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REVIEW ARTICLE

Spinal Muscular Atrophy: Genetics And Recent Advancement In Gene Therapy

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ABSTRACT: Spinal muscular atrophy (SMA) is a devastating genetic disease occurring ~1 in every 10,000 live births globally with varied mortality rates based on different severity of the disease. It is a recessive neurodegenerative disease (ND) with various severity due to different ages of onset. There is also a wide range of symptoms experienced by the patients, from muscle weaknesses to abnormal breathing patterns. However, all symptoms share one specific genetic etiology - survival motor neuron (SMN) protein deficiency due to mutations or deletions of the SMN1 gene. In this review, the in-depth structural concept regarding aspects of SMA, including its cause at a molecular level, is summarized; the recent novel establishment of two existing gene therapies, Spinraza and Zolgensma, is discussed, regarding mechanism and limitations. We further propose possible goals to pursue novel developments of SMA therapy.

KEYWORDS: Biomedical And Health Sciences; Genetics and Molecular Biology; Neurodegenerative Disease; Spinal Muscular Atrophy; Gene Therapy.

Introduction

Spinal muscular atrophy (SMA), with a frequency of ~1 in every 10,000 global live births, is a group of neurodegenerative diseases symbolized by the dysfunction and degeneration of α-motor neurons in the brain stem's anterior horn and motor nuclei. This results in progressive atrophy and weakness in muscles controlling vital activities, including bulbar, respiratory, trunk, and limb muscles.[1–3] SMA has a wide range of severity depending on the time of disease onset and variations of the causative mutations.[1,2,4,5] There are mainly three types of classic SMA (type 1 to 3) and two less common types (type 0 the severest type, and type 4 the mildest type).[1] The mortality rate of SMA increases with growing severity and increasing age (for example, type 1 SMA has mortality rates of 37-94% at 1 year, 31-87% at 2 years, 26-72% at 4 years, 8-50% at 10 years).[6]

The history of the disease can be traced back to the description in 1891 by Guido Werdnig regarding 2 infant brothers and the discovery of 7 other cases by Johan Hoffmann from 1893 to 1900. The first descriptions of severe infantile SMA were done by Sylvestre in 1899 and Beevor in 1903. A milder form of SMA, in which patients maintain the ability to stand and walk and prolong life was first described in detail by Kugelberg and Welander in the 1950s. All the early descriptions stressed the significant pathology as anterior horn cell degeneration and the related muscular weaknesses. It was not until 1991 when the variability in severity and the multiple phenotypes were finally formalized into a classification scheme at an International Consortium on Spinal Muscular Atrophy, which highlighted 3 SMA types according to the highest motor function achievement such as standing and the age of disease onset. This is followed by subsequent modifications dividing type 3 into subcategories based on the age of onset, in addition to adding type 0 SMA for prenatal onset and death within weeks plus type 4 SMA to include adult-onset cases.[2]

Despite the variety of SMA diseases, all variations mentioned have genetic causes. Classic SMA (type 0 to 4) is an autosomal recessive disease related to SMN genes, with 95% of patients having a homozygous deletion of SMN1 from both paternal and maternal sources. The other 5% have compound heterozygous mutations, including an SMN1 deletion allele and an intragenic mutation in the other SMN1 gene.[1,7] The mutation leads to reduced expression of survival motor neuron protein (SMN), which results in the degeneration of α-motor neurons and consequent muscular weaknesses.[1] The review will surround the question of how genetic mutation leads to spinal muscular atrophy – the type of mutation and the advancements in gene therapies. A range of aspects regarding the mentioned SMA variations will be outlined in this review, and the most recent advancement in classic SMA gene therapy will be especially emphasized.

Discussion

Pathology of SMA:

Classic SMA is classified into three main phenotypes (type 1 to type 3) based on the symptom onset age, and the highest motor function achieved by patients. Additionally, type 0 and type 4 are the most severe and the least severe classifications, respectively. Subclassification is proposed for type 3, and in some literature, type 1 and type 2.[1–3,8] Common symptoms of classic SMA include areflexia and fasciculations due to pure motor neuronopathy and axonopathy. Sensation is preserved, and more severe patterns of proximal and lower extremities muscle weaknesses are observed symbolically.[1] Ocular and facial muscles usually remain unaffected.[3] It is also important to remember that severity may vary and change over time no matter the type of SMA, as the disease progresses.[8]

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- ***Type 0 SMA:***

Type 0 SMA (categorized as type 1A in some cases) is the most severe. It occurs among a limited number of patients who witnessed the antenatal onset of symptoms, including reduced or absent fetal movements in addition to severe signs such as contractures experienced at birth.[1] These rare cases mostly present with arthrogryposis multiplex congenita, profound hypotonia, and respiratory difficulties soon after birth.[3,8] The life expectancy of the patients is dramatically reduced; most cases would not be able to survive beyond one month of age if they remain untreated.[8]

