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Complex *SMN* Hybrids Detected in a Cohort of 31 Patients With Spinal Muscular Atrophy

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Abstract

Background and Objectives

Spinal muscular atrophy (SMA) is a recessive neuromuscular disorder caused by the loss or presence of point pathogenic variants in the *SMN1* gene. The main positive modifier of the SMA phenotype is the number of copies of the *SMN2* gene, a paralog of *SMN1*, which only produces around 10%–15% of functional SMN protein. The *SMN2* copy number is inversely correlated with phenotype severity; however, discrepancies between the SMA type and the *SMN2* copy number have been reported. The presence of *SMN2-SMN1* hybrids has been proposed as a possible modifier of SMA disease.

Methods

We studied 31 patients with SMA, followed at a single center and molecularly diagnosed by Multiplex Ligand-Dependent Probe Amplification (MLPA), with a specific next-generation sequencing protocol to investigate their *SMN2* genes in depth. Hybrid characterization also included bioinformatics haplotype phasing and specific PCRs to resolve each *SMN2-SMN1* hybrid structure.

Results

We detected *SMN2-SMN1* hybrid genes in 45.2% of the patients (14/31), the highest rate reported to date. This represents a total of 25 hybrid alleles, with 9 different structures, of which only 4 are detectable by MLPA. Of particular interest were 2 patients who presented 4 *SMN2-SMN1* hybrid copies each and no pure *SMN2* copies, an event reported here for the first time. No clear trend between the presence of hybrids and a milder phenotype was observed, although 5 of the patients with hybrid copies showed a better-than-expected phenotype. The higher hybrid detection rate in our cohort may be due to both the methodology applied, which allows an in-depth characterization of the *SMN* genes and the ethnicity of the patients, mainly of African origin.

Discussion

Although hybrid genes have been proposed to be beneficial for patients with SMA, our work revealed great complexity and variability between hybrid structures; therefore, each hybrid structure should be studied independently to determine its contribution to the SMA phenotype. Large-scale studies are needed to gain a better understanding of the function and implications of *SMN2-SMN1* hybrid copies, improving genotype-phenotype correlations and prediction of the evolution of patients with SMA.

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Glossary

MLPA = Multiplex Ligand-Dependent Probe Amplification; **NGS** = next-generation sequencing; **PSV** = paralogous sequence variant; **SMA** = spinal muscular atrophy.

Introduction

Spinal muscular atrophy (SMA) is a neuromuscular disease defined by progressive atrophy of the lower motor neurons of the spinal cord.[1] It is the second most common recessive disease with a pan-ethnic incidence of 1 in 11,000 live births and a worldwide carrier frequency of around 1:54.[2,3] The severity of symptoms and age at onset are highly variable among patients with SMA; however, the clinical features that usually lead to diagnosis are motor difficulties with further muscle respiratory and bulbar weakness in the more severe phenotypes. Based on age at onset and acquired motor milestones before treatment, 4 SMA types have been established.[4,5] Type 1 is the postnatal most severe form, with onset before 6 months of life. Patients are unable to sit independently, being referred to as "nonsitters", and usually show severe complications such as respiratory failure and premature death. This SMA form is subdivided into 3 subtypes: type 1a, with perinatal onset; type 1b, when symptoms appear between the first weeks and 3 months of life; and type 1c, characterized by onset before 6 months of life and the achievement of cephalic control.[5,6] Type 2 is considered the intermediate form with onset between 6 and 18 months, and patients are called "sitters" because they can sit but never walk autonomously. Depending on their motor milestones, we differentiate 2 subgroups: type 2a patients are considered weak sitters because they will lose the ability to stay seated, while type 2b patients usually present a later debut (around 12 months of life or after), maintain the ability to sit, and can achieve bipedestation (strong sitters).[4,5] In type 3 disease, patients attain the ability to walk without support, being referred to as "walkers." Based on the age at onset, patients are subdivided into type 3a, characterized by onset between 18 months and 3 years of age and losing ambulation in childhood; and type 3b, in which patients debut after 3 years of age and retain the ability to walk into adolescence or even adulthood.[7] Finally, type 4 disease is an uncommon SMA form with adult presentation in which patients may show some gait disturbances and mild muscle weakness in the second or third decade of life.[8]

At the molecular level, SMA is an autosomal recessive disorder caused by the loss of both copies of the *SMN1* gene (Survival Motor Neuron 1, OMIM *600354), consisting of a homozygous deletion in approximately 95% of patients. The remaining cases are due to heterozygous deletion in one *SMN1* allele and a point pathogenic variant in the other *SMN1* copy. In addition, and more rarely, patients with biallelic point pathogenic variants have also been reported.[9] *SMN1* is located in the 5q13 locus, a highly complex and repetitive region that also includes *SMN2* (OMIM *601627), its nearly identical paralogous gene that resulted from a gene duplication event. Owing to this event, the region is highly homologous and dynamic leading to frequent genomic rearrangements, such as deletions, duplications, and gene conversions (*SMN2-SMN1* hybrids).[10,11] Therefore, *SMN1* and *SMN2* are highly homologous and differ in only 16 nucleotide changes, known as paralogous sequence variants (PSVs).[12,13] PSV c.840C>T, located in exon 7, represents the major functional difference between both genes. This change promotes alternative splicing in the majority of *SMN2* pre-mRNA transcripts, producing exon 7 skipping and leading to a nonfunctional and highly unstable protein (SMN-Δ7). As a consequence, each *SMN2* copy produces only 10–15% of functional full-length SMN protein.[13,14]

