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Genomic Variability in the Survival Motor Neuron Genes (SMN1 and SMN2): Implications for Spinal Muscular Atrophy Phenotype and Therapeutics Development

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Abstract: Spinal muscular atrophy (SMA) is a leading genetic cause of infant death worldwide that is characterized by loss of spinal motor neurons leading to muscle weakness and atrophy. SMA results from the loss of survival motor neuron 1 (SMN1) gene but retention of its paralog SMN2. The copy numbers of SMN1 and SMN2 are variable within the human population with SMN2 copy number inversely correlating with SMA severity. Current therapeutic options for SMA focus on increasing SMN2 expression and alternative splicing so as to increase the amount of SMN protein. Recent work has demonstrated that not all SMN2, or SMN1, genes are equivalent and there is a high degree of genomic heterogeneity with respect to the SMN genes. Because SMA is now an actionable disease with SMN2 being the primary target, it is imperative to have a comprehensive understanding of this genomic heterogeneity with respect to hybrid SMN1–SMN2 genes generated by gene conversion events as well as partial deletions of the SMN genes. This review will describe this genetic heterogeneity in SMA and its impact on disease phenotype as well as therapeutic efficacies.

Keywords: spinal muscular atrophy; copy number variation; SMN1; SMN2; modifier gene; precision medicine; therapeutics; gene conversion; hybrid gene

1. Introduction

Proximal spinal muscular atrophy (SMA) is a leading genetic cause of infant death worldwide. SMA is an early-onset disease that is characterized by the loss of α-motor neurons in the anterior horn of the spinal cord, i.e., lower motor neurons [1,2]. The incidence of SMA is 1 in 6000–10,000 live births [3–5]. SMA has a carrier frequency of 1:25–50 in most populations [5–8], although it is lower for some ethnic groups [9–12]. SMA results from the loss of α-motor neurons in the ventral spinal cord, leading to denervation and muscle weakness, with proximally innervated muscles being preferentially targeted. Following onset of symptoms, the denervation is progressive over time, as shown in SMA patients using motor unit number estimation (MUNE) and maximum compound muscle action potential amplitude (CMAP) analysis [13].

Based on age of onset and severity of the disease, SMA can be classified into five distinct phenotypes [14,15]. Type 0 SMA infants present with very severe hypotonia and require respiratory support from birth. These SMA infants usually do not survive beyond 6 months. Type I SMA (Online Inheritance in Man (OMIM) database #253300) patients have an age of onset before 6 months and they present with limb weakness due to hypotonia and the inability to sit independently. Abnormal respiratory patterns have been observed in type I SMA infants due to weakness in the intercostal muscles but not the diaphragm. These patients typically have shortened lifespans. Type II SMA (OMIM #253500) patients have an age of onset before 18 months. They are poor crawlers and weak sitters; most of
these patients can rarely stand and only with support. Their legs are generally weaker than their arms. These patients generally have a life expectancy into adulthood due to improvements in the standards of care. Type III SMA (OMIM #253400) patients have an age of onset greater than 18 months. These patients are able to walk with difficulty (waddling gait) and the legs are weaker than the arms. Type III SMA individuals generally live for a normal lifespan but some of them may require mobility support as the disease progress. Adult-onset (type IV) SMA (OMIM #271150) patients typically exhibit a slowly progressive limb weakness but the disease course is fairly benign.

