

Published in final edited form as:

Acta Neuropathol. 2013 October ; 126(4): 545–554. doi:10.1007/s00401-013-1161-2.

Progressive Amnestic Dementia, Hippocampal Sclerosis, and Mutation in *C9ORF72*

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Abstract

The most common cause of familial frontotemporal lobar degeneration with TAR DNA-binding protein-43 pathology (FTLD-TDP) has been found to be an expansion of a hexanucleotide repeat (GGGGCC) in a noncoding region of the gene *C9ORF72*. Hippocampal sclerosis (HpScl) is a common finding in FTLD-TDP. Our objective was to screen for the presence of *C9ORF72* hexanucleotide repeat expansions in a pathologically-confirmed cohort of “pure” hippocampal sclerosis cases (n=33), outside the setting of FTLD-TDP and Alzheimer’s disease (AD). Using a recently described repeat-associated non-ATG (RAN) translation (C9RANT) antibody that was found to be highly specific for c9FTD/ALS, we identified a single “pure” HpScl autopsy case with a repeat expansion in *C9ORF72* (c9HpScl). Mutation screening was also performed with repeat-primed polymerase chain reaction and further confirmed with southern blotting. The c9HpScl patient had a 14-year history of a slowly progressive amnestic syndrome and a clinical diagnosis of probable AD. Neuropsychological testing revealed memory impairment, but no deficits in other cognitive domains. Autopsy showed hippocampal sclerosis with TDP-43 immunoreactive neuronal inclusions relatively limited to limbic lobe structures. Neuritic pathology immunoreactive for p62 was more frequent than TDP-43 in amygdala and hippocampus. Frequent p62 positive neuronal inclusions were present in cerebellar granule neurons as is typical of *C9ORF72* mutation carriers. There was no significant FTLD or motor neuron disease. C9RANT was found to be sensitive and specific in this autopsy-confirmed series of HpScl cases. The findings in this patient suggest that the clinical and pathologic spectrum of *C9ORF72* repeat expansion is wider than frontotemporal dementia and motor neuron disease, including cases of progressive amnestic dementia with restricted TDP-43 pathology associated with HpScl.

Keywords

Hippocampus; *C9ORF72*; memory; neuropathology; frontotemporal lobar degeneration; C9RANT

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Competing Interests

Dr. Rademakers and Mrs. DeJesus-Hernandez have a patent on the clinical testing and therapeutic intervention for the hexanucleotide repeat expansion of *C9ORF72*.

INTRODUCTION

Hippocampal sclerosis (HpScl) in the elderly is characterized by selective neuronal loss and gliosis in CA1 sector and subiculum of the hippocampus, often associated with a slowly progressive amnesic syndrome [29]. HpScl is not uncommon in autopsy series of dementia of the elderly [10, 23]. The pathogenesis of HpScl is unknown, but it is hypothesized to be neurodegenerative and is often associated with neuronal inclusions containing TAR DNA binding protein-43 (TDP-43). [3] Neuronal loss in the same distribution can also be seen in patients with hypoxic-ischemic injury, but this is not associated with TDP-43 pathology [3, 14]. HpScl is frequent in other degenerative disorders, especially frontotemporal lobar degeneration with TDP-43 pathology (FTLD-TDP) [13]. When HpScl is the only neuropathologic finding sufficient to cause dementia, such that it cannot be explained by FTLD or Alzheimer's disease (AD) pathology, it is considered "pure" HpScl [2].

Investigation of genetic correlates of HpScl is a relatively new area of research. Mutations in the gene for progranulin (*GRN*) cause FTLD-TDP [5], while a genetic variant in the 3'-UTR of *GRN* in a putative micro-RNA binding site (SNP rs5848, T minor allele) is a risk factor not only for FTLD-TDP [25], but also for HpScl [9]. Pathologically, many individuals harboring *GRN* mutations have concomitant HpScl pathology [16]. The most common genetic cause of FTLD-TDP has been found to be an expansion of a hexanucleotide repeat (GGGGCC) in a noncoding region of the gene *C9ORF72* [8, 26]. The current recommended terminology for frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) caused by mutations in *C9ORF72* is c9FTD/ALS [8]. The clinical presentation of most patients with c9FTD/ALS is that of behavioral variant FTD, FTD with motor neuron disease or ALS, but other clinical phenotypes are also reported (e.g., psychosis or progressive aphasia) [12].

