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Quantitative Analysis of Survival Motor Neuron Copies: Identification of Subtle *SMN1* Mutations in Patients with Spinal Muscular Atrophy, Genotype-Phenotype Correlation, and Implications for Genetic Counseling

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Summary

Problems with diagnosis and genetic counseling occur for patients with autosomal recessive proximal spinal muscular atrophy (SMA) who do not show the most common mutation: homozygous absence of at least exon 7 of the telomeric survival motor neuron gene (*SMN1*). Here we present molecular genetic data for 42 independent nondeleted SMA patients. A nonradioactive quantitative PCR test showed one *SMN1* copy in 19 patients (45%). By sequencing cloned reverse-transcription (RT) PCR products or genomic fragments of *SMN1,* we identified nine different mutations in 18 of the 19 patients, six described for the first time: three missense mutations (Y272C, T274I, S262I), three frameshift mutations in exons 2a, 2b, and 4 (124insT, 241-242ins4, 591delA), one nonsense mutation in exon 1 (Q15X), one Alu-mediated deletion from intron 4 to intron 6, and one donor splice site mutation in intron 7 (c.922+6T→G). The most frequent mutation, Y272C, was found in 6 (33%) of 18 patients. Each intragenic mutation found in at least two patients occurred on the same haplotype background, indicating founder mutations. Genotype-phenotype correlation allowed inference of the effect of each mutation on the function of the SMN1 protein and the role of the *SMN2* copy number in modulating the SMA phenotype. In 14 of 23 SMA patients with two *SMN1* copies, at least one intact *SMN1* copy was sequenced, which excludes a 5q-SMA and suggests the existence of further gene(s) responsible for ~4%–5% of phenotypes indistinguishable from SMA. We determined the validity of the test, and we discuss its practical implications and limitations.

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Introduction

Autosomal recessive spinal muscular atrophy (SMA) is a neuromuscular disorder caused by the degeneration of the anterior horn cells of the spinal cord, which results in symmetric proximal muscle weakness. Patients with SMA were classified into three groups according to age at onset and maximum motor function (International SMA Consortium 1992; Zerres and Rudnik-Schöneborn 1995). Type I SMA (Werdnig-Hoffmann; MIM 253300) is the most severe form of the disorder, with onset at <6 months of age, inability to sit or walk, and death typically before the age of 2 years; it is the most common genetic cause of infant mortality in northern Europeans. Type II SMA (MIM 253550) is the intermediate form of the disorder, with onset at <18 months of age; patients are never able to walk unaided, and life expectancy is significantly reduced. Type III SMA (Kugelberg-Welander; MIM 253400) is the mildest form of the disorder; patients are able to walk independently, they often become wheelchair bound during youth or adulthood, and life expectancy is probably not much reduced. Childhood-onset SMA affects ~1/10,000 newborns, with a carrier frequency of 1/50.

The SMA region on 5q13 that includes the disease locus for SMA types I–III shows a complex genomic structure, including a 500-kb duplication and inversion (Lefebvre et al. 1995). Four genes, all present in at least two copies, telomeric and centromeric, have been identified within the critical region: the survival motor neuron gene (*SMN*) (Lefebvre et al. 1995), the neuronal apoptosis inhibitory protein gene (*NAIP*) (Roy et al. 1995), a subunit of the basal transcription factor (BTF2p44; Bürglen et al. 1997; Carter et al. 1997), and a putative RNA-binding protein (H4F5; Scharf et al. 1998). All four genes show homozygous absence of their telomeric copies in SMA patients, with higher frequencies for *SMN1* and lower frequencies for H4F5, *NAIP,* and BTF2p44.

The two *SMN* copies (*SMN1* and *SMN2*) are highly homologous, displaying only five base-pair differences

within their 3′ ends (Bürglen et al. 1996*a*). Two of these base-pair exchanges, in exons 7 and 8, allow us to distinguish *SMN1* from *SMN2* on DNA and RNA level and are currently used for direct diagnosis of SMA. Eighty percent to 98% of patients with SMA show homozygous absence of at least exon 7 of *SMN1* (Δ7SMN1) with slightly higher frequencies in types I and II than in type III (Bussaglia et al. 1995; Chang et al. 1995; Cobben et al. 1995; Hahnen et al. 1995; Lefebvre et al. 1995; Rodrigues et al. 1995; Parsons et al. 1996; Velasco et al. 1996; Simard et al. 1997). Different mechanisms, deletions, and gene conversions have been shown to be responsible for the absence of *SMN1* on SMA chromosomes (Hahnen et al. 1996; van der Steege et al. 1996; Burghes 1997; Campbell et al. 1997; DiDonato et al. 1997). Deletions were usually found in those SMA patients with severe phenotype, whereas gene conversions resulting in an increase of *SMN2* copy number were usually associated with mild phenotypes. In rare cases, homozygous absence of *SMN1* has been reported in asymptomatic individuals (Cobben et al. 1995; Hahnen et al. 1995; Wang et al. 1996). Consequently, doubts about *SMN1* as the SMA-determining gene have arisen but have been eliminated by the identification of several independent intragenic *SMN1* mutations (Bussaglia et al. 1995; Lefebvre et al. 1995; Brahe et al. 1996; Bürglen et al. 1996*b*; Parsons et al. 1996; Hahnen et al. 1997; McAndrew et al. 1997; Rochette et al. 1997; Talbot et al. 1997; Parsons et al. 1998; Wang et al. 1998).

The SMN protein is ubiquitously expressed, interacts with several other proteins and itself, and has an essential role in the spliceosomal snRNP biogenesis (Fischer et al. 1997; Liu et al. 1997; Lorson et al. 1998). The identification of several missense mutations has proved to be very helpful in disclosing the role and function of the SMN protein. Lorson et al. (1998) have shown that the self-oligomerization capacity of the SMN protein is decreased in patients carrying missense mutations in exon 6 of *SMN1*.

The finding of intragenic mutations in patients who retained *SMN1* (nondeleted SMA patients) has been considerably improved by the establishment of a PCR-based quantitative gene dosage test of the *SMN1* copy number prior to mutation analysis (McAndrew et al. 1997; Rochette et al. 1997). This test allows distinction between patients with SMA carrying one or more *SMN1* copies. On the basis of the high frequency (~90%) of homozygous absence of *SMN1* exon 7 found in patients with SMA and according to Hardy-Weinberg equilibrium, 99.7% of all patients with SMA must carry at least one Δ7SMN1 allele on one chromosome.

McAndrew et al. (1997) found only one *SMN1* copy in 6 (8%) of 76 patients referred as individuals with potential SMA; in 2 of 6 patients the intragenic mutations were identified. In a second study, in 4 (12%) of 32 patients, one *SMN1* copy was identified, and in 2 patients, intragenic mutations were found (Rochette et al. 1997). In a previous study in which we used SSCP and subsequent sequence analysis, we found two different missense mutations in exon 6 of *SMN1* in 3 (13%) of 23 nondeleted SMA patients (Hahnen et al. 1997).

The question of how many of the typical nondeleted SMA patients carry intragenic *SMN1* mutations is still unanswered. In this article, we present an analysis of 42 typical patients with SMA who retained at least one *SMN1* copy. A quantitative nonradioactive competitive PCR showed that 45% of these patients were heterozygous for Δ7SMN1, and in all cases but one we identified the mutation. From the practical point of view, the *SMN1* heterozygosity test is an important diagnostic tool and is essential for risk calculations. We analyzed 73 SMA carriers and 42 homozygously unaffected sibs of patients with SMA to determine the validity of the test.

Subjects, Material, and Methods

Patient Samples

All patients fulfilled the diagnostic criteria for proximal SMA defined by the International SMA Consortium (1992) and Zerres and Rudnik-Schöneborn (1995). Informed consent was obtained from all subjects. Forty-two typical patients with SMA (11 type I, 8 type II, and 23 type III) who did not show homozygous absence of *SMN1* exons 7 and 8 were included in this study. Permanent lymphoblastoid cell lines were established for RNA isolation from all patients with one *SMN1* copy (except patient 1006) and from 14 patients with two *SMN2* copies. Some of these patients have been clinically described in detail (Hahnen et al. 1995, 1997). The missense mutations T274I and S262I, found in patients 1378, 2549, and 935, have been reported elsewhere (Hahnen et al. 1997).

Carriers and Noncarriers

Seventy-three carriers (parents of patients with SMA) in whom de novo mutations could be excluded, either by family history (multiplex SMA families) or by haplotype analysis with Ag1-CA and C212 and molecular analysis of *SMN* as described elsewhere (Wirth et al. 1997), and 42 homozygously unaffected sibs of patients with SMA, as determined by haplotype analysis including Ag1-CA and C212 (Wirth et al. 1995, 1997), were used to determine the validity of the nonradioactive quantitative *SMN* test.

DNA Isolation and Haplotype Analysis

DNA was isolated from blood samples or permanent lymphoblastoid cell lines by the salting-out method (Mil-

ler et al. 1988). The multicopy polymorphic markers Ag1-CA (DiDonato et al. 1994) and C212 (Melki et al. 1994) were used for haplotype analysis as described elsewhere (Wirth et al. 1995).