As patients with SMA lack *SMN1*, they are dependent on residual *SMN2* production of functional SMN protein, and therefore, the *SMN2* copy number has been described as the main modifier of disease severity. The higher the number of *SMN2* copies, the less severe the SMA phenotype because more functional SMN protein is being produced.[10,15] Thus, the *SMN2* copy number is used as a prognostic factor to predict phenotype and guide therapeutics. Typically, SMA type 1 patients have 2 copies of *SMN2*, type 2 patients present 3 copies, type 3 patients have 3 or 4, and type 4 patients have 4 or more *SMN2* genes.[15] However, this correlation is not absolute, and discordant patients have been described, including both better-than-expected and worse-than-expected phenotypes.[15,16]

To date, some *SMN2*-positive modifiers associated with milder SMA phenotype have been discovered, such as variants c.835-44A>G and c.859G>C (p.Gly287Arg), located in intron 6 and exon 7, respectively. Both changes significantly increase the inclusion of *SMN2* exon 7, subsequently producing more functional SMN protein.[17-19] Nevertheless, only a small number of patients have been described to carry these changes, including 51 patients with SMA carrying c.859G>C and 12 patients with c.835-44A>G.[20]

In addition, to positive modifier variants, *SMN2-SMN1* hybrid genes have also been proposed as putative-positive modifiers in patients with SMA because some studies have shown that hybrid genes seem to be associated with milder phenotypes.[21] The proportion of *SMN2-SMN1* hybrid genes among patients with SMA has been described to be around 5%.[22-24] Hybrids were originally defined as *SMN* copies with exon 7 from *SMN2* and exon 8 from *SMN1* because the majority of routine techniques for SMA diagnosis only assess these exons of the

SMN genes. However, sequencing analysis also allowed the description of other, more complex, hybrid structures.[21,22,25]

This work aims to characterize in detail the *SMN* genetic architecture of a cohort of 31 patients with SMA and to establish the correlation between the structure of the hybrid genes and the phenotype of the patients.

Methods

Study Participants

We studied a total of 31 patients with SMA followed and treated at the Raymond Poincaré Hospital (Garches, France). Clinical data of the patients were collected and curated by clinicians from the same center. Patients were classified into SMA types according to age at onset, clinical severity, and maximal achieved motor milestones before receiving any modifying therapies. Exceptionally, patient 25 was diagnosed and treated at a presymptomatic stage, due to a prior case in his family.

DNA samples from the Généthon DNA bank (Evry, France) were extracted from peripheral blood using standard methods. All molecular procedures were performed at the Hospital Vall d'Hebron.

Standard Protocol Approvals, Registrations, and Patient Consents

The study was approved by the Clinical Research Ethics Committee of Hospital Vall d'Hebron (Comité de Ética de Investigación con Medicamentos del Hospital Universitari Vall d'Hebron [PR(AG)229/2018]). All participants or their legal caregivers signed written informed consent.

Molecular *SMN1* and *SMN2* Characterization

All patients were genetically confirmed as SMA by the P021 Multiplex Ligand-Dependent Probe Amplification (MLPA) kit from MRC Holland,[9] which determines the *SMN1* and *SMN2* copy number, and the results were replicated in a second laboratory. Subsequently, a detailed molecular characterization was performed using a previously described next-generation sequencing (NGS) method,[12] which covers the entire *SMN1* and *SMN2* gene sequence. This high coverage approach not only allows copy number evaluation but also the identification and quantification of PSVs, hybrid structures, and known positive modifier variants (c.835-44A>G and c.859G>C).

Hybrid Characterization

To establish hybrid structures, we used the previously published list of PSVs,[12] except for PSV8 in position chr5: 69371448, originally described as a PSV.[13] This PSV is currently considered by our group as a common variant due to its higher-than-expected occurrence in 368 SMA patients with a homozygous *SMN1* deletion studied by NGS (Blasco-Pérez et al., in preparation). Accordingly, we defined an *SMN2-SMN1* hybrid gene as an *SMN* gene that presents a T at position c.840 (PSV12; if a C is present, the gene is considered *SMN1*) and at least 1 PSV typical of *SMN1*. The hybrid structure was determined based on the study of the PSVs and their AB ratio (allelic frequency of the PSVs in each sample) in the NGS data.[12]

To better characterize the hybrid genes, we applied computational techniques and specific PCRs to phase the PSVs of each *SMN* copy in the patients with hybrid genes. On the one hand, we applied the WhatsHap package[26] in the NGS data, indicating the number of *SMN2* copies in each case to avoid the program assuming a diploid genome. Specifically, using the function "whatshap_polyphase" of the package, we obtained the phased genotypes of each variant, and with "whatshap_stats," we identified the blocks of variants that phased together. This bioinformatic tool uses the variability of the samples to phase and define the alleles. However, the distance between variants should be less than the NGS read length (250 bp).

