Chapter 5
The Pathology of FXTAS

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Abstract In 2002 a syndrome of tremor, ataxia, cognitive decline, and the presence of unique ubiquitin staining intranuclear inclusions in the brain was discovered in premutation males carrying an expansion of between 55 and 200 CGG trinucleotide repeats on the *FMR1* gene. This clinical syndrome is now known as fragile X-associated tremor/ataxia (FXTAS) and has been found in both male and female carriers of the expanded premutation allele. The goal of this chapter is to summarize what is known about the anatomical pathology associated with the fragile X premutation and particularly in those individuals with FXTAS. Neuropathology in FXTAS was initially found in the central nervous system, but recent evidence has demonstrated pathological features, including intranuclear inclusions, in the peripheral nervous system, the enteric nervous system, and the neuroendocrine system. The precise cellular dysfunctions that underlie these pathologic features are currently under intense investigation with the goal of prevention and treatment of this devastating disorder.

Introduction

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a late-onset, progressive neurodegenerative disorder that affects many carriers of an *FMR1* premutation, an expanded trinucleotide repeat sequence (CGG) in the 5′ untranslated region (5′ UTR) of the *FMR1* gene. The gene is polymorphic, and in unaffected individuals there are roughly 5–45 CGG repeats, while individuals with a premutation carry an allele of 55–200 CGG repeats and show a 2- to 8-fold increase in levels of the *FMR1* mRNA (see Chapter 6).

Patients with FXTAS typically show cerebellar ataxia, tremor, cognitive deficits, peripheral neuropathy, autonomic dysfunction, and psychiatric involvement (see Chapters 1 and 3). The disorder is thought to arise from a toxic RNA gain of function

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that is caused by over-expression of the expanded CGG *FMR1* mRNA in premutation carriers (Allen et al. 2005; Kenneson et al. 2001; Tassone et al. 2000). Magnetic resonance imaging (MRI) also shows that patients with FXTAS have mild to moderate brain atrophy in both cerebrum and cerebellum and white matter changes in cerebrum and cerebellum. Increased T2 signal intensity in the middle cerebellar peduncles (MCP) is commonly found in subjects affected by FXTAS (Brunberg et al. 2002; see Chapter 4). Finally, the neuropathological hallmark of FXTAS is the presence of eosinophilic intranuclear inclusions in both neurons and astrocytes. These inclusions are found throughout the brain and in the autonomic nervous system as well as in non-nervous system tissues (e.g., pancreas). In light of these unique findings, the proposed diagnostic criteria for FXTAS have been revised and now include the presence of intranuclear inclusions as a major criterion (Hagerman and Hagerman 2004; Jacquemont et al. 2004).

Intranuclear Inclusions

Intranuclear inclusions are the distinctive pathological finding among premutation carriers affected by FXTAS. They have also been observed in a knock-in mouse model of the *FMR1* premutation (see Chapter 8). Observed initially in human brain tissues (Greco et al. 2002), eosinophilic intranuclear inclusions are widely distributed in both neurons and astrocytes, being found in many different regions throughout the brain, including the frontal cortex, hippocampus, ependymal cells, choroid plexus, brain stem nuclei, and cerebellum (Greco et al. 2002, 2006). Given the significance of clinical symptomology related to the limbic system, it is important to note that the highest percentage of inclusions in FXTAS cases is in the hippocampus. Immunohistochemically, these inclusions stain positive for ubiquitin, lamin A/C, and a number of heat-shock proteins (Iwahashi et al. 2006). They stain negative for tau isoforms, α-synuclein, and polyglutamine peptides and appear to reflect a new class of nuclear inclusion disorder as compared to other triplet repeat disorders, such as Huntington's disease and some of the spinocerebellar atrophies (SCA). FXTAS is also distinct from neuronal intranuclear inclusion disorder (NIID) (reviewed by Hagerman and Hagerman 2004). Furthermore, these inclusions do not contain any single predominant protein species; the most prominent protein accounts for only roughly 7% of the total protein mass (Iwahashi et al. 2006). Also noteworthy is that in patients with FXTAS, and in contrast to patients with CAG repeat degenerative disorders and inclusions, the protein product of the *FMR1* gene, FMRP, is structurally normal and present at relatively normal or only slightly reduced expression levels, due to the fact that the expanded CGG repeat occurs in a non-coding portion (5′ UTR) of the gene.