While spinal motor neurons are the primary cell type affected in SMA, other types of cells aside from the motor neurons may also be affected by SMA [16,17]. For example, there are immature myoblasts present within muscles of SMA patients [18] and type I SMA patients tend to have smaller myotubes [19]. In addition to motor neuron degeneration, axonal degeneration of sensory neurons has also been observed in patients with severe SMA but not in milder forms of the disease [20,21]. Imaging and electrophysiology studies have shown degeneration of the thalamic nuclei within the cerebrum of type I SMA patients [22,23]. Type I SMA patients also manifest cardiac abnormalities including bradycardia and septal defects [24], while heart abnormalities are not observed in milder, types II and III SMA patients [25]. Distal digital necrosis of the blood vessels occurs in type I SMA patients [24,26]. Type I SMA patients also show an abnormal increase in pancreatic islet α cells, leading to abnormal glucose levels in some patients [27]. Other metabolic manifestations observed in SMA include abnormalities in fatty acid metabolism in SMA patients [28–30] and elevated serum leptin levels [31]. The multisystem nature of SMA tends to be more clinically prominent in more severe forms of the disease. It is unclear at present if these systemic clinical manifestations are a consequence of motor neuron dysfunction but it is important, nevertheless, to consider these systemic clinical manifestations in the care for and treatment of SMA patients.

2. Genetics and Biology of SMA

The SMA gene locus [32] maps to the 5q13 region of chromosome 5 (reviewed in [33]). Within this region there lies a 500 kilobase (kb) inverted segmental duplication that is unique to human lineages [34–36]. There are four genes within this segmental duplication region (Figure 1): SMN (survival motor neuron; [37]), NAIP (neuronal apoptosis inhibitor protein; [38]), GTF2H2A (general transcription factor IIH, p44; [39,40]) and SERF1A (small EDRK-rich factor 1A, H4F5A; [41]). These duplicated genes are either identical to their partner gene (SERF1B), differ by a small number of nucleotides but still produce functional genes (SMN2) or are pseudogenes (ΨGTF2H2B and ΨNAIPΔ5).
In more than 95% of cases, proximal SMA results from the loss of SMN1 but retention of SMN2, regardless of clinical severity [37]. In more severe types of SMA, other genes within this region of segmental duplication (such as NAIP, GTF2H2A and SERF1A) may also be lost, but not always [39,40,44–47]. Intragenic mutations in SMN1 [37,48] account for the remaining 5% of SMA cases (see Section 8) providing additional evidence to support SMN1 as the gene responsible for SMA. In SMA patient-derived cell lines as well as in patient tissues, SMN protein levels are inversely correlated with disease severity [49–56]. SMN is a ubiquitously expressed protein that is required for the assembly of diverse ribonucleoprotein (RNP) complexes, including small nuclear RNPs (snRNPs) required for spliceosome assembly and messenger RNPs (mRNPs) needed for transport of mRNAs along axons [48,57,58]. While it is well established that SMN is required for RNP assembly, it still remains to be resolved which types of RNP are affected in SMA and how motor neurons are preferentially affected.

The major functional difference between these two SMN genes is a C-to-T transition in exon 7 (SMN2 c.850C>T) [39,60]. While translationally silent, this position on exon 7 is in the middle of an exonic splicing enhancer (ESE) sequence important for the inclusion of exon 7 in SMN transcripts (Figure 1). This ESE is disrupted in SMN2, thereby causing the exclusion of exon 7 (SMNΔ7) from the majority (~90%) of SMN2-derived mRNAs. The resultant SMNΔ7 protein is unstable and is unable to associate with itself [61–63]. Some SMN2 mRNAs contain exon 7, depending on cell type, and can produce some full-length, functional SMN proteins. The SMNΔ7 protein is still partially functional since transgenic overexpression of SMNΔ7 in severe SMA mice partially ameliorates their phenotype [64].
3. SMN2 as a Disease Modifier for SMA

The number of SMN2 copies in the human genome varies between 0 and 8. Numerous studies have demonstrated an inverse relationship between SMN2 copy number and disease severity in SMA [13,37,49,50,54,65–94]. Patients with milder forms of SMA have higher SMN2 copy numbers than severe SMA patients. SMN2 copy number is being used as a prognostic tool to guide therapeutic strategies and care plans for SMA patients across the spectrum of phenotype severity [95,96]. The variability in SMN2 copy number within the SMA patient population and its relationship to disease severity makes it an ideal target for therapeutics development.