- ***Type 1 SMA:***

Around 50% of patients have SMA type 1 (Werdnig-Hoffman disease), which has symptom onset within the first 6 months and the most severe symptoms.[1,8,9] It usually appears with serious hypotonia – the infant appears to be symbolically floppy, with progressive swallowing difficulties and decreased voluntary movements.[1,8,9] Due to intercostal and chest wall muscle weakness and relatively preserved diaphragm movement, patients demonstrate a bell-shaped chest deformity with a paradoxical breathing pattern.[3] Tongue fasciculations are also presented in some cases,[3] and congenital heart defect is also featured.[8] SMA type 1 patients fail to achieve the major motor developmental milestone of sitting in their lifetime.[9] The life expectancy of patients is significantly compromised as SMA type 1 is the leading genetic cause of infant mortality, with a life expectancy under 2 years and a median survival of around 10 months if left untreated.[1,3,8,9]

- ***Type 2 SMA:***

Patients with SMA type 2, also known as Dubowitz's disease, are characterized by a milder onset of symptoms between the ages of 6 and 18 months.[8,9] The disease first presents diminished development in reaching gross motor skills as developmental milestones, although the patients can maintain an unaided sitting position.[1,8,9] It is also noted that the ability to sit independently may be lost later, and the patients can never stand or walk unaided.[3,8] Patients usually have similar or less severe patterns of muscle weakness observed in SMA type 1; additionally, the development of scoliosis, including Kyphoscoliosis, is observed, complicating respiratory impairment such as restrictive lung disease if no orthopedics intervened.[1,8] Examination suggests tongue atrophy and fasciculations, dysphagia, and sometimes minipolymyoclonus presented;[3] the ability to cough and clear airway secretions would compromise progressively.[8] Patients with SMA type 2 can mostly survive beyond adulthood; 68.5% of this historic cohort survived to age 25 and over 20 years for a median lifespan.[1,3,8,9] However, aggressive supportive management is usually required, particularly for gastrointestinal and respiratory complications, once adolescents have entered adolescence.[8]

- ***Type 3 SMA:***

SMA Type 3 (Kugelberg-Welander disease), characterized by the ability to stand or walk independently and presents with an onset at about 18 months of age, comprises about 15% of cases.[1–3,8,9] Roughly 50% of SMA type 3 patients will lose the ability to walk unaided around the age of puberty with disease progression,[1,3] some may become wheelchair dependent around middle age.[8] Unlike SMA type 1 and 2 patients, most type 3 patients would not experience profound respiratory muscle weakness and have an unaffected life expectancy.[3] Instead, they are featured with fatigue and proximal weakness, including the tendency to fall, abnormal gait, and struggle to climb stairs.[1,3] There are also emerging studies indicating cognitive impairment experienced in some SMA type 1 or 2 patients, which is not found among SMA type 3 or 4.[1] SMA type 3 is further categorized into two subgroups: 3a characterized with symptoms onset between 18 to 36 months; 3b with onset after 3 years old.[2,3,8]

- ***Type 4 SMA:***

At the mildest end of the phenotype spectrum is SMA type 4, characterized by typical symptom onset after the age of 20 years old with relatively milder weakness of lower extremities.[2,8] Type 4 patients mostly have a normal life span and preserve good ambulation with the ability to walk independently throughout their lifetime.[8]

Mechanism of SMA:

The most evident clinical manifestation of classic SMA, especially skeletal muscle weakness, is due to the interruption of the SMA motor unit, including the motor neurons and the muscle fibers they innervated. Developmentally immature SMA motor units are especially vulnerable to early and sudden degeneration involving all motor neuron parts. In humans, α-motor neurons are connected to skeletal muscles via neuromuscular junctions (NMJs) and receive inputs from proprioceptive neurons from the dorsal root ganglia. Survival motor neuron (SMN) deficiency in SMA results from multiple impairments in this loop, ultimately leading to motor neuron degeneration.[1]

Specifically, in mouse models and SMA patients, SMA motor neuron degeneration includes the withdrawal of the terminal motor axon from the neuromuscular junction (NMJ) postsynaptic terminal, degeneration of proximal motor axons, loss of cell body synaptic inputs, and motor neuron cell body death. This is followed by loss of myofibers after long-term denervation with replacement of fibro-adipose tissue. Some specific defects include slowed acquisition of motor neuron excitatory synaptic inputs and the maturing of motor neuron firing patterns, impairments in motor axon radial growth, Schwann cell ensheathment, and myelination.[1]

The abnormal firing pattern of motor neurons (MNs) is due to the reduction of presynaptic glutamate transmission onto MNs. The input resistance for SMA MNs is increased due to neurotransmission blockage. However, their firing frequency is paradoxically declined as Kv2.1 (a potassium ion channel protein) expression is reduced via a reduction in glutamate release from proprioceptive synapses, which is a homeostatic response to altered activity. As Kv2.1-mediated repolarizing currents are reduced, reduction in spiking frequency leads to longer action potentials and resultant sustainment of depolarized voltages between spikes. Extended depolarization would maintain voltage-gated sodium channels in their inactivated state and decrease their availability for action potential generation. Such reduction of MN spiking ability is a non-cell autonomous result of decreasing excitatory synaptic drive from

proprioceptive neurons. Thus, SMA MNs have a significantly low firing frequency, which is suggested to be triggered by the dysfunction of sensory-motor synapses. It is also important to mention that although reduced glutamate release at sensory-motor synapses leads to abnormal firing patterns, it does not cause MN death.[10]

