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Identification of Proximal Spinal Muscular Atrophy Carriers and Patients by Analysis of SMN^T and SMN^C Gene Copy Number

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Summary

The survival motor neuron (SMN) transcript is encoded by two genes, SMN^T and SMN^C. The autosomal recessive proximal spinal muscular atrophy that maps to 5q12 is caused by mutations in the SMN^T gene. The SMN^T gene can be distinguished from the SMN^C gene by base-pair changes in exons 7 and 8. SMN^T exon 7 is not detected in ~95% of SMA cases due to either deletion or sequence-conversion events. Small mutations in SMN^T now have been identified in some of the remaining nondeletion patients. However, there is no reliable quantitative assay for SMN^T, to distinguish SMA compound heterozygotes from non-5q SMA-like cases (phenocopies) and to accurately determine carrier status. We have developed a quantitative PCR assay for the determination of SMN^T and SMN^C gene-copy number. This report demonstrates how risk estimates for the diagnosis and detection of SMA carriers can be modified by the accurate determination of SMN^T copy number.

Introduction

Proximal spinal muscular atrophy (SMA) is an autosomal recessive disorder resulting in the loss of α motor neurons in the spinal cord. SMA has an estimated incidence of 1/10,000, with a carrier frequency of 1/40–1/60 (Pearn 1980; Melki et al. 1994). The recessive proximal childhood SMAs can be classified clinically into three groups. Type I (Werdnig-Hoffmann) is the most severe form, with onset at <6 mo of age and with death typically at <2 years of age. Type II SMA patients display an intermediate severity, with onset at <18 mo of age and with an inability to walk. Type III (Kugelburg-Welander) individuals are able to walk independently and have a relatively mild phenotype, with onset at >18 mo of age.

All three forms of SMA have been mapped, by linkage analysis, to 5q11.2-q13.3 (Brzustowicz et al. 1990; Melki et al. 1990; Simard et al. 1992; Brahe et al. 1994; Burghes et al. 1994*a;* Wirth et al. 1994; Wirth et al. 1995*a*), to a region that contains multiple copies of genes and markers (Francis et al. 1993; Kleyn et al. 1993; Carpten et al. 1994; Melki et al. 1994; Lefebvre et al. 1995; Roy et al. 1995; Thompson et al. 1995). Analysis of these multicopy markers showed specific alleles associated with SMA, loss of a copy of the marker in ~50% of severe SMA cases, and deletion of all copies of the marker on some SMA chromosomes (Burghes et al. 1994*b;* DiDonato et al. 1994; Melki et al. 1994; Lefebvre et al. 1995; Wirth et al. 1995*b*).

Three cDNAs have been reported that detect deletions in SMA patients (Lefebvre et al. 1995; Roy et al. 1995.; Thompson et al. 1995). Neuronal apoptosis inhibitory protein (NAIP) and XS2G3 are deleted in 50% of type I SMA patients (Roy et al. 1995; Thompson et al. 1995), which most likely represents the extent of the deletion on severe SMA chromosomes (Wirth et al. 1995*b;* Simard et al., in press). The third cDNA, the survival motor neuron, is encoded by SMN^T and SMN^C, two nearly identical genes that can be distinguished by base changes in exons 7 and 8 (Lefebvre et al. 1995; van der Steege et al. 1995). The SMN^T gene is not detectable in ~95% of SMA patients, either because of conversion of sequences in the SMN^T gene to those in the SMN^C gene or as a result of SMN^T gene deletions (Hahnen et al. 1995, 1996; Lefebvre et al. 1995; Rodrigues et al. 1995; van der Steege et al. 1996; Velasco et al. 1996; DiDonato et al. 1997*b*). Several small mutations in the SMN^T gene have been reported in patients without a deleted or sequence-converted SMN^T allele. These mutations include disrupted splicing of exon 7 (Lefebvre et al. 1995), deletion of 4 bp (Bussaglia et al. 1995) or 5 bp (Brahe et al. 1996) in exon 3, an 11-bp duplication in exon 6 (Parsons et al. 1996), and a clustering of missense mutations in exon 6 (Lefebvre et al. 1995; Hahnen et al. 1997; Talbot et al. 1997). These mutations provide strong evidence that SMN^T is the primary SMA-determining gene.

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The absence of detectable SMN^T exons 7 and 8 in SMA patients is being utilized as a powerful diagnostic test for SMA (van der Steege et al. 1995). Although the test has a sensitivity of ~95%, the assay is not quantitative, and it cannot detect individuals with heterozygous deletions of SMN^T. Thus, it is not possible to identify SMA carriers and to distinguish between a non-5q SMA-like patient (in whom both chromosomes contain SMN^T) and a compound heterozygote 5q SMA patient (in whom SMN^T is absent on one chromosome and an unknown alteration in the SMN^T gene is present on the other chromosome). Since SMN^T is homozygously deleted in 95% of 5q SMA cases, then, according to Hardy-Weinburg equilibrium, virtually all the remaining 5q SMA individuals should have a heterozygous deletion. The marker Ag1-CA (C272) lies at the 5′ end of the SMN genes (Burglen et al. 1996) and has been shown to vary, from zero to three copies, on a chromosome (DiDonato et al. 1994; Wirth et al. 1995*b*). This indicates that the copy number of SMN genes also varies on chromosomes. Previous attempts to determine the copy number of the SMN^T gene have measured the ratio of SMN^T to SMN^C; however, this has serious drawbacks.