On the other hand, we used 2 gene-specific PCRs for each *SMN* gene to sequence all PSVs by standard Sanger sequencing; PCR 1 included PSV1 to PSV9 and PCR 2 from PSV9 to PSV16 (PCR 2 was previously described[20]). Specificity was achieved by the differential amplification of PSV9 (Chr5:69371499A/C), which has an adenine in *SMN1* and cytosine in *SMN2*. If a patient does not show heterogeneity for PSV9, the PCRs will not be informative because all the patient's alleles will amplify in the same reaction. Primer sequences and PCR conditions are provided in eTable 1.

Genotype-Phenotype Correlation

To define the expected phenotype based on the *SMN2* copy number, we used the following model: SMA type 1b has 2 copies of *SMN2*; type 1c, type 2a/b, and type 3a have 3 copies; and type 3a/b has 4 copies.[15] Patients whose phenotype could not be assigned according to this model were considered discordant.

Data Availability

Deidentified data are available on reasonable request.

Results

Clinical and Molecular Characterization of Patients

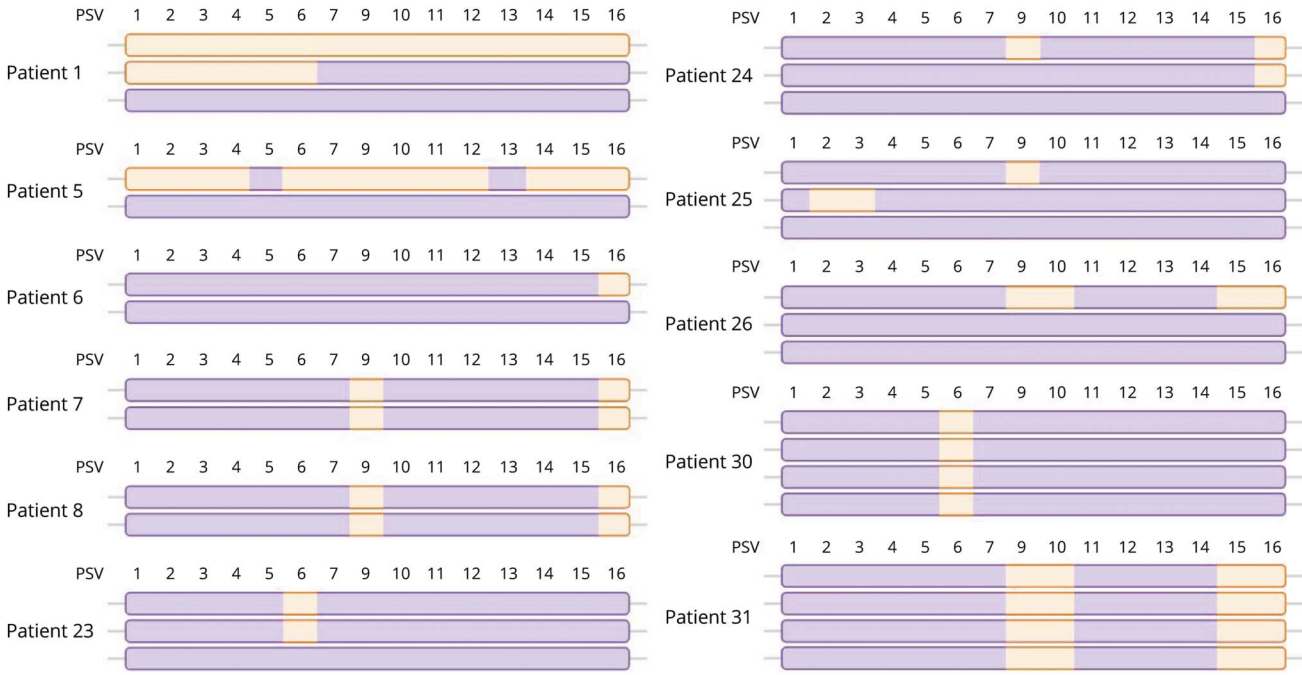
The majority of patients in this cohort were classified as SMA type 2a (29%, 9/31), type 3a (22.6%, 7/31), or type 1c (22.6%, 7/31). The remaining patients were SMA type 2b (16.1%, 5/31) and type 1b (6.5%, 2/31), and 1 proband was detected presymptomatically (3.2%) based on a family history of SMA. Our population included patients from different geographic origins and ethnicities, and most were of African (13/31) and European origin (10/31). Among them, there were 2 pairs of siblings (patients 7 and 8; patients 16 and 17)

Table Clinical and Molecular Data of the 31 Patients With SMA Included in the Study, Including General Characteristics of Patients, *SMN1/2* Genotypes, and SMA Phenotype

