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LETTER TO THE EDITOR

Neuronal intranuclear (hyaline) inclusion disease and fragile X-associated tremor/ataxia syndrome: a morphological and molecular dilemma

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Sir,

We read with interest the article by Jun Sone and collaborators (Sone *et al.*, 2016) describing clinico-pathological features of adult-onset neuronal intranuclear (hyaline) inclusion disease (NIID). The authors describe in detail the clinical phenotype and define 'dementia' and 'limb-weakness' dominant groups. They also confirm the usefulness of skin biopsy demonstrating characteristic hyaline, ubiquitin and p62-positive intranuclear inclusions for the antemortem diagnosis of NIID.

We have also been confronted with the morphological dilemma of identifying characteristic hyaline intranuclear neuronal and/or astroglial inclusions in 10 post-mortem brains obtained from brain donors who gave their consent to use the brain tissue for research purposes (Table 1): do these findings correspond to NIID, do they represent a mere incidental finding accompanying another neurodegenerative disease or are they related to an adult form of fragile X-associated tremor/ataxia syndrome (FXTAS), a late-onset neurodegenerative disorder presenting with a wide spectrum of motor (tremor, ataxia, parkinsonism), cognitive and psychiatric symptoms in patients carrying a premutation (55–200 CGG repeats) at the fragile X mental retardation 1 gene (*FMR1*) (Hagerman and Hagerman, 2004).

As the authors state in their article, both diseases share clinical and neuropathological features. Indeed, they are

Table 1 Characteristics of patients with hyaline intranuclear inclusions at post-mortem neuropathological investigation corresponding to NIHID, FXTAS or as incidental forms

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10
Gender	Female	Female	Female	Male	Male	Male	Male	Female	Female	Male
Age at onset (years)	77 (dementia)	At least 82	49	47	55	NA	76	73	70	-
Age at death (years)	78	84	62	48	59	86	76	78	95	97
Disease duration	Unknown	At least 2 years	13 years	10 months	4 years	>2 years	1.5 months	5 years	25 years	-
Leading clinical syndrome	Parkinsonism, dementia	Dementia	Ataxia	Rapidly progressive ataxia, parkinsonism	Dementia and MND	Dementia	Rapid progressive dementia and gait disorder	Dementia, tremor, ataxia	Hypoacusia, tremor, ataxia, dementia	Asymptomatic
Family history	No	Yes	Yes (cephalic tremor)	Yes (FFI)	No	No	No	Yes	Yes	Yes
Diagnostic category	NIHID	NIHID	NIHID	Incidental NIHI	Incidental NIHI	Incidental NIHI	Incidental NIHI	FXTAS	FXTAS	Premutation carrier
Brain weight, g	860	1045	1300	1335	1015	-	1400	1130	1030	1190
Neuronal nuclear inclusions	+ +	+ +	+ +	+ (hippocampus)	+ + (Purkinje cells)	+ + hippocampus, + basal ganglia and anterior cingular cortex, ± temporal cortex and inferior olives	+ + hippocampus, + cortical areas, basal ganglia and Purkinje cells	Isolated	+ +	-
Glial nuclear inclusions	+ + +	+ + +	+ + +	-	-	-	+	Isolated	+ + +	-
FMR1 premutation	Na	No	No	No	No	No	No	Yes	Yes	Yes
No. of CGG repeats (normal range 6–44)	Na	30	20/31	30	30	41	30	65	77	60
Co-existing neuropathology	CJD	ARTAG, SVD	Rosai-Dorfmann	FFI	CJD (VV)	Multiple infracts, AD-related pathology BB III, Thal 2, CAA, AGD I	CJD (MM), AGD III, BB III, Thal I, CAA	Mild AGD I, PART def, SVD	Advanced AD A3B3C3	Mild AD A3B1C2
Skin biopsy	No	No	No	No	No	No	No	No	No	No

A, B, C score = A amyloid phase according to Thal, B Braak neurofibrillary stage, C neuritic plaque score according to CERAD (each score ranges from 0 to 3); AD = Alzheimer's disease neuropathological changes; AgD = argyrophilic grain disease; ARTAG = aging-related tau astrogliopathy; BB = Braak and Braak neurofibrillary stage; CAA = cerebral amyloid angiopathy; CJD = Creutzfeldt-Jakob disease; FFI = fatal familial insomnia; FXTAS = fragile X-associated tremor/ataxia syndrome; MM = methionine homozygosity at codon 129 of the PRNP gene; MND = motor neuron disease; NIHI = neuronal intraneuronal hyaline inclusions; NIHID = neuronal intraneuronal hyaline inclusion disease; PART = primary age-related tauopathy; SVD = small vessel disease; VV = valine homozygosity at codon 129 of the PRNP gene; Assessment of inclusions: – absent; + mild amount of inclusions at 100× magnification; + + moderate; + + + abundant

