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Analysis of the *C9orf72* repeat in Parkinson's disease, essential tremor and restless legs syndrome

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Abstract

The hexanucleotide expanded repeat (GGGGCC) in intron 1 of the *C9orf72* gene is recognized as the most common genetic form of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). However, as part of the clinical phenotype, some patients present with parkinsonism. The present study investigated the potential expansion or association of the *C9orf72* repeat length with susceptibility to Parkinson's disease and related disorders, essential tremor and restless legs syndrome. One restless legs syndrome patient was shown to harbor a repeat expansion, however on clinical follow-up this patient was observed to have developed frontotemporal dementia. There was no evidence of association of repeat length on disease risk or age-at-onset for any of the three disorders. Therefore the *C9orf72* hexanucleotide repeat expansion appears to be specific to TDP-43 driven amyotrophic lateral sclerosis and dementia.

Keywords

C9orf72; expanded repeat; PD; ET; RLS; genetic association

Introduction

Expansion of short DNA repeat sequences are a well-established pathomechanism for neurodegenerative disease (e.g. spinocerebellar ataxias and Huntington's disease; HD) [1, 2]. The expansions can occur within the coding (Polyglutamine disorders such as HD) or

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Disclosure statement

RR and MD-J have a patent on the discovery of the hexanucleotide repeat expansion in C9ORF72. All other authors report no conflict of interest.

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non-coding regions (e.g. Friedreich's ataxia). Recently we and others identified an expansion of a hexanucleotide repeat (GGGGCC) in intron 1 of the as yet uncharacterized *C9orf72* gene which can result in the presentation of a clinical phenotype of amyotrophic lateral sclerosis (ALS) and/or frontotemporal dementia (FTD) [3, 4].

The *C9orf72* hexanucleotide expanded repeat mutation is now recognized as the most frequent genetic cause of ALS, with estimates of over 20% of familial and 4% of sporadic ALS in the US Caucasian population carrying the repeat expansion based on our sequential Mayo Clinic series [4]. In addition, this mutation is one of the most common causes of familial FTD and is estimated to be responsible for 3% of sporadic disease [4]. Genetic screening for the repeat expansion has widened the clinical phenotype associated with this form of neurodegenerative disease to include a number of clinical Alzheimer's disease (AD) patients, although at autopsy most have been confirmed as a TDP43-proteinopathy [5, 6].

Taken together, these findings suggest that the clinical phenotype for *C9orf72* repeat expansion can be somewhat heterogeneous with elements from both the spectrum of dementia and movement disorder syndromes. Indeed some patients within *C9orf72* repeat expansion present with parkinsonism as part of their symptomatic manifestation and report a family history of Parkinson's disease (PD) [6–9]. In addition, some evidence is emerging that genes involved in ALS risk may also be important genetic factors in parkinsonism [10–13]. To investigate the potential role of the *C9orf72* repeat length in the movement disorders spectrum, we assessed the frequency of expansions in PD, restless legs syndrome (RLS) and essential tremor (ET). Additionally, in those patients without a repeat expansion, we evaluated the association of repeat length with disease risk and age at disease onset.

Subjects and Methods

We employed three independent series of patients with different movement disorders: one series of 676 patients with PD, a second series of 280 patients with RLS and a third series of 106 patients with ET. A total of 1,356 controls were also included in this study, and characteristics of each of these four groups are summarized in Table 1. Patients were examined and observed longitudinally by a movement disorders neurologist and diagnosed according to published criteria. Unrelated control individuals (spouses, caregivers or friends) were free of personal or familial history suggestive of movement disorders. All subjects were collected at the Mayo Clinic Florida and were Caucasian. The Mayo Clinic ethical review board approved the study and informed consent was provided by all participants.

Genetic and Statistical Analysis

DNA was extracted from venous blood or frozen brain tissue using standard methods. Assessment for expansion and allele genotyping of the *C9orf72* repeat was performed employing fluorescent-labeled primer PCR with capillary electrophoresis on an ABI 3730 Genome Analyzer and analyzed with Genemapper software as previously described [4]. Associations of total number of repeats and maximum number of repeats on one allele with disease (PD vs. control, ET vs. control, RLS vs. control) were evaluated using logistic regression models adjusted for age and gender. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated. Associations of total number of repeats (combined sum of both alleles) and maximum number of repeats on one allele with age at onset were examined using linear regression models adjusted for gender, separately in patients with PD, patients with ET, and patients with RLS. Regression coefficients and 95% CIs were estimated, with age at onset considered as the outcome variable. In logistic and linear regression analysis, both total number of repeats and maximum number of repeats on one allele were considered as continuous variables in order to examine linear trends and also as categorical variables to

evaluate potential non-linear trends. As a categorical variable, we divided number of repeats and maximum number of repeats into five categories based on sample quintiles in order to evaluate a general potential nonlinear trend, and also into two categories based on the 95th percentile (i.e. >95th percentile s. 95th percentile) in order to examine the effect of presence of the greatest number of repeats. In order to adjust for multiple testing, we utilized a Bonferroni adjustment for all statistical tests that were performed in relation to the same outcome (disease or age at onset), after which p-values 0.0028 were considered as statistically significant. All statistical analyses were performed using R Statistical Software (version 2.14.0; R Foundation for Statistical Computing, Vienna, Austria).

