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ORIGINAL ARTICLE

Molecular diagnosis of non-deletion SMA patients using quantitative PCR of SMN exon 7

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ABSTRACT

The telomeric survival motor neuron (SMN^T) gene is a valuable molecular diagnostic tool for childhood-onset spinal muscular atrophy (SMA) as homozygous deletions of SMN^T exon 7 ($\Delta 7SMN^T$) are present in ~94% of patients. In this report, we provide the first comprehensive study of 32 unrelated non-deletion SMA patients. Quantitative polymerase chain reaction (PCR) studies established that 90% had two intact copies of SMN^T exon 7 suggesting that these patients do not have 5q SMA. Once 5q SMA is confirmed, the SMN^T gene can be screened for subtle mutations. Using single strand conformation analysis, we identified two missense mutations (P245L and Y272C) in exon 6 of the SMN^T gene of two SMA patients shown to have a single copy of SMN^T exon 7. Y272 is most likely critical for SMN^T function as it is a target for recurring mutations and is associated with type I SMA. These results emphasize the need for dosage analysis in the differential diagnosis of 5q SMA in non-deletion patients, consistent with extensive clinical heterogeneity and some genetic heterogeneity in this disease. Homozygosity or heterozygosity for a $\Delta 7SMN^T$ allele confirms the diagnosis of 5q SMA with greater precision than clinical examination alone.

Keywords: 5q SMA, non-deletion patients, quantitative PCR, point mutations

INTRODUCTION

The spinal muscular atrophies (SMA) are a heterogeneous group of disorders caused by selective degeneration of the anterior horn cells of the spinal cord (1). Over 70% of all SMAs are autosomal recessive childhood-onset forms associated with symmetrical, proximal muscle weakness (2). Three types of childhood-onset SMA have been recognized and are distinguished by age of onset, maximum motor function and age at death (3). Rapidly progressing type I SMA (Werdnig–Hoffmann disease) is the most severe form striking within the first few months of life and it is the second most common monogenic cause of infant mortality. The major clinical feature distinguishing chronic forms of SMA is that type II patients never walk unaided and have a reduced life expectancy while type III (Kugelberg–Welander disease) individuals are frequently mobile for long periods of their lives. Childhood-onset SMAs affect 1 in 10 000 newborns and ~1 in 40 to 1 in 60 individuals are carriers of this disease (2,4).

The locus for SMA types I, II and III has been localized to a complex region of chromosome 5q13 which contains multiple copies of microsatellite markers, expressed pseudogenes and genes (4–13). At least three genes have been localized to the critical SMA region, survival motor neuron (SMN), neuronal apoptosis inhibitory protein (NAIP) and p44 (9,10,14,15). Two copies of the SMN gene are present, centromeric SMN (SMN^C) and telomeric SMN (SMN^T), the result of an inverted duplication of ~500 kb (9). Mutations in SMN^T are responsible for all three forms of 5q-linked childhood-onset SMA. The majority of these mutations involve deletions of at least exon 7 of the SMN^T gene with world-wide frequencies ranging from 87 to 98% of all SMA patients (9,16–27). The NAIP gene is present in multiple and variable copy number; however, exons 5 and 6 appear to be unique to the telomeric intact NAIP gene (10). Homozygous deletions of at least exon 5 of the intact NAIP gene has been observed in ~55% of type 1 and 12% of chronic SMA patients (10,18,19,22,24–27). Finally, the p44 gene, which codes for a subunit of the TFIIH transcription factor, is also duplicated and homozygous dele-

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tions within the telomeric p44 gene have been identified in 73% of type I compared with 9% of chronic SMA patients (15). Taken together, most type I SMA patients are homozygous for large scale deletions involving the entire SMN, the intact NAIP and the p44 genes present in the telomeric element (13,15,22,26). In contrast, the majority of type II/III patients have SMA alleles which harbor intragenic SMN^T deletions or sequence conversions between the SMN^C and SMN^T copy genes (9,16,24–26,28,29). The broad spectrum of disease presentation in chronic SMA is most likely due to the interaction between different SMA alleles (26).

