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Clinico-pathological features in amyotrophic lateral sclerosis with expansions in C9ORF72

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Intronic expansion of the GGGGCC hexanucleotide repeat within the *C9ORF72* gene causes frontotemporal dementia and amyotrophic lateral sclerosis/motor neuron disease in both familial and sporadic cases. Initial reports indicate that this variant within the frontotemporal dementia/amyotrophic lateral sclerosis spectrum is associated with transactive response DNA binding protein (TDP-43) proteinopathy. The amyotrophic lateral sclerosis/motor neuron disease phenotype is not yet well characterized. We report the clinical and pathological phenotypes associated with pathogenic *C9ORF72* mutations in a cohort of 563 cases from Northern England, including 63 with a family history of amyotrophic lateral sclerosis. One hundred and fifty-eight cases from the cohort (21 familial, 137 sporadic) were post-mortem brain and spinal cord donors. We screened DNA for the *C9ORF72* mutation, reviewed clinical case histories and undertook pathological evaluation of brain and spinal cord. Control DNA samples ($n = 361$) from the same population were also screened. The *C9ORF72* intronic expansion was present in 62 cases [11% of the cohort; 27/63 (43%) familial, 35/500 (7%) cases with sporadic amyotrophic lateral sclerosis/motor neuron disease]. Disease duration was significantly shorter in cases with *C9ORF72*-related amyotrophic lateral sclerosis (30.5 months) compared with non-*C9ORF72* amyotrophic lateral sclerosis/motor neuron disease (36.3 months, $P < 0.05$). *C9ORF72* cases included both limb and bulbar onset disease and all cases showed combined upper and lower motor neuron degeneration (amyotrophic lateral sclerosis). Thus, clinically, *C9ORF72* cases show the features of a relatively rapidly progressive, but otherwise typical, variant of amyotrophic lateral sclerosis associated with both familial and sporadic presentations. Dementia was present in the patient or a close family member in 22/62 cases with *C9ORF72* mutation (35%) based on diagnoses established from retrospective clinical

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case note review that may underestimate significant cognitive changes in late disease. All the *C9ORF72* mutation cases showed classical amyotrophic lateral sclerosis pathology with TDP-43 inclusions in spinal motor neurons. Neuronal cytoplasmic inclusions and glial inclusions positive for p62 immunostaining in non-motor regions were strongly over-represented in the *C9ORF72* cases. Extra-motor pathology in the frontal cortex ($P < 0.0005$) and the hippocampal CA4 subfield neurons ($P < 0.0005$) discriminated *C9ORF72* cases strongly from the rest of the cohort. Inclusions in CA4 neurons were not present in non-*C9ORF72* cases, indicating that this pathology predicts mutation status.

Keywords: amyotrophic lateral sclerosis; C9ORF72; dementia; neurodegeneration

Abbreviations: ALS = amyotrophic lateral sclerosis; FTD = frontotemporal dementia; FTLD = frontotemporal lobar degeneration; MND = motor neuron disease

Introduction

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive neurodegenerative disorder affecting the motor neurons in the cerebral cortex, brainstem and spinal cord. Progressive destruction of motor neurons leads to a clinical syndrome of muscle weakness, wasting and paralysis resulting in death typically within 2–3 years. Only one drug, riluzole, extends survival and its effects are of modest impact. The understanding of disease pathogenesis is gradually increasing, particularly in relation to genetically determined subtypes of ALS (Ferraiuolo *et al*., 2011), but there is poor understanding of the basic mechanisms of motor neuron injury in sporadic ALS.

ALS affects around six people per 100 000 in the UK with 5–10% of cases having familial disease, usually with autosomal dominant inheritance. Onset is usually in the 6th or 7th decade, although familial cases frequently have a younger age of onset. Mutations in a growing number of genes including superoxide dismutase 1 (*SOD1*), TAR DNA binding protein (*TARDBP*), fused in sarcoma (*FUS*), valosin-containing protein (*VCP*), factor-induced gene 4 (*FIG4*), angiogenin (*ANG*), ubiquilin 2 (*UBQLN2*) and optineurin (*OPTN*) have been shown to be causative in ~30% of adult-onset familial ALS and in a smaller proportion of sporadic ALS cases (Ticozzi *et al*., 2011). In addition, genome-wide association studies have identified variants in several other genes, including ataxin 2 (*ATXN2*) and *UNC13A*, that are associated with increased risk of developing sporadic ALS (Lambrechts *et al*., 2003; van Es *et al*., 2009; Elden *et al*., 2010). Understanding how variations in these genes cause motor neuron degeneration is key to improving our understanding of disease pathophysiology and to the development of more powerful neuroprotective therapies.

In addition to the genes described earlier, genetic linkage to several other genomic regions, including 9p21 (ALS-FTD), 18q21 (ALS3) and 20p13 (ALS7), has been demonstrated in familial ALS cases (Ticozzi *et al*., 2011). The chromosome 9p21 locus has been intensively investigated in recent years by researchers interested both in ALS and the related condition frontotemporal dementia (FTD). This locus was first described in families with a high proportion of concurrent ALS and FTD (Hosler *et al*., 2000) and the locus was more recently refined in ALS cases to a 20 single nucleotide polymorphism common haplotype spanning a 140-kb segment (Mok *et al*., 2011). Linkage to 9p21 has generated particular interest because it provides strong evidence for the association between ALS and frontotemporal lobar degeneration (FTLD), which share common neuropathological features and have significant clinical overlap (Fecto and Siddique, 2011). In addition, the 9p21 locus was estimated to account for nearly half of familial ALS cases and one-fifth of sporadic ALS cases in the Finnish population (Laaksovirta *et al*., 2010). However, direct sequencing of the coding regions of the three genes at this locus (*MOBKL2B*, *C9ORF72* and *IFNK*) did not reveal any pathogenic variants.

Two groups have now separately identified the gene associated with the 9p21 linkage as a GGGGCC hexanucleotide repeat expansion in intron 1 of the gene *C9ORF72* (NM018325.2) (DeJesus-Hernandez *et al*., 2011; Renton *et al*., 2011). Following demonstration of segregation with ALS in two families, Renton *et al*. (2011) subsequently found the expansion in 46.4% of familial and 21% of sporadic ALS cases in the Finnish cohort and 38.1% of familial ALS cases from USA, Italy and Germany. DeJesus-Hernandez *et al*. (2011) similarly demonstrated segregation of the *C9ORF72* expansion in a large autosomal dominant ALS/FTD kindred (VSM-20). Subsequent screening of a larger cohort of FTD and ALS cases identified the expansion in 11.7% of familial and 3% of sporadic FTD cases, and 23.5% of familial and 4.1% of sporadic ALS cases. The length of the expansion varied between 6.5–12 kb (700–1600 repeats), while the majority of controls contained two repeats.

Neuropathology in both FTLD and ALS cases with *C9ORF72* expansions showed TDP-43-positive neuronal and glial inclusions and a higher proportion of nuclear RNA foci in frontal cortex and spinal cord neurons. No unique clinical phenotype was associated with this subtype of ALS or FTD.

We sought to confirm the relevance of this important finding in a large cohort of patients with ALS/motor neuron disease (MND) from Northern England, and to investigate clinical and pathological differences between cases with and without the repeat expansion. These patients represent a well-characterized cohort of 563 ALS cases, including 63 familial ALS index cases, with serial clinical assessment performed every 2–3 months throughout the disease course. Post-mortem pathological evaluation was available in 28% of these cases.

