ORIGINAL CONTRIBUTION

C9orf72 Hexanucleotide Repeat Expansions as the Causative Mutation for Chromosome 9p21–Linked Amyotrophic Lateral Sclerosis and Frontotemporal Dementia

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Objective: To further assess the presence of a large hexanucleotide repeat expansion in the first intron of the *C9orf72* gene identified as the genetic cause of chromosome 9p21–linked amyotrophic lateral sclerosis and frontotemporal dementia (c9ALS/FTD) in 4 unrelated families with a conclusive linkage to c9ALS/FTD.

Design: A repeat-primed polymerase chain reaction assay.

Setting: Academic research.

Participants: Affected and unaffected individuals from 4 ALS/FTD families.

Main Outcome Measure: The amplified *C9orf72* repeat expansion.

Results: We show that the repeat is expanded in and segregated perfectly with the disease in these 4 pedigrees.

Conclusion: Our findings further confirm the *C9orf72* hexanucleotide repeat expansion as the causative mutation for c9ALS/FTD and strengthen the hypothesis that ALS and FTD belong to the same disease spectrum.

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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are adult-onset neurodegenerative diseases generally characterized by a rapid progression after symptom onset. Amyotrophic lateral sclerosis is the most prevalent motor neuron disease worldwide, where the selective degeneration of upper and lower motor neurons in the brain and spinal cord lead to progressive paralysis and death, typically of respiratory failure within 3 to 5 years of symptom onset.[1] Frontotemporal dementia is the second most common form of presenile dementia after Alzheimer disease and is characterized by the degeneration of neurons in the frontal and anterior temporal lobes leading to behavioral dysfunction and impairment in executive functions and language.[2] Interestingly, up to 15% of patients with ALS have FTD,[3] and a further 30% have evidence of cognitive impairment, suggesting that these 2 conditions have a common genetic background.

Although most ALS cases are sporadic, approximately 5% are familial and show a mendelian pattern of inheritance.[4] Of these, the most frequently identified cause is mutations in the *SOD1* gene[5] (15%-20%), followed by the *TARDBP*[6] and *FUS*[7] genes (1%-3% each). Additionally, mutations in other genes have been linked to familial ALS but these appear to be very rare.[8] On the other hand, up to 50% of patients with FTD have family members with dementia and/or cognitive and behavioral changes.[9] Similarly, genetic variations in the *MAPT*[10] and *PRGN*[11] genes explain almost 50% of familial FTD cases and are considered as the main genetic cause of FTD. Variations in other genes, including *CHMP2B*, *VCP*, *TARDBP*, and *FUS*, contribute to less than 5% of all FTD cases.[12]

Growing evidence indicates that ALS and FTD are 2 phenotypic manifestations of a common underlying genetic cause. The identification of ubiquitinated TDP-43–positive inclusions as a common pathological hallmark of patients with ALS and FTD first contributed to the merging of the ALS and FTD fields.[13] Most importantly, the co-occurrence of ALS and FTD within the same families and sometimes within the same individuals was repeatedly reported, particularly over the last 5 years, strongly implicating common genetic components for these 2 conditions. Indeed, linkage analyses of extended pedigrees in which both ALS and FTD segregate have led to the identification of a very robust locus on the chromosome 9p21.[14-16] Very recently, a

