ALMS1 Protein-Associated Cardiomyopathy: Initial Studies Using Drosophila as a Model System

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ALMS1 PROTEIN-ASSOCIATED CARDIOMYOPATHY: INITIAL STUDIES USING DROSOPHILA AS A MODEL SYSTEM

By

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Abstract: Alström syndrome is a rare autosomal recessive disorder caused by mutations in the ALMS1 gene. ALMS1, located on chromosome 2p13, encodes for a large protein of length 4,169 amino acids. The biological role of ALM1 has been implicated in the function of primary cilia and intracellular trafficking. Mutations in ALMS1 are often nonsense or frameshift mutations and primarily affect exons 8, 10, and 16 of the gene. Alström syndrome is characterized by early onset childhood obesity, dilated cardiomyopathy (DCM), bilateral sensorineural hearing loss (SNHL), type 2 diabetes mellitus (T2D), and insulin resistance. With over 1,200 cases of Alström syndrome reported globally, ALMS1 is a rapidly growing area of interest. This Honors in the Major thesis seeks to use Drosophila melanogaster as a model organism for demonstrating how ALMS1 mutation affects the heart and can lead to the development of cardiomyopathy. Further, much is still unknown about ALMS1 structure and function. Alström syndrome can differ in severity, making a diagnosis difficult. This thesis aims to highlight current clinical findings involving ALMS1 and Alström syndrome presentation. By better understanding ALMS1, early genetic screening, diagnosis, and management of Alström syndrome can be obtained.

Keywords: Alström syndrome, ALMS1, ALMS1 gene, ALMS1 protein, dilated cardiomyopathy, DCM, bioinformatics

Introduction

The ALMS1 Protein

Alström Syndrome Protein 1 (ALMS1) is a large protein-encoding gene located on human chromosome 2p13. The ALMS1 gene is comprised of 23 exons and encodes for a protein 2 that is 4,169 amino acids in length. ALMS1 is a ubiquitous protein with multiple existing alternate splice forms. Although the biological function of ALMS1 is not fully understood, it has been associated with the formation of cilia, intracellular trafficking, and cell cycle regulation (Álvarez-Satta et al. 2015, Jaykumar et al. 2018, Li et al. 2007, Nerakh and Ranganath 2018). Localized on the basal bodies and centrosomes of ciliated cells, ALMS1 has been further implicated in the function of primary cilia (Ferkol and Leigh 2012).

Over 300 ALMS1 mutations have been reported, of which contribute to a wide variety of
phenotypic and biological abnormalities (Nerakh and Ranganath 2018). These mutations most commonly occur in exons 8, 10, and 16, referred to as mutation hot spots, with exon 8 accounting for 49% of documented cases of ALMS1 mutation (Álvarez-Satta et al. 2015). Most of these mutations are frameshift or nonsense mutations, causing the premature truncation of proteins (Marshall et al. 2015). Some case studies have found that consanguinity increases the prevalence of mutations in the ALMS1 gene and resulting genetic disorders (Laxer et al. 2016, Saadah et al. 2021).

Alström Syndrome

Alström syndrome – a rare, autosomal recessive disorder – is associated with mutations in the ALMS1 gene and impacts multiple organ systems (Choudhury et al. 2021). Alström syndrome includes clinical findings such as cone-rod dystrophy, type 2 diabetes mellitus (T2D), childhood obesity, dilated cardiomyopathy (DCM), hyperinsulinemia, and bilateral sensorineural hearing loss (SNHL). Organ dysfunction is also commonly seen in patients with Alström syndrome, including the kidneys, lungs, and liver (Hearn 2019). Alström syndrome belongs to a growing class of genetic disorders titled ciliopathies, with an incidence ranging from 1 to 9 affected individuals per 1,000,000 (Choudhury et al. 2021, Álvarez-Satta et al. 2015, Badano et al. 2006). As Alström syndrome varies in severity, there is no treatment for the disorder aside from symptom management. By addressing complications that arise from an Alström syndrome diagnosis, treatments currently focus on enhancing life quality and life expectancy (Choudhury et al. 2021).