It is also shown that restricted embryonic motor axon radial growth and impaired Schwann cell ensheathment are the initial cellular features of severe SMA. Radial growth of axons is the first step in implementing Schwann cell ensheathment, followed by segregation and myelination. Impaired radial growth is found in most SMA motor axons to various degrees. There has also been an establishment regarding preferential postnatal degeneration of the smallest and most developmentally delayed axons. As the neurons are extremely small in diameter and not myelinated, they are particularly vulnerable to rapid degeneration, which may be the cause of the early and abrupt clinical decline of infantile SMA patients.[11]

Genetics Basis of SMA:

The source of the above defects can all be traced back to SMN deficits. A mouse model demonstrates that MN dysfunction (reduced firing pattern) is due to SMN deficits in proprioceptive neurons, which compromises sensory-motor synapses.[10] The selective degeneration is also the result of relatively diminished survival motor neuron (SMN) expression.[11] Therefore, most clinical manifestations point to SMN deficiency as the major cause of SMA disease mechanisms. The SMN gene encodes SMN protein expression; mutations in the *SMN* gene lead to reduced expression levels of the SMN protein, which generates a series of abnormal neurological events as mentioned above, contributing to various symptoms of SMA.

Around 95% of patients with SMA have homozygous deletions in both paternal and maternal *SMN1* at position 5q13, regardless of their phenotypes.[1,3,7,9] The remaining ~5% of the patients carry an *SMN1* point mutation and a deletion in the other SMN allele.[1,3,7,9] Also, biallelic small-scale mutations may occur in any SMN1 exons. Still, rarely.[1,12] De novo mutations happen at a relatively high rate of ~2% due to the instability of this 5q region.[12] Genuine deletions of *SMN1* often occur for more severe phenotypes; this includes SMA type 1.[1,12,13] Whereas for relatively milder types, such as type 2 and type 3, gene conversion usually occurs, which is the change of the *SMN1* exon 7 to that of *SMN2*.[1,12,13]

- ***SMN1 and SMN2 genes:***

The presence of two *SMN* genes in humans is due to the intrachromosomal duplication of ~500 kb segment at the 5q13.3 locus on chromosome 5.[14] *SMN1* contains nine exons, 1, 2a, 2b, 3, 4, 5, 6, 7, 8, with exon 8 remaining untranslated (Figure 2). *SMN2* is the resultant centromeric copy of *SMN1*, which differs by five nonpolymorphic nucleotides within the 3' region, in addition to a C-to-T transition in exon 7 of *SMN2*, that have the favor of skipping exon 7 during splicing, resulting in the majority of *SMN2* products being one of the truncated isoform called SMN**Δ**7.[15,16]

SMN gene encodes for survival motor neuron protein, which is necessary for survival, as name-sake.[16,17] SMN protein is ubiquitously expressed and is needed by all cells and muscle types, localized to both nuclear and cytosolic compartments, not limited to neurons.[3,16,18] *SMN1* codes for SMN, while *SMN2* mainly produces the truncated protein isoform SMN**Δ**7 because of the predominant skipping of exon 7, which is highly unstable as it is quickly subjected to the ubiquitin-proteasome pathway for degradation (Table 1).

Table 1: Comparing two isoforms of SMN relevant to the condition of spinal muscular atrophy.[16]

SMN gene	SMN protein isoform	Splicing	Functionality of protein	Expression	Localization
SMN1	Full-length SMN (FL-SMN)	Exons 1, 2a, 2b, 3, 4, 5, 6, 7, 8	Functional SMN protein	The expression of both isoforms is high during development, however decreases when into the adult CNS.	Nuclear gems and cytosolic (axons, dendrites, and synapses)
SMN2	SMNΔ7	Exons 1, 2a, 2b, 3, 4, 5, 6, 8 (exon 7 is skipped during splicing)	SMA disease-causing protein, as degradation signal is introduced due to the changes in C-terminal		Nuclear accumulation

There are also many other isoforms of SMN, including SMN6B, SMNΔ**5, and a-SMN[20], which are not mentioned in the table due to their relative lack of relevance to the disease of spinal muscular atrophy, the key topic of this review. (adapted from H. Chaytow et al. 2018).*

Mainly transcribed from the telomeric *SMN1* gene, full-length SMN is translated into a 38-kDa SMN protein containing 294 amino acids after mRNA processing.[1,16] SMN has distinct functional and binding domains (all domains are specified in Figure 1)[1,19]; therefore, mutations in all domains have been significantly linked to SMA, meaning the whole structure of the protein is critical for the functioning of humans.[19]

