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Investigation of FUS R495x Mutations Associated With Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease that results in dysfunction and death of motor neurons throughout the body. The disease has an insidious onset and results in death due to atrophy of the motor neurons in the respiratory system causing asphyxiation. Previous research has firmly established that genetics play a critical role in determining the likelihood that an individual would develop the heritable familial ALS (fALS) and even sporadic ALS (sALS), which does not show typical heritability. While there is little known about how genes affect the pathology of ALS, multiple proteins have been correlated with the progression of ALS. Fused in Sarcoma (FUS) is a DNA/RNA binding protein that has recently been linked with ALS, and as such, its role in ALS has not been broadly investigated at the moment. Patients with the R495x mutation in FUS have been shown to have a much more aggressive pathogenesis than those that do not have that mutation in the FUS gene. Site directed mutagenesis and restriction digests were done to properly express the R495x FUS mutant in a mammalian vector. Systematic characterization of individual FUS mutations will contribute to the understanding of the unique onsets and pathogeneses and provide a much more in depth understanding of how the FUS protein contributes to ALS.

1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease that results in the death of motor neurons in the affected individual. The ALS Association reports that there are approximately 5,000 people diagnosed every year with ALS in the U.S. alone. It is also widely reported that at any given time there are 20,000 individuals with ALS in the U.S.[1] ALS is a progressive disease, resulting in paralysis and death for all suffering individuals. In fact, only 23.4% of diagnosed patients survive for greater than 5 years post diagnosis. [2]

Due to the nature of the motor neuron loss, ALS has a much larger impact on the public aside from the direct impact, as the progressive disease makes it impossible for the affected individual to independently go about everyday duties. ALS progresses to a state where the diagnosed are unable to take care of themselves and always results in the death of the diagnosed individuals through the death of the motor neurons that are responsible for respiration or through the death of other vital neurons. After the symptoms of ALS begin to progress, the patient's lack of self-sufficiency in addition to their medical care requirements becomes not only an emotional burden to those close to them but a financial one as well. The average ALS diagnosis results in over 1.4 million dollars spent on care. Approximately 10% of this cost is paid out of pocket paid by the family totaling around $130,000 per ALS diagnosis.[3] The stress that financial burden has on the loved ones of individuals that are diagnosed with ALS is immense, as well as the recorded guilt that the caregivers feel for being able-bodied and unable to affect the disease pathogenesis in any meaningful way.[4] It has even been recorded that friends of the patients were not comfortable being left with the patient by themselves.[4] With all of that in mind, there is a huge detrimental effect of ALS on more than just the patients, and even individual patients have large ramifications for the public.

Currently there are four medications approved by the FDA for the treatment of ALS and for this reason it is incredibly important for research towards understanding the disease to continue.[1] ALS is very difficult to diagnose and typically takes approximately 12 months to diagnose from the time the patient first starts experiencing symptoms of the disease.[5] Furthermore, the disease progression and pathogenesis varies widely among different individuals that experience the disease. Thus, patients are often not the best informed on timelines of disease progression or what symptoms they should expect to be aggressive first. Protein aggregation is one of the most prominent theories to what causes ALS and why it has so much variability in its progression for different individuals diagnosed with the disease.[6] FUS, TDP-43, and at least 7 other proteins have been identified to play a role in protein aggregation and motor neuron toxicity. Each of these proteins have a number of mutations that have also been linked to the disease and knowing more about them will shed light on the large disease variability in ALS.

2. Background

Fused in Sarcoma (FUS) is an RNA/DNA binding protein that acts as a nuclear protein at steady state.[8] Previous research on the FUS protein [8] has found that FUS expressed in yeast would form aggregates in the cytoplasm leading to cytotoxicity and cell death in the yeast cells.[6] Furthermore, it was discovered by the same study that while the researchers were able to find a lot of information about FUS aggregate formation in yeast and there are differences between mammalian cells and yeast, the same regions of FUS that are responsible for aggregation in yeast are also responsible for the aggregation in mammalian cells by using domain mapping experiments.[6] These researchers also determined that FUS cytoplasmic immunoreactivity is detectable in a broad spectrum of fALS and sALS and was even present in cases where there are no FUS mutations.