Materials and methods

Cases with amyotrophic lateral sclerosis and controls

DNA was extracted from 42 familial ALS index cases and 363 patients with sporadic ALS from the Sheffield MND Blood DNA Biobank. Additional DNA samples were isolated from 21 familial ALS index cases and 137 sporadic ALS cases in the Sheffield Brain Tissue Bank. In total, 563 ALS cases were screened: 63 familial ALS index cases and 500 sporadic ALS cases. Familial ALS cases were defined as individuals with one or more first or second degree relatives with a confirmed diagnosis of ALS. All 563 cases were reviewed by a senior consultant neurologist (C.J.M. or P.J.S.) and diagnosed with definite or probable ALS, as defined by the El Escorial criteria (Brooks *et al*., 2000). A full family history was taken from each patient. Patients with known mutations in *SOD1* ($n = 14$), *TARDBP* ($n = 5$), *FUS* ($n = 4$), *ANG* ($n = 1$), *OPTN* ($n = 1$), charged multi-vesicular protein 2B (*CHMP2B*) ($n = 4$) and vesicle associated membrane protein 2B (*VAPB*) ($n = 1$) were included in the *C9ORF72* screening. DNA was extracted from blood using the Nucleon™ Blood and Cell Culture Genomic Extraction kit (Tepnel) according to the manufacturer's protocol, while DNA was extracted from fresh frozen cerebellar samples using the Soft Tissue DNA Extraction Kit (Tepnel). Control DNA ($n = 361$) was extracted from blood donated by partners or unrelated carers of patients with ALS. All samples were from UK Caucasians. The South Sheffield Research Ethics Committee approved the study, and informed consent was obtained for all samples.

Determination of the clinical phenotype of patients with ALS with the C9ORF72 hexanucleotide expansion

Clinical notes of patients found to carry the *C9ORF72* expansion were reviewed in a systematic fashion to identify details of the disease phenotype including gender, age of onset, disease duration, disease variant, details of family history and the presence of any cognitive impairment. It should be noted that patients in this cohort only underwent formal cognitive evaluation when a clinical problem was identified and did not undergo routine serial neuropsychological evaluation during the disease course. Therefore, the recorded incidence of frontotemporal dysfunction in this cohort is likely to be an underestimate, as prospective neuropsychological studies show a higher prevalence of frontal lobe dysfunction in patients with ALS (Phukan *et al*., 2011).

Screening for the C9ORF72 hexanucleotide repeat sequence by repeat primed polymerase chain reaction

Genomic DNA (100 ng) was amplified using the primers and method described by Renton *et al*. (2011) with a minor adjustment to the primer ratio: Forward:Reverse:Anchor = 8:1:8. Detailed methodology is provided in the Supplementary Material. Fragments were analysed on an ABI3730 capillary analyser (Applied Biosystems, Life Technologies Corporation) using a 60-s injection time. Fragment data were analysed using Peak Scanner Software (Applied Biosystems, Life Technologies Corporation).

Neuropathological evaluation

The brain and spinal cord tissues were donated to the Sheffield Brain Tissue Bank for research, with the consent of the next of kin. The donation procedure and use of the tissue in this project were undertaken with research ethical committee approval. Tissue was available for detailed pathological evaluation from 19 of the 22 brain tissue bank cases with the hexanucleotide expansion of *C9ORF72*. These cases were compared with up to 96 (see below and Table 3) ALS cases, which were negative for the *C9ORF72* expansion and three neurologically normal controls. For some cases, one cerebral hemisphere, half the midbrain and brainstem, a portion of the cerebellum and segments of the spinal cord at various levels were rapidly frozen in liquid nitrogen at autopsy and stored at -80°C. The remainder of the CNS was formalin-fixed. For the other cases, only a portion of the cerebellum was frozen, and the whole brain and spinal cord were formalin fixed. Selected blocks (including lumbar, thoracic and cervical spinal cord, medulla, midbrain, hippocampus, and frontal, temporal and motor neocortex) were processed to paraffin.

In addition to routine neurohistology with tinctorial preparations, immunohistochemistry was performed for p62/sequestosome 1, TAR DNA-binding protein 43 (TDP-43), FUS, OPTN, CD68 and C9ORF72 (Supplementary Table 1) where paraffin tissue was available. The latter antibody is commercially available (Santa Cruz Biotechnology Inc.) and was selected on the basis that it labelled a protein of the molecular weight of C9ORF72 on western blotting (data not shown). Immunohistochemistry was performed on all available cases with the hexanucleotide expansion as well as three cases with sporadic ALS without the expansion and three neurologically healthy controls.

To characterize the distribution of pathology in cases with ALS in the Sheffield Brain Tissue Bank, the extent of p62-positive pathology was assessed. In all regions assessed, a single 6-μm section was examined and the number of neuronal cytoplasmic inclusions in the region of interest assessed semi-quantitatively as 0–4 (low), 5–9 (intermediate) or 10 or more (high). The regions of interest assessed were the anterior horn of the spinal cord, at mid-cervical and lumbar levels; the hypoglossal, dorsal vagal and ambiguus nuclei of the medulla; the dentate granule cell layer and CA4 subregion of the hippocampus and the frontal and motor cortices. In the cortical regions, quantification of neuronal cytoplasmic inclusions was carried out in 10 fields ($\times 25$ objective).

In addition, the number of neuronal cytoplasmic inclusions in the CA4 subregion of the hippocampus was assessed using the same semi-quantitative scheme on sections that had been immunostained for TDP-43 and OPTN.

Statistical methods

Differences between phenotypic characteristics of groups were determined by an independent-samples *t*-test. Differences between gender ratios and site of onset and differences in the ubiquitylated neuronal cytoplasmic inclusion load were calculated using a chi-squared (χ^2) test. A significance level of $P < 0.05$ was used for all tests.

Results

Genetic screening

ALS-associated pathological expansions in *C9ORF72* have been defined as >30 repeats, whereas the majority of controls have

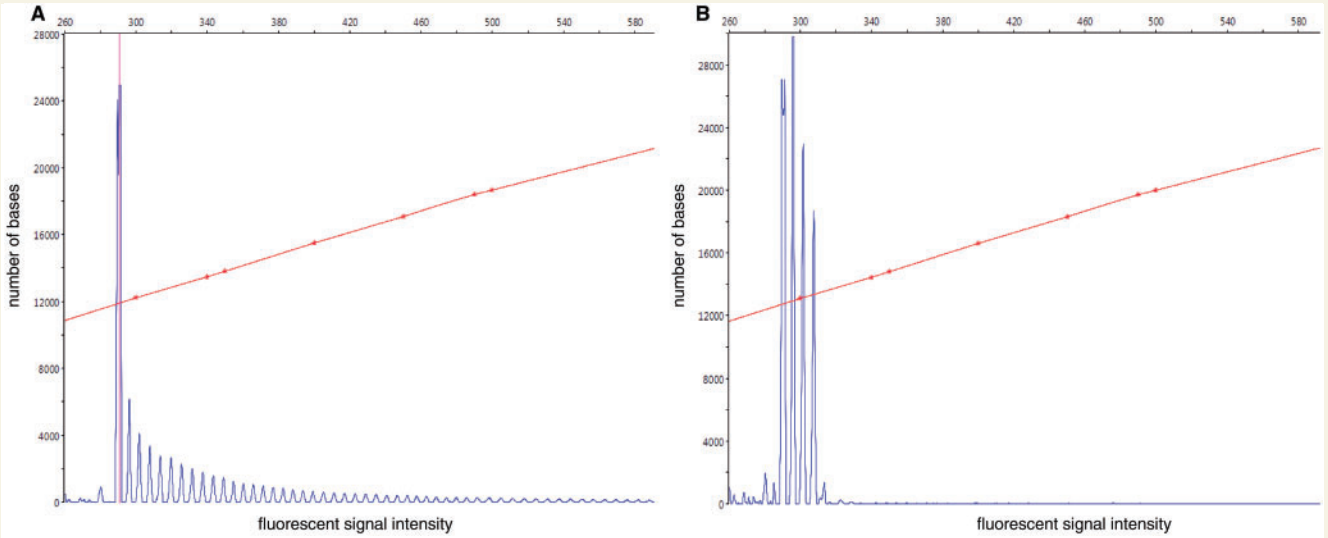

Figure 1 Graphical representation of fragments generated in repeat primed polymerase chain reaction displayed using Peak Scanner Software. Individual peaks represent fragments of increasing length at 6 bp intervals, corresponding to single GGGGCC repeats; 280 bp is minimum polymerase chain reaction product length. (**A**) Graph from a patient with ALS with a pathological expansion in *C9ORF72* showing exponentially tailing series of peaks consistent with a repeat length >30. (**B**) Graph from a patient with ALS without pathological expansion showing repeat length of approximately four repeats.