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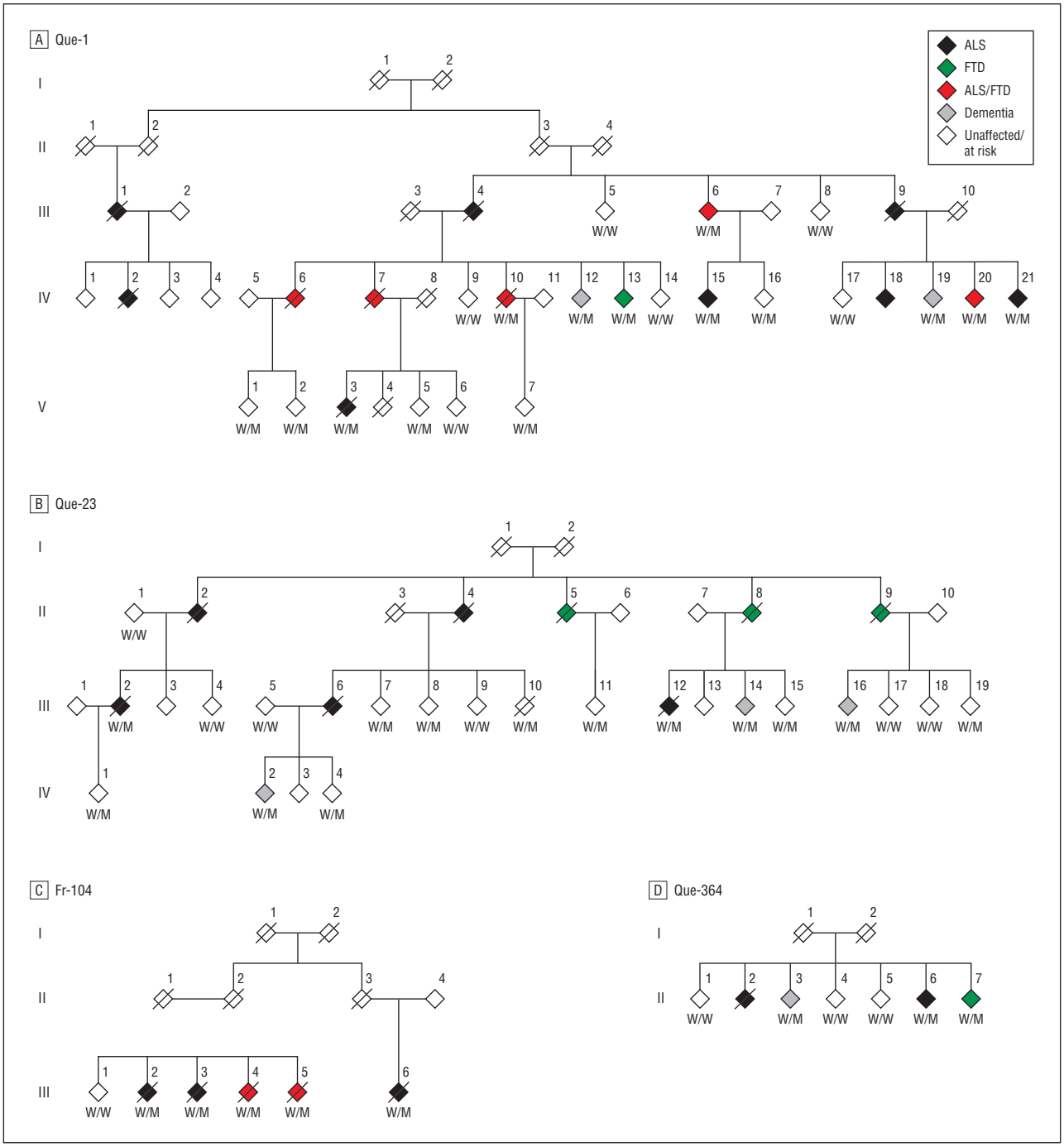

Figure. Pedigrees of the 4 families segregating the *C9orf72* hexanucleotide repeat expansion. Black diamonds indicate individuals with amyotrophic lateral sclerosis (ALS); green diamonds, individuals with frontotemporal dementia (FTD); red diamonds, individuals with both ALS and FTD; gray diamonds, individuals with preliminary signs of dementia; and white diamonds, unaffected or at-risk individuals. W indicates wild-type alleles; M, mutant alleles. Individuals with diagonal lines are deceased. Not all family members are shown to protect privacy.

large hexanucleotide repeat expansion in the first intron of the *C9orf72* gene has been identified as the genetic cause of chromosome 9p21-linked ALS/FTD (c9ALS/FTD) in families and the most common cause of familial ALS and FTD to date.[17-19]

We have previously reported 3 families with ALS/FTD from Canada and France with evidence of linkage to chromosome 9p21.[16] Herein, we show that the hexanucleotide repeat expansion in *C9orf72* is the underlying genetic defect in these 3 families as well as a fourth family from Quebec, Canada, and that this expansion segregated perfectly with the disease in these 4 families.

METHODS

PATIENTS AND SAMPLES

Four unrelated families including patients with ALS/FTD were included in this study (**Figure**). Three of them (Que-1, Que-23, and Fr-104) were previously linked to the c9ALS/FTD lo-

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Table. Clinical Details of *C9orf72* Expansion Carriers

Family	Individual	Diagnosis	Age at Onset, y	Site of Onset	Disease Duration, y
Que-1	III-6	ALS/FTD	81	Bulbar	Alive
Que-1	IV-10	ALS/FTD	60	Bulbar	5
Que-1	IV-12	Dementia	64	NA	Alive
Que-1	IV-13	FTD	67	NA	Alive
Que-1	IV-15	ALS	56	Spinal	Alive
Que-1	IV-18	ALS	68	Spinal	Alive
Que-1	IV-19	Dementia	62	NA	Alive
Que-1	IV-20	ALS/FTD	70	Unknown	Alive
Que-1	IV-21	ALS	74	Spinal	Alive
Que-1	V-3	ALS	48	Spinal	4
Que-23	III-2	ALS	46	Bulbar	3
Que-23	III-12	ALS	57	Spinal	4
Que-23	III-14	Dementia	54	NA	Alive
Que-23	III-16	Dementia	Unknown	NA	Alive
Que-23	IV-2	Dementia	57	NA	Alive
Fr-104	III-2	ALS	55	Spinal	2
Fr-104	III-3	ALS	56	Bulbar	Unknown
Fr-104	III-4	ALS/FTD	51	Bulbar	2
Fr-104	III-5	ALS/FTD	56	Bulbar	3
Fr-104	III-6	ALS	58	Spinal	5
Que-364	II-2	ALS	66	Spinal	2
Que-364	II-3	Dementia	Unknown	NA	Alive
Que-364	II-6	ALS	58	Spinal	Alive
Que-364	II-7	FTD	54	NA	Alive

