Analysis of the Therapeutic Strategies Used to Treat ALS Caused by TDP-43 Aggregation

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Abstract

Amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disorder that impairs motor functions, affects 1 in 50,000 people in the world, and almost 90% of patients diagnosed do not have any family history of the disease. ALS is a debilitating disease due to the limited effectiveness of treatments for most patients. However, researchers were able to identify one protein that malfunctions in almost 97% of patients: TDP-43. TDP-43 is a protein that regulates the process of transcription, and it is known to aggregate in the neurons of patients with ALS. Many researchers have decided to focus their therapeutic strategies on the protein aggregation using genetic therapies or small molecules. This study focuses on analyzing the benefits and limitations of the therapeutic strategies used to treat ALS caused by TDP-43 aggregation and identifying which method holds the most promise to target this proteinopathy.

What is ALS?

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that affects the function of motor neurons and, in most cases, leads to neuronal cell death. As neurons slowly deteriorate, they are unable to pass messages from the central nervous system (CNS) to the peripheral nervous system (PNS). The CNS is composed of the brain and spinal cord while the peripheral nervous system is made of the surrounding nerves that branch off from the CNS. When these neurons deteriorate, then it causes signals between adjacent cells to stop transmitting. Signals are the stimulus that produce muscle movement, so when the signals are disrupted, then muscles can weaken and twitch [1].

Doctors categorize ALS into two sub disorders: familial ALS and sporadic ALS. Familial ALS (fALS) is caused by hereditary genetic mutations such as mutations in the genes, superoxide dismutase 1 (SOD1) or chromosome 9 open reading frame 72 (C9ORF72), and it affects around 10% of patients. Sporadic ALS (sALS), however, is more prevalent amongst patients, and there are no common underlying causes derived from family history or environmental factors.

Some symptoms of ALS include muscle weakness, spasms, stiffness, and spasticity as well as involuntary movements, respiratory distress, and paralysis. By analyzing their symptoms, physicians are able to diagnose ALS patients, but due to the wide range of causes the disease has, they are unable to target the type of ALS their patients have using a standard treatment process[2]. Therefore, many FDA-approved medications for ALS target the broad neurotoxicity linked to the neuronal death in patients in order to slow the development of the patient's symptoms.

The most commonly-used medication for ALS is riluzole, whose function is to block the release of excess glutamate. Glutamate is an amino acid that can attach to two amino acid transporters in the neuron membrane, GLT1 and GLAST [3]. Build-up of glutamate in the synaptic cleft, or the space between two neighboring neurons, causes overactivation of glutamate receptors, and it is a common factor found in many cases of the disease. Therefore, this medication attempts to reduce the amount of the neurotransmitter present by activating a

G-protein transduction process that inhibits the release of glutamic acid, an amino acid used to form glutamate [4]. However, rather than only targeting glutamate receptors, riluzole has been seen to block the acetylcholine receptors in neuromuscular junctions, which would prevent signals from being sent to the muscles throughout the body [5]. This medication is, therefore, reducing the levels of glutamate but also exacerbating muscular degeneration in some ALS patients, showing a negative consequence of this treatment.

This is not the only issue that riluzole has been linked to. Since 1997, studies have linked it to higher levels of liver enzyme concentrations, sometimes 2-5 times over the normal limit [6], as well as rare cases of acute liver damage, and both have been continuously reported over the last two to three decades. Even though riluzole seems to increase the life expectancy of patients by two to three months, it induces at least one adverse effect in up to 50.3% of patients, including gastrointestinal disturbances and hepatotoxicity [7]. Overall, riluzole does not increase a patient's life expectancy drastically for the risks that come with treatment, so it cannot be labeled as the most effective treatment pathway for patients.

Riluzole is not the only medication that physicians prescribe; edaravone, both intravenous or oral, is another common treatment pathway. Rather than targeting neurotransmitters, edaravone focuses on providing cytoprotection to shield neurons from excess glutamate by detoxifying the surrounding reactive oxygen species (ROS). ROS are known to cause damage to the basic molecules such as DNA, proteins, and lipids that make up the cells, so it is important that their presence is regulated to prevent damage and exacerbation of ALS symptoms. One set of trials was completed on patients with ALS to test the effectiveness of intravenous edaravone treatment, and it was seen that disease progression was slowed by 30% after 24 weeks of treatment. However, this data set does come with drawbacks because the medication seemed to offer short-term benefits to a specific category of patients, and there are limitations to concluding the effectiveness of long-term edaravone treatment using short-term Revised ALS Functional Rating Scale (ALSFRS-R) scores (ALSFRS-R scores indicate the quality of life patients have based on where their symptoms fall on a scale determined by researchers) [8].

Even though the aforementioned trial did prove the effectiveness of this new treatment design, intravenous edaravone is not the only method used by doctors. Another trial specifically tested the effects of oral edaravone on patients who had not yet faced severe degeneration. It had positive outcomes for patients who had the least severe variations of ALS symptoms and had been diagnosed for at most 2 years; the rest of the patients who were not in this subgroup did not have as successful outcomes from the treatment [9]. Even though edaravone improves the health of many patients within certain types of ALS, the biggest obstacle that prevents the widespread use of edaravone is the increased cost compared to riluzole. A study conducted by the Canadian Agency for Drugs and Technologies in Health showed that in total using an incremental cost-effectiveness ratio, patients will pay approximately $1,957,200 for each year of increased life expectancy gained [10]. Edaravone increases the life expectancy of specific categories of ALS patients more effectively than riluzole, but the cost of the treatment relative to the rate of improvement may not be feasible for patients.