There are multiple interactions between various proteins and domains of the *SMN* gene. Encoded by exon 2, the basic/lysine-rich region has been found to have interaction with Gemin2.[16] The nucleic acid-binding domain coded by exon 2A and 2B overlaps with the binding site of Gemin2, which is also known as SMN-Interacting Protein 1 (SIP1).[19] The core complex formed by SMN-Gemin2 seems to be the key to most functions of SMN in vertebrates, including snRNP assembly, translation regulation, DNA recombination, and signal recognition particle biogenesis.[20–22] The Tudor domain provides the binding site for most proteins when interacting with SMN (Figure 1). To give an example, The SMN Tudor domain binds to the C-terminal arginine and glycine-rich tails of Smith core (Sm) proteins, which contain symmetrical demethylated arginine residues, therefore able to facilitate the assembly of spliceosomes (other specific proteins binding to Tudor is shown in Figure 1).[23–27]

Interactions with the YG box contribute to the molecular stability of SMN. The YG box is the tyrosine/glycine-rich region in the C-terminus of SMN; it is discovered to facilitate the oligomerization of SMN by forming a glycine-zipper structure,[24] which is crucial for the stability and subcellular

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localization of SMN. Additionally, the C-terminal sequences of SMN, including the YG box, also interact with Gemin3 (a dead-box helicase), ZPR1 (a zinc-finger protein required for localization of SMN to Cbs) and SIN3A (a transcription co-repressor).[28–31]

Exclusion of exon 7 during splicing leads to instability of SMN**Δ**7, resulting in protein degradation. The loss of amino acids coded by exon 7 cancels SMN interaction with Trimethylguanosine Synthase 1 (TGS1), which acts as a catalyst for the formation of 2,2,7-trimethylguanosine (TMG) cap structure at the 5' -end of the snRNAs, snoRNAs, and a subpopulation of mRNAs.[32,33] The QNQKE motif coded by exon 7 exports nuclear signal. The zebrafish SMN model (by Carrel, T. L. *et al.*) with mutations in the QNQKE motif conserves the snRNP assembly function but unsuccessful rescues motor axon defects.[34] Skipping of exon 7 adds a four-amino acid motif, EMLA, coded by exon 8, which acts as a degradation signal for SMN**Δ**7 and explains its unstable nature. Another factor resulting in the instability of SMN**Δ**7 is its reduced ability to oligomerize. SMN often exists in an oligomerized form, binding to itself through the YG box and multiple other proteins, including Gemin2. SMN without exon 7 is particularly unstable, attributed to the reduced ability to oligomerize, which adds to the creation of the degron motif and results in rapid degradation.[3]

The recognition of human disease modifier genes that act independently of SMN expressions such as *PLS3* (gene encodes for plastin 3, an actin-binding protein) and *NCALD* (gene encodes for neurocalcin-δ, a calcium-binding protein) signifies the importance of actin cytoskeletal dynamics, synaptic vesicle release, and endocytosis as consequences of SMN deficiency.[35–37] It is suggested that increased levels of plastin 3 reduce disease severity in female patients with SMA; in contrast, reduced NCALD expression may be protective as such may improve endocytic pathways at the NMJ.[1]

SMN can form a series of distinct protein complexes with different functions. As mentioned above, the functions of SMN are numerous, and it is impossible to introduce them all in this single section. The various functions of SMN throughout body cells and tissues may also explain why SMA patients experience muscular weakness atrophy and other defects in a wide range of organs, such as the heart, kidney, and liver.[3]

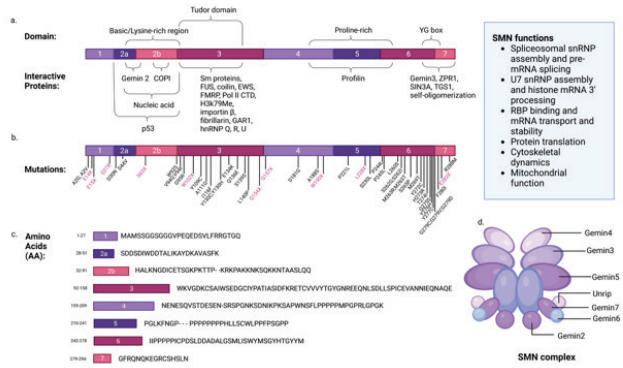

Figure 1: SMN protein structure. **a.** Full-length SMN protein has distinctive functional domains, which are labeled on top of the schematic; moving from the N-terminus to the C-terminus, there are a basic/lysine-rich domain, a Tudor domain, a proline-rich domain, and a YG box. These domains interact with various proteins, which are indicated below the schematic.[1,19] **b.** Location of SMA-associated intragenic missense and nonsense mutations within SMN1. The mutated residues in pink are nonsense mutations while the remaining black ones are missense mutations.[38] **c.** The amino acids, also known as residues corresponding to each exon are shown.[19] **d.** SMN protein complex. *(adapted from Genomic Variability in the Survival Motor Neuron Genes (SMN1 and SMN2): Implications for Spinal Muscular Atrophy Phenotype and Therapeutics Development by Butchbach, M. E. R. and Diverse role of Survival Motor Neuron Protein by Singh, R. N., Howell, M. D., Ottesen, E. W. & Singh, N. N.)*