Animal models such as zebrafish, fruit flies and mice have a single Smn gene which is orthologous to SMN1 [97,98]. Loss of Smn in mice (mSmn) leads to embryonic lethality or cell type-specific death, if using conditional gene knockout approaches [99–102]. Transgenic insertion of SMN2 rescues the embryonic lethality observed in mSmn nullizygous mice [103–105]. While two copies of SMN2 rescues embryonic lethality in mSmn-deficient mice, these mice develop a very severe SMA phenotype and die within 8 days after birth [103,104]. Those mSmn-deficient mice with 3–4 SMN2 copies exhibit a milder SMA phenotype than the two-copy SMN2 SMA mice [104,105]. If the SMN2 copy number is high (i.e., eight), then the resultant mSmn-deficient mice exhibit no signs of SMA and are phenotypically normal [103]. Introduction of SMN2 onto a Smn nullizygous background in zebrafish also rescues embryonic lethality in this animal model [106,107]. SMN2 CNV, therefore, is a major modifier of disease severity in SMA.

4. Measurement of SMN1 and SMN2 CNV

Because SMN2 copy number influences disease severity in SMA, there is prognostic value in accurate measurement of SMN2 copy number from patients being evaluated for SMA. Molecular diagnosis of SMA—i.e., loss of SMN1—has historically been made using a polymerase chain reaction (PCR)-based assay followed by digestion of the PCR product with specific restriction endonucleases (PCR-RFLP) [37,75]. Different types of genotyping assays—including radioactive PCR [49,65], fluorescent PCR [79], quantitative (real-time) PCR (qPCR) [76–78], competitive PCR/primer extension [80], denaturing high-performance liquid chromatography [81], multiplex ligation-dependent probe amplification (MLPA) [82–86,108], quantitative capillary electrophoresis fragment analysis [87], short-amplicon melt profiling [88], fluorescent multiplex PCR/capillary electrophoresis [89,90] and universal fluorescent triprobe ligation [91]—have since been developed to quantify SMN2 copy number in DNA samples from SMA patients. An important limitation of these established PCR-based copy number assays is the requirement for a parallel-run calibration curve to assign a necessary breakpoint that identifies placement of an ordinal SMN2 value. Additionally, these techniques cannot easily distinguish unit differences in SMN1 or SMN2 when the copy number is greater than three [78,85,109]; however, recent refinements to MLPA assays can accurately measure four or five copies of SMN1 or SMN2 [110]. Digital PCR (dPCR) can accurately measure SMN1 and SMN2 over a large range of unit copies (0–6) without the need for an external calibration curve [70,93,111–115]. Next-generation sequencing approaches have recently been shown to be useful for SMA carrier detection [116–120] as well as for SMN2 copy number measurements [120].

5. SMN1 to SMN2 Gene Conversions and Partial Deletions

Gene conversion is one mechanism to account for increased SMN2 copy number in the absence of SMN1 in SMA [121]. In this scenario, the SMN1 gene actually contains part of SMN2, in particular within exon 7 [46,122–126]. Gene conversion events between SMN1 and SMN2 have been observed by multiple groups using different approaches [89,93,122–125,127–133]. Gene conversion events may account for the inverse relationship between SMN2 copy number and disease severity in SMA (Figure 2). Deletion of SMN1 on both chromosomes is hypothesized to cause the more severe type I SMA. Milder forms of SMA result from conversion of SMN1 to SMN2 on one or both chromosomes (reviewed in [121]). Gene conversion events lead to the
generation of hybrid SMN genes, i.e. some portions are SMN1 while other sections of the gene are SMN2.

**Figure 2.** Relationship between SMN1–SMN2 gene conversion and disease severity in SMA. Adapted from [134].

Most gene conversion events occur at the canonical c.840C>T nucleotide difference at exon 7 [59,60]. There are, however, at least 15 other paralogous structural variants (PSVs) between SMN1 and SMN2 (Figure 3; [60,108,120,135,136]). Gene conversion events at exon 8 (SMN2c.1155G>A) as well as those within intron 6 (SMN2c.835-44G>A) and intron 7 (SMN2c.888+100A>G and SMN2c.888+215A>G) have been observed in SMA, as well as in control populations [127,129,133]. Some of these PSVs, such as c.835-44G>A and c.888+100A>G, can affect exon 7 inclusion in spliced SMN mRNAs [137,138]. Some hybrid SMN2 genes produce greater amounts of SMN protein than expected and SMA patients harboring these hybrid genes have milder than expected clinical phenotypes. Further characterization of these gene conversion events will aid in the understanding of the functional consequence of these hybrid genes on SMN expression.