The most recently approved medication for ALS is sodium phenylbutyrate-taur ursodiol, which received FDA approval in mid-2022. It targets the histone deacetylase inhibitor, which remodels chromatin (material that chromosomes are made of), and the alteration in the remodeling process of chromatin is one of the factors that leads to the progression of ALS [11]. The enzymes used in this remodeling process can reduce the expression of certain survival

genes and can impair the repair of DNA damage [12]. This medication has been tested in experimental models and was able to reduce neuronal death. The primary evidence for the effectiveness of sodium phenylbutyrate-taur ursodiol comes from the CENTAUR trial, which tested the new treatment on patients whose symptoms began less than 18 months before the start of the trial [13]. This trial showed that the administration of the medication for 6 months did decrease symptoms of ALS patients and was used to receive FDA approval [14]. However, since the development of this medication was recent, not enough analysis on its effectiveness has been conducted, so it is unknown what potential downsides it has.

The previous methods of treatment discussed have centered around medication that has already been FDA-approved, but research has been conducted on potentially new pathways for treatment such as genetic therapies. There are four specific types of gene therapy pathways that are being discussed for ALS treatment: inhibition of abnormally transcribed RNA using microRNA or antisense oligonucleotides, degradation of the abnormally transcribed RNA, removal or inhibition of mutant proteins, and genome editing. These therapeutic pathways are designed to target specific causes of ALS to ensure that they are the most effective they can be.

Researchers have focused on developing effective gene therapies for SOD1 mutations because it is one of the most common mutations that cause ALS, affecting 20% of patients with fALS. SOD1 mutations are mutations found in the superoxide dismutase type 1 gene, which is responsible for protecting cells against the damage caused by free oxygen radicals [15]. Researchers are proposing the potential of silencing SOD1 mutations or delivering compounds that can activate receptors to protect neurons from SOD1 toxicity. Overall, the new pathways for treatment have been effective in mice models, but there is no evidence yet about its effectiveness in humans or its feasibility.

Altogether, both the FDA-approved medication and the proposed treatments also target either genetic causes of ALS, found only in 10% of patients, or the aggregation of neurotransmitters in the synaptic cleft caused by underlying issues. However, these targeted causes are not shared by a majority of the patients, so these treatments cannot help a majority of patients.

What is TDP-43?

Even though a cause for ALS found in majority of patients has not been discovered yet, there is a common protein aggregation found in 97% of amyotrophic lateral sclerosis patients, both sALS and fALS, called transactive response DNA binding protein of 43 kDa (TDP-43). This aggregation is not a direct cause of neurodegeneration seen in ALS, but it can progress the disorder's symptoms. TDP-43's function is to regulate mRNA stability, and it is necessary for the translation and splicing of mRNA and the repression of cryptic exon inclusions [18,19]; its presence in the nucleus of the neuron is essential for cellular metabolism. When it aggregates in the cytoplasm, then it prevents the regulation of mRNA splicing and translation as well as nucleocytoplasmic transport [19,20]. This prevents proper protein production, which can inhibit important cellular functions from taking place.

To understand what causes TDP-43 aggregation, it is imperative to first analyze the structure of this protein. It contains two RNA-recognition motifs, called RRM1 and RRM2, that are rich in glycine, and it is where most mutations that cause TDP-43 aggregations are found [21]. However, aggregation of TDP-43 is not simply caused by mutations in the glycine-rich regions of the protein; it has also been linked to the misregulation of the protein's production. The

production of TDP-43 is highly regulated through an autoregulation process using cryptic exon repression of a 3' untranslated region on the TARDBP mRNA. When TDP-43 is overexpressed by the cell, the 3'UTR is activated, which results in the proximal poly-A site being excised from the mRNA; this temporarily stops the production of TDP-43 [18].

The structure of TDP-43, especially when misfolded, does lead to aggregation of the protein in the cytoplasm and partial loss of function. TDP-43 aggregation results in cellular stress as proven by the formation of stress granules and its co-localization with the cytoplasmic protein aggregates. Stress granules typically form to repress the translation of certain RNAs, and proteins that are involved in neurodegeneration interact with the granules [22]. Overall, aggregation of TDP-43 is a significant symptom in both fALS and sALS, and it can progress the neurodegeneration in patients. Genetic mutations causing mislocalization of TDP-43 also increases DNA damage because it impairs the localization of certain double-stranded break-repair proteins. This mislocalization of TDP-43, whether it's caused by genetic mutation or other factors, may also lead to the altered splicing reaction, in which exons from the target gene are joined in different combinations and form different but related mRNA versions [18]. Researchers remain divided on whether consequences of this protein aggregation are caused by the lack of the wild-type protein's function (loss-of-function) or the gain of a new function not seen in the wild-type protein (gain-of-function).

Loss of Function

The loss-of-function hypothesis states that the mutant TDP-43 loses some of its primary functions, and it is best tested using common genetic mutations found in TARDBP. Previous studies modeled various loss-of-function TARDBP mutations in non-mammalian and mammalian species (zebrafish, Caenorhabditis elegans) by knocking out the gene. They reported that all the subjects developed neurodegenerative properties; the same study was simulated in the Drosophila model by knocking down the A315T allele of TARDBP, and it garnered similar results [24]. Researchers who believe in the loss-of-function hypothesis were also supported by the modeling of the partial knockdown of TDP-43 in a transgenic mouse model because 601 mRNAs were changed, and 965 altered splicing reactions were tracked [25]. TDP-43 can, therefore, be causing neurodegenerative properties and ALS symptoms in these models since the reduced expression of the gene's products can become toxic for the neuron.