SMN mutations and SMA:

The SMN protein plays an important role in pre-mRNA processing. SMN complex promotes the biogenesis of spliceosomal snRNPs,[39–41] where SMN forms oligomers and interacts with Gemin2 directly via its N-terminus and Tudor domain with spliceosomal (Sm) proteins.[42,43] SMA disease severity was also correlated with the oligomerization of SMN, which is acquired before forming SMN complexes.[15] As seen in the previous section, many possible mutations can occur in SMN, and many are related to SMA. Mutations in the Tudor domain disrupt the interaction between SMN and Smith core (Sm) proteins, often found in SMA patients.[16] It was also demonstrated that motor neurons from a Type 1 SMA patient showed reduced Cajal bodies (Cbs - a complex where SMN localizes) and defects in the recruitment of SMN and snRNP for spliceosomal maturation.[44] Moreover, almost half of the missense mutations among SMA patients are found in the SMN YG box domain.[16] The YG box is needed for SMN self-oligomerization; proteins with mutations found in this motif, as shown in patients with SMA Type 1, have severe impairment of this association.[45]

SMN1 and *SMN2* are constitutively transcriptionally active and are regulated by multiple factors (transcription factors, multi-kilobase promotors, epigenetic determinants, and long non-coding RNAs).[1] SMA patients had deletions or point mutations in both copies of *SMN1* (encoding survival motor neuron protein (SMN)). In *SMN2*, A C-to-T transition in exon 7 inactivates an exonic splicing enhancer (ESE) while simultaneously forming an exonic splicing silencer (ESS), resulting in the exon 7 skipping during transcription, leading to the creation of shortened, non-functional SMN.[1,7] Splicing of *SMN2* pre-mRNA emphasis some of the intronic and exonic *cis-elements* and trans-acting factors that influence exon 7

inclusion.[1] Exon inclusion needs the recruitment of the U1 small nuclear ribonucleoprotein (snRNP) to the 5' splice site of intron 7 and the U2 snRNP, together with its auxiliary component U2AF, to the 3' splice site of intron 6. Such process is modulated by several critical serine/arginine (SR)-rich proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) that identify exonic and intronic splicing enhancers and silencers (ESE, ESS, ISE and ISS).[14] (Figure 2)

The alternative splicing of *SMN2* exon 7 is a crucial event in SMA pathogenesis. Exon 7 splicing is regulated by different *cis-acting* (intronic and exonic splice enhancer and silencing motifs) and *trans-acting* (RNA-binding proteins) factors,[14,46] and the single base substitution c.859G>C in the exon 7 encoding sequence of *SMN2* generates a new exonic splicing enhancer element that leads to increased full-length transcripts generation and reduced disease severity.[47] The frequency of including exon 7 in *SMN* mRNA may vary with different cell types, as normal motor neurons produce lower levels of full-length *SMN* mRNA from *SMN2* compared with other cells in the spinal cord.[48] The inefficiency in retaining *SMN2* exon 7 within motor neurons highlights the significantly low levels of SMN within such cell types and emphasizes that they are especially vulnerable to SMA.[48] (Figure 2). However, SMN2 can still produce a tiny quantity (10%) of full-length mRNA, resulting in functional, full-length SMN protein.[1]

Individuals present with different degrees of heterozygosity, which suggests activation of functional copies may be an attractive therapeutic intervention. Among individuals without SMA, there is at least one functional copy of SMN1 and various numbers of *SMN2* copies; 10-15% of the unaffected populations have no *SMN2* copies.[49] The copy number of *SMN2* is inversely correlated with SMA severity. SMA patients carrying four or more copies of *SMN2* demonstrate a less severe phenotype.[50] A large cohort study indicates that 80% of patients with SMA type 1 had either one or two copies of *SMN2*; three copies of *SMN2* are observed in 78% of SMA type 2 patients; three to four copies of *SMN2* is found in 93% of patients with SMA type 3.[49] There are also exceptions, for instance, five copies of *SMN2* have been detected among unaffected family members with homozygous *SMN1* deletions,[1] reinforcing the idea that *SMN2* copy number is not the solar modifier of disease severity, modifier genes such as *PLS3* and *NCALD* also contributes to the various severity as discussed.

As mentioned earlier, approximately 5% of all SMA cases associated with 5q13 are due to intragenic mutations within an *SMN1*, not simply the loss of *SMN1*. A table in an existing review lists the currently known SMA-associated intragenic mutations in *SMN1*, including missense, nonsense, and frameshift mutations. There are also intragenic mutations within the intronic region of *SMN1*, which can lead to aberrant splicing of S*MN1* pre-mRNAs.[38]