In an autopsy series of c9FTD/ALS, concomitant HpScl was frequent [21]. In addition, these cases presented with the characteristic c9FTD/ALS neuropathologic phenotype, namely, ubiquitin-positive, TDP-43-negative neuronal cytoplasmic inclusions in the cerebellum, hippocampus, and neocortex which are immunoreactive for ubiquitin-binding proteins sequestosome-1/p62 and ubiquilin-2 [6]. Recently, our group and a second independent group, uncovered an unconventional form of translation of the GGGGCC expanded repeat known as repeat-associated non-ATG (RAN) translation which leads to the generation of 3 alternating copolymer peptides [4, 20]. This pathology, termed C9RANT, is highly specific to the central nervous system of c9FTD/ALS [4].

In this study, our goals were to 1) screen pure HpScl cases with C9RANT immunohistochemistry [4] and 2) validate the results with genetic methods [8], in order to identify the frequency of pure HpScl cases harboring the *C9ORF72* repeat expansion. The findings presented in this report contribute to increasing evidence that the clinical and pathologic spectrum of disorders in *C9ORF72* is wider than previously suggested.

MATERIALS AND METHODS

Case material

The brain bank for neurodegenerative disorders at Mayo Clinic Jacksonville was queried for all cases with a pathologic diagnosis of HpScl with available frozen tissue for DNA extraction (n=280). Cases were excluded if there was a coexisting diagnosis of FTLD (n=73), AD (n=138), Lewy body disease (n=16). We also excluded other significant neurodegenerative diseases, such as progressive supranuclear palsy, multiple system atrophy, and corticobasal degeneration (n=22). A total of 33 cases met inclusion criteria and

only one case was found to have a *C9ORF72* repeat expansion (c9HpScl). The clinical information of the c9HpScl case was abstracted from the medical records.

Neuropathologic methods

At the time of neuropathologic examinations, brains were submitted for evaluation with the left hemibrain fixed in formalin and the right frozen at -80° . Fixed brain weight was calculated based upon doubling the weight of the left hemibrain. Using standardized dissection and sampling methods described previously [21], tissue samples were processed and embedded in paraffin blocks. The posterior hippocampus was used to evaluate C9RANT and TDP-43 immunoreactivity in all cases. Immunohistochemistry was performed on a DAKO Autostainer (Universal Staining System Carpinteria, California) with the pooled-peptide C9RANT antibody (Rb5823 1:5000, Mayo Clinic antibody)[4] and phospho-Serine 409/410 TDP-43 (1:5000 mouse monoclonal; Cosmo Bio Co., LTD.). Antigen retrieval was performed by steaming in deionized water for thirty minutes. To further characterize the *C9ORF72* mutation case, sections of frontal, temporal, parietal and motor cortex, hippocampus, amygdala, basal ganglia, thalamus, midbrain, pons, medulla, and cerebellum were evaluated using additional histologic methods. Additional immunohistochemical evaluations included: IBA1 (1:3000 rabbit anti-ionized calcium binding adaptor molecule 1, Wako Chemicals), Ubi1 (1:40,000 mouse monoclonal anti-ubiquitin, Millipore), p62 (1:250 mouse monoclonal anti-p62/sequestosome; BD Transduction Laboratories), CP13 (1:1000 mouse monoclonal anti phospho-tau, gift from Peter Davies), collagen IV (1:1000 rabbit polyclonal anti-Collagen IV, Rockland Inc.), and alpha-synuclein (1:3000 rabbit polyclonal anti-alpha-synuclein, Mayo Clinic antibody). Steam was used as an antigen retrieval for each of the additional antibodies; except for alpha-synuclein, which was incubating slides for 30 minutes in 98% formic acid, followed by steaming in deionized water for thirty minutes. Senile plaques and neurofibrillary tangles (NFT) were counted and severity of amyloid angiopathy scored with thioflavin S fluorescent microscopy, as previously described [22]. At the time of diagnosis a Braak NFT stage was assigned using thioflavin S [7]. Thal A β phase was assigned by retrospectively assessing neuropathologic reports to determine senile plaque distribution on thioflavin S stains of the cortices, posterior hippocampus, basal ganglia, and cerebellum [27]. The maximum Thal A β phase was assigned if senile plaques were found in: Phase 1- neocortex (*i.e.* mid-frontal, inferior parietal, or superior temporal cortex); Phase 2 – entorhinal cortex or CA1/subiculum of the hippocampus; Phase 3 – basal ganglia; Phase 4 – CA4 of the hippocampus; and Phase 5 – cerebellar molecular layer.