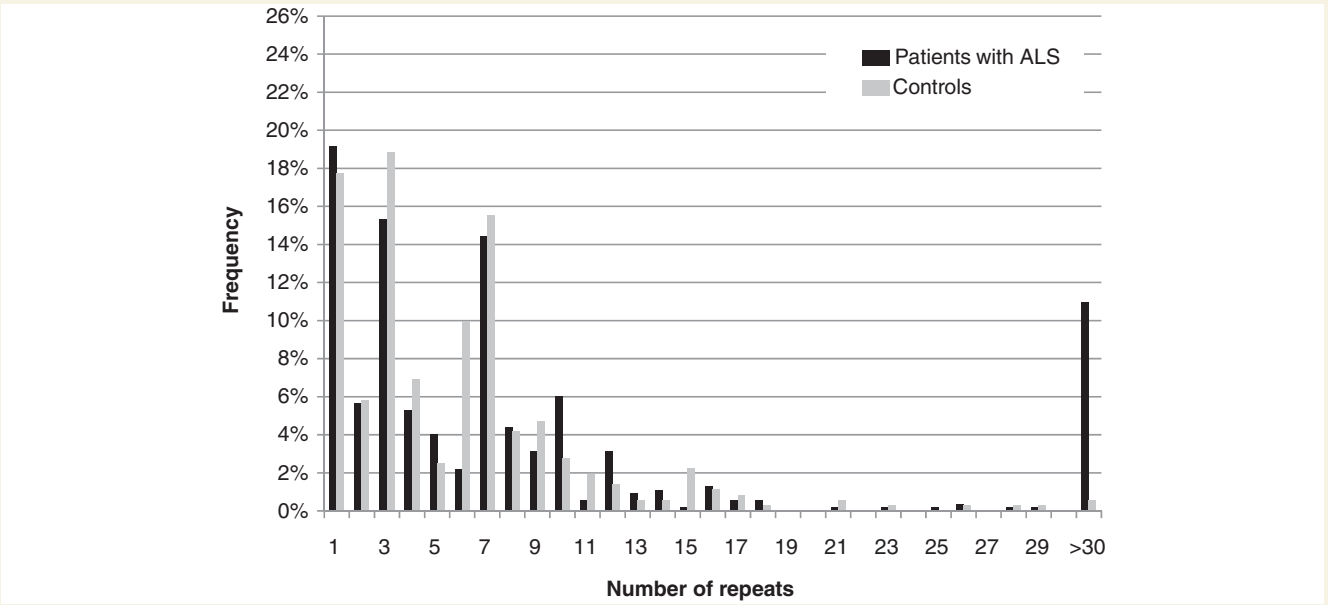

Figure 2 Graph showing the lengths of the GGGGCC hexanucleotide repeats in ALS cases ($n = 563$) and controls ($n = 361$); 62/563 (11%) of cases with ALS and 2/361 (0.6%) of control cases had repeat lengths of >30.

≤3 repeats (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). In our cohort of 563 ALS cases, 62 (11%) ALS cases (27 familial ALS, 35 sporadic ALS) were found to have expansions >30 repeats (Fig. 1). The median number of repeats in ALS cases without pathological expansions was four compared with five in controls (Fig. 2). The length of a pathologically expanded repeat (>30 repeats) cannot be accurately quantified by repeat primed polymerase chain reaction. This technique is only sufficient to segregate individuals with large expansions >30 from those without. An expanded repeat >30 was detected in two control cases (2/361 = 0.6%), a 76-year-old male and a 46-year-old female, neither of whom had any relevant past medical or family history.

Pathological expansions accounted for 27/63 (43%) of our familial ALS index cases. Analysis of family trees of all index cases revealed 13 families with a clear autosomal dominant pattern of inheritance and we were able to demonstrate segregation

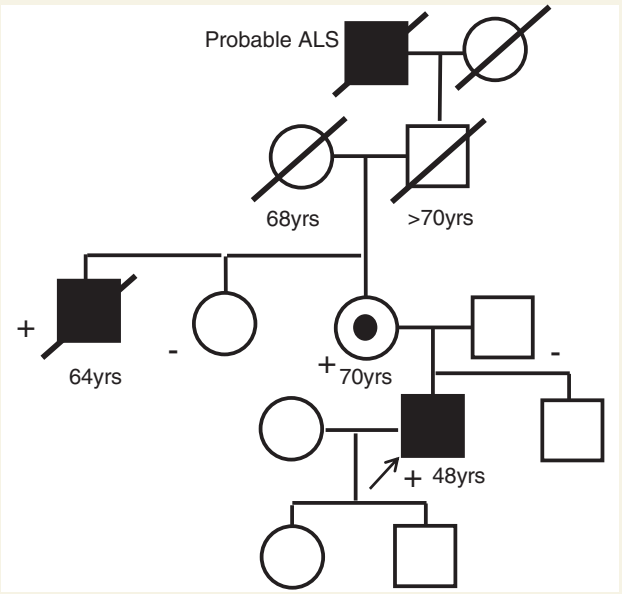

Figure 3 Family tree from a familial ALS case with the hexanucelotide repeat sequence in *C9ORF72*. The expansion segregated with disease in two cases and was present in an obligate carrier. + = carrier of expansion, − = confirmed absence of the expansion. Current age or age of death is shown where information was available.

of the expanded allele in one family with evidence of incomplete penetrance (Fig. 3). In familial ALS cases with previously identified mutations in *SOD1*, *TARDBP*, *FUS*, *ANG*, *CHMP2B* and *VAPB*, an expanded *C9ORF72* repeat >30 was identified in a case with an p.Ala321Val mutation in *TARDBP* (Kirby *et al*., 2010) and also in a case with a benign p.Gly174del alteration in *FUS* reported previously (Hewitt *et al*., 2010). An unreported case with a p.Glu322Lys *OPTN* substitution also showed an expansion.

Pathological expansions were also present in 35/500 (7%) of our cases with sporadic ALS. However, for 16/35 cases there was either a family history of dementia or other neurological or neuromuscular disease; or at least one parent died before the age of 70 years. It is therefore likely that a proportion of these cases represent unrecognized familial disease. For the remaining 19/35 sporadic ALS cases with the expansion, a complete family history was available and in these cases there was no family history of neurological disease and both parents lived into late old age. Therefore, it is likely that these cases represent true sporadic disease, although incomplete penetrance cannot be excluded. DNA from parents of these apparently sporadic cases was not available for analysis to confirm the occurrence of *de novo* mutations.

Clinical features of ALS cases with pathological expansions in C9ORF72

The clinical features of the cases with pathological expansions in *C9ORF72* are summarized in Table 1. The mean age of onset was 57.3 years (range 27–74 years). Of these, 60% of cases had limb onset disease, 31% had bulbar onset disease; 6% had multi-focal disease onset, one patient presented with dementia and for one patient the time of onset was not known. The mean disease duration from symptom onset in these patients was 30.5 months (range 7–60 months); three patients are alive at the time of writing and for one patient survival information was not available.

Clinical characteristics of patients with the *C9ORF72* expansion were compared with the remainder of the screened cohort (Table 2). The *C9ORF72* patients had a significantly lower age at onset than non-*C9ORF72* cases (mean age of onset 57.3 years, SD 8.9 years compared with mean 60.1 years, SD 12.3 years; $P = 0.03$, d.f. = 494, $t = 2.20$), but not significantly different to the overall familial ALS cohort ($P = 0.43$, d.f. = 107, $t = 0.78$). Likewise, patients with the *C9ORF72* expansion had a significantly shorter duration of disease than non-*C9ORF72* cases [mean (SD) duration of disease 30.5 (13.3 months) compared with mean (SD) 36.3 (28.5 months); $P = 0.01$, d.f. = 397, $t = 2.44$] but not significantly different to the overall familial ALS cohort ($P = 0.32$, d.f. = 100, $t = 1.00$). The subgroup of patients with sporadic ALS with the *C9ORF72* expansion differed from patients with familial ALS with the expansion, in that they had a significantly higher age of onset [mean 59.5 (SD 7.0 years) in sporadic ALS, 54.3 (SD 10.4 years) in familial ALS, $P = 0.03$, d.f. = 59, $t = 2.21$], but no difference in age of onset to the screened cohort overall. There was no difference between patients with sporadic ALS and patients with familial ALS with the expansion, with respect to duration of disease [mean (SD) 28.6 (12.0 months) in sporadic ALS, and 33.3 (14.8 months) in familial ALS, $P = 0.21$, d.f. = 56, $t = 1.26$].