**Figure 3.** Paralogous sequence variants (PSVs) between SMN1 and SMN2. The canonical PSV at exon 7 that functionally distinguishes SMN1 from SMN2 is highlighted in green.

Even though the relationship between SMN2 CN and disease severity is strong, there are exceptions to this inverse relationship. Some SMA patients displaying a type II or III clinical presentation only have two copies of SMN2 as opposed to the predicted SMN2 copy number for milder forms of SMA [139–141]. SMN2 sequencing identified the presence of a rare single-nucleotide variant (SMN2 c.859G>C) in exon 7 [139–141]. This variant regulates the splicing of SMN2 pre-mRNAs so that a greater proportion of SMN2 transcripts contain exon 7.

While most cases of SMA result from a complete loss of SMN1, partial deletions in SMN1 have been identified in some samples from SMA patients—as well as in healthy controls—using PCR [142,143], microsatellite analysis [85,143], MLPA [83,85,108,110,127,142], whole-genome
sequencing [120,135], long-range PCR [129] and dPCR [93]. Additionally, partial deletions have been observed in SMN2. The most common partial deletion of SMN1 or SMN2 encompasses exons 7 and 8 (SMN1/2Δ78) and is roughly 6.3 kb in length [120,135]. This partial deletion spans 6.3 kb of DNA, although it is possible that the sizes of these SMN1/2Δ78 partial deletions may be variable. Whole-genome sequencing revealed the presence of a 1.9 kb partial deletion in a single sample that spans exon 7 and part of the flanking intronic regions [120]. In addition to SMN1/2Δ78, partial deletions within the SMN genes have been observed in other regions of the SMN genes, including losses of exons 5 and 6 (SMN1Δ56) [144], of exons 2a through 5 (SMN1Δ2a5) [145] and of exons 1 through 6 (SMNΔ16) [85,143]. We and others [93,142] have detected partial deletions of exon 8 in SMN1 (SMN1Δ8). Even though this exon is downstream of the protein-encoding region of SMN1 mRNA, it may affect SMN1 mRNA stability as well as post-transcriptional gene regulation.

The reason for specific breakpoints to be favored in partial deletion of SMN1 is currently not known. There are numerous intrachromosomal repeats within human chromosome 5 [34], including within the SMA gene locus. Alu repeat elements are primate-specific, 300-base segments of repetitive DNA found throughout the human genome [146]. Within the SMN genes, there are numerous Alu repeat elements of different types [147]. Some of these Alu repeat elements may cause partial deletions of SMN1 (or SMN2) by nonallelic homologous recombination [148]. The most common partial deletion (6.3 kb) of SMN1/2, SMN1/2Δ78, is flanked by Alu repeat elements [135]. Other partial deletions of SMN1—such as SMN1Δ56 [144], SMN1Δ2a5 [145] and SMN1Δ16 [85,143]—are also flanked by Alu repeat elements. Alu/Alu-mediated rearrangements, therefore, may account for these partial deletions within SMN1.

