Characterization of survival motor neuron (SMN\textsuperscript{T}) gene deletions in asymptomatic carriers of spinal muscular atrophy

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Previous reports have established that the telomeric copy of the survival motor neuron (SMN\textsuperscript{T}) gene and the intact copy of the neuronal apoptosis inhibitory protein (NAIP) gene are preferentially deleted in patients with spinal muscular atrophy (SMA). Although deletions or mutations in the SMN\textsuperscript{T} gene are most highly correlated with SMA, it is not clear to what extent NAIP or other genes influence the SMA phenotype, or whether a small fraction of SMA patients actually have functional copies of both SMN\textsuperscript{T} and NAIP. To evaluate further the part of SMN\textsuperscript{T} in the development of SMA, we analyzed 280 asymptomatic SMA family members for the presence or absence of SMN\textsuperscript{T} exons 7 and 8. We report the following observations: (i) 4% of the sample harbored a polymorphic variant of SMN\textsuperscript{T} exon 7 that looks like a homozygous deletion; (ii) approximately 1% of the parents are homozygously deleted for both exons 7 and 8; (iii) one asymptomatic parent lacking both copies of SMN\textsuperscript{T} exons 7 and 8 displays a ‘subclinical phenotype’ characterized by mild neurogenic pathology; (iv) another asymptomatic parent lacking both SMN\textsuperscript{T} exons showed no signs of motor neuron disorder by clinical and neurodiagnostic analyses. The demonstration of polymorphic variants of exon 7 that masquerade as homozygous nulls, and the identification of SMA parents who harbor two disease alleles, serve as a caution to those conducting prenatal tests with these markers.

INTRODUCTION

Spinal muscular atrophy (SMA) is a motor neuron disease characterized by degeneration of spinal cord anterior horn cells and muscular atrophy. The autosomal recessive form of this disorder affects about 1:10 000 live births with a carrier frequency of 1/40–1/60. It is the most common fatal genetic disorder of infancy and the second most common childhood neuromuscular disease following Duchenne muscular dystrophy. Childhood-onset SMA is classified into three clinical subtypes: (i) SMA type I, or Werdnig-Hoffmann disease—the most severe phenotype, usually leading to early infant death; (ii) SMA type II, or the intermediate subtype—onset in early childhood often resulting in early impairment of walking; and (iii) SMA type III, or Kugelberg-Welander disease—onset in late childhood, with considerable clinical heterogeneity and the risk of wheelchair dependence in adult years. The gene for the three types of childhood-onset SMA has been mapped to a single locus on chromosome 5q13 by linkage analysis (1–3). Further studies using recombinant mapping methods identified a genetic interval of 1–2 cM flanked by new microsatellite markers (4–7,13). Several YAC contigs spanning the SMA gene region have been constructed by different research groups (5,8,9,11). Further refinement of the region was complicated by low-copy repeat sequences mapping in and around the SMA region (8,11–13). Gene-coding sequences likewise tend to exist in multiple copies within the disease gene region. In some cases pseudogenes map to the SMA region while the intact, presumably functional, copies map elsewhere in the genome (14).

Recently, independent laboratories reported two candidate genes in the SMA region: the survival motor neuron (SMN) gene and the neuronal apoptosis inhibitory protein (NAIP) (15,16). The survival motor neuron (SMN) gene exists as two highly homologous copies, a centromeric (SMN\textsuperscript{C}) and a telomeric form (SMN\textsuperscript{T}) which differ by only 2 bp in the entire 1.5 kb coding sequence. The SMN\textsuperscript{T} copy is homoyzogously missing in over 90% of SMA patients, whereas at least one copy of SMN\textsuperscript{C} is present in the vast majority of control DNA samples (15,17–19). SMN\textsuperscript{C} is not preferentially deleted in SMA patients, but is homozygously deleted in about 5% of samples tested. Expression of the SMN\textsuperscript{C}
gene appears to differ in SMA versus control samples. In samples lacking a copy of SMN\(^T\), there is a noticeable increase in the expression of an alternatively spliced SMN\(^C\) RNA transcript (15). Thus, while SMNC does not appear to be a target for SMA mutations, its pattern of expression may contribute to the pathophysiology of the disorder.