Patient no.	Ancestry	*SMN1* CN	*SMN2* exon 7 CN	Hybrid detection	Onset of manifestations (mo)	SMA type	Correlation
01	Caucasian	1[c]	2	Yes	24	3a	Better
02	European	0	2	No	10	1c	Better
03	South Asian	0	2	No	4	1c	Better
04	European	0	2	No	3	1b	Concordant
05	European	0	2	Yes	7	2a	Better
06	South Asian	0	2	Yes	3	1b	Concordant
07[a]	African	0	2	Yes	3	1c	Better
08[a]	African	0	2	Yes	7	2a	Better
09	Ashkenazi	0	3	No	12	2a	Concordant
10	European	0	3	No	11	1c	Concordant
11	African	0	3	No	8	2a	Concordant
12	Asian	0	3	No	12	2a	Concordant
13	African	0	3	No	9	1c	Concordant
14	Asian	0	3	No	9	1c	Concordant
15	African	0	3	No	36	2b	Concordant
16	European	0	3	No	16	2b	Concordant
17	European	0	3	No	4	2a	Concordant
18	European	0	3	No	14	3a	Better
19	European	0	3	No	8	2a	Concordant
20	African/European	0	3	No	10	3a	Better
21	European	0	3[d]	No	18	2b	Concordant
22	European	0	3[e]	No	31	3a	Concordant
23	African	0	3	Yes	9	1c	Concordant
24	African	0	3	Yes	14	2b	Concordant
25#	African-American (Caribbean)	0	3	Yes	0	PS	—
26[b]	African-American (Caribbean)	0	3	Yes	12	2a	Concordant
27	African	0	3	Yes	18	3a	Better
28	Latino	0	3	Yes	4	2a	Concordant
29	Latino/South Asian	0	3	Yes	17	3a	Better
30	African	0	4	Yes	24	2b	Worse
31	African	0	4	Yes	<36	3a	Concordant

Abbreviations: CN = copy number; PS = presymptomatic.
SMA type was assessed before treatment administration.
[a] These patients are siblings.
[b] These patients are cousins.
[c] The copy of *SMN1* presents the pathogenic variant c.332G>C p.(Ala111Gly).
[d] In addition, the patient carries a partial *SMN* gene comprising only exons 1 to 6 (*SMN1/2*Δ7-8).
[e] In addition, the patient presents another *SMN2* copy with an exon 7 deletion.

Figure 1 Schematic Representation of Solved Hybrid Alleles



Structure of the alleles in the 11 patients from group 1. Each bar represents 1 allele, *SMN1* is represented in orange, and *SMN2* in purple. Patient 1 presented 1 *SMN1* copy harboring the pathogenic variant c.332G>C. Note that PSV8 does not appear in the figure because it is no longer considered a PSV (see Methods for more details). PSV = paralogous sequence variant.

and a pair of cousins (patients 25 and 26), and the remaining patients with SMA were unrelated (see Table 1). Only 1 family of the cohort referred first-degree of consanguinity (patient 3).

All patients with SMA described in this study (20 males and 11 females) presented a homozygous deletion of *SMN1*, except for patient 1, who had a heterozygous *SMN1* deletion in one allele and the pathogenic variant c.332G>C p.(Ala111Gly) in the other.

Regarding the *SMN2* copy number, if we only consider the presence of *SMN2* exon 7, the majority of probands had 3 *SMN2* exon 7 copies (64.5%, 20/31), followed by 2 (25.8%, 8/31) and 4 copies (9.7%, 3/31). Moreover, patient 21 presented a partial *SMN* gene comprising only exons 1 to 6, and patient 22 showed an exon 7 deletion in one of his 4 *SMN2* copies (see Table 1 for details). Taking into account data from all *SMN2* exons and introns (MLPA and NGS), *SMN2-SMN1* hybrid genes were detected in almost half of our SMA cohort (45.2%, 14/31). These cases will be further discussed in the following section. Patients' characteristics are summarized in Table 1. We noticed that patients carrying hybrid genes were mostly of African origin. In the total cohort, 9 of the 13 patients of African origin presented *SMN2-SMN1* hybrid copies (69.2%), whereas only 5 of the 18 non-African patients had *SMN2-SMN1* hybrid genes (27.8%) (p = 0.0325 with the Fisher exact test).