The C9RANT antibody detects discrete neuronal inclusions, which were used to screen for C9RANT positivity throughout hippocampal subsectors and medial-temporal cortex. The TDP-43 antibody was used to screen for positivity, as well as classify previously described harmonized TDP-43 subtypes A–C [17].

Genetic methods

Genomic DNA was extracted from frozen brain tissue and screened for the *C9ORF72* expanded hexanucleotide repeat using repeat-primed PCR and southern blotting techniques as previously described [8]. Briefly, an initial assay of GGGGCC hexanucleotide repeat expansion was amplified for each case (n=33) using one fluorescently labeled PCR primer. Next, using an automated ABI3730 DNA analyzer, a fragment length analysis was performed using GeneMapper software (Applied Biosystems). If a case appears homozygous in the initial assay, they were then analyzed using a repeat primed PCR method. An individual with a repeat expansion will show a characteristic stutter amplification pattern on electropherogram.

Results

Pathogenetic screen

Of the 280 cases identified, 33 cases (12%) were not found to have coexisting FTLN, AD, or other significant neurodegenerative diseases. The demographics and pathologic information are summarized in Table 1. Of the final 33 HpScl there were 22 TDP-43 positive cases, 10 TDP-43 negative cases, and one case that lacked fixed tissue. Regardless of TDP-43 positivity, each case was screened with C9RANT in the hippocampus. One case was found to have C9RANT positivity with the highest burden of inclusions found in the dentate fascia and endplate (CA4). Occasional neuronal cytoplasmic inclusions (NCI) and “pre-inclusions” with granular somatodendritic C9RANT immunoreactivity could be found in the medial temporal lobe cortex (Fig. 1a, b).

Blinded to the C9RANT results, we found one out of the 33 cases with the characteristic stutter pattern in the repeat-primed PCR assay, indicative of an expansion in *C9ORF72* (Fig. 2a). Southern blot using DNA extracted from cerebellum was performed to confirm the presence of a pathogenic repeat expansion, which highlighted a smear of high-molecular weight bands (Fig. 2b). The C9RANT antibody identified the same case that was found to have a *C9ORF72* repeat expansion using genetic methods. In this series of HpScl cases that lacked neuropathology consistent with FTLN or advanced AD diagnoses, the C9RANT antibody was 100% sensitive and specific.

Clinicopathological Summary

Here, we give a clinicopathologic summary of the c9HpScl case identified in the pathogenetic screen. The c9HpScl case was a 76-year-old man with a final diagnosis prior to death of clinically probable AD, initially evaluated in 1995 at 72-years-of-age with a 10-year history of slowly progressive episodic memory impairment (age of onset = 62 years). The wife reported no change in personality or behavior, “He was socially active and plays piano, bridge, reads, walks, and shoots pool”. His mother had slowly progressive amnesic dementia with onset in her 70s and death at age 96. There was no other family history of dementia or ALS. His past medical history was notable for coronary artery disease with a 4-vessel coronary artery bypass graft in 1987. Cranial nerves were unremarkable; his palate moved symmetrically; and his tongue was midline. Although there were no EMG studies conducted, motor testing revealed preserved strength in upper and lower extremities. Word fluency was within normal limits, naming 13 words with the letter “F.” A Mini-Mental State Examination [11] revealed difficulties with the date, month, and day of the week. He also had mild difficulties with recall and scored a total of 24 out of 30. There were no signs of paranoia or psychosis. Neuropsychological evaluation showed executive/frontal functioning within normal limits and severe impairment in memory, especially for delayed recall (Table 2). Specific neuropsychological tests designed for the diagnosis of FTD were not conducted. Magnetic resonance imaging in 1995 showed diffuse cerebral volume loss and white matter changes consistent with age. The patient was further lost to follow-up in the five years preceding his death.