It has been shown that FUS is an RNA binding protein that is heavily involved in DNA repair and RNA biogenesis and transcription.[9] Furthermore, FUS has been implicated in the formation of stress induced compartments in sites of DNA damage and in stress granules. FUS binding at these compartments is a result of multiple factors, such as the type of damage that the cell was subjected to causing the problem, where the damage is on the DNA, where the DNA is in the cell, and any mutations that the FUS protein may have.[9] FUS assemblies have also been found to be liquid droplets that form liquid-liquid demixing in the cytoplasm or nucleoplasm where individual FUS proteins adhere to one another, changing the properties of the protein.[9] FUS may also play a significant role in forming liquid compartments at the sites of DNA damage and during stress. Research has shown that these liquid droplets that are formed by FUS have very different physical properties when they are formed by mutant FUS proteins, potentially leading to aggregation and other downstream effects.[9] However, the same study showed that in yeast FUS will form protein aggregates over time regardless of whether or not the protein contains mutations.[9] The hypothesis that was proposed is that the aggregation of FUS is concentration dependent as well as dependent on the conversion of FUS assemblies from a more liquid state to a more fibrous state.[9] Furthermore, the changing of the liquid state to fibrous state promotes the formation of aggregates by accelerating the rate by which the liquid assemblies convert to a fibrous state.[9]

Specific mutations of FUS have been linked to the different phenotypical expressions of ALS. Understanding the connections between the mutations and the phenotypical expression of disease onset and progression is vital for a complete understanding of ALS. The FUS protein has been found to interact both functionally and physically with survival motor neuron protein (SMN), a protein responsible for proper RNA splicing.[8] Mutation R495x has been identified to be a FUS truncation mutant associated with a severe ALS phenotype.[6,10] The R495x has been found to increase SMN protein binding three-fold, leading to FUS having an abnormally enhanced interactions with SMN, dysregulating SMN function, leading to increases in levels of small nuclear RNAs and a loss in Gems (nuclear compartment responsible for RNA processing), a nuclear hallmark of spinal muscular atrophy.[10] Furthermore, the R495x mutant has been determined to lead to delocalization from the nucleus to the cytoplasm.[10] Mutant FUS proteins have been found to lack the entire nuclear localization signal (NLS). The presence of the R495x mutation causes mislocalization to the cytoplasm in yeast and mouse models.[10]

The objective of this project was to introduce the R495x mutation into a mammalian plasmid to allow for future experimentation on mammalian cells. This plasmid would allow for the studying of acetylcholine (Ach), the neurotransmitter most involved in an action potentials that allow for muscular contraction, levels throughout the cell after the introduction of the plasmid. This was accomplished by doing a site directed mutagenesis, biotransformation, DNA plasmid amplification and purification, and introducing the plasmid to a mammalian vector.

3. Materials and Methods

3.1 Site Directed Mutagenesis

The R495x mutation was first introduced into a FUS plasmid using site directed mutagenesis with primers designed using Primer Blast and amplified using Polymerase Chain Reaction (PCR). A Kinase Ligase DAPA1 (KLD) treatment was completed to circularize the DNA after the PCR amplification.

A biotransformation was done to get the PCR plasmid to be picked up into E.coli NEB-85 cells using spectinomycin antibiotic selection for specificity, ensuring that only the cells that pick up the targeted pENTR223 plasmid would survive.

Bacterial colonies were then prepared for Sanger Sequencing (Genewiz). Colonies were collected and incubated overnight in LB media at 32 ℃. The following day the plasmids were retrieved from the bacteria using a Monarch Plasmid Miniprep Kit and submitted for sequencing. NCBI Nucleotide Blast was used to confirm sequencing.

3.2 Introduction of Mutation Into Mammalian Vector

A PCR based restriction site insertion was done to add BamH1 and Apa1 restriction sites to both sides of the mutation sequence. A co-digestion was then done on the sequence and the mammalian vector. A phosphatase treatment was done to the products of the PCR to ensure proper ligation into the vector. The vector was then biotransformed into *E.coli* then amplified and purified for sequencing. The purified plasmid was digested and ran out on a 1.2% agarose gel.

3.3 Cell Culture and Neuron Transfection

Introduction of the mutant plasmid to a micelle done, followed by the introduction of the micelle to NSC-34 motor neurons. We were using HEK-293 cell line to learn and practice cell culture. 10% heat inactivated fetal bovine serum was used as a growth media and 1:10 splits were done. During splitting the cells were rinsed with 1X PBS solution then trypsinized. They were then incubated at 37 degrees celsius with a 5% CO2 atmosphere for 3 minutes. A 1:10 split was done into new growth media.