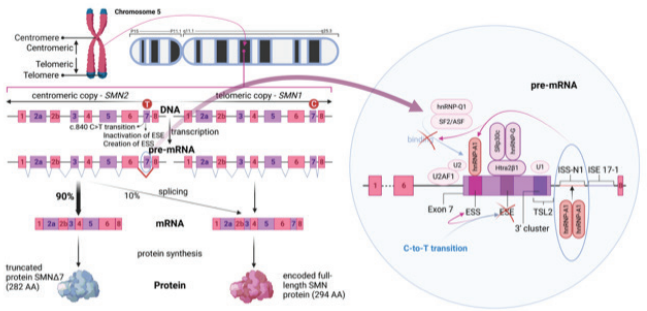

Figure 2: SMN gene transcription and splicing. The figure demonstrates and compares the splicing mechanism of *SMN1* and *SMN2*. The alternative splicing of *SMN2* that skips exon 7 is mainly because of the C-to-T transition that leads to the inactivation of ESE while simultaneously forming ESS. The binding of double hnRNP-A1 to ISS-N1 leads to the accumulation of hnRNP A1/A2 spreading alongside the SMN exon 7 and nearby introns, with consequent prevention of SF2/ASF, Tra2-b1, and other splicing factors binding, which are pivotal for exon 7 splicing; as well as the binding of hnRNP-Q1 that is essential for dendritic growth regulation. Due to the mis-splicing of exon 7, 90% of the protein produced by *SMN2* is truncated protein SMN**Δ**7, and only the rest of 10% are full-length proteins, whereas all proteins synthesized from *SMN1* are full-length SMN.[51]

Advancements in gene therapies:

- ***Antisense oligonucleotides - Modifying SMN2 splicing:***

A therapeutic strategy has been developed that alters the pathogenic splicing observed in patients with mutations. Oligonucleotide nusinersen appeared to be the first approved drug targeting SMA, which enhances the inclusion of exon 7 in *SMN2* mRNA transcripts as the problem with the *SMN2* gene is its alternative splicing that dis-includes exon 7 in mRNA.[18] Nusinersen binds to an intronic splice-silencing site in intron 7 of *SMN2* and suppresses other splice factors' binding. This increases the proportion of *SMN2*-mRNA that includes exon 7, resulting in a more functional, full-length SMN2 protein.[51] The development of nusinersen owns to the discovery of ISS-N1, a 15-nt long motif located right at the downstream of the 5'ss of exon 7, and deletion of ISS-N1 fully restores the inclusion of exon 7 in *SMN2* mRNA.[51] In 2006, when the report on the ISS-N1 target was published, Ionis Pharmaceuticals, in collaboration with the Krainer group, just happened to be working on developing an allele-specific oligonucleotide (ASO) based therapy for SMA. After a range of experiments, ISS-N1-targeting ASO appeared to be the most effective ASO and, therefore, became the first antisense target for SMA therapy to be validated independently. Then, after various clinical trials, nusinersen became the first FDA-approved drug for SMA and is named Spinraza.[52]

Spinraza has been approved for indications in adults and pediatric populations; it activates exon 7 splicing inclusion.[53,54] Nusinersen is an 18-mer 2'-O-(2-methoxyethyl) (MOE) phosphorothioate oligodeoxyribonucleotides (ODN), which is also known as ASO-10-27 5'-TCACTTTCATAATGCTGG-3'. This is a single-stranded DNA specifically designed for targeting an intron 7 site. ISS-N1 of the SMN2 comprises 15 nucleotides (CCAGCATTATGAAAG). CAGCAT is the first hnRNP A1 motif element, and TGAAAG is the second one. A strong ISS contains two juxtaposed weak motifs of hnRNP A1 to produce a strong splicing silencer. Two

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of the hnRNP A1/A2 binding to the intron 7 ISS and other sites result in the accumulation of hnRNP A1/A2 spreading alongside the SMN exon 7 and intron sequences. Such activity prevents the binding of SR proteins, SF2/ASF, Tra2-b1, and other splicing factors that are crucial for the recognition and then splicing activation of exon 7.[55] Nusinersen demolishes the binding of hnRNP A1/A2 molecules to the ISS-N1 of intron 7, facilitating exon 7 splicing activation and inclusion.[54] (Figure 3).

Two pathways are proposed for phosphorothioate antisense oligonucleotides (PS-ASOs) to enter, including the productive and non-productive pathways. Productive pathways guide PS-ASOs in accessing their targets; meanwhile, nonproductive pathways sequester PS-ASOs in saturable sinks. For PS-ASOs that reach the targets, the following uptake and internalization can be directed by cell-surface proteins, including receptors, through caveolin- or clathrin-dependent pathways or other endocytosis pathways. The endocytosis vesicle bud from the membrane and the scission of the plasma membrane are catalyzed by GTPase dynamin. Then, cargo-containing vesicles with PS-ASOs can fuse with early endosomes (EE) via transport along the cytoskeletal structures of actin or tubulin. Internalization of ASOs can be facilitated through the transportation from EE to late endosomes (LE) and lysosomes. PS-ASO accumulation is mainly in punctate structures within EE, LE, lysosomes (possibly equal to phosphorothioate bodies of cytoplasm), and Golgi-58K-related vesicles. This is where non-productive and productive uptake pathways may diverge, and most oligonucleotides are likely to be reserved in the non-productive pathway. In contrast, many PS-ASOs can be released from endosomal organelles and escape into the productive pathway. This requires entering the cellular nucleus and cytosol to act on target DNA. The specific mechanism of oligonucleotide uptake and cell trafficking is not fully discovered. Therefore, the above process is only proposed.[54]