The second candidate gene, NAIP, maps in the near vicinity of SMNT. NAIP likewise exists in multiple, highly homologous copies, although it appears that only one copy contains the full complement of exons. The intact copy of NAIP is homozygously deleted in about 40% of SMA type I cases and to a lesser extent in SMA II and III individuals (16,19). In the minority of SMA samples where no evidence was found for deletion of either SMNT or NAIP, several small insertion/deletion mutations have been found at the splice-site junctions of SMNT (15).

In summary, three types of evidence implicate SMN as the most critical gene influencing the onset of SMA: first, deletion of the 3'-end of SMNT is most highly correlated with incidence of SMA; second, a minority of SMA patients reveal small insertion/deletion mutations at splice-site junctions in SMNT whereas no disease-related point mutations have been reported for NAIP; and third, homozygous deletion of intact NAIP has been reported in asymptomatic parents of SMA patients (16), while recent evidence shows that SMNT is likewise missing in a small fraction of asymptomatic SMA siblings and parents (18,19; this manuscript).

From the sum of evidence available to date, it is still not clear whether all cases of SMA arise from deletions or alteration of the SMNT gene. Five to 10% of SMA samples reveal intact exons 7 and 8. As current single strand conformation polymorphism (SSCP) assays cannot distinguish the 5'-ends of the SMNT and SMNC genes, it is very difficult to screen for deletions specific for this region of the gene. Furthermore, the promoter sequences for SMNT have not yet been identified, nor has the full intronic DNA sequence been reported. Thus, it is not known whether the remaining 5–10% of SMA cases actually harbor deletions including the 5'-end of SMNT, or deletions or point mutations in the promoter or intronic sequences. If they do not, then a small proportion of SMA cases presumably arise from the inactivation of other genes, including but not necessarily limited to NAIP.

To evaluate further the part of SMNT in the etiology of SMA, we performed clinical assessment of individuals who are homozygous null for SMNT exons 7 and 8. The identification of such asymptomatic individuals might indicate that deletion of SMNT (exons 7 and 8) alone is insufficient to cause SMA symptoms, or alternatively, that compensating factors can, in rare cases, override the deletion and lead to a ‘normal’ phenotype. We screened 280 asymptomatic family members (214 parents and 66 siblings) of SMA patients for homozygous deletion of SMNT 7 and 8. Two asymptomatic parents from different families were identified who are missing both exons 7 and 8. One parent showed neurogenic abnormalities in electromyography (EMG) and muscle biopsy, consistent with subclinical SMA. The other parent, who has a normal EMG and muscle biopsy, was from a family that appeared to be unlinked to the chromosome 5q13 DNA markers (2,20). We now show that this family is actually consistent with chromosome 5q13 linkage and that the parent transmits different mutant chromosomes to two affected off-springs.

