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Molecular analysis of spinal muscular atrophy and modification of the phenotype by SMN2

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Purpose: This study describes SMN1 deletion frequency, carrier studies, and the effect of the modifying SMN2 gene on the spinal muscular atrophy (SMA) phenotype. A novel allele-specific intragenic mutation panel increases the sensitivity of SMN1 testing. **Methods:** From 1995 to 2001, 610 patients were tested for SMN1 deletions and 399 relatives of probands have been tested for carrier status. SMN2 copy number was compared between 52 type I and 90 type III patients, and between type I and type III patients with chimeric SMN genes. A fluorescent allele-specific polymerase chain reaction (PCR) -based strategy detected intragenic mutations in potential compound heterozygotes and was used on 366 patients. **Results:** Less than half of the patients tested were homozygously deleted for SMN1. A PCR-based panel detected the seven most common intragenic mutations. SMN2 copy number was significantly different between mild and severely affected patients. **Conclusions:** SMN1 molecular testing is essential for the diagnosis of SMA and allows for accurate carrier testing. Screening for intragenic mutations in SMN1 increases the sensitivity of diagnostic testing. Finally, SMN2 copy number is conclusively shown to ameliorate the phenotype and provide valuable prognostic information. ***Genet Med* 2002:4(1):20–26.**

Key Words: *spinal muscular atrophy, SMN, polymerase chain reaction*

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease caused by homozygous mutation of the SMN1 gene[1] on chromosome 5q13.[2–12] 5q-SMA has an incidence of 1/10,000 with an estimated carrier frequency of 1/50.[13–17] Deletion and gene conversion events result in a 95% to 98% rate of homozygous loss of the SMN1 gene in patients with classic SMA.[1,18–31] A further 1% to 3% of well-characterized SMA cases are the result of compound heterozygosity with an intragenic mutation and a deletion.[31,32] Clinically, SMA patients are classified as type I, II, or III, based on the severity of the disease and the age of onset.[33,34] SMA type I, also known as Werdnig-Hoffmann disease,[35,36] is the most severe presentation. Type I infants are hypotonic and have severe proximal muscle wasting due to a lack of α-motor neurons in the anterior horn of the spinal cord. These patients are diagnosed prenatally or within 6 months after birth. They never sit unaided and usually die within the first 2 years, most often due to respiratory muscle weakness. Patients with intermediate SMA type II develop the disease before 18 months of age. They are able to sit unaided, but do not stand or walk. SMA type III, also called Kugelberg-Welander disease, is less severe.[37,38] Type III patients are diagnosed after 18 months, are able to walk, can have children, and often live at least into the second or third decade.[39]

This study presents the clinical experience of 6 years of SMA testing in the Molecular Pathology Laboratory at The Ohio State University. Despite the fact that more than 95% of 5q-linked SMA patients lack any intact SMN1, it has been our experience that $<$43% of patients referred for diagnostic testing have the common SMN1 deletion. Although the remaining patients share some of the common features of SMA patients, including hypotonia, progressive muscle weakness, and loss of ambulation, they do not have chromosome 5q-linked proximal spinal muscular atrophy. For this reason, the molecular diagnosis is absolutely necessary for the clinical diagnosis of SMA and for accurate genetic counseling.

In an attempt to increase diagnostic sensitivity, we describe an efficient and inexpensive fluorescent allele-specific polymerase chain reaction (PCR) panel that allows for the rapid identification of the seven most common intragenic mutations found in compound heterozygotic SMA patients. This panel can easily be expanded or altered to meet the mutational profile of a specific population.

For autosomal recessive disorders, it is expected that parents will be obligate carriers of a mutant allele. However, there are two factors which complicate SMA carrier testing. One is the presence of de novo mutations, which occurs in approximately 2% of probands.[40] The other factor is the presence of single chromosomes with two copies of SMN1 confounding all dosage-based assays for SMN1 copy num-

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ber.[17,31,41] We present the results of the carrier testing of 399 familial relations of SMA probands and describe the population most interested in being tested. Approximately 5% of parents of homozygously deleted patients had two nondeleted copies of the SMN1 gene.

