C9ORF72 and UBQLN2 are genetic causes of ALS in New Zealand: A genetic and pathological study using banked human brain tissue


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C9ORF72 and UBQLN2 are genetic causes of ALS in New Zealand: A genetic and pathological study using banked human brain tissue

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Running title: ALS in New Zealand

Abstract

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease which causes progressive and eventually fatal loss of motor function. Here we describe genetic and pathological characterisation of brain tissue banked from 19 ALS patients over nearly 20 years at the Department of Anatomy and the Centre for Brain Research, University of Auckland, New Zealand. We screened for mutations in SOD1, TARDBP, FUS, and C9ORF72 genes, and for neuropathology caused by phosphorylated TDP-43, dipeptide repeats and ubiquilin. We identified two cases with C9ORF72 repeat expansions. Both harboured phosphorylated TDP-43 and dipeptide repeat inclusions. We show that dipeptide repeat inclusions can incorporate or occur independently of ubiquilin. We also identified one case with a UBQLN2 mutation, which showed phosphorylated TDP-43 and characteristic ubiquilin protein inclusions. This is the first study of ALS genetics in New Zealand, adding New Zealand to the growing list of countries in which C9ORF72 repeat expansion and UBQLN2 mutations are detected in ALS cases.

Keywords: Motor neurone disease, amyotrophic lateral sclerosis, TARDBP, brain bank, C9ORF72, ubiquilin
1. Introduction

The Neurological Foundation of New Zealand Human Brain Bank at the Centre for Brain Research, University of Auckland, was established in 1994 to collect brain and associated tissue from donors with neurological diseases including amyotrophic lateral sclerosis (ALS). With a growing number of genetic causes of ALS identified by researchers internationally we sought to examine the genetics and neuropathology of our ALS brain bank cases as a subset of ALS patients in New Zealand.

The genetic basis for approximately 18% of ALS cases is accounted for, with the GGGGCC expansion mutation in intron 1 of \( C9ORF72 \) alone comprising ~10% of all ALS. Exonic mutations in \( SOD1, FUS \) and \( TARDBP \), along with rarer ALS genes (\( UBQLN2, OPTN, TBK1, SQSTM1 \) etc.) found predominantly in FALS cases, comprise the remaining cases (Renton, et al., 2014). Despite the diverse genetic and environmental factors, ~97% of all ALS cases show a common neuropathology characterised by the deposition of phosphorylated TDP-43 aggregates (Neumann, et al., 2006). The presence of additional protein aggregates can indicate specific genetic causes of ALS. For example, in addition to TDP-43 pathology, \( C9ORF72 \) mutations also cause dipeptide repeat (DPR) protein aggregates in the cerebellum and hippocampus (Ash, et al., 2013, Mori, et al., 2013). Conversely, \( FUS \) and \( SOD1 \) mutant cases do not show TDP-43 proteinopathy, but rather deposition of the mutant proteins themselves (Mackenzie, et al., 2007, Vance, et al., 2009). Neuropathological analyses can therefore be used in conjunction with genetics to fully characterise ALS cases.

Here we examine genetic and pathological features of ALS in New Zealand using banked brain tissue. We identify \( C9ORF72 \) and \( UBQLN2 \) as genetic causes of ALS in New Zealand and confirm that cases with these mutations display characteristic neuropathological lesions. These findings will allow us to best utilise and share our existing bequests as well as establishing a platform for larger genetic and neuropathological studies of ALS in New Zealand.

2. Materials and Methods

2.1 Subjects, ethics and human tissue banking

Brain tissue was collected from 4 controls and 19 ALS cases through the Neurological Foundation of NZ Human Brain Bank from 1995-2013. Clinical and neuropathological diagnoses were performed by consultant neurologists and neuropathologists at Auckland City or Middlemore Hospitals, Auckland,
NZ. Informed written consent was obtained from all donors and next of kin. Ethical approval was granted by the University of Auckland Human Participants Ethics Committee.

2.2 DNA extraction from fixed and fresh brain tissue

One hundred mg fixed-frozen tissue and/or 300 mg fresh-frozen tissue from lateral cerebellum and/or rostral superior frontal gyrus was used. DNA was extracted from fresh tissue (n=10 cases) using standard phenol-chloroform-isoamyl alcohol extraction and DNA clean-up performed using Microclean (Microzone). DNA was extracted from fixed-frozen tissue (n=17 cases) using the QiaAmp FFPE Tissue Kit (Qiagen).

