Clinical/Scientific Notes

Johnathan Cooper-Knock, BA
Adrian Higginbottom, PhD
Natalie Connor-Robson, MSc
Nadhim Bayatti, PhD
Joanna J. Bury, MSc
Janine Kirby, PhD
Natalia Ninkina, MD, PhD
Vladimir L. Buchman, MD, PhD
Pamela J. Shaw, MD

C9ORF72 TRANSCRIPTION IN A FRONTOTEMPORAL DEMENTIA CASE WITH TWO EXPANDED ALLELES

Discovery of intronic hexanucleotide repeat expansions of the *C9ORF72* gene in a significant proportion of patients with amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD)[1,2] was an important step for research into these disorders. The *C9ORF72* genetic variant is more common than other described mutations, and, unlike patients with mutations in *SOD1*, *C9ORF72*-ALS clinically and pathologically resembles the more numerous sporadic form.[3] However, progress has been limited by lack of understanding of the function of the *C9ORF72* locus in health and disease. It is unknown whether the expansion causes disease by a gain of toxicity, whether it disrupts expression of the wild-type protein encoded by the *C9ORF72* gene, or some combination of both mechanisms.[1,2,4]

Case. Our case is a woman who presented with deteriorating handwriting at age 58 years. Later she developed features of frontal dysfunction and parkinsonism; she received a formal diagnosis of behavioral-variant FTD from a consultant neurologist. Two years after diagnosis, she has not developed motor weakness or denervation changes on EMG. The patient had a brother who died of ALS at age 63 years; no other family members are known to have had neurologic disease, although available information is limited. Several relatives died relatively young (<50 years) from non-neurologic causes, including her parents; she has no children.

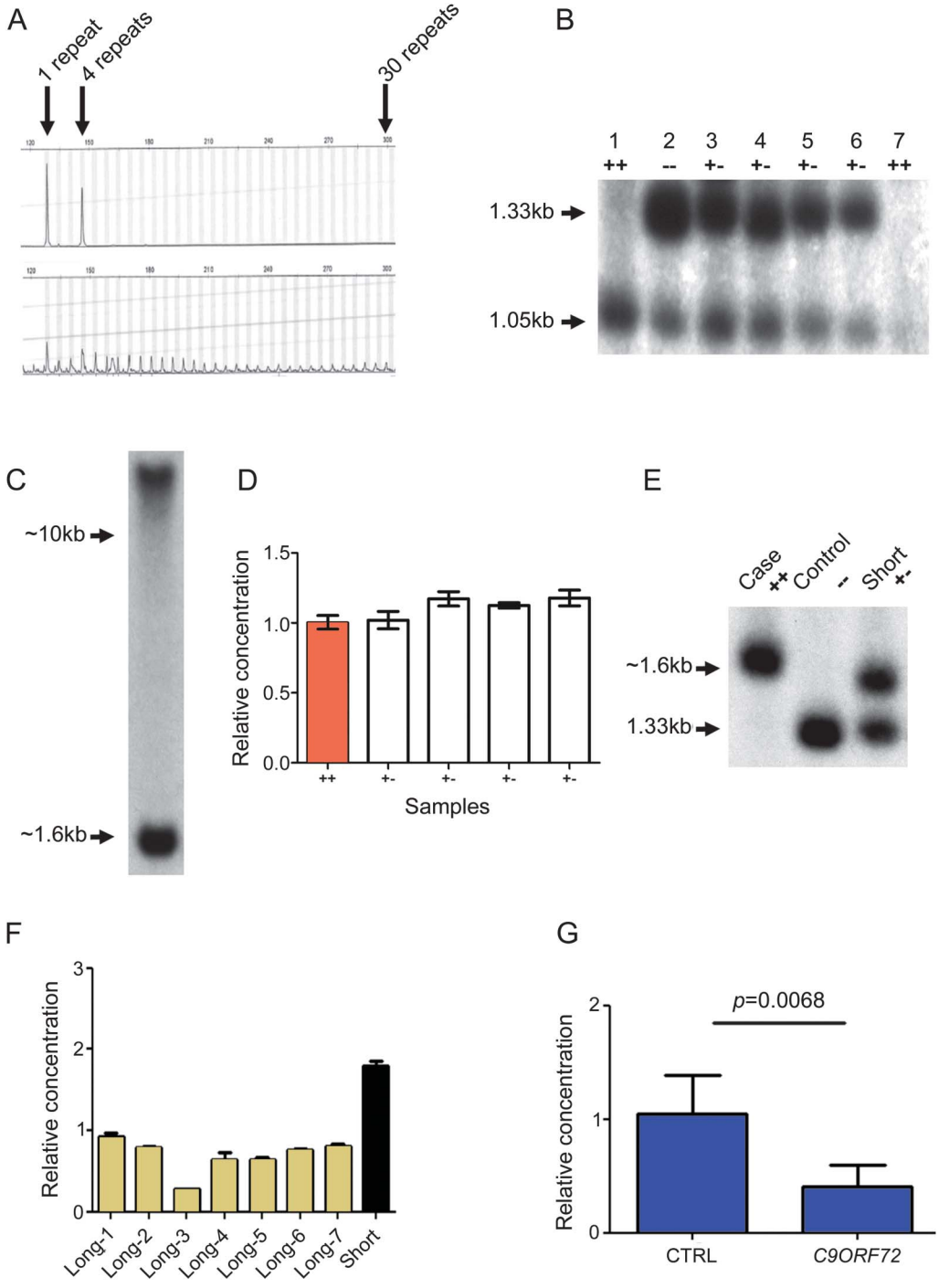
***Detection of 2 expanded C9ORF72* alleles.** PCR analysis of genomic DNA extracted from our patient's venous blood did not detect a normal-length *C9ORF72* allele (figure, A); similarly, Southern hybridization analysis[5] revealed no normal-length *C9ORF72* allele in venous blood or saliva (figure, B). Instead, 2 expanded alleles were detected of 50 ± 5 repeats and >2,000 repeats (figure, C).