Point mutations in the SMN^T gene have been identified in rare patients with at least one intact SMN^T gene, providing conclusive evidence that mutations in SMN^T are responsible for childhood-onset SMA (9,16,21,30–33). Database searches failed to reveal any homology to known genes and consequently, the function of SMN and its role in SMA etiology remains unclear (9). However, SMN has recently been implicated in RNA metabolism by virtue of its ability to bind the RGG box region of hnRNP U (34). SMN also interacts with itself, fibrillarin and several novel proteins. A monoclonal antibody against SMN localized the protein to the nucleus, in close association with coiled bodies, in structures that have been named gems for 'Gemini of coiled bodies' (34). To further our understanding of the function of SMN, we have analyzed 32 unrelated patients who lack homozygous deletions of SMN^T exon 7 (non-deletion patients) for subtle mutations in the SMN^T gene. This study is the first comprehensive report of the search for SMN^T point mutations in a large number of non-deletion patients. We identified two missense mutations, a novel mutation disrupting the poly-proline stretch in SMN^T exon 6 (codon 245) and a recurring mutation in codon 272 of exon 6 (9). Quantitative-PCR analysis indicated that the second SMA-allele in these patients was deleted for SMN^T exon 7. In addition, quantitative-PCR studies suggest that as many as 90% of non-deletion patients do not have 5q SMA.

MATERIALS AND METHODS

SMA patients

This study included 35 patients (two sib-pairs and one mother-child pair) from 32 unrelated SMA families referred to research (Hôpital Sainte-Justine; HSJ) or DNA diagnostic (The Children's Hospital of Eastern Ontario, CHEO; The Hospital for Sick Children, HSC) laboratories for SMA screening. All 35 patients retained at least one copy of SMN^T exons 7 and 8 (non-deletion patients).

Single-strand conformation analysis

Single strand conformation analysis (SSCA) was used to screen for variant migrating bands which might indicate the presence of a subtle mutation in the SMN^T gene. Each exon was PCR amplified using the primers (corresponding to flanking intronic sequences), annealing temperatures and $MgCl_2$ concentrations outlined in Table 1. Briefly, reactions contained 100 ng of genomic DNA, 200 μM dNTPs, 44 nM [α-^{32}P]dCTP (3000 Ci/mmol; ICN Pharmaceuticals, Inc.), 1 μM of each primer, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, $MgCl_2$, in a final volume of 25 μl. Initial denaturation was done at 94°C for 7 min and 1 U of *Taq* DNA polymerase (Gibco-BRL) was added at 80°C (Hot Start) using the PTC 100 cycler from MJ Research, Inc. This was followed by 35 cycles of 94°C, 30 s, annealing for 30 s and 72°C, 30 s. PCR products were diluted five-fold with 0.1% SDS and 10 mM EDTA, mixed with an equal volume of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), heated at 80°C for 5 min and rapidly cooled on ice. A total of 5 μl of the denatured sample was applied to a 0.5× MDE (AT Biochem), 0.6× TBE gel and run at 7W for 16 h at 4°C or room temperature on a S2 gel electrophoresis apparatus (Gibco-BRL). Gels were then dried and exposed to Fugi film overnight at –80°C in the presence of an intensifying screen.

Variant migrating fragments were excised from the dried gel, DNA eluted in water and 15 μl of the eluate was used for PCR amplification of exon 6. Exon 6 PCR products were gel purified with Geneclean II (Bio/Can) and sequenced directly using a dsDNA Cycle Sequencing kit (Gibco-BRL). To establish that the identified point mutations were in the SMN^T copy gene, long-range PCR spanning exons 6 and 7 of the SMN copy genes was done using the Expand™ Long Template PCR System (Boehringer Mannheim). Amplification of control (HSJ 28.6199) and patient (HSJ 72.4814 and CHEO 26027) DNAs (250 ng) was done using 0.3 μM of each primer, 350 μM dNTPs, 1× buffer 2 and 2.5 U of the thermostable *Taq* and Pwo DNA polymerase mix in a final volume of 50 μl. Initial denaturation was done at 94°C for 2 min. This was followed by 10 cycles of 94°C, 10 s, 56°C, 30 s, and 68°C, 4 min, followed by 30 cycles of 94°C, 10 s, 56°C, 30 s and 68°C, 4 min plus 20 s for each cycle. The final elongation step was for 7 min at 68°C. The 5′→3′ sequence of the forward primer was TACTTTTTGTTTACTGGATATAAAC, corresponding to sequences in intron 5 flanking exon 6 and the reverse primer used was that listed in Table 1 for exon 7. The ~6.2 kb PCR fragment was subcloned into pCR™2.1 using methods recommended by the supplier (Invitrogen). Recombinant clones were analyzed for SMN^C/SMN^T content by SSCA as described above.