The calculated fixed brain weight was 1140 grams (Table 1). There was mild cortical atrophy and ventricular dilation, especially the temporal horn of the lateral ventricle. The hippocampal formation, but not the amygdala was atrophic (Fig. 3a). The cerebellum was unremarkable. The substantia nigra and locus coeruleus had visible neuromelanin pigment. Microscopic inspection of the substantia nigra and locus coeruleus showed a normal neuronal population with no NFTs or Lewy bodies. There was severe neuronal loss and gliosis that was selective for CA1, with less neuronal loss in the subiculum and preservation of CA2/3, endplate and dentate fascia (Fig. 3d). Senile plaques were not detected in any

cortical region, hippocampus, amygdala, or cerebellum. There were only a few isolated NFTs in the frontal and temporal cortices and in the hippocampus and amygdala, but more numerous NFTs in the entorhinal cortex. As expected, no NFT pathology was found in the cerebellum. Mild amyloid angiopathy was detected in frontal and occipital cortices. Phospho-TDP-43 immunohistochemistry showed NCIs in the dentate gyrus of the hippocampus (Fig. 3b), but very sparse NCIs in frontal and temporal cortices and no significant cortical neuronal loss or gliosis. There were sparse TDP-43-positive dystrophic neurites and no neuronal intranuclear inclusions (NII) in the hippocampus, consistent with Type B [17]. The presence of hippocampal synaptic or fine neurites in the CA1 (Fig. 1c) was also noted in areas of relative neuronal preservation, a previously reported feature of many *C9ORF72*-associated FTLN cases [21]. TDP-43-positive perivascular glial inclusions (Fig. 1d), considered to be abnormal inclusions in astrocytic end-feet processes [15], were present in the hippocampus and entorhinal cortex, but very sparse in frontal and temporal cortices, nucleus accumbens, and amygdala. No evidence of motor neuron degeneration was present. We did not find TDP-43 pathology, neuronal loss, or Bunina bodies in the spinal cord.

In addition to TDP-43 positive pathology, there were also TDP-43-negative, p62/sequestosome-1-positive NCIs and dystrophic neurites (Fig. 3c). In all cortical and subcortical regions evaluated, p62-positive pathologic inclusions were more numerous than TDP-43-positive inclusions, as reported recently by others in c9FTD/ALS [1]. TDP-43 pathology was absent in the cerebellum, but C9RANT immunopositivity showed many NCIs in granule cells and occasionally in Purkinje cells (Fig. 4 a, b). As in other previously reported *C9ORF72* repeat expansion cases [1], p62 and ubiquitin immunohistochemistry revealed NCIs in both granule cells and Purkinje cells of the cerebellum (Fig. 4 c, d).

Discussion

This study identified a hippocampal sclerosis case with an expansion in *C9ORF72* that lacked FTLN, AD, or other significant neuropathology. The case was identified using a recently described antibody generated against an unconventional mechanism of translation-repeat-associated non-ATG translation that was validated using repeat primed PCR and southern blotting techniques, which was sensitive and specific in this series of HpScl cases. The clinical phenotype in the c9HpScl patient, namely, slowly progressive amnesic syndrome without behavioral or motor neuron signs, is distinctly different from the most common presentations in c9FTD/ALS [12]. Although the final clinical phenotype of this individual was unknown in the five years prior to his death, it is possible he developed FTD, but unlikely given the slowly progressive nature of the disease course and sparing of frontal cognitive domains at the last neuropsychological examination (i.e. attention/concentration). HpScl is more apt to be clinically misdiagnosed as AD than behavioral variant FTD [24, 29]. Pathologically, there was selective neuronal loss and gliosis in the hippocampus consistent with HpScl of the elderly, which fits well with the clinical presentation [23, 29]. TDP-43 inclusions were most abundant in limbic structures and sparse in the cortex. The cortical lesions were not associated with cortical neuronal loss or gliosis. Immunohistochemistry for ubiquitin and p62 in *C9ORF72*-associated HpScl of the elderly show that pathology can be more widespread than previously suspected based upon TDP-43 immunohistochemistry, with NCIs even in the cerebellar cortex. The greater propensity of p62 pathology compared to TDP-43 pathology in *C9ORF72*-associated HpScl suggests that TDP-43 may not be central to the *C9ORF72* disease mechanism, but rather another unknown polyubiquitinated substrate bound by p62.

In our previous clinicopathologic characterization of c9FTD/ALS, we reported that HpScl was common in c9FTLD-TDP (12 of 15), and some patients in this series also presented

with amnesic dementia that lead to clinical consideration of AD [21]. A large series of familial late-onset clinical AD patients screened for mutations in *C9ORF72*, found 6 of 771 patients with mutations [18]. Postmortem analysis of two patients showed pathology consistent with FTLD, with inconsistent concurrent Alzheimer type neuropathology. There is no mention in this report about whether the two patients with *C9ORF72* mutations and Alzheimer-like clinical presentation had HpScl. The reported frequency of 0.8% clinical AD patients with *C9ORF72* mutations was consistent with a recent report on a clinical AD patient series from our group, which found 0.9% of AD patients with an expansion mutation [28]. The frequency of *C9ORF72* in our pathologically-confirmed HpScl series is 3.0% if all cases are considered and 4.5% if only TDP-43 positive cases are included.

