The presence of heterogeneous nuclear ribonucleoproteins in frontotemporal lobar degeneration with FUS positive inclusions.

Priya Gami-Patel[1], Rina Bandopadhyay[1], Jack Brelstaff[1], Tamas Revesz[1], Tammaryn Lashley[1]

[1]The Queen Square Brain Bank for Neurological Disorders, Department of Molecular Neuroscience, Institute of Neurology, UCL, London

Corresponding author:

Dr Tammaryn Lashley,
Queen Square Brain Bank,
Department of Molecular Neuroscience,
Institute of Neurology,
London,
WC1N 3BG, UK

Email: T.Lashley@ucl.ac.uk

Abstract

Frontotemporal lobar degeneration with FUS-positive inclusions (FTLD-FUS) is a disease with unknown cause. Transportin1 (TRN1) is abundantly found in FUS-positive inclusions and responsible for the nuclear import of the FET proteins of which FUS is a member. The presence of all FET proteins in pathological inclusions suggests a disturbance of TRN1-mediated nuclear import. FUS also belongs to the heterogeneous nuclear ribonucleoprotein (hnRNP) protein family. We investigated whether hnRNP proteins are associated with FUS pathology implicating dysfunctional nuclear export in the pathogenesis of FTLD-FUS. hnRNP proteins were investigated in affected brain regions in FTLD-FUS using immunohistochemistry, biochemical analysis and the expression analysis. We demonstrated the presence of several hnRNP proteins in pathological inclusions including neuronal cytoplasmic inclusions and dystrophic neurites. Biochemical analysis revealed a shift in the location of hnRNP A1 from the nucleus to the cytoplasm. Expression analysis revealed an increase in several hnRNP proteins in FTLD-FUS. These results implicate a wider dysregulation of movement between intracellular compartments, than mechanisms only affecting the nuclear import of FUS proteins.

Key words: frontotemporal dementia, FUS, FET proteins, hnRNP, transportin, nanostring

1. Introduction

Recent advances in our understanding of the molecular mechanisms associated with frontotemporal lobar degeneration have shown that this heterogeneous group of diseases can be divided on the presence of the abnormal protein aggregates found in the pathological inclusions (Mackenzie et al., 2010). Fused in sarcoma (FUS) is a protein identified in pathological inclusions of patients clinically characterised with FTD and pathologically termed FTLD-FUS (Lashley et al., 2011; Munoz et al., 2009; Neumann et al., 2009)

It is still unknown how FUS plays a role in the pathogenesis of FTLD-FUS and which of its many known functions are disrupted that leads to the formation of pathological lesions. FUS is a multifunctional protein composed of 526 amino acids belonging to the FET family of proteins, which also includes Ewings sarcoma protein (EWS) and TATA-binding protein associated factor 15 (TAF15) (Aman et al., 1996; Crozat et al., 1993; Kovar, 2011; Law et al., 2006; Yang et al., 2010). The FET family of proteins are all ubiquitously expressed nuclear proteins, which are highly conserved with predicted roles in RNA transcription, processing, transport and DNA repair (Bertrand et al., 1999; Crozat et al., 1993; Kovar, 2011; Law et al., 2006; Perrotti et al., 1998). They are also found to shuttle between the nucleus and the cytoplasm and their nuclear import is mediated by their non-classical nuclear localisation signal, called PY-NLS which is recognised by the nuclear import protein transportin-1 (TRN1). The pathological lesions in FTLD-FUS have been found to contain TRN1 (Brelstaff et al., 2011), EWS and TAF15 in varying degrees (Davidson et al., 2013; Neumann et al., 2012), suggesting that the pathogenic mechanism in FTLD-FUS is related to the dysfunction of transportin-mediated nuclear import affecting all FET proteins that are transported by TRN1 (Neumann et al., 2012).