SMN2 is a copy of the SMN1 gene present in a duplicated region of chromosome 5q. Two exonic base changes differentiate SMN2 from SMN1,[1,42] and the variation in exon 7 of SMN2 is sufficient to greatly diminish the amount of full-length transcript and consequently the amount of full-length protein produced by the gene.[43,44] This base change affects an exonic splice enhancer site,[45] resulting in the diminished ability to correctly splice out intron 7. Absence of SMN2 does not cause spinal muscular atrophy, however SMN2 copy number has been correlated with modification of the SMA phenotype in a transgenic mouse model[46–48] and in humans, where increased copy number of SMN2 is associated with a more mild presentation of the disease.[27]

Several studies have shown that SMN2 gene copy number can modify the SMA phenotype.[17,23,26,28,31,47,48] We have performed a large-scale study comparing the SMN2 copy number between 52 type I and 90 type III patients. Our results conclusively demonstrate a greater number of copies of the SMN2 gene in type III versus type I patients.

Chimeric SMN alleles (exon 7^{SMN2}, exon 8^{SMN1}) were found by DiDonato et al.[29] to be more common in patients with mild SMA, suggesting that these alleles might be more mild than normal alleles.[27–29,49] Other studies found all SMA types represented in the population of patients with chimeric SMN genes.[22,23,25,26,30,50] An analysis of SMN2 copy number as it correlates to severity in SMA patients with chimeric SMN genes has not previously been reported. We hypothesized that SMN2 copy number might account for the variation in phenotype observed within the population of chimeric SMA patients and tested this by comparing SMN2 copy number between type I and type III patients with chimeric SMN genes.

PATIENTS AND METHODS

Patient clinical classification

We do not receive extensive clinical information about the majority of patients sent for diagnostic testing and almost never receive follow-up data. For these reasons, we were not able to reliably place all of our patients into SMA types I, II, and III. Lacking motor milestone information and dates of the death of these patients, we instead categorized 52 patient samples sent for molecular diagnosis between the ages of zero and 6 months as type I. Our group of 90 type III patients were taken from DNA samples of individuals classified as type III before enrollment in a gabapentin therapeutic drug trial. We realize that by analyzing the most severe and the most mild patients, we have chosen our data from the extreme ends of the diagnostic spectrum. This method was the only way for us to ensure the segregation of these populations, and we believe that these extremes legitimately represent acute and chronic SMA cases, without providing information about intermediate presentation.

Allele-specific PCR panel

A multiplex panel of allele-specific primers (Table 1) was used to preferentially amplify the mutant over the normal allele. For each primer pair, the nonallele-specific primer was fluorescently labeled. Part of the APC gene was used as an internal control and was amplified in every sample regardless of the presence of mutations to confirm that sufficient template was present in each reaction. Every gel had the following controls: blank, normal, and each of the seven mutations being tested. Amplification was performed on a Perkin Elmer 9600 thermocycler (Applied Biosystems, Foster City, CA) starting with a 5-minute denaturation at 94°C; followed by 30 cycles of 1 minute at 95°C, 2 minutes at 55°C, 3 minutes at 72°C; and concluding with a 7-minute extension at 72°C. Product was electrophoresed through a 5% LongRanger (FMC Bioproducts, Rockland, ME) polyacrylamide gel on an ABI Prism 377