2.3 Sanger sequencing and repeat-primed PCR

All coding exons and flanking sequence of SOD1 (Refseq NM_00454), exon 6 of TARDBP (Refseq NM_007375), and exons 6, 14 and 15 of FUS (Refseq NM_001170937) were amplified using standard PCR and directly sequenced with Big-Dye Terminator v1.1 on an ABI3130 DNA analyser (Applied Biosystems). Samples were screened for the C9ORF72 GGGGCC repeat expansion mutation using repeat-primed PCR (DeJesus-Hernandez, et al., 2011) and fragment analysis conducted on an ABI3130 DNA analyser and peaks visualized using Genemapper 4.0.

2.4 Neuropathology

Fifty micron free-floating sections were taken from fixed-frozen sensory-motor cortex blocks or hippocampus blocks and immunohistochemistry performed using antibodies listed in Supplementary material (Waldvogel, et al., 2008, Waldvogel, et al., 2006). Wide-field images were acquired using a Nikon Eclipse Ni microscope (20x magnification, 0.13 NA) and confocal images sourced using a Zeiss LSM 710 inverted confocal microscope (63x magnification, 1.4 NA, Z-step 0.34 μm) with ZEN 2012 software (Carl Zeiss). Maximum intensity Z-projections and orthogonal projections were generated using ImageJ software (http://imagej.nih.gov/ij/).
3. Results

3.1 Demographics of ALS in New Zealand

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=4)</th>
<th>All cases (n=19)</th>
<th>C9ORF72-negative (n=17)</th>
<th>C9ORF72-positive (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male: female (n)</td>
<td>50% (2):50%</td>
<td>47% (9): 53%</td>
<td>53% (9): 47%</td>
<td>0%:100% (2)</td>
</tr>
<tr>
<td>Family history of ALS (n)</td>
<td>-</td>
<td>26% (5)</td>
<td>18% (3)</td>
<td>100% (2)</td>
</tr>
<tr>
<td>Mean age at onset (years ± SD, range)</td>
<td>-</td>
<td>58.3 ± 14.9, 32-87</td>
<td>59.3 ± 15.9, 32-87</td>
<td>49.5 ± 3.5, 47-52</td>
</tr>
<tr>
<td>Mean age at death (years ± SD, range)</td>
<td>64.3 ± 2.9, 60-67</td>
<td>61.7 ± 13.5, 41-88</td>
<td>62.8 ± 14.3, 41-88</td>
<td>53.0 ± 0, 53-53</td>
</tr>
<tr>
<td>Mean post mortem delay (hours ± SD, range)</td>
<td>19.1 ± 6.9, 9-23.5</td>
<td>20.8 ± 17.1, 3.69*</td>
<td>20.8 ± 17.1, 3.69*</td>
<td>20.8 ± 17.1, 3.69*</td>
</tr>
</tbody>
</table>

*Excluding MN2 for which this information was unavailable.

A detailed clinical description of cases harbouring mutations can be found in Supplementary Material.

3.2 Genetic analysis of banked ALS cases

One of our cases had tested positive for UBQLN2 p.T487I mutation in life (patient IV:18 in (Williams, et al., 2012)). Another case tested negative for C9ORF72, SOD1, TARDBP and FUS during life. All other cases were screened for these genes using DNA extracted from brain. Fresh-frozen brain DNA, available for 10 cases, gave a high rate of sequencing success. Fixed-frozen brain DNA gave only ~50% viable sequence. We identified one fALS patient (MN18) with C9ORF72 repeat expansion (Fig. 1A), however DNA was not available from other affected family members to test segregation.

3.3 Neuropathology of banked ALS cases

We next conducted immunohistochemistry on all 19 cases. All showed classical phosphorylated TDP-43 deposits in the motor cortex (Fig. 1B), thus ruling out SOD1 and FUS gene mutations (Mackenzie, et al., 2007, Vance, et al., 2009). No phosphorylated TDP-43 inclusions were seen in controls, nor when phospho-TDP-43 primary antibody was omitted. We also screened using an antibody to the poly-glycine-proline DPR (Ash, et al., 2013). The C9ORF72-positive case (MN18) showed hallmark deposition of DPR aggregates in the dentate gyrus and cornus ammonis (CA) regions of the hippocampus (Fig. 1C a-d). A second fALS case (MN2) also showed DPRs. For this case, C9ORF72 repeat-primed PCR of fixed-frozen brain DNA had been unsuccessful, however follow-up with the
patient’s family revealed an affected offspring had tested positive. No DPR staining was seen in control hippocampus, any C9ORF72-negative ALS cases, nor when primary antibody was omitted. Finally, by screening using an antibody to ubiquilin 2 we confirmed that the case with UBQLN2 p.T487I mutation (MN17) showed deposition of ubiquilin in the hippocampus, particularly in the parahippocampal region and molecular layer of the dentate gyrus but not in the granular layer (Fig. 1D a-d). In cases with C9ORF72 mutation, ubiquilin-positive inclusions were observed in the CA regions and in both the granular and molecular layers (Fig. 1D e-h). Using double-label confocal microscopy we found that DPRs in the granular layer could be found alone, or decorating a core of ubiquilin (Fig. 1E). No ubiquilin staining was seen when primary antibody was omitted.