A highly efficient, flow-based isolation and purification of inclusions from postmortem FXTAS brain tissues has allowed for mass spectrometric analysis of the entire protein complement of the isolated inclusions as well as follow-up immunohistochemical analysis to conclusively identify more than 20 inclusion-associated proteins. Several proteins appear to be ubiquinated and/or polyubiquinated in these

purified inclusions, but ubiquinated proteins are the minority (Iwahashi et al. 2006). Ubiquitin is present within intracellular aggregates of a wide range of neurological disorders, not just FXTAS (Woulfe 2008). In the case of FXTAS, ubiquitin, a proteosomal degradation product, is utilized as a marker for isolation or detection of intranuclear inclusions by immunostaining (Greco et al. 2006, 2002; Iwahashi et al. 2006). Among the proteins identified within the inclusions are the RNA-binding protein, hnRNP A2, several intermediate filament (IF) proteins, including lamin A/C, the small heat-shock protein αB-crystallin, α-internexin, and other neurofilament (NF) proteins. The *FMR1* mRNA is present, but only as a minor component within the inclusions (Tassone et al. 2004a), and FMRP has not yet been found to be present in the inclusions. All of the proteins found in the inclusions are potential candidates for involvement in the RNA gain of function that may underlie FXTAS pathology (Hagerman and Hagerman 2004; Iwahashi et al. 2006).

The time course of inclusion formation relative to clinical onset of disease is not yet known, nor is it understood whether the intranuclear inclusions are directly causative of FXTAS pathophysiology and symptomology, or simply a reflection of the progression of the disorder. If the inclusion materials are active or neurotoxic, they may contribute directly to damage to the nervous system. However, it is also possible that the intranuclear inclusions may represent a protective mechanism, serving as a repository for disabled enzymes and their products as has been proposed for Huntington's disease (Bowman et al. 2005). Ongoing research is examining these possibilities.

It is unclear whether the *FMR1* premutation predisposes individuals to or accelerates the course of other degenerative diseases of the central nervous system (or vice versa), and this is also a topic of active investigation. A number of FXTAS cases that have come to autopsy showed Lewy body formation in the substantia nigra, whether or not Parkinson's disease (PD) was clinically identified (Greco et al. 2002). Two females carrying the FXTAS premutation who suffered early onset Alzheimer's

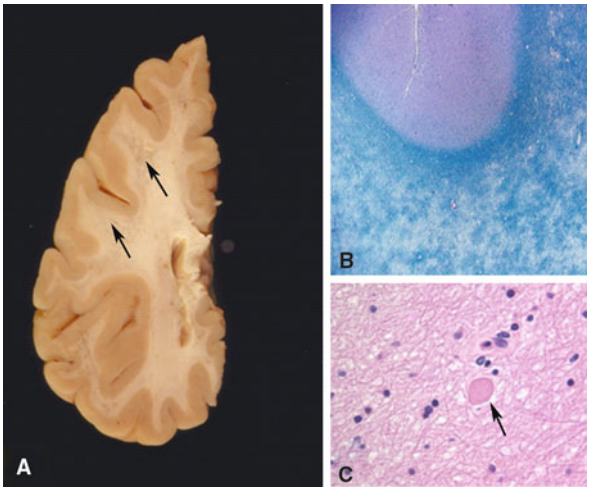

Fig. 5.1 (**a**) Severe subcortical white matter degeneration, as seen in some cases of FXTAS autopsy brains; (**b**) Corresponding patchy white matter loss, even affecting cortical U-fibers (×40, LFB–PAS stain); (**c**) Occasional swollen axons can be identified in cerebral and cerebellar white matter and middle cerebellar peduncles

disease (AD) symptomology along with tremor and ataxia showed histopathological features of both AD and FXTAS. The superior and middle temporal gyri in these two women showed as high a percentage of intranuclear inclusions as seen in the hippocampus (unpublished data). In a reported case of concurrent FXTAS and multiple sclerosis (MS), the patient showed patchy and diffuse signal intensity alterations in white matter on T2-weighted MRI scans. Histologically, there were numerous regions of demyelination as well as the presence of intranuclear inclusions (Fig. 5.1).

Brain Pathology

Gross Pathology

Gross abnormalities in the brain include varying degrees of cortical atrophy, patchy softening and loss of deep white matter, and brain stem atrophy, especially of the pons. When PD is concurrent, the substantia nigra is pale. There are no notable gross structural changes in the spinal cord. When AD is concurrent, cortical atrophy is often more prominent than that usually seen in FXTAS alone, although only a few cases of this type have been identified.