Table 1
Allele-specific primer sequence

Mutation	Forward primer	Reverse primer	Concentration (ng/μL)	Size (bp)
Internal control	APCMDE[54]	APCMDE-R[a,54]	32	230
800ins11	541C618[1,b]	5′-AAC AAA GTC ACA TAA CTA CAA A-3′	32	153/164[c]
542ΔGT	5′-GAA AGC CAA GTT TCA ACA GAT GAA AGA-3′	5′-GAG AGG TTA AAT GTC CCG A′3′[a]	45	199
A2G	5′-ATA CAC GCC ACA AAT GTG GGA-3′[b]	5′-CAC TGC CGC CGC TGC TCA TCC-3′	16	194
S2621	5′-GTA CCA TGA AAT TAA CAT AA-3′	541C618[1,b]	45	64
Q15X	121MA[a]	5′-GAA CAG CAC GGA ATC CTC CTA-3′	16	123
T2741	541C618[1,b]	5′-TGA CTG ATT ACT TAC CAT ATA ATA GCC AA-3′	16	107
Y272C	541C618[1,b]	5′-CCA TAT AAT AGC CAG TAT GAC-3′	16	94

[a]6-FAM labeled.
[b]TET labeled.
[c]153 bp for normal allele/164 bp for 11-bp insertion.

DNA Sequencer (Applied Biosystems) at 3 kV/h for 1.5 hours. GeneScan TAMRA 500 (Applied Biosystems) was used as a size standard. Intensity of bands were analyzed using GeneScan software (Applied Biosystems). Although the A2G primer pair produces a 194-bp band, a smaller more visible product is used for diagnosis.

Genomic DNA isolation and identification of SMN1 copy number

DNA was extracted from peripheral venous blood from patients by a simple salting out technique.[51] DNA was analyzed for SMA carrier status by an SMN dosage assay.[17] Paternity was confirmed in the cases of parents of affected patients found to have two copies of the SMN1 gene.

SMN2 dosage assay

Carrier analysis for the SMN1 and SMN2 genes was based on the protocol of McAndrew et al.;[17] however, the same primers were fluorescently labeled with 6-FAM. When testing for SMN2 copy number in patients deleted for SMN1, controls with zero copies of SMN1 and with 1, 2, 3, or 4 copies of SMN2 were run on each gel. Amplification was carried out in a Perkin Elmer 9600 thermocycler (Applied Biosystems) using the following conditions: 5 minutes denaturation at 94°C; followed by 21 cycles of 1 minute at 95°C, 2 minutes at 55°C, 3 minutes at 72°C; and concluding with a 7-minute extension at 72°C. Product was electrophoresed through a 5% LongRanger polyacrylamide gel (FMC Bioproducts) on an ABI Prism 377 DNA Sequencer (Applied Biosystems) at 3 kV/h for 1.5 hours. Dosage ratios were analyzed using GeneScan software (Applied Biosystems).

RESULTS AND DISCUSSION

SMN1 deletions

Since 1995, our laboratory has offered diagnostic SMN1 deletion analysis to detect the homozygous absence of the gene.[52] This deletion test was most often performed on DNA extracted from whole blood. Muscle biopsies, newborn blood spots, paraffin-embedded autopsy material, and fixed tissue on glass slides have been used for postmortem diagnosis. Postmortem molecular testing is often important for the confirmation of clinical diagnosis, thus allowing for accurate genetic counseling.

It has been the experience of this laboratory that the clinical symptoms of SMA are not sufficiently specific to make a reliable diagnosis. Patients that are sent for the molecular test generally have similar symptoms, including hypotonia, proximal muscle weakness, and loss of ambulation. These symptoms are not specific to 5q-spinal muscular atrophy. Of the 610 patients tested, 232 (38.0%) were deleted for exons 7 and 8 and 29 (4.8%) were deleted only for exon 7. The remaining 349 (57.2%) were not deleted. Our data indicate that the clinical diagnosis of SMA is not straightforward, and oftentimes the test is being ordered to exclude the disease. Muscle biopsies are sometimes ordered for hypotonic infants before the molecular test. Our data would suggest that such an invasive procedure should be reserved for infants with negative SMN1 deletion results.

