Poster

presentations

New genes and developments in diagnosis of the ataxias

Screening study of SCA-negative ataxia patients for presence of Friedreich's ataxia trinucleotide expansion mutation

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Background: Patients presenting with ataxia are routinely tested for a panel of expansion mutations in genes for the spinocerebellar ataxias (SCA1, 2, 3, 6, 7, 12 and 17). These patients predominantly have late disease onset and a dominant pattern of inheritance. By contrast, Friedreich's ataxia (FRDA) usually presents before age 25 and is recessively inherited. Therefore, a genetic test for FRDA is not routinely included in the diagnostic screening package for atypical ataxia patients, many of whom are also negative for all the routine screens and are thus lacking a formal genetic diagnosis. Since the discovery of the causative frataxin (FXN) gene in 1996, atypical and later onset cases of FRDA have been identified. This information led us to question whether the root cause of many of these idiopathic ataxia cases could be the FRDA mutation.

Objective: To screen over 2000 idiopathic ataxia patients for the FRDA mutation, and observe the genotype-phenotype correlation in any FRDA-positive cases. This is the largest population size of ataxic patients ever tested for FRDA. Our findings will allow us to decide in a more evidence-based manner whether FRDA testing should be included in the routine genetic screening package for ataxia patients of unknown etiology.

Methods: Molecular diagnostic tests can be used to detect the GAA expansion mutations and thus diagnose FRDA. Triplet-primed PCR was first used to detect the presence of the expansion. For samples where the expansion was discovered, long range PCR was performed to determine whether the subject was a carrier or homozygote for the GAA expansion, and thus whether the patient was FRDA-positive. The relevant clinical information was obtained to observe the genotype-phenotype correlation in positive cases.

Results: Out of 2021 idiopathic ataxia patients screened thus far, 45 have been found to carry the FRDA expansion on at least one allele by the TP-PCR method. Of these samples, 20 have so far been screened by Long Range PCR, with four FRDA positives confirmed, eleven FRDA carriers confirmed and five samples unconfirmed. The other 25 patients are still under investigation. Carriers will be screened for point mutations: if again negative, they will be tested for exonic deletions.

Conclusion: These results provide more evidence that patients who present with features typical of the SCAs, only rarely represent atypical presentations of FRDA. These results should be added to the guidelines for the genetic testing and counselling of ataxia patients.

Genetic and molecular mechanisms of the ataxias

A plant model for understanding the genetic and molecular mechanisms underlying triplet expansion disorders

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Triplet repeat expansions underlie several neuronal disorders and until recently they have been discovered only in humans. We have reported a GAA/TTC triplet repeat expansion associated genetic defect in a population of the model plant Arabidopsis thaliana and experimentally demonstrated that the repeat expansion causes the down regulation of the IIL1 gene, which harbours the expansion, which leads to a conditional growth defect (Sureshkumar et al, Science, 2009). Having discovered the first example for a triplet expansion associated genetic defect outside humans, we have exploited this system to ask questions, which are not easily feasible in the human systems. I will present our system, discuss the similarities with Friedreich's ataxia, explain the commonalities and demonstrate how the plant system can be an excellent complement to ongoing efforts to manage repeat expansion associated genetic defects.

Defining the pathogenic role of mitochondrial DNA mutations in neuronal degeneration in Friedreich's ataxia

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Friedreich's ataxia (FA) is an autosomal recessive neurodegenerative disorder elicited by epigenetic silencing of a GAA trinucleotide repeat expansion located within the first intron of the frataxin gene (FXN). FXN functions as a nuclear-encoded mitochondrial protein necessary for iron-sulfur cluster biosynthesis. Its deficiency has multiple effects within the cell including mitochondrial iron overload and increased sensitivity to reactive oxygen species (ROS). Current research suggests ROS contribute to the molecular pathogenesis of FA by causing oxidative stress, however, the consequences of ROS on the integrity of the mitochondrial genome remain unclear. Previous studies from other laboratories showed mitochondrial DNA damage in yeast expressing low levels of human frataxin and peripheral blood cells of FA patients. Building upon this finding, we hypothesize affected FA patient cells accumulate ROS within their mitochondria, causing increased mitochondrial DNA (mtDNA) mutations that have a pathogenic effect on the cell by impairing mitochondrial function, ultimately leading to neurodegeneration in FA cells.

