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Spinal Muscular Atrophy Genetic Testing Experience at an Academic Medical Center

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Approximately 94% of spinal muscular atrophy (SMA) patients lack both copies of *SMN1* exon 7. We report our SMA genetic testing experience (total 1281 cases), using SMA linkage analysis (32 families), SMA diagnostic testing by PCR-RFLP (restriction fragment length polymorphism) to detect the homozygous absence of *SMN1* exon 7 (and exon 8) (533 cases), and an assay to determine copy number of *SMN1* exon 7 (*SMN1* gene dosage analysis) (716 cases). *SMN1* gene dosage analysis is used for SMA carrier testing as well as for the confirmation of a heterozygous *SMN1* deletion in symptomatic individuals who do not lack both copies of *SMN1*. We conclude that comprehensive SMA testing, including *SMN1* deletion analysis, *SMN1* gene dosage analysis, and linkage analysis, offers the most complete evaluation of SMA patients and their families. *(J Mol Diag 2002, 4:53–58)*

Autosomal recessive spinal muscular atrophy (SMA) is a disorder that affects motor neurons in the anterior horn of the spinal cord.[1,2] SMA is the second most common lethal autosomal recessive disease after cystic fibrosis in the western hemisphere, and is clinically classified into three types based on the severity of symptoms. Type I SMA (Werdnig-Hoffmann disease) is the most severe form, type III SMA (Kugelberg-Wellander disease) is the mildest form, and type II SMA is an intermediate form. Localization of the SMA critical region made linkage analysis possible as the first genetic test for SMA.[3–5]

SMA of all three types is associated with deletions and point mutations in the Survival Motor Neuron 1 gene (*SMN1* or *SMNt*).[6] *SMN1* and its centromeric homologue, *SMN2* (or *SMNc*), lie within the telomeric and centromeric halves, respectively, of a large inverted repeat on chromosome 5q13.[6] *SMN1* and *SMN2* coding regions differ in only one base in exon 7.[7] This single-base C-to-T substitution in *SMN2* exon 7 affects the activity of an exonic splice enhancer and the splicing pattern of *SMN2* mRNA,[8] resulting in a lower level of full-length SMN protein from *SMN2* compared to *SMN1*.[9,10]

Taking advantage of the fact that approximately 94% of clinically typical SMA patients lack both copies of *SMN1* exon 7 (reviewed by Wirth[11]), a relatively sensitive, cost-effective polymerase chain reaction (PCR) method of testing for SMA was developed.[12] This test, herein referred to as the SMA diagnostic test, is used widely in the United States, including in our laboratory. It detects homozygous deletions of *SMN1* exon 7 (and exon 8) and can be used to confirm a diagnosis of SMA or for prenatal testing.[12] However, it has the following limitations: it cannot distinguish the types of SMA, since all three types of SMA typically show a homozygous absence of *SMN1* exon 7; it cannot distinguish SMA carriers from non-carriers, since both carriers and non-carriers are expected to have negative results; and a minority of SMA patients who have a small intragenic mutation in one *SMN1* allele instead of a homozygous *SMN1* deletion will have negative results.

To allow identification of carriers, a method to determine the copy number of *SMN1* was originally developed by McAndrew et al[13] and modified as a non-radioisotopic assay in our laboratory.[14] These methods are collectively referred to herein as *SMN1* gene dosage analysis. A one-copy *SMN1* result confirms carrier status in unaffected individuals or supports a diagnosis of SMA in an individual who has typical symptoms of SMA and does not lack both copies of *SMN1* exon 7. In this article, we describe our SMA genetic testing experience, including SMA diagnostic testing, *SMN1* gene dosage analysis, and SMA linkage analysis.

Materials and Methods

Subject Samples, DNA Isolation, SMA Diagnostic Test, and Linkage Analysis

Testing was performed in the Molecular Pathology Laboratory of the University of Pennsylvania during the period from April 1992 to June 2000. The subject population referred for SMA genetic testing was widely distributed in the U.S. with the majority being from the Northeast region.