Nusinersen improves respiratory and motor development, showing greater improvements among younger children.[56] Until 2020, there were more than 8000 SMA patients undergoing nusinersen therapy worldwide; noticeably, patients with late-onset SMA type 2 demonstrate dramatic motor improvement after the treatment;[8] in addition, no difference in treatment effect is shown between children with two or three SMN2 copies.[56] However, the price is high for such treatment, with $750,000 for the first year of treatment followed by $375,000 for each subsequent year; additionally, the drug's long-term effect is still unknown, while there are several restrictions on the use of nusinersen.[8] One restriction is that preclinical studies propose a discrete time window in neuromuscular development when it is the most effective period to increase SMN levels, suggesting earlier the treatment better the outcome with pre-symptomatic treatment. However, SMA newborn screening programs have not yet been widely performed.[8] Furthermore, the injection of nusinersen is performed via intrathecal administration, which has the risk of exacerbating respiratory compromise related to knee-to-chest flexion posture during the lumbar puncture procedure, headache, and CSF leakage. As the current dosing regimen requires treatment injection every 3-4 months, repeating such a procedure can pose challenges among some SMA patients with severe scoliosis. Therefore it is likely that image guidance is needed for those patients for the lumbar puncture to be performed.[57]

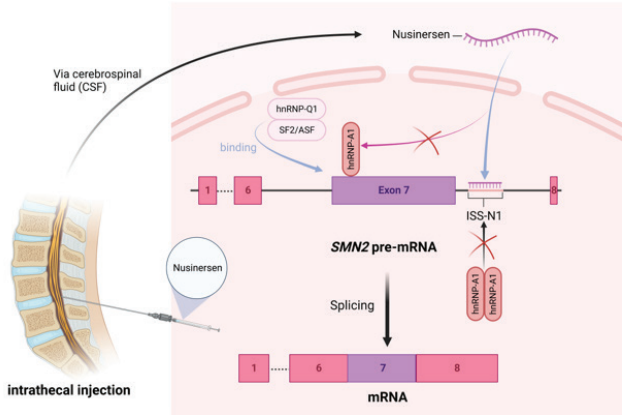

Figure 3: Nusinersen. The figure identifies the intrathecal injection of Nusinersen. The mechanism for how the drug gets into the nucleus is still unclear. However, we know it is first transferred via CSF. Nusinersen fixes *SMN2* splicing by preventing hnRNP-A1 from binding to ISS-N1, therefore allowing the binding of SR proteins and other splicing factors that are crucial for the splicing activation of exon 7, ultimately facilitating exon 7 splicing activation and inclusion.[61]

- ***Onasemnogene abeparvovec – Replacement of SMN1:***

In addition to fixing the splicing of *SMN2*, delivering the missing *SMN1* gene into patients' motor neurons is another alternative pathway for the development of gene therapy. Named Zolgensma, onasemnogene abeparvovec (AVXS-101) is a non-replicating, self-complementary, adeno-associated viral vector-9-based (scAAV9) gene therapy that delivers the relatively small size of *SMN1* gene to the motor neuron cells.[1,53,58,59] It is indicated for the usage of SMA patients under the age of 2, patients with type 1 SMA or have 3 or less than 3 copies of *SMN2*.[53] An adeno-associated virus (AAV) is a non-enveloped, single-stranded DNA virus which generally requires a helper virus for the completion of its life cycle and undergoes transduction in neurons. For scAAV9, vectors are packaged with double-stranded, self-complementary DNA, which manifests faster protein synthesis after transduction into the host cells. Gene transduction into the central nervous system (CNS) has remained a puzzle for other viral vectors due to the existence of a blood-brain barrier (BBB). However, this is not the case for AAV9, as it demonstrates the best transduction to the CNS compared to other AAV serotypes.[59] The coding region of AVEX-101 forms a double-stranded DNA template that delivers one or more copies of *SMN1* to the nucleus of target motor neurons. This is where the gene would reside as a non-integrating extra-chromosomal episome, enabling the continuous expression of the gene,[58] meaning encoding for functional full-length SMN protein to compensate for SMN deficiency of SMA patients. (Figure 4).