In our cohort of patients with the *C9ORF72* expansion, 5/27 (19%) familial ALS cases and 5/35 (14%) sporadic ALS cases had evidence on clinical and neuropsychological testing of FTD. An additional 12 cases had a family history of dementia in first- or second-degree relatives, six of whom had familial ALS and six of whom had sporadic ALS. Overall 22/62 (35%) of patients with the expansion had either a personal diagnosis of dementia or a family history of dementia in first- or second-degree relatives. In this cohort, routine neuropsychological assessment was not performed in the absence of a clinically apparent cognitive problem, so subclinical cognitive dysfunction was not evaluated.

Several of the patients carrying the hexanucleotide expansion in *C9ORF72* were either diagnosed with or had a family history of other non-dementia neurological or neuromuscular disease, particularly neurodegenerative disease: four patients had a family history of Parkinson's disease and one patient had a comorbid diagnosis of Parkinson's disease that was confirmed at post-mortem; thus 4/62 (6.5%) patients had either a diagnosis of Parkinson's disease or a family history of Parkinson's disease. Two patients had evidence of demyelinating disease and one further patient had a family history of multiple sclerosis; thus 3/62 (5%) patients had either a diagnosis or a family history of demyelinating disease. Other noteworthy findings included a patient with a family history of ALS, Charcot–Marie–Tooth disease and dementia; one patient with early onset cataracts which also occurred in his mother; one patient with a family history of Huntington's disease and one patient with a brother who died with a diagnosis of muscular dystrophy.

Table 1 Summary of phenotypic information from patients with the hexanucleotide repeat expansion of *C9ORF72*

Patient	Sex	Age at onset (years)	Duration of disease (months)	Site of onset	Variant of disease	Family history	Cognitive impairment	Family history and other noteworthy features
Brain Tissue Bank								
1	Male	69	26	Limb	ALS	Familial	None	Autosomal dominant pattern of familial ALS
2	Female	59	40	Limb	ALS	Familial	None	Autosomal dominant pattern of familial ALS
3	Male	42	50	Limb	ALS	Familial	None	Autosomal dominant pattern of familial ALS
4	Male	66	14	Bulbar	ALS	Familial	NA	Autosomal dominant pattern of familial ALS
5	Male	64	31	Limb	ALS	Familial	Undefined dementia	Nephew is Patient 27.
6	Female	62	24	Bulbar	ALS	Familial	None	Autosomal dominant pattern of familial ALS. Patient diagnosed with multiple sclerosis. Benign polymorphism Gly174del in FUS/TLS.
7	Female	56	43	Limb	ALS	Familial	Diagnosed FTD	Maternal family history of ALS, Charcot–Marie–Tooth and early onset undefined dementia.
8	Female	50	28	Bulbar	ALS	Familial	None	Autosomal dominant pattern of familial ALS. Patient carries a p.Glu322Lys substitution in *OPTN*
9	Male	47	19	Multi-focal	ALS	Familial	None	Autosomal dominant pattern of familial ALS. Patient and his father diagnosed with Parkinson's disease.
10	Female	63	43	Cognitive	ALS	Familial	Diagnosed FTD	Mother diagnosed with early onset undefined dementia. Brother diagnosed with ALS.
11	Female	61	58	Limb	ALS	Familial	None	Two sisters diagnosed with ALS
12	Female	65	12	Bulbar	ALS	Sporadic	None	
13	Female	67	26	Limb	NA	Sporadic	None	
14	Male	63	11	Limb	ALS	Sporadic	None	
15	Male	56	13	Bulbar	ALS	Sporadic	None	
16	Female	61	40	Bulbar	ALS	Sporadic	Undefined dementia	
17	Female	58	7	Limb	ALS	Sporadic	None	
18	Female	61	42	Limb	ALS	Sporadic	None	
19	Male	62	20	Bulbar	ALS	Sporadic	None	
20	Male	45	14	Limb	ALS	Sporadic	None	
21	Female	51	38	Multifocal	ALS	Sporadic	None	
Blood DNA Biobank								
22	Female	47	18	Limb	ALS	Familial	Diagnosed FTD	Autosomal dominant pattern of familial ALS
23	Male	61	38	Limb	ALS	Familial	None	Brother and sister diagnosed with ALS. Father diagnosed with Huntington's disease.
24	Female	27	14	Bulbar	ALS	Familial	None	Autosomal dominant pattern of familial ALS. Maternal family history of AD; paternal family history of undefined dementia.
25	Female	50	18	Bulbar	ALS	Familial	Undefined dementia	Father diagnosed with FTD-ALS. Autosomal dominant pattern of familial ALS
26	Female	48	52	Limb	ALS	Familial	None	Brother diagnosed with ALS. Maternal aunt diagnosed with Alzheimer's disease. Patient notably athletic.
27	Male	48	ALIVE	Bulbar	ALS	Familial	None	Maternal uncle is Patient 5. Maternal grandmother diagnosed with early onset dementia.
28	Female	61	22	Limb	ALS	Familial	None	Autosomal dominant pattern of familial ALS
29	Female	44	43	Bulbar	ALS	Familial	None	Autosomal dominant pattern of familial ALS
30	Female	61	43	Bulbar	NA	Familial	None	Paternal uncle diagnosed with ALS. Father diagnosed with Parkinson's disease.
31	Female	64	ALIVE	Limb	ALS	Familial	None	Brother diagnosed with ALS. Paternal grandfather diagnosed with undefined dementia.
32	Female	45	34	Bulbar	ALS	Familial	NA	Autosomal dominant pattern of familial ALS. Mother diagnosed with FTD-ALS. Maternal grandmother diagnosed with multiple sclerosis.
33	Male	51	17	Limb	ALS	Familial	None	Sister diagnosed with FTD-ALS. Patient and mother diagnosed with early onset cataracts.
34	Male	65	52	Limb	ALS	Familial	None	Nephew diagnosed with ALS. Patient notably athletic
35	Male	63	13	Multifocal	ALS	Familial	None	Paternal aunt diagnosed with ALS
36	Female	NA	NA	NA	NA	Familial	NA	
37	Female	37	58	Limb	ALS	Familial	None	Mother probable ALS. Patient has pAla321Val mutation in *TARDBP*
38	Male	56	24	Limb	ALS	Sporadic	None	

(continued)

Table 1 Continued

Patient	Sex	Age at onset (years)	Duration of disease (months)	Site of onset	Variant of disease	Family history	Cognitive impairment	Family history and other noteworthy features
39	Male	56	41	Limb	ALS	Sporadic	None	
40	Female	66	32	Bulbar	ALS	Sporadic	None	Father died of undefined dementia
41	Male	60	35	Bulbar	ALS	Sporadic	Undefined dementia	Mother diagnosed with Alzheimer's disease
42	Female	50	27	Limb	ALS	Sporadic	None	Father diagnosed with early onset dementia.
43	Male	60	32	Limb	ALS	Sporadic	None	Mother diagnosed with early onset dementia. Patient notably athletic.
44	Male	43	40	Limb	ALS	Sporadic	None	Patient notably athletic.
45	Male	64	28	Limb	ALS	Sporadic	None	
46	Male	74	36	Limb	ALS	Sporadic	None	Father and sister diagnosed with undefined dementia.
47	Male	58	60	Limb	ALS	Sporadic	None	Onset coincided with carpal tunnel syndrome therefore difficult to determine exact date.
48	Female	57	20	Limb	ALS	Sporadic	None	Demyelination noted on MRI of CNS.
49	Female	62	24	Limb	ALS	Sporadic	None	Maternal aunt diagnosed with Alzheimer's disease. Patient notably athletic
50	Female	57	21	Multifocal	ALS	Sporadic	None	
51	Male	60	27	Bulbar	ALS	Sporadic	None	
52	Male	63	ALIVE	Limb	ALS	Sporadic	Undefined dementia	Brother and father diagnosed with schizophrenia
53	Male	61	24	Limb	ALS	Sporadic	None	Mother diagnosed early onset Alzheimer's disease and Parkinson's disease. Patient notably athletic.
54	Male	51	21	Bulbar	ALS	Sporadic	None	Previous poliomyelitis which left him with wasted right leg
55	Male	50	27	Bulbar	ALS	Sporadic	None	
56	Female	59	22	Limb	ALS	Sporadic	Undefined dementia	
57	Female	71	24	Limb	ALS	Sporadic	Diagnosed FTD	
58	Female	65	40	Limb	ALS	Sporadic	None	Patient notably athletic.
59	Female	52	24	Limb	ALS	Sporadic	NA	
60	Female	63	28	Bulbar	ALS	Sporadic	NA	
61	Female	71	57	Limb	ALS	Sporadic	None	Cousin diagnosed with Parkinson's disease
62	Male	65	36	Limb	ALS	Sporadic	None	Brother suffered muscular dystrophy

NA = data not available.