Quantitative Multiplex PCR

The competitive PCR we used is similar to that described by McAndrew et al. (1997); however, we modified it in a few essential points. It is a nonradioactive, competitive PCR based on different *SMN* primers: the forward primer is a mismatch primer that lies adjacent to the 1-bp exchange in exon 7 (SMN7F: 5′-CTTCCTTTTATTTTCCTTACAGGGATT-3′) and creates a *Hin*fI restriction site in *SMN1,* whereas the reverse primer lies in intron 7 (SMN7R: 5′-TCCACAAACCATAAAGTTTTAC-3′). The uncut *SMN* PCR products are 135 bp; *SMN2* contains one *Hin*fI recognition site resulting in 101-bp and 34-bp fragments, whereas *SMN1* contains two *Hin*fI restriction sites resulting in three products of 78-bp, 34-bp, and 23-bp fragments after *Hin*fI digest. Furthermore, we constructed an *SMN* internal standard that has a deletion of 21 bp compared with the genomic fragment and gives two PCR fragments, 91 and 23 bp, respectively, after *Hin*fI digest. For the construction of the *SMN* internal standard, genomic DNA of an individual who retained only *SMN1* was amplified with the primers SMN7F and SMN7R; 0.5 μl of this PCR product was reamplified with the SMN7F and SMN7R-IS (5′-TCCACAAACCATAAAGTTTTACAATGCTGGCAGACTTACTCCT-3′) primers. Subsequently, PCR products were cloned into pCRII-TOPO cloning vector (Invitrogen) according to the supplier's protocol and used as *SMN* internal standard.

The competitive quantitative PCRs were performed in 25 μl reactions that contained 200 ng genomic DNA , 1 × Cetus PCR-buffer, 200 μM of each dNTPs, 1.5 U *Taq* polymerase (Life-Technologies), 3.4×10^{-3} pg of each cloned cystic fibrosis transmembrane conductance regulator (*CFTR*) internal standard (kindly provided by T. Prior and A. H. M. Burghes) and *SMN* internal standard, 2.5 pmol of each primer CF621F (5′-AGTCACCAAAGCAAGTACAGC-3′) and CF621R (5′-GGGCCTGTGCAAGGAAGTGTTA-3′) (Zielinsky et al. 1991), and 10 pmol of each primer SMN7F and SMN7R. Cycling conditions consisted of an initial denaturation at 94°C for 7 min followed by 23 cycles at 94°C for 55 s, 51.5°C for 55 s, 72°C for 1 min, and a final extension at 72°C for 7 min in a Perkin Elmer Cetus Cycler 9600. The PCR products (10 μl) were digested with 15 U *Hin*fI (Biolabs) for 3 h at 37°C. The samples were run on 10% native polyacrylamide gels at 8 mA for 3 h and silver stained as described by Hahnen et al. (1995), followed by three-dimensional scanning of the gel (BIORAD-imaging system) and densitometric analysis with ONE-Dscan (MWG). The ratios between *SMN1*/*CFTR*, defined as standardized *SMN1* (SSMN1) values, and *SMN2*/*CFTR* defined as standardized *SMN2* (SSMN2) values, were calculated.

It has to be noted that the *CFTR* genomic and internal standard PCR products were not always equal in intensity. However, for correct quantification it is essential to obtain nearly equal intensities of the two *CFTR* bands. In those cases where the *CFTR* genomic band showed more than a ±30% deviation from the *CFTR* internal standard, the PCR was repeated with an amount of genomic DNA that has been adjusted according to the percentage of deviation. In most cases this led to satisfactory results.

RNA Isolation and RT-PCR

RNA was isolated from Epstein-Barr virus transformed lymphoblastoid cell lines by using the TRIzol kit (Life Technologies). First-strand cDNA synthesis was performed with oligo-dT primers, 4 μg total RNA and M-MLV reverse transcriptase (Life Technologies) according to the manufacture's instructions. The single stranded cDNAs were amplified with 10 ng of each primer SMN1fwd (5′-ATCCGCGGGTTTGCTATG-3′) within exon 1 and SMN8rev (5′-TCAACTGCCTCACCACCG-3′) within exon 8, 200 μM each dNTP, 2.8 U Expand High Fidelity Polymerase (Boehringer) and 1xExpand HF buffer with 15 mM $MgCl_2$. Reactions were done in 50-μl volumes. PCR conditions included a 2-min initial denaturation at 94°C followed by 30 cycles of 15 s at 94°C, 30 s at 67°C, and 1 min at 72°C. The elongation time was extended for 20 s in each subsequent cycle, beginning at cycle 11, with a final extension of 7 min at 72°C, in a Perkin Elmer Cetus Cycler 9600.

PCR of Genomic DNA

Genomic DNA of patient 2598 was amplified from *SMN* intron 6 to exon 8 as described elsewhere (Hahnen et al. 1996). The single–base pair differences in exons 7 and 8 were shown by restriction digest PCR-based assay, as described by van der Steege et al. (1995).

Long-Range PCR

In patients 4259 and 3913, the genomic fragments including exons 4–8 were amplified with the Boehringer Expand High Fidelity System by using 250 ng of genomic DNA and 10 pmol of each primer, SMN4a (5′-ATCAGATAACATCAAGCC-3′), localized in exon 4, and 541C1120 (Lefebvre et al. 1995), localized in exon 8. Cycling conditions included a 2-min initial denaturation at 94°C followed by 30 cycles of 15 s at 94°C, 30 s at 60°C, and 8 min at 68°C and a final extension of

30 min at 68°C; the elongation time increased by 20 s per cycle beginning at cycle 11.

Cloning of PCR Products and Sequencing

RT-PCR and genomic PCR products of *SMN* were cloned into the pCRII-TOPO cloning vector (Invitrogen) according to the supplier's protocol. In general, 10–20 white colonies were analyzed for their *SMN1* or *SMN2* exon 8 content as described by van der Steege et al. (1995). Clones containing *SMN1* were cultured, and the DNA was purified with a miniplasmid kit (Biorad). Cycle sequencing was performed with the BigDye Terminator Reaction Kit (PE Applied Biosystems) according to the manufacturer's instructions by using vector or internal primers. The products obtained after cycle sequencing were purified through Centri-Sep columns (Applied Biosystems). An ABI Prism 377 machine was used for sequencing. Each mutation was detected in two independent clones; the new mutations were additionally confirmed by direct sequencing of RT-PCR products of *SMN*.

Restriction Digest Assay for Detection of the Y272C Mutation

For an easy and quick identification of the most frequent missense mutation Y272C (exon 6), we established a restriction-based assay of PCR products. The forward primer (SMN-272F: 5′-TCCCATATGTCCAGATTCTC-3′) lies within exon 6; the reverse mismatch primer (SMN-272R: 5′-TACTTACCATATAATAGCCAGTGTGA-3′) lies adjacent to nucleotide exchange A→G at position 848 in exon 6 and encompasses an additional 6 bp of intron 6. The Y272C allele creates a restriction site for *Tsp*45I that cuts the 101-bp PCR product into a 75-bp and a 26-bp fragment.

Eighty nanograms of genomic DNA, 10 pmol of each primer, 120 μM dNTP, 1 × Cetus PCR buffer, and 1 U *Taq* polymerase (Life-Technologies) were amplified by means of PCR. Cycling conditions consisted of an initial denaturation at 94°C for 7 min followed by 35 cycles at 94°C for 55 s, 54°C for 55 s, and 72°C for 1 min, and a final extension at 72°C for 7 min, in a Perkin Elmer Cetus Cycler 9600. PCR products were purified through Centri-Sep columns (Applied Biosystems) for better restriction efficiency. The PCR products were digested with 20 U *Tsp*45I (Biolabs) for 4 h at 65°C. The samples were separated on 3% (2% NuSieve/1% Agarose) ethidium bromide–stained agarose gels.

Statistical Analysis

Discriminant analysis was used to achieve an optimal separation of the group of carriers from the group of noncarriers. Discriminant analysis procedures implemented in the statistical program package SAS were used (SAS 1996).

The training set for the calibration step of discriminant analysis consists of 41 noncarriers (controls) and 70 carriers. One noncarrier and three carriers were considered outliers (see validity of the test as discussed later). Because the densities were not normally distributed in either group, a nonparametric approach was taken, and the performance of classification was evaluated by cross-validation methods. A minimal error count rate of one misclassification in both groups was achieved, with a nearest-neighbor method of $k = 4$; however, values of $k < 8$ showed similar results.

Results

Establishment of a Nonradioactive Gene Dosage Test for Determining the SMN1 *and* SMN2 *Copy Number*

On the basis of the method described by McAndrew et al. (1997), we established a nonradioactive quantitative analysis test that enabled us to determine the *SMN1* and *SMN2* copy numbers. We amplified exon 3 of *CFTR,* as genomic control, using the same primers as reported by McAndrew et al. (1997). For monitoring the efficiency of the PCR, we used a cloned *CFTR* internal standard (kindly provided by T. Prior and A. Burghes) and an *SMN* internal standard (see Subjects, Material, and Methods). In contrast to the methods of McAndrew et al. (1997), we amplified *SMN* exon 7 with a mismatch forward primer that creates a *Hin*fI restriction site in *SMN1,* whereas *SMN2* remains uncut at that nucleotide position, and a reverse primer localized in intron 7. In addition, the completeness of the restriction digest can be monitored by the existence of a further *Hin*fI restriction site within the genomic *SMN* PCR products. This site has been eliminated in the *SMN*-IS, but it contains the first restriction site equivalent to *SMN1.* Thus, the *Hin*fI restriction–based assay has two advantages compared with the *Dra*I restriction–based assay (van der Steege et al. 1995; McAndrew et al. 1997): (1) all *SMN*-PCR products are cut differently after *Hin*fI restriction digest, which allows us to recognize incompletely digested samples; and (2) it is much less expensive than *Dra*I. The PCR products were separated on native 10% polyacrylamide gels and visualized by silver staining. Densitometric measurements of the gels were scored with the One-Dscan software (MWG) (fig. 1).