Another study proved the aforementioned hypothesis in both C. elegans models and D. melanogaster models. The tdp-1 ortholog in C. elegans was silenced because it was similar in function and expression to TARDBP, and its loss led to defects in fertility, growth, and locomotion; transcriptional profiling was also utilized to prove the alterations in the expression of genes involved in RNA processing and protein folding [26]. This study was repeated in D. melanogaster subjects by knocking out the dTDP-43 ortholog, and it led to similar results of impaired locomotor activity and axonal loss, which are all indicators of neurodegeneration [27]. The symptoms and TDP-43 proteinopathy expressed in both non-mammalian and mammalian models resembles those of ALS patients. Therefore, it can be concluded that the mutation of the TDP-43 gene can lead to the loss of the protein's function, rather than the gain in novel toxic properties.

The loss-of-function hypothesis was also proven by identifying a set of proteins based on their function, association with ALS, and antibody availability and determining how TDP-43

aggregation can resemble TDP-43 knockdown through the impact on the proteins. The results showed that when simulating aggregation and TDP-43 knockdown, the chosen set of proteins' reaction to both resembled each other [28]. Altogether, the general mislocalization of TDP-43 can also be used as proof for the loss-of-function hypothesis. These aggregates are typically shifted from its normal location in the neuronal nuclei, and this can cause the protein to have impaired functions because its primary functions of mRNA splicing and repression of cryptic inclusions only take place in the nucleus before it migrates to the cytoplasm. Therefore, the protein becomes a hindrance to other cellular functions, which can eventually lead to neuronal degeneration.

Gain of Function

While the loss-of-function hypothesis has evidence to prove its existence, the gain-of-function hypothesis has also garnered an equal amount of support. The gain-of-function hypothesis is derived from the idea that the TDP-43 gene is overexpressed, which leads to the protein garnering additional properties. Some evidence for this hypothesis interprets the toxicity of TDP-43 as novel properties acquired due to the overexpression of TDP-43. These new properties can include the increased affinity to aggregation, mislocalization, and resistance to proteases or any modified binding interactions with other proteins, and these can all lead to neurodegeneration in models and patients.

Many studies have relied on the idea that the overexpression of TDP-43 is what causes the neurodegeneration in cells because *in vivo* experiments have shown that the aggregation caused by overexpression is toxic to neurons. This led to the hypothesis that TDP-43 may be gaining toxic properties not related to its primary function. However, another study was conducted in D. melanogaster and C. elegans models with human TDP-43 being expressed with mutations, and it led to similar results of nuclear and cytoplasmic toxicity as mentioned before due to TDP-43 mutations. In C. elegans, the expression of the NLS-mutant version of TDP-43 (type that affects the localization of the protein) was not toxic though it strictly accumulated in the cytoplasm. What both the worm and fly models proved was that the protein's toxicity due to overexpression is dependent on its RNA-binding domains. Therefore, the gain-of-function hypothesis may be dependent on its normal function related to RNA processing rather than on novel toxic properties [27].

One toxic gain-of-function mechanism that forms due to TDP-43 aggregation is the blockage of intracellular transport in neurons. These aggregates can be found throughout the neuron but it has been observed in both the axons and dendrites; the inhibition of axonal transport is a common symptom in ALS, so this would suggest a link between the aggregation and ALS. TDP-43 toxicity has also been linked to its RNA binding abilities because this function regulates its solubility, so when there is a lack of RNA, then inclusions of the protein begin to form [18]. Altogether, researchers are still determining whether the gain-of-function mechanism of TDP-43 leads to novel toxic properties or toxicity related to normal functions.

Conclusion

After presenting both sides of the argument, it is important to determine which one is more plausible because researchers can determine which therapy pathway can potentially be used to target TDP-43 aggregation only if they know how the protein's function is being affected. Since both hypotheses rely on different interpretations of similar pieces of evidence, the most

logical conclusion is that TDP-43 aggregation can lead to simultaneous gain-of-function and loss-of-function mutations in the TDP-43 protein. This can be explained by how loss-of-function mechanisms found in the protein can lead to aggregates of TDP-43 preventing primary functions, such as transcription and mRNA splicing, and this block can accumulate gain-of-function mechanisms such as the aggregates blocking any intracellular transport in the axons. It becomes a continuous cycle of loss-of-function and gain-of-function mechanisms feeding into each other, so it is most effective to consider the possibility of TDP-43 having pleiotropic properties expressed: gains of toxicity of TDP-43 and the loss of its original primary functions.

Potential Therapeutic Strategies

As established, TDP-43 aggregation is a shared proteinopathy found across both sALS and fALS patients, making it a critical discovery for treatment. Targeting this protein's mechanisms through different treatment pathways can potentially be more successful in increasing life expectancy of and decreasing the rate of neurodegeneration in patients. Researchers have focused on developing three distinct methods to target TDP-43 aggregation: gene therapies, antisense oligonucleotides, and small molecules and antibodies.

Gene Therapies

Gene therapy is a form of treatment that aims to manipulate or alter the expression of a gene in order to reverse the effects of diseases and, possibly, cure them [29]. It is a relatively new form of treatment since it was created in 1990 to treat a patient with severe combined immunodeficiency (SCID), but it has been very successful in its applications in treating other diseases.

There are two different methods of gene therapy that can be used by researchers: ex vivo and in vivo. In ex vivo gene therapy, target cells are removed from the patient's target tissue and edited using the therapeutic gene before being inserted again into the patient. In vivo gene therapy involves the insertion of the therapeutic gene into the patient's body, and the gene will be carried to its target tissue through the bloodstream or into the target tissue [30]. The therapeutic gene referred to in all the forms of gene therapy is carried using either a viral, bacterial, or lipid vector, which are all used depending on the outcome the researcher is aiming for; the viral vector most commonly used by researchers is the adeno-associated virus vector (AAV). In my opinion, the specificity of gene therapy has a promising future in the search for the cure of ALS, specifically in the cases caused by genetic mutations.