FUS is structurally characterized by an N-terminal serine, tyrosine, glycine and glutamine-rich region, an RNA recognition motif (RRM), a C2/C2 zinc finger motif, multiple RGG repeat regions and a nuclear localisation signal (NLS) at the extreme C-terminus. The C-terminal region of FUS contains multiple domains involved in RNA-protein interactions, while the N-terminus is involved in transcription activation (Prasad et al., 1994). Due to its distinct structure and function FUS also belongs to the heterogeneous nuclear ribonucleoproteins (hnRNPs) and is also known as hnRNP P2 (Calvio et al., 1995).

hnRNPs are a family of around twenty major polypeptides, hnRNPs A1 to U, which range in size from 34 to 120kDa (Pinol-Roma et al., 1988). Each protein contains at least one RNA-binding motif such as an RNA recognition motif (RRM), a hnRNP K homology domain or an arginine/glycine-rich (RGG) box (Dreyfuss et al., 1993; Krecic and Swanson, 1999). Some hnRNPs contain auxiliary domains with unusual amino acid compositions, which mediate protein-protein interactions (Cartegni et al.,

1996). Correlated with these diverse structural features a multitude of cellular functions have been ascribed to hnRNP proteins, including roles in DNA maintenance, recombination, transcription, processing of primary transcripts, mRNA nuclear export, subcellular localisation, translation and stability of mature mRNA (Busch and Hertel, 2012; Dreyfuss et al., 2002, 1993; Roy et al., 2014). hnRNPs A1 and A2 constitute 60% of the total protein mass of hnRNP particles, representing the most abundant nuclear proteins (Beyer et al., 1977). These proteins are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport. While all hnRNPs are present in the nucleus, some shuttle between the nucleus and the cytoplasm and have distinct nucleic acid binding properties. FUS, along with other hnRNP proteins, is exported from the nucleus, probably bound to mRNA, and is immediately re-imported once dissociated. Its M9 domain acts as both a nuclear localization and nuclear export signal (Macara, 2001; Xu and Massagué, 2004). However, FUS can be distinguished from other hnRNPs notably by the presence of an N-terminal peptide sequence that can serve as a transcriptional activation domain (Zinszner et al., 1994).

As FUS is a member of the hnRNP protein family we wished to investigate whether any other hnRNP proteins were associated with FUS pathology and if they could be implicated in the pathogenesis of FTLD-FUS. We studied the localization of proteins of the hnRNP family in affected brain regions in patients with FTLD-FUS and normal control brains by immunohistochemistry, biochemical analysis and investigated their expression using nanostring technology.

2. Material and Methods

2.1 Cases

Brains were donated to the Queen Square Brain Bank for Neurological Disorders, UCL Institute of Neurology, University College London; the MRC London Brain Bank for Neurodegenerative Diseases, Institute of Psychiatry, King's College, London, UK; Neuropathology Department, Århus Kommunehospital, Århus, Denmark and NeuroResource, UCL Institute of Neurology, University College London. All cases had previously been diagnosed as NIFID (6 cases) or aFTLD-U (6 cases) characterised as having pathological inclusions that were immunoreactive for FUS and ubiquitin, but negative for both tau and TDP-43, with cases of the NIFID subgroup also containing α-internexin positive inclusions (Lashley et al., 2011). Ethical approval for the study was obtained from the National Hospital for Neurology and Neurosurgery Local Research Ethics Committee.

2.2 Immunohistochemistry

Seven-micron-thick tissue sections from the hippocampus, frontal cortex and spinal cord where cut from the following cases listed in table 1 (NIFID 1-6 and aFTLD-U 1-6) and 6 neurologically normal controls. Sections were deparaffinised in xylene and rehydrated using graded alcohols. Immunohistochemistry for all antibodies required pre-treatment with pressure cooker for 10 minutes in citrate buffer pH6.0. Endogenous peroxidase activity was blocked (0.3% H_20_2 in methanol, 10 minutes) and non-specific binding with 10% dried milk solution. Tissue sections were incubated with the primary antibodies overnight at 4^0C, followed by biotinylated anti-rabbit IgG (1:200, 30 minutes; DAKO) and ABC complex (30 minutes ; DAKO). Colour was developed with di-aminobenzidine/H_20_2 (Lashley et al., 2011). Table 2 lists all antibodies used in this study with supplier and concentrations used.