Table 2 Comparison of phentotypic information from patients the hexanucleotide repeat expansion of *C9ORF72* and the overall screened cohort

Phenotype	*C9ORF72* cases ($n = 62$)	*C9ORF72* sporadic ALS cases ($n = 35$)	*C9ORF72* familial ALS cases ($n = 27$)	Non-*C9ORF72* ALS cases in the screened cohort ($n = 501$)
Mean (SD) age of onset (years)	57.3 (8.9)	59.5 (7.0)	54.3 (10.4)	60.1 (12.3)
Mean (SD) disease duration (months)	30.5 (13.3)	28.6 (12.0)	33.3 (14.8)	36.3 (28.5)
Limb onset (%)	60	66	51	56
Bulbar onset (%)	31	29	34	25
Gender ratio (males:females)	1:1.2	1.1:1	1:1.7	1.3:1

The patients with *C9ORF72* expansion had limb onset disease in 37/62 (60%) cases and bulbar onset disease in 19/62 (31% cases) (Table 2). These proportions are similar to those for the cohort without the *C9ORF72* expansion (56 and 25%, respectively). There was no significant difference in gender ratio ($P = 0.09$, d.f. = 1, $\chi^2 = 2.85$) or site of onset ($P = 0.74$, d.f. = 1, $\chi^2 = 0.10$) between the two groups although the patients with familial ALS with the expansion had a female preponderance (ratio males:females = 1:1.7), which was not present in the patients with sporadic ALS with the expansion (males: females = 1.1:1) or the screened cohort overall (males:females = 1.3:1).

Neuropathological features

All cases with the hexanucleotide repeat expansion of *C9ORF72* showed the classical molecular pathology of ALS (Figs 4 and 5). There was a marked loss of lower motor neurons in the anterior horns of the spinal cord and cranial nerve motor nuclei in the medulla. Bunina bodies were present in some residual motor neurons. All cases showed a moderate to marked microglial reaction on CD68 immunohistochemistry in the pyramidal tract at all levels (white matter underlying motor cortex, mid-crus cerebri,

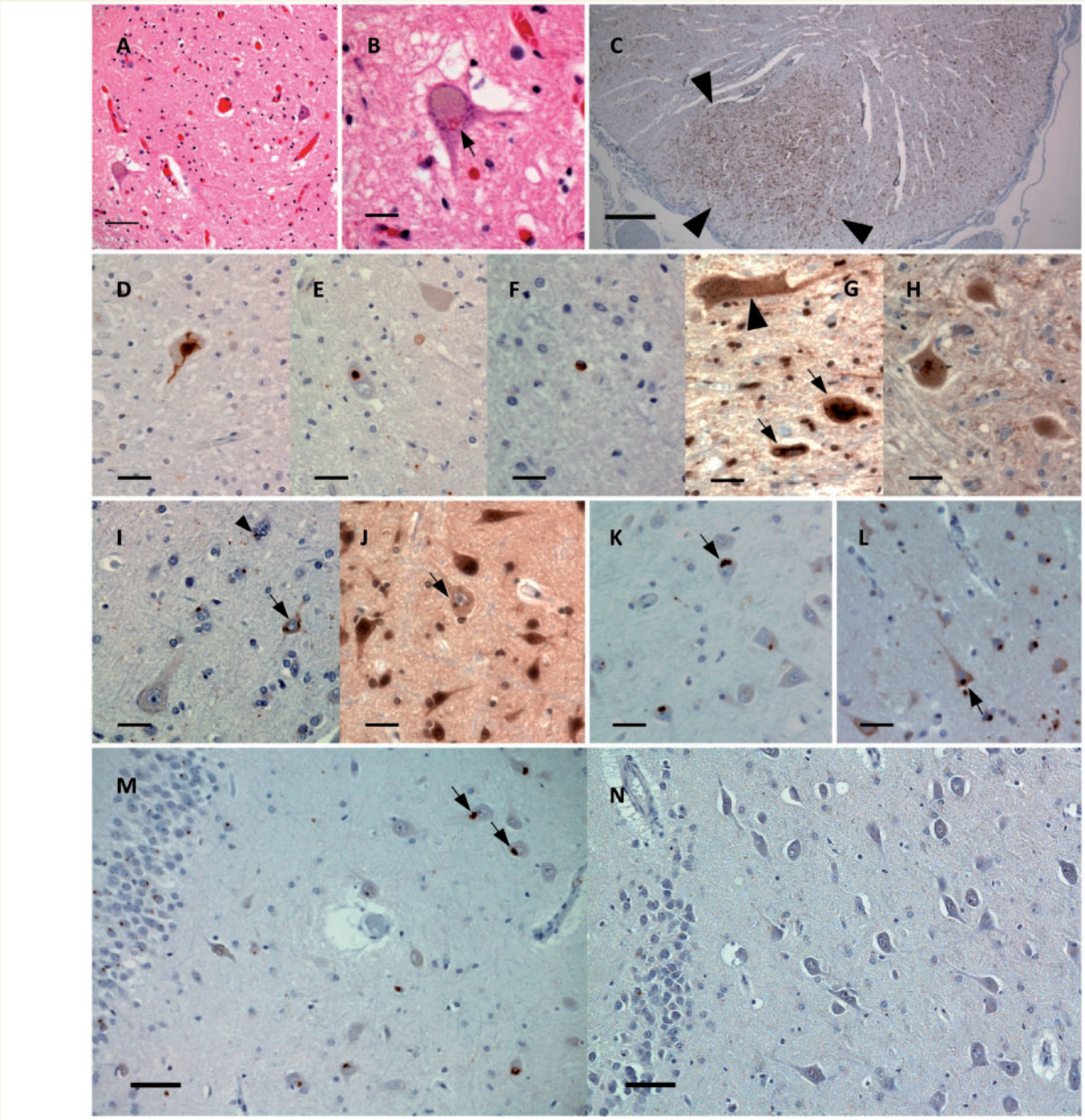

Figure 4 Histological examination of cases with the *C9ORF72* hexanucleotide repeat expansion reveals: depletion of motor neurons from the anterior horns of the spinal cord (**A**) with Bunina bodies (arrow) in residual neurons (**B**); Microglial activation in lateral corticospinal tracts (arrowheads, **C**); ubiquitylated neuronal (skein-like in **D** and compact in **E**) and glial (**F**) cytoplasmic inclusions in the anterior horns of the spinal cord; (**G**) TDP-43 positive skein-like neuronal cytoplasmic inclusions (arrows) and pre-inclusions (arrowhead) in the anterior horns of the spinal cord; (**H**) OPTN positive neuronal cytoplasmic inclusion in the anterior horns of the spinal cord; (**I**) ubiquitylated neuronal (arrow) and glial (arrowhead) cytoplasmic inclusions in the motor cortex; TDP-43 positive neuronal cytoplasmic inclusion in the motor cortex (arrow, **J**); ubiquitylated neuronal cytoplasmic inclusions (arrows) in the CA4 subfield of the hippocampus (**K**) and frontal neocortex (**L**). Lower power view of hippocampal CA4 subfield with adjacent dentate gyrus granule cells (*left*) reveals neuronal cytoplasmic inclusions (arrows) in CA4 of a case of ALS with *C9ORF72* hexanucleotide repeat expansion (**M**) and none in a case without this expansion (**N**). Preparations: haematoxylin and eosin, **A** and **B**; CD68, **C**; p62, **D–F**, **I**, **K–N**; TDP-43, **G** and **J**; optineurin, **H**. Scale bar = 20 μm (**B** and **F**); 30 μm (**D**, **E**, **G** and **H–L**); 60 μm (**A**, **M** and **N**); 500 μm (**C**).