The reproducibility of this modified method was checked in 10 independent PCR reactions on 10 different gels by use of DNA from a control with genotype 2/2, an SMA carrier with genotype 1/2, patients with SMA (homozygous Δ7SMN1) with genotype 1/1, and a control (homozygous Δ7SMN2) with genotype 1/1. The ge-

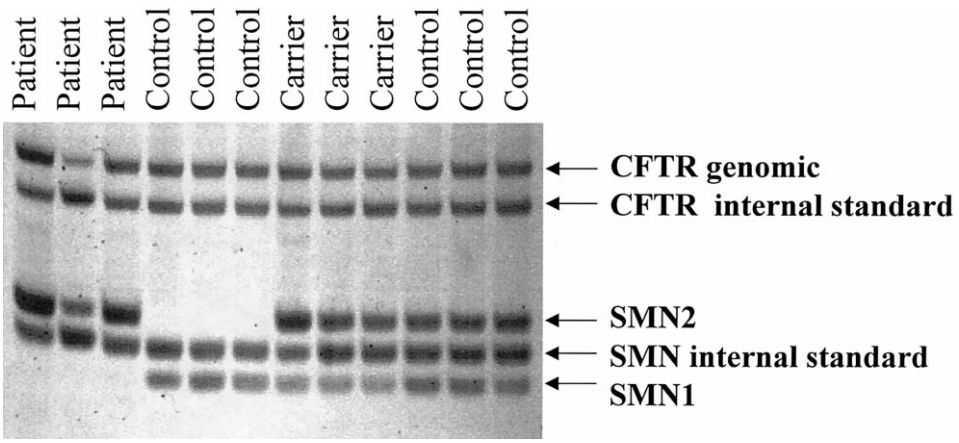

Figure 1 *SMN1* dosage analysis in patients, carriers, and controls. Example of a silver-stained PAA gel of the multiplex competitive PCR of genomic *SMN1, SMN2, SMN* internal standard, *CFTR*, and *CFTR* internal standard (see the Subjects, Material, and Methods section).

notype is defined as the number of marker alleles on each chromosome and is based on the haplotype analysis with the multicopy markers Ag1-CA and C212, both localized at the 5′ end of *SMN* (for details, see also Wirth et al. 1995). The mean ± SD SSMN1 and SSMN2 values are given in table 1. DNA from these four individuals was also used as a reference on all other gels.

Validity of the Heterozygosity SMN1 *Test*

This test represents a useful molecular genetic tool for heterozygosity screening in families with SMA as well as a screening test in the general population. To determine the validity of the test, we analyzed 73 carriers and 42 homozygously unaffected sibs of patients with SMA. The SSMN1 values obtained from the quantitative analysis are presented in figure 2. All but 1 of the 42 noncarriers presented two *SMN1* copies, with SSMN1 values in the range 0.65–1.33 (mean SSMN1 value ± SD: 1.01 ± 0.08). One of 42 presented three *SMN1* copies (SSMN1 value 1.61). Among the carriers, three patients definitively showed SSMN1 values in the interval corresponding to two *SMN1* copies. The remaining 70 carriers showed SSMN1 values between 0.21 and 0.67 (mean SSMN1 ± SD: 0.40 ± 0.07). On the basis of this data set, 4 (3.8%) of 115 carriers and noncarriers showed two *SMN1* copies per chromosome.

SSMN1 values of carriers and noncarriers show considerable scatter around their group means. There is a small region of overlap between both groups and a clear separation of group means (fig. 2). In a diagnostic situation, a test item is to be classified into either one of the two reference groups on the basis of empirical reference data and appropriate statistical procedures and interpretation.

Discriminant analysis is among the adequate procedures in situations like this. First, the reference data are evaluated (calibration step), and second, the test item is assigned to one or the other group and the uncertainty of the classification is calculated. However, the derivation of the optimal discriminant function may not be trivial. We chose a nonparametric approach and proved its robustness by extensive evaluations (see Subjects, Material, and Methods).

For the critical range of values $0.75 \geqslant \text{SSMN1} \geqslant 0.60$, the posterior probabilities for classification into either one group of carriers or noncarriers is given in table 2. Below and above this range, posterior probabilities are unity and zero, respectively.

Quantitative SMN *Analysis and Mutation Analysis in Nondeleted SMA Patients*

Five hundred twenty-five patients with SMA with typical SMA features and neurogenic findings on electromyogram (EMG) and muscle biopsy were screened for homozygous absence of *SMN1* exons 7 and 8 (table 3). Eleven (4.1%) of 270 type I, 8 (6.5%) of 124 type II, and 23 (17.6%) of 131 type III SMA patients retained at least one *SMN1* gene copy. Subsequently, the 42 nondeleted SMA patients were subjected to a quantitative analysis of *SMN1* and *SMN2* copy number analysis. Nineteen (45.2%) of 42 patients with SMA (6 type I, 6 type II, and 7 type III) showed a gene dosage corresponding to one copy of *SMN1,* whereas 23 (54.8%) of 42 patients with SMA (5 type I, 2 type II, and 16 type III) showed a gene dosage corresponding to two *SMN1* copies or within the overlapping interval. We assigned the three patients with SSMN1 values within the overlapping region to the group of patients carrying two

SMN1 copies (table 3). Five (22%) of 23 SMA patients with two *SMN1* copies showed homozygous absence of *SMN2* exons 7 and 8 (table 4).

Next we searched for small intragenic mutations within the *SMN1* copy. In 17 of 19 Δ7SMN1 heterozygous patients with SMA, lymphoblastoid cell lines were established for RNA isolation. In all patients, we identified the mutation by sequencing the complete coding region of *SMN1* from cloned RT-PCR products or by additional analysis of intronic sequences.

In 9 of 17 patients with SMA we identified three different missense mutations: Y272C (5 patients), T274I (3 patients), and S262I (1 patient), localized in exon 6 (table 4). All three mutations had been reported elsewhere (Lefebvre et al. 1995; Hahnen et al. 1997; McAndrew et al. 1997; Rochette et al. 1997). The following six mutations are described for the first time in this study.

A nonsense mutation Q15X (exon 1) was found in two independent type I (3952) and type III (2385) SMA patients and is caused by a C→T substitution at nucleotide 78 that replaces glutamine by a premature stop codon. This replaces the 294 amino acid SMN protein by a very short truncated peptide of only 14 amino acids (fig. 3*a*).

In patients 19 (type II), 1927 (type III), and 2833 (type II), we identified three different frameshift mutations that were caused by a 1-bp insertion ATTCT→ATTTCT at nucleotide 122-124 (exon 2a) designated as 124insT, a 4-bp insertion ACCT→ACGTGTCT at nucleotide 241 (exon 2b) designated as 241-242ins4, and a 1-bp deletion of an A (CAAAT→CAAT) at nucleotides 589–591 (exon 4), designated as 591delA, respectively (figs. 3*b*–3*d*). All three frameshift mutations lead to premature stop codons 1, 2, and 26 amino acids, respectively, after the mutation.

Patients 4259 and 3919, both with type I SMA, showed an abnormal RT-PCR product of smaller size. Sequence analysis of *SMN1* cDNA clones showed a complete deletion of exons 5 and 6. To determine the exact breakpoints of the deletion, we performed a long-range PCR of genomic DNA from exons 4–8 in patient 4249 and his parents (fig. 4*a*). Two fragments were obtained in the patient and his father: one of 10 kb, corresponding to *SMN2,* and one of 3.4 kb, corresponding to the deleted *SMN1*. By cloning and sequencing the 3.4-kb fragment we identified the deletion breakpoint region between nucleotides 28621 and 28647 in intron 4 and between nucleotides 35282 and 35309 in intron 6 (corresponding to PAC125D9 sequence in reverted and complemented version; GenBank accession number U80017; Chen et al. 1998), which resulted in a 6.6-kb deletion that encompasses complete exons 5 and 6, intron 5, and part of introns 4 and 6. Both breakpoint ends lie within Alu-repetitive elements that share 82% identity between nucleotides 28544 and 28711 in intron 4 and nucleotides 35205 and 35372 in intron 6 and show a 100% identical 27-bp deletion breakpoint region (fig. 4*b*). Both Alu repeats are oriented *3′–5′*. The 26-bp core Alu se-

