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Somatic expansion of the *C9orf72* hexanucleotide repeat does not occur in ALS spinal cord tissues

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Abstract

Objective
To test for somatic *C9orf72* hexanucleotide repeat expansion (HRE) and hexanucleotide repeat length instability in the spinal cord of amyotrophic lateral sclerosis (ALS) cases.

Methods
Whole and partial spinal cords of 19 ALS cases were dissected into transversal sections (5 mm thick). The presence of *C9orf72* HRE was tested in each independent section using Repeat-Primed PCR and amplicon-size genotyping. Index measures for the testing of mosaicism were obtained through serial dilutions of genomic DNA from an individual carrying a germline *C9orf72* HRE in the genomic DNA of an individual without a *C9orf72* HRE.

Results
None of the sections examined supported the presence of a subpopulation of cells with a *C9orf72* HRE. Moreover, the *C9orf72* hexanucleotide repeat lengths measured were identical across all the spinal cord sections of each individual patient.

Conclusions
We did not observe somatic instability of the *C9orf72* HRE in disease relevant tissues of ALS cases.

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Glossary

ALS = amyotrophic lateral sclerosis; **HRE** = hexanucleotide repeat expansion; **RPPCR** = repeat-primed PCR.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by rapid and progressive loss of motor neurons.[1] Although germline mutations in several genes have been identified, the *C9orf72* hexanucleotide repeat expansion (HRE) is currently one of the most prevalent and penetrant cause of ALS.[1] In the general population, *C9orf72* contains less than 30 GGGGCC repeats in the first intron, whereas in ALS cases the number of repeats ranges between hundreds to thousands.[1] Because it is difficult to precisely size the repeat length above 30,[2] many aspects of *C9orf72*-related ALS have not been thoroughly investigated.

Somatic mutations have been hypothesized as a possible cause of ALS in cases who do not have germline mutations in genes known to be associated with the disease.[3] Repeat sequences are particularly of interest for somatic mutation analysis because their emergent secondary structures can lead to expansion or contraction of repeat lengths.[4] It is also notable that the *C9orf72* HRE can lead to cell-to-cell transmission of dipeptide repeat proteins,[5] and as such, it is conceivable that a small population of *C9orf72* HRE cells nested in the nervous system could potentiate ALS.

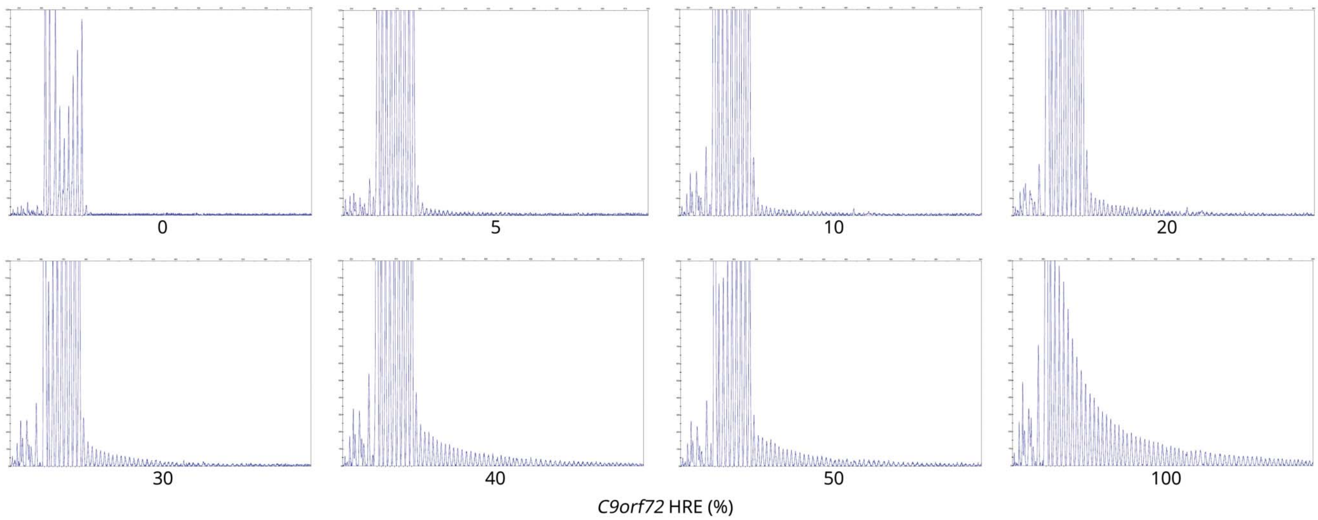
Recently, somatic recombination of *APP* has been demonstrated to occur in Alzheimer's disease neurons.[6] Because somatic expansion of *C9orf72* hexanucleotide repeats is a potential mechanism for ALS pathogenesis and because routine blood DNA testing would not identify such somatic events,[2] we tested DNA extracted from finely sectioned spinal cords of 19 patients with ALS for low levels of the *C9orf72* HRE.