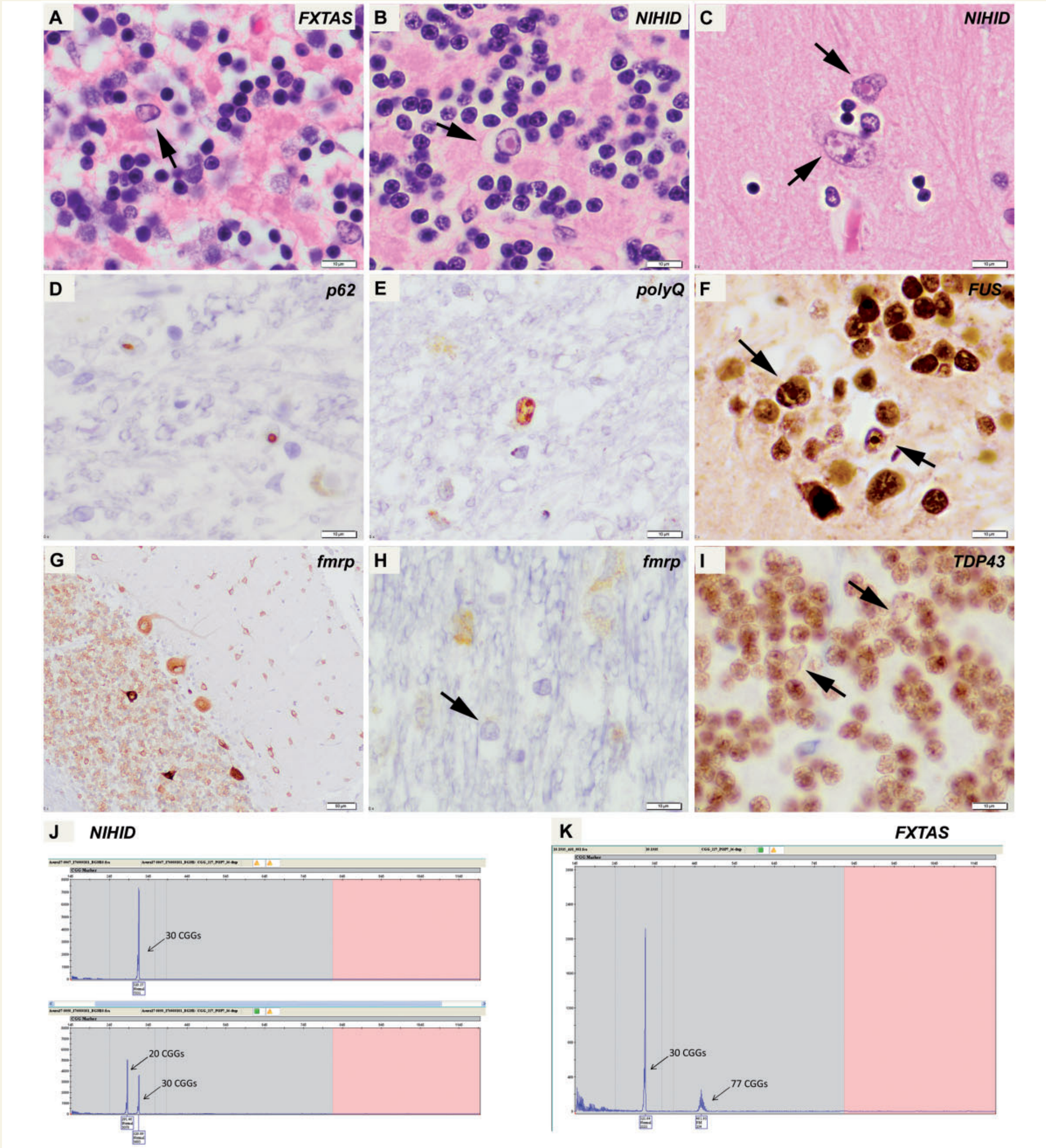

Figure 1 Morphological overlap of NIID and FXTAS. (**A–C**) Intranuclear hyaline inclusions in glial cells in cerebellar cortex are observed in FXTAS (**A**) and NIID (**B**), and also in neurons (**C**). **(D–I)** The immunohistochemical profile of the inclusions is identical in both diseases; inclusions are immunoreactive for p62 (**D**), rarely for polyQ (**E**), for FUS (**F**), and are negative for FMRP (**G** and **H**), and TDP43 (**I**). (**J** and **K**) Determination of CGG repeat track in the *FMR1* locus in patients with NIID (**J**) and in a FXTAS patient by means of genomic DNA sequencing.

indistinguishable morphologically in haematoxylin and eosin stained tissue sections (Fig. 1). Both disorders affect neurons and glial cells throughout the brain, including cortex, white matter, basal ganglia, hippocampus, brainstem, cerebellum and spinal cord (Fig. 1A–C). The intranuclear inclusions found in both disorders share immunohistochemical features: they are ubiquitinated, ubiquilinated, immunoreactive for p62 (Fig. 1D) and SUMO (Mori *et al*., 2012), they have several associated components (Pountney *et al*., 2008), a few show immunoreactivity for polyglutamine chains (Fig. 1E), and they are generally positive for FUS (Fig. 1F), but remain negative for TDP43 protein (Fig. 1I). Although immunostaining of inclusions by LAP2b has been also reported in FXTAS experimental setting (Sellier *et al*., 2017), we could not detect it in our human FXTAS and NIID cases; we only observed a nuclear membrane pattern (data not shown). This might be due to post-mortem delay, longer formalin fixation, or agonic factors, among others. In contrast to NIID in which intranuclear inclusions can be detected in skin biopsies, in FXTAS no inclusions have been detected in fibroblast cultures (Garcia-Arocena *et al*., 2010).