In this paper, we describe a competitive PCR strategy to determine the SMN^T and SMN^C gene-copy number. The assay uses an exon from the cystic fibrosis transmembrane regulator (CFTR) as a standard to determine the relative dosage of SMN^T and SMN^C and thus avoids bias due to fluctuations in the copy number of SMN^C. We demonstrate that this assay is capable of accurately distinguishing compound heterozygotes from non-5q SMA-like cases. In a type III patient who lacked one copy of SMN^T, we found a novel missense mutation in exon 6. Analysis of normal and carrier individuals by this assay clearly indicates that the copy number of SMN^T and SMN^C varies from zero to at least two per chromosome and that the majority of SMA carriers have a single copy of the SMN^T gene on their normal chromosome. This report demonstrates that it now is possible to directly identify SMA carriers and affected compound heterozygotes by the accurate determination of SMN^T copy number.

Material and Methods

Patient Samples and DNA Isolation

DNA was isolated from peripheral venous blood or lymphoblast cell lines by the salting-out procedure (Miller et al. 1988) or by organic extraction (Sambrook et al. 1989). A total of 76 patients (64 from Ohio State University [OSU], 8 from Hôpital Sainte-Justine, Montreal [HSJ], and 4 from the Hospital for Sick Children, Toronto [HSC]) with a potential diagnosis of SMA were analyzed to identify compound heterozygotes. These patients did not necessarily conform to all criteria defined by the SMA consortium, since most were referred for diagnostic purposes. A small number of the samples were collected for prior genetic studies, which did conform to the diagnostic criteria outlined by the international SMA consortium. We also performed the SMN^T and SMN^C copy-number assay on 54 normal, unselected individuals and on 79 SMA carriers previously characterized by Ag1-CA segregation analysis (DiDonato et al. 1994). Patient 4659 is a 24-year-old male diagnosed with SMA type III, by muscle biopsy, at age 14 years.

Synthesis of Internal Standards

Two internal standards were constructed for the SMN^T and SMN^C copy-number assay. These standards have internal deletions of 20–50 bp, so that they are amplified with the same primer pairs as their genomic counterparts but can be distinguished by size (Celi et al. 1993). Incorporation of equimolar amounts of internal standards in the competitive PCR reaction was used to standardize the amount of input genomic DNA and to monitor the efficiency of the reaction. Genomic DNA was used as a template to generate the in vitro–synthesized CFTR internal standard. The 50-μl PCR reaction contained 200 ng genomic DNA, 3 mM $MgCl_2$, 1 × *Taq* DNA polymerase buffer (USB), 200 μM each dNTP, 30 ng each of CF621F(5′-AGTCACCAAAGCAGTACAGC-3′; Zielenski et al. 1991) and CFTR-IS (5′-GGGCCTGTGCAAGGAAGTGTTAAGCTATTCTCATCTGCATTCCA-3′) primers, and 0.5 U *Taq* DNA polymerase (USB). Diluted plasmid DNA containing a portion of the SMN^T gene was used as a template to generate the in vitro–synthesized SMN internal standard. The 50-μl reaction contained components similar to those in the CFTR internal standard reaction, except that primers R111 (5′-AGACTATCAACTTAATTTCTGATCA-3′; Lefebvre et al. 1995) and SMN-IS (5′-CCTTCCTTCTTTTTGATTTTGTTTATAGCTATATAGACATAGATAGCTA-3′) were used. The reactions were denatured at 95°C for 5 min, then run for 35 cycles at 95°C 1 min, 55°C 2 min, and 72°C 3 min (Ericomp Thermocycler). The products were subcloned into pCR 2.1 (Invitrogen), and plasmid DNA was purified. We found the use of cloned plasmid DNA more reliable than diluted PCR products. The inserts were excised with *Eco*RI and were stored at −70°C.

SMN^T and SMN^C Copy-Number Assay

Two competitive PCR amplifications were performed. In the first reaction, the DNA concentration of each sample was determined by estimation of the number of genome equivalents, according to the method of Sestini et al. (1995). Approximately 200 ng genomic DNA (~60,000 genome equivalents), determined on the basis of spectrophotometric quantitation, was amplified in the

presence of 60,000 CFTR-IS competitor molecules with CF621F and CF621R (5′-GGGCCTGTGCAAGGAAGTGTTA-3′ [Zielenski et al. 1991]) primers. The 25-μl PCR reaction contained 3 mM $MgCl_2$, 1 × *Taq* DNA polymerase buffer (USB), 200 μM each dNTP, 15 ng each CF621 primer, 0.5 U *Taq* DNA polymerase (USB), and 1 μl (60,000 copies) CFTR internal standard. The reaction conditions consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C 1 min, 55°C 2 min, and 72°C 3 min. The products were electrophoresed on an 8% polyacrylamide gel and were stained with ethidium bromide. The band intensities were evaluated, and the target genomic DNA for the SMN-dosage assay was adjusted accordingly.

In the second competitive PCR reaction, R111 and CF621F primers (15 ng each) were end-labeled with 0.1 μl [γ^{32}P]ATP (10 μCi/μl; Amersham) and T4 DNA kinase (Gibco BRL) at 37°C for 20 min. The 25-μl PCR reaction contained 200 ng genomic DNA, 3 mM $MgCl_2$, 1 × *Taq* DNA polymerase buffer (USB), 200 μM each dNTP, end-labeled forward primers, 15 ng each of CF621R and X-7 Dra (5′-CCTTCCTTCTTTTTGATTTTGTTT-3′; van der Steege et al. 1995) primers, 0.5 U *Taq* DNA polymerase (USB), and 1 μl (60,000 copies) each of CFTR and SMN internal standards. The reaction conditions consisted of an initial denaturation at 95°C for 5 min, followed by 16 cycles of 95°C 1 min, 55°C 2 min, and 72°C 3 min. The PCR product (8 μl) was digested with 20 U *Dra*I (New England Biolabs) overnight at 37°C. An SMA-patient sample with a homozygous deletion of SMN^T always was run as a control, to check for complete digestion with *Dra*I. The digested samples were run on a 6% denaturing polyacrylamide gel and were quantitated by autoradiography. Hyperfilm-MP (Amersham) was preflashed with a unit (Amersham) to ensure linearity of film response (Laskey and Mills 1975). The gel was exposed for 16–24 and 48–72 h at −70°C. Densitometry of the bands was performed on a Shimadzu CS-9000. The genomic SMN^T/genomic CFTR and genomic SMN^C/genomic CFTR ratios were determined for all samples. Since there are two copies of CFTR per genome, the SMN/CFTR ratio was used to determine the relative dosage or copy number of SMN^T and SMN^C genes.