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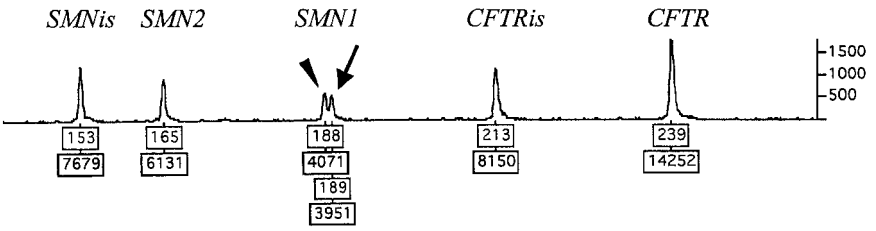


Figure 1. An example of an ABI 373a electropherogram showing a one-base-larger *SMN1* peak (actual size 188 bp, shown as 189 bp, **arrow**) in addition to the expected *SMN1* peak (actual size 187 bp, shown as 188 bp, **arrowhead**), each corresponding to one *SMN1* copy by gene dosage analysis. Each peak corresponds to PCR products of an *SMN* internal standard (*SMNis*), *SMN2* genomic sequence (*SMN2*), *SMN1* genomic sequence (*SMN1*, with a doublet), a *CFTR* internal standard (*CFTRis*), and *CFTR* genomic sequence (*CFTR*), from left to right, respectively. Numbers under each peak represent approximate product size in bp (**top**) and fluorescence intensity measured as an area under curve (**bottom**).

Rare samples were from outside the U.S., including Canada, Mexico, and the Middle East. Subjects referred for SMA genetic testing included patients clinically suspected to be affected by SMA, family members of SMA patients, individuals without a family history of SMA (typically those who marry someone with a family history of SMA), and fetuses of parents who had affected children or a family history of SMA. In addition, we tested five fetuses and two neonates with arthrogryposis. Patients were categorized into SMA type I, type II, and type III based on the clinical diagnosis provided by the ordering clinicians or genetic counselors according to standard criteria.[1] If a patient had clinical features that overlapped two types of SMA, then type I-II or type II-III was assigned to such a patient by the referring clinician. Adult-onset SMA, so-called type IV, is not a well-established category and many regard it as a variant of type III. Therefore, we included adult-onset SMA in the type III category.

Genomic DNA was extracted from peripheral blood, prenatal samples [chorionic villous sampling (CVS) tissues and amniocytes, before and/or after culture], or fresh frozen tissue using the Puregene DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, MN) and from formalin-fixed, paraffin-embedded tissue sections using the QIAmp Tissue Kit (Qiagen, Inc., Valencia, CA). The SMA diagnostic test for homozygous deletions of *SMN1* exon 7 (and exon 8 until March 1997) was performed as described previously.[12] Prenatal samples were analyzed in duplicate since July 1998. SMA linkage analysis was performed as described previously.[3,4,15]

SMN1 Gene Dosage Analysis and Genetic Risk Assessment

SMN1 gene dosage analysis was originally developed by McAndrew et al[13] and modified as a non-radioisotopic assay in our laboratory.[14] All samples were analyzed in duplicate. Briefly, exon 7 of *SMN1* and *SMN2* was coamplified with exon 4 of the cystic fibrosis transmembrane regulator gene (*CFTR*), an *SMN1* internal standard, and a *CFTR* internal standard using fluorescently labeled primers. PCR products were then digested with *Dra*I, which digests only *SMN2* exon 7 PCR products, followed by analysis on the ABI 373a Sequencer (Applied Biosystems Inc., Foster City, CA) or the ABI 310 Genetic Analyzer (Applied Biosystems Inc.).[16]

Genetic risk assessment was performed routinely as an integral part of SMA genetic testing in our laboratory. Remaining risk of being a carrier was analyzed and reported in cases with two or more copies of *SMN1* by gene dosage analysis, using estimates of various *SMN1* normal and disease allele frequencies (Ogino S, Leonard DGB, Rennert H, Ewens WJ, Wilson RB, submitted).