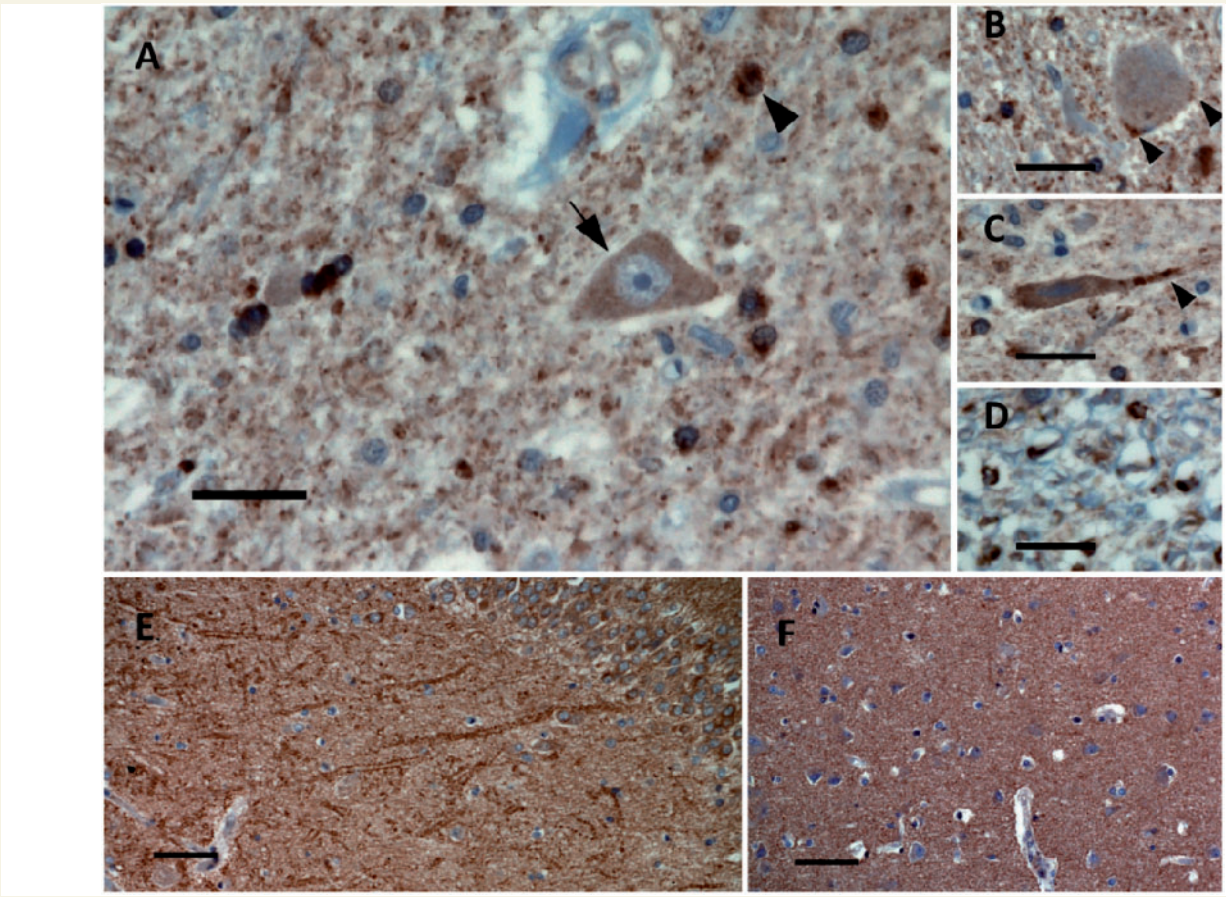

Figure 5 Immunohistochemistry for C9ORF72 shows similar features in neurologically healthy controls and individuals with motor neuron disease with and without the hexanucleotide repeat expansion. The spinal cord stained with C9ORF72 antibody shows: (**A**) Coarse granular staining of the spinal cord anterior horn neuropil with variable cytoplasmic labelling of motor neurons (arrow) and perinuclear labelling of small glial cells (e.g. arrowhead); patches of more intense labelling (arrowheads) on the surfaces of some motor neurons (**B**) that is often more marked in neuronal processes (**C**). In white matter (dorsal column) there is staining around axons corresponding to portions of myelin sheath (**D**). Granular staining of the neuropil with more prominent 'streaks' and only pale neuronal expression is seen in the hippocampus (CA4 subregion with dentate gyrus granule cell layer top right; **E**). Granular neuropil labelling with lesser staining of neurons is also seen in the neocortex (frontal cortex; **F**). Scale bar = 20 μm (**B** and **F**); 30 μm (**A–D**); 60 μm (**E** and **F**).

medullary pyramids, and lateral and anterior corticospinal tracts) indicating corticospinal tract degeneration.

Immunohistochemistry for p62 revealed neuronal and glial cytoplasmic inclusions in the anterior horns of the spinal cord, cranial nerve motor nuclei and motor cortex. These were also detectable using both TDP-43 and OPTN immunohistochemistry. Results of the quantitation of neuronal cytoplasmic inclusions using p62 immunohistochemistry are presented in Table 3. In general terms, there was a significantly higher load of neuronal cytoplasmic inclusions in all regions in individuals with the *C9ORF72* repeat expansion compared with ALS cases without this expansion.

The most striking feature of cases bearing the *C9ORF72* hexanucleotide expansion was the degree of extra-motor p62 positive neuronal cytoplasmic inclusion pathology in contrast to the relative paucity of this finding in cases without the expansion. This feature was most pronounced in the CA4 and CA3 subregions of the hippocampus and, to a lesser extent, the frontal neocortex and dentate gyrus of the hippocampus. To avoid potential bias, hippocampal and frontal neocortex slides prepared for p62 immunohistochemistry from 11 cases with and 11 cases without the expansion were randomly selected and reassessed blind to case identity and *C9ORF72* gene status. For the frontal neocortex, low, intermediate and high levels of neuronal cytoplasmic inclusions were seen in 0, 1 and 10 cases with the expansion and 10, 1 and 0 cases without the expansion, respectively ($\chi^2 = 20$, d.f. = 2, $P < 0.0005$). For the dentate fascia of the hippocampus, low, intermediate and high levels of cytoplasmic inclusions were seen in 3, 1 and 7 cases with the expansion and 9, 0 and 2 cases without the expansion, respectively ($\chi^2 = 6.778$, d.f. = 2, $P = 0.034$). For the CA4 subfield of the hippocampus, high levels of neuronal cytoplasmic inclusions were seen in all 11 cases with the hexanucleotide expansion and low levels in all 11 cases without the expansion ($\chi^2 = 22.000$, d.f. = 1, $P < 0.0005$). The slides were assessed independently by a second neuropathologist who assigned all 22 cases correctly into groups with

Table 3 Numbers of cases with low, intermediate and high levels of neuronal cytoplasmic inclusion pathology in cases with and without the *C9ORF72* hexanucleotide repeat expansion in different regions of the CNS

C9ORF72 repeat expansion	Total number of cases				Per cent			Chi-squared test		
	Low	Intermediate	High	Total	Low	Intermediate	High	χ^2	d.f.	*P*-value
Lumbar cord anterior horns										
Present	3	6	7	16	19	38	44	23.657	2	<0.0005
Absent	32	10	1	43	74	23	2			
Cervical cord anterior horns										
Present	7	5	3	15	47	33	20	6.243	2	0.044
Absent	32	6	2	40	80	15	5			
Medulla motor nuclei										
Present	8	1	6	15	53	7	40	7.800	2	0.02
Absent	12	3	0	15	80	20	0			
Dentate gyrus of hippocampus										
Present	0	0	17	17	0	0	100	36.780	2	<0.0005
Absent	66	8	22	96	69	8	23			
Hippocampus CA4 subfield										
Present	0	0	17	17	0	0	100	67.501	1	<0.0005
Absent	93	0	0	93	100	0	0			
Frontal neocortex										
Present	0	1	18	19	0	5	95	74.495	2	<0.0005
Absent	92	1	1	94	98	1	1			

