A Positive Modifier of Spinal Muscular Atrophy in the *SMN2* Gene

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Spinal muscular atrophy (SMA) is a common autosomal-recessive motor neuron disease caused by the homozygous loss of the *SMN1* gene. A nearly identical gene, *SMN2*, has been shown to decrease the severity of SMA in a dose-dependent manner. However *SMN2* is not the sole phenotypic modifier, because there are discrepant SMA cases in which the *SMN2* copy number does not explain the clinical phenotype. This report describes three unrelated SMA patients who possessed *SMN2* copy numbers that did not correlate with the observed mild clinical phenotypes. A single base substitution in *SMN2*, c.859G>C,, was identified in exon 7 in the patients' DNA. We now show that the change creates a new exonic splicing enhancer element and increases the amount of full-length transcripts, thus resulting in the less severe phenotypes. This demonstrates that the c.859G>C substitution is a positive modifier of the SMA phenotype and that not all *SMN2* genes are equivalent. We have shown not only that the SMA phenotype is modified by the number of *SMN2* genes but that *SMN2* sequence variations can also affect the disease severity.

The autosomal-recessive disorder proximal spinal muscular atrophy (SMA types I, II, and III [MIM 253300, 253550, and 253400]) is a severe neuromuscular disease characterized by degeneration of alpha motor neurons in the spinal cord, which results in progressive muscle weakness and paralysis. SMA is the second most common fatal autosomal-recessive disorder after cystic fibrosis, with an estimated prevalence of 1 in 10,000 live births.[1] Childhood SMA is subdivided into three clinical groups on the basis of age of onset and clinical course.[2,3] Type I SMA (Werdnig-Hoffmann) is characterized by severe, generalized muscle weakness and hypotonia at birth or within the first three months. Death from respiratory failure usually occurs within the first two years. Children with type II SMA are able to sit, although they cannot stand or walk unaided, and typically survive beyond four years. Type III SMA (Kugelberg-Welander) is a milder form, with onset during infancy or youth, and patients with type III SMA learn to walk unaided and have prolonged survival rates. Type III SMA is further subdivided into two groups: type IIIa (onset before 3 years of age) and type IIIb (onset at age $\geq$ 3 years). Patients presenting with the first symptoms of the disease at the age of 20–30 years are classified as type IV, or proximal adult type SMA.

The survival motor neuron (*SMN*) gene (Entrez Gene ID no. 6606) comprises nine exons and has been shown to be the primary SMA-determining gene.[4] Two almost identical *SMN* genes are present on 5q13: the telomeric or *SMN1* gene (MIM 600354), which is the SMA-determining gene, and the centromeric or *SMN2* gene (MIM 60127). The *SMN1* gene exon 7 is absent in about 95% of affected patients, although small, more subtle mutations have been identified in the remaining patients. Although mutations of the *SMN1* gene are observed in the majority of patients, no phenotype-genotype correlation was initially observed because *SMN1* exon 7 is absent in the majority of patients, independent of the type of SMA. This is due to the fact that routine diagnostic methods do not distinguish between a deletion of *SMN1* and a conversion event whereby *SMN1* is replaced by a copy of *SMN2*. Several studies have shown that the *SMN2* copy number modifies the severity of the disease.[5–9] The copy number varies from zero to three copies in the normal population, with approximately 10%–15% of this population having no *SMN2*. However, patients with the milder type II or III SMA have been shown to have more copies of *SMN2* than type I patients. The majority of patients with the severe type I form have one or two copies of *SMN2*; most patients with type II have three *SMN2* copies; and most patients with type III have three or four *SMN2* copies. Three unaffected family members of SMA patients with confirmed *SMN1* deletions were shown to have five copies of *SMN2*.[10] This inverse dose relationship between *SMN2* copy number and disease severity has also been supported by the SMA mouse model.[11,12] Mice lacking the endogenous mouse *Smn* gene but expressing two copies of the human *SMN2* gene develop severe SMA and die within one week of age. However, mice that express multiple copies of *SMN2* do not develop the disease.