Mutations in *C9ORF72* have been reported in seemingly sporadic cases of FTLD and ALS [8]. The family history of this patient included amnesic disorder in her mother (presenting with symptoms in her 70s), but there was no history of dementia or motor neuron disease in other family members. Although there was no autopsy confirmation, the patient's mother died in her late 90s, which is more than a decade later than our patient. While it is impossible to make any conclusions from these two individuals, it is of interest that the possibility of anticipation, with earlier age of onset in subsequent generations, has been raised in other c9FTD/ALS series [12].

The pathogenesis of HpScl in our patient is unknown, but most evidence favors a neurodegenerative disease process. He had no history of seizures. He had coronary artery disease and had a 4-vessel coronary artery bypass graft, but there was no history of cognitive deficits after surgery. The presence of TDP-43 and p62 NCIs and dystrophic neurites in the hippocampus and to a lesser extent in other brain regions supports a neurodegenerative origin for HpScl. It is of some interest that microvascular TDP-43-related pathology was also present [15]. The significance of TDP-43 inclusions in astrocytic end-feet of microvessels remains to be determined; however, we speculate that this pathology may compromise microvascular function and contribute to neuronal loss in regions known to be vulnerable to hypoxic-ischemic injury.

This case adds increasing evidence that the clinical and pathologic spectrum of disorders associated with mutations in *C9ORF72* is wider than merely FTD and ALS. Screening for mutations in *C9ORF72* should be considered in patients with slowly progressive amnesic dementia who have a positive family history, especially if other biomarkers (*e.g.*, apolipoprotein E genotype, neuroimaging, or cerebrospinal fluid analyses) do not support a diagnosis of AD [19]. Further studies are needed to identify the underlying molecular mechanisms responsible for clinical and pathologic heterogeneity of c9FTLD/ALS.

Acknowledgments

We are grateful to all patients, family members, and caregivers who agreed to brain donation, without which these studies would have been impossible. We also acknowledge expert technical assistance of Linda Rousseau and Virginia Phillips for histology, and Monica Castaneda-Casey for immunohistochemistry. We would like to thank Martha Purdy, John Gonzalez, and Beth Marten for brain bank coordination. This research was funded by Mayo Foundation (Jacoby Professorship of Alzheimer Research); National Institutes of Health (P50-AG16574, P50-NS72187, P01-AG03949, R01-AG37491, R01-NS080882 and R01-AG026251); The ALS Therapy Alliance; and the State of Florida Alzheimer Disease Initiative. MEM is supported by a fellowship from the Robert and Clarice Smith and Abigail Van Buren Alzheimer's Disease Research Program.

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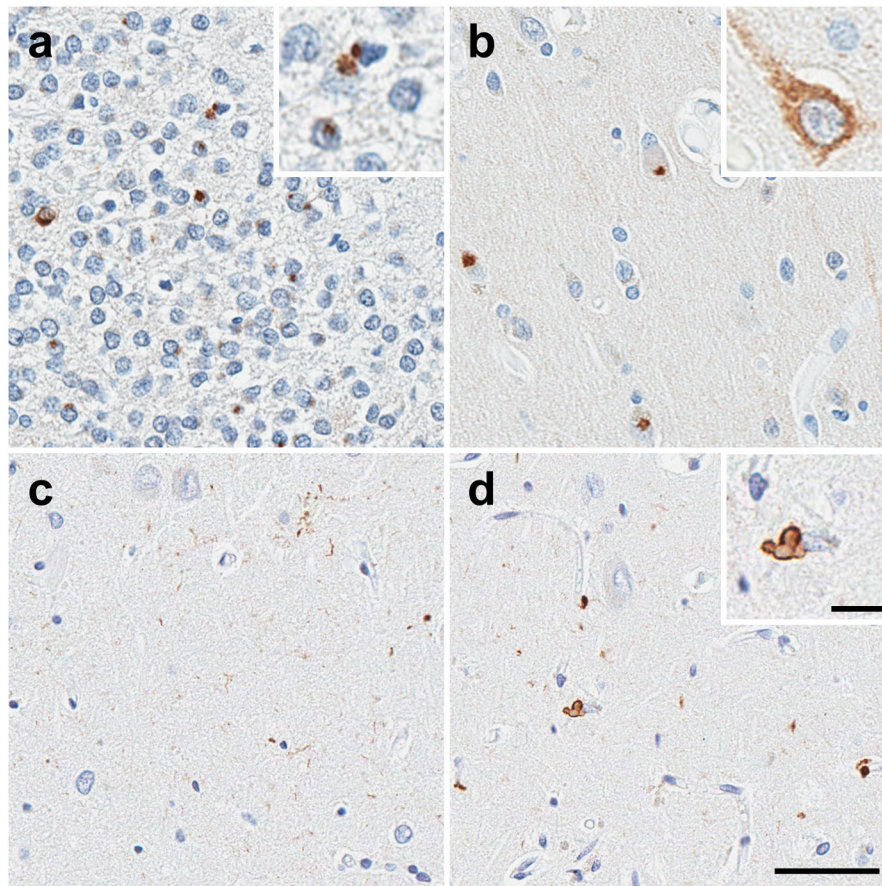