Quantitative-PCR

To confirm the diagnosis of 5q SMA in non-deletion patients, quantitative PCR of exon 7 of the SMN genes was done as described in McAndrew *et al.* (31). Briefly, co-amplification of SMN exon 7 and exon 4 of the CFTR gene was carried out in the presence of internal standards harboring 20–50 bp internal deletions of SMN exon 7 and CFTR exon 4 amplification products. To distinguish SMN^C from SMN^T exon 7, PCR products were digested with *Dra*I (35). Dried sequencing gels were exposed in a phosphor screen for 72 h, scanned with a PhosphorImager SI™ (Molecular Dynamics) and PCR products quantified using the ImageQuant™ software. In general, the intensity of one copy of SMN was ~40–50% the intensity of one copy of CFTR. Results are presented as a ratio of SMN^C or SMN^T exon 7 to CFTR exon 4 volume (integrated intensity of all the pixels in the given PCR fragment). Results from one experiment are provided in Table 2; however, identical results were obtained in at least two independent experiments.

RESULTS

Single-strand conformation analysis

To identify variant migrating bands indicative of possible subtle mutations, SSCA was done for each of the nine exons

Table 1. Primers for SSCA of the SMN gene

Exon	Forward primer (5′→3′)	Reverse primer (5′→3′)	T(°C)	Concentration $MgCl_2$ (mM)
1	taagaagggacggggcccca	gaagaagggtgctgagagcgc	56	1.0
2a	ctgattaaacctatctgaacatg	cgtatgttatcaattccctttcca	56	2.5
2b	ttctgtgcaccaccctgtaac	aaggactaatgagacatctttg	58	2.0
3	cgagatgatagtttgccctc	catctagtctctgcttccag	56	1.5
4	acacccttataacaaaaacctgc	ctggaaaactttcatagaagg	56	1.5
5	cctatcatattgaaattgg	caaattgtatgtgaaagca	50	1.5
6	ctttttgtttactggatat	acaaagtcacataactaca	50	1.5
7	agactatcaacttaatttctgatca	ccacaaaccataaagttttacaa	56	1.5
8	gtaataaccaaatgcaatgtgaa	ctacaacacccttctcacag	56	1.0

Forward primers for exons 3, 7 and 8 correspond to primers 8C (16), R111 and 541C960 (9), respectively. Reverse primer exon 8 corresponds to primer 541C1120 (9). Primers for exons 1, 2, 4, 5 and 6R were designed from our own unpublished sequence data; the remaining primers were designed from published sequence (51).