The assay was performed at HSJ, with the following modifications. The CF621F and R111 primers were end-labeled with [γ^{33}P]ATP from ICN. The PCR conditions included a hot start with the addition of 1 U *Taq* DNA polymerase (Gibco BRL), followed by 20 cycles of 94°C 1 min, 55°C 1 min, and 72°C 1 min (MJ Research thermocycler). The PCR products were digested with *Dra*I (Gibco BRL). The dried gels were exposed in a phosphor screen for 72 h and were scanned by use of a PhosphorImager SI™ (Molecular Dynamics). Products of each PCR reaction were quantitated by use of the ImageQuant™ software. Thus, the SMN^T and SMN^C copy-number assay was performed independently by two groups and was quantitated by two different methods. Similar ratios were obtained, which demonstrates the versatility of this method.

The quantitative PCR assay to measure SMN^T and SMN^C gene copy number provides clear advantages over existing methods; however, a few technical points should be addressed, since the assay is subject to the potential problems of quantitative PCR. It is important to control for the amount of input genomic DNA, to avoid inaccuracies in the quantitation of copy number (Celi et al. 1994). We observed a small degree of variability in the amplification efficiency of SMN-IS, especially when comparing DNA samples extracted by different methods. Therefore, we recommend the use of normal, carrier, and affected controls prepared by the same extraction method as is used for the samples being tested. Both autoradiography and phosphoimaging were effective and accurate methods to quantitate copy number, but standard precautions, such as preflashing the film and monitoring exposure times, must be taken, to ensure the linearity of film response for autoradiography.

PCR Amplification of SMN for Mutation Detection

PCR primers used for amplification of SMN exons were selected on the basis of published intron/exon boundary sequences (Burglen et al. 1996) and primer sequences kindly provided by Dr. Judith Melki (Hôpital des Enfants Malades, Paris). For samples with one copy of SMN^T, each SMN exon was amplified from genomic DNA and was subjected to SSCP analysis as described elsewhere (Parsons et al. 1996). SMN exon 6 was PCR amplified by use of 30 ng each of primers 541C618 (5′-CTCCCATATGTCCAGATTCTCTTG-3′) and EX63 (5′-AAGAGTAATTTAAGCCTCAGACAG-3′) in a 50-μl reaction mixture containing 1 U *Taq* polymerase (Perkin Elmer), 0.5 mM each dNTP, 3 mM $MgCl_2$, and 1 × PCR buffer (670 mM Tris, 100 mM β-mercaptoethanol, 166 mM ammonium sulfate, 67 mM EDTA, and 0.5 mg BSA/ml). The PCR reaction consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min, with a final extension for 8 min at 72°C.

Heteroduplex Analysis

To allow heteroduplex formation, PCR products were heated to 95°C for 5 min and then incubated at 37°C for 30 min. Fifteen microliters of the PCR product was mixed with 2.5 μl of 6 × MDE™ gel loading buffer and was electrophoresed on a 50-cm vertical, 0.8-mm-thick MDE™ gel (FMC) for 15 h at 1,000 V. The gel was stained in a solution of 0.6 × Tris-borate EDTA con-

taining 1 μg ethidium bromide/ml and was photographed under UV light.

Reverse-Transcriptase–PCR (RT-PCR)

Total RNA was isolated from peripheral blood lymphocytes by use of TRIzol™ Reagent (Gibco BRL). First-strand cDNA synthesis was performed with 2 μg total RNA, oligo(dT), and Superscript II RNase H⁻ Reverse Transcriptase (200 U/μl; Gibco BRL), according to the manufacturer's instructions. The single-stranded cDNAs were PCR amplified by use of 30 ng each of the exon 6 forward primer 541C618 and the exon 8 reverse primer 541C1120 (5′-CTACAACACCCTTCTCACAG-3′), with reaction mixture components and PCR conditions identical to those used for PCR amplification of SMN exon 6.

Subcloning and Sequencing

PCR and RT-PCR amplification products were subcloned into a TA cloning vector (Invitrogen), according to the manufacturer's instructions. Sequencing of DNA purified by use of Wizard Minipreps (Promega) was performed with the dsDNA Cycle Sequencing System (Gibco BRL). Sequencing reaction products were analyzed by use of a 5% denaturing polyacrylamide gel. The mutation was detected on both DNA strands in multiple subclones.

Results

We describe a quantitative PCR-based method to measure the copy number of SMN^T and SMN^C genes in order to detect individuals with a heterozygous deletion of the SMN^T gene. Since homozygous deletions of SMN^T account for ~95% of SMA cases, the detection of a single copy of SMN^T in a patient with clinical features consistent with SMA would support a diagnosis of SMA. Detection of a single copy of SMN^T in an asymptomatic individual would identify that person as an SMA carrier.

