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C9orf72 repeat expansions in South Africans with amyotrophic lateral sclerosis

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ABSTRACT

The hexanucleotide repeat expansion in the *C9orf72* gene is the most common genetic variant found in individuals with sporadic amyotrophic lateral sclerosis (ALS), occurring at a frequency of between 7 and 11% in cohorts of European ancestry. While limited data suggest that *C9*-expansions (> 30 repeats) are less frequent in African-Americans with ALS, there is no data on the frequency of *C9*-expansions among ALS subjects residing in Africa. We therefore investigated the frequency of this expansion mutation (using repeat-primed PCR) in a cohort of 143 South Africans (SA) with ALS. The cohort included different genetic ancestry subgroups who self-identified as black African (n = 24), Cape mixed-African (M/A) (n = 65), white European ancestry (n = 51), and Indian ancestry (n = 3). Three M/A individuals had a family history of ALS (2%) and all had normal *C9orf72* alleles. Of the 140 individuals with sporadic ALS who were successfully genotyped, 10 (7%) carried pathogenic *C9*-expansions; four white and six M/A ancestry individuals, respectively. Our results highlight the importance of including Africans in genetic studies aimed at unravelling the genomic architecture in ALS and suggest pathogenetic mechanisms other than the *C9orf72* expansion in black Africans with ALS.

1. Introduction

The hexanucleotide repeat expansion in the *C9orf72* gene is the most common genetic variant identified in individuals with familial amyotrophic lateral sclerosis (ALS) and fronto-temporal dementia (FTD) [1,2]. The normal (wild-type) repeat region comprises < 10 repeats and > 30 repeats are considered pathogenic [3]. Although the function of the C9orf72 protein remains largely unknown, and it is not clear whether the pathogenic consequence of the *C9orf72* expansion is related to loss or gain of function, it does appear to be relevant to the underlying pathogenesis of ALS in a substantial proportion of familial and sporadic cases [3]. A prominent pathogenic mechanism in *C9orf72* associated ALS is proposed to be related to nuclear inclusions formed by the expanded repeats encoding dipeptide repeat proteins [4].

The frequency of pathogenic *C9orf72* repeat expansions, hereafter referred to as *C9*-expansions, among sporadic ALS cases is much lower compared to those with a family history of ALS. In addition, the frequencies of *C9*-expansions differ substantially among different European populations ranging between 4% in Italy and 21% in Finland, although most frequently ranges between 7 and 10% among sporadic ALS cases with European genetic ancestry (in [3,5,6]). There is little data regarding *C9*-expansions among African-Americans with ALS and no published data from Africa [3]. As the *C9orf72* expansion mutation is the most common variant found in ALS cohorts we proceeded to assess the frequency of this expansion mutation in a cohort of South Africans with ALS.

Abbreviations: ALS, Amyotrophic lateral sclerosis; FTD, Fronto-temporal dementia; SA, South Africa(n); PLS, Primary lateral sclerosis; PMA, Progressive muscular atrophy; FA, Flail arm; M/A, Mixed-African; RP-PCR, Repeat primed polymerase chain reaction; ANOVA, Analysis of variance; US, United States; HD, Huntington's disease

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2. Methods

2.1. Study subjects

One hundred and forty-three South African (SA) patients attending ALS clinics at two academic centres in the Cape Town region, Groote Schuur Hospital and Tygerberg Hospital, underwent genotyping for the *C9orf72* repeat expansion. Subjects were diagnosed by a neurologist and categorized as either classic ALS or one of the phenotypic subtypes: primary lateral sclerosis (PLS), progressive muscular atrophy (PMA) or flail arm (FA) ALS [7]. Participants categorized themselves according to the following SA racial census categories (http://www.statssa.gov.za): indigenous black African (17%), Cape mixed-African ancestry (M/A) (44%), white European genetic ancestry (36%), or SA Indian ancestry (2%). Cape M/A is a term used in South Africa for persons of predominantly Khoisan (> 60% genetic contribution) and black African genetic ancestry as well as smaller genetic contributions from Europeans more than Southeast Asians [8]. White refers to individuals of European genetic ancestry. The black Africans are largely Xhosa-speaking migrants from the Eastern Cape region and 'Indian' refers to those with recent ancestors immigrating from the subcontinent. Family history was obtained after a structured interview according to a standardized data capture form. Non-ALS South African controls (SA-controls) of black African and Cape mixed-African ancestry included 25 individuals with myasthenia gravis (MG) and 20 healthy individuals [9,10].