Table 2. Quantitative PCR of SMN exon 7 of non-deletion SMA patients

	SMN^C	SMN^T	Copy no.		
Patient ID	CFTR	CFTR	SMN^C	SMN^T	Clinical/referral information
CHEO 21503	0.52	0	3	0	type III SMA/limb girdle[a]
HSJ 72.4814	0.50	0.25	2	1	type I SMA, Y272C missense mutation[b]
CHEO 26027	0.13	0.10	1	1	type III SMA, P245L missense mutation
HSC 12092	0.18	0.19	1	1	type I SMA, subtle mutation not found
CHEO 19965	0.32	0.34	2	2	Arthrogryposis, rule out 5q SMA
HSC 5525	0	0.39	0	2	rule out SMA
HSC 10037	0.39	0.40	2	2	rule out type I SMA, died 1 mth of age
HSC 5430	0.19	0.40	1	2	rule out type I SMA
HSC 12241	0.55	0.39	3	2	rule out type I SMA, died 5 mths of age
HSC 6208[c]	0.16	0.37	1	2	rule out type I SMA
HSC 4506[c]	0.19	0.35	1	2	rule out type I SMA
CHEO 27170	0.16	0.52	1	3	referred as type I SMA
CHEO 29334	0.41	0.44	2	2	referred as type I SMA
CHEO 30325	0.34	0.37	2	2	referred as type I SMA
HSJ 51.6933	0.19	0.42	1	2	referred as type I SMA
HSJ 77.4830	0.19	0.40	1	2	referred as type II SMA
CHEO 21422	0.33	0.33	2	2	referred as type III SMA
HSJ 41.6310	0.23	0.49	1	2	type III SMA, consanguinity
CHEO 21265	0.19	0.40	1	2	referred as type III SMA
CHEO 31009	0.16	0.33	1	2	referred as type III SMA
HSJ 43.6993	0	0.37	0	2	referred as type III SMA
HSJ 73.4815	0.41	0.43	2	2	referred as type III SMA
CHEO 31801.0	0.16	0.35	1	2	type III SMA/CMT[d]
HSJ 16.6865	0.40	0.45	2	2	type III SMA/CMT
HSJ 65.7317	0	0.42	0	2	type III SMA/CMT
HSJ 13.6570	0.22	0.45	1	2	type III SMA/limb girdle[e]
HSJ 46.6249[c]	0.44	0.47	2	2	type IV SMA, adult onset
HSJ 46.6248[c]	0.46	0.50	2	2	type IV SMA, adult onset
CHEO 28484	0.17	0.36	1	2	type IV SMA, adult onset
HSC 11538	0.19	0.40	1	2	type IV SMA, adult onset
CHEO 21258	0.21	0.45	1	2	type IV SMA, adult onset, probably AD[f]
HSJ 66.7318	0.47	0.47	2	2	type IV SMA, adult onset, probably AD[f]
CHEO 31801.1	0.36	0.39	2	2	type IV SMA/CMT[d]
CHEO 28744	0.20	0.41	1	2	type IV SMA/Kennedy syndrome
HSJ 57.5949	0.32	0.30	2	2	type III SMA
HSJ 28.6199	0.45	0.47	2	2	normal control
HSJ 28.6201	0.38	0.33	2	2	normal control
HSJ 23.5095	0.19	0.38	1	2	normal control

[a]Non-deletion status was determined by SSCP analysis (9).
[b]The paternal and maternal SMN^T/CFTR ratios were 0.l8 and 0.40, respectively, consistent with paternal inheritance of $\Delta 7SMN^T$ and maternal inheritance of Y272C.
[c]HSJ 46.6249 and HSJ 46.6248 are sibs; HSC 4506 and HSC 6208 are sibs.
[d]CHEO 31801.1, diagnosed with Charcot–Marie–Tooth (CMT) is the mother of 31801.0.
[e]Haploidentical normal sib.
[f]Probably autosomal dominant, positive family history.

