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Molecular Pathogenesis of FXTAS

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Abstract

Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) is a late-adult-onset neurodegenerative disorder that affects individuals who carry a premutation CGG-repeat expansion (55-200 CGG repeats) in the 5' untranslated portion of the fragile X mental retardation 1 (*FMRI*) gene. Affected individuals display cognitive decline, progressive intention tremor, gait ataxia, neuropathy, psychiatric symptoms, and Parkinsonism; the severity of both clinical and neuropathological phenotypes is positively correlated with the extent of the CGG expansion. Overexpression of the expanded CGG-repeat mRNA results in a direct gain-of-function cellular toxicity that is believed to form the pathogenic basis for FXTAS. This mechanism is entirely different from the mechanism giving rise to fragile X syndrome, which is due to transcriptional silencing and consequent loss of *FMRI* protein. Much of the research in the field has focused on understanding the link between the pathogenic *FMRI* mRNA and the potential proteins that interact with it.

Keywords

Neurodegeneration; fragile X; autism; neuropathy; trinucleotide; CGG

Introduction

Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) is a late-onset neurodegenerative disorder affecting many older male and some female carriers of a premutation CGG-repeat expansion (55-200 CGG repeats) in the fragile X mental retardation 1 (*FMRI*) gene. (1-8) Larger expansions of the highly polymorphic repeat (>200 CGG repeats; full mutation) are usually associated with transcriptional silencing of the *FMRI* gene and consequent loss of *FMRI* protein (FMRP), (1,9-13) which plays an important role in synaptogenesis and synaptic plasticity. (14) Absence of the protein leads to fragile X syndrome, a neurodevelopmental disorder and the leading monogenic form of cognitive impairment and autism. (Clinical reviews: 15,16-18)

Epidemiological studies have revealed that nearly one in 3,000 males, and a smaller number of females, is at risk of developing FXTAS. (3,5,19,20) Affected individuals display cognitive decline, progressive intention tremor, gait ataxia, peripheral neuropathy, psychiatric symptoms, and Parkinsonism, and the severity of both clinical and neuropathological phenotypes are positively correlated with the extent of the CGG expansion. (21-27) Magnetic

resonance imaging (MRI) reveals white matter disease, diffuse brain atrophy, and high signal lesions (T2/FLAIR) of the middle cerebellar peduncles (“MCP sign”). (4,28) Furthermore, immunohistochemical staining of post mortem brain tissue from FXTAS cases reveals loss of axons and myelin, significant astroglial activation in subcortical white matter, as well as Purkinje cell loss in the cerebellum. (5,22,28) Additionally, ubiquitin-positive intranuclear inclusions (Figure 1) are found in both neurons and astroglial cells in wide distribution in post mortem brains of FXTAS patients. (5,28) Moreover, the number of the inclusions is positively correlated with the CGG number. Inclusions of similar morphology, albeit distinct composition, are found in several other neurodegenerative diseases. (29-31)

The discovery of FXTAS among adult carriers of premutation *FMRI* alleles has revealed an entirely separate molecular mechanism from that operating in fragile X syndrome. In contrast to the complete transcriptional silencing of *FMRI* in full mutation alleles, premutation carriers produce up to eight-fold more *FMRI* mRNA over normal levels in blood leukocytes, despite expressing slightly to moderately less FMRP. (32-35) The expanded CGG-repeat mRNA *per se*, present at elevated levels, is now thought to exert a toxic “gain-of-function” that leads to FXTAS. (1,2,5) Many of the features of FXTAS, particularly those involving neuropathology, have been recapitulated in mouse (36-38) and fly (39-43) models of FXTAS. A review of the animal work is the subject of a companion article. (44)

RNA toxicity in FXTAS

Initial evidence for an mRNA-mediated pathogenesis of FXTAS was based on patterns of clinical involvement, where it was demonstrated that only individuals who were premutation carriers, with elevated levels of the expanded CGG-repeat mRNA, developed FXTAS. (2-4, 28,32) Adults with full mutation alleles, which are generally transcriptionally silent (no RNA or protein) have not been reported to develop FXTAS. (1) Further, although there is some diminution of FMRP levels in the upper portion of the premutation range (>100 CGG repeats), (33,35) most cases of FXTAS tend to occur in the ~70-120 CGG repeat range where FMRP levels are only slightly reduced. Again, older adults with fragile X syndrome, whose protein levels are often undetectable, do not appear to get FXTAS thus ruling out an FMRP-loss mechanism. In this regard, a similar argument had earlier been made for a possible RNA-based mechanism for primary ovarian insufficiency, (45) which affects a substantial fraction of women who are carriers of premutation alleles.