To test the reliability and reproducibility of the assay, we measured the SMN^T copy number in a normal individual and in a known SMA carrier determined by haplotype analysis (DiDonato et al. 1994). The samples were amplified in 10 separate PCR reactions, electrophoresed on different gels, and quantitated by densitometry. The values from the densitometric scans and the calculated SMN^T/CFTR and SMN^C/CFTR ratios are shown in table 1. The mean ± SD SMN^T/CFTR and SMN^C/CFTR ratios for the normal individual were 0.68 ± .08 and 0.62 ± .06, respectively, which represents two copies of SMN^T and two copies of SMN^C. The SMA carrier had mean ± SD SMN^T/CFTR and SMN^C/CFTR ratios of 0.28 ± .06 and 0.29 ± .06, respectively, which is consistent with one copy of SMN^T and one copy of SMN^C. (It should be noted that, although we characterized an SMA carrier with one copy of SMN^C, not all carriers have one copy of SMN^C.) There was no overlap in the SMN^T/CFTR or SMN^C/CFTR ratios, within 2 SD of the mean, between the normal and carrier individuals. The described quantitative PCR assay demonstrates high precision between different gel runs and therefore can distinguish reliably between individuals with one and two copies of SMN^T and SMN^C.

We then studied a population of patients referred for SMA diagnostic testing who were not homozygously deleted for SMN^T exons 7 and 8 by either SSCP analysis or the restriction-enzyme assay. Seventy-six nondeletion samples were analyzed by use of the quantitative PCR assay, and the results for those individuals with a single copy of SMN^T are shown in table 2. A heterozygous deletion of SMN^T was detected in 6/76 (~8%) individuals. Heteroduplex or SSCP analysis of the single SMN^T copy present in these patients revealed abnormal bands in 3/6 patients. Mutations have been detected in 2/6 individuals (1 type I SMA [Parsons et al. 1996] and 1 type III patient described below), and the other individual (type I SMA) is currently under investigation. We did not detect abnormal patterns by SSCP or heteroduplex analysis of genomic DNA in 3/6 individuals.

We now describe one of the six patients with a single copy of SMN^T by gene-dosage analysis (patient 4659; table 2 and fig. 1*A* and *B*). SSCP and heteroduplex analysis of SMN exons PCR amplified from genomic DNA in this type III SMA patient demonstrated an abnormal band in exon 6. Sequence analysis of the patient's exon 6 subclones revealed a guanine-to-thymine transversion at nucleotide 818, producing a substitution of isoleucine for serine (S262I) at a conserved residue in the deduced protein (fig. 1*C*). In order to determine whether the variant exon 6 sequence was contained within the telomeric or centromeric copy of SMN, the patient's lymphocyte RNA was amplified by RT-PCR using an exon 6 sense primer (541C618) and an exon 8 antisense primer (541C1120) and then was subcloned and sequenced. The patient's subclones containing SMN^T were identified by restriction-enzyme digestion, and sequence analysis of these clones confirmed that the mutant transcripts were derived from SMN^T. The nucleotide change was not observed in 200 normal chromosomes. These results, in combination with the dosage data demonstrating that the patient possesses only one copy of SMN^T, strongly indicate that this type III SMA patient has two different SMN^T mutations: one SMN^T allele has been deleted, whereas the other contains a missense mutation (S262I) in exon 6.

SMN^T/CFTR and SMN^C/CFTR ratios were determined for 79 SMA carriers and 54 normal individuals, to establish this assay as a valid method to distinguish between SMA carriers with one copy of SMN^T and normal individuals with two copies; for example, figure 2

Table 1

Reproducibility of SMN^T and SMN^C Copy Number

Type	No. from Densitometric Scans for				SMN^T/CFTR[a]	SMN^C/CFTR[a]
	CFTR	CFTR-IS	SMN^T	SMN^C		
Normal	118,967	114,311	71,537	69,543	.60	.58
	107,932	113,891	76,390	73,369	.71	.68
	64,006	73,251	48,313	33,911	.75	.53
	60,206	74,688	32,022	34,595	.53	.57
	54,790	55,420	36,279	31,484	.66	.57
	49,754	48,086	38,716	35,801	.78	.72
	126,412	117,640	85,921	81,091	.68	.64
	96,863	98,561	66,955	64,919	.69	.67
	49,246	37,091	29,185	30,693	.59	.62
	26,518	46,443	19,126	17,042	.72	.64
					(.67 ± .08)	(.62 ± .06)
Carrier	47,802	53,273	14,930	14,214	.31	.30
	52,047	63,934	14,330	12,972	.28	.25
	73,957	74,941	13,279	13,395	.18	.18
	17,460	22,132	3,553	4,064	.20	.23
	63,749	81,768	18,218	16,557	.29	.26
	120,059	102,099	46,225	46,732	.39	.39
	29,175	38,230	8,531	8,325	.29	.29
	29,373	40,148	7,720	9,890	.26	.34
	58,207	62,618	18,274	20,373	.31	.35
	45,177	47,462	15,158	14,638	.34	.32
					(.28 ± .06)	(.29 ± .06)

[a] Data in parentheses are mean ± SD values.

shows the expected results of the SMN^T and SMN^C copy-number assay for normal, carrier, and affected individuals from several families. Two different populations of SMA carriers previously characterized by multicopy markers in the SMA region were analyzed in this study. Fifty-five SMA carriers analyzed at OSU had a mean ± SD SMN^T/CFTR ratio of 0.27 ± 0.07 (one carrier individual had two copies of SMN^T; table 3). Twenty-two French Canadian SMA carriers (from HSJ) had a mean ± SD SMN^T/CFTR ratio of 0.19 ± .03 (one was homozygously deleted for SMN^T). Interestingly, analysis of SMN^C copy number in SMA carriers revealed