Results

All samples were screened for allele repeat genotyping and subsequently all homozygous carriers were subjected to a second PCR-based assay for repeat expansion. No repeat expansions were found in the series of patients with PD or ET, or in the large control series. The screening of patients with RLS revealed one carrier with an expansion of the *C9orf72* hexanucleotide repeat. This 61 year old man was seen in the neurology clinic for evaluation of abnormal sensation in his feet of approximate 10 month duration. He characterized his symptoms as feeling like “I am walking on water”. His symptoms were more pronounced at rest and relieved by walking. His neurological examination was essentially normal. EMG study demonstrated only the presence of mild chronic right L5 radiculopathy. He was diagnosed with RLS and was placed on clonazepam. On subsequent clinical follow-up two years later he had developed personality and behavioral changes and was given a diagnosis of frontotemporal dementia. The patient survives 4 years after the onset of his behavioral symptoms and no signs of ALS are present based on verbal communication with the patient’s spouse.

Allelic analysis of the PD, ET, RLS and control series demonstrated a wide range of repeat lengths (2–23 repeat units); the most frequent allele in the overall series of patients and controls contained 2 repeat units (20.9%), followed by 8 (18.8%) and 5 (18.3%) repeat units (Table 2). Two alternate analytical approaches were employed for association testing; 1) using the combined sum of the two alleles as an individual total allele score and 2) using the longest repeat allele as a dominant model. As displayed in Table 3, there was no evidence of an association between number of repeats and risk of PD, ET or RLS after adjustment for multiple testing (P 0.0028 considered significant) when considering number of repeats as a continuous variable, as a five-level categorical variable based on sample quintiles, or as a binary categorical variable based on the 95th percentile. There was a non-significant difference in risk of ET across quintile-based total of number repeat categories (P=0.041), however this finding is of questionable biological significance given that it was driven by a higher risk of ET in individuals with 5–7 and 11–13 repeats but not observed in the 8–10 and >13 repeat groups. There was no significant association between number of repeats and age-at-onset of any of the three movement disorders (all P 0.24, Supplemental Table 1).

Discussion

The identification of a hexanucleotide repeat expansion in the *C9orf72* gene as a frequent cause of both ALS and/or FTD suggests a possible role in other dementias or related movement disorders. This hypothesis is supported by the recent observation of repeat expansions in patients with clinical diagnoses of AD and dementia with Lewy bodies [5, 6]. Herein we demonstrate that the *C9orf72* repeat expansion is not a common mechanism of disease for PD and related movement disorders. Given the relatively small size of the ET (n=106) and RLS (n=280) patient series we cannot definitively exclude the possibility of a role for the *C9orf72* repeat in disease risk, however another recent study has also shown an

absence of expansion repeats or association with the *C9orf72* repeat allele and disease susceptibility in a series of PD patients [14].

Studies to date appear to show that expansion of the *C9orf72* hexanucleotide repeat is specific to TDP-43 pathology-associated dementia and ALS. Our recent article on the role of the normal repeat length allele on susceptibility to FTD/ALS further suggest the length of the non-expanded repeat does influence the clinical disease phenotype [15]. However a number of other questions remain to be answered including resolution of the critical repeat number threshold for disease presentation, the diversity in clinical phenotype of FTD or ALS and the pathogenic mechanism associated with *C9orf72* expansion-related disease, whether toxicity is due to gain of function supported by the identification of nuclear RNA foci or a loss of function with the observed loss of expression of a specific *C9orf72* isoform [4].

The present study's findings of a lack of association of normal allele repeat length with disease risk or an effect on age-at-onset would support the conclusion that variation in the *C9orf72* gene does not play a major role in the susceptibility to the wider spectrum of movement disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Patient characteristics

Variable	PD patients (N=676)	ET patients (N=106)	RLS patients (N=280)	Controls (N=1,356)
Age	76 ± 11 (33 – 101)	76 ± 11 (45 – 96)	70 ± 14 (24 – 108)	73 ± 12 (22 – 100)
Gender				
Male	424 (62.7%)	50 (47.2%)	123 (43.9%)	568 (41.9%)
Female	252 (37.3%)	56 (52.8%)	157 (56.1%)	788 (58.1%)
Age at onset	69 ± 11 (28 – 94)	67 ± 14 (20 – 89)	64 ± 15 (20 – 104)	

- The sample mean ± SD (minimum – maximum) is given for age and age at onset. Information was unavailable regarding age at onset for 99 PD patients, 1 ET patient, and 4 RLS patients.