Dilated Cardiomyopathy (DCM)
For most patients diagnosed with Alström syndrome, congestive heart failure (CHF) occurs secondarily to dilated cardiomyopathy (Corbetti et al. 2014). Dilated cardiomyopathy (DCM) is a progressive heart disease characterized by left ventricular enlargement and subsequent systolic dysfunction (Weintraub et al. 2017, Lakdawala et al. 2012, Mathew et al. 2017). Left ventricular dilation in DCM patients occurs in the absence of both coronary artery disease (CAD) and abnormal loading conditions such as hypertension (Elliott 2000, Japp et al. 2016). Despite enlargement of the left ventricular, normal wall thickness is seen in patients with DCM (Mestroni et al. 2015). Compared to other cardiomyopathies, DCM is the leading cause for heart transplants in children and adults (Towbin et al. 2006, Lakdawala et al. 2012). DCM also can lead to sudden cardiac death (SCD) in pediatric patients (Hamilton and Azevedo 2009). High mortality rates are associated with DCM among both adult and pediatric patient populations (Mestroni et al. 2015).

Dilated cardiomyopathy has a myriad of both genetic and nongenetic causes (Japp et al. 2016). Nongenetic etiologies of DCM including autoimmune disorders, myocarditis, and environmental factors such as exposure to toxins (McNally and Mestroni 2017, Reichart et al. 2019, Bakalakos et al. 2018). Initially, genetics were thought to be involved in 2% of DCM cases, but recent studies estimate 35% of DCM cases to have familial inheritance (Fuster et al. 1981, Lakdawala et al. 2012, Reichart et al. 2019). 50% of DCM cases have unknown etiology and are referred to as idiopathic in nature (Mathew et al. 2017). Research shows that DCM has marked locus heterogeneity, with over 60 genes that affect the sarcomere, cytoskeleton, or nuclear envelope having been associated with the disease (Mestroni et al. 2015, Japp et al. 2016).

Diagnosis of dilated cardiomyopathy may begin with a family history of the disease, as well as a physical examination of the patient (Bakalakos et al. 2018). Electrocardiography (ECG)
may also reveal abnormalities in heart rhythm, but patients with DCM can have normal ECG results. Some noted ECG abnormalities include bundle branch blockages, septal Q waves, isolated T waves, and ventricular tachycardia, either sustained or non-sustained (Elliot 2000). Ultimately, echocardiography is needed for a patient to be diagnosed with DCM.

Echocardiographic findings may involve enlarged cardiac chambers, left ventricular dilation, and dysfunction in the ability of the heart to properly contract (Pinamonti et al. 2019, Bakalakos et al. 2018). Other useful techniques for diagnosing DCM include phenotyping of the affected family, magnetic resonance imaging (MRI) of the heart, stress testing, and blood testing that can reveal renal, cardiac, and hepatic functions (Elliot 2000, Reichart et al. 2019, Lakdawala et al. 2013, McNally and Mestroni 2017).

Dilated cardiomyopathy affects around 60% of patients with Alström syndrome and commonly presents as acute congestive heart failure within early infancy (Joy et al. 2007). DCM, nevertheless, may develop at any point during life (Blanco et al. 2017, Joy et al. 2007). Although patients with DCM see improved cardiac function during childhood, they may experience sudden recurrences of CHF throughout adolescence and adulthood (Marshall et al. 2007). Alström syndrome is considered high risk for the eventual development of DCM (Marshall et al. 2007).

**Methods**

*Drosophila melanogaster*, or the common fruit fly, was determined to be the ideal model organism for this thesis. Many scientists, such as Thomas Hunt Morgan, have chosen *D. melanogaster* to conduct their work. There are conserved genes in *D. melanogaster* that regulate aspects of development and have been found to be homologous to those involved in the human disease process (Tolwinski 2017). *D. melanogaster* moreover has a rapid generation time and is
associated with a low maintenance cost. As such, *D. melanogaster* is a considered an ideal model organism for studying both genetics and developmental biology.

In the initial stages of this project, tin-GAL4 was chosen as a driver and was crossed to UAS-mcherry/CyO. Tinman, also referred to as tin, is homologous with the human Nkx2-5 gene and is expressed in the precardiac mesoderm. Absent or nonfunctional tinman genes have been associated with cardiac deformities, as tin is expressed in both cardial and pericardial cells. Tinman has been found to be a requirement for the specification of both the heart and visceral muscles in *D. melanogaster* (Bodmer 1993). *D. melanogaster* were crossed for 10 days in a 3:1 virgin female to male ratio, with adult flies being removed and placed in a separate vial before offspring eclosure. Vials were kept at 25° Celsius. Upon imaging using fluorescent microscopy, tin-GAL4 was not successful at isolating the *D. melanogaster* heart tube. Differentiating the heart tube from other muscle, such as the musculature of the intestine, was not possible.