In adult forms of NIID presenting with dementia, intranuclear inclusions are more frequently detected in glial cells than in neurons (Munoz-Garcia and Ludwin, 1986; Takahashi-Fujigasaki, 2003). This is also seen in adult patients with FXTAS. Greco *et al*. (2006) observed in a post-mortem series of 11 FXTAS patients that the number of inclusions in neurons and astrocytes increases with increasing CGG repeats. The authors suggested that the CGG repeat is a powerful predictor of neurological involvement. Ariza *et al*. (2016) noted the frequent involvement of Purkinje cells in a study of 66 post-mortem cases and observed that the number of inclusions in the cerebellum increased with age. However, in one of our cases with 77 repeats and long disease duration (90 years old and 25 years of disease duration), we observed a high number of inclusions, suggesting that disease duration might also influence the development of inclusions.

The pathogenic mechanisms inducing neurodegeneration and the development of intranuclear inclusions in *FMR1* premutation carriers are largely unknown, as is the case for NIID. A gain-of-function 'toxicity' of the abnormal CGG-expanded *FMR1* mRNA was first proposed for FXTAS (Hagerman, 2013). It has been shown *in vitro* and in animal models that the expanded *FMR1* mRNA recruits RNA binding proteins with subsequent loss or impairment of their function (Sofola *et al*., 2007; Sellier *et al*., 2010; Muslimov *et al*., 2011). The exact time sequence of protein recruitment and whether these interactions are direct or mediated by other factors are still unknown. It has been shown that there is a repeat-associated non-AUG (RAN) translation of the expanded repeats that leads to expression of the polyglycine- and polyalanine-containing peptides, FMRpolyG and FMRpolyA, respectively (Todd *et al*., 2013). Specifically, a recent study reports a disease phenotype in transgenic mice expressing FMRpolyG, but not in mice with sole expression of *Fmr1* expanded RNA (Sellier *et al*., 2017). Despite the increasing evidence of the role of RAN translation products in the pathogenesis of FXTAS, the available disease models have limitations and RNA toxicity cannot be totally excluded. Nevertheless, the CGG knock-in mouse model recapitulates much of the pathology seen in patients, including increased expression of *FMR1* mRNA, decreased fragile X mental retardation protein (FMRP, encoded by *FMR1*) ubiquitin-positive intranuclear inclusions and evidence of motor and spatial processing deficits (Willemsen *et al*., 2003; Hunsaker, 2013). Interestingly, inclusions are not immunoreactive for FMRP in our human cases, either in FXTAS or in NIID (Fig. 1G and H).

Taken together these data raise some questions. First, what is the pathogenic role of the inclusions? In most adult NIID and FXTAS cases, despite the presence of widespread inclusions, there is no prominent neuronal loss. It is likely that neuronal dysfunction predominates and the inclusion may have a protective role in mitigating nuclear toxicity caused by abnormal proteins, as has been suggested for Huntington's disease or even for Lewy bodies in Parkinson's disease. Second, what is the relationship between the CGG expansion and the intranuclear inclusion? While some authors observed an increase in the number of inclusions with higher repeats, in two of our FXTAS cases (Cases 8 and 9), a similar number of repeats induced single inclusions in Case 8 and abundant and widespread inclusions in Case 9. This suggests that other factors such as age at death (95 versus 78 in our cases, respectively) or disease duration may influence the development of inclusions. Moreover, trinucleotide repeat expansions have not been found in NIID cases. However, in both NIID and FXTAS some inclusions stain with antibodies directed to polyglutamines (CAG repeats) or even ataxin 3. While this may represent a cross-reaction, it suggests that a long peptide chain may be attached to the inclusion. In any case, FMRP is not an integral part of the nuclear inclusion in either FXTAS or NIID (Fig. 1H). In agreement with this observation, a computational model has shown that FMRP does not have a strong sequestration propensity for the CGG repeat RNA (Cirillo *et al*., 2013; Botta-Orfila *et al*., 2016). This may be also related to compartmentalization of proteins and that RNA is transcribed in the cytoplasm and then accumulated in the nucleus.

Undoubtedly, FXTAS markers are needed to therapeutically target the cellular/neuronal dysfunction. What is intriguing to us is the existence of NIID pathogenicity, with a completely unknown molecular substrate. NIID research can certainly benefit from the FXTAS field, although it has to be elucidated whether these represent two completely different entities as suggested by their genotype and whether they share pathogenic mechanisms leading to the same intranuclear hyaline inclusion formation.

In conclusion, NIID and FXTAS may be clinically and morphologically indistinguishable, and even with the possibility of genetic testing for CGG expansion, there is an urgent need for the development of more specific inclusion markers, the elucidation of the role of *FMR1* and FMRpolyG or RNA regulating proteins in the development of inclusions and the identification of disease specific biomarkers.

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