Perhaps the most important findings in support of an RNA gain-of function pathogenesis for FXTAS is the presence of *FMRI* mRNA within the intranuclear inclusions in the CNS. (22, 28,46) Using biotinylated riboprobes specific for the *FMRI* 5' UTR, *FMRI* mRNA was detected within inclusion-bearing nuclei isolated from a human post mortem FXTAS brain. This was the first direct evidence in humans for involvement of the *FMRI* RNA in the pathogenic mechanism for FXTAS. This observation parallels the prior observations of mRNA-containing intranuclear foci in the myotonic dystrophies, DM1 (CUG repeat) and DM2 (CCUG repeat). (47,48) The myotonic dystrophies represent the paradigm for RNA gain-of-function toxicity. In both DM1 and DM2, the CUG and CCUG repeat-containing mRNAs interact with, and sequester the CUG-binding protein muscleblind-like 1 (*MBNL1*), leading to the aberrant splicing events associated with disease formation. Based on this model (Figure 2), research has focused on understanding the link between the pathogenic *FMRI* mRNA, and the potential proteins that interact with it.

A second important finding was that a cellular phenotype could be induced in neuroepithelial (SK) cells in culture by transfecting the cells with plasmids that retain only the expanded CGG repeat (~95 CGG repeats) and adjacent 5' untranslated region, with the *FMRI* promoter and FMRP coding region replaced by a viral (CMV) promoter and GFP reporter. (49) The principal

feature of the phenotype was the collapse of the lamin A/C nuclear architecture; the lamin A and C isoforms, produced by the *LMNA* gene (OMIM*150330) are found within the intranuclear inclusions. (31; See: below) Further, the abnormal cellular phenotype was only induced when the CGG-containing reporter gene was expressed, since inactivation of the viral promoter eliminated the effects of the CGG repeat. This last observation is analogous to the absence of evident clinical features of FXTAS in individuals with full mutation alleles, where the *FMR1* gene is transcriptionally silent. A second important component of the cellular phenotype was the formation of α B-crystallin-positive intranuclear, despite the absence of any *FMR1* coding sequence. These results demonstrated that a non-coding CGG-5'UTR transcript is sufficient to recapitulate at least two major features of the FXTAS cellular phenotype. Moreover, the 99 CGG construct was able to induce inclusion formation in primary glial progenitor cells. In contrast, cells transfected with 30 CGGs did not form inclusions. Interestingly, massive cell death was observed in the cells transfected with 99 CGGs; however very little of the cell death could be attributed to apoptotic pathways. (49)

Components of the intranuclear inclusions of FXTAS

The fact that RNA is not intrinsically toxic implies that there must be one or more protein effectors of the gain-of-function effects of the expanded CGG-repeat mRNA. To address this issue, a combination of gel-based methods and tandem mass spectrometry (MS) were utilized to identify more than 20 proteins in the inclusions. (31) Among the proteins found within the inclusions were several stress-response ("heat shock") proteins, including HSP27, HSP70, and α B-crystallin. The presence of these proteins suggests that there is an overall cellular stress induced by the expanded CGG-repeat mRNA. While this observation is not unexpected, the nature and origin of the primary stress are currently not known.

Also included in the list of proteins are the lamin A/C isoforms, the presence of which provided the impetus for the investigation of the lamin architecture mentioned above. The presence of the lamin isoforms within the inclusions, coupled with the altered nuclear lamin architecture, provide a potentially important link to the clinical features of FXTAS; namely, the prominence of peripheral neuropathy among those who have the core features of FXTAS (tremor and gait ataxia), and even among those who are carriers of premutation alleles, but who do not have FXTAS. (19,24,50,51) Mutations in the *LMNA* gene are known to give rise to a form of axonal Charcot-Marie-Tooth (CMT) peripheral neuropathy (CMT Type 2B1; OMIM #605588), suggesting that the neuropathic features of FXTAS may represent at least in part a functional laminopathy.

