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.
Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by rapid and progressive loss of motor neurons. 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. 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. Because it is difficult to precisely size the repeat length above 30, 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. Repeat sequences are particularly of interest for somatic mutation analysis because their emergent secondary structures can lead to expansion or contraction of repeat lengths. It is also notable that the C9orf72 HRE can lead to cell-to-cell transmission of dipeptide repeat proteins, 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. 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, 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.
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 \textit{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\cite{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.

\textit{C9orf72} HRE reactions
\textit{C9orf72} HRE genotyping\cite{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)\cite{9} was performed on all sampled sections of each patient to assess for the \textit{C9orf72} HRE and to estimate the lengths of \textit{C9orf72} alleles in each section. GeneMapper v4.0 (Applied Biosystems) was used to visualize and estimate reaction fragment sizes. Lengths of \textit{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 \textit{C9orf72} alleles, which were plotted to assess variation within normal-length \textit{C9orf72} hexanucleotide repeat lengths.

HRE mosaicism index measures
Genomic DNA from a patient previously established as a \textit{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 \textit{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 \textit{C9orf72} HRE diluted in wild-type DNA displayed unique profiles on RPPCR fragment sizing.
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, 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 and that if instability does occur, it is restricted to expanded alleles. In C9orf72 expression vectors, the number of hexanucleotide repeats has been reported to contract or expand above a critical number of repeats. 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; 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.
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 (continued)

| Name                     | Location                                      | Role       | Contribution                                                                 |
|--------------------------|-----------------------------------------------|------------|------------------------------------------------------------------------------|
| Claire S. Leblond, PhD   | Pasteur Institute, University Paris Diderot, Sorbonne Paris Cité, Paris, France       | Author     | Design and concept of study; experimental procedures; analysis and interpretation of the data; and drafting the manuscript for intellectual content |
| Hélène Catoire, PhD      | McGill University, Montréal, QC, Canada       | Author     | Experimental procedures; and drafting the manuscript for intellectual content |
| Kathryn Volkening, PhD   | Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada. | Author     | Experimental procedures; and drafting the manuscript for intellectual content |
| Michael Strong, MD, FRCP, FANN, FCAHS | Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada. | Author     | Clinical assessment of patients; experimental procedures; and drafting the manuscript for intellectual content |
| Lorne Zinman, MD, MSC, FRCP | Sunnybrook Health Sciences Centre, Toronto, ON, Canada. | Author     | Clinical assessment of patients; and drafting the manuscript for intellectual content |
| Janice Robertson, PhD    | University of Toronto, Toronto, ON, Canada.  | Author     | Drafting the manuscript for intellectual content                             |
| Patrick A. Dion, PhD     | McGill University, Montréal, QC, Canada       | Author     | Design and concept of study; interpretation of the data; and drafting or revising the manuscript for intellectual content |
| Guy A. Rouleau, MD PhD, FRCP | 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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