Figure 1.

C9RANT immunohistochemistry in the hippocampus (**A**) shows the highest number of neuronal inclusions (arrows) in the dentate fascia. (**B**) Occasional neuronal cytoplasmic inclusions (NCI, inset) and “pre-inclusions” with granular somatodendritic C9RANT immunoreactivity (inset) could be found in the medial temporal lobe cortex. TDP-43 immunohistochemistry in the CA1 sector of the hippocampus shows (**C**) synaptic or fine neurites in areas of relative neuronal preservation, as well as (**D**) TDP-43-positive perivascular glial inclusions. They can be seen as a bi-lobed inclusion (inset) or as dense perivascular deposits (arrowhead). (*bar* 50 μ m A–D, *insets* 100 μ m)

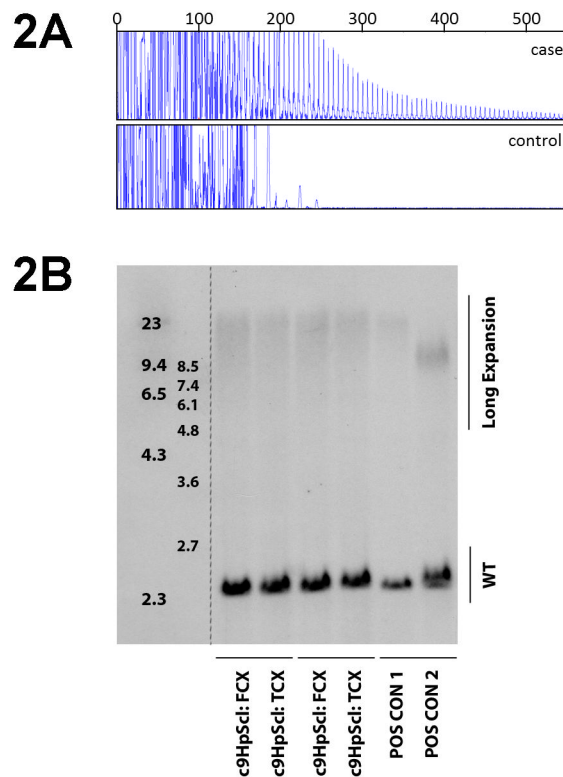


Figure 2.

(A) The electropherogram plot of repeat-primed PCR reaction products zoomed to 2,000 relative fluorescence units to show stutter amplification. Our case and a control (*C9ORF72*-negative) with HpScl are graphically represented in the upper and lower panel, respectively. (B) Southern blotting of two expanded repeat carriers and one noncarrier using genomic DNA extracted from cerebellum. Lane 1 shows DIG-labeled DNA Molecular Weight Marker II (Roche) with fragments of 2,027; 2,322; 4,361; 6,557; 9,416; 23,130 bp. Lane 2 shows DIG labeled DNA Molecular Weight Marker VII (Roche) with fragments of 1,882; 1,953; 2,799; 3,639; 4,899; 6,106; 7,427; and 8,576 bp. Lane 3 shows a positive control using DNA extracted from an expanded repeat lymphoblast cell line shows the expected 2.3 kb wild-type allele with expanded repeats showing an additional allele around 5.5 kb (arrow). The HpScl noncarrier control only shows the 2.3 kb wild-type allele (lane 4), while the chromosome 9 frontotemporal dementia positive-control and chromosome 9 HpScl case show an additional allele from at around 8.5 kb (lanes 5 & 6). Note the smear of high molecular weight bands in DNA extracted from cerebellum suggesting somatic instability of the repeat.