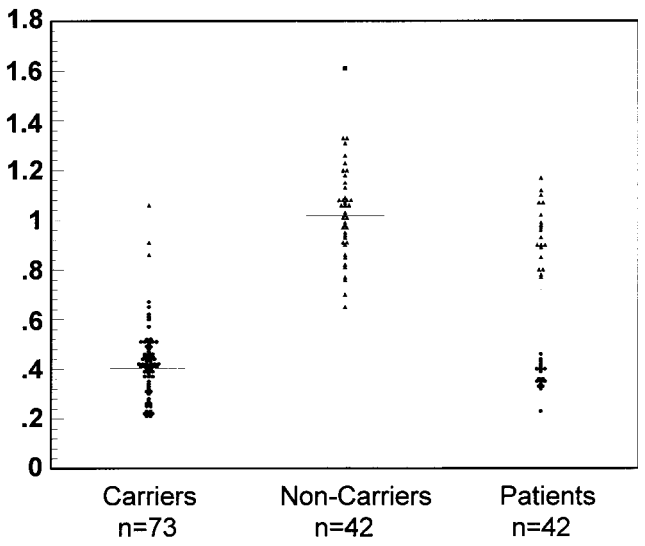

Figure 2 Plot of the SSMN1 values determined in carriers, noncarriers, and patients with SMA who failed to show homozygous absence of *SMN1*. Circles correspond to one *SMN1* copy, triangles to two *SMN1* copies, and squares to three *SMN1* copies, and the open rhombus represents the values within the uncertain interval between one and two *SMN1* copies (0.60–0.75). The average values for one *SMN1* and two *SMN1* copies are marked with a horizontal line.

Table 1

Reproducibility of *SMN1* and *SMN2* Copy Number

Subject	Genotype[a]	SSMN1 Values (mean ± SD)	*SMN1* Copies	SSMN2 Values (mean ± SD)	*SMN2* Copies
Control	2/2	.87 ± .14	2	1.39 ± .09	2
Carrier	2/1	.31 ± .05	1	.82 ± .08	2
SMA patient (homozygous Δ7SMN1)	1/1	…	…	.85 ± .08	2
Control (homozygous Δ7SMN2)	1/1	.91 ± .07	2	…	…

[a] According to Ag1-CA and C212 marker alleles.

Table 2

Posterior Probabilities for Classification into the Carrier or Noncarrier Group

Test Value of SSMN1	Classified into Group	Posterior Probability Carrier	Posterior Probability Noncarrier
≤.60	Carrier	1.0000	.0000
.61	Carrier	.7455	.2545
.62	Carrier	.7009	.2991
.63	Carrier	.6373	.3627
.64	Carrier	.6373	.3627
.65	Carrier	.6373	.3627
.66	Noncarrier	.4677	.5323
.67	Noncarrier	.3694	.6306
.68	Noncarrier	.3694	.6306
.69	Noncarrier	.3694	.6306
.70	Noncarrier	.3694	.6306
.71	Noncarrier	.2265	.7735
.72	Noncarrier	.1633	.8367
.73	Noncarrier	.1633	.8367
.74	Noncarrier	.1277	.8723
≥.75	Noncarrier	.0000	1.0000

NOTE.—Based on nonparametric discriminant analysis with prior probabilities of .50 (SAS Program; see Subjects, Material, and Methods section).

quence responsible for homologous and nonhomologous recombinations (Rüdiger et al. 1995) is localized 4 bp upstream of the 27-bp deletion breakpoint region. This is the first Alu-mediated large deletion within *SMN1* identified so far.

Sequencing of *SMN1* RT-PCR products from patient 2598 showed no full-length *SMN1,* but only the isoform Δ7SMN1 cDNA, which indicates that a splice site mutation or deletion of exon 7 must have occurred. PCR amplification of genomic DNA from intron 6 to exon 8, followed by cloning and sequencing of *SMN1* clones, disclosed a T→G nucleotide exchange at position 6 within the intron 7 donor splice site (c.922+6T→G; fig. 3*e*).

For patients 1006 and 4133, no RNA was available; SSCA of each *SMN* exon in patient 1006 failed to show any aberrant band. Haplotype analysis in the family of patient 4133 displayed the same haplotype for Ag1-CA and C212 as found for the other five patients carrying the missense mutation Y272C (table 4). Because of the high frequency of this mutation, we established a PCR-based assay by using a mismatch primer that allows easy detection of this mutation (see Subjects, Material, and Methods). Patient 4133 showed the typical pattern for patients or carriers with the Y272C mutation (fig. 5). In summary, nine different mutations in 18 unrelated patients with SMA were found: three missense mutations, Y272C (6 patients), T274I (3 patients), and S262I (1 patient); one nonsense mutation Q15X (2 patients); three frameshift mutations, 124insT (1 patient), 241-242ins4 (1 patient), and 591delA (1 patient); one deletion of exons 5 and 6 (2 patients); and one splice site mutation, c.922+6T→G (1 patient).

Sequence Analysis of SMN1 *in Patients with SMA with Two* SMN1 *Copies*

Twenty patients with typical SMA each had two *SMN1* copies. In three further cases (2544, 1377, and 2245), the SSMN1 values lie within the overlapping intervals. Although it is very unlikely that these SMA patients, all but one of whom have nonconsanguineous parents, have 5q-unlinked SMA, we decided to sequence *SMN1* to eliminate any doubt concerning a possible case with two subtle mutations. RNA was available from 14 of 23 patients, which allowed us to sequence at least one copy of the complete coding region of *SMN1*. These 14 patients also include patients 2544 and 2245 (with values within the overlapping interval) and all patients with homozygous deletions of *SMN2*. In all cases we identified an intact *SMN1* (table 4). In addition, in four families, a second unaffected sib showed identical haplotypes within the SMA region, compared with the affected sib. In all cases in which DNA from parents was available for testing, two *SMN1* copies were found. All together, these findings give strong evidence of the existence of gene(s) responsible for ~4%–5% of phenotypes indistinguishable from SMA.

Correlation of Intragenic SMN1 *Mutations,* SMN2 *Copy Number, and Severity of SMA*

To look for evidence of founder mutations, we performed haplotype analysis with the multicopy markers Ag1-CA and C212 and determined the parental origin of the subtle mutation by quantitative analysis of *SMN1* in the parents (table 4). In all 17 of 19 cases in which the parents were available, one parent was Δ7SMN1 heterozygous and the other had two *SMN1* copies, which indicates that the latter parent must be the carrier of a subtle mutation. In each case in which identical mutations were identified in at least two independent patients, these were found on identical haplotypes, providing evidence of founder mutations (table 4). The missense mutation Y272C was found in 6 (33%) of 18 patients, representing the most frequent intragenic mutation. It was identified in all three types of SMA and was always present on the same haplotype: 28/29 alleles for C212 and 108/112 alleles for Ag1-CA. The consensus haplotypes for the other mutations are given in table 4.

To answer the question whether the severity of SMA in patients with identical intragenic mutations but different phenotypes is influenced by the *SMN2* copy number, we also determined the number of *SMN2* copies. In

Table 3

Total No. and Frequency of Mutations Found in Patients with Type I, Type II, or Type III SMA

SMA Type	No. of Patients	No. (%) of SMA Patients						
		Homozygous for Δ7SMN1	Nondeleted SMA	Heterozygous for Δ7SMN1	With at Least Two SMN1 Copies	With Subtle Mutations in SMN1	With at Least One Δ7SMN1	With Mutations on Both Chromosomes
I	270	259 (96)	11 (4.1)	6 (2.2)	5 (1.9)	6 (2.2)	265 (98.1)	265 (98.1)
II	124	116 (93.5)	8 (6.5)	6 (4.8)	2 (1.6)	6 (4.8)	122 (98.4)	122 (98.4)
III	131	108 (82.4)	23 (17.6)	7 (5.3)	16 (12.2)	6 (4.6)	115 (87.8)	114 (87)
Total	525	483 (92)	42 (8)	19 (3.6)	23 (4.4)	18 (3.4)	502 (95.6)	501 (95.1)

the six patients who carry the Y272C mutation, there were two *SMN2* copies for patients with type I SMA (patients 1307, 1023, 3551), two and three *SMN2* copies for type II SMA (patients 44 and 4133, respectively), and three *SMN2* copies for type III SMA (patient 1030), which indicates that this is a severe mutation and that the severity of SMA is influenced by the *SMN2* copy number. The T274I mutation was found in one patient with type II (patient 1378) and two patients with type III SMA (patients 2549 and 2692), with one *SMN2* copy in patients with type II and two *SMN2* copies in patients with type III. Thus, patient 1378 has type II SMA despite carrying only one *SMN2,* whereas patients 2549 and 2692 developed type III SMA in the presence of only two *SMN2* copies, which indicates that T274I is a mild mutation. Similarly, patient 935, with the missense mutation S262I, has type III SMA even in the presence of only one *SMN2,* which indicates that this is a very mild mutation.

Patients 4259 and 3919, with *SMN1* mutations encompassing larger deletions (exons 5 and 6), and patient 3952, with a nonsense mutation in exon 1 (Q15X), developed type I SMA in the presence of two *SMN2* copies, which indicates that this mutation acts similar to a deletion of the complete *SMN1.* However, patient 2385, who carries the same nonsense mutation (Q15X), showed three or four *SMN2* copies and thus developed type III SMA. Patient 2598, with the splice site mutation in intron 7, shows a type III SMA in the presence of two *SMN2* copies, which suggests that the skipping of *SMN1* exon 7 only is a mild mutation.