Onasemnogene abeparvovec is currently served as a frozen kit as it needs to be thawed for 4 hours at room temperature or 12 hours in the refrigerator before using it. Then, the contents of the medication vial are drawn up to the needed dose into a

syringe and delivered to the patient within 8 hours of drawing via intravenous (IV) injection. The manufacturer recommends to administer the drug only as a single-dose slow IV infusion through a peripheral venous catheter over an hour by using an infusion pump. Patients should receive systemic corticosteroids equivalent to 1 mg/kg/day of oral prednisolone, starting one day before onasemnogene abeparvovec administration and for 30 days in total. Then liver function should be checked; according to the assessing result, corticosteroids are then either tapered off or continued over the next 28 days and only tapered off after all assessment results are within normal limits.[59] Despite there are continuous promising results demonstrated via clinical trials, including significant improvement in motor and survival in a phase 1/2a trial, the FDA has recently placed a partial hold on intrathecal Zolgensma administration among SMA patients between 2 to 5 years old due to safety concerns.[8]

Onasemnogene abeparvovec comes with a price of $2.125 million for the one-time dose. Besides the high price tag, adverse effects are reported regarding abnormal liver function, as corticosteroids are needed to ensure and maintain the liver's normal function. There are also cardiac and hepatic toxicities associated with mortality at a dose of 2.4×10^{14}vg/kg or higher in animal models, even though this has not been observed in human patients.[58,59] In addition, due to the nature of the human immune system, patients may generate anti-AAV9 antibodies. Therefore, testing of both liver function and anti-AAV9 antibodies need to be performed.[59]

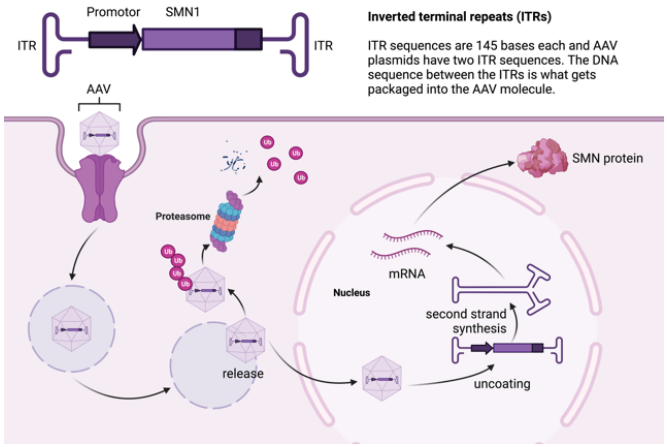

Figure 4: Onasemnogene abeparvovec. The figure demonstrates two possible outcomes for onasemnogene abeparvovec (AVXS-101). Packed in scAAV9, the *SMN1* gene enters the motor neuron cell via receptors, and after going through a series of events, early endosome, late endosome, and lysosome, the package is released. Then, the packaged gene is either mediated by Ubiquitin (Ub) for degradation via proteasome or enters the nucleus via nuclear pore entry. The gene package is uncoated within the nucleus, and mRNA is produced after second strand synthesis, producing fully functional SMN protein.[1] *(adapted from Spinal muscular atrophy by Mercuri et al. 2022)*

Conclusion

SMA is a devastating neurodegenerative disease, and we are lucky to have the boost of research and the number of therapies established in the 20th century. The two gene therapies discussed in this review complement each other. Nusinersen mainly aims for type 2 patients, both adults and children, while onasemnogene abeparvovec indicates for type 1 patients under age 2. Unlike other treatments, gene therapy targets the original genetic mutation, either delivering the *SMN1* gene that is deleted or fixing the splicing pattern of *SMN2*, both aimed to increase the production of needed SMN protein, which the level is significantly low in SMA patients.

It cannot be denied that the outcome of both two treatments is exceptional. However, this also comes with a significantly high price that is merely affordable for normal middle-class families. Additionally, there are various side effects of the two drugs, which can also compromise patients' health as some are closely linked with liver abnormal functioning. Furthermore, as the therapeutics are all relatively new, the long-term effects of the drug, especially nusinersen (need to be injected repeatedly), are still unknown. Given that there are continuously emerging new SMA patient cases with new mutations and phenotypes, the original phenotypes might also evolve due to the continuous nature of the disease; gene therapy development targeting all aspects of the disease is challenging.

Therefore, while being appreciative of having various therapeutics targeting such devastating disease, many still need to be done. The action mechanism of the oligonucleotide has yet to be elucidated, and more studies are required to unravel the complex molecular processes to improve the treatment. By knowing the pathway for nusinersen to reach the target, researchers may be able to improve the efficiency of the therapy or update the method of drug deliverance to reduce the pain endured by patients. Furthermore, the long-term side effects of both therapies shall be closely observed and tracked by patients of various ages and severities. This may assist with developing later-stage therapies to suppress potential harmful side effects. This is especially crucial for nusinersen as it requires multiple doses over a prolonged period, fortunately, there is a follow-up study called SHINE that is collecting long-term safety data regarding nusinersen.[56] As the two mentioned therapies only mainly focus on type 1 and 2 patients, gene therapy or altered existing gene therapy variations may be considered to be developed for the remaining SMA types patients. Additionally, given the devastating symptoms and mortality rate of SMA, especially for type 1 and 2, SMA newborn screening shall be promoted clinically and by governmental authorities. Health practitioners may also inform parents of the risks regarding SMA to provide them with opportunities to make informed decisions regarding newborn screening of their children. Ideally, SMA newborn screening shall be widely performed worldwide in the near future to achieve early diagnosis to perform pre-symptomatic treatment, which is shown to have the best treatment result. Finally, given the high cost of both therapies mentioned, hopefully, there will be public or governmental economic support or funds from charities and organizations to financially aid patients and their families.

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