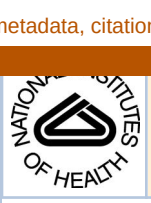

Published in final edited form as:

Mov Disord. 2014 May ; 29(6): 827–830. doi:10.1002/mds.25838.

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Financial disclosure/conflict of interest:
None of the authors have anything to disclose relative to the research covered in the submitted manuscript.

Financial Disclosures
Dr. Nuytemans, nothing to disclose.
Ms. Inchausti, nothing to disclose.
Dr. Beecham, nothing to disclose.
Dr. Wang, nothing to disclose.
Dr. Dickson is an editorial board member of Annals of Neurology, Parkinsonism and Related Disorders, Journal of Neuropathology and Experimental Neurology, and Brain Pathology. He is editor in chief of American Journal of Neurodegenerative Disease, and International Journal of Clinical and Experimental Pathology. Grant support: P50-AG16574, P50-NS72187, and P01-AG03949.
Dr. Trojanowski serves as an Associate Editor of Alzheimer's & Dementia. He may accrue revenue on patents submitted by the University of Pennsylvania wherein he is inventor including: Modified avidin-biotin technique, Method of stabilizing microtubules to treat Alzheimer's disease, Method of detecting abnormally phosphorylated tau, Method of screening for Alzheimer's disease or disease associated with the accumulation of paired helical filaments, Compositions and methods for producing and using homogeneous neuronal cell transplants, Rat comprising straight filaments in its brain, Compositions and methods for producing and using homogeneous neuronal cell transplants to treat neurodegenerative disorders and brain and spinal cord injuries, Diagnostic methods for Alzheimer's disease by detection of multiple MRNAs, Methods and compositions for determining lipid peroxidation levels in oxidant stress syndromes and diseases, Compositions and methods for producing and using homogenous neuronal cell transplants, Method of identifying, diagnosing and treating alpha-synuclein positive neurodegenerative disorders, Mutation-specific functional impairments in distinct tau isoforms of hereditary frontotemporal dementia and parkinsonism linked to chromosome-17: genotype predicts phenotype, Microtubule stabilizing therapies for neurodegenerative disorders, and Treatment of Alzheimer's and related diseases with an antibody. He is co-inventor on patents submitted the University of Pennsylvania wherein he is inventor that have generated income he has received from the sale of Avid to Eli Lily including: Amyloid plaque aggregation inhibitors and diagnostic imaging agents. Finally, he receives research support from the NIH (AG 10124, AG 17586, AG-19724AG 024904, NS053488, AG029213).
Dr. Lee has received funding for travel and honoraria from Takeda Pharmaceutical Company Ltd.; has received speaker honoraria from Pfizer Inc., BMS and Merck; may accrue revenue on patents re: Modified avidin-biotin technique, Method of stabilizing microtubules to treat Alzheimer's disease, Method of detecting abnormally phosphorylated tau, Method of screening for Alzheimer's disease or disease associated with the accumulation of paired helical filaments, Compositions and methods for producing and using homogeneous neuronal cell transplants, Rat comprising straight filaments in its brain, Compositions and methods for producing and using homogeneous neuronal cell transplants to treat neurodegenerative disorders and brain and spinal cord injuries, Diagnostic methods for Alzheimer's disease by detection of multiple MRNAs, Methods and compositions for determining lipid peroxidation levels in oxidant stress syndromes and diseases, Compositions and methods for producing and using homogenous neuronal cell transplants, Method of identifying, diagnosing and treating alpha-synuclein positive neurodegenerative disorders, Mutation-specific functional impairments in distinct tau isoforms of hereditary frontotemporal dementia and parkinsonism linked to chromosome-17: genotype predicts phenotype, Microtubule stabilizing therapies for neurodegenerative disorders, and Treatment of Alzheimer's and related diseases with an antibody; and receives research support from the NIH NIA PO1 AG 17586-10, PO1 AG-032953, NINDS P50 NS053488-02, NIA UO1 AG029213-01; and from the Marian S. Ware Alzheimer Program.
Dr. Mash reports Stock Ownership of DEMERx, Inc.
Dr. Frosch is a member of the external Scientific Advisory Committee for Northwestern University ADC, NIA/NIH grant funding
Dr. Foroud is a consultant for Univ of Penn (NIA Genetics of Alzheimer's Data Storage), an NIH SREA Reviewer for an NHGRI CSR study section; Natl Advisory Council on Alcohol Abuse & Alcoholism; Univ of Colorado. She is on the Scientific Advisory Board for the Center for Antisocial Drug Dependence (award DA011015) and has received funding from AAAS honoraria and NIH grant.
Dr. Honig is a consultant for Johnson & Johnson; Eli Lilly; receives research support from Eli Lilly, Johnson & Johnson, Genentech, Allon, Elan, Pfizer, NIH/NIA, Alzheimer Association, Alzheimer Drug Discovery Foundation.
Dr. Montine, nothing to disclose.
Dr. Dawson is chair of the Scientific Advisory Board of the Bachmann Strauss Dystonia and Parkinson's disease foundation. He is on the Medical Advisory Board of the Society for Progressive Supranuclear Palsy and is a member of the Program Committee for the 2012 World Parkinson's Congress. He has received funding from NIH/ NINDS/ NIDA/ MSCRF/ AHMMRF /JPB /CPT.
Dr. Martin, nothing to disclose.
Dr. Scott is co-inventor on US Patent Number 8,088,587, Genetic variants increase the risk of age-related macular degeneration, assigned to Duke University and is a member of the Parkinson Study Group Scientific Review Committee.
Dr. Vance is a member of the Scientific Advisory Board of the National Parkinson Foundation. He may accrue revenue on patents submitted by the Duke University wherein he is inventor including discoveries of genes causing Charcot-Marie-Tooth disease and Methods for identifying an individual at increased risk for developing coronary artery disease. He has research support from NIH 1P50NS071674-02 and Hope for Vision. In 2013 Dr. Vance received honoraria from AAN and royalties from Athena Diagnostics for Charcot-Marie-Tooth Disease.