The *SMN1* gene produces a full-length transcript, whereas the *SMN2* gene produces predominantly an alternatively spliced transcript (exon 7 deleted) encoding a protein (SMNΔ7) that does not oligomerize efficiently and is unstable.[13,14] The inclusion of exon 7 in *SMN1* transcripts and exclusion of this exon in *SMN2* transcripts is caused by a single nucleotide difference at +6 in *SMN1*

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exon 7. Although the C-to-T change in *SMN2* exon 7 does not change an amino acid, it does disrupt an exonic splicing enhancer (ESE) or creates an exon silencer element (ESS), which results in the majority of transcripts lacking exon 7.[14,15] Therefore, SMA arises because the *SMN2* gene cannot fully compensate for the lack of functional SMN when *SMN1* is mutated. However, a small amount of full-length transcript generated by *SMN2* accounts for a milder type II or III phenotype when the copy number of the *SMN2* gene is increased.

In addition to the *SMN2* copy number, other modifying factors influence the phenotypic variability of SMA. There are very rare reports of families in which markedly different degrees of disease severity are present in affected siblings with the same *SMN2* copy number. Differences in splicing factors may allow more full-length expression from the *SMN2* gene and account for some of the variability observed between discordant siblings.[16] It was also found that in some rare families with unaffected SMN1-deleted females, the expression of plastin 3 (*PLS3*, T-plastin or T-fimbrin [MIM 300131]) was higher than in their SMA-affected counterparts.[17] PLS3 was shown to be important for axonogenesis and therefore may act as a protective modifier. The identification of such gene modifiers not only provides important insight into pathogenesis of SMA but may also identify potential targets for therapy. Several drugs have been shown to increase *SMN2* expression in SMA patient-derived cell lines and are being studied in ongoing clinical trials.[18–20]

Although the existence of discordant phenotypes within a family and asymptomatic carriers with less than five copies of *SMN2* implies that additional genes are influencing the severity of SMA, another source of phenotypic variation may actually be due to the differential expression of the *SMN2* gene. It has generally been assumed that all *SMN2* genes are equivalent. We now present three cases that clearly indicate that a single base change in the *SMN2* gene increases the amount of full-length transcripts generated and positively modifies the disease phenotype.

The affected case 1 individual is a 42-year-old patient with type IIIb SMA who had normal early motor milestones but walked on her toes for as long as she could remember. She participated normally in activities while in grade school but recalled always being slow, and she often had to use a hand assist while going up and down steps. By high school she noticed slight weakness in her hips, but this was thought by her physician to be a normal variation. Her disease declared itself in her early 20s, when her husband witnessed her fall and became convinced that her leg strength was not normal. She sought neurologic consultation, and a DNA analysis demonstrated a homozygous absence of *SMN1* exon 7, confirming the diagnosis of SMA. She had noticed no definite change in her strength in the decade prior to her presentation to the SMA clinic. Her examination revealed very slight levoscoliosis but no other abnormalities. Her cranial nerves were normal. Peripheral strength testing, performed with the use of the Medical Research Council (MRC) scale for muscle strength, revealed grade 4 strength (normal being grade 5) in the shoulder abductors, with grade 4+ elbow flexors, grade 4 elbow extensors, and grade 5 or normal wrist extensors and flexors. In the legs, her hip flexors, hip abductors, and hip extensors were all grade 2 (not antigravity), with grade 4 knee flexors, grade 5 knee extensors, and grade 4 ankle dorsiflexors and evertors. Her gait was stable, with significant hyperlordosis. She was areflexic, with an otherwise normal neurologic examination.

Dosage testing was performed in order to determine the *SMN2* copy number[6] and revealed the absence of detectable *SMN2* exon 7. To our knowledge, the finding (0 *SMN1*/0 *SMN2* genotype) has never been reported in SMA and was assumed to result in fetal demise, given that there would be no protein produced from the *SMN2* gene to protect the motor neurons. Because this genotype was not consistent with the mild phenotype, we concluded that the absence of *SMN2* was most likely technical in nature. Sequence variations have been shown to inhibit primer binding during the PCR, and therefore a different set of primers was utilized for both the sequencing of *SMN2* and the determination of *SMN2* copy number. A homozygous substitution of guanine by cytosine at nucleotide 859 (c.859G>C) was found in *SMN2* exon 7 at the +25 position (Figure 1). This change replaces a glycine by an arginine at codon 287 (p.G287R). This variant was first detected on a normal chromosome of an unaffected carrier when it resulted in an abnormal single-strand conformation polymorphism (SSCP) pattern.[21] This change was also reported to affect the melting curve of a quantitative real-time PCR assay based on the use of a specific hybridization probe.[8] The nucleotide change was shown to be localized within the annealing region of the hybridized probe assay and therefore altered the melting curve.

