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Construction of ALS-linked TDP-43 Mutants D169G and A315T to Examine Mechanisms of NCS-34 Motor Neuron Cell Death

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a terminal neurodegenerative disorder with a mean survival of 2-5 years after diagnosis. In ALS, the neurons innervating voluntary muscles retract and die, leading to muscle atrophy and loss of limb function. This neuronal death eventually reaches the lungs, causing death by suffocation. One protein implicated in ALS-related neuron death is TAR DNA Binding Protein 43 (TDP-43). TDP-43 is a highly conserved, RNA-binding protein that has been shown to form aggregates in the cytoplasm of cells in 90% of ALS cases. The aggregation of the protein is attributed to sequence mutations that lead to its erroneous location in the cytoplasm and enhance its ability to form the detrimental aggregates. Only two of the over fifty identified TDP-43 mutations are outside the domain responsible for protein-protein interactions. One of them is an Aspartate-169 to Glycine (D169G) mutation, which is located in the first RNA-recognition domain, suggesting that a protein with this mutation could utilize a unique pathogenic mechanism. The Alanine-315 to Threonine (A315T) mutant is located in a toxic membrane binding region within the prion-like domain of TDP-43, and if part of the subdomain is removed, there is evidence it prevents TDP-43 toxicity. The A315T mutation is also associated with higher lethality. The aim of this research is to create two TDP-43 mutants with D169G and A315T substitutions via site-directed mutagenesis and then transfect the constructs into a motor neuron cell line NSC-34 for overexpression. Transfected cells will be imaged to document rate of morphological changes, and rate of cell death will be quantified by spectrographic analysis. These data will be entered into a databank to collect a broad range of data pairing specific mutations with the physiological effects they produce.

1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is the most common motor neuron (MN) disease,[1] affecting 2-8 per 100,000 people around the world.[2] The vast majority of ALS patients have no family history of ALS and are classified as sporadic ALS patients (sALS).[1] Patients who do have family history of ALS are classified as having familial ALS (fALS).[1] Patients suffering from ALS experience progressive degeneration of upper and lower motor neurons.[2] Mean survival after diagnosis is 2-5 years,[2] with death due to suffocation caused by paralysis of the respiratory system.[3] The exact mechanism leading to MN death remains elusive. According to the leading hypothesis, the primary cause of MN death is likely proteomic inclusions within the cytoplasm of MN and glia which disrupt cell function and eventually kill the cell.[2,5,8] A second hypothesis suggests that endoplasmic reticulum (ER) stress could create a lethal cascade involving unfolded protein response (UPR).[2] When the ER is stressed due to large quantities of misfolded proteins, it triggers UPR to refold or degrade those proteins. The UPR pathway, however, is hypothesized to becomes overwhelmed and degradative pathways become backed up, killing the cell.[2] ER stress and UPR could also lead to disruptions in protein homeostasis, which is another hypothesized mechanism of cell death.[9] With fewer functioning proteins caused either by UPR or the inclusions sequestering functional proteins, the cell might be unable to maintain homeostasis and die. Lastly, another implicated mechanism is astrocyte induced necroptosis of MN.[10] For an unknown

reason, ALS astrocytes initiate an MLKL/RIP1 dependent cell death pathway in the MNs, causing their plasma membranes to break down, releasing the protein aggregates.[10] Regardless of mechanism, a variety of mutated proteins are implicated in ALS MN death. Superoxide dismutase 1 (SOD1), C9orf72, TAR DNA Binding Protein 43 (TDP-43) and Fused in Sarcoma (FUS) are the most common proteins associated with the incidence of fALS.[1] They are found mutated and in cytoplasmic inclusions. These inclusions are likely involved in a prion-like spread of disease from cell to cell within the individual.[1]

TDP-43 is a highly conserved RNA-binding protein.[1] It's structure includes an N-terminal domain, two RNA-binding domains, and a low-complexity domain at the C-terminus.[1] TDP-43 plays a critical role in protein homeostasis as it has over 40,000 RNA binding sites it can bind within the genome found via consensus sequence analysis and is involved in the splicing regulation of over 950 RNAs, the binding of long non-coding RNA, miRNA biosynthesis, stress granule formation, and 3' UTR binding.[9] Upon knockdown of TDP-43, 283 RNAs are downregulated, and over 600 RNAs change expression patterns.[9] TDP-43 is found in 97% of pathological inclusion from ALS and frontotemporal dimension (FTD) patients.[9] TDP-43 is a major component of ubiquitinated inclusion in ALS patients,[4,5] and can be found cleaved, phosphorylated, ubiquitinated, or misfolded. One TDP-43 mutation associated with ALS is D169G, which is the only mutation in the RNA Recognition Motif (RRM1),[4,6] and one of two mutations outside of the low-complexity domain, with the other mutation being found in non-ALS patients.[6] The D169G mutation is linked with increased stability of the RRM1 domain, which leads to an increased rate of cleavage by caspase 3.[4] The cleaved fragment is 35kD and is called TDP-35, which is the pathogenic C-terminus of TDP-43.[4] This is the only study which proposes a mechanistic hypothesis for the D169G mutation.