4. Results

Site directed mutagenesis was done using an antibiotic resistant plasmid and confirmed by the presence of colonies in Figure 1. The cells were worked up and the plasmids submitted for sequencing and the presence of the mutation was confirmed shown in Figure 2. Evidence of the successful biotransformation of the mammalian vector is shown by the bacterial colonies present in Figure 3. A restriction digest was done to determine which colonies would be valuable to submit for sequencing. The gel showed 3 colonies that had the appropriate bands, and those colonies were sequenced (Figure 4). To prepare for transfecting the mammalian vector it was necessary to learn and practice cell culture techniques; Figure 5 shows the successful culturing of HEK-293 cells.

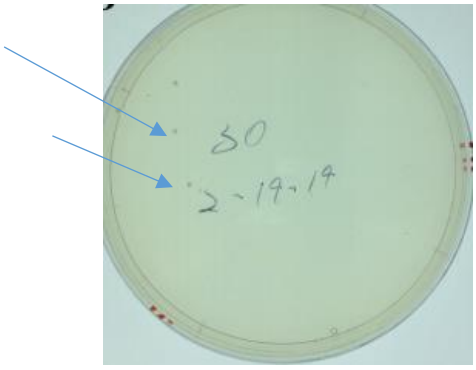

Figure 1: Plate showing the presence of bacterial colonies after the biotransformation.

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Query  868   CAAGGCCTGGGTGAGAATGTTACAATTGAGTCTGTGGCTGATTACTTCAAGCAGATTGGT  927
                                                 |||||||||| ||||||||||
Sbjct  1     NNNNNNNNNNNNN-----------------------TGATTACTTCA-GCAGATTGGT  34

Query  928   ATTATTAAGACAAACAAGAAAACGGGACAGCCCATGATTAATTTGTACACAGACAGGGAA  987
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35    ATTATTAAGACAAACAAGAAAACGGGACAGCCCATGATTAATTTGTACACAGACAGGGAA  94

Query  988   ACTGGCAAGCTGAAGGGAGAGGCAACGGTCTCTTTTGATGACCCACCTTCAGCTAAAGCA  1047
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95    ACTGGCAAGCTGAAGGGAGAGGCAACGGTCTCTTTTGATGACCCACCTTCAGCTAAAGCA  154

Query  1048  GCTATTGACTGGTTTGATGGTAAAGAATTCTCCGGAAATCCTATCAAGGTCTCATTTGCT  1107
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  155   GCTATTGACTGGTTTGATGGTAAAGAATTCTCCGGAAATCCTATCAAGGTCTCATTTGCT  214

Query  1108  ACTCGCCGGGCAGACTTTAATCGGGGTGGTGGCAATGGTCGTGGAGGCCGAGGGCGAGGA  1167
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  215   ACTCGCCGGGCAGACTTTAATCGGGGTGGTGGCAATGGTCGTGGAGGCCGAGGGCGAGGA  274

Query  1168  GGACCCATGGGCCGTGGAGGCTATGGAGGTGGTGGCAGTGGTGGTGGTGGCCGAGGAGGA  1227
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  275   GGACCCATGGGCCGTGGAGGCTATGGAGGTGGTGGCAGTGGTGGTGGTGGCCGAGGAGGA  334

Query  1228  TTTCCCAGTGGAGGTGGTGGCGGTGGAGGACAGCAGCGAGCTGGTGACTGGAAGTGTCCT  1287
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  335   TTTCCCAGTGGAGGTGGTGGCGGTGGAGGACAGCAGCGAGCTGGTGACTGGAAGTGTCCT  394

Query  1288  AATCCCACCTGTGAGAATATGAACTTCTCTTGGAGGAATGAATGCAACCAGTGTAAGGCC  1347
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  395   AATCCCACCTGTGAGAATATGAACTTCTCTTGGAGGAATGAATGCAACCAGTGTAAGGCC  454

Query  1348  CCTAAACCAGATGGCCCAGGAGGGGGACCAGGTGGCTCTCACATGGGGGGTAACTACGGG  1407
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  455   CCTAAACCAGATGGCCCAGGAGGGGGACCAGGTGGCTCTCACATGGGGGGTAACTACGGG  514

Query  1408  GATGATCGTCGTGGTGGCAGAGGAGGCTATGATCGAGGCGGCTACCGGGGCCGCGGCGGG  1467
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  515   GATGATCGTCGTGGTGGCAGAGGAGGCTATGATCGAGGCGGCTACCGGGGCCGCGGCGGG  574

