Synopsis of the Ph.D. thesis on

Study the association of micro-RNA with mitochondria in Fragile X-associated tremor/ataxia syndrome (FXTAS) and their role in regulation of neuronal cell death

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INTRODUCTION

FXTAS is a late onset inherited neurodegenerative disorder characterized by progressive intention tremor, gait ataxia and cognitive decline [1],[2]. Nearly,1 in~3000 male and 1 in ~5000 female can be affected by FXTAS and disease symptoms get more pronounced with the age [3]. FXTAS is caused an expansion of 55 to 200 CGG repeats (known as premutation) at the 5'UTR of the *FMR1* gene located on the long arm of X chromosome [4]. The expanded CGG repeats are transcribed into RNAs that titrate specific RNA binding proteins such as the DROSHA/DGCR8 complex involved in regulation of the processing of microRNAs (miRNAs) [5]. Consequently, expression of various miRNAs are altered in FXTAS [6],[7]. CGG repeats embedded in the 5'UTR of *FMR1* are translated in to a toxic polyglycine-containing protein, FMRpolyG, through initiation via a non-canonical ACG start codon located upstream of the repeats [8]–[10]. However, it is still not understood if CGG RNA and/ or FMRpolyG protein contribute to mitochondrial alterations leading neuronal cell dysfunctions and death [11]. Importantly, recent findings suggest that mitochondrial dysfunctions, including loss of mitochondrial membrane potential, ATP and mitochondrial transcripts and proteins are associated with FXTAS pathogenesis [11]–[13]. We have recently shown decreased expression levels of mitochondrial transcripts in FXTAS leading to altered mitochondrial supercomplexes assembly and individual complex activity in cellular models and transgenic mice expressing expanded CGG repeats [14]. However, the molecular mechanisms regulating mitochondrial dysfunction in FXTAS conditions are not well understood.

miRNAs belong to a class of noncoding RNAs important for post transcriptional regulation of mRNAs by partial base-pairing of miRNA-mRNA mediated by the RNA-induced silencing complexes (RISC) [15],[16]. Studies from last decade have shown evidences of organelle specific localization of miRNAs and presence of crucial RISC component like Ago2 in the mitochondria [17],[18]. We and others have also reported that specific nuclear encoded miRNAs translocate to mitochondria (referred as mito-miRs) under specific stimuli and can regulate mitochondrial functions [19],[20]. As miRNAs expression and mitochondrial functions are altered in FXTAS, we investigated mito-miRs and their potential role in dysregulated mitochondrial bioenergetics in FXTAS *in vitro* cellular model. Interestingly, we identified a specific population of miRNAs which was altered in mitochondrial fractions in cells expressing expanded CGG repeats. Among these miRNAs, we analyzed the role of miR-320a, which was specifically enriched in mitochondria in FXTAS condition. Finally, transfection of miR-320a mimic showed increased OXPHOS activity, suggesting a crucial role of miR-320a in FXTAS pathology.

HYPOTHESIS

Mitochondrial dysfunctions are one of the mor hallmarks associated with FXTAS pathology. Further, growing evidences have confirmed the presence of nuclear encoded RNA species in the mitochondria including miRNAs, referred as mito-miRNAs. The transport of nuclear encoded

miRNA to the mitochondria is required for optimal mitochondrial function. We hypothesized that in FXTAS condition, where secondary RNA structures (hairpin, duplexes) sequester several RNA binding proteins including proteins involved in miRNA biogenesis (e.g. DROSHA/ DGCR8). This may lead to decreased pool of miRNAs and also affect translocation of miRNA to mitochondria. Thus, the role of mito-miRNAs in modulation of mitochondrial functions should be considered and needs to be investigated further to understand FXTAS pathogenicity.