Microscopic Brain Pathology

At the present time, the intranuclear inclusions of FXTAS lack a distinctive molecular identity. On H&E stains, they are discrete, hyaline-appearing, eosinophilic, round to slightly ovoid bodies (Fig. 5.2). They typically measure 2–5 μm in diameter and are almost unanimously single, and are only very rarely double within a nucleus. They are periodic acid-Schiff (PAS), silver, tau, and neurofilament (NF) negative but stain positively for ubiquitin. Although inclusions have been identified in neurons throughout the brain, they have not been seen in Betz cells of the motor cortex and in only one case have been found in Purkinje cells of the cerebellum. Purkinje cell loss beyond that expected with otherwise normal aging and axonal swellings/torpedoes are commonly seen in FXTAS. Bergmann gliosis accompanies Purkinje cell loss in the cerebellum (Greco et al. 2006, 2002). Inclusions are also present in neurons of the dentate nucleus and astrocytes throughout the cerebellum. When clinical PD has been diagnosed, cytoplasmic Lewy bodies are seen in pigmented neurons of the substantia nigra, and when FXTAS coexists the intranuclear inclusions can be seen in the pigmented neurons of the substantia nigra, whether or not cytoplasmic Lewy bodies are present. In both symptomatic male and female premutation carriers who also carry a diagnosis of AD (i.e., diagnostic features of AD based on established criteria) (Braak and Braak 1991; CERAD; NIA-Reagan), the intranuclear inclusions of FXTAS can be seen in pyramidal neurons of the hippocampus that also contain neurofibrillary tangles.

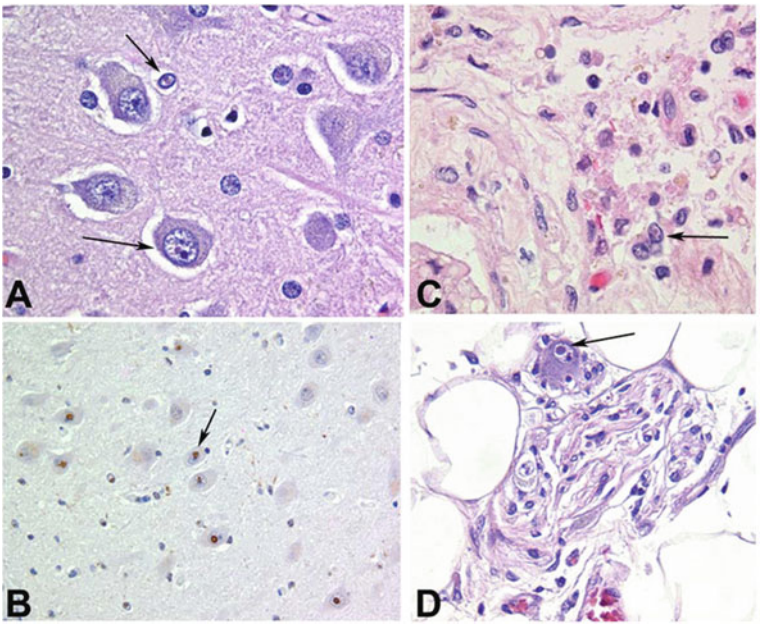

Fig. 5.2 (**a**) Neuronal and astrocytic intranuclear inclusions, CA4 (×400, H&E stain). (**b**) Ubiquitin immunoperoxidase stain showing numerous intranuclear inclusions, CA4, of hippocampus (×200). (**c**) Intranuclear inclusions, similar to those seen in the nervous system, are identified in Leydig cells (testosterone producing) of the testicles (×200, H&E). (**d**) Epicardial fat pad autonomic ganglion cell harboring an intranuclear inclusion (×200, H&E)

In astrocytes, intranuclear inclusions are usually surrounded by a clear halo, although this may be an artifact of tissue preparation. They are present diffusely in both protoplasmic astrocytes of gray matter and fibrillary astrocytes of white matter of the brain and spinal cord. They are also seen in pituicytes, the modified astrocytes of the posterior pituitary gland (Louis et al. 2006). Inclusions are also present in cells of the choroid plexus and ependyma, both of which have astrocyte lineages. In contrast, they are rarely present in microglia and have not been identified in oligodendrocyte nuclei or endothelial cells of the brain. The appearance of intranuclear inclusions is similar between males and females and between brain and spinal cord. While the spinal cord is otherwise grossly unremarkable, intranuclear inclusions have been identified in astrocytes and autonomic neurons of the intermediolateral column of the spinal cord but not in anterior horn cells (Gokden et al. 2008; Greco et al. 2002, 2006). Gokden et al. (2008) have also observed intranuclear inclusions in paraspinal sympathetic ganglia.