One study specifically focused on improving one symptom of ALS, the loss of the integrity of corticospinal motor neurons (CSMN), by targeting an enzyme called ubiquitin C-terminal hydrolase-L1 (UCHL1), which maintains the levels of free ubiquitin in neurons. Free ubiquitin allows for normal nervous system development and rapid responses to cell signaling, and the deterioration of this reaction is what leads to some ALS symptoms. The analysis of mice who lacked the function of all UCHL1 showed early and selective degeneration in their CSMN due to misfolded SOD1 toxicity and TDP-43 aggregation. By using adenovirus-mediated retrograde transduction (binding of the AAV to receptors on the surface of the axon to begin receptor-mediated mechanism), the researchers were able to reduce the extent of the loss of

integrity due to misfolded SOD1 toxicity and mutated human TDP-43; this was measured by analyzing the neuronal integrity and the stability of the cytoskeleton of the cell in the two different mouse models [31]. Another study targeted the human frameshift mutation protein 1 (UPF1) by increasing its production after discovering that it expresses protective effects in a rat paralysis model. They recreated ALS symptoms by inducing the expression of mutated TDP-43 in the models in order to introduce UPF1 using an adeno-associated virus vector. Analysis of the rat models after UPF1 treatment showed that they regained forelimb motor function, and this helped to prove the use of UPF1 as a therapeutic strategy to target the symptoms of ALS induced by the expression of mutant TDP-43 [32]. These two strategies targeted mutations found in enzymes that protected cells from TDP-43 aggregation, and the specificity of its genomic material made it more successful in animal models.

Another study analyzes the effect of using an AAV9 vector compared to a new version of the vector to monitor the CNS transduction of genomic material in neonatal and adult rats because it was the standard procedure of other researchers. When targeting neuron cells specifically, the vectors must include a promoter that is specific to the cell-type, so a synapsin promoter, which is neuron-specific, was used. However, they determined that the AAV9 vector was targeting other tissues along with the CNS since they found traces of the promoter in the liver 4 weeks after the first round of results. This is because with the vector, synapsin becomes neuron-selective, not neuron-specific. They also tested a variant of the adenovirus vector, called AAV-PHP.B, that was genetically engineered to enhance its efficiency and carry the TDP-43 gene. The therapeutic agent was injected intracerebroventricularly and intravenously to target the expression of TDP-43 in the CNS, but results showed that the wide-scale intravenous delivery was most advantageous for efficient expression of TDP-43 through AAV-PHP.B because it increased the strength and specificity of the promoter [33]. Though AAV-PHP.B is a more effective method of transport of genetic material, it is significantly more expensive than AAV9, which decreases the feasibility of making it a widespread solution. Therefore, future research in this field should focus on either decreasing the production cost of the vector or finding a less expensive but equally effective variant of AAV9.

Gene therapies were also used by researchers to analyze the effectiveness of targeting TDP-43 and its relationship with other proteins such as SARM1, which is essential for the degeneration of axons (a symptom of ALS). One study analyzed the effects of knocking out the SARM1 ortholog in Drosophila, and it showed that the axons were prevented from being degenerated and cut for an additional 50 days, proving SARM1's connection to neurodegeneration [34]. This study proves how significant SARM1's effect is on the timeline for neurodegeneration and how it can be applicable in the analysis of ALS treatment. One study used TDP-43 to manipulate the levels of SARM1 in neurons through the presence of Stathmin-2 (STMN2). TDP-43 mediates the mRNA splicing of STMN2, which is a protein that is severely reduced in ALS and can be linked to the aggregation of TDP-43. Loss of STMN2 was replicated in mouse models and at first, results showed that there was a connection between motor neuropathy with the protein loss. STMN2 is coregulated with another protein called NMNAT2, which can stimulate axon protection if overexpressed and can also inhibit the function of SARM1. However, due to TDP-43 aggregation, STMN2 is not regulated efficiently, which negatively impacts the regulation of NMNAT2 leading to the expression of SARM1 in the patient's neurons. This specific study proposed the theoretical strategy to target the regulation of STMN2 to overexpress NMNAT2 in order to inhibit the function of SARM1 in hopes of reducing motor neuropathy in patients [35]. The results from this specific study shows that an indirect gene

therapy pathway that impacts a related protein not TDP-43 could be effective as a standard therapy, but to confirm, the next step would be to run analysis in human trials. The one limitation to this pathway is that it fails to address the aggregation of TDP-43 already present in the neuron.

Researchers also utilized the deletion or suppression of the expression of certain proteins related to TDP-43 in order to control the toxicity of the protein. One study found that the most successful suppressor of TDP-43 toxicity was the deletion of DBR1, which coded for an RNA lariat debranching enzyme [36]. Intronic lariats accumulate in the cytoplasm, which prevents TDP-43 from interfering with essential cellular RNA and RNA-binding proteins.The various gene therapies that researchers have developed in order to provide potential treatment for TDP-43 toxicity; they have all shown positive results, but they still have both benefits and adverse effects like most potential treatment pathways.

Benefits

Gene therapy is a very specific treatment option because it is able to target a certain cause of ALS and, therefore, is only applicable to a certain class of patients who meet specific criteria. Since this pathway targets certain genes that affect TDP-43 in some manner, the patient must also have that correlation between the target genes and TDP-43. This is to ensure that the gene therapy does not worsen the patient's symptoms. Patients must also pass many diagnostic tests to confirm that the patient fits all the criteria to receive the gene therapy, and this can increase the success rate of the therapy since it can more effectively address the symptoms of the patients. The current medication used addresses the general symptoms of all ALS patients, so it is not guaranteed that it will be as successful in all patients; with treatment targeting smaller categories of patients, researchers can adjust their methods in order to target the cause of the disease in patients.