Table 2

Total number of repeats and maximum number of repeats on one allele in PD patients, ET patients, RLS patients, and controls

Variable	PD patients (N=676)	ET patients (N=106)	RLS patients (N=280)	Controls (N=1,356)
Total number of repeats				
Continuous *	7 (2, 4, 11, 32)	8 (4, 6, 12, 24)	8 (4, 4, 12, 28)	8 (2, 4, 11, 40)
Categorical **				
4	214 (31.7%)	25 (23.6%)	83 (29.6%)	425 (31.3%)
5-7	125 (18.5%)	28 (26.4%)	57 (20.4%)	249 (18.4%)
8-10	153 (22.6%)	20 (18.9%)	64 (22.3%)	311 (22.9%)
11-13	85 (12.6%)	21 (19.8%)	30 (10.7%)	168 (12.4%)
>13	99 (14.6%)	12 (11.3%)	46 (16.4%)	203 (15.0%)
>18	34 (5.0%)	5 (4.7%)	11 (3.9%)	64 (4.7%)
Maximum number of repeats on one allele				
Continuous *	5 (2, 2, 8, 23)	5 (2, 4, 8, 21)	5 (2, 2, 8, 18)	5 (1, 2, 8, 23)
Categorical **				
2	214 (31.7%)	25 (23.6%)	83 (29.6%)	425 (31.3%)
3-5	140 (20.7%)	29 (27.4%)	66 (23.6%)	282 (20.8%)
6-7	78 (11.5%)	15 (14.2%)	25 (8.9%)	153 (11.3%)
8	115 (17.0%)	18 (17.0%)	60 (21.4%)	262 (19.3%)
>8	129 (19.1%)	19 (17.9%)	46 (16.4%)	234 (17.2%)
>13	39 (5.8%)	4 (3.8%)	9 (3.2%)	55 (4.1%)

* The sample median (minimum, 25th percentile, 75th percentile, maximum) is given.

** Total number of repeats and maximum number of repeats on one allele were categorized using the sample quintiles and also using the sample 95th percentile.

Table 3
Association of number of repeats with disease: comparisons of PD patients, ET patients and RLS patients with controls

Variable	Association of number of repeats with disease					
	PD patients vs. controls		ET patients vs. controls		RLS patients vs. controls	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Total number of repeats						
Continuous variable *	1.01 (0.92, 1.11)	0.83	1.03 (0.84, 1.26)	0.80	1.01 (0.89, 1.16)	0.84
Categorical variable (quintiles) **		0.99		0.041		0.77
4	1.00 (reference)		1.00 (reference)		1.00 (reference)	
5-7	1.00 (0.76, 1.32)		1.87 (1.06, 3.29)		1.21 (0.83, 1.75)	
8-10	0.97 (0.75, 1.26)		1.06 (0.58, 1.94)		1.10 (0.77, 1.58)	
11-13	1.02 (0.74, 1.40)		2.06 (1.12, 3.79)		0.93 (0.59, 1.47)	
>13	0.98 (0.73, 1.32)		0.99 (0.50, 2.02)		1.18 (0.79, 1.75)	
Categorical variable (95 th percentile) **						
>18 (vs. 18)	1.03 (0.66, 1.60)	0.90	1.00 (0.39, 2.54)	0.99	0.83 (0.43, 1.60)	0.58
Maximum number of repeats on one allele						
Continuous variable *	1.02 (0.95, 1.10)	0.63	1.06 (0.91, 1.23)	0.47	1.00 (0.90, 1.11)	0.96
Categorical variable (quintiles) **		0.76		0.32		0.53
2	1.00 (reference)		1.00 (reference)		1.00 (reference)	
3-5	1.00 (0.76, 1.30)		1.72 (0.98, 3.00)		1.24 (0.86, 1.77)	
6-7	0.99 (0.72, 1.38)		1.64 (0.83, 3.19)		0.86 (0.53, 1.40)	
8	0.88 (0.67, 1.17)		1.12 (0.60, 2.11)		1.21 (0.84, 1.74)	
>8	1.10 (0.83, 1.45)		1.35 (0.73, 2.51)		1.03 (0.70, 1.53)	
Categorical variable (95 th percentile) **						
>13 (vs. 13)	1.42 (0.92, 2.20)	0.11	0.92 (0.33, 2.59)	0.87	0.79 (0.39, 1.63)	0.53

ORs, 95% CIs, and p-values result from logistic regression models adjusted for age and gender. P-values 0.0028 are considered as statistically significant after a Bonferroni adjustment for multiple testing (18 tests).

* ORs correspond to a 5 unit increase for total number of repeats and to a 3 unit increase for maximum number of repeats on one allele.

** Total number of repeats and maximum number of repeats on one allele were categorized using the sample quintiles and also using the sample 95th percentile. OR=odds ratio. CI=confidence interval.