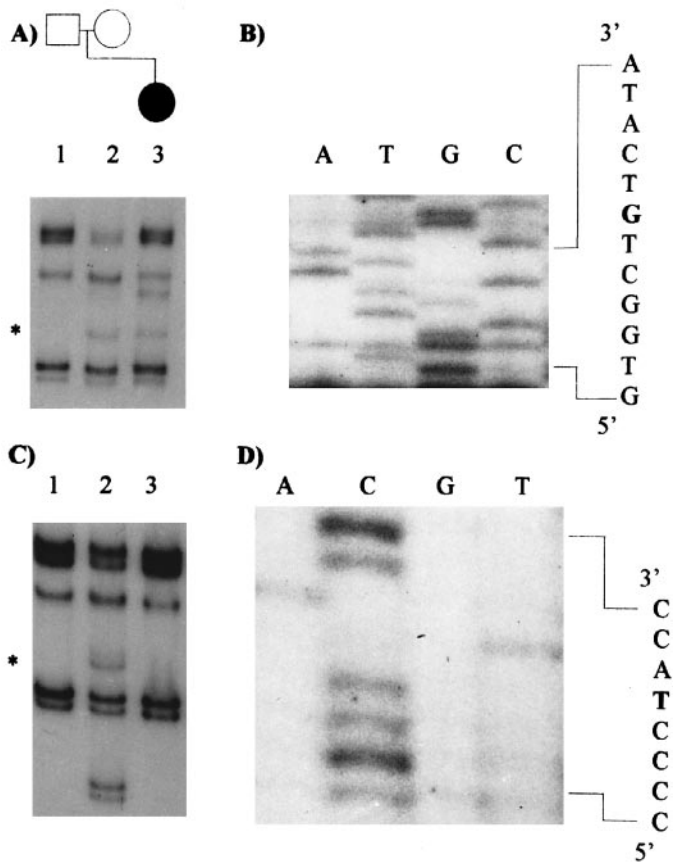

Figure 1. SMN^T point mutation analysis of patients HSJ 72.4814 (A and B) and CHEO 26027 (C and D). SSCA results for SMN exon 6 for a type I SMA family consisting of the father (lane 1), mother (lane 2) and affected child HSJ 72.4814 (lane 3) is shown in (**A**) and results for three unrelated, non-deletion SMA patients (CHEO 26027 in lane 2) are shown in (**C**). Variant SSCA fragments are identified by the asterisk. Sequence of the variant fragment from the type I SMA patient HSJ 72.4814 (B) and from the type III SMA patient (CHEO 26027 (**D**) are shown. These compare with normal SMN^T sequence 5′ GTGGCTATCATA 3′ (**B**) and 5′ CCCCCACC 3′ (D). HSJ 72.4814 has an A→G substitution in codon 272 which replaces a tyrosine for a cysteine (Y272C) while CHEO 26027 has a C→T substitution in codon 245 which replaces a proline for a leucine (P245L).

(1–2a, 2b–8) of the SMN gene for 32 unrelated non-deletion SMA patients. DNA from a P1 (P3996) (12) and a PAC (215P15; unpublished data) clone was used to identify SSCA fragments corresponding to the SMN^T and SMN^C copy genes, respectively. Migration patterns were identical except for the previously identified polymorphisms in exons 7 and 8 used to distinguish SMN^C and SMN^T (9). Analysis of these individuals revealed two different variant SSCA fragments in exon 6 of two patients (Fig. 1). We did not observe any differences in the SSCA profile of all nine exons in each of the remaining 30 unrelated patients (data not shown). The variant SSCA fragments from a type I (HSJ 72.4814; Fig. 1A) and a type III (CHEO 26027; Fig. 1C) patient were excised and sequenced directly. Patient HSJ 72.4814 had an A→G substitution of nucleotide 848 (Fig. 1B) which replaces a tyrosine for a cysteine at codon 272. This mutation has been designated Y272C and was independently identified by Lefebvre *et al.* (9). The variant Y272C fragment was present in only one of HSJ 72.4814's parents (maternal origin) indicating that this patient is a compound heterozygote (Fig. 1A). Quantitative PCR results indicated that the paternal SMA allele harbored a deletion of SMN^T exon 7 (see Table 2 legend). CHEO 26027 had a proline to leucine substitution at codon 245 (CCA→CTA; Fig. 1D) which has been designated P245L. Absence of either variant SSCA fragment in 31 SMA patients and 50 unrelated controls indicated that Y272C and P245L were not rare polymorphisms (62 SMA and 100 normal chromosomes; data not shown). Finally, analysis of cloned DNA from long PCR products spanning exons 6 and 7 of the SMN gene indicated that the Y272C and P245L mutations co-segregated with SMN^T exon 7 (data not shown).

Quantitative-PCR

As we had identified SMN^T point mutations in only two of 32 unrelated non-deletion patients studied, quantitative PCR was undertaken to confirm the diagnosis of 5q SMA. Worldwide studies indicate that >94% of all childhood-onset SMA patients have homozygous deletions of SMN^T exon 7 ($\Delta7SMN^T$) indicating that the $\Delta7SMN^T$ allele frequency is ~0.97. If these patients have 5q SMA, the majority of them should have one $\Delta7SMN^T$ allele. Results of dosage analysis for SMN exon 7 are summarized in Table 2. We identified a single $\Delta7SMN^T$ allele in 3 patients (10%). SMN^T point mutations have been identified for two of these, namely HSJ 72.4814 and CHEO 26027. In one patient, CHEO 21503, we observed a homozygous deletion of SMN^T exon 7 not previously detected by the single strand conformation polymorphism (SSCP) test described by Lefebvre *et al.* (9). In another patient, CHEO 27170, we detected three copies of SMN^T exon 7, while patient HSC 12241 had three copies of SMN^C exon 7.