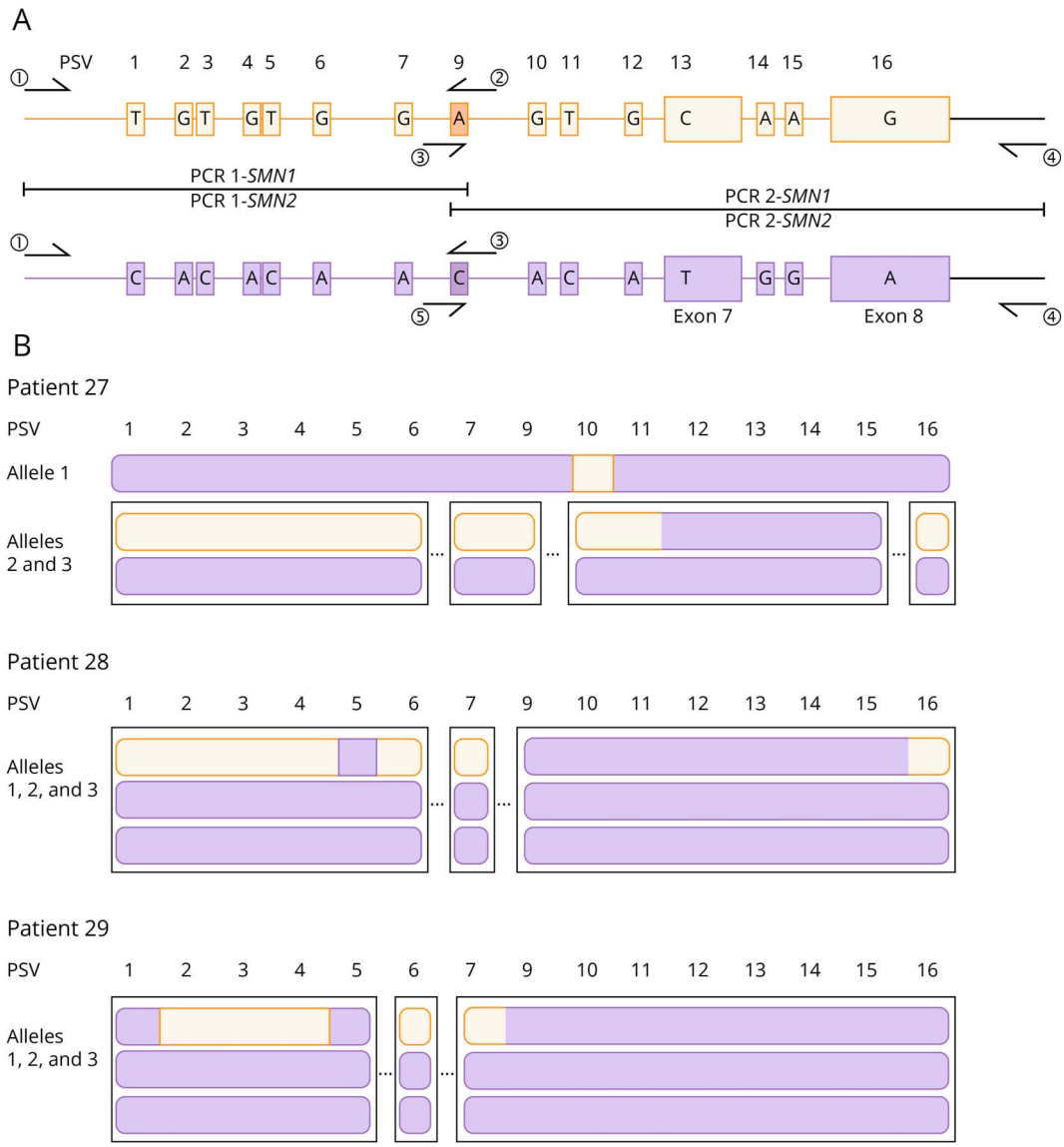
Hybrid Characterization

From the total of 14 probands with *SMN2-SMN1* hybrid genes, haplotype phasing and specific PCRs were able to fully resolve the structure of all the alleles in 11 patients, whereas the other 3 cases were partially solved (see below).

Regarding the former group (n = 11), 4 patients had 1 hybrid copy, 5 had 2, and the other 2 had 4 *SMN2-SMN1* hybrid copies, representing a total of 22 hybrid genes. Considering the total number of *SMN* alleles in this group (n = 31, 1 *SMN1,* 8 *SMN2*, and 22 *SMN2-SMN1* hybrids; Table 1), 71% were hybrids. In addition, patient 5 presented PSV12 (c.835-44A>G) as part of her hybrid copy, considered a positive modifier.[18] Detailed characterization of these solved *SMN2-SMN1* hybrid structures is shown in Figure 1.

On the other hand, not all the alleles of the latter group (n = 3) could be completely solved. In patient 27, MLPA results showed zero *SMN1,* 2 *SMN2*, and 1 hybrid copy, resulting in a total of 3 *SMN* copies. NGS data defined that the *SMN* copies of this patient had 11 PSVs from *SMN1,* 9 at an AB ratio of around 33%, and 2 at around 66% (see Figure 2B). This result indicated that the hybrid copies in this patient were more complex than previously identified by MLPA. Both specific PCRs of *SMN2* (using PSV9) only amplified 1 *SMN* copy, which included all PSVs from *SMN2* except for PSV10 from *SMN1*, thus resolving one of the hybrid alleles in this patient. The specific *SMN1* PCRs amplified the remaining 2 *SMN* copies, which we can state are also hybrid alleles because both