SIGNIFICANCE OF THE STUDY

The proposed research work is highly innovative as it is focused on identifying novel mechanisms explaining mitochondrial dysfunction and brain degeneration in human. This is also a pioneering study since microRNAs and mitochondria have never been linked in FXTAS, and mitochondria dysfunction is a key component of majority of neurodegenerative disorders including FXTAS. Furthermore, this study may have significant implications beyond FXTAS as neurodegenerative diseases represent an increasing burden and challenge to society and are one of the main causes of death. FXTAS shares some neuropathology features with a number of other neurodegenerative diseases, including Parkinson disease, spinocerebellar ataxias, as well as, but in a lesser extent, Alzheimer disease and other dementia. The proposed research on FXTAS will enrich our knowledge on other neurodegenerative disorders caused by repeat expansion. It would also define site specific association of mitochondria and associated miRNA in pathologic progression of FXTAS. Moreover, the miRNA is known to be stable in the body fluids like serum, urine and plasma, the unique pattern of miRNA may serve as novel prognosis, and marker for the progression of the disease.

PROPOSED OBJECTIVES

- **Objective-1:** Analysis of FMRpolyG induced toxicity in FXTAS condition (in vitro cell line model system.)
- **Objective-2:** Alteration in mitochondrial function, dynamics and physiology due to expressed expanded CGG repeats.
- **Objective-3:** Study the miRNA dysregulation at cellular levels and its association with mitochondria.
- **Objective 4:** Understanding the roles of identified miRNAs in mitochondrial dysfunctions in FXTAS condition.

RESULTS (OBJECTIVE WISE)

Objective 1.

Expression of expanded CGG repeats decreases cellular viability in vitro.

- The ectopic expression of both ATG FMRpolyG-GFP and 5'UTR FMR1 CGG99X significantly decrease cellular viability n HEK293, U87MG and SH-SY5Y. In conscience to this, CGG repeats further lead to increase in extra cellular release of LDH in HEK293, U87MG and SH-SY5Y cells.
- The expression of 5'UTR FMR1 CGG99X shows increased casapse-3/7 activity as compared to vector transfected cells in HEK-293 and SHSY-5Y neuronal cells.
- These results suggest that the expression of CGG repeats increases cellular toxicity and induces caspase dependent apoptosis, in the cells derived from different origin.
- CGG repeats translated via RAN translation and forms toxic protein called FMRpolyG. FMRpolyG forms nuclear inclusion which can be visible as puncta of GFP in the nucleus under fluorescence microscope. The cells having nuclear inclusions show altered morphology as compared to vector transfected cells.
- FMRpolyG also forms cytosolic aggregates, which get colocalize with mitochondria as seen under confocal microscope. Immunoblotting against GFP in the mitochondrial fraction further confirms interaction of FMRpolyG with mitochondria.

Objective 2.

FMRpolyG alters mitochondrial functions in cell lines, mice model and patients.

We further hypothesized that interaction of FMRpolyG with mitochondria may modulate mitochondrial functions.

- Transfection of FXTAS premutation constructs lead to decreased ATP levels in HEK293, SH-SY5Y cells. Brain cells from FXTAS transgenic mice model also shows decreased ATP level as compare to control mice.
- Mitochondrial membrane potential decreased in HEK293 and SH-SY5Y cells transfected with ACG 99XGly FMR as compared to control cells.

Reduced ATP levels may be due to dysfunction of mitochondrial respiratory chain complexes. Hence, Mitochondrial supercomplexes assembly and individual complex activity were checked by Blue-Native PAGE.

- Mitochondrial supercomplexes assembly gets altered in SH-SY5Y, HEK293 cells and in the neurons derived from FXTAS transgenic mice brain.
- Translation of expanded CGG repeats cause significant decrease in complex I (NADH dehydrogenase) and complex IV (Cytochrome Oxidase) activity as compared to control in SH-SY5Y, HEK293 and neurons derived from FXTAS transgenic mice brain.

- Mitochondrial DNA copy number shows no significant change in FXTAS premutation condition in HEK293 and SH-SY5Ycells. In contradictory to this, the level of mitochondrial transcripts decreased in HEK293, SH-SY5Y cells under both CGG repeats transfected condition.
- RNA samples derived from cerebellum region of FXTAS patient brain also showed depleted levels of majority of mitochondrial transcripts such as 16S rRNA, ND1, ND2, ND3, ND5, COX1, COX2, COX3 and ATP6. These results clearly indicate that mitochondrial respiratory chain dysfunction is outcome of decreased mitochondrial transcripts levels.

Objective 3:

FMRpolyG translated due to translated CGG repeats causes altered pattern of mitochondrial associated miRNAs.