Table 2

Detection of SMA Patients with One Copy of SMN^T by Dosage Analysis

Patient[a]	SMA Type	SMN^T/CFTR	SMN^C/CFTR	No. of Telomeric Copies	No. of Centromeric Copies	Mutation
Control	Normal	.58	.55	2	2	. . .
OSU 367	I	.24	.53	1	2	11-bp duplication[b]
OSU 4659	III	.23	.25	1	1	S262I[c]
OSU 284	II/III	.13	.10	1	1	Not detected
OSU 379	III	.33	1.01	1	3	Not detected
HSC[d]	I	.19	.18	1	1	Not detected
FC 72[e]	I	.25	.50	1	2	Abnormal SSCP pattern[f]

[a] Of the 64 patients screened at OSU, the 4 listed here had a heterozygous deletion of the SMN^T gene.
[b] Reported by Parsons et al. (1996).
[c] Novel exon 6 missense mutation dercribed in the Results section.
[d] One of the four HSC samples had a single copy of the SMN^T gene.
[e] One of the eight HSJ nondeletion patients had one copy of the SMN^T gene, by dosage analysis.
[f] L. R. Simard and C. Rochette (unpublished data).

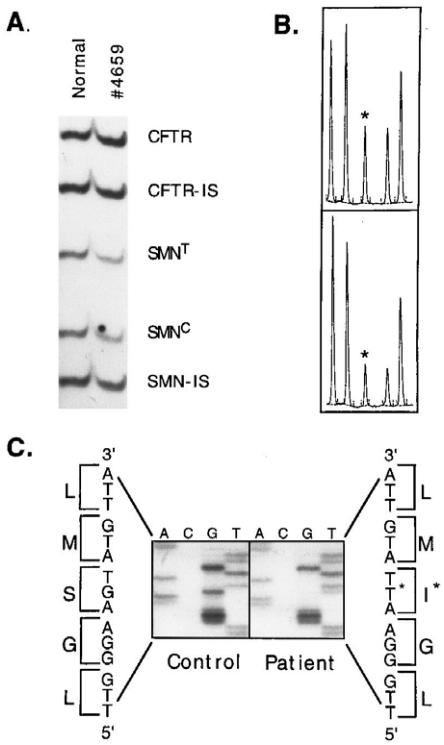

Figure 1 Dosage and sequence analysis of a compound-heterozygote 5q SMA patient. *A,* Autoradiograph of competitive PCR products digested with *Dra*I and run on a 6% denaturing polyacrylamide gel. Equal amounts of genomic DNA were added to each reaction, and the SMN^T and SMN^C bands from patient 4659 were half the intensity that was seen in the normal control. The SMN^T/CFTR ratios of the normal individual and patient 4659 were 0.51 and 0.25, respectively, demonstrating that the patient has half the normal dosage, or one copy of SMN^T. *B,* Densitometric scan of the gel shown in *A*. The order of peaks, from left to right, is CFTR, CFTR-IS, SMN^T, SMN^C, and SMN-IS. An asterisk (*) denotes the SMN^T peak in the normal control (*top panel*) and the patient (*bottom panel*). The area of the patient's SMN^T and SMN^C peaks is half that of the normal control's. *C,* Comparison of nucleotide and deduced amino acid sequences in the normal control's and the patient's SMN^T subclones. Patient 4659 has a G→T transversion that produces a substitution of serine by isoleucine at codon 262. The base change is denoted by an asterisk (*).

that 27/79 (~35%) had three or four copies of SMN^C, indicating that a large number of chromosomes in this population have two copies of SMN^C on one chromosome.

Fifty normal, unselected individuals had a mean ± SD SMN^T/CFTR ratio of 0.66 ± .07 (table 3). One normal individual had one copy of SMN^T, which is consistent with the 1/40–1/60 carrier frequency for SMA in the population. We also found three normal individuals with three copies of SMN^T. SMN^C-dosage analysis in normal individuals revealed that only 1 (1/53, or ~2%) had three copies, 23 had two SMN^C genes, 25 had one SMN^C gene, and 4 had no copies of SMN^C. The results of this analysis clearly demonstrate the limitations of utilizing SMN^C as a standard to determine dosage of SMN^T.

Finally, SMN^T and SMN^C copy number was determined for individuals from several interesting SMA families (Burghes et al. 1994*a;* DiDonato et al. 1997*b*). In one case, an asymptomatic type II/III SMA carrier with a homozygous deletion of SMN^T was shown to have four copies of SMN^C, by the quantitative PCR assay (II.1; fig. 3). However, we observed another asymptomatic carrier deleted for SMN^T who had two SMN^C genes (table 3). We also investigated three cases of haploidentical siblings from SMA families with discordant phenotypes. We found no difference in SMN^T or SMN^C copy numbers (I.4 and I.5; fig. 3).

Discussion

Accurate dosage analysis is necessary in order to identify SMA carriers and to distinguish SMA compound heterozygotes from non-5q SMA-like cases. Both of these diagnostic applications require a method that can differentiate between individuals with one and two copies of the SMN^T gene. We applied a quantitative PCR assay that uses an exon of the CFTR gene as a standard to determine the copy number of the SMN^T and SMN^C genes. The assay also incorporates the use of two internal standards (CFTR-IS and SMN-IS) to monitor the efficiency of the PCR reaction and to ensure that equal amounts of target genomic DNA are added to each tube. Similar quantitative PCR approaches have been used successfully to identify deletions in the insulin-receptor gene (Celi 1994), to detect duplications in Down syndrome patients (Celi 1994), and to quantitate oncogene amplification (Sestini et al. 1994, 1995).