### RESULTS

Table 1 shows the results of screening 280 asymptomatic family members of SMA patients for the presence or absence of SMNT exons 7 and 8. The sample consisted of unaffected parents and siblings. All SMA families meet diagnostic criteria established by the “International SMA Consortium” (22). Twelve individuals were lacking both copies (homozygous null) of SMNT exon 7 only, and two individuals were homozygous null for both SMNT exons 7 and 8. Figure 1 shows three families in which unaffected family members are apparently missing both copies of the SMNT exon 7 band. In each of these families, the SMNT exon 7 band (lower closed arrow in the upper panel) is present in one parent (open symbol), missing in the affected proband (black symbol), and missing in one asymptomatic parent and one sibling (stippled symbol). Analysis of SMNT exon 8 in these families (see upper closed arrow in the lower panel) shows that only the affected proband is homozygously missing this exon. Closer inspection of the exon 7 profiles suggests the presence of two additional bands labeled ‘a’ and ‘b’ in the asymptomatic family members with apparent deletions. Band ‘a’ was separated from the overlapping SMNC exon 7 band on a longer gel and its DNA sequence was determined. Band ‘a’ was shown to contain a ‘C’ nucleotide at base pair position 873 denoting the SMNC locus (data not shown). The same band contains an A to G polymorphism in the intronic sequence 96 bp 5’ to the start of exon 7. This polymorphism alters the band migration such that the SSCP pattern mimics the pattern of a homozygous null for SMNT exon 7. The band ‘b’ sequence is the exact complement of band ‘a’, verifying that bands ‘a’ and ‘b’ are the two complementary strands of the same locus. Haplotype analysis of families 2532 and 7322 using CA-repeat microsatellite markers flanking the SMN gene locus (13) are shown in Figure 2. The combination of SMN and microsatellite genotypes shows that affected probands inherit two SMNT null alleles while the healthy siblings inherit a null allele from one parent along with a polymorphic variant of SMNT exon 7 from the other parent. Thus, the asymptomatic siblings appear to be homozygous nulls by SSCP analysis, but actually inherit a normal, presumably functional copy of SMNT exon 7. Analysis

<table>
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<th>Type</th>
<th>n (n = 100)</th>
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<tr>
<td>Exon 8 only</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Exons 7+8</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2 (0.7%)</td>
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SSCP analysis was performed to detect the presence or homozygous absence of SMNT exons 7 and 8 (15). This assay does not distinguish between the presence of one versus two copies of either exon. The sample consists of 214 parents and 66 siblings. The ‘type’ of SMA (I = Weddington Hoffmann disease/severe SMA; II = intermediate type; III = Kugelberg Welander disease/mild SMA) is indicated at the top of the Table. n = the number of asymptomatic family members analyzed.
unlinked family, or an autosomal dominant SMA family. It is interesting that in the absence of SMN autosomal recessive inheritance where the mother is homozygous and phenotypic data, it is clear that this family is a case of showed evidence of fasciculation. In view of the full DNA marker revealed a mild neurogenic atrophy of muscle fibers and her EMG muscle biopsy and EMG from the mother. Her muscle biopsy and her affected brother have inherited identical disease alleles as asymptomatic parents who are also missing both copies of SMNT exon 7. Bands ‘a’ and ‘b’ are the complementary strands of a polymorphic variant of the SSCP band specific for SMNT exon 7.

Figure 1. SSCP analysis of SMNT exons 7 and 8 in three SMA families. YAC clone Y99C11 contains the telomeric copy, but not the centromeric copy of SMN. YAC clone Y920C9 contains both copies. Human-hamster hybrid cell line HHW105 contains chromosome 5 as its sole human component (23). Closed arrows indicate SSCP bands specific for SMNT exon 7 (top panel) and exon 8 (bottom panel). Open arrows indicate SMNT specific bands. For exon 7, the upper set of bands are barely discernable, but arrows are included for reference. In the three pedigrees; filled symbols indicate the affected proband; open symbols indicate asymptomatic parents; and stippled symbols indicate asymptomatic family members who are also missing both copies of SMNT exon 7. Bands ‘a’ and ‘b’ are the complementary strands of a polymorphic variant of the SSCP band specific for SMNT exon 7.

of the remaining six asymptomatic family members (five parents and one sibling) with apparent exon 7 deletions likewise revealed polymorphic variants of the SMNT specific exon.

Figure 3 shows the SSCP results from a family with an asymptomatic parent who is a homozygous null for SMNT exons 7 and 8. In this family the proband is affected with SMA type II. His mother is clinically asymptomatic witnessed by a thorough neurological examination and by a demanding professional career and active life-style. The maternal uncle is affected with neurological examination and by a demanding professional career and active life-style. The maternal uncle is affected with sensorimotor peripheral neuropathy in EMG and no abnormalities in fiber size or distribution in muscle biopsy (see Materials and Methods). These results show no evidence of motor neuron disorder. Therefore the father is asymptomatic for SMA by both clinical examination and electrophysiological and histopathological studies.