Dosage testing with a new set of primers showed that the patient had two copies of *SMN2*, both positive for the c.859G>C. A missense substitution of arginine for the glycine is a significant change in amino acid structure and is predicted to alter the three-dimensional conformation of the SMN protein and may therefore result in a less functional protein.[22] However, Hua and Zhou found that a random C-terminal tag of five or more amino acids downstream of exon 6 was sufficient to promote cytoplasmic localization of the SMNΔ7.[23] Wolstercroft et al. demonstrated that the SMN exon 7 sequence is not specifically required; rather, this region functions as a nonspecific tail that facilitates proper localization.[24] The authors showed that a variety of native and synthetic C-terminal amino acid sequences can restore wild-type SMN subcellular localization patterns when fused to SMN exons 1–6. Given that the majority of type I patients have two copies of *SMN2* and this patient presented with a mild type IIIb phenotype, we hypothesized that the substitution not only causes assay problems but may potentially be a positive modifier. Although the C-to-T at +6 in *SMN2* exon 7 normally results in the production of *SMN2* transcripts

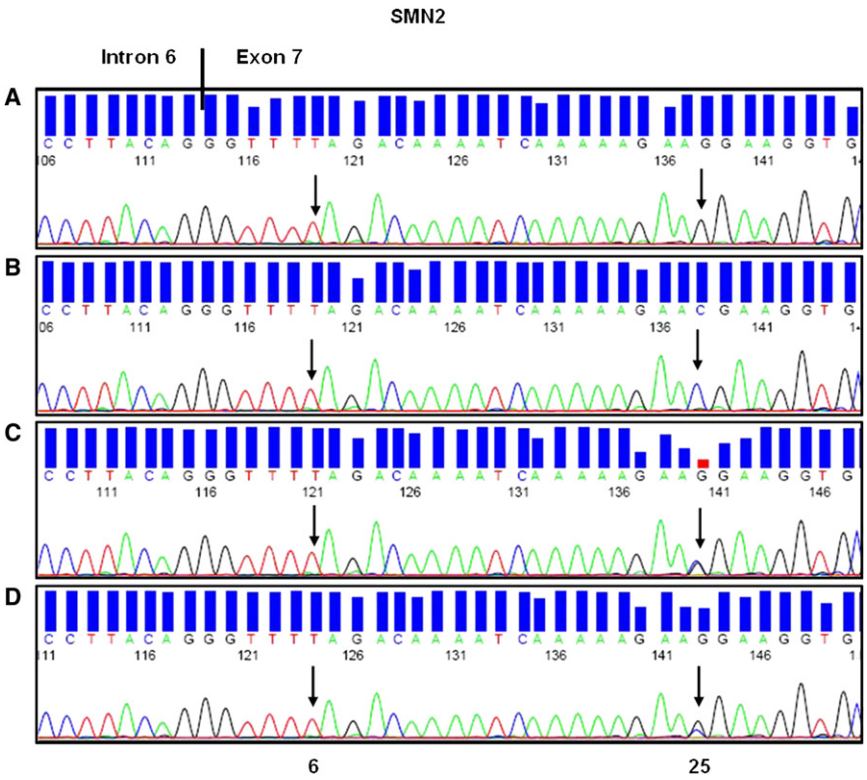

Figure 1. *SMN2* Sequence Analysis of SMA Cases

The inclusion of exon 7 in *SMN1* transcripts, as well as the exclusion of this exon in *SMN2* transcripts, is caused by the C-to-T change difference shown at nucleotide position 6 in *SMN2* exon 7. The substitution of guanine by cytosine at nucleotide 859 (c.859G>C) is shown at nucleotide position 25 in *SMN2* exon 7.

