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CORRESPONDENCE

An ALS case with 38 (G4C2)-repeats in the C9orf72 gene shows TDP-43 and sparse dipeptide repeat protein pathology

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The (G4C2)-hexanucleotide repeat expansion in chromosome 9 open reading frame 72 *(C9orf72)* is the most common mutation linked to amyotrophic lateral sclerosis (ALS), as it accounts for 51.6% of the familial ALS cases and 9.6% of the sporadic ALS cases [1, 2]. This repeat expansion also underlies approximately 25% of the familial frontotemporal lobar degeneration (FTLD) cases [12]. The pathogenic (G4C2)-repeat length is estimated to range from hundreds to thousands of repeat units, whereas neurologically healthy controls usually show a repeat length of only 2–30 (G4C2)-repeats [3]. However, the threshold for the repeat length to drive or aggravate ALS pathology is still under debate. Some ALS or FTLD cases show an intermediate (G4C2)-repeat length of 30–90 (G4C2)-repeats in peripheral blood DNA [4, 5, 7, 11], while, in contrast, some 30–70 repeat carriers did not develop symptomatic disease [6, 8]. Several cases with this so-called intermediate repeat length in peripheral blood DNA have been thoroughly investigated, showing somatic instability of the repeat with a mixture of intermediate and long repeat lengths (from hundreds to thousands of repeats) throughout the brain (mosaicism) [5, 7, 8, 11]. Dipeptide repeat proteins (DPRs) resulting from unconventional repeat-associated non-ATG translation of the (G4C2)-hexanucleotide repeat expansion exhibit distinct types of inclusions within neurons [10]. These pathological lesions were investigated for some of these (G4C2)-mosaic carriers, showing full-blown DPR pathology throughout the brain, comparable to non-mosaic carriers with a long repeat length [5, 7, 8]. One intermediate repeat case [30 (G4C2)-repeats] without mosaicism in the examined frontal cortex and cerebellum was previously reported [6]. This 30-repeat

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case was clinically unaffected and showed only sparse DPR pathology and no transactive response DNA-binding protein 43 kDa (TDP-43) pathology.

Here, we present a case of a 64-year-old man referred to the neuromuscular reference center of the University Hospitals of Leuven (UZ Leuven). The diagnosis of spinal onset probable ALS in the right arm was based on the revised El Escorial criteria and the Awaji algorithm. He did not show any cognitive or behavioral impairment and there was no family history of ALS or FTLD. Analysis of the *C9orf72* repeat expansion in peripheral blood DNA by triplet repeat primed PCR (TP-PCR) revealed a 38 (G4C2)-repeat length on one allele (Supplementary Fig. 1) and a five (G4C2)-repeat length on the other allele. The patient died 28 months after disease onset. Autopsy was carried out in accordance with the UZ Leuven ethics committee with written informed consent. Frozen and fixed tissue was stored in the UZ Leuven biobank. Macroscopically, the brain had a normal appearance with mild atherosclerosis and a weight of 1524 g. The anterior nerve roots were atrophic. Microscopically, ALS was confirmed by the presence of TDP-43 inclusions in spinal cord motor neurons and in layer V neurons of the primary motor cortex, as well as in neurons of the thalamus (Fig. 1) and other brain regions indicated in Supplementary Table 1. Neurofibrillary tangle (NFT) pathology matched Braak-NFT-stage I and was—in the absence of amyloid plaques and cerebral amyloid angiopathy—considered as definite primary age-related tauopathy. No obvious cerebral small vessel disease and no brain infarction or hemorrhage was observed.