Also among the species present in the inclusions are at least three RNA binding proteins, hnRNP A2, MBNL1, and *pur* α (31,52). While for none of these proteins is there evidence of functional importance in FXTAS pathogenesis, the work of Jin and co-workers (52) in *Drosophila* suggests that the CGG-induced neurodegenerative phenotype can be rescued by overexpression of *pur* α . Based on the broad range of functions of *pur* α in DNA replication/repair, transcription, and translation, coupled with the early postnatal lethal phenotype of *pur* α knockout in mice, (Review: 53) it is somewhat surprising that the phenotype of FXTAS is both late adult-onset and incompletely penetrant. However, a *pur* α -coupled mechanism might be consistent with a moderately lowered level of *pur* α due to partial sequestration. Additional work will be needed in mouse models in order to define the role (if any) of *pur* α in FXTAS. Interestingly, *pur* α was not observed in the inclusions by MS, though low levels of that protein, not detectable by those methods could still be revealed through immunohistochemical methods. HnRNP A2, another of the RNA binding proteins identified by MS, (31) was also shown to exhibit a rescue phenotype in *Drosophila*. (43) It is possible that partial sequestration of hnRNP A2 may lead to altered transport of its target mRNAs and cause cellular dysregulation.

A third RNA binding protein, MBNL1, found in the FXTAS inclusions, plays a prominent role in myotonic dystrophy; however, as yet, there is no evidence for a functional role of this protein in FXTAS. MBNL1 has not been shown to bind CGG repeat mRNA directly; however recent evidence implicates it as a more general modulator of repeat RNA toxicity. (54) Those researchers were able to induce neurodegeneration by overexpressing an untranslated CAG repeat mRNA in *Drosophila*. Surprisingly, overexpression of MBNL1 exacerbated the effect, while downregulation attenuated the CAG induced neurodegeneration – an effect opposite to that observed with MBNL1 sequestration in the DM model. This study was not only important in demonstrating that RNA toxicity may be a component spinocerebellar ataxia 3 (SCA3), traditionally thought to be strictly a polyglutamine disorder, but also suggested that MBL1 may have distinct roles in disease pathogenesis depending on the particular trinucleotide expansion. Finally, in contrast to its involvement in myotonic dystrophy, MBNL1 did not lead to mis-splicing of a target reporter construct, suggesting a distinct pathogenic mechanism in CAG RNA-induced neurodegeneration. (54)

Another significant finding was the absence of any single, predominant protein species in the inclusions. (31) This argues against a mechanism in which the mass accumulation of one or more specific proteins leads to inclusion formation and cell death; a result that stands in contrast to Huntington disease (huntingtin), the tauopathies (tau protein), or Parkinson disease (α -synuclein). (29,31) In addition, very few of the isolated proteins were found to be ubiquitinated, with no evidence of polyubiquitination; a result that stands in contrast to other neurodegenerative disorders in which accumulation of ubiquitinated, aberrant protein species in inclusions is thought to overwhelm the proteasomal degradation pathway. (review: 55) However, it is possible that accumulation of a protein in the inclusions in even small quantities could be triggering downstream pathways leading to cell death. (31)

The case for mRNA toxicity in FXTAS was further strengthened through the creation of a 98 CGG knock-in mouse. (38,44) Using homologous recombination, those researchers inserted a human 98 CGG repeat into the 5'UTR of the mouse *FMR1* gene to study the stability and methylation of the allele, as well as the pathogenesis. Through selective breeding, mice were produced with varying numbers of repeats spanning the premutation range. Using real-time PCR, they found 2 to 3.5-fold elevated *FMR1* mRNA in brain compared to wild type mice, consistent with the situation in humans. (46) Although no gross abnormalities were observed in 20-72 week old premutation mice, ubiquitin-positive inclusions were widely distributed throughout the brains. The inclusions were only observed in nuclei and, in contrast to post mortem brains from FXTAS patients, none was seen in astrocytes. (38)