(A) Wild-type *SMN2* sequence chromatogram.

(B) Case 1, showing a homozygous substitution of guanine by cytosine at nucleotide 859 (c.859G>C) at nucleotide position 25.

(C) Case 2, showing a heterozygous substitution of guanine by cytosine at nucleotide 859 (c.859G>C) at nucleotide position 25. The patient has one wild-type *SMN2* copy.

(D) Case 3, also showing the substitution of guanine by cytosine at nucleotide 859 (c.859G>C) at nucleotide position 25. The patient has two wild-type *SMN2* copies.

predominantly lacking exon 7 (by disrupting an ESE or creating an ESS), the c.859G>C may act to partially restore normal exon splicing and produce more full-length *SMN2* transcript; e.g., by creating a new ESE or disrupting an ESS. ESEs are *cis*-acting elements that stimulate splicing and are often required for efficient intron removal, whereas ESSs inhibit splicing. Many ESEs act as binding sites for serine/arginine-rich proteins (SR proteins), a family of essential splicing factors, and promote splicing by recruiting spliceosomal components. Each SR protein recognizes a distinct functional sequence motif. We used ESEfinder 3.0, which includes four motif matrices of SR proteins (SF2/ASF, SC35, SRp40, and SRp55), to score wild-type and mutant sequences (Figure 2).[25,26] The c.859G>C specifically creates a new high-score SF2/ASF motif. As shown in Figure 2, there is now a new SF/ASF2 motif with a score of 3.50 created by the mutant sequence. The wild-type score is 0.60, which is below the threshold, usually set at 1.88. We concluded that a SF2/ASF recognition sequence created by the substitution may have allowed for increased full-length transcript and thus resulted in a milder phenotype than predicted from the *SMN2* copy number alone.

For further investigation of the functional significance of the new SF2/ASF motif, a transient transfection experiment was performed as previously described.[27] The c.859G>C was introduced by site-directed mutagenesis into an *SMN2* minigene plasmid. The variant, *SMN2*, and *SMN1* minigene plasmids were separately electroporated into human embryonic kidney (HEK) 293 cells, and RNA was isolated from the cells 2 days later. After reverse transcription, the variant, *SMN1*, and *SMN2* cDNAs were amplified for 25 cycles in the presence of α-^{32}P-dCTP, and the labeled PCR products were separated on a 6% polyacrylamide gel, followed by phosphorimage analysis. The exon 7 inclusion level as a percentage of the total spliced mRNA was determined. As shown in Figure 2, the c.859G>C substitution showed a significant increase of exon 7 inclusion, to 70%, in comparison to an inclusion level of 50% for the wild-type *SMN2* minigene ($n = 3$, $p \leq 0.01$). We note that although the precise extent of exon 7 inclusion differs between minigenes and intact genes, the greater inclusion of the exon in *SMN1* than in *SMN2* is preserved. In addition, changes in ratios of exon-included to exon-skipped isoforms are accurately reflected in semiquantitative, radioactive reverse transcriptase-PCR done in the linear range of amplification.[14,27]

There is another high-score SF2/ASF motif beginning at position 33 of exon 7 in both *SMN1* and *SMN2*, shown in Figure 2A.[28] However, this does not appear to be a functional SF2/ASF element, given that mutating it in an *SMN1* minigene had no effect on exon 7 inclusion and had little or no effect in the context of *SMN2* (M.L. Hastings, J.A. Calarco, and A.R.K, unpublished data). In addition, the motif may not be accessible to SF2/ASF, because two nucleotides (AT) at its 3′ end are part of an RNA secondary structure element (TSL2) that promotes exon 7 skipping. It remains possible that the new motif created by c.859G>C is recognized by SF2/ASF in conjunction with the preexisting motif at position 33 of the exon. Also of note, the new presumptive SF2/ASF motif beginning at position 25 overlaps the Tra2β motif beginning at position 22, and the nucleotide change would be expected