***Investigation of C9ORF72* mRNA expression.** qRT-PCR analysis of RNA extracted from venous blood cells in our patient and several patients with expansions of >2,000 repeats in 1 *C9ORF72* allele and the absence of expansion (i.e., <20 repeats) in another allele revealed similar levels of *C9ORF72* mRNA in all samples (figure, D). Southern hybridization identified a lymphoblastoid cell line derived from a *C9ORF72*-ALS patient with one expanded *C9ORF72* allele of similar size (45 ± 5 repeats) to the smaller expansion in our case (figure, E). qRT-PCR of RNA extracted from this cell line and other lines with expansions of >2,000 repeats in 1 *C9ORF72* allele revealed that *C9ORF72* mRNA levels were twice as high in the line with the smaller expansion (figure, F). Ethics committee approval and written consent was obtained for all biosamples.

Discussion and conclusions. Identification of a patient with 2 expanded *C9ORF72* alleles is an important step in the study of *C9ORF72* disease. Her disease severity, defined by age at onset and disease duration, is not remarkably different from other *C9ORF72*-positive patients.[6] Notably, the length of expansion is different in her 2 *C9ORF72* alleles, which allowed us to explore the effect of shorter expansions on *C9ORF72* mRNA expression. We and others have shown that the majority of *C9ORF72* neurodegeneration patients carry a repeat expansion of >2,000 repeats,[5] although it has been suggested that more than 30[1,2] or even as little as 20 to 22 repeats in 1 *C9ORF72* allele are pathogenic.[7] A recent study has reported hypermethylation of a CpG island 5′ to the repeat sequence, which did not occur in samples with intermediate-length expansions of up to 43 repeats.[8] If this is the mechanism underlying reduced mRNA expression, then it should not affect smaller repeat sizes.

Previously published[2] and our own data (figure, G) demonstrate that the level of *C9ORF72* mRNA is reduced in blood cells from patients with long expansions of >2,000 repeats, suggesting that expression of RNA from the expanded allele is compromised. In a lymphoblastoid cell line from a patient with a short expansion in 1 *C9ORF72* allele, estimated as 45 ± 5 repeats by Southern hybridization, the level of *C9ORF72* mRNA was approximately double the level in cell lines from patients with long expansions (figure, F). This suggests that an allele with a short pathogenic expansion is normally expressed, but this finding might reflect a compensatory increase in transcription of the normal allele. However, since our patient with 2 expansions does not possess a normal allele, such

Figure ***C9ORF72*** **expansion size and** ***C9ORF72*** **mRNA expression in lymphoblastoid cells and venous blood**