Abbreviations: ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia; NA, not applicable.

cus.[16] Informed written consent was obtained from all participating family members, and the study was approved by the ethics committee of the Centre Hospitalier de l'Université de Montréal, Montreal, Quebec. Patients with ALS met the diagnosis of definite or probable ALS as defined in the El Escorial criteria.[20] All patients with FTD exhibited cerebral atrophy at neuroimaging. Some individuals had preliminary signs of dementia; others had only FTD without any motor involvement (Figure). The clinical descriptions of the Que-1, Que-23, and Fr-104 families have been previously reported.[16] Briefly, these 3 pedigrees contain 32 affected individuals, 16 with ALS, 4 with FTD, 7 with ALS/FTD, and 5 with dementia. Since the first description of these 3 families,[16] 11 additional individuals became affected with ALS, FTD, or both (8 new cases in Que-1 and 3 in Que-23). The fourth pedigree (Que-364) contains 2 individuals with ALS, 1 with FTD, and 1 with dementia.

C9orf72 HEXANUCLEOTIDE REPEAT ANALYSIS

Genomic DNA was extracted from blood samples or lymphoblastoid cell lines using standard methods. To assess the presence of an expanded hexanucleotide repeat in the *C9orf72* gene, we performed a repeat-primed polymerase chain reaction (PCR) assay using the FastStart PCR Master Mix (Roche) and the previously optimized assay conditions.[17,18] The repeat was amplified in all affected and unaffected individuals from these 4 pedigrees for whom DNA was available. The PCR products were analyzed on an ABI 3730 sequencer with GeneMapper software version 4.0 (Applied Biosystems). Individuals carrying the expansion showed a characteristic pattern with a 6–base pair periodicity (eFigure, http://www.archneurol.com).

RESULTS

We amplified the *C9orf72* hexanucleotide repeat in 4 unrelated families including patients with ALS and/or FTD (Figure). Using the repeat-primed PCR method, we show that this repeat is expanded and segregated with the disease in these 4 pedigrees. We also show that some at-risk individuals carry the *C9orf72* expansion but did not develop any disease signs, probably because they are still younger than the age at onset (Figure). This expansion was absent from 190 French Canadian neurologically healthy individuals (data not shown). Altogether, 36 affected individuals carry the expansion: 18 with ALS (50.0%), 5 with FTD (13.8%), 7 with ALS/FTD (19.4%), and 6 with preliminary signs of dementia (16.6%). The average age at onset in 22 patients for whom clinical records were available was 59.9 years (range, 46-81 years) with an average disease duration of 3.3 years (range, 2-5 years) (**Table**). The site of onset of ALS was bulbar in 6 patients and spinal in 9 patients. In family Que-1, the average age at onset was 48 years in the fifth generation, which was younger than that of 8 patients from the fourth generation (65 years; 95% CI, 60.2-69.9) and that of the third generation (81 years).

COMMENT

In 2007, our group reported 3 relatively large ALS/FTD pedigrees with genetic linkage to chromosome 9p21.[16]

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In this study, we report the hexanucleotide repeat expansion in the noncoding region of the *C9orf72* gene as the underlying genetic cause of ALS/FTD in these 3 families as well as a newly recruited family with ALS/FTD. We show that the repeat expansion segregates perfectly with the disease in these 4 families and is absent from a cohort of French Canadian controls (data not shown), suggesting that this expansion is fully penetrant.

However, given that the repeat-primed method we and other groups have used[17-19] cannot establish the exact sizes of the expanded alleles, we could not establish a genotype-phenotype correlation and determine the link between allele sizes and disease severity. For now, we only noticed a trend toward younger age at onset in 3 generations of the family Que-1. Although clinical information was not available in all affected individuals of this family, this trend suggests the presence of anticipation in this family that could possibly be explained by the instability of this hexanucleotide, as its number may increase over the generations. However, we did not observe this anticipation in the other 3 families, which could be related to the small size of these families, notably Fr-104 and Que-364, or the lack of clinical information.

The other caveat of the repeat-primed assay is that the exact threshold leading to disease could not be established. Indeed, the development of other methods that can accurately size the large alleles, such as Southern blotting or long-range PCR assays, is needed to better understand the correlation between the allele sizes and disease parameters such as age at onset, site of onset, and disease duration. This accurate sizing is also essential to assess whether this repeat is unstable and whether this instability occurs on the paternal or the maternal alleles.

In summary, we report herein the hexanucleotide expansion in the *C9orf72* gene as the genetic cause of c9ALS/FTD in 4 families with a French and French Canadian ethnicity. Our data further confirm this hexanucleotide repeat expansion as the causative mutation for c9ALS/FTD. Although the mechanism by which these large expansions lead to neurodegeneration has yet to be identified, the identification of the *C9orf72* gene as a common cause of ALS and FTD strengthens the hypothesis that these diseases are 2 phenotypic ends to the same spectrum and suggests that additional genes causing both ALS and FTD are likely to be identified in the future. Altogether, these newly identified genes would increase our understanding of these diseases and hopefully lead to the development of therapeutic strategies.

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