6. SMN2 Copy Number and Therapeutic Efficacy

The Food and Drug Administration has approved three therapeutic agents for SMA patients: nusinersen (Spinraza™, Ionis Pharmaceuticals (Carlsbad, CA, USA) and Biogen (Cambridge, MA, USA) [149,150]), onasemnogene abeparvovec (Zolgensma™, AveXis (Bannockburn, IL, USA) and Novartis (Basel, Switzerland) [151]) and risdiplam (Evrysdi™, Genentech (South San Francisco, CA, USA) and Roche (Basel, Switzerland) [152]). Nusinersen and risdiplam act by increasing exon 7 inclusion in SMN2 transcripts while onasemnogene abeparvovec replaces full-length SMN mRNA and protein. Since there is a strong relationship between SMN2 copy number and disease severity, accurate and rapid measurements of SMN2 copy number are often used to identify treatment options and regimens for children with SMA [96,153,154]. Accurate and rapid measurement of SMN2 CN is particularly essential to guide decisions around timing and treatment choice for SMA infants identified by newborn screening. The impact of SMN hybrid genes and partial deletions on the responsiveness of these therapeutics has not yet been determined, but these atypical SMN genes are predicted to affect therapeutic efficacy, especially for nusinersin and risdiplam, as they are dependent on endogenous SMN2.

7. Intragenic Mutations in SMN1 and SMA

As mentioned earlier, approximately 5% of all cases of SMA linked to 5q13 result from intragenic mutations within SMN1 as opposed to the loss of SMN1. Table 1 provides a list of the currently known SMA-associated intragenic mutations in SMN1. The SMA-associated intragenic mutations located within the exons can be either missense, nonsense or frameshift mutations. Additionally, there are intragenic mutations within the intronic regions of SMN1, which can cause aberrant splicing of SMN1 pre-mRNAs.
Table 1. Intragenic mutations in SMN1 that have been identified in SMA patients. The nucleotide position for the mutation starts relative to the initiation codon for DNA (NM_022874.2) or amino acid for protein (NP_075012.1).

| Type of Mutation | Mutation | Phenotype | Reference(s) |
|------------------|----------|-----------|--------------|
| Nonsense mutations | p.E14X | I | [127,155] |
|                   | p.Q15X | I, II, III | [127,144,155,156] |
|                   | p.Q27X | I | [157] |
|                   | p.S63X | I | [157] |
|                   | p.W102X | II, III | [158,159] |
|                   | p.Q154X | III | [160] |
|                   | p.Q157X | II | [160,161] |
|                   | p.W190X | I | [162] |
|                   | p.L228X | I | [127,155,163,164] |
|                   | p.Q282X | I | [165] |
|                   | p.R288X | II | [166] |
|                   | c.-7-9del | III | [127] |
|                   | c.19delG | I | [164] |
|                   | c.22dupA | I, II, III | [127,155,163,164,167,168] |
|                   | c.48_55dupGGATTCG | I | [87] |
|                   | c.56delT | II | [127,155,168] |
|                   | c.81+1dupG | II | [166,170] |
|                   | c.90_91insT | I, II | [144,162] |
|                   | c.98delT | I | [170] |
|                   | c.100delT | N/A | [171] |
|                   | c.109dupA | N/A | [158] |
|                   | c.124insT | I | [144] |
|                   | c.198_214del | N/A | [172] |
|                   | c.208_209ins4 | III | [144] |
|                   | c.241-242ins4 | III | [144] |
|                   | c.286delG | I | [166] |
|                   | c.312dupA | III | [162] |
|                   | c.314_317dup | III | [172] |
|                   | c.401_402delAG | I | [164] |
|                   | c.411delT | I | [162] |
|                   | c.429_435del | I | [171] |
|                   | c.430del4 | I, II, III | [173] |
|                   | c.431delC | I | [171] |
|                   | c.439_443del | I | [159,174] |
|                   | c.472del5 | I | [174] |
|                   | c.509_510delGT | N/A | [158] |
|                   | c.524delC | N/A | [175] |
|                   | c.542delGT | II | [176,177] |
|                   | c.549delC | N/A | [178] |
|                   | c.551_552insA | I | [164] |
|                   | c.585dupT | I | [172] |
|                   | c.591delA | II | [144] |
|                   | c.627_628ins65 | I | [161] |
|                   | c.722delC | I | [171] |
|                   | c.734_735insC | I | [160,175] |
|                   | c.735_736insA | N/A | [178] |
|                   | c.740dupC | N/A | [179] |
|                   | c.744delC | I | [127] |
|                   | c.770-780dup11 | I | [158,160,162,172,175] |
|                   | c.773insC | III | [179] |
|                   | c.811_814dupGGCT | I | [127] |
|                   | c.813ins/dup11 | I, II | [176,179,180] |
|                   | c.819_820insT | I | [157,181,182] |
Table 1. Cont.