It was decided to use Hand-GAL4, a cardiac-specific tissue driver, in place of tin-GAL4. Hand is a regulatory region that regulates the expression of GAL4, which is a transcription factor. A Hand-GAL4 stock was produced and recombined with Ubi-β-tubulin-GFP. Ubi-β-tubulin-GFP is a green fluorescent protein (GFP) tagged β-tubulin, a microtubule subunit. The hand-GAL4, Ubi-β-tubulin-GFP recombinant was then combined with UAS-tdtk on the second chromosome. UAS-tdtk is a red fluorescent membrane marker that delineates heart tubes. The hand-GAL4, Ubi-β-tubulin-GFP; UAS-tdtk stock was crossed with a control - RFP - and ALMS1 RNAi lines. Other RNAi lines were crossed to this driver stock, including patronin, msp3, msp300, and α-tubulin.

RNAi lines were set up and left for 10 days, with adults being removed at 5 days as to not confuse with the offspring. Female fruit flies were selected that lacked the CyO and Stu traits.
Flies were mounted on to 24X40-1.5 glass slides using Canada balsam. A drop of Canada balsam was placed at the center of each slide using a 200 μl pipette tip. Canada balsam was beneficial to use because it is a clear, viscous substance and is not known to be toxic to *Drosophila melanogaster*, despite being a flammable liquid. Adult flies were kept alive during imaging as to see the heart tube beating. 6 slides in total were prepared – 3 using female flies with the RFP control, and 3 using female flies with the ALMS1 RNAi line. Flies were analyzed using a Nikon Eclipse Ti microscope and NIS Elements Imaging Software. The wings for each fly were either spread away from the *D. melanogaster* body or were removed in totality. Imaging used a 20X objective and both EGFP and TRITC lasers. Imaging also used a fps of 493.9 and a time of 2.0 ms. Each slide was analyzed under microscopy for a total of 30 minutes. The 6 slides were analyzed for a total of 3 hours.

To analyze the images, ND2 files were converted to .png files using NIS Elements Viewer. ImageJ from the National Institutes of Health (NIH), USA, was further used to interpret the time in seconds between each heartbeat for both RFP and ALMS1 samples. 3 images were interpreted for each of the 6 flies, for a total of 18 images. The total distance for each image was a 1.04 second interval. This interval was set as the scale on ImageJ and the length between the peak of each heartbeat was determined. Analysis was done using Excel, and for each of the images the average value, standard deviation, and median value were calculated. Values were converted from seconds to milliseconds by multiplying the seconds value by 1000.

**Results**

**ALMS1 RNAi Images**

For the first ALMS1 sample, the average distance between heartbeats was 0.239 seconds,
or 239 milliseconds. The standard deviation was 5 seconds, and the median value was 0.21
seconds, or 210 milliseconds. For the second ALMS1 sample, the average distance was 213
milliseconds, with a standard deviation of 22 milliseconds and a median value of 210
milliseconds. For the third ALMS1 sample, the average in milliseconds was 176. The standard
deviation was 24 milliseconds, and the median value was 170 milliseconds. For all three ALMS1
samples, the average distance between heartbeats was 209 milliseconds, with a standard
deviation of 17 milliseconds and a median value of 210 milliseconds.

**RFP Images**

In the first RFP sample, the average distance between heartbeats was 141 milliseconds.
The standard deviation was 14 milliseconds, and the median value was 140 milliseconds. For the
second RFP sample, the average was 156 milliseconds, the standard deviation was 17
milliseconds, and the median value was 160 milliseconds. For the final RFP sample, the average
value was 137 milliseconds, the standard deviation was 23 milliseconds, and the median value
was 130 milliseconds. For all three RFP samples, the average was 144 milliseconds, with a
standard deviation of 4.9 milliseconds and median value of 140 milliseconds.
Figure 1: ALMS1 sample 2 image 2 (left) compared to RFP sample 2 image 1 (right). Images were captured using NIS Elements Imaging Software, with each image representing a distance of 1.04 seconds.