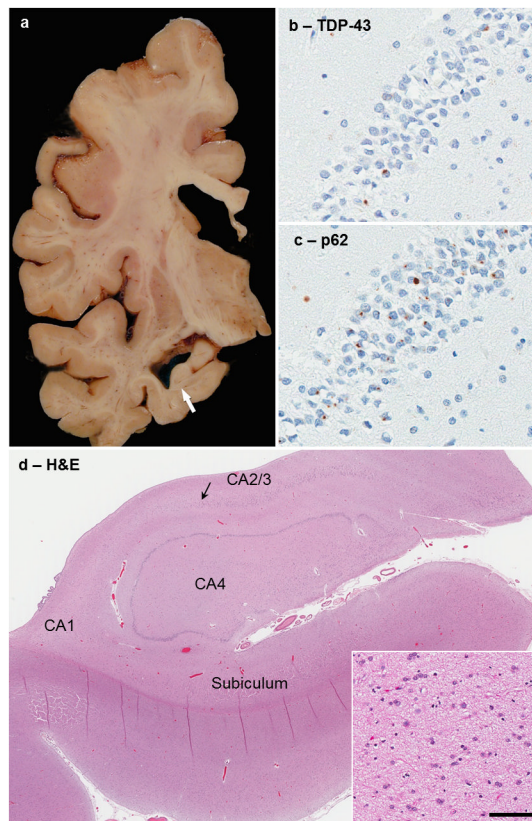


Figure 3.

(A) Macroscopic findings in c9HpScl shows atrophic hippocampus (arrow) in the CA1 region. (B) TDP-43 staining of the hippocampus shows few neuronal cytoplasmic inclusions (NCIs) and no intranuclear inclusions (NIIs). (C) p62 immunohistochemistry of the same region shows many NCIs and scattered NIIs in dentate fascia. (D) An H&E of the anterior hippocampus shows focal CA1 and subiculum pathology, while CA2/3 pyramidal neurons remain resistant (arrow shows transition to neuronal dropout). Macroscopic findings correlate with underlying neuronal loss and gliosis (*inset* shows higher magnification of neuronal loss and gliosis characteristic of HpScl in of CA1). (*bar* 45 μ m for B&C at 40 \times magnification, 1150 μ m for D at 1 \times magnification, and 90 μ m for *inset* at 20 \times magnification)