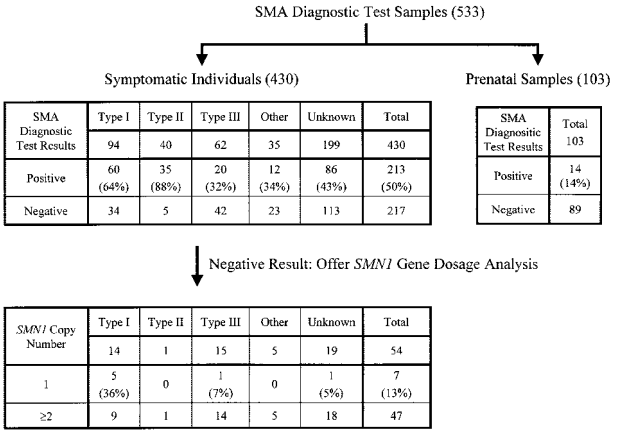
Sequence Analysis for SMN1 PCR Products

The PCR products from five samples showing the expected *SMN1* peak and a one-base-larger peak on ABI 310 or 373a electropherograms by *SMN1* gene dosage analysis (Figure 1) were sequenced. First, PCR was performed by the same method used for the SMA diagnostic test. Then, PCR products were purified from the *SMN1* bands on agarose gels and sequenced with the *SMN* forward primer, R111[6] and the *SMN* reverse primer, X-7 Dra[12] using the BigDye Terminator Reaction Kit (Applied Biosystems Inc.) and the ABI 310 Genetic Analyzer (Applied Biosystems Inc.).

Results

SMA Diagnostic Test: Symptomatic and Prenatal Testing

A total of 533 SMA diagnostic tests were performed: 430 for symptomatic individuals with a suspected diagnosis of SMA and 103 for prenatal diagnosis (Figure 2). Of the 430 tests on symptomatic individuals, 213 were positive (a homozygous deletion of exon 7). Of the 213 positive patients, 16 were positive only for an exon 7 deletion but negative for exon 8, whereas 110 were positive for deletions of both exon 7 and exon 8. The other 87 patients were tested only for an exon 7 deletion with positive results. There were no symptomatic individuals with an exon 8 deletion without an exon 7 deletion. When the subjects were divided into SMA types, type II patients had the highest percentage of positive results (88%; 35 of 40), followed by type I (64%; 60 of 94), type I-II (44%; 7 of 16), type II-III (33%; 4 of 12) and type III (32%; 20 of 62). SMA type was unknown in 199 cases (46%). Reasons for unknown SMA type included a lack of description by the



SMA Diagnostic Test Results	Type I	Type II	Type III	Other	Unknown	Total
	94	40	62	35	199	430
Positive	60 (64%)	35 (88%)	20 (32%)	12 (34%)	86 (43%)	213 (50%)
Negative	34	5	42	23	113	217

SMA Diagnositc Test Results	Total 103
Positive	14 (14%)
Negative	89

SMN1 Copy Number	Type I	Type II	Type III	Other	Unknown	Total
	14	1	15	5	19	54
1	5 (36%)	0	1 (7%)	0	1 (5%)	7 (13%)
≥2	9	1	14	5	18	47

Figure 2. A flow chart of the results of SMA diagnostic tests and *SMN1* gene dosage analyses for symptomatic and prenatal cases.

health care provider and atypical symptoms precluding classification by clinicians.

The 103 samples for prenatal SMA diagnostic testing consisted of 31 CVS samples without prior culture, 10 cultured cell samples derived from CVS, 42 cultured cell samples derived from amniotic fluid, 12 amniotic fluid samples without prior culture, 3 cultured cell samples not specified as CVS or amniotic fluid derived, and 5 other prenatal samples. In all, 14 of 103 prenatal cases were positive and 89 were negative for a homozygous *SMN1* deletion by the SMA diagnostic test (Figure 2). To exclude significant maternal DNA contamination, polymorphic short tandem repeat (STR) analysis was performed (data not shown).