In this study, we present detailed bioinformatic analyses of the mutation spectrum such as insertions and deletions and the transition to transversion ratio, and the frequency of mtDNA mutations in different cellular FA models. Specifically, we determined the mtDNA mutation frequency by deep sequencing the mitochondrial genomes of FA patient and unaffected control primary fibroblasts, cerebrum and cerebellum tissues, and iPSC-derived neurons. In parallel, we used qPCR to measure mtDNA damage and determined mitochondrial function in FA patient and unaffected fibroblasts and iPSC-derived neurons. Lastly, we utilized an isogenic zinc-finger nuclease (ZFN) corrected FA cell line generated in our laboratory to determine whether increased frataxin expression will have a protective effect against mtDNA mutations and improve mitochondrial function. Our qPCR analyses show increased mtDNA damage in FA fibroblasts compared to unaffected control fibroblasts. In addition, deep sequencing results from the mitochondrial genomes of fibroblasts indicate that the mtDNA mutation frequency is increased within the mitochondrial genomes of FA affected individuals compared to unaffected controls. Importantly, results of this study will provide insights into the contribution of pathogenic mtDNA mutations to mitochondrial phenotype in FA fibroblasts and neuronal cells, as well as their effect on the cellular and molecular phenotype of FA. We believe these results will advance our understanding of the molecular mechanism of neurodegeneration in FA and direct the development of novel disease therapies.

MLH3 isoform 2 does not make the cut in Friedreich ataxia GAA•TTC repeat somatic expansion

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Friedreich ataxia (FRDA) is a progressive neurodegenerative disorder caused by GAA·TTC repeat expansion in the first intron of the frataxin (FXN) gene and is the most common inherited ataxia. Disease severity correlates to the length of the expanded repeats and the consequent reduction of FXN mRNA. We aim to refine our knowledge of the somatic repeat expansion process that causes FRDA. Previously, we have shown that the expansion rate is associated with transcription within the repeat and requires DNA mismatch repair enzymes, MutSβ and the subsequent action of a MutL complex. We established that the necessary MutL complex is the heterodimer of MutL Homologue one (MLH1) with MLH3, which is known as MutLγ. Our studies indicate a pivotal role for MLH3 in GAA•TTC expansion, specifically a single isoform, MLH3 isoform 1 (MLH3iso1). MLH3iso2 does not have an endonuclease domain and does not contribute to expansion in our human cell model. We show that FRDA patient derived cells that we have examined also express MLH3iso1, like our model cells. All of the known MutL complexes require MLH1, while MLH3 is a constituent of only the MutLγ complex. Consequently, when considering therapeutic targets to halt GAA•TTC expansion in FRDA, switching isoforms of MLH3 is much more attractive than targeting other DNA mismatch repair enzyme.

Helper dependent adenoviral vector (HdAV) genetic correction of expanded GAA repeats in Friedreich's ataxia patient specific induced pluripotent stem cells (iPSCs).

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Background/Hypothesis: The expanded GAA repeats in the frataxin (FXN) gene form the genetic basis for Friedreich's ataxia (FRDA), and understanding the genetic network related to the repeats would offer tremendous insight on the pathology of FRDA. Gene profiling studies such as microarrays often are confounded by individiual genetic backgrounds, but this bias can be reduced or eliminated by using isogenic cell lines. So far, there have been no reported gene profiling studies performed on FRDA isogenic cell lines. We aim to establish an isogenic FRDA cell line that only differs in repeat length, which allows for more effective and reliable gene profiling studies.

Methods: We utilized the HdAV gene correction method, which is an adenoviral based gene targeting approach (see PMID: 21596650). This approach allows for long homology arms (up to 30 kb) that increase gene targeting efficiency, as well as minimal off target effects compared to nuclease based methods (Zinc finger nucleases or TALENs). FRDA patient specific iPSCs were infected with HdAV containing a correction vector for the expanded GAA repeats. iPSC clones were screened for GAA repeat length.

Results: The corrected iPSC clones (isongenic to parent clone with unaffected GAA lengths) show restored frataxin mRNA and protein expression levels that are comparable to iPSC clones from unaffected individuals. The histone activation marks infected in FRDA cells such as H3K9 acetylation and repressing marks such as H3K9 di- and tri- methylation all are restored to levels found in iPSCs from uninfected individuals. We have further differentiated the corrected iPSCs into neurons and RNA-seq for gene expression profiling of the isogenic cell lines is currently in progress.

Conclusions: We have established an isogenic FRDA iPSC line where the expanded GAA repeats are corrected to six repeats, a repeat length representative of unaffected individuals. We have also differentiated the isogenic iPSCs into neurons. Gene profiling studies on isogenic cell lines will provide insight on the disease pathology and lead to future therapeautic opportunities.