The appearance of intranuclear inclusions by electron microscopy is similar in neurons and astrocytes and appears as non-membrane-bound collections of granulofilamentous material (Greco et al. 2002). The filaments appear as straight rod-like proteins arranged in a haphazard manner (Gokden et al. 2008; Greco et al.

2002). The ultrastructural appearance of these inclusions is, however, otherwise not particularly informative.

Morphometric Analysis: Neuronal and Inclusion Counts

Percentages of neurons and astrocytes containing intranuclear inclusions have been determined only in one study, for frontal cortex, hippocampus, and the ventral pontine region of 8 male FXTAS patients and 10 normal (no neurological disease), age-matched control subjects. Quantification of inclusions was carried out using a computer-based imaging and cell-counting system (StereoInvestigator, MBI, Inc., Williston, VT) on H&E-stained slides. The number of neurons and astrocytes with intranuclear inclusions (actual counts and percentages) is presented in Table 5.1 (summary of Tables 3–5 from Greco et al. 2006). No inclusions were seen in control cases.

In general, more intranuclear inclusions were observed in astrocytes than in neurons (the hippocampal CA1 subregion was an exception, but the pyramidal cell layer was counted, which contains relatively few astrocytes compared to neurons relative to the cortex), although there was a great deal of variability across subjects. Statistical correlations (Spearman's rho) were calculated between histological findings and molecular measures. Significant positive correlations were present between the percentages of both neurons and astrocytes with inclusions in several brain regions and the number of CGG repeats. However, correlations between percentage inclusions and peripheral blood leukocyte *FMR1* mRNA or FMRP levels were not statistically significant. This last observation is not surprising in view of the large differences between expression levels in brain and blood and the region-specific differences in *FMR1* mRNA levels in brain (Tassone et al. 2004b). Most striking was the clinical–molecular correlation that showed a significant decrease in age of death with increasing CGG repeat length (i.e., the greater the CGG repeat number, the earlier the age of death; Greco et al. 2006).

Table 5.1 Percent of neurons and astrocytes with intranuclear inclusions in FXTAS patients

Brain region	Percentage of neurons with inclusions	Percentage of astrocytes with inclusions
Frontal cortex		
Gray matter	4.4 ± 1.4	16.7 ± 3.8
White matter	22.0 ± 6.4	5.0 ± 1.9
Hippocampus		
Pyramidal neurons	10.1 ± 2.8	10.3 ± 4.2
Granule cells	2.1 ± 1.0	26.6 ± 6.1
Hilar neurons	11.0 ± 3.6	28.3 ± 6.2
Pontine nuclei	0.2 ± 0.1	20.8 ± 3.1

Summary of data contained in Tables 3–5 in Greco et al. (2006).

White Matter Pathology

White matter changes seen on MRI studies include non-specific, subcortical, patchy regions of increased T2 signal intensity in the cerebrum. In a high percentage of FXTAS cases, increased T2 signal intensity is present in the middle cerebellar peduncles (Brunberg et al. 2002 and see Chapter 4) and can also be seen in the deep cerebellar white matter and brain stem.

When these regions are examined microscopically using histologic and immunochemical stains, abnormal areas of white matter show spongiosis, axonal degeneration, and myelin loss. The same histological features are identified in damaged white matter of the cerebellum (Greco et al. 2006, 2002). In cases with the most severe cerebral white matter changes, scattered fibrillary astrocytes are greatly enlarged by irregular expansion of cytoplasm that contains lysosomal debris. These same cells may also contain intranuclear inclusions. Rare axonal spheroids have been identified in spongiotic middle cerebellar peduncles on H&E and neurofilament stains. The middle cerebellar peduncles may also show myelin pallor on LFB–PAS stain (Greco et al. 2006).

Peripheral Nervous System

While the intranuclear inclusions of FXTAS were first identified in neurons and astrocytes of the brain in 2002 (Greco et al. 2002), systemic locations of the inclusions in the peripheral nervous system and other tissues are rapidly being cataloged and published in the medical literature.