To investigate the somatic instability of the *C9orf72* repeat expansion, TP-PCR was performed on DNA extracted from the frontal cortex, temporal cortex, parietal cortex, occipital cortex, thalamus, hypothalamus, cerebellum and the spinal cord (Fig. 1 and Supplementary Fig. 1). TP-PCR results did not show somatic instability and mosaicism of the 38-(G4C2) repeat length in all investigated regions. This is evidenced in the TP-PCR electropherograms by the premature ending of the typical sawtooth pattern at 38 units, instead of the presence of a tail of stutter amplification characteristic for *C9orf72* repeat expansion carriers. Other genes that could be causative for ALS were excluded by whole-genome sequencing (WGS) (Supplementary Table 2). Moreover, this so-called 38-repeat case is a carrier of a risk haplotype thought to be associated with *C9orf72* diseases, since homozygosity for allele A of SNP rs3849942 and rs4879515 was shown by WGS [9, 14]. Four other *C9orf72* repeat expansion carriers were included in this study for comparison and showed the characteristic tail of stutter amplification, not ending at 38 units, on the TP-PCR electropherogram of DNA extracted from peripheral blood and/or cerebellum. Therefore, these cases were considered as long repeat length cases (Fig. 1 and Supplementary Fig. 1). By immunohistochemical analysis, sparse DPR pathology was detected throughout the central nervous system (CNS) of the 38-repeat case in comparison to the abundant DPR pathology detected in the CNS of the four long repeat length cases (Fig. 1 and Supplementary Table 1 and Fig. 2). The pathology of the four examined DPRs had the same relative abundance (poly(GA) > poly(GP) > poly(GR) > poly(PR)) as shown in the long repeat length cases (Supplementary Table 1). Immunohistochemistry for p62 (as used by neuropathologists for diagnostic purposes) showed single DPR-like inclusions in the frontal cortex, thalamus and cerebellum, distinguishable from other p62 positive material (Supplementary Fig. 3). However, p62 inclusions were less abundant than poly(GA) lesions in those regions. Accordingly, poly(GA) immunohistochemistry appears to be better for the neuropathological diagnosis of non-mosaic intermediate repeat length cases.

Somatic instability and mosaicism was described in reported cases carrying an intermediate repeat length, except for one case [6]. This case, a clinically unaffected 84-year-old 30-repeat length carrier, showed a stable repeat size in the investigated frontal cortex, and cerebellum and sparse DPR pathology was found in the hippocampus, cerebellum, frontal and temporal cortices, but in no other brain region. No TDP-43 lesions were observed and the risk haplotype was not tested. In contrast, our non-mosaic 38-repeat case showed, besides sparse DPR pathology in all investigated CNS regions (indicated in Supplementary Table 1), also TDP-43 pathology, explaining the clinical picture of ALS. Whereas in the previously reported 30-repeat case no inclusions were detected in the thalamic nuclei, we observed DPR inclusions predominantly in the thalamus and the cerebellum compared to other neuroanatomical regions. Such a vulnerability of the thalamus for DPR pathology would be in line with the fact that *C9orf72* carriers show significant atrophy in the thalamus compared to sporadic ALS patients [13]. On the other hand, since some studies suggest that DPR pathology may precede the TDP-43 pathology, the reported 84-year-old 30-repeat case could still have developed ALS at a later age [1].

In conclusion, this case, carrying a 38 (G4C2)-repeat length in the *C9orf72* gene and carrier of a *C9orf72* risk haplotype, showed the typical clinical picture of ALS, demonstrating that in this case the 38 (G4C2)-repeat length was presumably sufficient to trigger the development of TDP-43 pathology and ALS. Accordingly, non-symptomatic carriers with a minimum repeat length of 38 repeats should be considered to be at higher risk to develop ALS. Besides, autopsied cases with sparse DPR/p62 pathology should be considered as possible *C9orf72* intermediate repeat length carriers. This warrants the importance of investigating more intermediate repeat length carriers to clarify the threshold of the (G4C2)-repeat length for the onset of ALS.

