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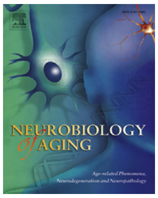

Screening a UK amyotrophic lateral sclerosis cohort provides evidence of multiple origins of the *C9orf72* expansion[☆]

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ABSTRACT

An expanded hexanucleotide repeat in the *C9orf72* gene is the most common genetic cause of amyotrophic lateral sclerosis and frontotemporal dementia (C9ALS/FTD). Although 0–30 hexanucleotide repeats are present in the general population, expansions >500 repeats are associated with C9ALS/FTD. Large C9ALS/FTD expansions share a common haplotype and whether these expansions derive from a single founder or occur more frequently on a predisposing haplotype is yet to be determined and is relevant to disease pathomechanisms. Furthermore, although cases carrying 50–200 repeats have been described, their role and the pathogenic threshold of the expansions remain to be identified and carry importance for diagnostics and genetic counseling. We present clinical and genetic data from a UK ALS cohort and report the detailed molecular study of an atypical somatically unstable expansion of 90 repeats. Our results across different tissues provide evidence for the pathogenicity of this repeat number by showing they can somatically expand in the central nervous system to the well characterized pathogenic range. Our results support the occurrence of multiple expansion events for C9ALS/FTD.

1. Introduction

Expansions of a GGGGCC hexanucleotide repeat in the first intron and/or promoter region of *C9orf72* are the most frequent known monogenic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) in populations of European descent (DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Majounie et al., 2012; Renton et al., 2011). Repeats ranging in size from 0 to 30 are found in the general population, whereas pathogenic expansions range between 500 and 4500 repeats, with considerable somatic instability (Beck et al., 2013; DeJesus-Hernandez et al., 2011;

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van Blitterswijk et al., 2013b). The finding of a conserved haplotype spanning approximately 200 kb around pathogenic expansions may result from either a common founder, or a “risk” haplotype predisposing to expansion (Beck et al., 2013; Majounie et al., 2012; Mok et al., 2012; Pliner et al., 2014). How the *C9orf72* expansion leads to disease is as yet unknown, proposed mechanisms include the following: (1) loss of *C9orf72* function; (2) RNA toxicity associated with the presence of nuclear repeat expansion RNA foci; and (3) protein toxicity caused by dipeptide repeats originating from the translation of the repeat expansion (Ling et al., 2013).

We present genetic and clinical data from *C9orf72* screening in a UK ALS cohort. We confirm the clinical features previously associated with *C9orf72* expansions and report an atypical expansion with approximately 90 repeats in blood which shows somatic instability within and between tissues. Our analysis of multiple tissues shows the repeat significantly expanded in the central nervous system (CNS) to over 3000 hexanucleotides.

Our data, together with recently reported cases, strongly support the hypothesis that *C9orf72* repeat expansions occur in different individuals in the context of the permissive risk haplotype (Dols-Icardo et al., 2014; Van Blitterswijk et al., 2013b). This finding sheds light on the origins of the *C9orf72* repeat expansion and has important implications for pathomechanisms of *C9orf72* ALS/FTD, diagnostic testing, and genetic counseling for *C9orf72* expansions.

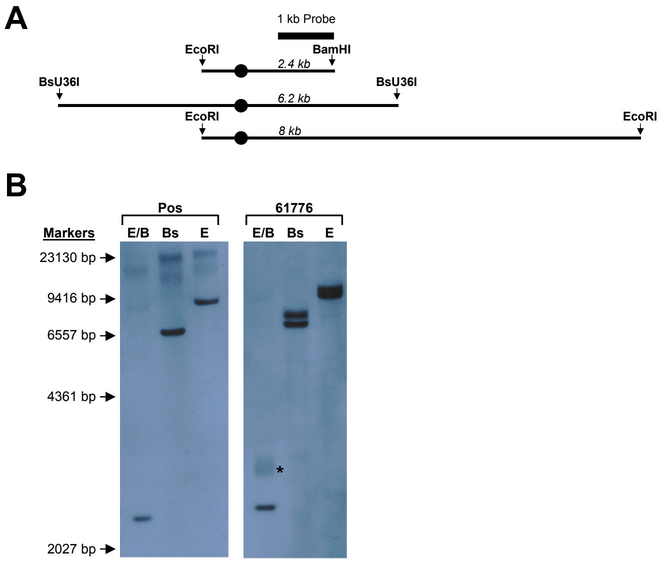

Fig. 1. Case 61776 shows a somatically unstable repeat. (A) Map of the *C9orf72* locus illustrating the probe and the restriction sites used for single-copy 1-kb probe Southern hybridization. The site of the hexanucleotide expansion is indicated with a circle. (B) Southern hybridization performed on a typical bimodal *C9orf72* repeat expansion (Pos) and case 61776 with the following restriction digestions: EcoRI/BamHI (E/B), Bsu36I (Bs), and EcoRI (E). The somatic mosaicism in 61776 is evident in the E/B digest lane (marked with *).