Autonomic System

Inclusions have been observed in paraspinal sympathetic ganglion, ganglion cells of adrenal medulla, ganglion cells of the myenteric plexus of the stomach, and ganglion cells of a subepicardial ganglion. Also, intranuclear inclusions have been identified in dorsal root ganglion neurons in the spinal cord (autonomic neurons), but not in the ventral root (Gokden et al. 2008). Symptoms corresponding to this autonomic pathology may include mega-esophagus, constipation, bladder spasms, orthostasis, hypertension, and sexual dysfunction (see Chapters 1 and 9).

Peripheral Nerve

Non-specific features of axonal degeneration have been seen in nerve examined at autopsy. Inclusions have not been observed by light microscopy. Clinically, neuropathic features are seen in male premutation carriers (Berry-Kravis 2007), and peripheral neuropathy of variable severity is noted in individuals with FXTAS with reduced peripheral nerve conduction velocity (Soontarapornchai et al. 2008). The possible causes of this dysfunction are unknown.

Skeletal Muscle

Light microscopy, including histochemical and enzyme staining, has shown no pathological changes. Ultrastructural examination has yielded no distinctive abnormalities.

Neuroendocrine

In a limited number of cases (one male and one female), intranuclear inclusions within the anterior and posterior pituitary have been identified and may be associated with dysregulation of neuroendocrine function (Gokden et al. 2008; Greco et al. 2007; Louis et al. 2006). Similar findings have been made for the CGG KI mouse (see Chapter 8). This observation is of particular interest in view of the elevated cortisol levels found in FXTAS, as well as an increased incidence of anxiety disorders and depression (Bourgeois et al. 2009; Hunter et al. 2008; Rodriguez-Revenga et al. 2008).

Testicular pathology has been documented in two cases of FXTAS stained with H&E, including tubular fibrosis, decreased numbers of Leydig cells, and decreased spermatogenesis. Sertoli cells were abundant in the tubules along with a scant number of germ cells and spermatozoa that were remnants of germ maturation, but these changes were comparable to those seen in normal age-matched controls. There were also eosinophilic intranuclear inclusions in a small percentage of the Leydig cells in both cases as well as in the myoid cells of the tubular walls. Inclusions within the Leydig cells may be related to decreased levels of testosterone in some younger males with FXTAS who suffer premature erectile dysfunction. The presence of intranuclear inclusions in myoid cells in testicular connective tissue compartments in FXTAS is intriguing. The tunica propria of the testicle is a component of the tunica albuginea and it is the middle of the 3 layers of the fibrous capsule beneath the scrotal skin that protects and supports the testes. Among other cellular components, the tunica albuginea contains myofibroblasts. In the tunica propria smooth muscle cells are involved in contractile and transport functions. Inclusions in these cells suggest that other cell populations outside of the nervous system may also have inclusions. This finding raises the possibility of identifying easily accessible diagnostic tissue for biopsy, and such tissue samples could be used for diagnostic purposes or for monitoring therapeutic responses to treatment (Greco et al. 2007).

Summary

Since the initial discovery in 2002 that male premutation carriers with a clinical syndrome of tremor, ataxia, and cognitive decline showed a unique intranuclear inclusion disorder in pathological studies, there have been further studies elucidating the histologic, molecular, and biochemical features of the inclusions. The peripheral nervous system, specifically the autonomic system, is clearly involved,

as is the neuroendocrine system. Cellular dysfunctions that underlie these pathologic features are currently under intense investigation with the goal of prevention and treatment of this devastating disorder.

Abbreviations

DRPLA	Dentatorubropallidoluysian atrophy
SBMA	X-linked spinobulbar muscular atrophy (Kennedy's disease)
NIID	Neuronal intranuclear inclusion disease
SCA	Spinocerebellar ataxia
AD	Alzheimer's disease
PD	Parkinson's disease
5′ UTR	5′ Untranslated region
MS	Multiple sclerosis
FXTAS	Fragile X-associated tremor/ataxia syndrome
FMR1	Fragile X mental retardation gene
FMRP	Fragile X mental retardation protein
H&E	Hematoxylin and eosin stain
HD	Huntington's disease
PAS	Periodic acid-Schiff stain
LFB	Luxol fast blue stain
MCP	Middle cerebellar peduncle
IF	Intermediate filament
NF	Neurofilament

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