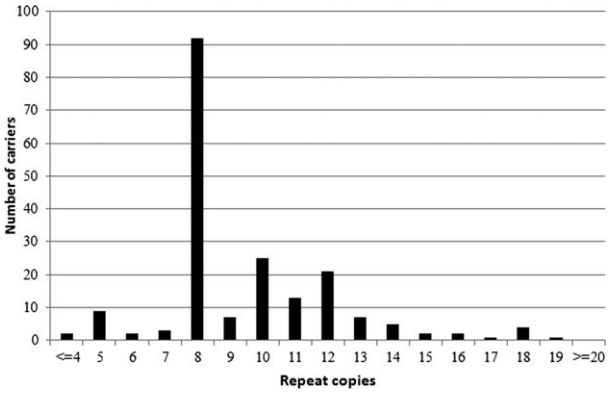

FIG. 1. Histogram of autopsy confirmed PD data set. Histogram of the maximum number of *C9ORF72* RCs (x-axis) for 196 rs3849942 T-positive, autopsy-confirmed PD cases.

Comparing frequencies of intermediate repeat carriers between the previously reported clinically diagnosed data set (14 of 889) and the present autopsy-confirmed data set revealed a significant difference between the 2 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 data set (~0.8%), we had a chance of >98% of observing at least 1 carrier in 488 autopsy-confirmed individuals, indicating that we would have likely observed the intermediate repeat if it existed in the autopsy-confirmed PD cases.

Discussion

We recently described the intermediate-sized *C9ORF72* repeats (20-30 repeats in size) as a risk factor for PD in two large clinically diagnosed data sets.[16] Only a small number of large expansions (>30 RCs) have been found in PD, suggesting that repeats over 30 copies are not a common cause of PD (~0.2% of ~3,500 tested). However, additional intermediate repeat carriers (20-30 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 data set 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 on a 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 PD patients, these observations might also suggest possible concomitant diseases in these families. Patients with symptoms reminiscent of Parkinson-plus syndromes, dementia within 1 year of PD onset, or positive family history of FTD were excluded from the previously reported clinically diagnosed data set. Though not specifically ascertained in this data set, 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 researchers observed 1 carrier of the *C9ORF72* expansion (of 377 patients with LB-positive alpha-synucleinopathy). Because they did not use any exclusion criteria, the neuropathologic evaluation in this individual also showed transactivation response DNA-binding protein 43 kDa pathology with frontotemporal lobar degeneration features. In combination with the described family history for ALS, it suggests that 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 RCs.[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 RC carriers will still present with pathological indications of disease. This concept gets some support from our analyses in the clinically diagnosed data sets,[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 20 RCs in this group than generally reported thus far (<0.5% vs. 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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