| Type of Mutation | Mutation | Phenotype | Reference(s) |
|------------------|----------|-----------|--------------|
| Missense mutations | p.A2G | II, III | [127,155,158,160,176] |
|                  | p.A2V | III | [129,157,182] |
|                  | p.D30N | II | [156] |
|                  | p.D44V | III | [156] |
|                  | p.W92S | I | [157,181–183] |
|                  | p.V94F | I | [184] |
|                  | p.V94G | II | [172] |
|                  | p.G95R | III | [156,178] |
|                  | p.Y109C | III | [127] |
|                  | p.A111G | I | [156] |
|                  | p.I116F | I | [160,175,185] |
|                  | p.Y130C | III | [158,186] |
|                  | p.Y130H | III | [186] |
|                  | p.E134K | I, II | [127,156,187] |
|                  | p.Q136E | I | [185] |
|                  | p.S139S | N/A | [158,159] |
|                  | p.L141P | I | [157] |
|                  | p.D181G | N/A | [188] |
|                  | p.A188S | I | [170] |
|                  | p.P221L | I | [87] |
|                  | p.S230L | II, III | [127,165,168,171] |
|                  | p.P244L | III | [171] |
|                  | p.P245L | III | [189] |
|                  | p.L260S | II | [172] |
|                  | p.S262G | III | [156] |
|                  | p.S262I | III | [65,158,190] |
|                  | p.M263R | I | [172] |
|                  | p.M263T | II | [162] |
|                  | p.S266P | II | [158] |
|                  | p.M269T | III | [160] |
|                  | p.Y272C | I, II, III | [144,156,162,164,170,172,189,191] |
|                  | p.H273R | II | [158] |
|                  | p.T274I | II, III | [71,144,170,190] |
|                  | p.G275S | III | [172] |
|                  | p.Y276H | I | [157,192] |
|                  | p.Y277C | II, III | [127,129,168,182] |
|                  | p.G279C | II, III | [178,193] |
|                  | p.G279V | I | [194] |
|                  | p.G279D | N/A | [143] |
|                  | p.F280I | N/A | [178] |
|                  | p.R288M | I, II | [168,195,196] |
| Splice-site mutations | c.*3+3A>T | I | [184] |
|                  | c.628-140A>G | N/A | [161] |
|                  | c.834T>G | I | [162] |
|                  | c.835G>A | III | [197] |
|                  | c.835C>A | I | [157] |
|                  | c.835T>G | I | [127] |
|                  | c.867T>G | I | [179] |
|                  | c.868-11del7 | I | [37] |
|                  | c.888+3delAGAG | I | [37,172] |
|                  | c.922+3del4 | I | [97] |
|                  | c.922+6T>G | III | [144] |

SMN is a highly conserved protein containing 294 amino acids (in humans) with multiple domains (Figure 4). There are three regions within SMN1—located within exons 2A (K rich domain), 3 (tudor domain) and 6 (YG box)—that are highly conserved
evolutionarily [58]. Even though SMA mutations have been linked throughout SMN1, a greater proportion of SMA-associated intragenic point mutations are localized within these evolutionarily conserved regions (Figure 4). These conserved regions are required for self-oligomerization (YG box) as well as interactions with Sm proteins (tudor domain) and gemin-2 (K rich domain).

As mentioned previously, the loss of Smn in animal models is embryonically lethal and SMN2 rescues this embryonic lethality but results in an SMA phenotype whose severity depends on SMN2 copy number. Transgenic introduction of SMA intragenic missense mutations—specifically SMN1(A2G) [199], SMN1(A111G) [200], SMN1(D44V) [201], SMN1(T74I) [201] and SMN1(Q282A) [201]—into severe SMA mice (two copies of SMN2 on an mSmn nulligous background) improves the motor phenotype of severe SMA mice but does not completely ameliorate the SMA phenotype in these mice. These observations suggest that SMN genes harboring these point mutations are partially functional.