Query  1468  GACCGTGGAGGCTTCCGAGGGGGCCGGGGTGGTGGGGACAGAGGTGGCTTTGGCCCTGGC  1527
             |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  575   GACCGTGGAGGCTTCTGAGGGGGCCGGGGTGGTGGGGACAGAGGTGGCTTTGGCCCTGGC  634

Query  1528  AAGATGGATTCCAGGGGTGAGCACAGACAGGATCGCAGGGAGAGGCCGTAT          1578
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  635   AAGATGGATTCCAGGGGTGAGCACAGACAGGATCGCAGGGAGAGGCCGTATTAATTAGCC  694
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Figure 2: The sequence returned from the lab at NC State showing the successful mutation of the FUS protein with the R495x mutation. The arrow in the figure highlights the confirmed mutation in the sequence.

Figure 3: Plate showing mammalian vector with mutation in colonies grown in the biotransformation step after the restriction digest.

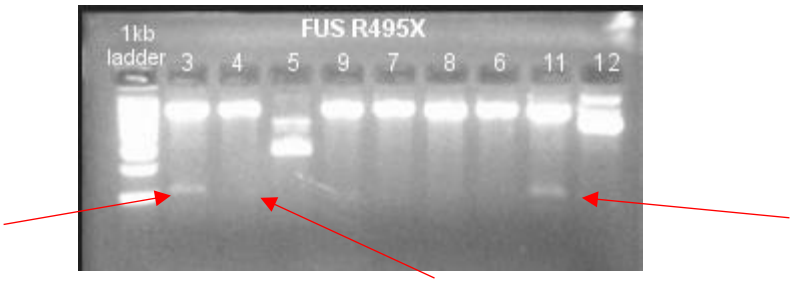

Figure 4: Figure shows the agarose gel visualization using ethidium bromide confirming the presence of the mammalian vector as well as the FUS mutation sequence in wells 3, 4, and 11, however it is difficult to see the confirmation in well 4 on the image. The arrow shows the expected FUS length that is visualized at 1600 bp much further down the gel than the whole plasmid as FUS is much smaller. The red arrows point at the bands that confirm the presence of the FUS sequence. Well 4 is very difficult to see in the image and was more easily visible in person.

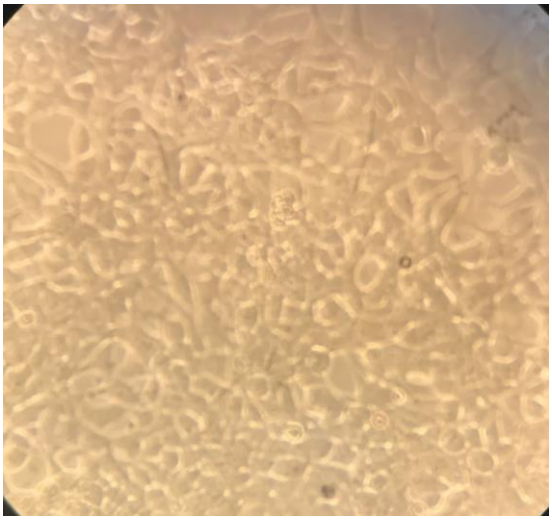

Figure 5. Cultured HEK-293 cells that have not been transfected at a greater than 90% confluency.

5. Discussion

The R495x mutation was successfully introduce into the mammalian vector. Issues arising through the process included the annealing temperature of the mutant not being cited correctly so the PCR needed to be troubleshot in order to determine the temperature that worked for the PCR. Structure function viability data was unable to be obtained because the mutant FUS genes were not properly introduced into a micelle prior to being introduced into neurons. HEK-293 cell line was successfully split and cultured but the transfection of the mutant into the cell line was not achieved

6. Conclusions

Through the use of site directed mutagenesis, biotransformation, sanger sequencing, restriction digests and agarose gel visualization our research group was able to subclone our mutant FUS sequence into a mammalian vector. Further work in this project is necessary in order to understand the specific mechanism of how FUS mutants could be altering the normal motor neuron cellular function. In order to understand how ALS functions it is necessary to understand the effect the biological components of the neurons are having on all pathways that are specifically implicated in motor function. Future directions of this research should focus on researching the role that Acetylcholine (Ach) plays in

ALS and how the R495x mutation specifically affects that pathway. Acetylcholine is the major neurotransmitter associated with motor movement and muscular contractions and has not been studied much when looking at ALS as a disease. Investigation of Ach concentrations resulting from mutations in FUS could shed a significant amount of information of the potential pathways leading to motor neuron toxicity.

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