SMN1 Gene Dosage Analysis

The primary purpose of *SMN1* gene dosage analysis was SMA carrier testing, which was performed on a total of 663 asymptomatic individuals (Table 1). Of these individuals, 191 individuals had no family history of SMA and 472 had a family history of suspected or definitive chromosome 5q13-linked SMA. Among individuals without a family history, the percentage of individuals with a one-copy result was 1.6%, which is comparable to the ~2% carrier frequency in the general population. Among parents whose affected children lacked both copies of *SMN1* (0-copy *SMN1*) (n = 118), 110 had one copy of *SMN1*, confirming a carrier status. Of the remaining eight parents who had two copies of *SMN1*, five had two copies of *SMN1* on one chromosome 5 and a deletion mutation of *SMN1* on the other chromosome 5, also referred to as the 2 + 0 genotype.[14] Two other parents with two copies of *SMN1* had one copy of *SMN1* on each chromosome 5 (1 + 1 genotype) and had passed a *de novo* deletion mutation associated with a crossover to their affected children. The 2 + 0 or 1 + 1 genotype was confirmed by a combination of *SMN1* gene dosage analysis and SMA linkage analysis.[14]

Three asymptomatic individuals from independent families were shown to have one *SMN1* copy and no *SMN2* copies. Therefore, only one copy of *SMN1*, without any *SMN2* copies is apparently sufficient for a normal phenotype. All of them had an affected child with a homozygous *SMN1* deletion, but with *SMN2* copies present.

The second purpose of *SMN1* gene dosage analysis was for the detection of heterozygous *SMN1* deletions to support a diagnosis of SMA in symptomatic individuals with a negative SMA diagnostic test result (Figure 2). For this purpose, we tested a total of 54 samples that initially were negative for homozygous *SMN1* deletions. The percentage of heterozygous *SMN1* deletions (one copy of *SMN1*) was highest for type I SMA patients (36%; 5 of 14) but was low for all other SMA types.

We also performed *SMN1* gene dosage analysis on four prenatal samples from three different families, where the affected child was negative by the SMA diagnostic test and subsequently was found to have one copy of *SMN1* by gene dosage analysis. These prenatal *SMN1* gene dosage analyses were combined with linkage analyses (see SMA Linkage Analysis). Two prenatal samples showed one copy of *SMN1* and the other two showed two copies of *SMN1*.

Table 1. *SMN1* Gene Dosage Analyses for SMA Carrier Testing

	Number of cases							
		Family history of SMA						
		Parent of a child with SMA and the child has						
SMN1 copy number	No family history	0-copy *SMN1*	1-copy *SMN1*	Negative diagnostic test	Not been tested	Other family history	Subtotal	Total
1	3	110	0	2	39	117	268	271
2	174	8*	3	6	8	157	182	356
3	12	0	1	0	1	20	22	34
4	2	0	0	0	0	0	0	2
% 1-copy results	1.6%	93%	0%	25%	81%	40%	57%	48%
Total	191	118	4	8	48	294	472	663

Results are listed by type of family history.

*Five individuals showed the "2 + 0" genotype and two showed the "1 + 1" genotype. The "2 + 0" genotype refers to two *SMN1* exon 7 copies on one chromosome 5 and an exon 7 deletion on the other chromosome 5. The "1 + 1" genotype refers to one *SMN1* exon 7 copy on each chromosome 5. The other individual's genotype could not be determined because additional family members were not available for testing.

SMA Linkage Analysis

Linkage analysis was performed for a total of 32 families, with 2 to 12 members per family. For 22 families, linkage analysis was performed before the SMA diagnostic test was developed. However, linkage analysis continues to be useful even with the availability of the SMA diagnostic testing and *SMN1* gene dosage analysis. Linkage analysis is particularly important for families with an affected child who has a homozygous deletion of *SMN1* exon 7 and a parent with two copies of *SMN1*.[14] We used linkage analysis in combination with *SMN1* gene dosage analysis in six such families. Four of the six parents with two copies of *SMN1* were carriers with a deletion allele plus two copies of *SMN1* on the other chromosome 5, and the remaining two parents had two copies of *SMN1* and their children had a *de novo* deletion associated with a cross-over event. Linkage analysis was also used in five cases in four independent families with an affected child having a non-deletion mutation in one *SMN1* allele, to determine whether a new fetus would be likely to inherit the same disease alleles as the affected child. In three cases both the maternal and paternal haplotypes of the fetus were shared with the affected child, in one case only the paternal haplotype was shared with the affected child, and in the remaining one case neither haplotype was shared with the affected child.