Ethics approval for this study was obtained from the University of Cape Town's Health Sciences Faculty research ethics committee (HREC 351/2016).

2.2. DNA extraction and determination of C9orf72 repeat length

Genomic DNA from ALS and SA-control subjects was extracted from buffy coats of nucleated cells obtained from anticoagulated whole blood. Three DNA samples with pathogenic *C9orf72* expansions (ND11252*A1, ND11081*A1 and ND10284*A1) were donated by the Coriell Institute of Medical Research and included as positive controls.

2.2.1. Repeat primed PCR (RP-PCR)

For ALS subjects, a two-step protocol was designed for characterising the *C9orf72* G_4C_2 hexanucleotide repeat in the first intron. Step one involved amplification of the expanded alleles by RP-PCR using primer sequences and reaction conditions described by Renton et al. [1] which allows for the detection of up to about 30 repeats. The second step allowed for the amplification of unexpanded alleles only (up to 12 repeats) and the determination of zygosity by combining the fluorescently labelled sequence specific primer from the RP-PCR and a sequence specific primer designed with Primer3Plus [11] (5'-CACAGTA CTCGCTGAGGGTG-3'). This second reaction was optimized to include 50 ng genomic DNA, 1× Failsafe Premix J buffer (Lucigen), 1.25 U GoTaq G2 flexi DNA polymerase (Promega Corporation) and 10 pmol of each primer in a 15 μL reaction volume. Cycling conditions involved an initial denaturation at 95 °C for 2 min followed by 35 cycles consisting of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min. Amplification was concluded with a final extension at 72 °C for 10 min.

Amplified products were analysed on a 3130*xl* Genetic Analyzer with a GeneScan™500 internal size standard using standard electrophoresis conditions for fragment analysis on a 36 cm capillary array with POP7 polymer (Thermo Fisher Scientific). Electropherograms were visualised with GeneMapper v4 software and the fragment sizes recorded. The smaller allele sizes were confirmed for the sequence specific amplified products, using the BigDye™ terminator v3.1 cycle sequencing kit according the manufacturer's instructions and capillary electrophoresis on a 3130*xl* Genetic Analyzer (Thermo Fisher Scientific).

The repeat sizes were calculated by creating a bin set for the sequence specific PCR with the smallest peak corresponding to two repeats. Additional bins were created in 6 bp increments. The repeat primed primer binds exactly three repeats in addition to a partial repeat (C_4G), however, it will also bind and amplify two repeats and it is therefore not possible to distinguish two and three repeats with this primer set [12]. Sizes were verified by applying a formula to the fragment sizes obtained from the chromatograms which takes primer and flanking sequences into account and incorporates a correction factor to allow for the mobility shifts observed with capillary electrophoresis.

In samples with low DNA concentrations and indeterminate results with the above mentioned RP-PCR protocol, we used the AmplideX™ PCR/CE *C9orf72* kit (Asuragen, Austin, USA) according to the manufacturers' instructions to determine the expanded repeat sizes (analytic range 2–145 repeats).

2.2.2. Whole genome sequencing (WGS)

We were able to examine the WGS data of a sample of South African ALS (n = 25) and control subjects (n = 45) [9,10], for *C9orf72* repeat expansions and the surrogate 'risk' marker, rs3849942 T allele, which is associated with the C9-expansion haplotype [12,13]. The *C9orf72*-allele length was determined from PCR-free 30× coverage WGS data using ExpansionHunter [14] which validated the RP-PCR results from 25 ALS subjects.

2.3. Statistical analyses

Allele frequencies were calculated in Microsoft® Excel for Mac version 16.18. Group comparisons of continuous variables were compared with Student's t-test (2 groups) or one-way ANOVA (> 2 groups). A Wilcoxon matched-pairs signed rank test was performed to compare allele frequencies between the two control groups (MG and healthy controls) and a Fischer exact test was used to compare *C9*-risk allele frequencies between black vs M/A groups. A 2-sided p value < .05 was considered significant. Graphs were generated in Prism 7 for Mac OS X version 7.0c.

3. Results

C9orf72 repeat length was successfully determined in 143 samples. The cohort contained three subjects with familial ALS (2%) (2 families with either PLS ± progressive aphasia (one family), and PMA at presentation (two individuals from the same family).