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 Biosytems). 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 Biosytems).

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.

Acknowledgments

Samples from the National Cell Repository for Alzheimer's Disease (NCRAD), which receives government support under a cooperative agreement grant (U24 AG21886) awarded by the National Institute on Aging (NIA), were used in this study. The authors thank contributors, as well as patients and their families, whose help and participation made this work possible.

Funding sources for this study:

This project was supported by: NS039764 and NS071674 (GWB, WKS, ERM, LW, KN, DMD, JMV), P50 NS072187, P50 AG016574 (DWD); P50 NS053488 (JQT, VMYL); NeuroBioBank HHSN271201300028C (DCM), NS38377 and the JPB Foundation (TMD); P01 AG007232 (LSH); AG06781, AG005136 and NS062684 (TJM); P01 AG007232 (LSH); R01NS37167, P30AG10133 and U24AG021886 (TF) and P50 AG005134 and P50 NS038372 (MPF).

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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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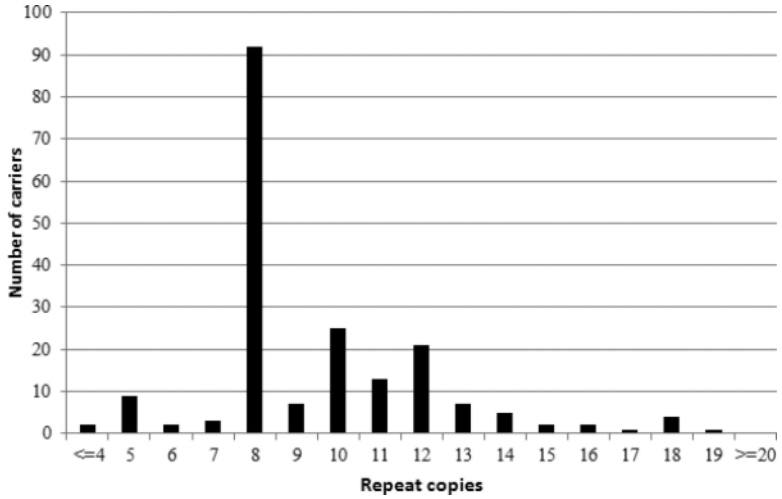

Figure 1. Histogram of autopsy confirmed PD dataset

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