On their own, none of these intragenic missense SMN1 mutations can rescue the embryonic lethality of the loss of Smn in mice [199–201]. In zebrafish models for SMA where zSmn is knocked down with an antisense morpholino oligonucleotide [202], intragenic SMA missense mutations cannot rescue the motor axon deficits observed in these fish. These observations also support that these patient-derived point mutations are not fully functional. Interestingly, addition of both an N-terminal missense mutation and a C-terminal missense mutation can fully rescue the embryonic lethality caused by the loss of Smn in mice [203]. These intragenic complementation studies demonstrate that SMN must be oligomeric in order to function completely.

8. Silent Carriers and Compound Heterozygotes in SMA

Most parents of children with SMA are both carriers harboring one copy of SMN1. Interestingly, multiple independent studies have identified SMA carriers who have two copies of SMN1 [65,204,205]. In fact, one study identified 4.3% of the SMA carrier parents within their cohort as having two copies of SMN1 [205]. It is hypothesized that these so-
called silent carriers have two copies of SMN1 on one allele (i.e. the duplication allele) and zero copies of SMN1 on the other allele (i.e., the deletion allele; Figure 5). In other words, the two copies of SMN1 in a silent carrier have a cis allelic distribution (SMN1:2+0) as opposed to having two alleles each with a single copy of SMN1 (the trans allelic distribution; SMN1:1+1), which would be phenotypically normal.

![Figure 5. Allelic organizations of SMN1 copies within normal (SMN1:1+1), silent carrier (SMN1:2+0), carrier (SMN1:1+0) and SMA (SMN1:0+0) patients.](image)

Luo et al. [206] recently identified two small variants within SMN1—SMN1g.27134T>G (also known as SMN1c.*3+80T>G) and SMN1g.27706_27707delAT (also known as SMN1c.211_*212del)—that are tightly associated with silent carriers. The allelic frequencies of these variants were higher than expected in certain ethnic populations, such as the Ashkenazi Jewish and African American populations [206]. Another group identified an association between either of these variants and silent carriers in about 20% of their cohort, suggesting that there may be other variants in SMN1 associated with silent carriers [128]. Alternatively, some instances of silent SMA carriers may not be linked with any structural variant within SMN1.

It is essential to develop diagnostic tools which can detect silent SMA carriers, as standard assays cannot readily distinguish SMN1:2+0 cases from SMN1:1+1. To facilitate the development of genotyping assays that can identify silent carriers, the Genetic Testing Reference Materials Coordination Program has identified a set of reference samples containing structural variants linked with silent carriers [207]. Recently, targeted next-generation and whole-genome sequencing approaches have identified silent carriers using the SMN1g.27134T>G single nucleotide polymorphism [117,120,208]. A quantitative PCR assay has also been developed to identify silent carriers using this variant [209]. It should be noted that most of these approaches will most likely not identify all silent SMA carriers, as some instances may not be linked with any structural variant within SMN1.

Compound heterozygosity, wherein the SMA phenotype results from two different types of genetic event on each allele, can help explain discordant phenotypes within the families of SMA patients with differing phenotypic severities. One of the first cases of compound heterozygosity in SMA was identified by detailed analysis of haplotype markers [210]. In most cases, compound heterozygosity results from the deletion of one SMN1 allele and an intragenic mutation within the other allele [133,155,166,179,192]. There have been cases where two different types of intragenic mutations, i.e., a frameshift mutation and a missense mutation, in SMN1 occur on the same allele (cis) [87]. With new advances in molecular diagnostic tools, the genetics of complex cases of SMA can be resolved with relative ease.