DISCUSSION

We describe two individuals with homozygous null deletion of telomeric SMN exons 7 and 8 who demonstrate no clinical symptoms of SMA. In one case, an SMA type III brother and his asymptomatic sister appear to inherit identical ‘disease alleles’, one of which is transmitted to the son with type II SMA. EMG and muscle biopsy show that the sister has mild neurogenic pathology consistent with a mild, ‘asymptomatic’ manifestation of SMA. Barring an undetected de novo mutation in the affected brother, this result suggests that other factors, genetic or environmental, may compensate for the loss of SMNT function. If genetic in nature, such factors presumably map outside the immediate disease gene region. The second individual is an asymptomatic father of SMA children who is homozygous null for SMNT exons 7 and 8. In contrast to the aforementioned asymptomatic carrier, this individual shows no sign of SMA either by clinical examination or by neurodiagnostic studies. Interestingly, either of his homologues, in combination with the maternal SMA homologue, leads to typical SMA in the offspring.

The data from both families are consistent, therefore, with the notion that deletion of SMNT exons 7 and 8 per se is not sufficient to account for the clinical symptoms typical of SMA. Several explanations could account for this observation. First, homozygous deletion of SMNT exons 7 and 8 is normally sufficient to produce SMA symptoms, but in rare cases, compensating factors protect against the manifestation of disease symptoms. Such factors might include a quantitative or qualitative alteration of the SMNT gene; a third SMN homologue; or an unlinked, background gene product. Second, the majority of SMA homologues are likely to be homozygous null for the entire SMNT coding sequence. In rare circumstances, deletion, gene conversion or unequal recombination may produce a partial SMNT gene which is truncated, converted or recombined with the 3’ portion of SMNC. This variant SMN protein may retain sufficient function to alyl the onset of symptoms. It is noteworthy that no groups have successfully identified deletion breakpoints within the SMNT gene. Third, the majority of SMA mutations may include critical DNA sequence (which is not...
Figure 2. Haplotype analysis of two SMA families which segregate a polymorphic variant of the SMN exon 7 PCR product. Pedigree symbols are the same as Figure 1. CA-repeat microsatellite markers flanking the SMN locus were used for haplotyping (13). The symbols (–*) indicate the polymorphic allele detected in the intron preceding SMN exon 7 (bands ‘a’ and ‘b’ in Fig. 1). The SMN genotypes are listed immediately below each pedigree symbol. The SMN locus is flanked in the centromeric direction by locus D5S1414 and the telomeric direction by locus D5S1408. Individual homologues are separated by a vertical line. The polymorphic variant is indicated by (–*) and a deletion is indicated by (–).

Figure 3. An asymptomatic SMA parent lacking both copies of SMN exons 7 and 8. The control YAC clones and hybrid cell lines, arrows, and pedigree symbols are described in Figure 1. The proband and his maternal uncle are diagnosed with SMA type II and type III, respectively, based upon muscle biopsy and EMG. The mother is clinically asymptomatic. The two affected individuals, as well as the asymptomatic mother of the proband, are lacking both copies of SMN exons 7 and 8.

Deleted in these two asymptomatic parents) in addition to the SMN and NAIP gene sequences. This interpretation implicates yet another gene product in the development of SMA.

The identification of polymorphic variants of SMN exon 7 that masquerade as null alleles provides a cautionary note to the interpretation of prenatal and diagnostic tests. In those cases where only SMN exon 7 is missing, it will be important to distinguish null alleles from polymorphic alleles. This is particularly important when the assay is used in prenatal diagnosis where false positives may lead to unnecessary termination of pregnancy.

Figure 4. Haplotype analysis of SMA family 6376. CA-repeat microsatellite markers D5S1414 and D5S1408 flank the SMN locus. The affected individual II-3 and the asymptomatic individual II-4 share identical haplotypes for both homologues and are both missing exons 7 and 8 as shown in Figure 3.