The clinical characteristics of sporadic ALS cases are summarized according to *C9orf72* repeat length in Table 1. Men were more frequent in the sample (64%). The median age at onset was 55 years (interquartile range (IQR) 47–66) and was younger in men compared to women (54 vs 59 years, p = .023) and in those with African genetic ancestry; 50 years in black African-, 54 years in Cape M/A-, and 61 years in white patients ($p < 1 \times 10^{-4}$). Among those with African genetic ancestry (black + M/A; n = 89) the disease phenotype at presentation was largely ALS (n = 74; 83%) and the most frequent clinical anatomical region involved first by history was lumbosacral (n = 42; 47%) followed by cervical involvement (n = 37; 42%) and bulbar onset occurred in 12% (n = 10).

In the overall cohort who were genotyped (n = 143), 93% of the cases had *C9orf72* alleles within the normal range (< 30 repeats) and the distribution of the normal allele sizes was not significantly different between the ancestry groups (Fig. 1, one-way ANOVA test p = .51). Of the sporadic ALS cases (n = 140), 10 (four white/European genetic ancestry and six Cape M/A patients), carried pathogenic *C9*-expansions of > 30 repeats (7%) and none of these were black ALS cases. There was no difference in the ages at symptoms onset between those with normal repeat allele sizes (< 20) and those with C9-expansions (> 30) (p = .87). All three ALS cases with familiy members with ALS (Cape M/A genetic ancestry) had normal *C9orf72* alleles (≤ 7 repeats). Therefore, among those who self identified as Cape mixed-African ancestry, the

Table 1
Clinical characteristics of South African ALS patients according to *C9orf72* allele repeat size.

Clinical characteristics	< 20 repeats	> 30 repeats
	n (%)	n (%)
Disease phenotype		
ALS	123 (86)	8 (7)
PMA	9 (6)	1 (11)
PLS	7 (5)	1 (14)
FA	4 (3)	0
Clinical presentation		
Lumbosacral	65 (45)	7 (11)
Cervical	56 (39)	2 (4)
Bulbar	18 (13)	1 (6)
Respiratory	3 (2)	0
Behavioral	1 (1)	0
Genetic ancestry		
Black African	24 (17)	0
Cape mixed-African	65 (45)	6 (9)
White	51 (36)	4 (8)
Indian	3 (2)	0

Legend: n refers to the number of individuals with one allele in a particular size range.

frequency with *C9*-expansions of > 30 repeats was 9%. Three of the Cape M/A subjects with pathogenic C9-expansions underwent WGS, and we confirmed the *rs3849942 T/T* genotype which associated with the *C9orf72* risk haplotype.

All 45 SA-control subjects had unexpanded *C9orf72* alleles. In this group, the distribution of normal allele sizes was not significantly different between MG and healthy controls (Supplementary Fig. 1; $p = .98$). Furthermore, among 142 chromosomes without C9-expansions the *rs3849942 T* allele frequencies were similar among the M/A and black African genomes (T allele frequency 16% M/A group vs 18% black group; $p > .99$).

4. Discussion

This clinic-based series of South Africans (SA) with ALS showed a similar frequency of *C9orf72* expanded alleles overall compared to most European cohorts. However, although those with Cape mixed-African genetics had a similar frequency to some European cohorts (6/65; 9%) none of the 24 black Africans carried an expanded *C9*-allele. This is interesting as the Cape mixed-African subpopulation derives most of their genetic ancestry from Khoisan and black Africans. Few data are available in black populations with ALS. Two cohorts from North America (US) included 71 and 49 black people, respectively, of whom four (3%) had an expanded *C9*-allele [6,15]. Although not directly comparable, but to provide some context, previous studies [in 8] and a pharmacogenetic study by our group in [16] suggested similar European admixture proportions (≈25%) among the Cape mixed-African genetic ancestry group and African-Americans. Combining the US data with ours show that 10/206 (5%) individuals with sporadic ALS and African genetic ancestry have been found to have the expanded *C9*-alelle which may be attributed to European genetic admixture.

We found a similar frequency of *C9*-expansions among the white cases with European genetic ancestry (8%) compared to a recent large European cohort (10%) [5], but this frequency varies considerably among Europeans (4% to 21%) [3]. *C9*-expansion frequencies of ≈5% have been reported in ALS cases from India and East-Asia [3] and rarely in ALS cases from mainland China (0.3%) [17]. Although these frequencies reflect the prevalence of the expanded *C9*-allele among individuals without a family history of ALS (sALS), European populations also appear to have a higher frequency of familial ALS in their samples (10–13%) [6,18,19] compared to Africans (this report; 2%), Indians and Chinese (1–3%) [17,20]. This observation may reflect the lower frequency of expanded *C9orf72* expansion mutations, but it may also be the result of non-biological or socio-economic effects resulting in lower life expectancy and poorer access to specialist diagnostic health care. However, it is noteworthy that both this African cohort (Cape M/A and black), and a report from China [17] showed lower frequencies of bulbar onset ALS compared to cohorts with European genetic ancestors (13–14% vs 25–30%) [6,15]. Although some European ancestry cohorts showed associations with *C9*-expansions and bulbar onset ALS and earlier age at onset [21], others [12] and this study did not replicate this finding.