9. Intrafamilial Variation in SMA Clinical Presentation

In some SMA families with more than one affected sibling, intrafamilial variability in clinical presentation has been observed [211,212]. In fact, recent analysis of a patient database curated by Cure SMA found 15.2% of SMA siblings to be discordant with respect to phenotypic severity [213]. These siblings have the same SMN2 copy number but have
differing clinical presentations [65,214–217]. This would suggest that there are additional genetic modifiers of SMA disease severity aside from SMN2. It is important to identify and characterize these novel modifiers for the development of new SMA biomarkers and targets for the development of therapeutic strategies for SMA [218].

Plastin-3 (PLS3) was one of the first SMN2-independent modifier genes identified for SMA. On examining the transcriptomes of SMA families with discordant siblings, PLS3 mRNA levels were found to be higher in females with milder SMA than those siblings with a more severe SMA clinical presentation [219–224]. The mechanism by which PLS3 expression is altered in these discordant families remains to be resolved. Reduction in the expression of PLS3-interacting proteins coronin 1C (CORO1C) [225] and calcineurin-like EF-hand protein 1 (CHP1) [226] improves neurite outgrowth in motor neurons from SMA model systems. A link between CORO1C and CHP1 levels and disease severity, however, has yet to be shown in SMA patients. In some families, female siblings with a more severe SMA phenotype had higher PLS3 mRNA levels than their more mildly affected siblings, suggesting that the protective effects of PLS3 on SMA patients may be age- and sex-dependent or incompletely penetrant [222]. In the Smn2B−/− mouse model for SMA, SMA mice on a C57bl/6J genetic background lived, on average, over 30% longer than those mice on a FVB/N background [227]. Interestingly, Pls3 levels were elevated in SMA mice on a C57bl/6J genetic background when compared against Smn2B−/− mice on a FVB/N genetic background. Ectopic overexpression of PLS3 improved the survival and phenotype of SMA mice in some cases, but not all [228–231].

By using an approach combining linkage analysis with transcriptomics, Neurocalcin-D (NCALD) was found to be a potential modifier gene within a cohort of discordant SMA cases [232]. Targeted sequencing of the NCALD region identified a 17 bp deletion within the promoter region of this gene in these discordant SMA patients, which led to reduced levels of NCALD mRNA and protein.

Whole-exome sequencing of a discordant SMA family, where one sibling presented a milder SMA phenotype than the other sibling, even though they both had two copies of SMN2, identified point mutations in the Tolloid-like 2 (TLL2) gene in the sibling with the milder phenotype [233]. TLL2 acts as an activator of myostatin (MSTN; growth differentiation factor 8), which inhibits skeletal muscle growth. The TLL2 point mutations identified in the milder sibling are predicted to reduce MSTN activation. MSTN inhibitors (such as SRK-015) have shown therapeutic benefit in mouse models for SMA and are currently in clinical trials with SMA patients [234].

Neuritin 1 (NRN1; cpg15) is an SMN-interacting protein present in neurons which promotes neurite outgrowth. Overexpression of NRN1 in various animal models for SMA showed increased motor neurite outgrowth [235]. Yener et al. [223] recently showed elevated NRN1 mRNA levels in a mildly affected sibling within a discordant family. The molecular basis for increased NRN1 expression in this case remains to be resolved.

10. Conclusions

SMA results from the loss of SMN1, but retention of its paralog SMN2 copy number can modulate disease severity in SMA. SMN2 copy number is becoming an inclusion criterion for many clinical trials for SMA. Additionally, SMN2 copy number can be used to help guide the type of care SMA patients will receive. Because of this relationship, SMN2 is a primary target for the development of therapeutics for SMA [236,237]. Numerous targets of SMN2 gene regulation—including promoter activation, increased inclusion of exon 7 and protein stabilization—are currently being developed to increase SMN2 expression. Given the genomic heterogeneity of SMN1 and SMN2, it will become very important to comprehensively assess the thes genes in individual SMA patients, as some of them may harbor SMN1–SMN2 hybrid genes or partial SMN1/2 deletions that may affect the therapeutic efficacy of both current and future therapeutics.

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