Table 4 Numbers of cases with *C9ORF72* hexanucleotide repeat showing low, intermediate and high numbers of neuronal cytoplasmic inclusions in the hippocampal dentate gyrus granule cell layer and CA4 subfields using immunohistochemistry for p62, TDP-43 and OPTN

CNS region	p62, *n* (%)	TDP-43, *n* (%)	OPTN, *n* (%)
Dentate gyrus			
Low	0 (0)	10 (58.8)	13 (81.25)
Intermediate	0 (0)	3 (17.6)	2 (12.5)
High	17 (100)	4 (23.5)	1 (6.25)
CA4			
Low	0 (0)	15 (88.2)	4 (25)
Intermediate	0 (0)	1 (5.9)	2 (12.5)
High	17 (100)	1 (5.9)	10 (62.5)

and without the hexanucleotide expansion on the basis of p62-immunoreactive pathology in the CA4 hippocampal subfield. There was thus 100% inter-rater agreement between the assessments of the two observers. Next, the proportion of the neuronal cytoplasmic inclusion pathology seen in the hippocampus that was detected by TDP-43 and OPTN immunohistochemistry was investigated (Table 4). This was markedly less than was seen on p62 immunohistochemistry; in the CA4 subregion, OPTN antibodies detect greater numbers of inclusions than TDP-43 antibodies. The converse is true for the dentate gyrus.

The hippocampal and neocortical p62 labelling took the form of neuronal and glial inclusions in all cortical layers with very few dystrophic neurites, corresponding to Type B according to the harmonized classification system for FTLD-TDP pathology (Mackenzie *et al.*, 2011).

A single case with the hexanucleotide repeat expansion showed combined ALS and multiple sclerosis pathology. This case had previously been demonstrated to also have a benign polymorphism (p.Gly174del) in the gene *FUS* and has been described elsewhere (see Case 4, Hewitt *et al.*, 2010).

Immunohistochemistry for C9ORF72 revealed fine punctate staining throughout the grey matter structures of the CNS. Neuronal cell bodies showed only pale to moderate intensity of staining. There were occasional patches of staining on the cell membrane that was more prominent on axonal hillocks. Neuronal nuclei were negative for C9ORF72. This pattern of staining was reminiscent of that seen in preparations for synaptic markers. There was positive staining of smaller glial cells, mostly having the appearance of oligodendrocytes. In white matter tracts, there was partial circumferential staining around axons, with some

granularity to the staining pattern. In the hippocampal pyramidal cell layer, there was coarse staining of the neuropil that was most intense in the CA4 and CA3 subfields. The intensity of this staining was much less marked in CA2 and CA1. In the hippocampus, the intense C9ORF72 labelling was seen in the same regions that also demonstrated ubiquitylated neuronal cytoplasmic inclusions. However, no difference in the pattern of immunostaining for C9ORF72 was observed between cases with and without the hexanucleotide expansion or in the neurologically healthy control cases.

Discussion

In keeping with the recent reports (DeJesus-Hernandez *et al*., 2011; Renton *et al*., 2011), we find significant variability in the presentation of ALS in patients carrying the *C9ORF72* hexanucleotide expansion so that patients with the expansion manifest the full range of the known ALS phenotype. In addition, we have identified a number of interesting associations between ALS and other neurological diseases in this group of patients. This may suggest that the effect of *C9ORF72* expansions is modified by other genetic or environmental factors to produce variability in the ALS phenotype and even in the area of the nervous system affected. Importantly, we find that with the exception of the granule cells of the dentate fascia, carriers of the *C9ORF72* hexanucleotide repeat expansion account for the extra-motor pathology in our series.

Patterns of inheritance

Hexanucleotide expansions of *C9ORF72* account for a large proportion (43%) of familial ALS cases in our cohort from the North of England and many of these families exhibit clear autosomal dominant inheritance. The high incidence of the *C9ORF72* repeat expansions in apparently sporadic cases is an important observation to explain. It may represent a genuinely inherited pathogenic genotype with variable penetrance or it may represent a high rate of new mutation consistent with the suggestion that the *C9ORF72* gene may be inherently unstable (Renton *et al*., 2011). That incomplete penetrance can occur is demonstrated by a family within our cohort where the expansion is present in the index case and his maternal uncle who are both patients with ALS, and in the mother of the index case who is disease free into old age (Fig. 3). It is noteworthy that for a proportion of the apparently sporadic cases there is a family history of neurological disease; it seems likely that some of these cases represent unrecognized or undisclosed familial disease. In particular, some of the patients with relatives who developed dementia may in fact have familial ALS/FTD. It is interesting that, with regard to age of onset and gender ratio but not disease length, the patients with sporadic ALS with the *C9ORF72* expansion are distinct from the patients with familial ALS with the expansion. The earlier age of onset of familial ALS cases with the mutation may suggest the occurrence of genetic anticipation, although this could not be confirmed in this study as the precise length of the pathological repeat sequences has not yet been determined.

Of note, we have now identified a pathogenic change in 42/63 (67%) of our familial cohort. In two cases, an expansion in *C9ORF72* was found to coexist with another identified putative mutation linked to ALS. That the expansion was absent from most of our patients with a previously identified mutation is supportive of the independent pathogenesis of those changes. An expansion was found in one case (Patient 37) who had a p.Ala321Val change in *TARDBP*. The pathogenesis of mutations in the C-terminal region of TARDBP is well established (Sreedharan *et al*., 2008; Kirby *et al*., 2010) and it is notable that the remainder of patients in our cohort with described changes in *TARDBP* did not carry the *C9ORF72* expansion. It is interesting that the age of onset in this patient was relatively young (37 years), perhaps consistent with a synergistic effect of both genetic changes on the disease pathogenesis. However, the disease duration in this patient was relatively long at 58 months. Since mutations in *TARDBP* and expansions of *C9ORF72* are both associated with neuronal cytoplasmic TDP-43 inclusions, it is conceivable that they may be affecting the same pathway in a non-additive manner. Unfortunately, no post-mortem material was available from this case.

An unreported familial ALS case with a p.Glu322Lys *OPTN* substitution also showed a *C9ORF72* expansion. While this substitution has been reported as a polymorphism in sub-Saharan African samples (Liu *et al*., 2008), it was absent from 375 neurologically normal Caucasian controls from the same geographical region. This patient had bulbar onset ALS, with onset of disease aged 50 years and a disease course of 29 months.

Dementia

An association between ALS and FTD is well established (Phukan *et al*., 2007). However, a previous population study reported that the incidence of dementia (5%) in relatives of patients with ALS was only slightly higher than in controls (Huisman *et al*., 2011) and only in first-degree relatives. ALS is thought to be the common end-point of various disease mechanisms (Ferraiuolo *et al*., 2011) and it is possible that only some of these mechanisms result in an associated dementia. Thus, disease heterogeneity may have meant that a stronger association in some patients within this population study was masked by a lack of association in other patients. In our cohort of patients with an expansion in *C9ORF72*, 35% either had a diagnosis of dementia or a family history of dementia. We propose that the subtype of ALS caused by hexanucleotide repeat expansions of *C9ORF72* shows a striking association with dementia clinically. This proposal is strongly supported by our finding of extra-motor neuropathology in all *C9ORF72* expansion cases examined at autopsy, in combination with a relative paucity of extra-motor pathology in cases found to have normal repeat lengths of *C9ORF72*. This hypothesis would be supported further by routine cognitive testing of ALS cases during life, with subsequent correlation with post-mortem neuropathology. The TDP-43 proteinopathies ALS, ALS with FTLD and pure FTLD-TDP have often been seen as a continuum of disease at both the clinical (Lillo and Hodges, 2009) and pathological levels (Mackenzie and Feldman, 2005; Geser *et al*., 2009). This has raised the question of what governs where on this spectrum

a patient with a TDP-43 proteinopathy will manifest disease pathologically, and how they will present clinically. Our data suggest that repeat expansions of *C9ORF72* have a strong influence on the clinical phenotype and the spectrum of pathology. The precise mechanism(s) by which the repeat expansion has this effect needs to be elucidated, and it will clearly be of interest to compare the distribution of pathology in brains of FTLD-TDP cases with and without the repeat expansion.