The second TDP-43 mutation of interest is A315T. A315 is part of a membrane-interacting subdomain in the TDP-43 low complexity domain and is associated directly with neuron death and increased TDP-43 stability.[4,7] It is also adjacent to the amino acids located at 307-311 which, if removed, appear to diminish TDP-43 toxicity.[7] Its proximity to these amino acids and its presence in the membrane-binding domain might be responsible for its cell death mechanism. One study found that overexpression of A315T, however, was less toxic than WT TDP-43 overexpression in Drosophila neurons, if both had the same levels of overexpression.[11] However, further increase of A315T overexpression leads to almost identical results of cell death and axonal protein aggregates.[11] Whether this difference in expression is due to the disease model or an actual difference in disease propagation in mutant A315T remains unknown. No further studies proposing mechanisms of action for this mutant have been performed.

Once the mutants are transfected into the NCS-34 mouse MN line, data on individual mutant disease progression can be collected. D169G may promote a different mechanism of disease incidence and progression as compared to other ALS-related mutations. Thus, once the mutant is transfected into a MN cell line, the cells should exhibit exponential disease progression as TDP-35 levels build and protein homeostasis is disrupted. A315T, however, is known for its ability to cause cell death, and as such, will likely have a more rapid disease progression in the MN cell lines.

2. Methods

2.1 Bacterial Transformation

WT plasmid stocks were acquired from DNASU (clone ID FLH258208.01L). Competent cells were transformed to take up the wildtype plasmid. Briefly, 1µL of stock plasmid was added to competent cells. Cells were incubated on ice for thirty minutes, followed by a 30sec, 42°C heat shock, and a 2 min ice incubation. 250µL of S.O.C. medium was added to the cells and the cells incubated for 1h, shaking at 37°C. The cells were then plated on 50µg/mL kanamycin plates and grown overnight.

2.2 Plasmid Isolation

Plasmids were purified from bacterial cultures using the Invitrogen™ PureLink™ Hipure Plasmid Maxiprep kit. Briefly, individual colonies were isolated and grown in individual 2mL LB media (50µg/mL kanamycin) for 8-10h in 37°C shaker. 1mL of culture were transferred to 1L flasks with 125mL of LB media (50µg/mL kanamycin). Cultures were grown overnight for 13-15h at 37°C. Bacteria were lysed and plasmids purified using the kit protocols. Plasmid sequence was confirmed via sequencing.

2.3 Site-Directed Mutagenesis

Mutant plasmids were created using New England Biolabs® Q5 Site-directed Mutagenesis Kit. Plasmid backbone primers include M13 primers. Substitution mutagenesis primers were designed using the NEBaseChanger program. D169G primers include 5' CATATGATAGGTGGACGATGG 3' and 5' TCGCTGTGACATTACTTTC 3' with a Tm of 59°C. A315T mutants used 5' GAACTTTGGTACGTTCAGCATTAATC C 3' and 5' ATCCCACCACCCATATTAC 3' with a Tm of 62°C. PCR conditions include: 98°C for 30sec, 25 cycles of 98°C for 10sec, primer Tm for 30sec, and lastly 72°C for 1min and 40sec, a final extension of 72°C for 2min and a holding temperature of 4°C. L PCR product was then cleaned with KLD treatment. 1µL of PCR product was mixed with 5µL of 2X KLD reaction buffer, 1µL of 10X KDL enzyme mix, and 3µL of nuclease-free water. Mixture was incubated at room temperature for 5min. Successful mutant formation was verified through Sanger sequencing.

2.4 PCR

Sequences of restriction cut sites, ApaI and BamHI, were added to TDP-43 via PCR based amplification to allow for subcloning of desired sequences into the mammalian vector pEGFP-C1. This was done using the New England Biolabs® Q5 Site-directed Mutagenesis Kit. The forward ApaI site insertion primer was 5' GGATCCGGGCCCAATGTCTGAATATATTCGGGTA 3' and reverse BamHI site insertion primer, primer was 5' CTAATCGGATCC CTACAACATTCCCCAGCCAGAAGA 3'. The insertion primer for ApaI was also used to correct frameshift during subcloning, and the BamHI primer was used to add a stop codon to the end of the TDP-43 sequence.