Since this assay uses CFTR as a standard to determine SMN^T and SMN^C copy number, the problems of using the variable SMN^C gene as a standard are avoided. Previous attempts to estimate the copy number of SMN^T and SMN^C have been based on measurement of the SMN^T/SMN^C ratio (Matthijs et al. 1996; Velasco et al. 1996; Schwartz et al. 1997). The copy number of SMN^C was determined in parents of SMA patients in cases in which it was assumed that only one copy of SMN^T was present (Velasco et al. 1996); thus this assay is limited to quantitation of SMN^C in obligate carriers. In another study, a nonradioactive SSCP assay was used to determine SMN^T copy number by means of the SMN^T/SMN^C ratio (Matthijs et al. 1996). An elegant solid minisequencing method was used to determine the SMN^T/SMN^C ratio on normal and SMA chromosomes (Schwartz et al. 1997). However, these methods are not suitable for carrier analysis or detection of compound heterozygotes, because they rely on the ratio of SMN^T/SMN^C without the use of an external standard. This ratio is effected by the variation in SMN^C copy number that occurs in the

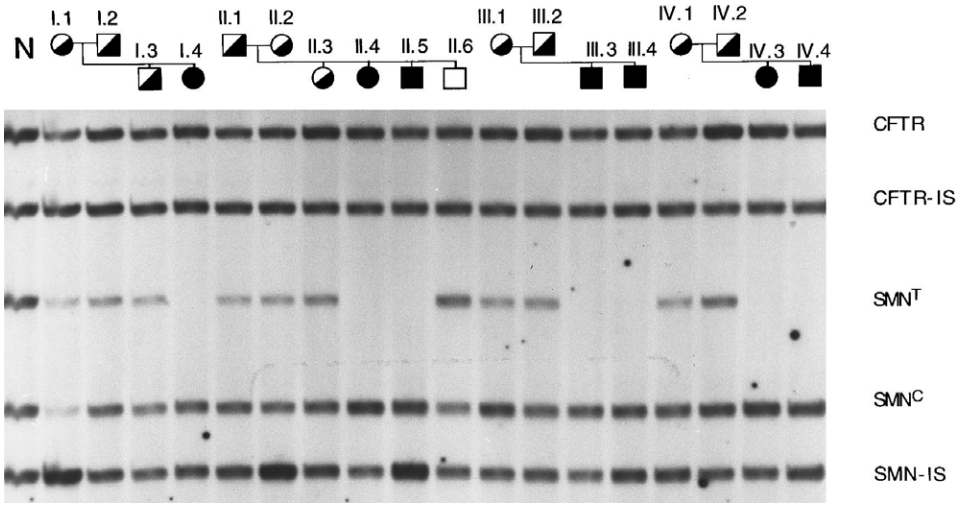

Figure 2 SMN^T-dosage analysis of SMA carriers and their families. All members of these pedigrees were characterized previously by linkage analysis. Families were diagnosed as follows: "I" (type I SMA), "II" (type II/III SMA), "III" (type II SMA), and "IV" (type II SMA). The normal control is represented as "N." SMA carriers are represented as half-blackened boxes, whereas affected SMA patients are represented as completely blackened boxes. The SMN^T/CFTR ratios for SMA carriers are half the ratio for the normal control, whereas affected SMA patients are deleted for SMN^T.

normal population, and it does not account for the possibility of more than one SMN^T gene on a chromosome.

Utilizing the competitive PCR assay, we quantitated the SMN^T copy number in patients with clinical features consistent with SMA without homozygous deletions of SMN^T. We found that a majority (~92%) had normal SMN^T dosage. Although SMA patients with two identical, nondeleted SMN^T alleles were identified in cosanguinous Spanish families (Bussaglia et al. 1995), the estimated frequency of this event is rare in a random population. Although further clinical evaluation may have excluded some of these patients from SMN^T-dosage testing, the results of our analysis clearly indicate that the clinical features of SMA are shared by other neuromuscular disorders. In addition, we did not detect abnormal patterns by SSCP or heteroduplex analysis in 3/6 patients identified with a single copy of SMN^T. Possible explanations include the following: the mutation may be outside of the screened region, there may be a duplication or a deletion of a region other than exon 7, or individuals with one copy of SMN^T may be SMA carriers. We would expect ~2% (1.5/76) of this population to be SMA carriers, and therefore 1 or 2 of these patients could be an SMA carrier with another neuromuscular disorder.

Our assay rapidly identified potential SMA 5q compound heterozygotes and defined a limited population of patients for SMN^T mutation analysis. We identified a novel exon 6 missense mutation in a type III SMA patient with one copy of SMN^T. The serine-to-isoleucine substitution at codon 262 in exon 6 introduces an amino acid with a bulkier side chain and the net loss of a hydroxyl group. The phenotype of this chronic type III SMA patient correlates well with the possibility that he has a combination of a severe (deleted) and a milder (S262I missense mutation) SMN^T allele. This serine residue is conserved in both mouse (DiDonato et al. 1997*a*; Viollet et al. 1997) and *Caenorhabditis elegans* (cosmid C41G7; GenBank accession Z81048) SMN^T proteins. This mutation was identified simultaneously by Hahnen et al. (1997). Talbot et al. (1997) report a clustering of missense mutations in a C-terminal dodecapeptide region, highly conserved in *Saccharomyces pombe* and *C. elegans* homologues, which may be an important binding domain. The S262I missense mutation identified in a type III SMA patient lies just upstream of this region, indicating that the conformation of the region upstream of the dodecapeptide is also important for SMN function.