These data clearly show that the severity of the phenotype is determined by the mutations from both homologs and the *SMN2* copy number. Nevertheless, two multiplex families, the first with three affected brothers and the genotype Y272C/Δ7SMN1 and the second with two affected brothers and the genotype T274I/Δ7SMN1, showed the same number of *SMN2* copies despite phenotypic discrepancies, types IIIa and IIIb (table 5). According to Zerres and Rudnik-Schöneborn (1995) patients with type IIIa SMA have an age at onset of <3 years, and patients with type IIIb SMA, >3 years, the latter showing a mild course with a high probability of remaining ambulant for decades. This suggests that the severity of SMA is also influenced by factors still unknown.

Identification of a De Novo Gene-Conversion Event by Quantitative Analysis of SMN Gene Copies

In a previous study we reported a 2% de novo mutation frequency in patients with SMA (Wirth et al. 1997). In all but one family, we identified a paternal origin of the de novo mutation by haplotype analysis with the multicopy markers Ag1-CA and C212, which showed paternal allele loss of these markers. In one case, family G, the only difference between the affected and unaffected haploidentical sibs is the homozygous absence of *SMN1.* We concluded that a gene-conversion event is responsible for the de novo mutation. By quantitative analysis we were now able to show that the unaffected brother carries two *SMN2* copies besides one *SMN1* copy, whereas his affected brother carries three *SMN2* copies, which indicates that *SMN1* has been converted into *SMN2.* Furthermore, we determined the parental origin of the de novo mutation. Whereas the father carries one *SMN1,* the mother has two *SMN1* copies, which indicates that the de novo mutation must be of maternal origin. Finally, we confirmed the paternal origin of the de novo mutation in the other six families reported elsewhere (Wirth et al. 1997). In all six families, the fathers carried two *SMN1* copies, whereas the mothers had only one *SMN1* copy.

Discussion

Quantitative SMN1 *Analysis in Nondeleted SMA Patients*

It is now well known that homozygous absence of at least exon 7 of *SMN1* is the major defect in patients with SMA. Overall, the deletion frequency is in the range 92%–100% in type I, 82%–100% in type II, and 78%–100% in type III (Bussaglia et al. 1995; Chang et al. 1995; Cobben et al. 1995; Hahnen et al. 1995; Le-

Table 4

Quantitative, Mutation, and Haplotype Analysis in Nondeleted Type I, Type II, or Type III SMA Patients

Patient Sex	DNA Number	SMA Type	SSMN1 Value	*SMN1* Copies	SSMN2 Value	*SMN2* Copies	Type of Mutation[a]	Mutation	Haplotype C 212 (P/M)	Haplotype Ag1-CA (P/M)	Parental Origin of Mutations	FH[b]
1, F	1307	I	.40	1	1.15	2	Missense	Y272C	31–28 29	102–108 112	M	0/1/0
2, F	1023	I	.39	1	1.10	2	Missense	Y272C	28 29–25	108 112–114	P	0/2/0
3, M	3551	I	.46	1	1.29	2	Missense	Y272C	28 29–34	108 112–106	P	0/0/0
4, F	44	II	.34	1	1.46	2	Missense	Y272C	28 29–29	108 112–110	P	1/0/0
5, M	4133	II	.43	1	1.59	3	Missense	Y272C	27 29–28 29	114 116–108 112	M	0/0/0
6, M	1030	III	.35	1	2.06	3	Missense	Y272C	28 29–29 30	108 112–110	P	2/2/0
7, M	1378	II	.40	1	.61	1	Missense	T274I	del –25 29	del –108 114	M	2/0/0
8, M	2549	III	.33	1	1.09	2	Missense	T274I	32–25 29	100 108 114	M	1/0/0
9, M	2692	III	.32	1	1.03	2	Missense	T274I	25 29 28	108 114 104	NA	0/0/0
10, M	935	III	.42	1	.54	1	Missense	S262I	27–33	110–102	P	0/1/0
11, F	3952	I	.35	1	1.05	2	Nonsense	Q15X	34–27 28	106–114 118	M	0/0/0
12, F	2385	III	.33	1	1.80	3	Nonsense	Q15X	27 28 29	108 110 114 118	NA	NA
13, M	19	II	.44	1	1.19	2	FS	124insT	29–24 31	108 114–100 112	M	0/0/0
14, M	1927	III	.40	1	1.83	3	FS	241-242ins4	29 30–22 30	108 110–114	P	1/1/0
15, F	2833	II	.36	1	1.13	2	FS	591delA	29 31 33 34	106 106 108	NA	1/0/0
16, F	4259	I	.41	1	1.17	2	Deletion	Exons 5/6	28 30–32	108 108–100	P	0/0/0
17, M	3919	I	.23	1	1.48	3	Deletion	Exons 5/6	28 30–26 28	108 108–108 112	P	0/0/0
18, F	2598	III	.36	1	1.29	2	Spl site	c.922+6T→G	32–28 29	102–110 114	M	0/0/0
19, M	1006	II	.35	1	1.50	3	RNA NA	…	28 30–29 31	108 112–108 116	M	0/1/0[c]
20, M	3612	I–II	1.02	2	del	…	…	None	34 26 30	108 108 112	…	0/0/0
21, M	2395	III	.98	2	del	…	…	None	27–28	114–112	…	0/1/1
22, M	2161	III	1.07	2	del	…	…	None	30 27–27	114–108 112	…	0/1/0
23, M	2544	III	.62	1/2	del	…	…	None	27 28 30	108 114 118	…	NA
24, F	2128	III	.78	2	del	…	…	None	29–34	108–106	…	0/1/1[d]
25, M	1058	I	.96	2	.65	1	…	None	26 28–28	108 116–112	…	0/1/1
26, M	1377	I	.68	1/2	.45	1	…	ND	30–25 29	108–114	…	0/1/0
27, F	621	I	.77	2	1.05	2	…	ND	29–28 30	110–108 114	…	0/1/1
28, M	1151	I	1.12	2	.80	2	…	ND	28 30–27	108 114–112	…	0/1/0
29, F	2192	I	.80	2	.58	1	…	ND	27 30 32 35	100 102 108 112	…	NA
30, M	751	II	1.10	2	.62	1	…	ND	29–23 28	112–114	…	0/1/0
31, M	968	III	.90	2	.68	1	…	None	29 32 34	106 112	-	NA
32, M	2869	III	.99	2	1.30	2	…	ND	24 26 27 28	108 110 114	…	NA
33, F	938	III	.89	2	1.15	2	…	ND	28 29–24 27	108 116–108 114	…	0/3/0
34, M	2436	III	.86	2	.49	1	…	None	31 32–23 25	100 106–108 114	…	0/0/0
35, M	3488	III	.90	2	1.00	2	…	None	26 27 28	108 114 114	…	0/0/1
36, F	2495	III	.93	2	.53	1	…	ND	23 24 26	102 110 112	…	0/0/0
37, M	2188	III	.97	2	1.21	2	…	None	34 36–26 28	100 106–112	…	0/0/0
38, M	2245	III	.73	1/2	.40	1	…	None	24 27 32 36	102 106 110 112	…	NA
39, F	1514	III	1.17	2	.46	1	…	ND	31 33 34 35	102 106 108 110	…	NA
40, M	2272	III	1.07	2	.70	1	…	None	27 28 33	102 108 114	…	NA
41, M	235	III	.80	2	.59	1	…	None	27 29 29	108 114 116	…	NA
42, M	3630	III	.90	2	1.88	3	…	None	32 32 32 32?	106 106 106 106?	…	NA

NOTE.—NA = not available, ND = not determined, P = paternal, and M = maternal. Haplotype carrying the intragenic mutation is marked with underlined allele numbers.

[a] FS = frameshift, and Spl site = splice site.

[b] FH = family history. Data are no. of haploidentical affected siblings/no. of nonhaploidentical unaffected siblings/no. of haploidentical unaffected siblings.

[c] Pseudodominant family, affected father.