2.5 Ligation

PCR product was purified using Promega Wizard® SV Gel and PCR Clean-Up System according to kit protocol. The purified PCR product, as well as the mammalian vector pEGFP-C1, then underwent a double digest. 3ug of DNA was incubated with 5µL of Cutsmart buffer, 1µL ApaI, and sufficient double deionized water to make 50µL reactions. Reactions were incubated overnight at 25°C. Then 1µL of BamHI-HF was added and incubated for 6h at 37°C. Digested samples were run on a 1% agarose gel with 4µL ethidium bromide at 50V. TDP-43 vector bands of 1.2kbp and pEGFP-C1 bands between 4-5kbp were excised and purified using Invitrogen™ PureLink™ Quick Gel Extraction Kit according to kit protocol. Purified samples were then ligated using T4 ligase.

2.6 Subcloning

Ligations were then biotransformed (see 2.1 Cell Transformation). After biotransformation, six colonies were selected from each sample to undergo DNA extraction using Invitrogen™ PureLink™ Quick Plasmid Miniprep Kit according to kit protocols. Successful mutant formation was verified through a diagnostic double digest, then by Sanger sequencing. The diagnostic digest followed the same procedure as the restriction digest, only incubation times were reduced to 15 min.

3. Results

Successful mutagenesis was confirmed for mutation D169G via Sanger sequencing on the PCR product and subsequent alignment of D169G and wild type TDP-43 (Figure 1). The Sanger sequencing primer used was the M13 universal primer. The second Sanger sequencing primer used was the mid-sequence primer 5' GCCTTTGCCTTTGTTACATT3'. A315T failed to prime properly due to loss of sequencing buffer while in transit to Sanger sequencing.

Figure 1. Alignment of TDP-43 sequence (Query) and Sanger sequence of D169G (Sbjct) using BLAST nucleotide alignment tool. Mutation is boxed in red.

Successful addition of restriction sites ApaI and BamHI were confirmed via a double digest followed by gel electrophoresis. Both the WT TDP-43 and D169G showed bands at 1.2kbp (Figure 2A), indicating successful excision of insert. This also indicates addition of the cut sites, as a 2.4kbp band would be expected if the addition had failed because the ApaI and BamHI sites already within the bacterial plasmid are 2.4kbp apart when the TDP-43 insert is within the plasmid. The mammalian vector was successfully linearized in preparation for ligation as the digest contains a band between 4000bp and 5000bp (Figure 2B). Undigested pEGFP-C1 was used as control. The multiple bands on the undigested pEGFP-C1 indicate differing levels of supercoiling.