Table Description of the ALS patient cohort

Individual	No. sections	Age	Sex	Site of onset	Germline mutations
ALS01	108	50	M	Left hand	None
ALS02	75	69	F	Right hand	None
ALS03	96	62	F	Bulbar	None
ALS04	70	79	M	Right leg	None
ALS05	118	78	M	Bulbar	*NEK1* p.P318L
ALS06	78	58	F	Right hand	None
ALS07	78			Left foot	None
ALS08	88			Left hand	None
ALS09	82			Right foot	None
ALS10	22	66	M		None
ALS11	28	62	M		None
ALS12	30	61	M		*TBK1* p.L306I, *CCNF* p.E396D, *SPG11* p.R1992Q
ALS13	17	57	M		None
ALS14	38	66	F		None
ALS15	11	78	F	Bulbar	None
ALS16	21	62	M		None
ALS17	31	71	M		None
ALS18	22	69	F		None
ALS19	31	67	F		*SPAST* p.R221C

Abbreviation: ALS = amyotrophic lateral sclerosis.
Site of onset refers to the initial location of ALS symptoms, Sections refer to the number of ~5 mm spinal cord samples generated from each spinal cord. A total of 1,053 unique sections were tested.

Figure 1 *C9orf72* HRE mosaicism RPPCR profiles



Genomic DNA from an individual with germline *C9orf72* HRE was diluted in genomic DNA from an individual without germline *C9orf72* HRE at various percentages. HRE = hexanucleotide repeat expansion; RPPCR = repeat-primed PCR.

Methods

Samples

The spinal cords from 19 ALS cases were included in this study. DNA obtained from prior blood samplings of these cases established them all to be negative for the *C9orf72* HRE. Samples were collected from 3 institutions: the Montreal Neurological Institute and Hospital in Montréal, Québec; the Sunnybrook Health Sciences Centre in Toronto, Ontario; and the ALS Clinic at the London Health Science Centre in London, Ontario. Average patient age at donation was 65.9 years, with a male-to-female ratio of 1.29. A targeted sequencing approach[7] was used to test for rare (minor allele frequency < 0.001) protein-altering germline mutations in genes known to be ALS risk factors. Information regarding the ALS cases is listed in table.

Standard protocol approvals, registrations, and patient consents

All participants signed an informed consent form that was approved by the ethical review boards of institutions that contributed the material.

Tissue sectioning and DNA extraction

Spinal cords were manually portioned into transverse sections of approximately 5 mm thickness. Sections were then separated along the coronal plane into dorsal and ventral halves, with only the ventral areas being used in the present study. Each ventral portion was separated into left and right ventral horns. Genomic DNA was extracted using standard salting-out methods from approximately half of both the left and right ventral portions of every section available from each spinal cord.

C9orf72 HRE reactions

C9orf72 HRE genotyping[8] was performed on blood DNA samples (or sampling of the cervical area of the cerebellum if blood was not available) to accurately size germline hexanucleotide repeat alleles. Repeat-primed PCR (RPPCR)[9] was performed on all sampled sections of each patient to assess for the *C9orf72* HRE and to estimate the lengths of *C9orf72* alleles in each section. GeneMapper v4.0 (Applied Biosystems) was used to visualize and estimate reaction fragment sizes. Lengths of *C9orf72* hexanucleotide repeat amplicons were measured using GeneMapper compared to the GeneScan-500 LIZ Size Standard (Applied Biosystems). Peaks from the RPPCR profiles were chosen based on the genotyping method results to represent *C9orf72* alleles, which were plotted to assess variation within normal-length *C9orf72* hexanucleotide repeat lengths.