2. Methods

We screened a cohort of 452 ALS patients who had not been screened for other genetic causes of ALS, from University College London Partners Motor Neuron Disease clinics (London, UK). Patients gave written consent, and the project was approved by the local ethical review committee. DNA was extracted from blood and flash-frozen tissues using standard techniques.

Fibroblasts were generated from a 3-mm skin punch biopsy taken under local anesthetic following informed consent. Biopsies were dissected into 1 mm pieces and cultured at 37 °C, 5% CO$_2$ in DMEM, 10% FBS, 1% L-Glutamine, 50U/mL penicillin, and 50 μg/mL streptomycin until fibroblasts were seen to grow out from the explants. Media changes were performed every 3 days. When fibroblasts reached confluency, they were detached from culture dishes using TrypleE (Invitrogen) and transferred to larger culture vessels for further expansion. Cells (1×10^7) were used for genomic DNA extraction using the standard techniques.

Repeat-primed polymerase chain reaction expansion screening and single-nucleotide polymorphism (SNP) genotyping was performed on blood-derived DNA as previously described (Beck et al., 2013; Majounie et al., 2012). Genetic screening data for 350 patients were previously reported (Beck et al., 2013). Two Southern hybridization methods for expansion sizing were performed using: (1) an oligonucleotide probe consisting of 5 GGGGCC repeats (Beck et al., 2013); or (2) a single-copy 1 kb probe (Fratta et al., 2013) that anneals adjacent to the repeat (see Supplementary Table 1 for PCR-primer sequences used to derive the single-copy probe). Three different restriction enzyme digestions were used in the single-copy probe method to produce “normal” bands of different sizes: EcoRI/BamHI double digest: 2.4 kb; BsU36I: 6.2 kb; EcoRI: 8 kb (Fig. 1A); the 6.2 kb and 8 kb bands compress the large somatic smears in expansion-positives for unambiguous detection, whereas the 2.4-kb band allows accurate sizing.

Case 61776 was donated to the NeuroResource tissue bank, UCL Institute of Neurology. Routine neuropathologic assessment was carried out in the Queen Square Brain Bank for Neurological Disorders (Lashley et al., 2011). Formalin-fixed and paraffin-embedded tissue sections (7 microns thick) were immunostained as previously described using commercially available antibodies to the following proteins: TDP-43 (Abnova, Taipei City, Taiwan; 1:800), p62 (BD Transduction Laboratories, Oxford, UK; 1:200), ubiquitin (Dako, Ely, UK; 1:200), α-synuclein (Vector, Peterborough, UK; 1:50), Aβ (Dako; 1:100), tau (AT8 clone; Autogen Bioclear, Wiltshire, UK; 1:600), CD68 (Dako; 1:150), and GFAP (Dako; 1:1000) as previously described (Lashley et al., 2011). Sections were also immunostained with novel antibodies able to detect each of the proteins generated by non-ATG dependent translation of the noncoding repeat expansion. Antibodies, made in rabbits by Biogenes (Germany), were generated against (Gly-Ala)$_7$, (Gly-Pro)$_7$, (Gly-Arg)$_7$, (Pro-Arg)$_7$, or (Pro-Ala)$_7$ peptides.

Fluorescence in situ hybridization was performed with 2′-O-methyl RNA probes (Integrated DNA Technologies): (GGCCCC)4 for sense and (GGGGCC)4 for antisense RNA foci, 5′ labeled with Cy3 or Alexa488, respectively. In parallel with staining with NeuN (ABN78, Millipore; 1:250) to identify neurons was performed as previously described (Mizielinska et al., 2013).

3. Results

3.1. Clinical features and C9orf72 screening of a UK ALS cohort

We screened *C9orf72* hexanucleotide repeats in 452 ALS patient blood-derived DNA samples using repeat-primed polymerase chain

Table 1
Summary of clinical features of the general cohort and *C9orf72* expansion positive individuals

	C9orf72 expansion negative patients[a] (n = 422)	*C9orf72* expansion positive patients[b] (n = 30)
Male:female	1.55	2.00
Age at onset ± SD (y)	59.3 ± 12.5	54.6 ± 8.1
Bulbar onset (%)	27.3	26.1
Positive family history (%)	3.8	30.0

Key: SD, standard deviation.

[a] Site of onset and age of onset missing for 81 patients and gender missing for 62 patients.

[b] Site of onset and gender missing for 7 patients and gender missing for 3 patients.