2.3 Double-label Immunofluorescence

This was applied to tissue sections of selected brain regions using an anti-FUS antibody in combination with the hnRNP A1 antibody that showed positive staining in the single stain preparations. After appropriate pre-treatment tissue sections were incubated with the secondary antibodies Alexa Fluor 488 and Alexa Fluor 568 (Molecular Probes, 1:300) for one hour at room temperature. 4'-6-diamidino-2-phenylindol (DAPI) was used for nuclear counterstaining. Sections were viewed with a Leica TCS4D confocal microscope using a 3-channel scan head and argon/krypton laser.

2.4 Biochemical fractionation and immunoblot analysis

Two biochemical fractionation methods were employed to separate the cytosol from the nuclear fractions. Firstly a method for sequential extraction of proteins with increasing insolubility was adapted from Neumann et al, 2009. For this study we have analysed the frontal cortex grey matter from NIFID (cases 2,3 and 5) and an aFTLD-U case (case 5). Neurologically normal (n= 4) and Alzheimer's disease cases (n=3) were also used as controls (Lashley et al., 2011; Neumann et al., 2009).

Protein concentration was determined by the BCA protein assay (Pierce, UK) and 10µg of high-salt buffer from each case were loaded onto 10% Bis-Tris gels (Invitrogen, UK) and run at 200V with MES (Invitrogen, UK) buffer under reducing conditions. Following electrophoresis, the proteins were transferred onto Hybond P membrane (GE Healthcare, UK) for 2hr at 40V. The membranes were blocked with 5% milk (Marvel) in PBS containing 0.1%Tween (PBS-T) and probed overnight with hnRNP A1 and β-actin antibodies. The membranes were washed in PBS-T three times for 5min each with shaking followed by incubating the blots with polyclonal HRP-conjugated secondary antibody (Santa Cruz, USA) at 1:2000 dilution for 30 min at room temperature. Following this, the membranes were washed thoroughly (three times for 5 min each with shaking). The specific bands were visualised by enhanced chemiluminesence (Pierce) and captured onto Biorad (Kodak, USA) membranes. The densities were quantified using Image J and the results expressed as a ratio.

2.5 Nuclear and cytoplasmic fraction analysis

One hundred mg of tissue was cut into small pieces and washed with 1XPBS containing protease inhibitors and centrifuged at 500g for 5min. Tissue was homogenised with a tissue grinder in "cytoplasmic extraction reagent" (Thermo scientific) according to manufacturer's instructions to separate nuclear and cytoplasmic fractions (NE-PER nuclear and cytoplasmic extraction reagents: Thermo Scientific). Protein was measured using BCA protein assay (Pierce). 30µg and 10µg protein from the cytosolic and nuclear extracts were run for each sample respectively (NIFID cases, 2, 3 and 5; aFTLD-U case 5; 3 AD cases) on 10% BIS-Tris gels (Invitrogen) and transferred onto hybond-P membrane (Amersham biosciences, VWR), blocked with 5% non-fat milk for 1hr at room temperature, incubated with mouse monoclonal primary antibody, hnRNPA1 (1:1000, Abcam) overnight at 4C. Protein loading was checked with beta actin antibody (monoclonal 1: 5000; Sigma) after stripping the blot with stripping solution (Thermo Scientific). For both antibodies HRP-conjugated anti-mouse secondary (Santa Cruz) was used at 1:2000 dilution. Densitometric images after ECL detection were captured onto Kodak-X-OMat films (Sigma). Band intensity was quantitated

using Image J Software and the graph and statistics were calculated using Graph-Pad Prism. One-way Anova was used to determine statistical significance between the sample groups.