Each of the suggested gene therapies have also been supported by multiple trials of the treatment in mice models that replicate the pathology of the target category of ALS patients. This shows that the effectiveness of the treatment has been proven repeatedly. The most significant benefit of gene therapies is that it does not have to be repeated numerous times; once the vector has been delivered to the target neurons, then the effects can remain for extended periods of time because this treatment manipulates the genetic material of the neuron. This is a benefit that previous medication has not been able to offer since it targets enzymes in the neuron, instead, so the effects could not last as long. Therefore, the genetic therapies can be more effective treatments compared to the general medication.

Drawbacks

Although genetic therapies do have multiple significant benefits, they also present many limitations to the treatment of patients. Because these therapies target a specific subset of ALS, they cannot be applied to the general population of ALS patients. Though this is beneficial since it can increase success rates, it also makes treatment more inefficient, time-consuming, and expensive because more effort is spent in deducing which gene may be causing or increasing the toxicity of TDP-43. The gene therapies analyzed are targeting the symptoms of ALS rather than ridding the patient of the cause of the symptoms of the disease, so creating treatment to

target each specific gene related to the toxicity of TDP-43 can make the process more inefficient.

The other drawback of gene therapies is that it can only treat patients with fALS presenting genetic mutations. This therapy is only applicable in cases of ALS where the patient presents with genetic mutations that are related to their symptoms, but this is only found in around 10% of patients. There are two distinct barriers present in this situation: sequencing and developing the treatment. To apply gene therapy, the patient's genome must be analyzed to discover the mutation that could be causing TDP-43 toxicity. But since many genes could be connected, multiple variants of the treatment must be developed with new delivered genetic material and adjustments of the vectors. Therefore, the patients who will benefit from this treatment is a very small amount since each proposed treatment targets a specific cause that is found in a minute percentage of patients who suffer from ALS.

In conclusion, genetic therapies present a new form of treatment that can target the specific causes of the symptoms of ALS, which can make it more effective; however, as most proposed treatments, it has significant benefits and drawbacks because of the complicated etiology of ALS.

Antisense Oligonucleotides

Antisense oligonucleotides (ASO) are single-stranded DNA designed to be complementary to certain sections of target mRNA in order to bind effectively; they are typically used to regulate gene expression through, for instance, the inhibition of mRNA translation [37]. They were developed in 1978 when papers showed that if synthetic oligonucleotides were complementary to mRNA, they could inhibit viral replication. One of the most well-known applications of ASOs is in nusinersen, a drug used to treat spinal muscular atrophy.

ASOs act by causing RNA cleavage, RNA blockage, mediated cleavage, RNA interference, or splice modulation in order to manipulate the expression of certain genetic material. Similar to gene therapies, the oligonucleotides are also typically transported in vectors, either viral or bacterial, in order to protect them from degradation and ensure that their transport is completed. ASOs are administered either through intravenous infusion, subcutaneous injections, or direct injections through the bloodstream [38]. Researchers determined that synthetic oligonucleotides are a possible subset for gene therapies when treating ALS symptoms. However, this paper analyzes ASOs separately in order to emphasize this treatment's effectiveness.

Research shows how the administration of anti-SOD1 ASOs through either direct delivery or AAV9 showed promise in certain animal models. The results of administration of subpial injections of the ASO using viral vectors showed that the progression of ALS can be prevented or completely stopped in the models depending on whether the injection is given before or after the onset of the disease. Therefore, the use of anti-SOD1 ASOs seems to be more effective in reducing the symptoms of the disease compared to the previous gene therapies mentioned, proving the significance of the development of oligonucleotide-based therapy in the search for ALS treatment. This research also proves the success of adenovirus vectors in the effective delivery of ASOs to the neurons [39]. This specific study is able to identify which method of delivery should be used with ASOs, and it shows how compared to gene therapies, which were delivered more successfully using a AAV9 variant, ASOs need a direct delivery without a vector.

However, the application of this specific treatment is limited due to the immunogenicity present in humans in response to viral vectors.

Another study shows how ASOs can be used in combination therapies to target the levels of TDP-43 without reducing the levels of STMN2. Previously, when toxic TDP-43 cytoplasmic aggregation was targeted using Ataxin-2-ASOs or small molecules, the levels of Stathmin-2 (STMN2) decreased while the toxicity of TDP-43 decreased. STMN2 levels are reduced in patients with ALS due to lack of mediation of its mRNA splicing, so this dated method may contribute to ALS pathogenesis. However, the study provides a solution by combining the two previous therapies to target the levels of TDP-43 with an ASO to block STMN2 cryptic splicing [40]. This treatment uses an indirect treatment pathway as described before as a type of gene therapy since the ASO targets a protein that affects TDP-43 aggregation. It can target both the loss-of-function and gain-of-function effects seen in the ALS pathology, which can make it increasingly effective. It is also more effective since it ensures that symptoms are not exacerbated in patients by ensuring that surrounding proteins impacted by TDP-43 are also monitored.