- The role of microRNAs(miRNAs) have been emerging during neuronal development, maintenance and optimal functions. Their aberrant expressions have been observed in in neurodegenerative diseases such as Parkinson, Alzheimer and Huntington diseases.
- To check the effect of translated CGG repeats on expression levels of mitochondrial associated miRNAs under premutation condition, HEK293 cells were transfected with premutation constructs. 24 hours of post transfection, cells were collected followed by mitochondria isolation. RNAs were isolated from the mitochondrial fractions and total cell fraction.
- NGS was performed for small RNA analysis. As expected, most of the miRNAs were getting down regulated in ATG 99XGly FMR and 5'UTR *FMR1* CGG99x conditions in total cell. Interestingly, mitochondrial fraction showed altered association of miRNAs with mitochondria in both conditions.

Objective 4:

Bioinformatic analysis of targets miRNAs gets altered at mitochondria under FXTAS condition

- We identified targets of both population of miRNAs gets, (1) up regulated and (2) down regulated at mitochondria under CGG repeats transfected condition. All the targets were clustered and analysed by using DAVID platform.
- Bioinformatic analysis shows that majority of the targets were associated with brain tissue. Biological functions of the majority of the targets involves axon guidance, serotonergic, synapse protein phosphorylation, and RNA binding.
- Bioinformatic analysis of cellular localization of targets shows its presence at nucleus, mitochondria, axon, neuronal cell body, synapse and dendrites. These results further confirm involvement of brain and mitochondrial associated targets under FXTAS condition.
- The mitochondrial associated miRNAs in FXTAS condition targeting mitochondrial genes were further validated by qPCR. Four candidate miRNAs hsa-miR-181a, hsa-miR-221, hsa-

miR-320a, and hsa-miR-4485 were screened from NGS data analysis. The expression levels of all miRNAs at mitochondria and in total cells were analysed in HEK293 by qPCR. All the candidate miRNAs showed differential associated pattern with mitochondria as compare to their total cell enrichment.

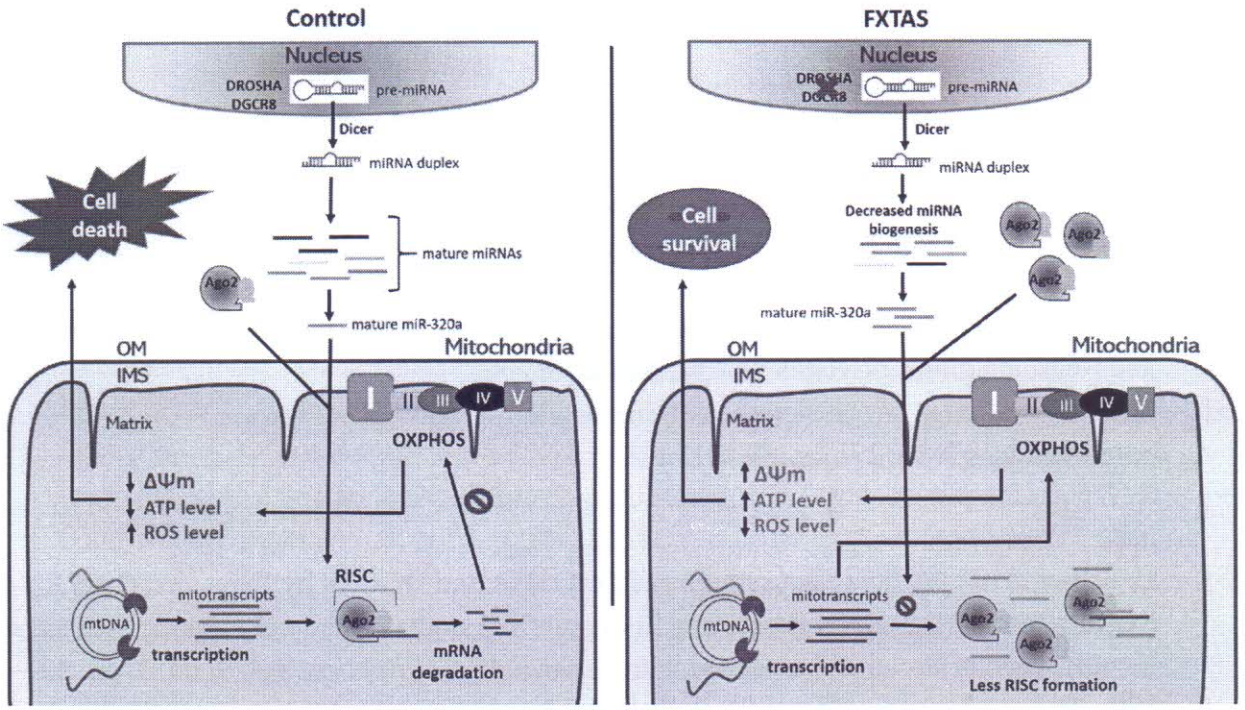
Mito-miR, miR-320a translocates to mitoplast in FXTAS condition