2.6 Nanostring nCounter assay and analysis

Total RNA was extracted from the frontal and temporal cortices of 5 FTLD-FUS cases (NIFID cases 1,2,3 and 5; aFTLD-U 4) and 6 neurologically normal controls using the RNeasy kit (Qiagen) as per the manufacturer's instructions and RNA quality was evaluated using an Eppendorf Nanospectrophotometer. 100 ng of total RNA from each sample was analyzed with the NanoString nCounter analysis system (NanoString Technologies, Seattle, WA) using a pre-designed codeset. The codeset contained 17 probes for detection of the genes of interest (*FUS, TAF15, EWS, TNPO1, hnRNP A1, hnRNP A2/B1, hnRNP C, hnRNP D, hnRNP F, hnRNP G, hnRNP H1, hnRNP H3, hnRNP L, hnRNP M, hnRNP R, hnRNP U)*. Probes were designed according to the manufacturer's design principles (Geiss et al., 2008), including screening for inter- and intra-reporter and capture probe interactions, and selection for probes with optimal melting temperatures (Geiss et al., 2008). The laboratory running the assay was blinded to the diagnosis. To avoid run-order bias, samples of cases or controls were randomly assigned to plates. Raw counts were subjected to a technical normalization and normalized to the geometric mean using nSolver Analysis Software v2.0 (NanoString). Biological normalization using reference genes (*CLTC, GAPDH, GUSB, HPRT1, PGK1, TUBB*) included in the CodeSet was performed. Statistical analysis of Nanostring data was performed using Graphpad Prism 5 software (GraphPad Software Inc.).

3. Results

Cases used for this study have been described clinically and pathologically in previous publications (Brelstaff et al., 2011; Lashley et al., 2011; Rohrer et al., 2011). The presence of EWS, TAF15 and the hnRNP proteins were investigated in the hippocampal and frontal cortical areas that have previously been shown to be affected by FUS pathology (Lashley et al., 2011).

3.1 EWS and TAFF15 immunohistochemistry

TAF15 showed staining of normal neuronal and glial nuclei in all cases and controls, although the intensity of the neuronal staining was dependent on formalin fixation time; the shorter the fixation time was the higher the intensity of the normal staining was. Both aFTLD-U and NIFID subtypes of FLTD-FUS showed strong TAF15 staining of neuronal cytoplasmic inclusions (figure 1, tables 3 and 4) and intranuclear inclusions previously seen with FUS antibodies. Antibodies against EWS showed nuclear staining of both neuronal and glial cells. It was noted that not all cases showed normal physiological nuclear staining of EWS and the numbers of inclusions stained varied between

cases. However the two subtypes of FTLD-FUS investigated here showed EWS positive inclusions (figure 1, tables 3 and 4).

3.2 hnRNP immunohistochemistry

The presence of hnRNP proteins (A1, A2/B1, C, D, E, F, G, H, I, L M and U) in pathological inclusions was investigated in the hippocampal granular cell layer, CA1-CA4 hippocampal subregions, entorhinal cortex, frontal cortex and spinal cord and/or medulla wherever available. The presence of hnRNP proteins in pathological deposits were assessed as present (+) or absent (-) in the regions investigated (tables 3 and 4), as their frequency compared to FUS positive inclusions was low. All hnRNP proteins investigated were found in the both neuronal and glial nuclei in all FTLD-FUS cases regardless of their subtype (NIFID or aFTLD-U) and in neurologically normal controls. In cases previously diagnosed as NIFID hnRNP proteins A1, C, D, G, I and L were found in pathological inclusions (figure 2). hnRNP A1 was observed in neuronal cytoplasmic inclusions in the entorhinal cortex and frontal cortex in all 6 NIFID cases examined (figure 2a-b). hnRNP proteins C, D, G, I and L were observed in pathological inclusions in a number of NIFID cases (table 3, figure 2). It was noted that the majority of the hnRNP staining was observed in NIFID case 5 which lacked hippocampal FUS positive inclusions but contained sparse hippocampal hnRNP inclusions. hnRNP proteins A2/B1,E, F, H, M and U were not found in any pathological inclusions associated with disease in NIFID.

Cases previously diagnosed as aFTLD-U showed the least amount of hnRNP staining (table 4) pathological inclusions only being positive for hnRNP A1, hnRNP D and hnRNP I. These positive inclusions were sparse compared to the number of inclusions found to be positive with FUS. No pathological inclusions were found to be positive with hnRNP A2/B1, C, E, F, G, H, L, M or U.