Since SMA is one of the most common lethal genetic disorders, with a carrier frequency of 1/40–1/60, development of a rapid, direct carrier test would be beneficial to many families. SMA carrier testing presently is being done by linkage analysis and is subject to the potential problems of recombination events, de novo mutations, and the difficulty of obtaining DNA samples from various family members. The advantage of our assay is that it directly measures SMN^T copy number and circumvents these problems. The SMN^T gene-dosage assay identified a single copy of SMN^T in most SMA carriers, except for one that was homozygously deleted and one that had two copies of SMN^T. Possible explanations for this finding include a de novo mutation (Melki et al. 1994, Wirth et al. 1995*b*), somatic mosaicism, or one

Table 3

SMN^T and SMN^C Copy Number in Normal Individuals and SMA Carriers

Type	No. of Individuals	Genotype (SMN^T, SMN^C)	SMN^T/CFTR[a]	SMN^C/CFTR[b]
Normal	3	3, 1	.92 ± .12[c]	.22 ± .06
	1	2, 3	.62	.91
	23	2, 2	.67 ± .08	.64 ± .07
	22	2, 1	.67 ± .06	.27 ± .04
	4	2, 0	.60 ± .10	0
	1	1, 3	.30	1.10
SMA carriers:				
OSU:				
SMA I	1	2, 1	.76	.38
	4	1, 3	.28 ± .09	1.06 ± .13
	6	1, 2	.33 ± .06	.68 ± .09
	5	1, 1	.27 ± .08	.32 ± .09
SMA II	8	1, 3	.26 ± .05	.99 ± .10
	7	1, 2	.23 ± .07	.62 ± .11
	2	1, 1	.29 ± .05	.26 ± .01
SMA II/III	2	1, 3	.29 ± .18	1.09 ± .04
	4	1, 2	.25 ± .10	.63 ± .14
	1	1, 1	.27	.27
SMA III	3	1, 4	.25 ± .06	1.40 ± .09
	6	1, 3	.28 ± .03	1.02 ± .15
	5	1, 2	.31 ± .05	.70 ± .12
	2	1, 1	.28 ± .04	.29 ± .02
HSJ:				
SMA I	2	1, 2	.21 ± .02	.41 ± .08
	2	1, 1	.18 ± 0	.17 ± .02
	1	0, 2	0	.41
SMA II	1	1, 3	.21	.57
	3	1, 2	.20 ± .01	.37 ± .03
SMA III	3	1, 3	.22 ± .02	.62 ± .07
	9	1, 2	.19 ± .04	.41 ± .09
	2	1, 1	.17 ± .01	.18 ± 0

[a] The average for 50 normal individuals with two copies of SMN^T was .66 ± .07; for the OSU sample, the average for 55 carriers with one copy of SMN^T was .27 ± .07, whereas, for the HSJ sample, for 22 carriers with one copy of SMN^T it was .19 ± .03.

[b] SMN^C copy number was calculated for OSU samples by dividing the SMN^C/CFTR ratio by .34 (average SMN^T/CFTR of .27 + 1 SD, as the maximum amplification of one copy of SMN). It should be noted that this method may underestimate SMN^C copy number in some cases, since the SMN^C/SMN^T ratio is not equivalent.

[c] The quantitative PCR assay was repeated in four separate reactions to confirm these results.

chromosome with two SMN^T genes. Since this carrier individual has two affected children with the same haplotype, a de novo mutation is unlikely. Although we cannot distinguish between the latter two possibilities, the case of two SMN^T genes on one chromosome is likely, on the basis of our finding of 3/106 normal chromosomes with two SMN^T genes.

Analysis of SMN^T copy number in 54 normal individuals revealed several interesting points. First, one carrier individual with a single SMN^T gene was identified, which is consistent with the estimated carrier frequency of 1/40–1/60. Second, the marker Ag1-CA lies at the 5′ end of the SMN gene, and this marker varies on chromosomes (DiDonato et al. 1994; Wirth et al. 1995*b*). This would indicate that the copy number of SMN also varies on chromosomes. Quantitation of SMN^T copy number in normal individuals revealed that 3/53 had three copies of SMN^T, indicating that two copies are present on one chromosome. Velasco et al. (1996) previously reported a carrier mother and fetus with two SMN^T genes on one chromosome. Multiple copies of SMN genes on one chromosome may have arisen by either unequal crossing-over or a sequence-conversion event (Hahnen et al. 1996).

The finding of two SMN^T genes on a single chromosome has serious counseling implications, because a carrier individual with two SMN^T genes on one chromosome would be misdiagnosed by SMN^T copy-number

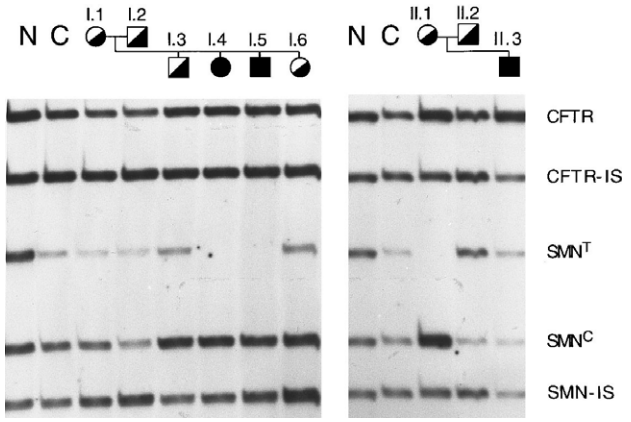

Figure 3 SMN^T- and SMN^C-dosage analysis of two atypical SMA families. Family I (type II SMA) has been described previously, by Burghes et al. (1994*a*), as SMA6. Although linkage analysis demonstrated that individuals I.4 and I.5 are haploidentical, they have remarkably different phenotypes. Individual I.4 had onset at 1 year of age and never walked, whereas individual I.5 was still able to walk at 20 years of age. Individuals I.4 and I.5 have a homozygous deletion of SMN^T and have SMN^C/CFTR ratios of 0.95 and 0.88, respectively. Since both siblings have three copies of SMN^C, the different phenotypes cannot be explained by a difference in SMN^C copy number. Family II (type II/III SMA) represents a case of an asymptomatic carrier parent (II.1) homozygously deleted for SMN^T. The SMN^C/CFTR ratio is consistent with four copies of the SMN^C gene. In table 2, individual II.3 is designated as "OSU 284"; and he currently is being investigated to detect a mutation in the single copy of SMN^T present. Normal and carrier controls are represented as "N" and "C," respectively. Carrier individuals are represented by half-blackened boxes, whereas affected individuals are represented by completely blackened boxes.