HRE mosaicism index measures

Genomic DNA from a patient previously established as a *C9orf72* HRE carrier was diluted in genomic DNA from an ALS patient without the HRE to generate a percentage of HRE within a sample (0%, 5%, 10%, 20%, 30%, 40%, 50%, and 100%). These dilutions were index measures for the testing of *C9orf72* HRE mosaicism within a section; their RPPCR profiles enabled us to assess the sensitivity of the method for each HRE dilution. RPPCR fragment length profiles were visually compared between every spinal cord section and the mosaicism index measures.

Data availability statement

The authors confirm that the data necessary for confirming the conclusions of this study are available within the article and its supplementary material. Raw data is available upon request.

Results

Mosaicism detection

Varying proportions of the *C9orf72* HRE diluted in wild-type DNA displayed unique profiles on RPPCR fragment sizing (figure 1). We were able to detect as low as 5% mosaicism based on the profiles generated by our assay.

Spinal sample testing

A total of 1,053 individual sections were tested by RPPCR in the spinal cords of patients with ALS. No section showed evidence of *C9orf72* HRE at or above a 5% mosaicism level in any of the spinal cords tested. All sections from the same spinal cord showed the same profile of RPPCR fragments, and RPPCR peaks (chosen by the amplicon genotyping method sizing) showed that repeat sizing did not significantly change across a spinal cord (figure 2).