3.3 FUS and hnRNP A1 double immunohistochemistry

hnRNP A1 positive pathological inclusions were observed in the entorhinal and frontal cortex in all NIFID cases and two aFTLD-U cases (tables 3 and 4). Double label immunohistochemical analysis with anti-FUS showed that the hnRNP A1 and FUS co-localised in the same neuronal cytoplasmic inclusions and neuropil threads (figure 3). No hnRNP A1 positive inclusions were found in the granular cell layer of the dentate gyrus in any case.

3.4 Cellular re-localisation of hnRNP A1 in FTLD-FUS

In addition to observing hnRNP A1 in the FUS-positive pathological inclusions, we observed an increase in hnRNP A1 in the cytoplasm of neurons compared to that seen in normal controls in the frontal and temporal cortices (figure 3, arrows). This shift in protein localisation was not observed with other hnRNP proteins tested where the proteins were localised to the nucleus in both

FTLD-FUS (data not shown) and normal controls. Nuclear and cytoplasmic biochemical fractionation was carried out to determine whether there were increased levels of hnRNP A1 in the cytoplasm of FTLD-FUS compared to normal controls.

A crude biochemical sequential fractionation was performed to separate the nuclear fraction from the cytoplasmic fraction (figure 4a). Probing the cytoplasmic fractions with an anti-hnRNP A1 antibody showed a significant increase in cytoplasmic hnRNP A1 in FTLD-FUS compared to cytoplasmic expression of hnRNP A1 in both normal controls and Alzheimer's disease cases (figure 4b). This was repeated using a commercially available kit (Thermo scientific) to separate the cytosol from the nuclear fraction (figure 4c) which confirmed the previous observations in that there is an increase of hnRNP A1 protein expression in the cytoplasm of FTLD-FUS cases compared to normal controls and Alzheimer's disease.

3.5 Expression of hnRNP proteins in FTLD-FUS

The expression of *FUS, TAF15, EWS, TNPO1, hnRNP A1, hnRNP A2/B1, hnRNP C, hnRNP D, hnRNP F, hnRNP G, hnRNP H1, hnRNP H3, hnRNP L, hnRNP M, hnRNP R, hnRNP U* were analysed using Nanostring technology for high-sensitive capture of mRNA transcripts (see supplementary table 1). The frontal and temporal cortices where analysed in FTLD-FUS (NIFID and aFTLD-U) and compared to frontal and temporal cortices from normal control cases with no pathological abnormalities. The normalised expression showed no significant difference in expression of *FUS, TAF15, EWS, TNPO1, hnRNP A1, hnRNP C, hnRNP F, hnRNP G, hnRNP H1, hnRNP H3, hnRNP L, hnRNP M and hnRNP R,* whereas a significant increase in expression of *hnRNP A2/B1, hnRNP D*, and *hnRNP U* was seen in FTLD-FUS cases (figure 5).

4. Discussion

In this study we have demonstrated that hnRNP proteins, associated with nuclear export, can be found in neuronal cytoplasmic inclusions and dystrophic neurites indicating an involvement of these proteins in the pathogenic mechanism of FTLD-FUS. The current suggested pathogenic mechanism of FTLD-FUS proposes that hypomethylation of the FET proteins, including FUS leads to an increased binding of TRN1 to these proteins causing a dysregulation of their TRN1-associated nuclear import (Dormann et al., 2012). We suggest that a broader dysfunction of not only nuclear import but also a disturbance of the nuclear export mechanism may be a contributing factor to disease pathogenesis. In our study we were able to detect hnRNP A1, D, G, I and L in pathological deposits in neurons, although only hnRNP A1 was shown through double label fluorescence immunohistochemistry to be co-localised with FUS in neuronal cytoplasmic inclusions and dystrophic neurites. hnRNP D, G, I and L were found in pathological deposits in the hippocampus of cases which were previously shown to