Another important focus of the mouse study was on the biological components of the intranuclear inclusions. Using immunohistochemistry, Willemsen and co-workers (38) did not see any FMRP in the inclusions; however they did find heat shock protein HSP40, and the 20S catalytic complex of the proteasome. They also found the 20S complex co-localized with ubiquitin in many of the inclusions. However, many other proteins involved in neurodegenerative disorders, including MAP2, tyrosine tubulin, MAP1B, actin, SUMO1, GFAP, TAU, neurofilament, nucleolin, HSP27, HSP60, and HSP72, did not co-stain with inclusions, which suggests novel neuropathogenic processes in the FXTAS mouse model. (29,38)

The potential for therapeutic intervention

Although some treatment options are available for patients with FXTAS, essentially all are based on alleviation of symptoms, but do not target the underlying pathogenic processes. A targeted therapeutic agent directed towards the expanded CGG-repeat mRNA is clearly a plausible treatment option. Knockdown of the RNA itself using either small interfering RNA

(siRNA) or antisense DNA analogs is an approach that is currently under study. One caveat with the use of siRNA is that its site of action in the cytoplasm would not address any nuclear-specific toxic events. An alternative approach to therapeutic intervention would be to target one or more steps in the pathogenic process that is induced by expression of the CGG-repeat mRNA; elucidation of the steps in this process is currently underway in a number of laboratories. In this regard, researchers recently were able to design a small molecule, cell permeable ligand, which binds both CUG and CAG repeat mRNAs and disrupts their interactions with MBNL1. (56) Thus, to the extent that MBNL1 sequestration is important for disease pathogenesis (e.g., in DM), this approach has merit.

From the perspective of targeting RNA-protein interactions in FXTAS, as in the DM model, it is important to recognize that the consequence of a specific protein-RNA interaction may not be sequestration. Whereas the CUG expansions in DM may exceed several thousand repeats, disease-causing expansions in FXTAS may be as little as two-three fold above normal repeat lengths; thus, the trigger for disease pathogenesis may not be loss of function (sequestration), but gain of function, as would be the case were an RNA-protein interaction to result in protein activation. One classic example of such an activation is the interaction of RNA-stimulated double-stranded protein kinase (PKR) with double-stranded (e.g., viral) RNA. Although studies to date suggest that PKR itself is not involved with FXTAS pathogenesis, (57) the principle remains viable.

Conclusion

As with other neurodegenerative disorders, several important questions remain to be resolved with regard to the molecular pathogenesis of FXTAS. Why do some individuals not appear to get FXTAS? When, during the course of disease, does intervention need to occur in order to substantially reverse the course of the illness? Also, in treating existing disease, how much improvement might be expected? Lastly, does the absence of full penetrance of the disorder imply the co-occurrence of other environmental or genetic factors? Resolution to these questions will substantially improve our ability to design rational treatment approaches for FXTAS. With this disorder, we have the distinct advantage of knowing both the responsible gene and the primary pathogenic trigger – the expanded CGG-repeat mRNA. This knowledge provides avenues of approach to the more complete characterization of the clinical phenotype, (18) the creation of appropriate animal models, (44) and the development of targeted therapeutic agents.

Acknowledgments

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Figure 1. Cortical neuron and adjacent astrocyte demonstrating ubiquitin-positive intranuclear inclusions in post-mortem brain tissue from a male who died with FXTAS. Original magnification, 1,000 $\times$. Image courtesy of Dr. Claudia Greco.

Figure 2.

Two limiting models for RNA-triggered pathogenesis in FXTAS. In the sequestration model, as typified by myotonic dystrophy, excessive protein binding to the triplet repeat limits availability of those proteins to carry out other cellular functions; pathogenesis arises as a consequence of a functional protein deficiency. In the trigger model, interactions between proteins and the triplet repeat lead to activation of the protein; e.g., through phosphorylation, leading to activation of downstream signaling cascades.