Diagnostic testing for intragenic mutations

Although many researchers have identified intragenic mutations in the SMN1 gene in compound heterozygotic patients,[53] testing for these mutations is not performed on a routine diagnostic basis. The difficulties in screening for intragenic mutations include the fact that there is no highly prevalent mutation found in these patients, and that mutations are found throughout the gene. Adding to the complexity of finding intragenic mutations is the presence of SMN2, which makes for technical difficulties in identification of mutant sequence in SMN1 through the background of normal sequence from the copy gene. Also important is that newly identified mutations must be shown to be present on the SMN1 gene and not on SMN2. Several mutations were found more often than others, and these mutations have not been found in the SMN2 gene.[31,32] This finding led us to develop a rapid and inexpensive assay that detects the more common intragenic mutations. The test entails the amplification of patient DNA using a panel of allele-specific primers that preferentially amplify the mutant allele. Seven mutations that have been observed more than once are detected (Fig. 1), based on mismatches in the 3' end of the primer that are homologous to the mutant sequence (Table 1). Amplification of the APC gene is used as an internal control to confirm that sufficient template is present and amplification conditions are appropriate. The 800ins11 primer pair also acts as an internal control. This primer set is not allele-specific. Rather, it detects the mutation based on an 11- bp size differential; a 153-bp band if a normal allele is present, and a 164-bp band indicates a mutation. The 153-bp product acts as an amplification control, because this primer pair should amplify at least one normal SMN2 allele. If any of the seven mutations are identified, SMN1 copy number is determined to confirm the compound heterozygosity of the sample.

This panel was used to screen 366 patients shown not to homozygously lack the SMN1 gene based on results of the diagnostic deletion test.[52] Ninety-five percent to 98% of SMA patients homozygously lack the SMN1 gene. Approximately 43% of the patients screened were deleted. Therefore, we estimated that 44% of the patients tested by our laboratory actually had SMA. Based on this estimation, we expected to find between one and three intragenic mutations. One mutation was identified (T274I). The patient with this mutation was subsequently found to possess only a single copy of the SMN1 gene and was, therefore, determined to be a compound heterozygote. This panel detects only the seven most common intragenic mutations, representing approximately 60% of all intragenic mutations reported in the literature.[53] It is possible that less common mutations were present in patients for which mutations were not identified by this assay. This multiplex panel was able to detect every patient previously shown to have one of these common mutations. For this reason, we believe that it is an effective and appropriate diagnostic assay to increase the sensitivity of SMA testing. Because of the low intra-

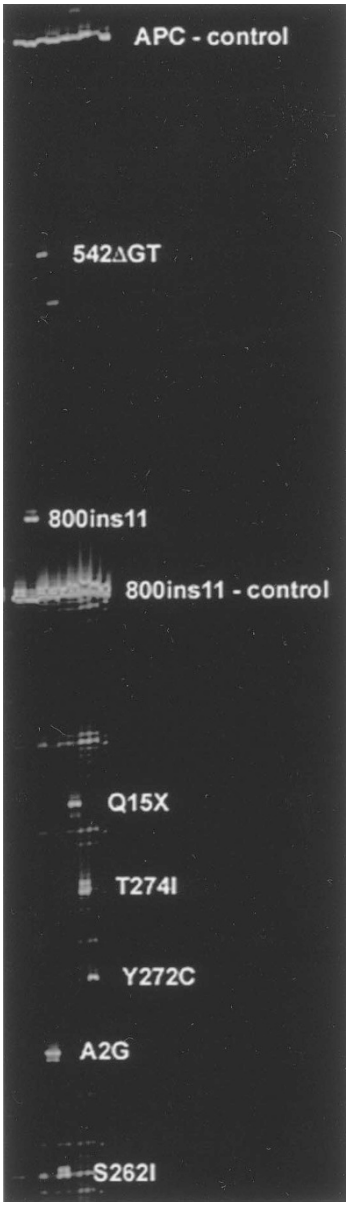

Fig. 1 Fluorescent allele-specific multiplex PCR panel detects seven intragenic mutations in the SMN1 gene in patients but not in controls. The presence of a band indicates a mutation. APC and 800ins11 controls amplify in every sample and confirm the presence of sufficient template in the reaction.

genic mutation rate, this application might be reserved for patients with a very high probability of having proximal spinal muscular atrophy.