Figure 2 Schematic Representation of Partially Solved Hybrid Alleles

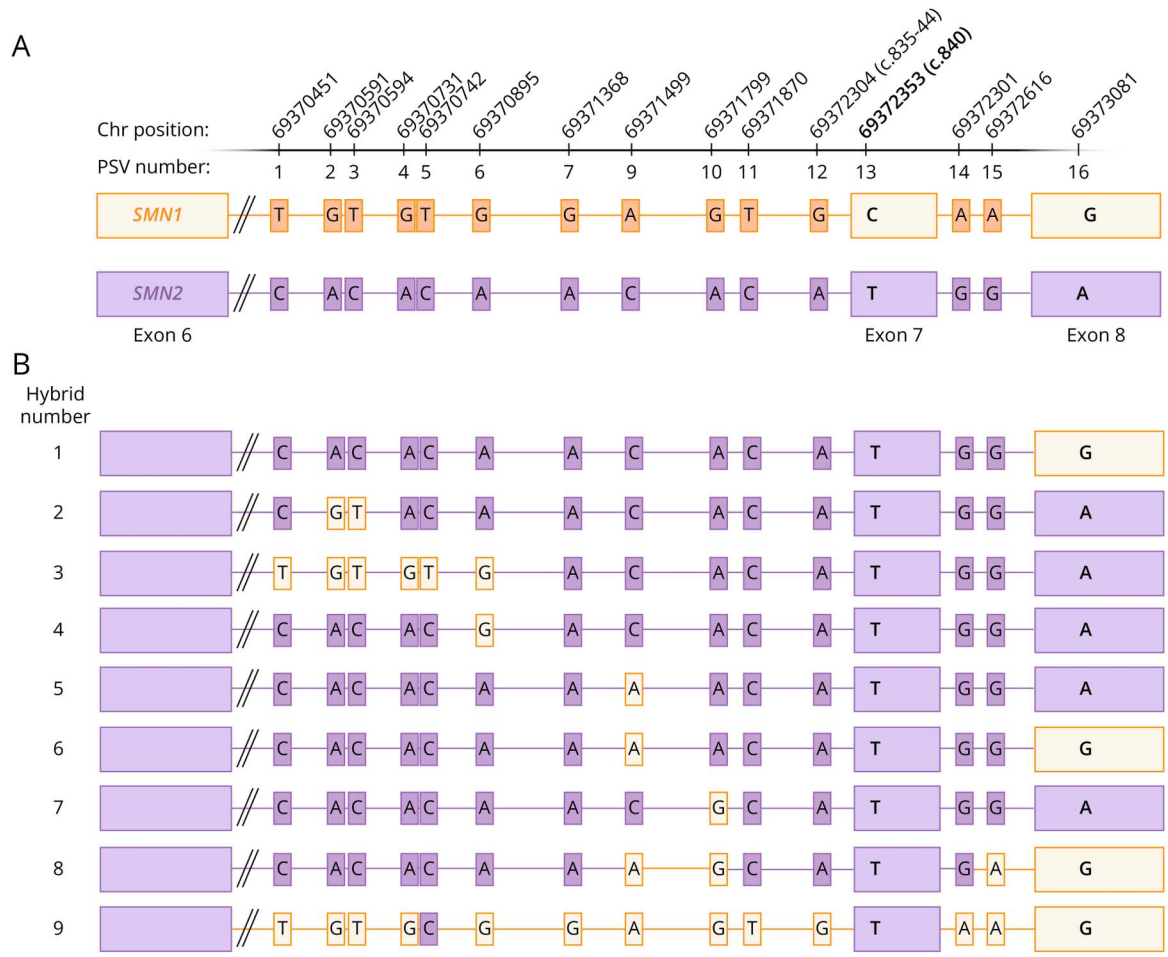


(A) Depiction of the specific PCRs designed for each *SMN* gene, one including PSV1 to PSV9 (PCR 1) and the other PSV9 to PSV16 (PCR 2). Primer numbers 1 and 4 are common for both *SMN* genes, while primer numbers 2 and 3 are specific for *SMN1* and primer numbers 5 and 6 for *SMN2*. For more details, see eTable 1. (B) Schematic representation of the different alleles of patients 27, 28, and 29, whose hybrid structures were partially solved with the methodology used. In patient 27, allele 1 was fully solved as a hybrid structure containing only PSV10 from *SMN1*. By haplotype phasing, in alleles 2 and 3, we were able to define 4 blocks of PSVs, albeit the connection between the blocks cannot be defined (dashed lines). In patients 28 and 29, haplotype phasing established 3 blocks of PSVs, but the connection between the blocks cannot be solved for any of these alleles. Depending on these connections, both patients could present from 1 to 3 hybrid copies. Note that PSV8 does not appear in the figure because it is no longer considered a PSV (see Methods for more details). PSV = paralogous sequence variant.

contained PSV9 from *SMN1*. However, we were unable to resolve the exact structure of each copy. Nevertheless, haplotype phasing defined 4 blocks of PSVs, although the connection between them could not be established. As in the previous case, patient 28 presented zero *SMN1*, 2 *SMN2*, and 1 hybrid copy by MLPA, while NGS results showed 7 PSVs from *SMN1* at an AB ratio of around 33%. As PSV9 of *SMN1* was not present in this patient, it was not possible to perform specific PCRs. However, haplotype phasing partially solved the allele structures by defining 3 blocks of PSVs (see Figure 2B). Based on these results, it is not possible to know how many of his *SMN* copies are hybrid alleles; nevertheless, it could range between 1 and 3. Finally, patient 29 presented zero *SMN1* and 3 *SMN2* copies by MLPA, and NGS results revealed 5 PSVs from *SMN1* at an AB ratio of around 33%, indicating the presence of at least 1 hybrid copy. As in the previous case, specific PCRs could not be applied and haplotype phasing defined 3 blocks of PSVs (see Figure 2B). Again, the genotype of this patient could include from 1 to 3 hybrid copies.