analysis. If both a prior probability of 1/40–1/60 of being an SMA carrier in the general population and a conditional probability of ~2% of carrying two SMN^T genes on one chromosome (4/185 chromosomes; i.e., 3/106 normal chromosomes and 1/79 "normal" chromosomes from SMA carriers) are assumed, then a normal SMN^T/CFTR ratio on the basis of gene-dosage analysis would reduce the risk of being an SMA carrier to 1/2,000–1/3,000. Thus, although the finding of normal dosage significantly reduces the risk of being a carrier, our results show that there is still a small recurrence risk of future affected offspring for individuals with normal dosage.

Last, quantitation of SMN^C gene-copy number in normal individuals revealed that 1/53 had three copies, 23/53 had two copies, 25/53 had one copy, and 4/53 were homozygously deleted for SMN^C. The observed genotypes for SMN^C in our normal population were 1.9% (three copies), 43.4% (two copies), 47.2% (one copy), and 7.5% (no copies). The expected genotypes, on the basis of Hardy-Weinburg for a three-allele system, are 2.5% (three copies), 46.9% (two copies), 41.3% (one copy), and 9.3% (no copies). The observed genotype ratios do not differ significantly from the expected genotype ratios ($\chi^2 = 1.6$). It should be noted that this assay does not distinguish between the genotypic groups (1,1) or a single copy of SMN^C on each chromosome from (2,0) or two copies of SMN^C on a single chromosome. Because no normal individuals with four copies of SMN^C were observed, the precise estimation of the frequency of the (2,0) genotype by use of the maximum-likelihood method is not possible. However, the frequency of the (2,0) genotype would be predicted to be negligible. A larger number of individuals will have to be typed to allow accurate assessment of these genotype frequencies.

An interesting finding from these studies is that 27/79 (4/21 type I and 23/58 types II and III) SMA carriers had three or four copies of SMN^C, indicating that there were two copies of SMN^C on one or both chromosomes, whereas only 1/53 (~2%) of the normal individuals had three copies of SMN^C. Previous studies, using the marker Ag1-CA, demonstrated a correlation between the number of copies of Ag1-CA and SMA phenotype (DiDonato et al. 1994; Wirth et al. 1995*b*). Since Ag1-CA lies at the 5′ end of the SMN genes, this implies that type I patients have deleted chromosomes and that type II/III patients have one deleted chromosome and a gene conversion on the other chromosome. The study by Velasco et al. (1996), which demonstrated that three copies of SMN^C predominated in type II/III families is consistent with this interpretation. Therefore, the increase in SMN^C copy number reported here provides evidence to support previous work indicating that a large number of type II/III SMA chromosomes contain gene conversions as opposed to deletions (DiDonato et al. 1994, 1997; Wirth et al. 1995*b;* Hahnen et al. 1996; van der Steege et al. 1996). We currently are preforming a more detailed analysis of gene conversion, using this dosage assay to determine the proportion of gene conversions in different SMA types.

Determination of SMN^C gene-copy number in SMA family members led to some interesting observations. Individual II.3 (fig. 3) previously had been identified as an affected patient who was not homozygously deleted for SMN^T, on the basis of SSCP and restriction-enzyme analysis (DiDonato et al. 1997*b*). These same tests demonstrated that individual II-1, the asymptomatic carrier mother of II-3, was completely deleted for SMN^T. Several reports describe the existence of unaffected individuals with a homozygous deletion of SMN^T (Cobben et al. 1995; Hahnen et al. 1995; Wang et al. 1996). This asymptomatic carrier has four copies of SMN^C, which could suggest that the number of copies of SMN^C in this individual compensates for the lack of SMN^T. However, this situation does not apply to all asymptomatic individuals, since we observed (1) only two SMN^C genes in a second asymptomatic carrier (table 3) and (2) SMA individuals with four copies of SMN^C. In addition, our

group (Burghes et al. 1994*a*) and others (Müller et al. 1992; Rudnik-Schöneborn et al. 1994) have described SMA families in which two sibs have remarkably discordant phenotypes. In the cases analyzed in this study (SMA6 and SMA14 [Burghes et al. 1994*a*] and SMA75 [DiDonato et al. 1997*b*]), no difference in SMN^C copy number was detected between the affected and unaffected/mildly affected individuals (I.4 and I.5; fig. 3), indicating that a change in SMN^C copy number is not the mechanism responsible for discordant phenotypes in these families. Studies at the RNA and protein levels should elucidate whether the critical component in the unaffected/mildly affected individuals is the amount of full-length SMN produced.

In conclusion, we report a powerful, rapid quantitative PCR assay and demonstrate its clinical application for detection of compound-heterozygote 5q SMA patients and SMA carriers. The quantitative SMN^T and SMN^C copy-number assay increases the sensitivity of diagnosis of SMA and allows for direct carrier testing. This assay now can be used to quantitate SMN^C and SMN^T genes in SMA families, to provide insight into the frequency and mechanisms of gene-conversion events.

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