Carrier testing

A carrier test for SMA was developed in our laboratory in 1997,[17] which compares the ratios of the coamplified products of the SMN1 and CFTR genes. This competitive radiolabeled PCR reaction is optimized such that the copy numbers of the SMN1 and SMN2 genes can be determined in comparison to the two copies of the CFTR gene. Currently, 399 individuals have been tested for carrier status. Parents were most often tested as shown in Table 2. Unlike most autosomal recessive disorders, one cannot assume that parents of affected children are obligate carriers. De novo mutations[40] and the presence of chromosomes with two copies of SMN1[41] allow for the possibility of affected individuals born to parents with normal SMN1 copy number. Ninety-five percent of the parents tested were shown to be carriers (Table 2). The remaining individuals present a diagnostic challenge. Because we cannot distinguish the de novo events (which confer a very low recurrence risk) from two-copy SMN1 chromosomes (which confer the normal 25% recurrence risk) based solely on the SMN1 dosage test, linkage studies, dosage testing of extended family members, or monosomal hybrid studies would be helpful in providing accurate genetic counseling to such parents.

The second most common population requesting carrier testing are the unrelated spouses of carriers. Three percent (3 of 100) of unrelated spouses were positive for carrier status, indicating a carrier frequency of 3%, which is in agreement with the previously described frequency of 1 of 50.[16] Eighty-eight aunts and uncles of probands were tested of which 41 (47%) were carriers. Additionally, we found that 73% (27 of 37) of sibs, 21% (3 of 14) of first cousins, and 40% (8 of 20) of grandparents were carriers. None of these values are different from what would be expected from a recessive model with a 2% de novo mutation rate (Table 2).

Because more than 57% of the patients tested are not deleted for the critical exon 7, we only performed carrier tests on families known to have an SMN1 deletion. There are two important reasons for our policy. It is possible that one would perform dosage testing on a point mutation, which may result in false negatives. Another reason is that the clinical diagnosis is not straightforward. One may, therefore, perform dosage testing on the carrier of a phenotypically similar disorder, thereby falsely reducing the recurrence risk.

SMN2 copy number

Previous studies state that 5% of unaffected people homozygously lack SMN2.[1] In this study, 14 of 97 normal controls with at least two copies of SMN1 had zero copies of SMN2, indicating a much higher percentage (14.4%). The difference between studies may be attributed to variation between populations. The majority of normal individuals have one or two copies of

Table 2
Comparison of carrier data with recessive model of inheritance

Relation to proband	*N*	Mean	95% CI	Expected for relationship
Parents	107	0.953	(0.913, 0.994)	0.980
Unrelated spouse	100	0.030	(0, 0.064)	0.020
Aunts/uncles	88	0.466	(0.360, 0.572)	0.490
Sibs	37	0.730	(0.580, 0.880)	0.654
Grandparents	20	0.400	(0.165, 0.635)	0.490
Cousins	14	0.214	(0, 0.353)	0.245

CI, confidence interval.

SMN2 (Fig. 2). It was also found that 4% of this sample of the normal population had three copies of the SMN1 gene.

To determine whether mild SMA patients have more SMN2 copies than severe patients, we have performed a large-scale comparison of SMN2 copy number in SMA patients with disparate ages of onset, including 52 type I cases that were sent for molecular diagnosis before they were 6 months old and 90 patients who were at least 18 years of age and diagnosed as having type III SMA. The results are shown in Table 3 and clearly demonstrate that there are more copies of SMN2 in mild SMA cases compared with severe cases based on a chi-squared test ($P < 0.0001$). Interestingly, 100% of type III patients had at least three copies of SMN2 and 20 of 90 had four copies. This finding is in contrast to only 3.8% of type I patients with three copies, whereas none had more than three copies. No type III patients were identified with one or two copies of SMN2. These data indicate that the presence of three or more copies of SMN2 is clearly correlated with a milder phenotype. Based on this information, it can be concluded that the presence of one or two copies of SMN2 predicts a severe phenotype, and three or more copies of SMN2 is a good prognostic indicator that a patient will at least sit unaided and live more than 2 years.