DISCUSSION

The identification of SMN^T as the SMA-determining gene (9,16) provided a valuable molecular diagnostic tool especially as homozygous deletions of SMN^T exon 7 ($\Delta7SMN^T$) are present in ~94% of all childhood-onset SMA patients (27,36). In addition, homozygous $\Delta7SMN^T$ alleles have also been documented in some adult-onset SMAs (37–39) as well as some SMAs with atypical clinical features including SMA associated with congenital heart defects (40), arthrogryposis multiplex congenita (41) or other exclusion criterion (42) established by the International SMA Consortium (3). Consequently, the determination of a homozygous $\Delta7SMN^T$ state serves to confirm the diagnosis of 5q SMA with greater precision than clinical examination alone. The extensive clinical heterogeneity documented for 5q SMA raises the possibility that the variability in deletion frequencies observed between studies (range 87–98%) may be due to the inclusion of some non-5q SMA patients in these studies rather than ethnic differences (9,16–27). This problem becomes critical in the differential diagnosis of presumptive 5q SMA patients who lack homozygous deletions in SMN^T (non-deletion patients). To address this, we have utilized the quantitative PCR assay recently presented by McAndrew *et al.* (31) to determine the copy number of SMN^T exon 7 in a series of 32 unrelated, non-deletion SMA patients.

As mentioned above, >94% of all SMA patients are homozygous for $\Delta7SMN^T$, indicating that the $\Delta7SMN^T$ allele

frequency is ~0.97. Consequently, the majority of non-deletion patients, if they have 5q SMA, must be genetic compounds with one $\Delta 7SMN^T$ allele and one allele with an as yet unidentified mutation in the SMN^T gene. Dosage analysis of SMN^T exon 7 provides a powerful tool to confirm the diagnosis of 5q SMA in these patients (31). Quantitative-PCR of SMN exon 7 for 32 unrelated affected individuals suggests that as many as 90% of these patients do not have 5q SMA. This sample includes severe, mild and adult-onset forms of SMA as well as some patients referred for differential diagnosis and support the existence of genetic heterogeneity in SMA. In one patient, we identified a homozygous deletion of SMN^T exon 7, not detected by SSCP, indicating that false negative results can be obtained using SSCP. An estimate of 4% false positives using this technique has also been reported (43). Taken together, DNA-based enzymatic analysis (35) provides a more accurate molecular diagnostic test. We cannot exclude the possibility that patient HSC 12242 has 5q SMA as we identified three copies of SMN^C and two copies of SMN^T exon 7. This result may indicate the presence of a SMN^C:SMN^T chimeric gene; chimeric genes have been associated with 5q SMA (9,16,24–26,28,29). This possibility is being investigated.

The assumption that the majority of non-deletion SMA patients should have one $\Delta 7SMN^T$ allele may not be valid in situations of consanguinity or in populations isolated either by geography, language or culture which have experienced genetic drift or a founder effect. Indeed, a type II SMA patient was shown to be homozygous for a 4 bp deletion in exon 3 of the SMN^T gene consistent with consanguinity (16). Homozygosity for subtle mutations in the SMN^T gene is also possible in Turkish (23), Reunion island (44,45), Egyptian Karaite (46) and French Canadian (47) SMA populations. However, if consanguinity, genetic drift or founder effect are responsible for homozygosity of a given subtle mutation in the SMN^T gene, then genetic linkage should confirm this by demonstrating identical maternal and paternal SMA haplotypes. We were able to exclude 5q SMA for patient HSJ 13.6570 with the identification of a 5q haploidentical unaffected sibling. While consanguinity has been reported for patient HSJ 41.6310, analysis of 5q DNA markers has not yet been done in this family. Finally, genetic linkage studies for five of the remaining nine French Canadian SMA patients (HSJ series), with two copies of SMN^T exon 7, established that the maternal and paternal SMA chromosomes were distinct, confirming our conclusion that these patients do not have 5q SMA.