(A) Genotyping PCR of a wild-type control and our patient. The shaded lines represent numbers of repeats from 1 to 30. Thus the upper panel shows a heterozygous control with 2 normal-length alleles of 1 and 4 repeats. No normal-length allele of less than 30 repeats is detected in our patient, as shown in the lower panel. (B, C) Southern hybridization–based detection of the *C9ORF72* allele. (B) Analysis of DNA extracted from venous blood of 5 *C9ORF72*-positive patients and 1 control. The 1.33-kb band corresponds to an *Eco*RI/*Xba*I fragment derived from a nonexpanded locus. This band is present in patients with a single *C9ORF72* expansion (lanes 3–6, +−) and in normal controls without an expansion (lane 2, −−) but is absent in the patient with 2 expanded alleles (lanes 1 and 7, ++) in both venous blood (lane 1) and saliva (lane 7). The 1.05-kb band is an internal control to show that the absence of 1.33-kb band is not due to a low amount of DNA loaded or its inability to hybridize with the labeled probe. (C) A longer gel allows sizing of both the alleles in the patient with 2 expanded alleles in venous blood. Bands are seen at ~1.6 kb and >12 kb, suggesting that 1 *C9ORF72* allele of the patient carried 50 ± 5 repeats and the other >2,000 repeats. (D) qRT-PCR for *C9ORF72* mRNA in venous blood cells from *C9ORF72*-positive patients; error bars illustrate 95% confidence intervals. Concentration is plotted relative to the concentration in the case with 2 expanded alleles (++). (E) Southern hybridization–based detection of small-size expansion in the *C9ORF72* allele. DNA was extracted from venous blood derived from our case with 2 expanded alleles (left lane), a normal control (middle lane), and a patient with expansion in 1 *C9ORF72* allele of similar length to the

compensation could not explain why *C9ORF72* mRNA expression in her blood cells is equivalent to patients carrying 1 normal allele (figure, D). Thus we conclude that the presence of ~50 copies of the repeat does not significantly affect *C9ORF72* gene transcription or mRNA stability in vivo. If shorter repeats are indeed pathogenic, our evidence suggests that this is unlikely to be mediated by haploinsufficiency.

From the Sheffield Institute for Translational Neuroscience (SITraN) (J.C.-K., A.H., N.B., J.J.B., J.K., P.J.S.), University of Sheffield; and the School of Biosciences (N.C.-R., N.N., V.L.B.), Cardiff University, UK.

Author contributions: The study was conceived and designed by J.C.-K., V.B., and P.J.S. Data acquisition was carried out by J.C.-K., A.H., N.C.-R., N.B., J.J.B., N.N., and V.L.B. Data analysis and interpretation was performed by J.C.-K., A.H., N.C.-R., N.B., J.J.B., J.K., N.N., V.L.B., and P.J.S. The manuscript was critically revised by J.C.-K., A.H., V.L.B., and P.J.S. The study was supervised by N.N., V.L.B., and P.J.S.

Study funding: Supported by grants from the Wellcome Trust (075615/Z/04/z) to V.L.B. and EU Framework 7 (Euromotor No259867) and the SOPHIA project (funded by JPND and MRC) to P.J.S. and J.K. J.C.-K. holds an MND Association/MRC Lady Edith Wolfson Fellowship award (MR/K003771/1). P.J.S. is an NIHR Senior Investigator. Biosample collection was supported by the MND Association and the Wellcome Trust (P.J.S.).

Disclosure: J. Cooper-Knock is supported by an MND Association/MRC Lady Edith Wolfson fellowship award (MR/K003771/1). J. Kirby is supported by an FP7 grant EuroMOTOR (no. 259867) and by Joint Programme for Neurodegenerative Disease (JPND) grant SOPHIA. P. Shaw is supported by NIHR as a Senior Investigator and an FP7 grant EuroMOTOR (no. 259867) and Joint Programme for Neurodegenerative Disease (JPND) grant SOPHIA. V. Buchman is supported by a research grant from the Wellcome Trust (075615/Z/04/z). A. Higginbottom, N. Connor-Robson, N. Bayatti, J. Bury, and N. Ninkina report no disclosures. Go to Neurology.org for full disclosures.

Received March 31, 2013. Accepted in final form July 2, 2013.
Correspondence to Dr. Shaw: pamela.shaw@sheffield.ac.uk

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smaller expansion in our case (right lane). Bands correspond to genomic fragments carrying 50 ± 5 repeats (left lane), 45 ± 5 repeats (right lane, top band), or no repeats (middle lane and right lane, bottom band). (F) qRT-PCR for *C9ORF72* mRNA in lymphoblastoid cells derived from patients carrying 1 *C9ORF72* allele with repeat expansion. Levels of *C9ORF72* mRNA are approximately double in the patient with a shorter repeat length compared to patients with >2,000 repeats; error bars illustrate 95% confidence intervals. (G) qRT-PCR for *C9ORF72* mRNA in venous blood cells from *C9ORF72*-positive patients and age- and sex-matched controls (CTRL). Levels of *C9ORF72* mRNA are significantly reduced in the patients compared to controls.