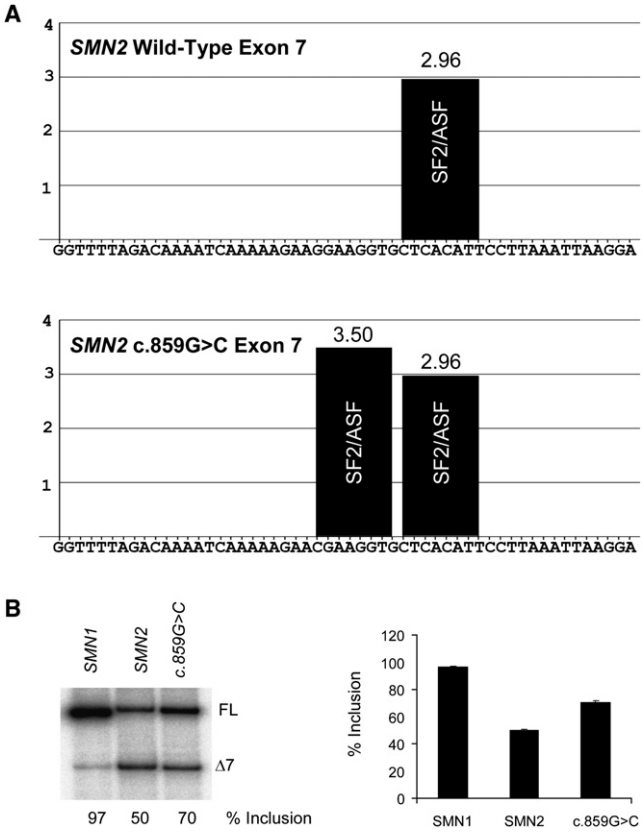

Figure 2. c.859G>C Creates a Putative SF2/ASF Motif
(A) ESEfinder3.0, which includes four motif matrices of SR proteins, SF2/ASF, SC35, SRp40, and SRp55, predicts a strong SF2/ASF motif CGAAGGT with a score of 3.50 that was created by c.859G>C. Note that the wild-type sequence GGAAGGT score is 0.60 and the standard threshold is usually set at 1.88.
(B) c.859G>C in the context of the *SMN2* minigene showed a significant increase of exon 7 inclusion during pre-mRNA splicing in comparison to the wild-type *SMN2* minigene.

to weaken or disrupt the binding of this SR-like protein, which recognizes a purine-rich motif.[16] Because of the overlap of the motifs, we do not expect that both proteins could bind simultaneously, so perhaps a loss of Tra2β binding is more than compensated for by a gain of SF2/ASF binding. We have not ruled out the possibility that a splicing activator other than SF2/ASF recognizes an element created or strengthened by the mutation or that the mutation disrupts the binding of a putative repressor to a greater extent than it disrupts the binding of Tra2β.

Because the c.859G>C substitution was initially identified in a type IIIb patient with only two *SMN2* copies, we decided to screen all of our discrepant cases, whereby the *SMN2* copy numbers predicted more severe disease presentations than observed. We had previously identified another seven of these cases, and we now found the same substitution in two additional unrelated patients in this group.

The second case was a 31-year-old woman with SMA type IIIa. Early motor milestones were normal (crawling by 9 months, standing with support by 15 months, walking independently by 18 months), but at 2–3 years of age, her gait became unsteady and she preferred to hold on to furniture while waking. She required assistance in ambulation from 5–6 yrs of age and ultimately was completely wheelchair dependent by age 12. She underwent scoliosis surgery at 14 years of age. At the time of her initial evaluation in the SMA clinic (at age 26), she had good respiratory function and could minimally assist with transfers, but she required full assistance for most activities of daily living, including bathroom activities and dressing. She could still sit unsupported, get from a sitting position to a lying position, and partially roll. She had significant hip and knee contractures. She also had significant arm weakness, with 3 grade shoulder abductors and elbow extensors and grade 4 wrist extensors and finger extensors. She could not lift her arms to shoulder level, had moderate elbow contractures, and was unable to fully extend her fingers. She had grade 0 hip and knee extensors. Deep tendon reflexes were absent. DNA testing initially indicated that she had a 0 *SMN1*/1 *SMN2* genotype, which was not consistent with her milder type IIIa presentation. With utilization of the redesigned *SMN2* primers, dosage testing revealed a second copy of *SMN2*, which was positive for the variant (Figure 1). Because we have observed the 0 *SMN1*/2 *SMN2* genotype in 90% of our type I patients, this finding in case 2 provided additional support for a positive modifying effect of the *SMN2* change.