lack FUS pathology (Lashley et al., 2011). It is of note that of the two different FTLD-FUS subtypes investigated here those originally diagnosed as NIFID showed hnRNP A1, D, G, I and L proteins in FUS-negative deposits whereas those diagnosed as aFTLD-U typically had hnRNP A1 positive deposits. Furthermore, in addition to hnRNP A1 co-localising with FUS-positive inclusions, our immunohistochemical studies also showed that in comparison with normal controls, hnRNP A1 accumulated in a diffuse manner in the cytoplasm of neurons. This prompted us to perform biochemical analysis and we have shown a shift in cellular localisation of hnRNP A1 from the nucleus to the cytoplasm in FTLD-FUS compared to normal controls, suggesting that the normal function of this protein could be impaired due to its cellular re-localisation. mRNA expression analysis of *FUS, TAF15, EWS* and *TRN1* showed no increase in expression between FTLD-FUS and normal controls, supporting the notion that a reduced expression of TRN1 does not play a role in the pathogenesis of FTLD-FUS. However, of the 11 hnRNP proteins investigated the expression of *hnRNP A2/B1, hnRNP D* and *hnRNP U* was significantly increased compared to normal controls, although hnRNP A2/B1 and hnRNP U were not present in the pathological inclusions, hnRNP D was found in the occasional FUS-negative pathological deposit. The significance of the increased expression of hnRNP A2/B1, hnRNP D and hnRNP U requires further investigation. However, hnRNP A2/B1 overexpression plays a role in biogenesis and transport of mRNA in cancer (Yan-Sanders et al., 2002; Zhou et al., 1996). It is integral to cell proliferation and protein synthesis showing an increase in expression to cellular injury, and early indication of cell damage (Rajpurohit et al., 1994; Zhou et al., 1996). hnRNP D, also known as AU-rich element RNA-binding protein (AUF1), is an extensively studied AU-rich binding protein (AUBP). AUF1 has been shown to regulate mRNA turnover function to promote rapid mRNA degradation. AUF1 comprises a family of four related protein isoforms, with each isoform displaying multiple and distinct functions including the ability to target mRNA stability or decay, transcriptional activation of certain genes controlled by their different subcellular locations, expression levels, and post-translational modifications (Moore et al., 2014). Whilst hnRNP U has been shown to act as an accessory protein in DNA strand repair following oxidative damage (Dutta et al., 2015).

FUS belongs to the FET family of proteins and we have confirmed previous findings showing that the other members, TAF15 and EWS, are present in the FUS-positive inclusions found in FTLD-FUS (Neumann et al., 2011). Protein interaction studies have revealed that FET proteins are able to interact with each other forming protein complexes, therefore any disruption of the nuclear import of FUS would also result in accumulation of TAF15 or EWS in the inclusions (Kovar, 2011; Pahlich et al., 2008). Due to its distinct structure and function FUS also belongs to the hnRNP family and is also known as hnRNP P2 (Calvio et al., 1995). FUS, along with other hnRNP proteins, is exported from the nucleus by binding to mRNA, and, once dissociated from it FUS is immediately re-imported into the

nucleus via the TRN1 mediated process (Brelstaff et al., 2011; Dormann et al., 2010). According to a current hypothesis hypomethylated FET proteins have an increased binding affinity for TRN1 resulting in the build-up of such FET-TRN1 complexes in the cytoplasm overtime (Dormann et al., 2012). The presence of both FUS and TRN1 in neuronal intranuclear inclusions, as confirmed by our previous study, indicates that such abnormal FET-TRN1 complexes are able to shuttle into the nucleus (Brelstaff et al., 2011). The presence of normal nuclear FUS staining in a proportion of neurons with cytoplasmic and/or intranuclear inclusions indicates that FET proteins are able to dissociate from TRN1, consistent with the finding that hypomethylation of the FET protein only slightly increases their binding affinity to TRN1 (Dormann et al., 2010; Lashley et al., 2011; Neumann et al., 2012). One can hypothesise that due to the increased binding of FUS (and the other FET proteins) to TRN1 in the cytoplasm and nucleus will also compromise the nuclear export of FUS. This may also result in a disruption of the nuclear export of other hnRNP proteins, as under normal conditions shuttling hnRNPs are able to bind to each other through their auxiliary domains forming protein complexes. We found that hnRNP A1 was present in FUS-positive neuronal cytoplasmic inclusions in both NIFID and aFTLD-U. A previous study failed to show the presence of hnRNP A1 in pathological inclusions in two subtypes of BIBD and aFTLD-U; however it did not investigate this in NIFID (Neumann et al., 2012).