This study sought to test whether the variation in severity between patients with chimeric SMN genes was correlated with SMN2 copy number by analyzing the number of SMN2 genes in one type I and 10 type III patients who lacked exon 7 and retained exon 8 of SMN1, while possessing exon 7 of SMN2. We observed that every chimeric type III patient had at least three copies of SMN2, whereas the only type I chimeric patient tested had two copies. There were two additional chimeric patients who were 8 and 8.5 months at molecular diagnosis (possible type I) that had two copies of SMN2. If chimeric alleles were milder than normal SMN2 alleles, we might have expected to find some type III patients with two copies of SMN2. SMN2 copy number modifies the phenotype in deleted patients[17,23,26,28,31,47,48] as well as in compound heterozygotes.[32] These data are suggestive that the variation in phenotype between patients with chimeric SMN genes is also correlated with SMN2 copy number and not with the fact that chimeric alleles are inherently mild. Our population does not contain a large proportion of type I chimeric patients. It would be interesting to analyze SMN2 copy number in a population with a larger number of such patients.

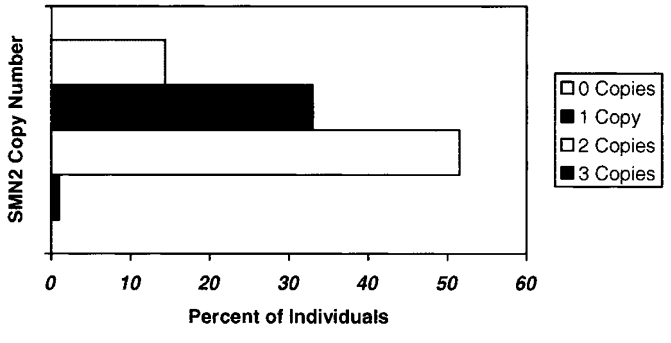

Fig. 2 Variation of SMN2 copy number in normal individuals with at least two copies of SMN1.

Table 3
SMN2 copy number in type 1 compared with type 3 SMA patients

	Type I	Type III	Total
1 Copy SMN2	7 (13.5%)	0 (0%)	7 (4.9%)
2 Copy SMN2	43 (82.7%)	0 (0%)	43 (30.3%)
3 Copy SMN2	2 (3.9%)	70 (77.8%)	72 (50.7%)
4 Copy SMN2	0 (0%)	20 (22.2%)	20 (14.1%)
Total	52	90	142

CONCLUSIONS

The experience from 6 years of spinal muscular atrophy testing leads us to the following conclusions. There is a high frequency of patients with some symptoms consistent with SMA that are negative for the standard SMA deletion test. This fact emphasizes the importance of the molecular test in the diagnosis of spinal muscular atrophy. To further increase the sensitivity of molecular testing, a cost-effective and rapid PCR-based intragenic mutation panel has been described. There is great interest in SMA carrier testing, particularly by parents of affected patients, which has uncovered the interesting finding that 5% of parents possess two intact copies of SMN1 and require linkage analysis or monosomal hybrid studies for accurate risk assessment. With such a high rate of SMA-like patients testing negative for the common deletion, we recommend that carrier testing be reserved for at-risk relatives of patients that are positive for the SMN1 deletion. Our data indicate that, if a molecular diagnosis of SMA is not made in a family, then carrier testing will be performed on the wrong gene in more than half of the cases. Finally, SMN2 copy number has been shown to clearly correlate with the clinical type of SMA. Our comparison of severe type I and mild type III patients is the largest study to date and demonstrates that SMN2 can provide prognostic information.

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