There are rare cases in which *C9*-expansion alleles have been found in European controls (16/10,992, 0.1%) [6,12,18,21]. In addition, low frequencies of *C9*-expansion alleles were found in Europeans with other neurodegenerative diseases including Huntington's disease-like syndromes (7/421; 1.6%) [3]. A study confined to black South Africans ($n = 97$), in which *C9orf72* alleles were sized in a lab-based sample of possible "Huntington disease" (HD) phenocopies (both *HD1* and *HD2* mutations were excluded), found all the *C9orf72* repeat alleles in that cohort within the 2–11 repeat range [22]. Here we add an 45 additional SA-controls with African genetic ancestry, who underwent WGS, and found no *C9orf72* expansion mutations.

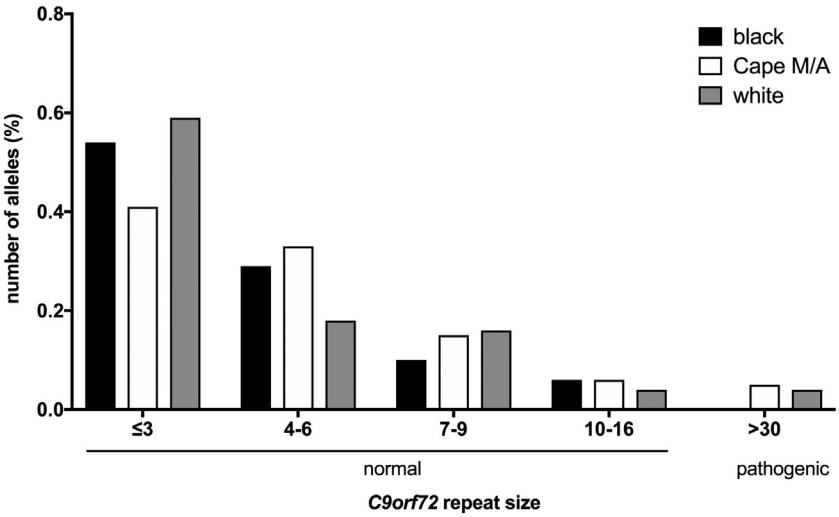

Fig. 1. Distribution of *C9orf72* alleles in each South African subpopulation according to repeat size. Normal refers to ≤30 repeats and pathogenic refers to > 30 repeats. Three individuals of SA 'Indian' ancestry are not shown (all had normal alleles).

Majounie et al. studied the haplotype of the region adjoining the expanded *C9orf72* locus and found evidence to suggest a common ancestral founder effect in which Europeans with the *C9*-expanded allele shared at least part of the haplotype [6]. However, using more polymorphic microsatellite markers within a 300 kb region flanking the *C9orf72* locus, Beck et al. reported that the *C9*-expanded alleles were found on several unrelated haplotypes suggesting mutiple mutational events and arguing against a shared common ancestor [12]. In this report the three individuals with mixed African genetic ancestry and the *C9* mutation were also homozygous for the C9-associated *rs3849942* Tallele despite a similar frequency of the T allele among the South African sub-populations.

A potential limitation of our study is that we have not confirmed our RP-PCR results with Southern blotting. However, positive controls with known expanded C9-alleles were included in each experiment and the accuracy of the assay was confirmed by independent sequencing of 3 samples. It is worth highlighting that we found false 'intermediate alleles' when RP-PCR was performed on samples with low concentrations of DNA (results not shown), but these were resolved using a commercial kit which was optimized for low quantities of DNA.

5. Conclusion

This ALS cohort comprising 89 individuals with African genetic ancestry showed no *C9* expansion mutations among black South Africans with ALS, but the Cape M/A group, who have European admixture, showed similar frequencies to European populations. We highlight the importance of including populations from Africa and Asia in studies aimed at unravelling the genomic architecture in ALS.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jns.2019.04.026.

Declarations of interest

None.

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