Dense cytoplasmic staining was also evident with hnRNP A1 immunohistochemistry and, although an increase in expression of hnRNP A1 mRNA was not evident, biochemical analysis showed an increase in cytoplasmic hnRNP A1 in FTLD-FUS suggesting a disruption of its normal functions. Although the focus of the current study was to investigate the hnRNP proteins involved in nuclear export, the presence of hnRNP A1 in the pathological inclusions adds evidence that defective TRN1-mediated nuclear import is not restricted to FET proteins as hnRNP A1 is also a cargo protein of TRN1 (Lee et al., 2006). Therefore investigating whether TRN1 mediated nuclear import of hnRNP A1 is regulated by arginine methylation in a similar fashion to the FET proteins remains to be shown.

Previous studies have shown that there are subtle differences in the staining patterns of the FET proteins with EWS being present in a proportion of FUS-positive inclusions in aFTLD-U cases, whereas in NIFID and BIBD the EWS staining is more consistent and inclusions are more robustly labelled (Davidson et al., 2013; Neumann et al., 2011). This was mirrored with our hnRNP immunohistochemistry with NIFID showing more hnRNP proteins in inclusions than aFTLD-U. It was also noted that TRN1, TAF15 and EWS were always found in pathological FUS-immunoreactive inclusions. However, hnRNP D, G, I and L were found in regions and deposits where no FUS pathology was seen. It is of note that for the present study tissue was only available from previously

diagnosed subtypes NIFID and aFTLD-U. Cases previously diagnosed as BIBD are a rarer subtype of FTLD-FUS and weren't available for this study.

Disruption of nuclear import mechanisms can result in the redistribution of proteins from the nucleus to the cytoplasm, which has been strongly implicated in the pathogenesis of FUS proteinopathies (Lagier-Tourenne et al., 2010; Wang et al., 2008). The hypothesis that cytoplasmic redistribution of FUS is central to the pathogenesis of FTLD-FUS is supported by several studies showing that FUS is recruited into stress granules due to cellular stress disrupting nuclear transport ALS-FUS and FTLD-FUS (Dormann et al., 2010) and cell culture experiments demonstrating that modification to the nuclear localisation signal of FUS disrupts the binding to TRN1 resulting in failure of the nuclear import of FUS in familial ALS-FUS (Dormann et al., 2010). However, this is a time dependent mechanism as the FUS protein accumulates in neuronal cytoplasmic and intranuclear inclusions but also normal nuclear staining of FUS remains suggesting that FUS continues to be imported into the nucleus (Lashley et al., 2011).

In summary this study has demonstrated the co-accumulation of hnRNP A1 in a proportion of FUS-positive inclusions in the NIFID and to a lesser extent in aFTLD-U subtypes of FTLD-FUS. The accumulation of hnRNP D, I, G and L involved in nuclear export are found in pathological deposits negative for FUS and may play a role in the pathological process. Subtle differences in the pathogenic pathways maybe involved in the different FTLD-FUS subtypes as more hnRNP proteins were found deposited in the NIFID subtype. Hypomethylation of the FET proteins increasing the binding affinity to TRN1 has been suggested as a possible mechanism involved in FTLD-FUS. Through the increased binding affinity of FUS to TRN1, whether the FUS-TRN1 complex is sequestered in neuronal cytoplasmic or nuclear inclusions, the involvement of FUS in the nuclear export mechanisms will be impaired. Therefore we suggested that a disruption of the nuclear export pathways should also be considered as potential mechanisms in FTLD-FUS.

Disclosure statement

The authors have no conflict of interest to disclose.

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and 5) were provided by the MRC London Neurodegenerative Diseases Brain Bank. NIFID case 2 was provided by Neuropathology Department, Århus Kommunehospital, Århus, Denmark.