The third case was a 29-year-old male type IIIb patient who had normal early motor milestones and development. At approximately age 12, he was noted to have "skinny legs" and began having trouble keeping up in gym class. He had difficulty running while in high school, but did not feel there was definitely something wrong until age 22, when he realized he was not gaining strength despite an intensive weight lifting program. He sought medical attention, and electrodiagnostic studies revealed chronic neurogenic changes. DNA testing revealed a homozygous deletion of SMN1 genes, and a diagnosis of SMA was made. His medical history was otherwise remarkable only for restless leg syndrome, slight depression, and mild attention deficit disorder. Examination at the time of presentation to the SMA clinic revealed a normal general examination and normal cranial nerves with no tongue fasciculations. Peripherally, he had very mild proximal upper extremity weakness, with grade 4 strength in the shoulder abductors and 4+ strength in the elbow flexors. All other upper extremity groups were normal (i.e., grade 5). In the legs, his hip flexors and knee extensors were grade 2, knee flexors were grade 4, and ankle dorsiflexors and ankle everters were grade 5. His gait was slow but stable with significant genu recurvatum. He was areflexic, and the rest of his neurologic examination was normal. DNA testing initially indicated that he had a 0 *SMN1*/2 *SMN2* genotype. Because we had not observed this genotype in our type IIIb population, the patient was retested with the new primer set, and he was shown to have a third

copy of *SMN2* with the c.859G>C. Although the majority of our type III patients have three *SMN2* copies, the milder type IIIb patients often have four *SMN2* copies. In our previous studies of type III patients; 76% had three, 24% had four, and 1% demonstrated five copies of the *SMN2* gene.[9]

Because this mutation was detected in our discrepant milder cases, we also screened 41 unselected type I patients, and we did not identify the mutation in any of the patients with a more severe phenotype. We also tested a discordant SMA family (SMA family 6), which included siblings who were positive for the homozygous *SMN1* deletion and had the same *SMN2* copy number but markedly different clinical manifestations.[29] The family had one daughter with the type II phenotype and a son with a mild type IIIb presentation, who was still ambulant at 20 years of age. Both siblings were negative for the variant. Lastly, we tested 139 unaffected individuals, who were all previously shown to have 2 *SMN1*/2 *SMN2* genotypes, and all of the 278 *SMN2* alleles were negative for the c.859G>C.

In this report, we have shown a positive modifying effect of the c.859G>C substitution in three milder cases of SMA, in which the *SMN2* copy number did not correlate with the clinical phenotype. This change had been previously reported and shown to cause methodological issues, which we also observed. Upon further examination, however, we now show that the change apparently creates a new ESE site, increasing the amount of full-length transcript and thus resulting in a milder phenotype than that predicted from the *SMN2* copy number alone. Our study suggests that the c.859G>C substitution is likely to be an important modifier accounting for some of the exception patients, given that it has been observed by other investigators and we have identified the substitution in three unrelated discrepant cases, the first of which had two copies of the variant. Furthermore, there appears to be a selective advantage in this change, given that we did not observe it in any of our type I or unaffected individuals. This is also true of the *SMN2* copy number; elevated SMN2 copies (>3) are rarely found in type I patients and unaffected individuals. There are additional genes outside the SMA region that modify the disease severity, as has been demonstrated by rare cases with discordant siblings with identical *SMN2* copy numbers. However, it should not be assumed that all *SMN2* genes are equivalent, and sequence changes found within the *SMN2* gene should be further investigated for potential positive or negative effects on *SMN2* transcription and posttranscriptional RNA processing. Importantly, the cases presented here also support a potential therapeutic benefit in increasing the *SMN2* gene expression in order to decrease the severity of the disorder.

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Web Resources

The URLs for data presented herein are as follows:

Align-GVGD, http://agvgd.iarc.fr/references.php
Entrez Gene, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene/
ESEfinder 3.0, http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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