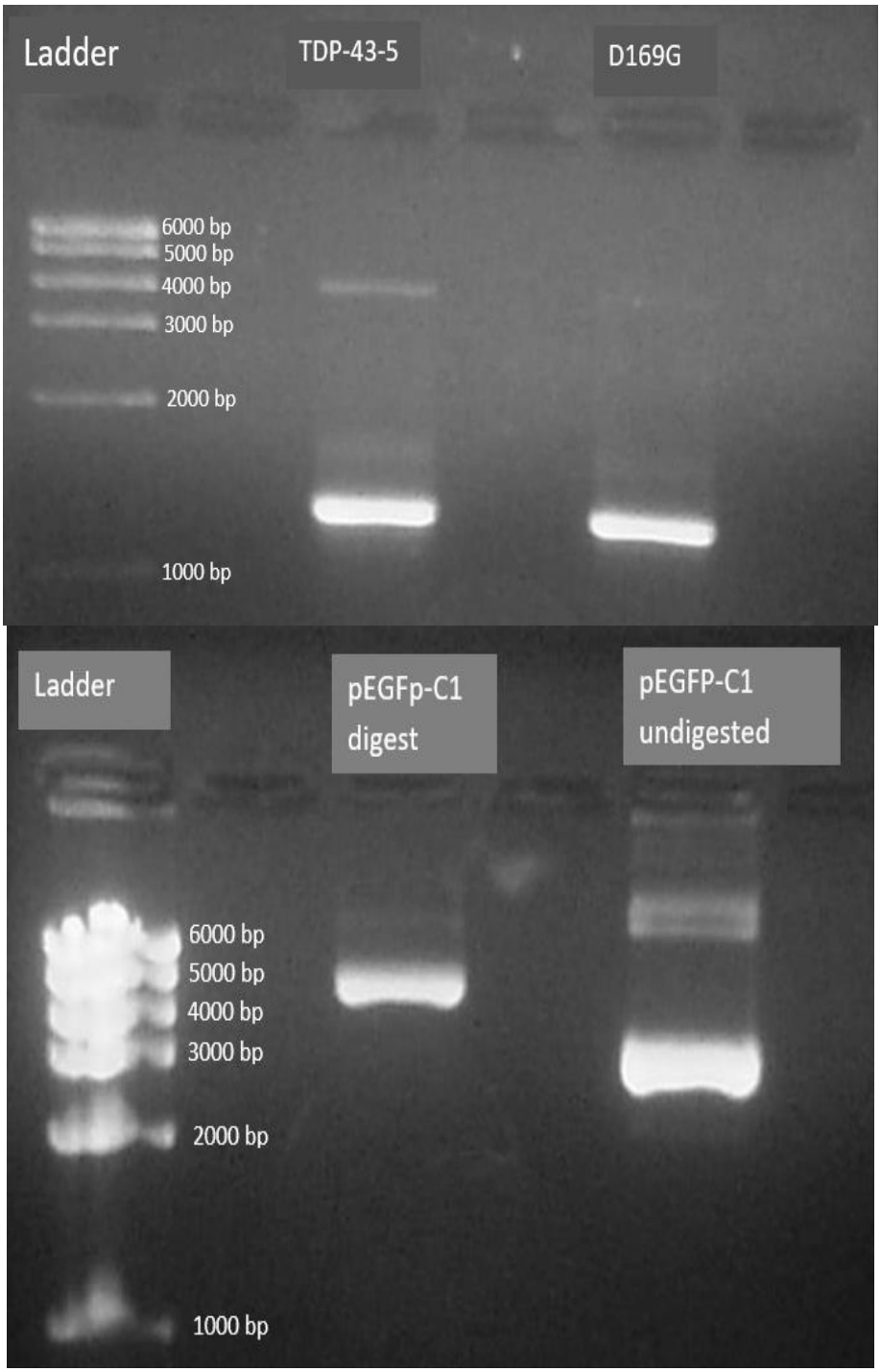

Figure 2. A. ApaI and BamHI double digest on Wild Type TDP-43 (TDP-43-5) and mutant D169G. Bands at 1200 bp indicate successful insertion of restriction sites as well as successful digestion. B. Gel of ApaI and BamHI double digest on mammalian vector pEGFP-C1. The band between 4000 and 5000 bp indicates successful linearization. Undigested pEGFP-C1 was used as control. Multiple bands indicate differing levels of supercoiling.

Ligations failed to produce two bands, indicating unsuccessful insertion of the TDP-43 mutant (Fig. 3). A subset of the samples were subjected to Sanger sequencing to confirm successful subcloning, but no insert was found.