Identification of chemical agents that alter Frataxin protein processing

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Frataxin (FXN) is a mitochondrial protein involved in iron sulfur cluster biosynthesis. FXN is encoded by a nuclear gene to yield a precursor protein (pFXN) that is processed, upon mitochondrial import, into an intermediate form (iFXN) by removal of an N-terminal transit peptide. Within the mitochondria, another transit peptide is cleaved off iFXN to yield the functional mature form, mFXN. Diminished levels of mFXN cause the hereditary disease Friedreich's Ataxia. We surveyed the effect of a set of mechanistically diverse pharmacological agents on FXN processing. Surprisingly, several agents led to distinct changes in the amounts of different forms of FXN, including increasing the relative and absolute abundance of iFXN with no effect on mFXN. Our data suggest that care must be taken when interpreting chemically-induced increases in total FXN levels, to ascertain that they reflect an increase in functional mFXN. Insight into the pathways that control these processing events will be presented.

Identification, characterization and cloning of a full-length frataxin antisense transcript, FAST-1.

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It has previously been reported that a frataxin antisense transcript, FAST-1, is overexpressed in FRDA patient fibroblasts. However, a lack of detailed information about the FAST-1 gene, including the size, sequence and position of the transcription start and stop sites, has hindered understanding of its potential role in Friedreich ataxia (FRDA). Therefore, we have further investigated FAST-1 in FRDA cells and mouse models.

Firstly, using northern blot hybridization of human fibroblast RNA with two riboprobes, we have identified two distinct bands of approximately 500bp and 9kb, representing potential FAST-1 transcripts. To further characterize FAST-1, we have performed 5'- and 3'-RACE experiments, followed by cloning and sequencing. This analysis has resulted in the identification of a full-length polyadenylated FAST-1 sequence. The 5'- and 3'-ends map to nucleotide positions +164 and -359 of the FXN gene (relative to TSS1 = +1), respectively, giving a total length for this FAST-1 transcript of 523bp. This may correspond to the northern blot band that we determined to be approximately 500bp in size. Interestingly, the start position of this FAST-1 transcript is within a known CTCF binding site.

Subsequently, using a robust qRT-PCR method to quantify FAST-1 expression levels, we have confirmed the original report of increased FAST-1 levels in human FRDA fibroblasts, and we further quantified FAST-1 levels in FRDA mouse model cell lines and tissues. However, no consistently altered patterns of FAST-1 expression were identified in relation to FXN expression. Our full-length FAST-1 clone will be useful for further studies of potential FRDA molecular disease mechanisms.

The role ferredoxin in Fe-S cluster assembly

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The assembly of Fe-S cluster is an essential pathway whose disruption is associated with severve diseases, most notably Friedreich's Ataxia. De novo synthesis of Fe-S clusters is a highly coordinated process involving a desulphurase enzyme, Fe-S scaffold proteins, ferredoxin, frataxin, and other accessory proteins, whilst the targetting and transfer of clusters to their final protein acceptor requires chaperones and glutaredoxin. To study the roles of these different components, we employ structural and biochemical methods to elucidate how and why the different components interact. In recent work, we have elucidated the structural details of how ferredoxin interacts with the enzyme complex and devised assays to test its function in electron transfer. Our results suggest that ferreodxin is involved in electron transfer in Fe-S clsuter assembly and explain how ferredoxin transfers electrons to the enzyme.

Targeting $(GAA)_n$ repeats and Frataxin protein in Friedreich's ataxia

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Expansion of $(GAA)_n$ repeats in the first intron of the Frataxin (FXN) gene is associated with reduction of mRNA and protein levels and the development of Friedreich's ataxia disease (FRDA). $(GAA)_n$ repeats form non-B-DNA structures, including intramolecular triple-helix H-DNA, which contribute to repeat instability and inhibition of FXN gene expression. Studies have also shown that the FXN protein levels can be regulated by proteasome degradation. Here we aimed to explore two different strategies for obtaining normal levels of FXN protein. First, we mapped H-DNA and higher order structure formation at pathological $(GAA)_n$ repeats in plasmids by using structural- and chemical probing assays. Also, we examined binding of modified oligonucleotides to these repeats to establish their effect on the formation of different DNA structures. Our results demonstrate that sequence-specific binding of modified oligonucleotides at expanded $(GAA)_n$ repeats abolishes H-DNA formation, as indicated by single strand- and triplex-modifying probes. Then we identified the Trim32 protein, with potential E3 ligase activity, as a factor elevating FXN protein levels. Trim32 function is crucial during neuronal differentiation, indicating that this protein has an essential role in the same cell types affected in FRDA patients. We found that in HeLa cells, overexpressing FXN (wt), stabilization of the Frataxin protein is observed when co-expressed with Trim32. Moreover, co-localisation studies using FXN-GFP and Trim32-CFP shows that part of the protein pool co-localizes within mitochondria when overexpressed in HeLa cells. Further results along these lines will be presented.