Direct sequence analysis of the SSCP bands may be necessary for verification of the exon subforms, particularly when novel bands are present. Alternatively, a PCR-enzyme digestion method can be used to confirm the SSCP results (21).

Two research groups have recently reported deletion of the SMN gene in unaffected family members of SMA patients...
Figure 5. Homozygous absence of SMN \textsuperscript{T} exons 7 and 8 in an asymptomatic SMA parent. The symbols are the same as Figure 1. Both affected children and the asymptomatic father are missing exons 7 and 8 of SMN \textsuperscript{T}.

Figure 6. Haplotype analysis of SMA family 1116. Haplotype analysis was performed as described in Figure 2. The asymptomatic father (I-2) and his two affected offspring are lacking both copies of SMN \textsuperscript{T} exons 7 and 8. The asymptomatic mother and the two unaffected offspring are heterozygous for the loss of SMN \textsuperscript{T} exons 7 and 8. The affected son (I-3) inherits the paternal homologue (2 7 3 - - 1) while the affected daughter (I-4) inherits the other paternal homologue (1 1 9 - - 2).

MATERIALS AND METHODS

Family ascertainment and diagnosis

SMA families were obtained through Muscular Dystrophy Association clinics in the United States and through an international group of collaborators. All cases of SMA were diagnosed according to consensus criteria established by an international SMA consortium (22). Family 6376 was referred by a local genetic counselor. The 3 year old proband was diagnosed with SMA type II at 18 months old. The maternal uncle is 32 years old and diagnosed with SMA type III (Kugelberg-Welander disease) at 14 years of age. The mother is 33 years old and clinically asymptomatic. She lives an active life-style including a demanding professional occupation and regular aerobic exercise. Her muscle biopsy shows mild neurogenic atrophy and the EMG shows presence of fasciculation consistent with SMA. Family 1116 includes two affected children diagnosed at ages 13 and 11 with SMA type III. All cases of SMA were confirmed by EMG and muscle biopsy. The asymptomatic father had clubfeet at birth but exhibits no sign of motor disability at age 60. His electrophysiological studies show mild motor conduction slowing and reduced sensory amplitude consistent with axonal sensory-motor...
peripheral neuropathy. His EMG shows no active denervation. His muscle biopsy shows normal fiber size (no atrophy or hypertrophy) and no fiber type grouping. These studies indicate no sign of motor neuron disorder.

Haplotyping analysis

Microsatellite markers D5S1411, D5S1413, D5S1414 and D5S1408 were used to genotype SMA families 6376 and 1116 according to a previously published procedure (13).

SSCP assays

PCR primers for exon 7 (R111 and 540–541) and exon 8 (541C960 and 541C1120) were derived from previously published sequences (15). One hundred ng of genomic DNA was added to 25 µl of reaction mix containing 25 µM of primers, 2.5 mM MgCl2, 1 U of Taq polymerase, and 2.5 µCi of [α-32P]dCTP. The PCR reaction includes an initial 11 cycles of 30 s at 94°C, followed by 24 cycles of 30 s at 94°C, 55°C and 72°C each and a final step of 5 min at 72°C. Four µl of the PCR product were added to an equal volume of denaturing dye containing 96% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. The mixtures were denatured at 95°C for 10 min then chilled on ice and loaded on a 0.5×MEDE gel (MDE3 TM; AT Biochem, PA). The gel was run at 4 W for 18–20 h in 4°C followed by autoradiography.

DNA sequence analysis

To verify the exon subforms of the SSCP bands, we excised the bands directly from the SSCP gel. The single-stranded DNA retained in the gel was eluted by soaking in 20 µl of TE buffer (10 mM Tris and 1 mM EDTA) for 2 h in room temperature. Five µl of the eluent was used for PCR amplification. The purified PCR products were then DNA sequenced using the original oligonucleotide primers and a DyeDeoxy terminator cycle sequencing kit (Applied Biosystem).

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REFERENCES