TDP-43 proteinopathies have been linked to the de-repression and inclusion of cryptic exons in the mRNA that it is splicing, which can lead to the loss of neuronal proteins such as STMN2. Targeting the STMN2 cryptic exon using ASOs can lead to an increase in STMN2 expression, and this study shows that through the ASO-mediated reduction of either TDP-43 or stathmin-2 mRNA in induced pluripotent stem cells can also restore axonal regeneration. Oligonucleotides can also bind to pre-mRNA in cells to reduce the levels of toxic proteins, and this allows the ASO to edit both the exons and introns of the RNA before it is edited into mRNA [41]. This can increase the effectiveness of treatment against TDP-43 since it targets cryptic exons, whose inclusion is caused by TDP-43 aggregation. Another study shows that using multiple small effectors, which are cells that respond to stimulus, multiple ASOs, or small nuclear RNA (snRNA) can be packaged to target multiple cryptic exons simultaneously [42]. It also introduces a different form of combination therapy where rather than either indirectly targeting or partially using gene therapy, multiple vectors with genetic material are delivered. The one limitation is that we do not yet understand how this method functions in human models, so it could have unknown side effects.

Benefits

ASOs are a type of genetic therapy as described in the previous section, but it focuses on inactivating or silencing certain genes. Because of its increased specificity, they are very successful in the process of silencing genetic mutations. They can lead to the restoration of protein function and expression, reduce the expression of toxic proteins found in the cell, or modify the expression of the proteins; therefore, it can target both gain-of-function and loss-of-function mutations. This is important in order to address the pleiotropic characteristics that TDP-43 proteinopathies show. The studies mentioned before are the most effective in silencing genetic mutations in forms of either individual targeting or combination therapy of both the TDP-43 aggregation and the levels of STMN2 in the neuron.

Drawbacks

Although ASOs are effective in targeting mutation, it requires continuous dosing to maintain the response to the inactivation. This may be undesirable for many patients since

many methods of dosing of ASOs are invasive such as subcutaneous, intravenous, intrathecal, or subpial. Undergoing this treatment several times, therefore, becomes a disadvantage of the use of ASOs since patients sometimes undergo other gene therapies only once. The cellular uptake of the ASOs also cannot be controlled even as the delivery of the ASOs become more precise because of its stability in biological fluids. Even though the implementation of liposomal delivery has improved the uptake, it still presents a disadvantage since it is not certain whether the ASO is delivered accurately and effectively. Another drawback that ASO therapy developments face is the accurate delivery of the oligonucleotide to its action site because if it's being delivered intravenously, then it has to travel through the bloodstream to reach the neuron, but it can face many challenges to reach its destination.

In conclusion, ASOs are effective methods of treatment for ALS caused by TDP-43 aggregation because it is specific and can inhibit both gain-of-function and loss-of-function mutations. It was important to analyze the specificity of its mechanisms to understand what makes it unique compared to the other gene therapies. However, even though many forms of ASO treatments work more effectively than the current medication, it still presents significant benefits and drawbacks that must be considered.

Small Molecules and Protein Drugs

Small molecules are the molecules found in drugs and are designed to interact with target proteins in a specific way, and they were created during the golden age of drug discovery with the first ones produced being antidepressants and antipsychotics in the 20th century. They were recently introduced as a potential therapeutic for ALS because of their easy administration and effectiveness. However, there is no way to group the different small molecules together based on their function because each small molecule interacts with each protein differently; many, for instance, are capable of crossing the blood-brain barrier in order to target large proteins in the CNS and alter their activity.

One study proposes the use of calcium channel agonists to protect against neuromuscular dysfunction that is seen in many ALS patients. A channel agonist is a substance that binds to the channel receptors of the cell, in this case the neuron, to cause a specific biological response. When the ALS mutation, mutTARDBP, was expressed in zebrafish larvae, the characteristics of its motor function prior to treatment and after treatment using the calcium channel agonists, FPL 64176 or Bay K 8644 were stabilized after treatment, showing how effective this treatment is in animal models and its potential in human models [43]. It is a simple method of treatment, and this can increase its accessibility to patients when it is applied to human models because more people will find it easier to administer.

Another study analyzed the use of small molecule medication in the treatment of ALS by studying the impact of methylene blue (MB) and dimebon on TDP-43 aggregation. MB is a medication used to treat methemoglobinemia, in which hemoglobin slowly loses its ability to carry oxygen, while dimebon is an antihistamine that has also been tested for its therapeutic potential in Alzheimer's. These two small molecule medications have been successful in phase II Alzheimer clinical trials, and their combined use reduced the TDP-43 aggregations in neuroblastoma cell lines by 80% [44]. Therefore, it can be concluded that both MB and dimebon may have success in ALS treatment, but this can only be proved after a trial on human models

has been conducted. The only challenge would be the administration of both small molecule drugs to ensure cellular uptake remains similar to the trials conducted.

However, sometimes the small molecules analyzed do not necessarily have to be created but can be proteins already found in the body; these protein drugs function in a manner similar to small molecule drugs because they have high specificity to their target, which improves their cellular uptake. Patients with both fALS and sALS caused by TDP-43 aggregation have impaired autophagosome formation and the accumulation of glutamate receptors. One study showed that an anticoagulation-deficient form of activated protein C, which is a glycoprotein that controls blood clotting, can reduce the presence of these defects in induced motor neuron models; proteostasis and low glutamate levels are both also accomplished in gain- and loss-of-function fALS models with C9ORF72 [45]. Therefore, this treatment method does have some increased benefits due to its specificity and simple mechanisms. Compared to the other solutions, this protein drug utilizes a blood test to determine whether a patient fits the criteria.