4. Discussion

This study is the first to explore the genetics and neuropathology of ALS in New Zealand. With a total population of only 4.5 million, and an estimated current cohort of 300 people living with ALS, ALS research in New Zealand to date has been on a small scale. However after 20 years of banking brain tissue, and with growing national and international support for ALS research, the time was ripe for characterisation of our bank as a foundation for growth of a New Zealand ALS research programme.

Using combined genetic and neuropathological screening we identified two C9ORF72 mutation carriers and a UBQLN2 p.T487I carrier. The C9ORF72-positive cases showed abundant deposition of DPRs in the hippocampus. Although TDP-43 proteinopathy correlates better with regional neurodegeneration (Mackenzie, et al., 2013), DPRs are pathognomonic for C9ORF72-linked disease thus staining for DPRs can augment genetic screening. Before the discovery of DPRs it was found that C9ORF72-positive cases could also be distinguished by the presence of p62 and ubiquilin protein pathology in the hippocampus and cerebellum (Al-Sarraj, et al., 2011, Brettschneider, et al., 2012). Although TDP-43 inclusions can be ubiquilin-modified (Deng, et al., 2011) they are rare in hippocampus before late stage (Brettschneider, et al., 2013) therefore hippocampal ubiquilin pathology is considered a surrogate for DPRs (Brettschneider, et al., 2012, Mann, et al., 2013). We showed using double-label immunofluorescence that polyGP DPRs are ubiquilin-modified in the dentate granular layer, while ubiquilin inclusions in the molecular layer were DPR-negative. Ubiquilin inclusions have also been seen in the hippocampus and spinal cord in ALS caused by
UBQLN2 gene mutation (Deng, et al., 2011, Williams, et al., 2012), and indeed we detected hippocampal ubiquilin inclusions in case MN17 with UBQLN2 p.T487I mutation.

While this study was essential for characterising our banked tissue for future use, we screened only a small number of patients who may not necessarily be representative of the wider New Zealand ALS cohort. For instance, 5 of our 19 cases (26%) had a family history of ALS while most studies report that 5-10% of ALS is familial. Their over-representation in our brain bank may suggest that familial ALS patients are more highly motivated to bequeath tissue for research. For this reason, the inclusion in our cohort of a case with a rare UBQLN2 mutation should not be over interpreted. The frequency of C9ORF72 mutation in our cohort, 2 of 5 familial cases (40%), agrees with the expected frequency of 37.6% for European populations (Majounie, et al., 2012).

This study profiles ALS genetics and pathology in a small New Zealand cohort. Moving forward, we seek to press for inclusion of New Zealand ALS patients in international cohort studies and clinical trials.

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Figure legend

Figure 1. Phosphorylated TDP-43, C9ORF72 dipeptide repeat, and ubiquilin pathology in New Zealand ALS patients.

(A) Repeat-primed PCR showed C9ORF72 repeat expansion in MN18. (B) Phospho-TDP-43 inclusions were seen in the motor cortex of all ALS patients. p-TDP-43 neuronal cytoplasmic inclusions (NCI) (a, arrow), threads (b) skeins (c) and dystrophic neurites (DN, d). Scale bars 20 µm. (C) Poly-GP DPRs were seen in MN2 and MN18 hippocampus. Both cases showed abundant NCI in
pyramidal cells of CA1-4 (a) and granule cells of the dentate gyrus (DG) (c). Pyramidal cells with diffuse staining with or without concomitant NCI (b), dipeptide-containing DN (d), and dot-like intranuclear inclusions (a, arrow) were seen occasionally. Scale bars 20 µm. (D) Ubiquilin inclusions were seen in the hippocampus of three ALS patients. MN17, harbouring a UBQLN2 p.T487I mutation, showed abundant neuritic inclusions in the molecular layer of the DG (a, enlarged in b). Parahippocampal gyrus (c, enlarged in d) had the highest load of neuritic inclusions and occasional pyramidal NCI (d, arrow head). The granular layer of the DG, devoid of inclusions in MN17, showed abundant NCI in C9ORF72 repeat expansion cases MN2 and MN18 (e left, enlarged in g). In these cases the CA regions and the molecular layer of the DG showed diffuse and aggregated neuritic ubiquilin and DN (e right, enlarged in f). Occasional pyramidal NCI were seen in the CA regions (arrow head). Scale bars 20 µm (b,d,f-h,) or 50 µm (a,c,e). (E) Colocalisation of ubiquilin and DPRs. In MN2 and MN18 ubiquilin inclusions were found with (a,b, arrow heads) or without (a, arrow) a shell of DPRs. DPRs without a ubiquilin core were also seen (a, red arrow). Main images are maximum intensity z-projections, panels are orthogonal projections. Scale bars 20 µm.