Figure 2: ALMS1 sample 3 image 3 (left) compared to RFP sample 3 image 1 (right). Each image represents a 1.04 second interval and the distance between the peak of each heartbeat was calculated using ImageJ software.
Discussion

Bioinformatics

Initial bioinformatics analyses of the ALMS1 gene suggest that certain regions like the ALMS motif have conserved functions. Found at the C-terminus of ALMS1, the ALMS motif is a stretch of 130 amino acid residues. The ALMS motif is the only region of ALMS1 that, when compared to other proteins in the human genome, demonstrates sequence similarity (Hearn 2019). Mammalian orthologs of C10orf90, otherwise known as fragile site-associated tumor suppressor (FATS), have been found to share sequences with the ALMS motif. FATS is an E2-independent ubiquitin ligase with a role of stabilizing and promoting p53 pathway activation when DNA is damaged (Yan et al. 2014, Hearn 2019, Song et al. 2015). The ALMS motif also shares similarity with the sequence of CEP295/KIAA1731. CEP295 has implicated roles in the assembly of centrioles, as well as potential binding of microtubules (MTs) (Knorz et al. 2010, Chang et al. 2016, Fu et al. 2016, Hearn 2019). Proteins with mutations that cause the ALMS motif to be absent have been found to be unstable in nature and prematurely undergo degradation (Tsai et al. 2018, Kamal et al. 2020).

Using proteomic analysis, ALMS1 has been found to interact with mitotic RNA polymerase II (RNAPII) (Möller et al. 2012, Hearn 2019). RNAPII primarily assists in the transcription of protein-encoding genes found in eukaryotic organisms (Möller et al. 2012). ALMS1 has further been identified by BioID to have a potential interactor/substrate relationship with the E3 ubiquitin ligase SCFβ-TrCP1 (Coyaud et al. 2015). SCFβ-TrCP1 targets substrates to be degraded by proteasomes. BioID also has revealed a potential interactor/substrate relationship between ALMS1, and a protease called separase. Separase is essential during mitosis as it
mediates sister chromatid separation during the transition from metaphase to anaphase (Hearn
2019, Agircan et al. 2016, Sun et al. 2009). Much is still unknown about how ALMS1 potentially
interacts with other proteins under pathological conditions.

**Future Studies**

If there was additional time to complete this thesis, there is more analysis that could be
conducted. Further analysis would include statistical analyses to determine the significance of
the determined distance between heartbeats in milliseconds. A probability value, or p-value, test
would be conducted to determine the probability that either the hypothesis is true or should be
rejected. A p-value \( \leq 0.05 \) would be considered statistically significant and would indicate strong
evidence against the null hypothesis that there is no difference in the hearts of fruit flies with
RFP and ALMS1 RNAi lines. A statistically significant result would provide support for the
research hypothesis.

Further analysis would include a t-test to compare the means of the samples to the null
hypothesis. Additional testing could include a pacing assay of cardiac function, Semi-Automated
Optical Heartbeat Analysis (SOHA), atomic force microscopy (AFM) to monitor the mechanical
properties of the *D. melanogaster* heart, as well as histological methods to assess the
development of the heart and the heart’s structural integrity. Measurements of the heart wall
thickness can be determined using reconstructed microCT tomogram, and this may demonstrate
if dilated cardiomyopathy (DCM) is present in the adult fruit flies with the ALMS1 RNAi.

**Conclusion**

It was found that *Drosophila melanogaster* with the ALMS1 RNAi had a slower, on
average, heartbeat than the RFP control. The average distance, in milliseconds, between the heartbeats of the ALMS1 RNAi fruit flies was 209, with the average distance between the RFP heartbeats being 144. The median value for the ALMS1 RNAi fruit flies was 210 milliseconds, and the median value for the RFP fruit flies was 140 milliseconds. Further testing would include statistical analyses, such as p-tests, to determine the statistical significance of these findings. Future testing should also include a larger sample size and include both male and female fruit flies to analyze if there is a sex difference between the linear heart tubes of *D. melanogaster*. Additional testing could include Micro Computed Tomography (Micro-CT), as well as a histological analysis of hearts obtained from *D. melanogaster* larvae.

**Literature Cited**


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