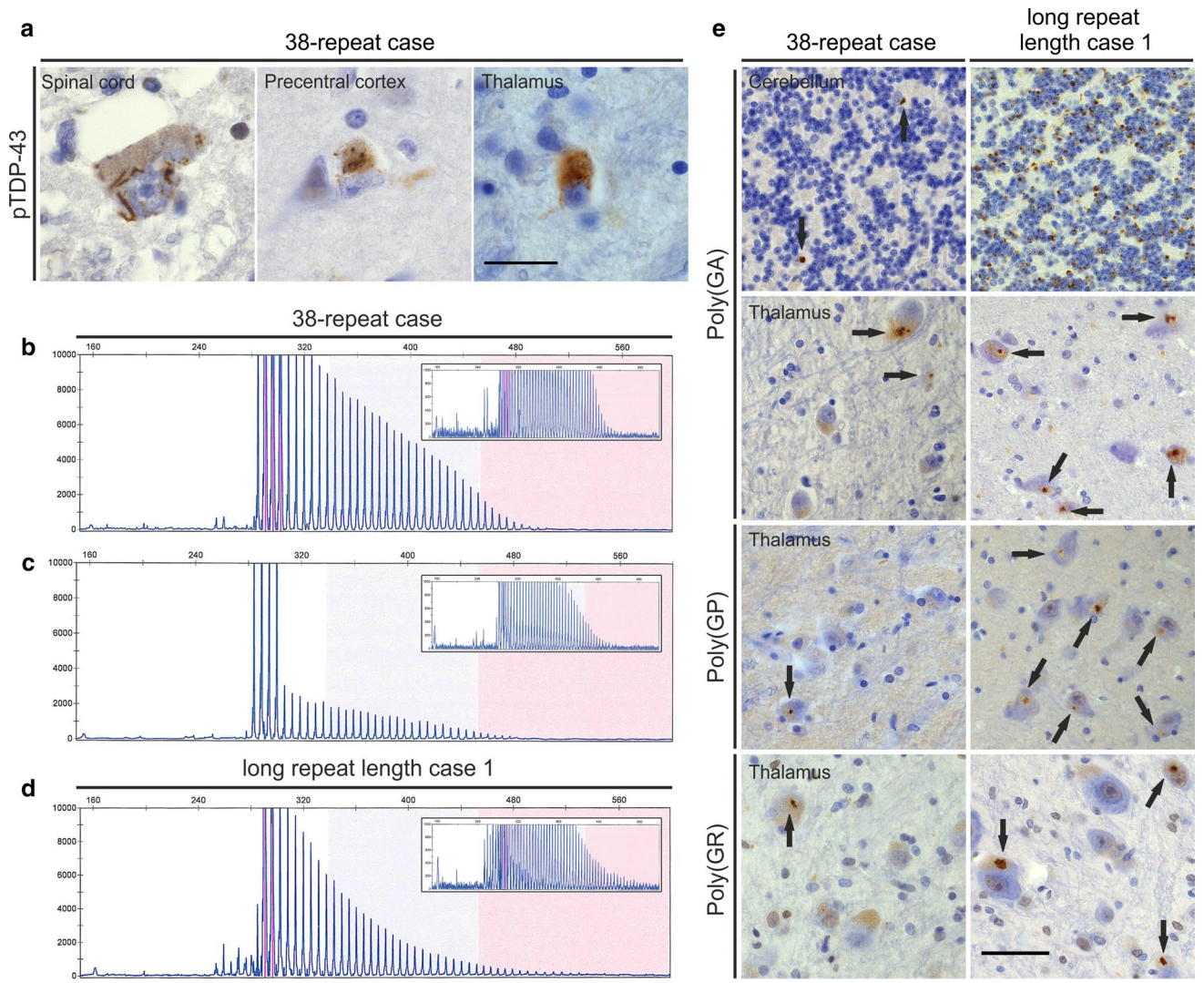

Fig. 1 Characterization of the 38-repeat case. TDP-43 pathology affecting a motor neuron of the lumbosacral spinal cord, a neuron of the precentral cortex and the thalamus of the 38-repeat case is shown as detected by an antibody against phosphorylated TDP-43 (pTDP-43) (see Supplementary information) (**a**). TP-PCR electropherograms from cerebellar (**b**) and thalamic (**c**) DNA of the 38-repeat case demonstrate a 38 (G4C2)-repeat length. TP-PCR result of cerebellar DNA of long repeat length case 1 (**d**) shows the presence of a tail of stutter amplification. The *x*-axis corresponds to the length from the GeneScan-500 Rox and the *y*-axis to relative fluorescent units. Poly(GA), poly(GP) and poly(GR) pathology in cerebellum and thalamus of the 38-repeat case and long repeat length case 1 is shown (**e**). Arrows indicate the inclusions. Obviously, fewer inclusions are present in the 38-repeat case. To avoid artificial blurring of the poly(GA) inclusions and to obtain clear pictures, no additional formic acid pretreatment was performed. Scale bars represent 25 µm (**a**) and 50 µm (**e**)

Disclosures

RV's institution has a clinical trial agreement (RV as PI) with AbbVie (USA), Biogen (USA), Genentech (USA), Novartis (Switzerland), and Roche (Switzerland). PVD participated in advisory board meetings for Genzyme (USA), Pfizer (USA), Biogen (USA), Cytokinetics (USA), Mitsubishi Tanabe (Japan), CSL Behring (USA), Alexion Pharmaceuticals (USA). DRT received consultant honorary from GE-Healthcare (UK), and Covance Laboratories (UK), speaker honorary from Novartis Pharma AG (Switzerland), and collaborated with Novartis Pharma AG (Switzerland), Probiodrug (Germany), GE-Healthcare (UK), and Janssen Pharmaceutical Companies (Belgium).

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