[d] Consanguineous family.

febvre et al. 1995; Rodrigues et al. 1995; Parsons et al. 1996; Velasco et al. 1996; Simard et al. 1997). These discrepancies may be partly due to different ethnic origin and partly to misdiagnoses but are essentially due to true differences between the three SMA phenotypes. McAndrew et al. (1997) and Rochette et al. (1997) recently suggested use of the quantitative analysis of *SMN1* for determining Δ7SMN1 heterozygous patients before performing intragenic mutation analysis. Both articles reported analysis of patients who were referred for routine diagnosis without being sufficiently investigated clinically. Consequently, they found one *SMN1* in only

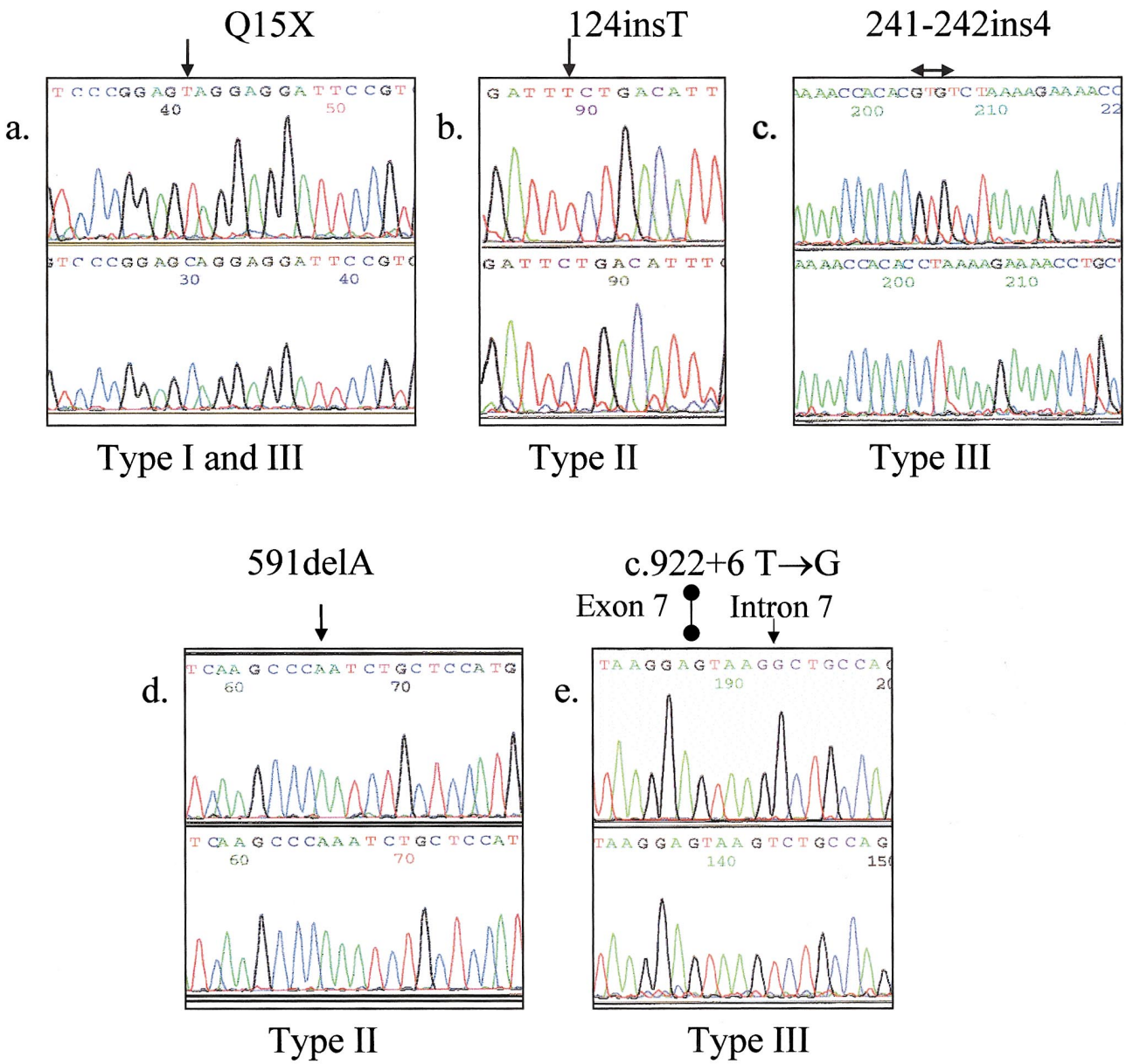

Figure 3 New intragenic *SMN1* mutations in patients with SMA. Mutant sequences of patients (*upper*) *a*, 3952; *b*, 19; *c*, 1927; *d*, 2833; *e*, 2598; and control sequences (*lower*) are shown. Arrows indicate the positions of the mutations. Bars indicate exon-intron boundaries.

6 (8%) of 76 (McAndrew et al. 1997) and 4 (12%) of 32 (Rochette et al. 1997), and in 3 of 6 and 2 of 4 intragenic mutations, respectively.

To obtain reliable data concerning the frequency of intragenic mutations among nondeleted SMA patients, we included in this study only patients with well-characterized SMA with typical features of proximal SMA, which was supported by EMG and muscle biopsy findings. The distribution of patients without the deletion among the three types of SMA in this study is 4.1% type I, 6.5% type II, and 17.6% type III SMA (table 3) and corresponds to our previously reported frequencies of nondeleted SMA patients (Hahnen et al. 1995). In contrast to the low frequency of heterozygous SMA patients identified by McAndrew et al. (1997) and Rochette et al. (1997), we determined a heterozygous Δ7SMN1 status (one *SMN1* copy) in 45.2% of nondeleted SMA patients (six type I, six type II, and seven type III). Furthermore, our data show that quantitative analysis followed by sequencing of the complete coding region is very efficient and allows the identification of almost all mutations (18 of 19). The only Δ7SMN1 heterozygous

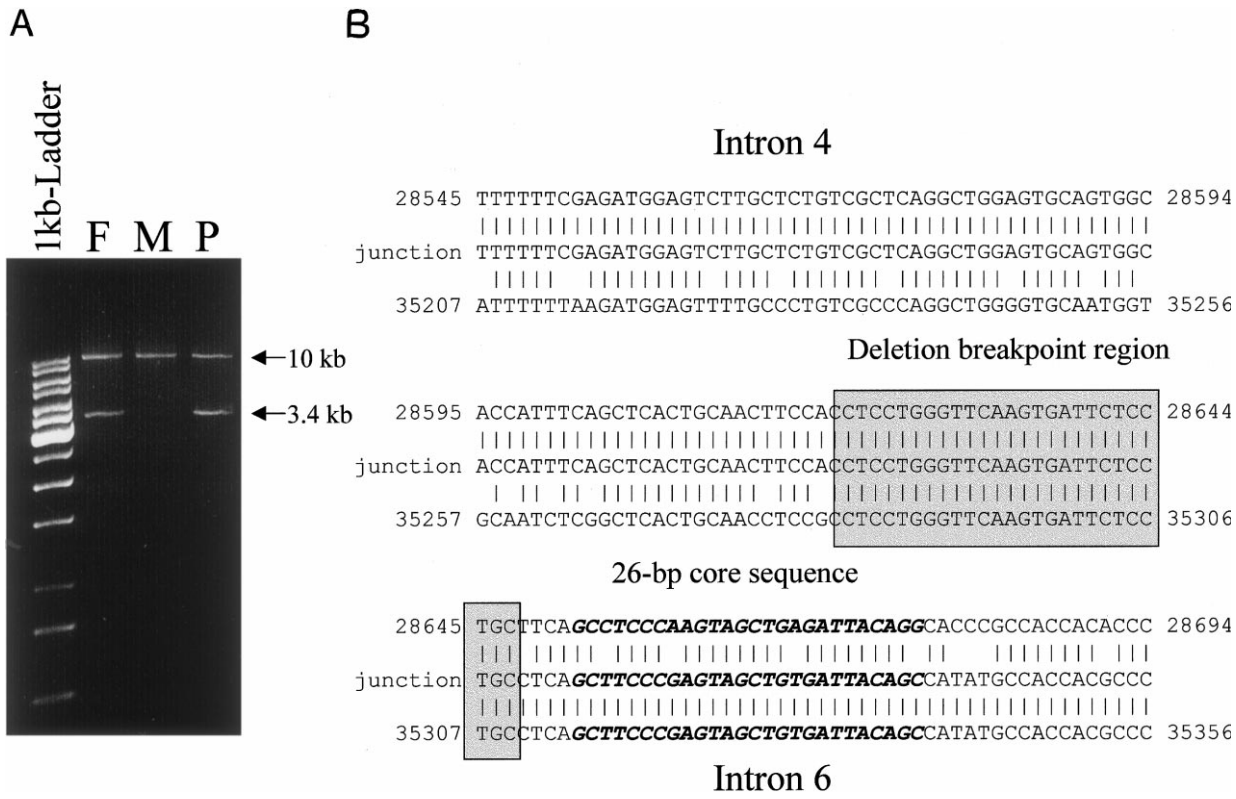

Figure 4 Alu-mediated deletion in patient 4259 with SMA. *A,* Long-range PCR products between exons 4 and 8 from father (F), mother (M), and patient (P) were separated on ethidium bromide–stained agarose gels. The normal, full-length SMN-PCR product is 10 kb, whereas the deleted product is 3.4 kb. The size marker is the 1-kb ladder (MBI-Fermentas). *B,* Sequence homology of the Alu-repetitive elements in introns 4 and 6 to the junction fragment of patient 4259 is shown. The deletion breakpoint region is marked with a gray box; the 26-bp core sequence is written in italics and bold letters.

SMA patient (patient 1006) in whom we did not identify the mutation is the son of an affected father, who showed homozygous absence of *SMN1;* therefore the son is an obligate carrier of Δ7SMN1. In addition, because no RNA was available, the result is not conclusive.

In a previous study, we analyzed 23 patients with typical SMA by SSCA of each exon, followed by sequencing, and identified the mutation in 3 (13%) of 23 patients (Hahnen et al. 1997). The actual study led to the identification of mutations in six other patients of the same cohort. None of the mutations was a deletion or inversion of a complete exon. This clearly shows the limitations of SSCA compared with direct sequencing. Because the assignment of the mutation requires the sequencing of cloned *SMN1* gene products and because the coding region of *SMN1* is small (<1 kb), it is far more sensible to perform sequencing directly instead of performing SSCA after the quantitative analysis.