Other neurological disease

Several of the patients carrying the hexanucleotide expansion in *C9ORF72* were either diagnosed with or had a family history of other neurological, particularly neurodegenerative disease. This is illustrated by the case with both ALS and multiple sclerosis confirmed at autopsy. Previous work has suggested that the clinical spectrum of ALS may be wider than initially recognized. In particular, mutations in the *VCP* gene appear to cause a clinical spectrum including inclusion body myositis, FTD and Paget's disease (Johnson *et al*., 2010). ALS has also been reported to coexist with glaucoma in a patient with a mutation in the *OPTN* gene, although the authors went on to suggest that this may have been a coincidence (Maruyama *et al*., 2010). Analysis of our cohort suggests that expansion of *C9ORF72* may also produce a broad clinical phenotype although we have not yet demonstrated segregation of the expansion with non-ALS neurological disease.

A previous population study on the Island of Guam described coexistence of Parkinson's disease and ALS both within individuals and within families (Yanagihara *et al*., 1983). However, the results of other population studies investigating the coexistence of more than one neurodegenerative disease have been inconsistent. A recent population-based study suggested that the rate of Parkinson's disease among relatives of patients with ALS was not significantly different from controls (Huisman *et al*., 2011). However, it is noteworthy that among our patients with the hexanucleotide repeat sequence, the frequency of Parkinson's disease in relatives (6.5%) is much higher than in either the disease or control population from the Huisman study (0.9%). One of our patients with the *C9ORF72* expansion was diagnosed with both Parkinson's disease and ALS. His Parkinson's disease developed at a relatively early age of 38 years but was otherwise clinically typical, showing a good response to L-DOPA therapy; he had a deep brain stimulator inserted aged 47 years. Post-mortem he had neuropathological features of both Parkinson's disease and ALS including cell loss from the substantia nigra and 6/6 Braak grade α-syncluclein pathology with substantial staining both in the substantia nigra and in neocortical regions.

The coexistence of multiple sclerosis and ALS has been previously reported (Hader *et al*., 2008; Ismail *et al*., manuscript under review) and an increased incidence of multiple sclerosis in the offspring of patients with ALS has also been reported (Hemminki *et al*., 2009). The association of patients with ALS with the *C9ORF72* expansion and multiple sclerosis in our cohort is consistent with these reports and raises the possibility that the association between ALS and multiple sclerosis may be related to this genotype. The diagnosis of Huntington's disease in the father of a patient carrying the *C9ORF72* expansion is especially interesting given that Huntington's disease is also related to an aberrant repeat sequence. Perhaps a common underlying mechanism has led to the *de novo* occurrence of nucleotide repeat sequences in both of these individuals.

Neuropathological features

Qualitatively, the neuropathological features associated with hexanucleotide repeat expansion of *C9ORF72* are those of classical ALS with ubiquitylated, TDP-43 and OPTN positive neuronal and glial cytoplasmic inclusions in upper and lower motor neurons as well as glia. This is seen in combination with Bunina bodies and degeneration of the pyramidal tracts. What is striking is the association of pyramidal cell pathology with the expansion in the hippocampus and frontal neocortex, while very little such pathology is seen in ALS cases without the repeat expansion. This differentiation is less marked in the context of the granule cells of the hippocampal dentate gyrus, a structure that is generally believed to be one of the most sensitive extra-motor structures to TDP-43 proteinopathy (Mackenzie and Feldman, 2003; Takeda *et al*., 2009). The extra-motor pathology seen is best characterized as type B according to the scheme of Mackenzie *et al*. (2011).

The subdivision of ALS cases into two subgroups on the basis of neuropathological involvement of extra-motor (principally hippocampal and frontotemporal neocortex) has been described elsewhere (Nishihira *et al*., 2008). Data presented in this report indicate that repeat expansions in *C9ORF72* represent a major molecular basis for this pathological dichotomy.

It appears from our data derived from a large pathological cohort of ALS autopsies that the finding of extra-motor neocortical pathology, in particular ubiquitylated neuronal cytoplasmic inclusions in the CA4 subfield of the hippocampus is a relatively reliable indicator of the presence of a hexanucleotide repeat expansion in *C9ORF72*. Given that these changes can be detected at autopsy with relative ease, this neuropathological feature may be used to guide genetic investigation for the repeat expansion and thereby inform genetic counselling and further research. Interestingly, along with the characteristic hyaline conglomerate inclusions found in some patients with *SOD1* mutations (Ince *et al*., 1998), this report adds another important element to neuropathological predictors of genotype in ALS. It is also noteworthy that in the hippocampus, the majority of the neuronal cytoplasmic inclusion pathology that is evident on p62 immunohistochemistry is not apparent on TDP-43 immunohistochemistry. In fact, for the CA4 subregion (where pathology is most specific for the repeat expansion), OPTN is a more sensitive marker than TDP-43, although neither is as sensitive as p62. This raises the significant issue of what protein forms the ubiquitylated lesion in the majority of these neurons if it is not TDP-43.

Immunohistochemistry to C9ORF72 protein reveals multiple minute, puncta of labelling throughout the neuropil of grey matter structures having the appearance of synaptic labelling in neuronal processes. In this hippocampus, this is most marked in the regions that show pathology that is specific for the hexanucleotide repeat expansion. There is additionally labelling of glial cells, many of which appear to be oligodendrocytes, and around axons, corresponding to some regions of myelin. These constitute

initial observations, and the precise nature of C9ORF72 expression requires more detailed characterization, including finer localization studies, in order to begin to understand the function of the C9ORF72 protein in the CNS.

Potential mechanism of neurodegeneration in C9ORF72-related ALS

ALS and FTD now join a growing number of neurodegenerative disorders caused by expansions in repeat regions. In diseases such as Huntington's disease where the expansion is located in a coding region and is translated, the mechanism leading to neurodegeneration seems to be clear; toxic accumulation of the mutant protein that subsequently disrupts a variety of cellular processes. However, in other diseases, such as myotonic dystrophy type 1 and several of the spinocerebellar ataxias, as in *C9ORF72*, the pathological expansion is located in a non-coding region of the gene, suggesting that the RNA species itself may be toxic. Several lines of evidence are developing as to how RNA toxicity may be mediated (Todd and Paulson, 2009). In myotonic dystrophy type 1, the mutant RNA has been shown to sequester several splicing factors. This results in the formation of RNA foci within the cell nucleus and also leads to downstream messenger RNA splicing defects that are responsible for the features of muscle fibre atrophy and insulin resistance found in these patients (Fugier *et al*., 2011). Consistent with this as a potential mechanism occurring in ALS/FTD cases with expansions in *C9ORF72*, DeJesus-Hernandez *et al*. (2011) demonstrated the presence of increased numbers of neuronal RNA foci in ALS cases with the expansion. Additionally, levels of C9ORF72 protein in lymphoblastoid cell lines and frontal cortex samples taken from patients with FTD with pathological expansions were not significantly different to those from patients with FTD without expansions (DeJesus-Hernandez *et al*., 2011). The splicing factor sequestration hypothesis also links *C9ORF72* expansions to the TDP-43 pathology present in these cases; the latter being implicated in alternative splicing of multiple transcripts (Buratti and Baralle, 2001; Bose *et al*., 2008). However, to date little is known about the function of the C9ORF72 protein and it remains possible that defective splicing of its own messenger RNA or transcriptional silencing resulting in haploinsufficiency, might contribute to the neurodegenerative process.

Conclusion

This is the first detailed report of the clinical and pathological phenotypes in a large cohort of 62 ALS/MND cases with pathological hexanucleotide expansions in the *C9ORF72* gene. This genetic variant is common, accounting for 43% of familial ALS and 7% of sporadic ALS cases in this Caucasian cohort from Northern England. Clinical features of note include the prevalence of dementia in the ALS cases and close family members, and a younger age of onset and more rapid disease progression compared with other ALS/MND subtypes. Pathologically, these cases showed classical ALS changes, with TDP-43 positive inclusions. Extra-motor system pathology was strikingly over-represented in the C9*ORF*72 positive cases, suggesting that this genotype may be one predictor of an individual ALS patient's location along the anatomical spectrum of ALS pathology. Neuronal cytoplasmic inclusions within the CA4 hippocampal region may be sufficiently characteristic to allow prediction of mutation status.

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Supplementary material

Supplementary material is available at *Brain* online.

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