SMA Diagnostic Testing in Unusual Cases

One unusual case was a two-month-old white male who was diagnosed with sudden infant death syndrome (SIDS) at autopsy. However, the parents reported decreased movements of extremities and choking. After completion of the autopsy, SMA diagnostic testing showed a homozygous deletion of *SMN1* exon 7.

We tested seven samples from five fetuses and two neonates with clinical arthrogryposis. Only one case (a 6-week-old neonate) had a homozygous *SMN1* exon 7 deletion. One of the six negative cases, subsequently tested by *SMN1* gene dosage analysis, had two copies of *SMN1*.

Sequence Analysis of SMN1 PCR Products

Five samples showed the expected size *SMN1* peak (actual size 187 bp) and a one-base-larger peak (actual size 188 bp) on ABI 310 or 373a electropherograms by *SMN1* gene dosage analysis (Figure 1). The clinical features of these five cases included two symptomatic individuals (a 29-year-old pregnant woman with type III SMA and a 2-year-old boy with type II SMA) and three without symptoms. The two symptomatic individuals were initially tested for *SMN1* homozygous deletions with negative results and subsequently tested by *SMN1* gene dosage analysis. The three asymptomatic individuals included two with family histories of SMA and one without a family history of SMA. These samples were analyzed by sequencing of the *SMN1* exon 7 PCR product. All five cases showed the presence of two alleles, which differed in length by one base due to a single thymidine insertion in the pre-existing polythymidine tract (8T) in *SMN1* intron 6.

Discussion

Our laboratory has been using homozygous deletion analysis and *SMN1* gene dosage analysis for *SMN1* exon 7 only and not for exon 8. Our rationale is based on the critical functional importance of exon 7 in splicing.[17] A gene conversion event, in which a single nucleotide C→T change in the exon 7 sequence (from *SMN1* to *SMN2*), but without a change in the *SMN1* exon 8 sequence, has been shown to be associated with SMA[18,19] (reviewed by Burghes[20]). The significance of a homozygous deletion of only exon 8 without an exon 7 homozygous deletion is unknown. Two patients (type II and type III) with an *SMN1* exon 8 homozygous deletion but exon 7 present have been reported.[21] However, the possibility of missing a deletion located between the distal half of exon 7 and exon 8 cannot be excluded, because the method used was that of van der Steege et al,[12] which cannot detect a deletion limited to the distal half of *SMN1* exon 7. Therefore, an *SMN1* allele with a deletion limited to exon 8 but with an intact exon 7 does not necessarily constitute a disease allele.

In our laboratory, 88% of clinically-defined type II SMA patients lacked both copies of *SMN1*, compared to 64% of type I patients and 32% of type III patients. The reported percentages of SMA patients who lack both copies of *SMN1* are 96% (400 of 418) for type I SMA, 94% (241 of 257) for type II, and 86% (185 of 216) for type III (reviewed by Wirth[11]). These studies included only patients selected by the standard criteria with clinical manifestations typical of each type of SMA. By contrast, our results were derived from patients tested in a clinical molecular pathology laboratory. Many of the patients we tested had atypical clinical features, suggesting that their diseases might be similar to SMA but unrelated to *SMN1* mutations. Our results suggest that hypotonia with intermediate onset and severity is more likely due to 5q13-linked SMA than early or late onset hypotonia. On the other hand, among patients with negative SMA diagnostic test results whom we tested by *SMN1* gene dosage analysis, suspected type I patients had the highest percentage (36%) of one-copy results. Our results suggest that negative results in SMA diagnostic testing on possible type III SMA patients significantly decreases the likelihood of 5q13-linked SMA, as only 1 of 14 suspected type III SMA patients without a homozygous *SMN1* deletion who were subsequently tested by *SMN1* gene dosage analysis had one copy of *SMN1*. The positive predictive value of a one-copy *SMN1* result in a symptomatic individual depends largely on the degree of clinical diagnostic certainty, due to the relatively high prevalence (~2%) of carriers with one *SMN1* copy in the general population. Therefore, we have not recommended *SMN1* gene dosage analysis unless clinical suspicion for SMA remains high even after a negative SMA diagnostic test.