Another more recent and similar treatment method for ALS is antibodies, proteins created to counteract an antigen in the bloodstream. Monoclonal antibodies were first used to prevent kidney transplant rejection in 1986, and now it has a broad range of applications such as diagnosis, research, and disease treatment. They are able to recognize and target specific proteins in cells, and they have been used in multiple different cell lines such as cancer cells or cells found in the immune system. Antibodies can also be categorized as a protein drug when administered to alleviate the symptoms of ALS. One study investigated the effectiveness of the single-chain antibodies (scFv) that they generated in order to target the RRM1 of the TDP-43 protein in order to reduce its cytoplasmic aggregation. When delivered using an adenovirus vector, the antibodies were able to reduce toxic aggregation, neuroinflammation, motor defects, and cognitive impairment in mouse models [46]. These results suggest that this specific antibody may be useful in treating patients with ALS and that antibodies can be an effective method to target neurodegenerative disorders like ALS. However, because not as much research has been done on the use of antibodies to treat ALS, the full analysis of it as a treatment method cannot be accomplished, but its similarity to small molecules in function can allow for a proper analysis for both treatment models together.

Benefits

The current research on both small molecules and antibodies shows that when it targets TDP-43 aggregation, it can benefit both fALS and sALS models which shows its broad effectiveness. It also has as much success as other more established methods of treatment such as genetic therapy, so there should be a greater push for its use for two main reasons. Primarily, it is easier to administer because small molecules can be provided in the form of medication while patients are given monoclonal antibodies through intravenous injections. This is simpler than the administration of ASOs or genetic therapies because most do not require the use of different types of injections or viral vectors. Additionally, small molecules are already an accepted treatment model, so it will be easier to convince patients to consent to this new form of treatment compared to experimental genetic therapies. Small molecules and antibodies delivery models have already been established, but the model of genetic therapies change depending on the target tissue and the type of disease that is being treated. Overall, the advantages that small molecules and antibodies present as treatment methods are greater compared to genetic therapies in both the medical and societal sense.

Drawbacks

Though antibodies and small molecules both have significant advantages, the problems presented by TDP-43 aggregation cannot be solved using small molecules like proteins or antibodies because these treatment methods lack the specificity presented by other models. Typically, medication containing small molecules or antibodies induce off-target effects because they do not contain the explicit detail that both gene therapies and ASOs provide. Though they have been proven successful at the animal trial level, both methods have not yet been tested on human models. Another drawback present in the use of small molecules and antibodies is the need for repeated administration because its biological effects can wear off. Many patients may not prefer the need for repetitive treatment because it increases costs and discomfort.

In conclusion, the use of antibodies and small molecules is an effective treatment method because it is able to target the TDP-43 aggregation found in cell models. However, because it lacks specificity that the other models provide, the drawbacks and benefits of each treatment must be analyzed before being implemented in human models.

Potential Proposal

The treatment methods proposed previously by different research groups have all shown their success in fALS and/or sALS cases caused by TDP-43 aggregation. However, as previously described, they each present with some important drawbacks that must be improved upon in order to be the most effective treatment model for treating TDP-43 aggregation and ALS. In this section, I will propose a potential method that may solve the challenges presented by previous models.

GTPase Ran

The neuron has to maintain constant transportation of cellular products and waste while performing other important functions such as mRNA splicing, protein production, and receiving and sending signals to other neurons. However, when TDP-43 aggregates in the cytoplasm, it inhibits important functions such as nucleocytoplasmic transport carried out by the nuclear pore complex [20]. This can cause further aggregation and mislocalization of nucleoporins, which are the proteins that make up the nuclear pore complex, and transport factors, which can increase the cytoplasmic aggregation and mislocalization of TDP-43, creating a cycle of toxic protein production. However, there is one protein, GTPase Ran, which handles the nuclear transport of material in order for nuclear localization of TDP-43 and regulates nuclear transport using the nuclear pore complex; its accessory proteins are necessary for the accurate localization of TDP-43 [47]. This function makes GTPase Ran increasingly important in reducing the toxic properties of TDP-43 to target the symptoms of ALS.

Potential Proposal

This specific proposal has a two-part process that, together, can potentially lead to a successful outcome for ALS patients. First, the existing aggregates of TDP-43 must be broken down in order to clear the cytoplasm for the nuclear pore complex and the nucleocytoplasmic transport. Since TDP-43 aggregates will still be produced as the existing aggregates are removed, this must be carried out almost simultaneously with the second step. The most

effective way to break down these aggregates would be to utilize synthetic peptides that can be administered through capsules in the form of small molecule medication [48]. These peptides are able to reduce the levels of TDP-43 aggregation without inducing or completely preventing cell death, which suggests that it is the most effective method to accomplish the first step. Research has also shown that artificial peptides can be designed to target mutated domains of TDP-43, such as the low complexity domain, known to cause aggregation [49]. Therefore, synthesis of peptides is the most efficient method to clear mutated TDP-43 from the cytoplasm, and it would be delivered orally using nanoparticles (most common delivery model for peptides to neurons) and can achieve the wanted results.