FIGURE LEGENDS

Table 1: Demographic data of cases with FUS-positive inclusions. *aFTLD-U2 (son) is related to aFTLD-U6 (mother)

Table 2: Antibodies used in this study.

Table 3: Summary of hnRNP immunohistochemical staining in neuronal intermediate filament inclusion disease (NIFID). Qualitative analysis of the presence of hnRNP proteins in pathological deposits. '+' shows the depicted protein was found in a pathological deposits; '-' the depicted protein was absent from pathological deposits. NCI – neuronal cytoplasmic inclusions; NT – neuropil threads.

Table 4: Summary of hnRNP immunohistochemical staining in atypical frontotemporal lobar degeneration (aFTLD-U). Qualitative analysis of the presence of hnRNP proteins in pathological deposits. '+' shows the depicted protein was found in a pathological deposits; '-' the depicted protein was absent from pathological deposits. NCI – neuronal cytoplasmic inclusions; NT – neuropil threads.

Figure 1: FET pathology in FTLD-FUS. FET proteins are present in the pathological inclusions of NIFID (a, c,e,g,I, and k) and aFTLD-U (b,d,f,h,j, and l). FUS is present in neuronal cytoplasmic inclusions in the frontal cortex of NIFID (a) and motor neurons in the spinal cord (c), which was also seen in aFTLD-U cases (b and d). A similar staining pattern was seen with TAF15 (e-h) and EWS (i-l). Bar on a represents 40µm on a-b, e-f, i-j and 5µm on c-d, g-h, k-l.

Figure 2: hnRNP immunohistchemistry in NIFID (NIFID 4). Several hnRNP proteins were found in pathological deposits in FTLD-FUS. hnRNP A1 was observed in neuronal cytoplasmic inclusions in the frontal cortex (a and b). hnRNP D was found in neuronal cytoplasmic inclusions and in dystrophic neurites in the subiculum (c-f). hnRNP G was the only hnRNP protein to be found in the granular cells of the dentate fascia and dystrophic neurites (g and h). hnRNP I was found deposited in neuronal cytoplasmic deposits and dystrophic neurites (i-k). Bar in A represents 10µm in a, b, g and k; 20µm in h-j and f; 40µm in c-e.

Figure 3: Double immunohistochemistry and dense cytoplasmic hnRNP A1 staining (NIFID 4). Double immunohistochemistry with FUS and hnRNP A1 highlights the co-localisation of both proteins in neuronal cytoplasmic inclusions (double white arrows) and dystrophic neurites (single white arrows). hnRNP A1 immunohistochemistry in FTLD-FUS shows an increase in cytoplasmic hnRNP A1 compared to normal controls (arrows).

Figure 4: Figure 4: Biochemical fractionation of cytoplasmic extracts showing an increase of hnRNPA1 in the cytoplasmic fractions of FTLD-FUS cases compared to normal controls and AD cases. Biochemical fractionation was carried out using a sequential extraction from the frontal cortices from FTLD-FUS (NIFID 2 and 3; aFTLD-U 3 and 5), four pathologically normal controls and three AD cases (a). The amount of hnRNP A1 in the cytoplasmic extract was compared to the amount of β-actin found in each sample (b), a significant increase in hnRNP A1 was seen in the FTLD-FUS compared with normal controls and AD cases. Nuclear and cytoplasmic fractions were prepared using a commercially available separation kit (Thermo Scientific), confirming the increase of hnRNP A1 in the cytosolic fraction but with the retention of the hnRNP A1 in the nuclear fraction (c).

Figure 5: Nanostring expression analysis of hnRNP mRNA levels in FTLD-FUS. The expression of the FET, Transportin (TNP1) and the hnRNP mRNA levels were investigated in the frontal and temporal cortices of FTLD-FUS compared to normal controls. A significant increase in expression was identified for *hnRNP A2/B1*, *hnRNP D* and *hnRNP U*. No significant differences were found for other hnRNP mRNA's investigated.

Supplementary tables:

Supplementary table 1: Gene information table for nanostring analysis.

References

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