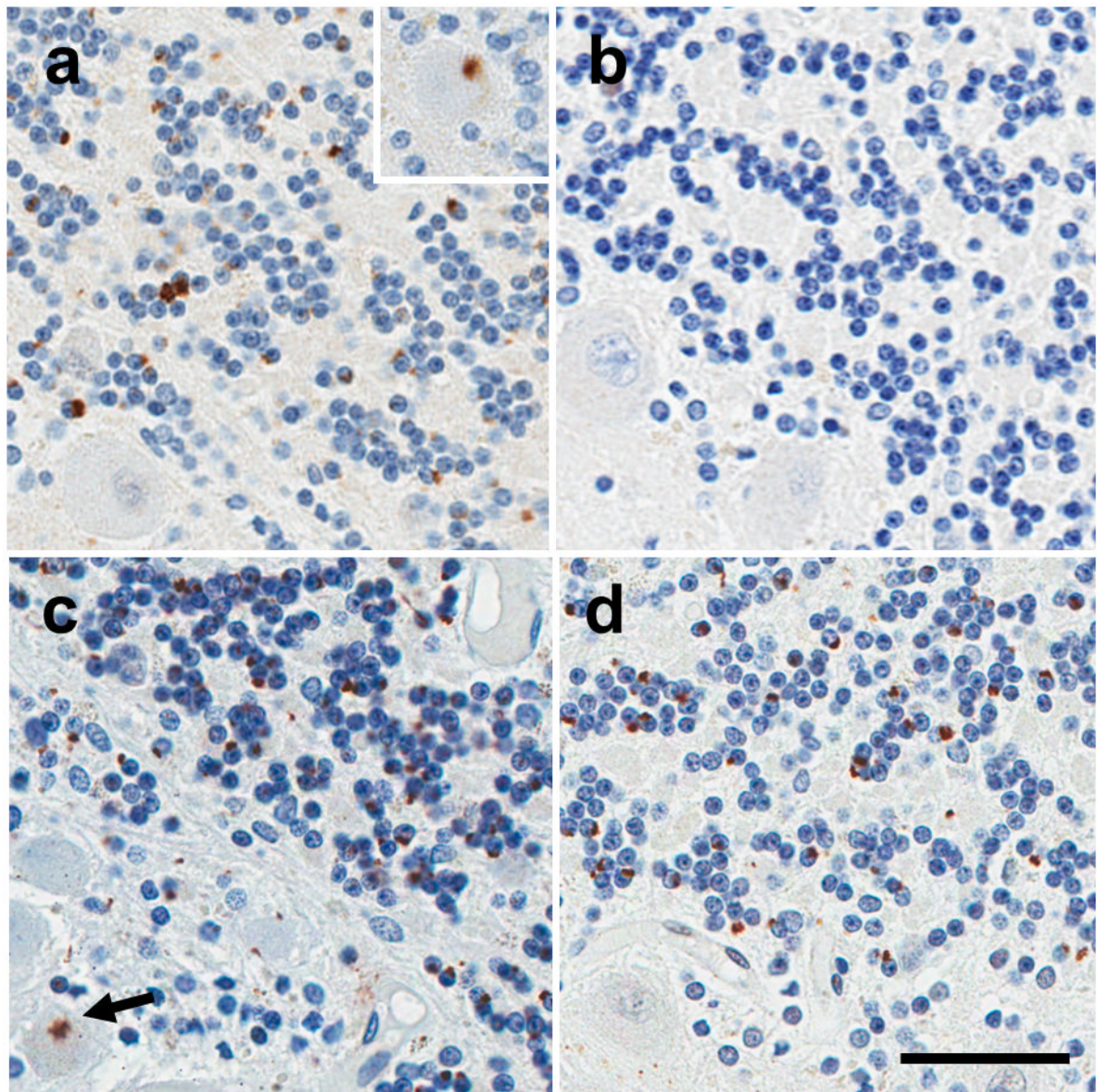


Figure 4. Immunohistochemistry of the cerebellum in c9HpScl shows (A) C9RANT immunopositivity shows many NCIs in granule cells, although occasional purkinje cells can be observed (*inset*). As expected (B), the cerebellum lacks TDP-43 pathology. (C) p62 immunohistochemistry shows many NCIs and occasional NIIs of the granule cell layer. A star-shaped NCI (arrow) was observed in the purkinje cell layer of the cerebellum with p62 staining, but not with (D) Ub1 staining. Ub1-positive NCIs were seen at moderate frequency, and no NII were observed. (*bar* 45 μ m)

Table 1

Summary of demographics and neuropathology in hippocampal sclerosis

	c9HpScl	HpScl TDP-43 positive	HpScl TDP-43 negative
Sample size, n.	1	21	10
Sex, Males (%)	1 (100)	9 (43)	3 (30)
Age at death, years	76	91 (87, 94)	88 (84, 93)
Brain weight, g	1140	1160 (1095, 1220)	1091 (978, 1250)
Braak NFT stage	III	II (II, III)	III (I, IV)
Thal amyloid phase	0	2 (2, 3)	2 (0, 2)
TDP-43 positivity	Positive	Positive	Negative
Type A (1), n. (%)	--	17 (81)	--
Type B (3), n. (%)	1 (100)	4 (19)	--
Type C (2), n. (%)	--	--	--

All data is presented as median (25th, 75th interquartile range) unless noted otherwise. One case lacked fixed tissue, therefore could not be evaluated for TDP-43 and is not presented in this table. Acronyms: HpScl - hippocampal sclerosis, TDP-43 – TAR DNA binding protein-43, g – grams.

Table 2Neuropsychological evaluation of *C9ORF72*-associated HpScl case (September 1995)

Test/Subtest	Percentile	Score
Wechsler Adult Intelligence Scale – Revised*		
Digit span	98	16
Vocabulary	95	15
Comprehension	84	13
Similarities	75	12
Block design	37	9
Digit symbol	25	8
Wechsler Memory Scale – Revised		
Verbal Memory	2	70
Visual Memory	7	77
General Memory	1	68
Attention/Concentration	91	120
Delayed Recall	1	67
Controlled Word Association Test		
Verbal Fluency	60	N/A
Trail Making Test B	75	N/A
Beck Depression Inventory	N/A	4

Acronyms: HpScl – hippocampal sclerosis, n/a – test not available in clinical records.