- We selected miR-320a for further exploration as this miRNA was previously reported as a mito-miR, and its protective roles during cellular stress and a tumor suppressor have been reported in some studies.
- RNase A protection assay was performed with mitochondria and mitoplast fractions from HEK293 cells followed by RNA isolation. qPCR analysis showed high levels of miR-320a in RNase A treated mitoplasts and was not degraded upon treatment with RNase A suggesting its localization in mitoplast.
- We performed ddPCR to further quantify the changes in abundance of miR-320a in mitochondria, mitoplast and whole cell fractions. Increased number of positive droplets (blue) and more copies/µl of miR-320a was observed in mitochondria and mitoplast fraction in 5'UTR FMR1 CGG99X transfected condition as compared to pEGFP-C1.
- The results were consistent with RT-qPCR results as observed in Fig 3A. Furthermore, ddPCR confirmed decreased levels of miR-320a under premutation conditions in whole cell lysate (Fig 3C). These results suggest that expression of CGG repeats may either induce degradation of miR-320a within the cytoplasm and/or enhance translocation to mitochondria, inside the mitoplast.

miR-320a improves mitochondrial transcripts levels and mitochondrial functions in FXTAS conditions

- To understand the impact of the decreased binding of miR-320a with Ago2 on mitochondrial functions, we first quantified the level of mitochondrial transcripts in FXTAS condition. The expression of 5'UTR FMR1 CGG99X showed decreased levels of mitotranscripts (Fig 5A), which is consistent with previous reports. Interestingly, we observed a rescue in levels of majority of mitotranscripts in presence of miR-320a mimic.
- cotransfection with miR-320a mimic corrects the deleterious effect of the CGG repeats on complex IV activity (Fig 5B). Further, transfection of miR-320a mimic enhanced the level of ATP in cells expressing 5'UTR FMR1 CGG99X (Fig 5C). Finally, we examined cellular ROS levels by DCFDA staining where rotenone (25µM for 2 hours) treated group taken as positive control. The increased ROS levels in premutation condition was not statistically significant; however, cotransfection with miR-320a mimic caused decreased levels of cellular ROS under 5'UTR FMR1 CGG99X (Fig 5D). Combining all the results, it can be inferred that miR-320a can rescue some of the mitochondrial dysfunctions in FXTAS condition.

SUMMARY



Schematic representation of comparative role of mito-miR, hsa-miR-320a in modulation of mitotranscripts levels, mitochondrial functions and cell death in control and FXTAS condition.

1. miRNAs are encoded from nuclear genome and the pre-miRNA with 2nt overhang are transported into cytosol, processed by DICER into miRNA duplexes and loaded into miRISC core component, Ago2. Ago2 along with its accessory components binds to target mRNA initiating the degradation or inhibition of translation to fine tune protein levels.
2. miRNAs can localize to various sub cellular compartments including mitochondria, stress granules, P bodies etc. and plays crucial role in site specific regulation of several proteins.
3. Transcripts encoded by mitochondrial DNA (mitotranscripts) are involved in formation of OXPHOS components.
4. Under normal condition, miR-320a localize to mitoplast and binds to Ago2. Ago2 along with miR-320a and its target mitotranscripts forms RISC assembly and regulates the levels of mitotranscripts causing mitochondrial dysfunctions and cell death.
5. The transfection of mir-302a mimic under premutation condition, mir-320a and Ago2 both localize to mitoplast in high abundance, but unable to form RISC assembly. This works in favor of the cell via increased OXPHOS activity and less ROS production.

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