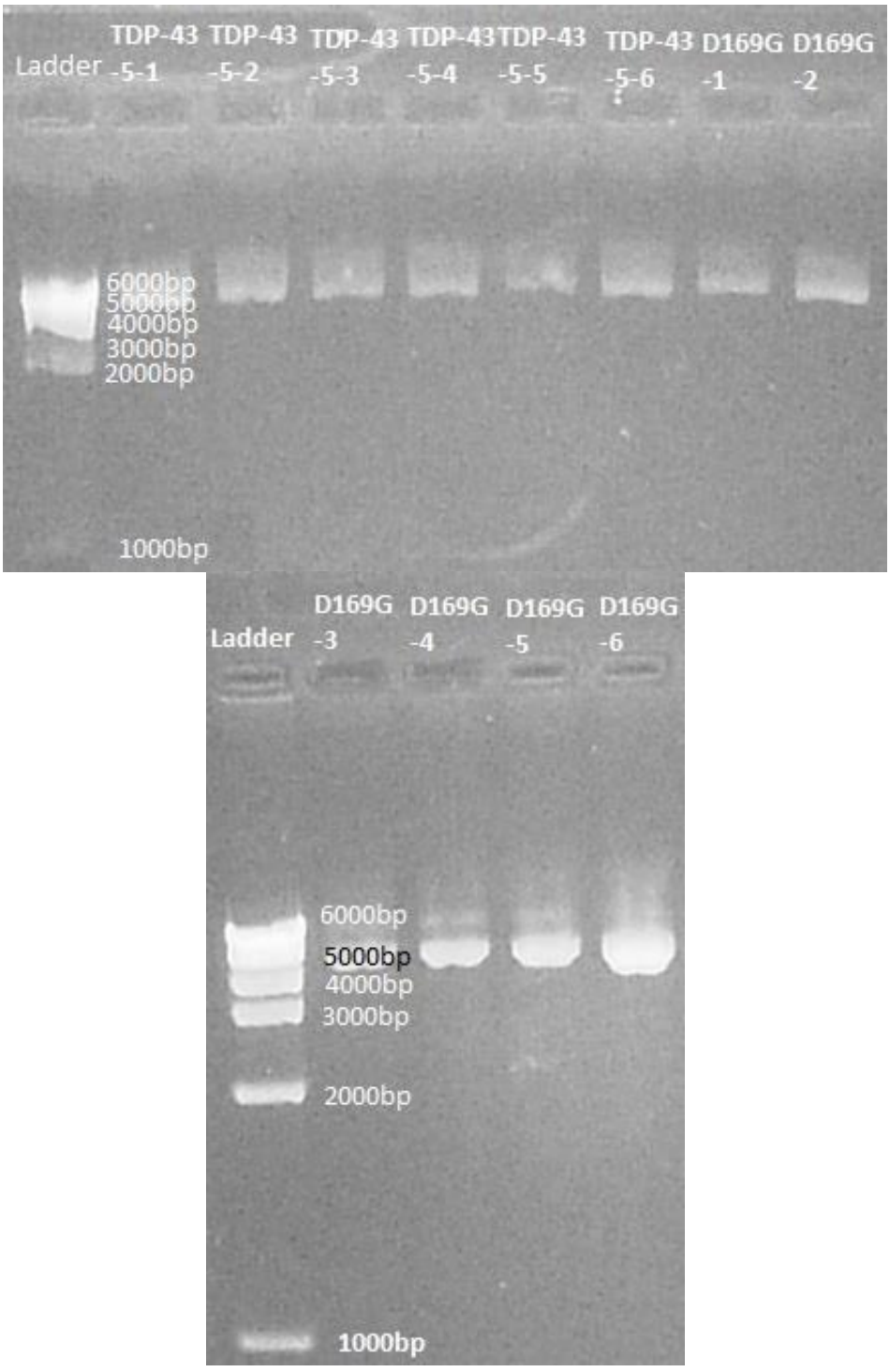

Figure 3. Diagnostic digests with ApaI and BamHI after TDP-43 and mammalian vector ligation. The absence of a band near the 1000bp mark indicates there is no TDP-43 insert within the mammalian vector. This was confirmed using Sanger sequencing.

4. Discussion

The success of the site-directed mutagenesis and the restriction digest show progress towards the creation of a mutant library. The restriction digest also shows the plasmid and inserts are ready for ligation. Difficulties with ligation are likely due to low yields of the gel extraction, with concentrations ranging between 15.7-18.2ng/µL. With low DNA concentrations, the probability of an insert being ligated into the mammalian vector is low. Optimization of the gel extraction will enable further progress.

The individual qualities of disease progression, mechanism of action, disease onset and disease characteristics are unknown for individual mutations. It has been hypothesized that all these characteristics can vary widely depending on the protein involved, and the specific mutation within that protein, which makes creating and characterizing these mutations vitally important to ALS research. For example, FUS mutations are known for early age of onset and quick disease progression while SOD1 is known more for associated mental deficits. This study is the very beginning of this process. The mutants created in this project could also be shared with other labs.

5. Conclusion

Before a systematic study of the mechanisms by which specific mutant TDP-43 aggregates promote cell death can be performed, mutant TDP-43 proteins need to be created. This study details progress towards the creation of the required library. With the success of multiple mutagenesis procedures and the isolation of both insert and vector by restriction digest, the project is only a few steps behind insertion of the mammalian vector into NCS-34 mouse motor neuron cell line for expression. Thereafter, studies on the effects of individual mutants on MN viability can be performed, thus furthering understanding of this complex and enigmatic disease. Future directions include reperforming ligation and subsequent subcloning and transfection of mammalian vectors containing mutant TDP-43 into the NCS-34 cell lines. The GFP within the pEGFP-C1 plasmid would show which cells had successfully taken up the plasmid, but would not be attached to the TDP-43 mutants as that could hinder mutant mechanisms of action such as aggregate formation. These cell lines would then undergo Celltiter 94 Aqueous One solution Cell Proliferation Assays to determine cell death rates. This data would be used to create a database of mutation-linked cell death assays which would be used, in collaboration with Dr. Brian Drawert (Assistant Professor of Computer Science at UNC Asheville), to build computational models to predict the effect of mutations on neuronal cell health and function.

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