The percentage of subtle mutations, 3.4%, can now be added to the percentage of homozygous absence of *SMN* exon 7, so that mutations on both chromosomes are found in 95.1% of patients with SMA (98.1% type I, 98.4% type II, and 87% type III) (table 3). On the basis of a heterozygosity frequency of 1:50 for an SMA mutation in the general population, the frequency for a subtle mutation is ~1:3,000, which would account for an incidence of ~1:35,000,000 for SMA with two subtle mutations.

Evidence for SMN1*-Unlinked SMA Patients*

Twenty-three patients with typical SMA showed two *SMN1* copies, which suggests an *SMN1*-unlinked SMA. All but 1 of the 23 SMA patients with two *SMN1* copies were derived from nonconsanguineous families, which makes the existence of intragenic mutations on both chromosomes extremely unlikely. Four of 23 patients, including the 1 from the consanguineous family, have 1 unaffected haploidentical sib. A homozygous absence of *SMN2* was found in 5 (22%) of 23 patients compared with only 3%–4% in the normal population. However, this proportion may be biased and caused by our special attention given to patients carrying homozygous absence of *SMN2* during the collection process of patients with

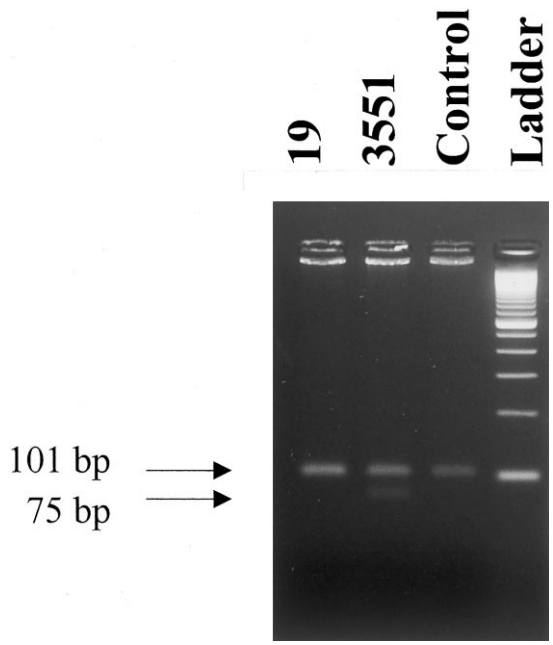

Figure 5 Restriction digest PCR-based assay for detection of the Y272C mutation. PCR product obtained from DNA of patient 3551, who carries the Y272C mutation, are cut with *Tsp*45I into 75-bp and 26-bp fragments; individuals without this mutation show a 101-bp uncut PCR fragment on a 3% ethidium bromide–stained agarose gel.

SMA in the past few years. The sequencing results of the complete coding region of the *SMN1* gene in 14 of 23 patients (including the 5 patients with homozygous absence of *SMN2*) showed an intact *SMN1* gene in each case. Furthermore, on the basis of our actual knowledge concerning the essential function of *SMN* in spliceosomal snRNP biogenesis (Fischer et al. 1997; Liu et al. 1997), the findings that the absence of *SMN* in knockout mice is lethal in the early embryonic state (Schrank et al. 1997) and that homozygous absence of both *SMN1* and *SMN2* has never been observed, obviously being a lethal state, indicate that patients with homozygous absence of *SMN2* must have at least one functional *SMN1*. This suggests that, in as little as 4%–5% (1.9% type I, 1.6% type II, and 13% type III) of all patients with SMA, gene(s) other than *SMN1* are very likely responsible for the SMA phenotype. We can speculate, on the basis of these findings, that other genes encoding SMN-interacting proteins, such as SIP1 and other not-yet-characterized proteins (Fischer et al. 1997; Liu et al. 1997), may be responsible for an SMA-like phenotype.

Intragenic SMN1 *Mutations*

In 18 of 19 heterozygous Δ7SMN1 patients, we identified nine different intragenic mutations, six of which were new. In more than half of the cases (10 of 18), we identified three different missense mutations, Y272, T274I, and S262I, that have been reported elsewhere in single cases (Lefebvre et al. 1995; Hahnen et al. 1997; McAndrew et al. 1997; Rochette et al. 1997). All three mutations are localized within exon 6 and affect the *SMN-SMN* oligomerization capacity (Lorson et al. 1998). The nonsense mutation, Q15X, and the three frameshift mutations, 124insT, 241-242ins4, and 591delA, lead to SMN-truncated products of various lengths and thus to nonfunctional SMN1 proteins.

Here we present the first Alu-mediated deletion within *SMN1* in two independent patients with SMA. It has been shown that Alu repeats are five times more abundant within a 131-kb region that encompasses *SMN* and *NAIP* than in genomic DNA in general (Chen et al. 1998). Alu repeats are frequently responsible for aberrant recombination leading to deletions, duplications, or inversions (Rüdiger et al. 1995). Rüdiger et al. (1995) have found that a 26-bp core sequence within the Alu repeat, which is highly conserved and shows homology to the 8-bp χ sequence known to stimulate recBC-mediated recombination in prokaryotes, is a hot spot of recombination in several disease genes. We have shown that the two Alu repeats in introns 4 and 6 (the deletion occurred between these two introns) were 86% homologous, both oriented 3′–5′, and that the deletion breakpoint region lies a few base pairs upstream of the 26-bp Alu core sequence. This finding is in agreement with the observation described for other disease genes, such as the α-globin gene cluster (Harteveld et al. 1997), hereditary nonpolyposis colon cancer (Nyström-Lahti et al. 1995), BRCA1 (Petrij-Bosch et al. 1997), and others, that Alu-mediated deletions occur near or at the core sequence. Alu-mediated deletions and duplications might be a frequent phenomenon in SMA, although this has not yet been reported. The existence of two highly homologous *SMN* copies, the variability in *SMN* copy number, and the lack of sequence discrepancies from exon 1 to intron 6 hamper the identification of deletions, duplications, and their breakpoints.

Here we present a splice site mutation that occurred within the donor splice site consensus sequence of intron 7 at position +6 (T→G) and resulted in *SMN1* transcripts lacking exon 7 (for further details see Lorson et al. [in press]). Two further splice site mutations caused by deletions in the acceptor splice site (c.868-10del7) and in the donor splice site (c.922+3del4) of exon 7 have been reported (Lefebvre et al. 1995). This splice site mutation in patient 2598 that produces Δ7SMN1 transcript only confers further evidence for the functional relevance of *SMN1* exon 7.

Genotype-Phenotype Correlation

The severity of the SMA phenotype is dependent on the mutations on both chromosomes (intragenic muta-

Table 5

Quantitative and Haplotype Analysis in Two Families with Siblings with Discordant SMA Phenotypes and Intragenic Mutations

Family and DNA Number	Sex	SMA Type	Genotype	Age			Motor Milestones (age [years])	SSMN1 Value	*SMN1* Copies	SSMN2 Value	*SMN2* Copies
				Current (years)	At Onset (years)	At Diagnosis (years)					
244:											
1029	M	IIIa	Δ7SMN1/Y272C	35	1.4	2.8	Wheelchair (26)	.27	1	1.83	3
1030	M	IIIb	Δ7SMN1/Y272C	31	5	10	Still walking	.35	1	2.06	3
1019	M	IIIb	Δ7SMN1/Y272C	39	16	18	Still walking	.21	1	1.83	3
614:											
2593/B1	M	IIIa	Δ7SMN1/T274I	20	1.4	2.8	Wheelchair (5)	.40	1	1.28	2
2549	M	IIIb	Δ7SMN1/T274I	24	3.5	4.1	Wheelchair (16)	.33	1	1.09	2

tion +Δ7SMN1 caused either by deletion or gene conversion of *SMN1*) and by the total number of *SMN2* copies. Four of the subtle mutations (Y272C, T274I, Q15X, and the Alu-mediated deletion of exons 5 and 6) were found in at least two independent patients with SMA, always on the same haplotype background, which indicates that they are founder mutations. The most abundant mutation, Y272C, was found in 6 (33.3%) of 18 independent patients with SMA types I–III. This mutation has been reported previously in two patients with type I SMA (Lefebvre et al. 1995; Rochette et al. 1997). Similar to the patient with type I SMA reported by Rochette et al. (1997), the three patients with type I SMA presented here carry two *SMN2* copies, whereas the patient with type III SMA carries three *SMN2* copies and the two patients with the intermediate form carry two and three *SMN2* copies, which confirms the role of *SMN2* in modulating the SMA phenotype (Lefebvre et al. 1995; Burghes et al. 1997; Campbell et al. 1997; McAndrew et al. 1997). However, the correlation is not absolutely linear, suggesting that other factors may also modulate the SMA phenotype. These data also show that Y272C must be a severe mutation, which is in agreement with results presented by Lorson et al. (1998), who showed that SMN1 protein containing the Y272C mutation leads to almost complete reduction of the self-oligomerization capacity.