References


Supplementary Material

Immunohistochemistry

Primary antibodies:

Phosphorylated TDP-43 (rabbit anti-phosphoserine 409/410, 1 in 50,000, #CAC-TIP-PTD-P02, RRID: AB_1961898, Cosmo Bio Co. Ltd., Tokyo, Japan); C9ORF72 dipeptide repeat proteins (rabbit anti-C9RANT, 1 in 15,000, #NBP2-25018, Novus Biologicals, Littleton, CO); Ubiquilin (mouse anti-PLIC-2 (QR-2) Ubiquilin 2 antibody (also detects ubiquilins 1 and 4), 1 in 1000, #SC-100612, RRID: AB_2272422, Santa Cruz Biotechnology, Dallas, TX). Antigen retrieval (microwaving 30 s in 0.1 M sodium citrate buffer, pH 4.6) was performed for phosphorylated TDP-43 only, before blocking.

Biotinylated secondary antibody (goat anti-rabbit, #B7389, RRID: AB_258613; or anti-mouse, #B7264, RRID: AB_258607, 1 in 1000, Sigma-Aldrich, St. Louis, MO) was applied overnight and ExtrAvidin peroxidase (1 in 1000, #E2886, Sigma-Aldrich) applied for 4 h at room temperature. Colour development with 3,3’-Diaminobenzidine (DAB) was allowed to proceed for 20 min. After de- and re-rehydration, sections were counterstained with cresyl violet (Nissl stain) for 15 min. For fluorescent immunolabelling, secondary antibodies were added overnight at room temperature (1 in 500, Alexa Fluor® 488-conjugated goat anti-mouse, #A11029, RRID: AB_2534088; and Alexa Fluor® 594-conjugated goat anti-rabbit, #A11012, RRID: AB_2534079, ThermoFisher Scientific, Waltham MA), followed by counterstaining with Hoechst 33342 (1 µg/mL, #H3570, ThermoFisher Scientific) for 15 minutes at room temperature and mounted with ProLong® Gold antifade mountant (#P36930, ThermoFisher Scientific).

Clinical features of C9ORF72 and UBQLN2 positive cases

Patient MN2

The patient was a European female presenting at age 52 with ALS of spinal onset, with wasting and weakness of the upper limbs progressing to quadriplegia. The patient developed a bulbar palsy and prior to death was severely dysphagic and almost entirely anarthric. The patient died at the age of 53. The patient’s grandmother was diagnosed with progressive muscular atrophy. The patient’s mother died at the age of 55, likely of FTD; her autopsy revealed frontal and temporal atrophy, diagnosed as
Pick’s disease but neither neurofibrillary tangles nor plaques were detected. The patient’s brother and two aunts also died of ALS.

*Patient MN18*

The patient was a European (Australian) female with onset at age 47, presenting with lower limb weakness. Concomitant FTD was not suspected. The patient died at the age of 53. The patient’s mother was diagnosed with early Alzheimer’s at age 60, and a maternal aunt was diagnosed with unspecified dementia. A maternal uncle was diagnosed with MND but was not known to have dementia.

*Patient MN17*

The patient was a European female, presenting with ALS of spinal onset at the age of 58 with distal upper limb weakness, progressing to severe weakness in all limbs and anarthria within 3 years. FTD was suspected clinically, developing fairly late, with confirmed executive dysfunction. The patient died at the age of 61. The patient’s mother, sister, and brother died of ALS aged 48, 38, and 43 years, respectively. The family history of ALS was extensive. At the time of her death, 9 of 22 of the patient’s family had died of ALS. No male-to-male transmission was seen, consistent with X-linked disease. This patient and kindred have been described in detail previously, during the patient’s life (Williams, et al., 2012).
A. Repeat-primed PCR

B. Motor cortex phospho-TDP-43

C. Hippocampus polyGP DPRs

D. Hippocampus ubiquilin

E. Hippocampus ubiquilin + polyGP DPRs
Highlights

- Genetic and neuropathological characterisation of 19 ALS patients in New Zealand
- \textit{C9ORF72} and \textit{UBQLN2} mutations are genetic causes of ALS in New Zealand
- Hippocampal ubiquilin distinguishes \textit{UBQLN2, C9ORF72}-negative and -positive cases
- Hippocampal dipeptide repeat inclusions surround or occur independently of ubiquilin