Globally, our approach has allowed the identification of 9 different hybrid structures from a total of 25 *SMN2-SMN1* hybrid alleles (see Figure 3). Only 4 of these *SMN2-SMN1* hybrid structures were detected by MLPA (44.4%, 4/9)

Figure 3 Paralogous Sequence Variants (PSVs) of the Different Hybrid Structures



(A) Scheme of the 15 PSVs, which differentiate *SMN* genes, named from 1 to 16, because PSV 8 has been excluded (for more details, see Methods). *SMN1* is colored orange, and *SMN2* is purple. (B) Details of the PSVs included in each *SMN2-SMN1* hybrid structure reported in our cohort. Hybrid structure numbers 1, 6, 8, and 9 are detectable by MLPA.

because they included PSV16 from *SMN1* located in exon 8. In our cohort, this represents that 14 of the 25 hybrid alleles were detectable by MLPA (56%). Moreover, we found 5 cases without any pure *SMN2* gene in which only *SMN2-SMN1* hybrid copies were present (patients 7, 8, 26, 30, and 31; Table 1). Specifically, patients 7 and 8, who were siblings, had 2 copies of hybrid structure number 6 (see Figure 3); patient 30 had 4 copies of hybrid structure number 4; and patient 31 presented 4 copies of hybrid structure number 8 (see Figure 3). Finally, patient 27 carried 3 different hybrid copies, which included hybrid structure number 7; however, it was not possible to characterize the remaining 2 alleles (see Figure 2A).

Genotype-Phenotype Correlation

We investigated the phenotypes of the patients taking into account *SMN2* exon 7 copies. From the whole cohort, 2 patients were excluded from the analysis: the presymptomatic case (patient 25) and patient 1, who carried a deletion and an *SMN1* sequence pathogenic variant (p.(Ala111Gly)). For the remaining patients (n = 29), more than half presented the expected phenotype when considering *SMN2* exon 7 copy number (65.5%, 19/29), 9 patients had a better-than-expected phenotype (31%, 9/29) and 1 a worse-than-expected phenotype (3.5%, 1/29).

Taking into account that some studies have proposed *SMN2-SMN1* hybrid genes as possible positive modifiers of SMA disease,[21] we also performed this analysis considering only patients carrying hybrid copies. Among these patients (n = 12, excluding patients 1 and 25, as explained above), 6 were concordant patients (50%, 6/12), 5 showed a better-than-expected phenotype (42%, 5/12), and 1 presented a worse-than-expected phenotype (8%, 1/12). No variants in the *SMN2* gene of these patients were found that could explain these phenotypic discrepancies.

Discussion

Using NGS and specific PCRs, we characterized the *SMN1* and *SMN2* genes in depth, including copy number, structure, and modifier variants in 31 patients with SMA followed at the same center (Hôpital Raymond Poincaré). The clinical

distribution of our patients was approximately one-third of each major SMA type (SMA types 1, 2, and 3); thus, initial conclusions can be drawn fairly equally for the different subgroups. It is worth noting that most of the type 1 cases studied were SMA type 1c (also called chronic SMA 1 or SMA 1 "long survivors") because most were recruited in a pretherapeutic era when only milder cases of SMA type 1 survived. Most of them carry 3 *SMN2* copies, which are associated with milder phenotypes than those carrying 2 *SMN2* copies.

Application of NGS to the *SMN* locus identified *SMN2-SMN1* hybrid genes in 14 of 31 patients with SMA, representing a frequency of 45.2% of hybrid genes in our cohort. This is much higher than previous literature reports, which ranged from 5 to 12%.[21-24,27-30] A high frequency (30%) of hybrid genes has also been reported in patients with SMA of Czech or Polish ancestry.[27]

The high frequency of *SMN2-SMN1* hybrid genes in our cohort could be partially explained by the NGS methodology applied in this study, which permitted a more thorough study of the *SMN1* and *SMN2* genes. Indeed, the NGS-based method allowed the identification of 5 hybrid structures, which would not have been detected by MLPA due to the intronic position of their *SMN1* PSVs (see Figure 3). This represents an increase in the hybrid detection rate of almost 80% in our cohort because 11 *SMN2-SMN1* hybrid alleles were detectable exclusively by NGS out of a total of 25 hybrid copies. In the majority of studies, hybrid detection was only based on the last 5 PSV positions[21,22,25] for which the most reported hybrid is the classic *SMN2*E1-7+*SMN1*E8.