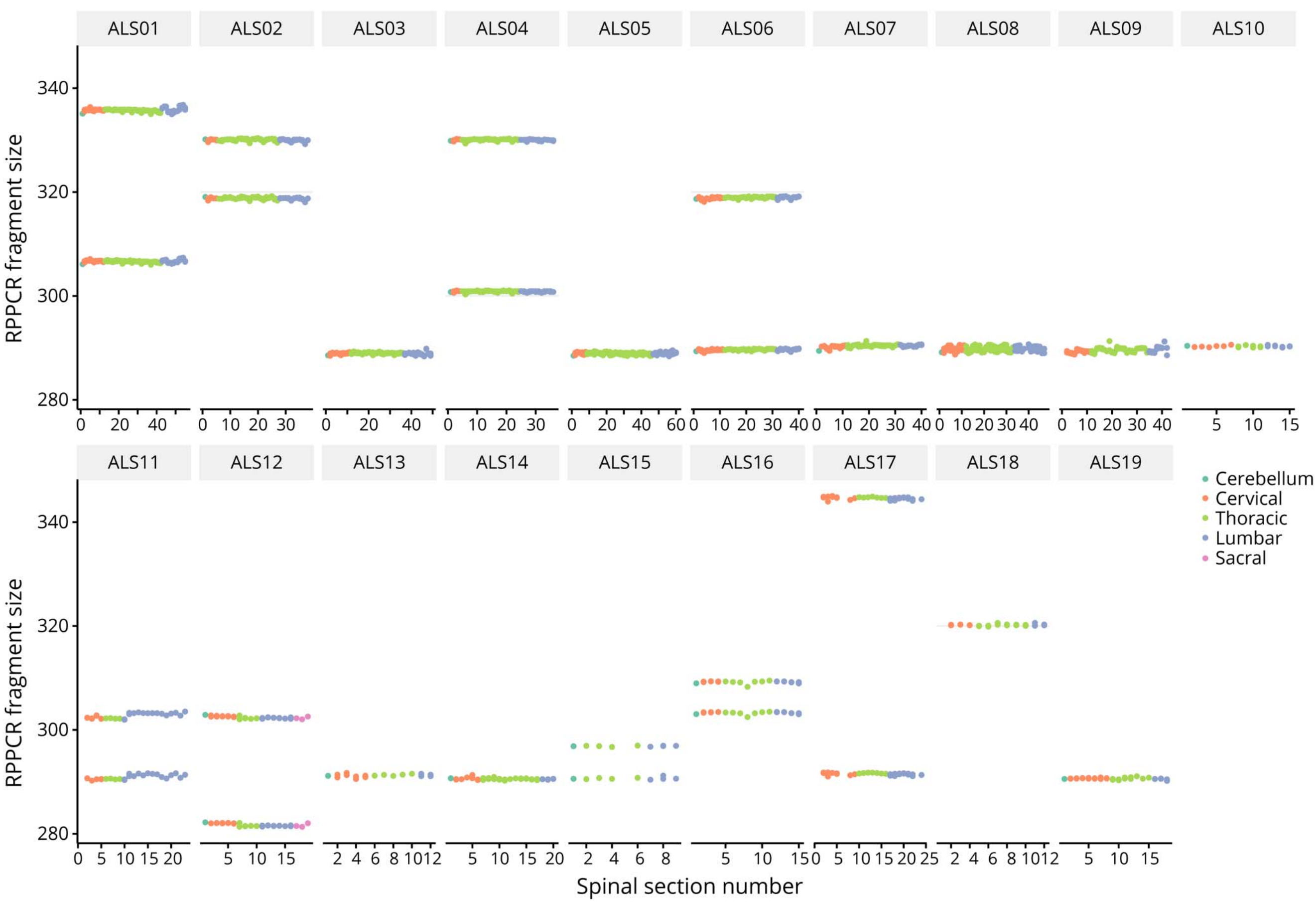
Discussion

Because of the high penetrance of the *C9orf72* HRE and the accumulation of repeat RNA fragments and dipeptide proteins,[1,9] its pathologic mechanism must have a strong (albeit time-dependent) effect. Therefore, there must be a threshold or concentration at which the products and effects of *C9orf72* HRE are toxic to cells and tissues. It is possible that low levels of *C9orf72* HRE not detectable by germline testing could be sufficient to cause disease through accumulation of products.

Our study did not find evidence for *C9orf72* HRE somatic expansion in the spinal cords of patients with ALS. This does not preclude the possibility that very low levels of expansion may exist in patients with ALS. However, as we were able to detect the levels of mosaicism at or above 5%, lower-frequency somatic mutations would have had to occur late in neural tissue development.

The lengths of *C9orf72* hexanucleotide repeats across all sections of the same spinal cord were identical. This result confirms that *C9orf72* hexanucleotide repeats are stable when in the normal range[10] and that if instability does occur, it is restricted to expanded alleles.[2] In *C9orf72* expression vectors, the number of hexanucleotide repeats has been reported to contract or expand above a critical number of repeats.[4] Changes in *C9orf72* hexanucleotide repeat length might occur more readily in artificial systems, and in human neural cells there may be a mechanism to prevent frequent alterations. Very large *C9orf72* HRE can exhibit a range of repeat lengths across tissues of an individual[10]; however, these pathogenic expansions likely occur in most or all cells of an individual and the exact number of repeats triggering the disease remains to be established.

Figure 2 RPPCR fragment sizes in the spinal cord sections of patients with ALS



RPPCR peaks representing the measured *C9orf72* HRE alleles were chosen based on the results of the amplicon genotyping method. ALS = amyotrophic lateral sclerosis; HRE = hexanucleotide repeat expansion; RPPCR = repeat-primed PCR.

Our study is limited by sample size, as it is difficult to acquire large numbers of spinal cords from patients with ALS. Based on our results, if somatic expansion occurs at the level detectable by our assays, it is likely that it does not account for a large proportion of ALS cases, not occurring in large clusters of neuronal cells. However, as we sampled exclusively from the ventral spinal cord, our assay did not test for somatic events in dorsal neurons or glial cells, which could be sources of pathogenic protein seeding.

Study of the *C9orf72* HRE remains difficult because of the technological limitations of sequencing GC-rich and repetitive regions of the genome. Techniques such as single cell and long-read sequencing may allow detection of very low-level somatic events and precise measurement of the *C9orf72* HRE length.

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Appendix Author contributions

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Guy A. Rouleau, MD PhD, FRCPC	McGill University, Montréal, QC, Canada	Author	Design and concept of study; interpretation of the data; and drafting or revising the manuscript for intellectual content

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