We obtained a positive result in 14% (14 of 103) of prenatal samples by the SMA diagnostic test. This is

significantly lower than the 25% positivity we would have expected by simple Mendelian genetics if we had tested only prenatal samples from carrier couples with heterozygous *SMN1* deletions. However, we also tested prenatal samples from carrier parents with new partners whose genetic status was unknown, as well as samples from parents whose previous affected children had not been tested for *SMN1* deletions. These prenatal samples are expected to have a lower positive rate than those from carrier parents with confirmed *SMN1* deletion mutations. Negative selection by early miscarriage of fetuses with lethal mutations, such as contiguous gene deletions that comprise deletions of both *SMN1* and *SMN2* in the 5q13 region, may also have contributed to the lower-than-expected percentage of positive results. There have been no documented cases of SMA with homozygous deletions of both *SMN1* and *SMN2*. Homozygous deletion of the mouse *SMN* homolog (Smn −/−) causes embryonic lethality, and introduction of the human *SMN2* gene partially rescues embryonic lethality in mice with symptoms resembling human SMA.[22,23]

There has been no report of a homozygous *SMN1* deletion in an infant who was suspected to have SIDS. One report described an infant who almost suffered sudden death at the age of 7 weeks due to diaphragmatic paralysis and was later diagnosed to have type I SMA.[24] Diaphragmatic paralysis and resulting respiratory distress was an initial manifestation of SMA in reports of six infants.[25–27] However, the status of *SMN1* in these infants has not been determined. We speculate that the cause of some clinically unexplained infant deaths may be SMA. Strictly speaking, the diagnosis of SIDS is inappropriate if a homozygous *SMN1* mutation confirms the diagnosis of SMA. More studies are necessary to draw a definitive conclusion about the frequency of undiagnosed SMA being misdiagnosed as SIDS.

Arthrogryposis represents a group of heterogeneous syndromes, all of which have in common joint contractures that are present at birth.[28] The major clinical form, arthrogryposis multiplex congenita (AMC), is caused by decreased fetal movements *in utero* due to a large number of conditions, including neuropathies, myopathies, connective tissue diseases, and conditions that limit the space within the uterus.[29] Burglen et al[30] showed that 6 of 12 patients with "neurogenic AMC" had homozygous deletions of *SMN1*. Bingham et al[31] showed that two of four patients with arthrogryposis, not otherwise specified, had homozygous deletions of *SMN1*. By contrast, Rudnik-Schöneborn et al[32] showed that two of two "neurogenic arthrogryposis" patients did not have homozygous *SMN1* deletions. We detected a homozygous deletion of *SMN1* in only one of seven cases referred for "arthrogryposis" (one negative case was AMC) and tested by the SMA diagnostic test. However, comparing these data is problematic because of the heterogeneous etiologies for arthrogryposis and the lack of uniform inclusion criteria in these studies.

We report five unrelated individuals who have two alleles that differ in length by a single base due to a single nucleotide thymidine insertion in a pre-existing polythymidine tract (8T) in *SMN1* intron 6. Two were symptomatic (one with type II SMA and the other with type III SMA) and the other three were asymptomatic. Interestingly, all five of the samples contained the expected "normal 8T allele" *SMN1* peak in addition to the one-base-larger 9T *SMN1* peak, and each peak corresponded to one copy of *SMN1* by gene dosage analysis. Despite careful scrutiny, we were unable to identify any individuals with only the 9T *SMN1* peak without the normal 8T *SMN1* peak. The biological significance of this single nucleotide insertion is unknown. It may merely be a polymorphism with no clinical significance.

In conclusion, comprehensive SMA testing, including SMA deletion analysis, *SMN1* dosage analysis and linkage analysis, offers the most complete evaluation of SMA patients and their families at this time.

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