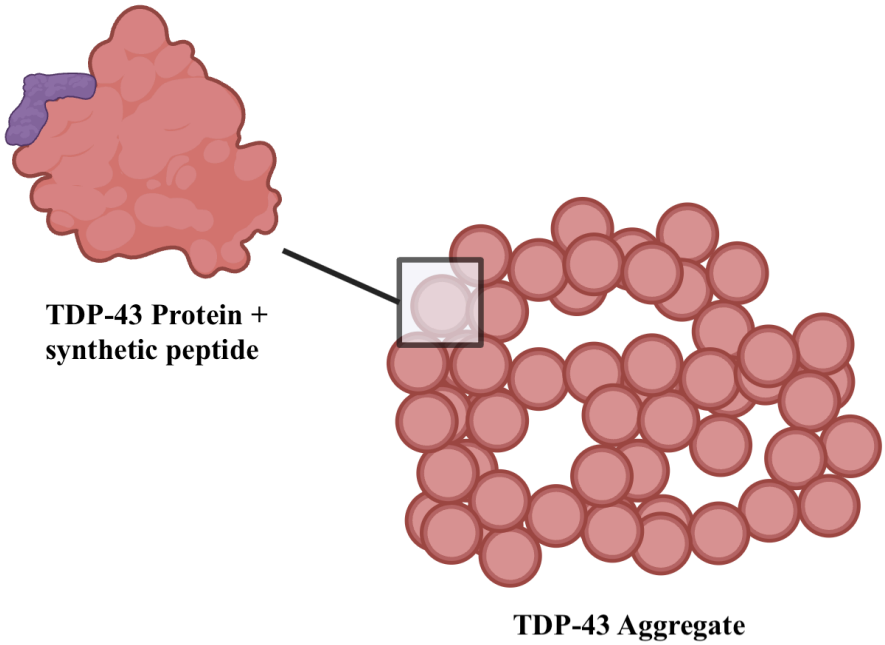

Figure 1: The first part of the proposal includes the breakdown of the TDP-43 aggregation using synthetic peptides. These peptides will target mutated domains of the protein in order to prevent aggregation.

The second part would include regulating GTPase Ran by increasing its expression to prevent TDP-43 from exiting the nucleus of the neuron. Levels of GTPase Ran are depleted in a case study of frontotemporal lobe dementia (FTLD) patients [47] , and FTLD shares TDP-43 pathology with sALS [50]. In both disorders, the protein shares a pathogenic role and exacerbates the symptoms by inhibiting necessary functions in neurons. Therefore, it can be hypothesized that the depletion of GTPase Ran can contribute to the mislocalization of TDP-43 seen in ALS. Increasing the levels of GTPase Ran can help decrease the formation of the toxic proteins in the cytoplasm. Research has shown that previously the inhibition of (G4C2)30 RNA, which has been linked to the increase in GTPase Ran, has been successful in patients with C9orf72 mutations [51]. However, this proposal was designed to be applicable to patients with sALS as well, so though it is successful for C9orf72 patients, this research cannot be applied to a broader

spectrum of patients. Instead, direct delivery of the genomic material that codes for the protein can be utilized to increase the production of this nuclear protein.

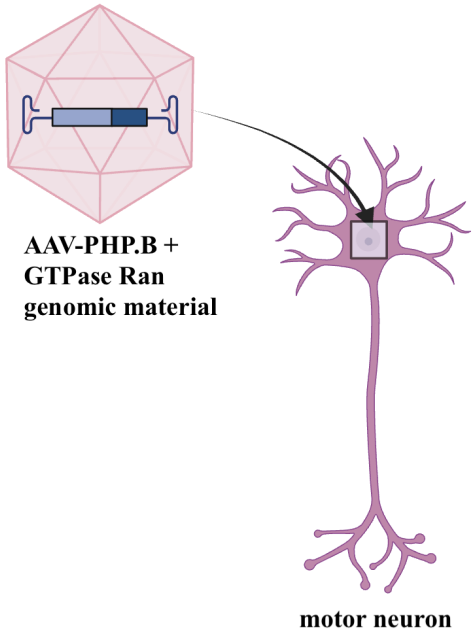

Figure 2: The second part of the proposal includes the direct delivery of the GTPase Ran genomic material into the motor neuron using an AAV-PHP.B vector. These vectors are best delivered intravenously in order to achieve the most effective CNS transduction. This method will produce GTPase Ran in motor neurons with depleted levels of the protein.

This treatment proposal would be more effective because it targets the issue of TDP-43 aggregation, which is not the protein but the mislocalization. It will be delivered using the bacterial vector, AAV9, since it has been shown to effectively reach neurons and produce the protein. However, the variant, AAV-PHP.B, may be more advantageous since it increased the expression of TDP-43 in the completed studies [33]. One limitation to this method would be the surplus of GTPase Ran in neurons. In the future, to tackle this challenge, the genomic material can also be packaged with an inducible promoter in order to better monitor the amount of GTPase Ran being produced. Additionally, though the location of TDP-43 will be better localized with this therapeutic pathway, a limitation may also be the structure of the protein if it has been mutated. Therefore, with further research, an additional step must also be added in order to resolve this obstacle. Overall, this potential treatment model could be beneficial in solving the challenges that other methods have faced, and it may prove how targeting the cause of ALS will be more effective than treating the symptoms.

Conclusion

Researchers have hypothesized that targeting TDP-43 aggregation using multiple therapeutic strategies (gene therapies, ASOs, small molecules, and protein drugs) could reduce symptoms of ALS and extend the lifespan of patients. The strategies developed so far have been analyzed in this paper, and overall, each has been effective in the animal models with

specific criteria. But the most important limitation to many of these therapeutic strategies was that they targeted patients with known genetic mutations. Many gene therapies are only effective in patients with mutations regarding the structure of TDP-43 while some ASO pathways use combination therapy in animal models with a STMN2 cryptic exon in order to lessen side effects. When implemented in patient models, then it may only be effective in patients with fALS, but a majority of patients suffer from sALS and have no family history of the disorder. Therefore, after analyzing the common thread in TDP-43 aggregation, a potential proposal was detailed in this paper that suggested the use of a two-part process: synthetic peptides delivered orally using nanoparticles to break down the present TDP-43 aggregation and direct delivery of the genetic material of GTPase Ran using AAV-PHP.B to increase the protein's levels. Though it is applicable to patients with sALS, the potential proposal that was described in this paper was also limited by the unregulated expression of GTPase Ran. Since ALS is a debilitating disease that still has no effective treatment, any therapeutic pathway that extends the lifespan of patients would be a huge advancement in the field.

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