Compared with the patients carrying the Y272C missense mutation, where the presence of two *SMN2* copies was found in type I SMA, the three patients with the T274I mutation developed type II SMA with one copy of *SMN2* and type III with two copies of *SMN2,* which suggests that T274I is a mild mutation. The S262I mutation seems to be an even milder mutation, because patient 935, carrying this mutation, developed type III SMA in the presence of only one additional *SMN2* copy. This is in agreement with the finding of Lorson et al. (1998), who showed that T274I and S262I lead to minor reduction of the self-oligomerization capacity of the SMN protein.

The two patients with Alu-mediated deletion including exons 5 and 6 of *SMN1,* and the one patient with nonsense mutation in exon 1 (Q15X), developed type I SMA in the presence of two *SMN2* copies, which indicates that *SMN1* is, in both cases, as expected, nonfunctional. However, patient 2385, who carries the same nonsense mutation (Q15X), shows three or four *SMN2* copies and has type III SMA. Patient 2598, with the donor splice site mutation in intron 7 of *SMN1,* has type III SMA in the presence of only two *SMN2* copies, which suggests that this mutation is a mild one. This is in agreement with the *SMN* self-oligomerization results obtained for Δ7SMN isoforms (Lorson et al. 1998). Finally, two of the patients with frameshift mutations developed type II SMA in the presence of two *SMN2* copies, whereas the third patient with a frameshift mutation developed type III SMA in the presence of three *SMN2* copies—again showing the role of *SMN2* as a modifier of the severity of SMA.

We also observed in two multiplex families intrafamilial discrepancies of SMA IIIa and IIIb, despite the fact that the sibs showed identical haplotypes and carried the same number of *SMN2* copies. This observation is in agreement with previous reports of affected and unaffected or phenotypically discordant haploidentical sibs that also suggested that factors other than *SMN2* copy number can modulate the SMA phenotypes (Cobben et al. 1995; Hahnen et al. 1995; Wang et al. 1996; McAndrew et al. 1997). In conclusion, the genotype-phenotype correlation studies of these patients clearly showed that the severity of SMA is determined by the *SMN1* mutations on both chromosomes, the *SMN2* copy numbers, and additional unknown factors.

Identification of a De Novo Gene-Conversion Event

Gene conversion has been postulated as a major mutational mechanism in SMA, leading to a replacement of *SMN1* by *SMN2* (Cobben et al. 1995; Hahnen et al. 1996; Burghes et al. 1997; Campbell et al. 1997;

DiDonato et al. 1997). De novo gene-conversion events in human genes have been described only twice before: 21-hydroxylase (Collier at al. 1993) and A γ-globin gene (Patrinos et al. 1998). Here we reanalyzed a family (G) with two sibs, one affected and one unaffected, showing identical haplotypes for all tested markers (Wirth et al. 1997). The only difference we found was the homozygous absence of *SMN1* in the affected sib, which must have occurred as a de novo event. Quantitative analysis showed a higher *SMN2* copy number in the affected child compared with the unaffected sib, providing evidence of a gene conversion of *SMN1* in *SMN2*.

The gene dosage test is also useful for the identification of de novo mutations, especially when linkage analysis remains inconclusive or no further sibs are available. We have previously reported de novo rearrangements in 7 (2%) of 340 patients with SMA (Wirth et al. 1997). In all but one case, we determined a paternal origin of the de novo rearrangements by finding allele loss of markers Ag1-CA and C212; in the remaining case (family G), the parental origin of the de novo mutation could not be identified, because no allele loss occurred. The quantitative *SMN1* analysis allowed the determination of a maternal origin of this de novo mutation. It is the second de novo mutation of maternal origin (Raclin et al. 1997).

Practical Implications and Limitations of the SMN1 *Heterozygosity Test*

First, from the practical point of view, the *SMN1* heterozygosity test is chiefly an important diagnostic tool for nondeleted SMA patients. Our test showed that 45.2% of nondeleted SMA patients with clinically well-defined type I–III SMA were heterozygous for Δ7SMN1, and, in all but one patient, an obligate carrier for whom no RNA was available, we identified intragenic *SMN1* mutations. This means that the heterozygosity test helps to confirm the diagnosis of SMA in patients with one *SMN1* copy. Consequently, a further search for the exact mutation can eventually be omitted in the routine diagnosis, because of high costs and labor-intensive procedures, and replaced by haplotype analysis for risk calculations. This strategy can be followed only when the SMA phenotype of the patient has been carefully investigated. Parsons et al. (1998) concluded that some of the patients referred to their laboratory for SMA testing without sufficient clinical investigation may be carriers by chance and may have neuromuscular disorders other than SMA. Second, the heterozygosity testing is essential for calculating risk of heterozygosity in the following groups: parents of patients with SMA where no material from the index patient is available, relatives of patients with SMA, and spouses.

By analyzing 73 carriers and 42 noncarriers, we determined the validity of the test. A major problem, which affected the validity of the test, is the existence of chromosomes carrying two *SMN1* copies (4%) and the difficulty of distinguishing between one and two *SMN1* copies in ~6% of carriers and noncarriers (overlapping interval $0.60 < \text{SSMN1} < 0.75$). The latter disadvantage might be overcome by use of methods based on real-time ongoing quantitative measurements, such as the TAQMAN system. However, we will present the practical implications and limitations based on our nonradioactive dosage assay, specifying for each group the factors that must be taken into consideration for risk calculation.

Group 1.—Parents of patients with SMA, where no material from the index patient is available. In some cases in which the patients died and no direct molecular genetic test was possible, the parents should be tested for their carrier status, only if the diagnosis of SMA in the child is typical according to the diagnostic criteria of the International SMA consortium. If both parents show a heterozygous state, the SMA diagnosis of the patient is most likely and a reliable prenatal diagnosis can be offered. When the patient had a typical SMA and only one parent is ΔSMN1 heterozygous, the other parent has a 45% chance of carrying an intragenic mutation. If the intragenic mutation is identified, a prenatal diagnosis can be offered. If both parents show at least two *SMN1* copies, it is extremely unlikely that the affected child had a 5q-SMA and it is advised to refrain from prenatal diagnosis.

Group 2.—Relatives of patients with SMA who show homozygous absence of *SMN1*. This category includes relatives such as sibs, aunts, or uncles who have a high a priori risk of being a carrier (risk to offspring >1:400, justifying prenatal testing). Use of the Δ7SMN1 heterozygosity test in relatives of patients with SMA will produce a false-negative or uncertain result due to two *SMN1* copies per chromosome and SSMN1 values within the overlapping interval in ~10%. However, if the relative turns out to be a carrier (SSMN1 values ≤ 0.60), the spouse should be tested for further risk calculation.

Although carrier testing is a helpful technique and may even be improved by methods that may allow a 100% distinction between one and two *SMN1* copies, it leads to a 4% false-negative result rate because of carriers with two *SMN1* copies per chromosome. The only way to overcome this drawback is by linkage analysis. Unfortunately, this is more expensive, requires testing of more family members, and is not always feasible.

Group 3.—Spouses of carriers or patients with SMA. A large number of prenatal diagnoses are currently undertaken in low-risk constellations because heterozygosity screening of spouses is not available. For example, there is a statistical risk for an affected child of 1:200 (carriers) and 1:100 (patients), given a carrier frequency

of 1:50 in the normal population, which leads to prenatal diagnoses in most pregnancies. With the possibility of heterozygosity testing, a spouse showing at least two *SMN1* copies with an SSMN1 value ≥ 0.75 has 96% probability of being a noncarrier (taking into consideration the frequency of two *SMN1* copies and theoretically of subtle mutations), and thus the probability of being heterozygous can be reduced to <1:1,000. In this case prenatal diagnostic testing should no longer be performed. If an individual shows SSMN1 values within the overlapping interval, the posterior probabilities should be calculated by discriminant analysis, also taking into consideration the a priori risk for each individual. Because of the relatively small sample size, we have not undertaken this approach. Further studies and a larger sample size are therefore needed.

It is not useful to investigate parents of patients with homozygous absence of *SMN1* who wish to know whether they are carriers by this quantitative test. On the basis of a 2% de novo mutation frequency in SMA patients (Wirth et al. 1997), 1% of SMA parents are not carriers of SMA. About 90% of SMA parents will show a gene dosage corresponding to one *SMN1* copy, and one can assume that they are carriers. Because the percentage of SMA parents for whom knowledge about their noncarrier status would be important is 1%, and thus far below the efficiency of the test (10% false-negative rate or, with improved techniques, 4%), the heterozygosity test would have no practical consequences and should not be offered. Furthermore, other risk factors, such as germ-line mosaicism (Campbell et al. 1998) have to be considered, and, therefore, it is very difficult to release a family with one carrier and an affected child from further prenatal testing. On the other hand, a direct *SMN* test of the fetus would always provide a highly reliable molecular diagnosis. Our data, obtained from *SMN1* dosage screening and searching for intragenic *SMN1* mutations in a collective of patients with well-defined SMA, provide the first reliable basis for risk calculations in families with SMA and will become an important diagnostic tool for genetic counseling.

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Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for type I SMA [Werdnig-Hoffmann disease, MIM 253300], type II SMA [MIM 253550], and type III SMA [Kugelberg-Welander disease, MIM 253400])

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