Of interest, the hybrid frequency is higher in patients of African ancestry (69.2%) compared with non-African patients (27.8%) (p = 0.0325). In agreement, some studies have shown that African populations tend to have a higher number of *SMN* gene copies than other populations, which could make them prone to nonallelic homologous recombination events and, as a consequence, to a higher prevalence of *SMN* hybrid genes, including *SMN2-SMN1* hybrids and vice versa (*SMN* hybrids that present c.840C and at least 1 PSV from *SMN2*).[31-34] Therefore, the large proportion of patients of African origin in our cohort could be another factor contributing to the high frequency of hybrids detected.

Our approach based on NGS, haplotype phasing, and specific PCRs allowed the full characterization of *SMN2-SMN1* hybrid alleles in 11 patients. In the remaining 3 cases, the hybrid structures were not fully solved because of the lack of informativeness of their *SMN* sequences (Figure 2). To overcome this limitation, long-read sequencing could be applied.[35] However, due to the high cost of third-generation sequencing techniques and the fact that high-molecular weight DNA is necessary, this approach would be, at least at present, restricted to cases that cannot be solved by our methodology.

Regarding the specific structures of these hybrids, the 9 different *SMN2-SMN1* hybrids were very diverse, with some having only 1 PSV from *SMN1* to others containing 13 PSVs from *SMN1* (Figure 3). All hybrid genes presented PSV12 of *SMN2* (c.840T), which categorically defines *SMN2* because it determines its functionality. Of interest, hybrid structure number 9 included the positive modifier c.835-44A > G[18] and mainly comprised PSVs from *SMN1*, with the exception of PSV5 and PSV13 (Figure 3). Patient 5 carried this hybrid copy, and 1 pure *SMN2* copy presented SMA type 2a, which is classified as better-than-expected. The beneficial effect of this variant has been described in the context of a pure *SMN2* gene[12,16,18]; however, our case suggests that this modifier may maintain the positive effect when present in combination with other PSVs in a hybrid structure. Another interesting case is patient 1, carrying the *SMN1* p.Ala111Gly variant, originally reported in a patient with type 1b/2 and 2 *SMN2* copies.[36] By contrast, our patient presented a type 3a phenotype also with 2 *SMN2* copies but being one an *SMN2-SMN1* hybrid (Figure 1; Table 1). Studies in mice pointed toward a possible mild effect of this variant complementing the *SMN2* influence.[37,38] However, the milder phenotype in our patient could also be explained by a positive effect of the hybrid allele. It is also interesting to note that we described 5 cases wherein all *SMN2* genes were *SMN2-SMN1* hybrid copies. In particular, as a novel finding, patients 30 and 31 presented 4 identical hybrid copies each (Figure 1).

In previous literature, hybrid *SMN* genes have been proposed to be associated with a milder phenotype.[21] In our cohort, although the number of patients is limited, 5 of the patients with *SMN2-SMN1* hybrids showed a better-than-expected phenotype and only 1 presented a worse-than-expected phenotype (Table 1). Thus, no clear correlation could be determined between the presence of hybrids and a milder phenotype. A large-scale study should be performed to draw firm conclusions; however, it would be necessary to take into account the wide variability of hybrid structures identified in our patients. Therefore, each hybrid structure should be studied independently to perform genotype-phenotype correlations because their contribution to the patient's phenotype or the response to *SMN2* modifying therapies could be different. For instance, patient 23 presented 3 *SMN2* genes, including 2 hybrid copies with only PSV6 from *SMN1*. This patient is classified as type 1c SMA, which is considered concordant but is the worst phenotype associated with patients harboring 3 *SMN2* copies.[15] In the same line, patient 30 presented the same hybrid structure in all 4 copies and developed type 2b SMA, which should be considered a worse-than-expected phenotype. On the other hand, some patients do not present *SMN1* PSVs or known positive modifiers, although may have better-than-expected phenotypes. This is, for example, the case of patients 2 and 3 (Table 1) with type 1c and 2 *SMN2* copies. Thus, unknown modifier genes/mechanisms independent of *SMN2* sequence may be still involved in *SMN* modulation or have an *SMN* independent mode of action to influence the phenotype.

To sum up, we characterized in-depth the *SMN2-SMN1* hybrid copies detected in 14 patients with SMA from a cohort of 31 cases. Our work revealed the high complexity of the hybrid structures detected, even within the same patient, and suggests that some hybrid structures could be beneficial, but others could negatively affect the patient's phenotype. A large-scale application of our methodology would allow a better understanding of the function of the different *SMN2-SMN1* hybrid copies. This would, therefore, improve genotype-phenotype correlations in patients with SMA, better predict the evolution of cases detected by newborn screening, and raise the possibility of addressing tailored therapeutics.

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Disclosure

E.F. Tizzano has served as a consultant and has participated on advisory boards for Novartis Gene Therapies, Inc., Biogen, Biologix, Cytokinetics, Novartis, and Roche, and research funding from Biogen/Ionis and Roche. S. Quijano-Roy is a site principal investigator for clinical trials of Biogen and Novartis Gene Therapies, Inc.; has served as a consultant and has participated on advisory boards for Novartis Gene Therapies, Inc., Biogen, and Roche; and has received travel and speaker honoraria from Biogen, Novartis, and Roche. Go to Neurology.org/NG for full disclosures.

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Appendix *(continued)*

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Continued

Appendix *(continued)*

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