The function of SMN remains unclear as database searches failed to reveal any homology to known genes (9). However, several features have come to light (Fig. 2); a putative nuclear localization signal (NLS) in exon 2b (48), a stretch of five prolines in exons 4 and 6, and 10 prolines in exon 5 (9) as well as a putative Y–G box (amino acids 268–279; exons 6 and 7) often present in the C-terminal region of RNP binding proteins (32). The identification of a putative NLS and a Y–G box is consistent with SMN's ability to bind the RGG box of hnRNP U and its nuclear localization in gems (34). Identification of point mutations in the SMN^T gene may provide further insight into its function. A combination of SSCA, DNA sequencing and long-PCR studies revealed two different SMN^T point mutations in patients HSJ 72.4814 and CHEO 26027. HSJ 72.4814 is a type I patient who inherited a $\Delta 7SMN^T$ allele from her father (as determined by dosage analysis) and a Y272C missense mutation in exon 6 of the

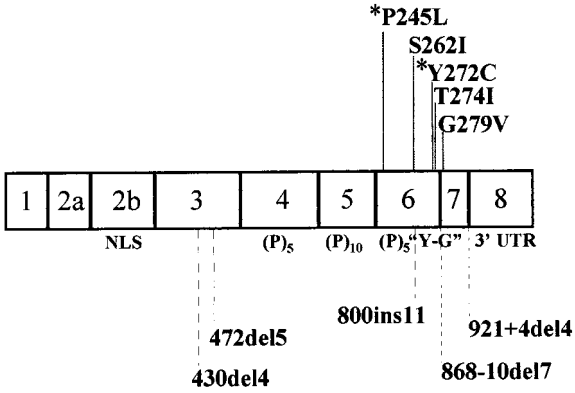

Figure 2. Schematic diagram of the nine exons of the SMN^T gene and summary of published point mutations to date. Exons are depicted as boxes and features of the gene are presented below. A putative nuclear localization signal (NLS) has been identified in exon 2b (48) as well as poly-proline stretches in exons 4 (P5), 5 (P10) and 6 (P5). Most of exon 7 and 8 code for the 3′ UTR. A putative Y–G box ('Y–G') involving amino acids 268–279 has recently been identified (32). Amino acids 268–278 are in exon 6 while 279 is the first amino acid of exon 7. The Y272C, 921+4del4 and 868–10del7 mutations were reported by Lefebvre *et al.* (9); 430 del4 by Bussaglia *et al.* (16); 272del5 by Brahe *et al.* (21); 800ins11 by Parsons *et al.* (30); S262I by McAndrew *et al.* (31) and Hahnen *et al.* (33); T274I by Hahnen *et al.* (33); and G279V by Talbot *et al.* (32). The missense mutations reported here are identified by an asterisk.

SMN^T gene from her mother. This mutation has been previously reported by Lefebvre *et al.* (9); however, as our patient is from the United States and Lefebvre's patient was part of the cohort from France, this is most likely a recurring mutation. CHEO 26027 is also a compound heterozygote with one $\Delta 7SMN^T$ allele, and one SMA allele harboring a P245L missense mutation which replaces the first proline of a poly-proline stretch in exon 6 with a leucine which contains a bulky aliphatic side chain. This non-conservative change may affect the folding of the SMN^T protein. These and other SMN^T point mutations reported to date are summarized in Figure 2. The SMA phenotype of the patients with the 921+4del4, 868–10del7 and Y272C mutations from Lefebvre *et al.* (9) were not reported. In general, frameshift mutations in combination with a $\Delta 7SMN^T$ allele have been reported in severe type I or type II SMA individuals (16,21,30). All of the missense mutations reported to date (9,31–33) are amino acids which are conserved in the mouse (47,49) and rat (49) *Smn* gene. Of these, three localize to a putative Y–G box (32), one (Y272C) is a recurring mutation (9, this paper), supporting the hypothesis that the Y–G domain is important for SMN^T function (32). The tyrosine at position 272 and the glycine at position 279 appear to be critical as substitutions of these amino acids were found in type I SMA patients. In contrast, the P245L, S262I and T274I mutations are associated with chronic forms of SMA. Finally, we did not detect a subtle mutation in the SMN^T gene of patient HSC 12092 using SSCA. Further dosage studies will be required to rule out the possibility of an intragenic deletion or duplication not involving exons 7 and 8. In addition, sequences outside those analyzed by the SSCA assay are currently being investigated.

This report provides the first comprehensive study of non-deletion SMA patients. Utilizing quantitative PCR of SMN exon 7, we estimate that as many as 90% of these patients do not have 5q SMA. This fact most likely explains the variability

in deletion frequencies observed between studies and as such, quantitative PCR provides an essential molecular diagnostic tool in the differential diagnosis of 5q SMA in non-deletion patients. In addition, we describe one novel and one recurring point mutation in exon 6 of the SMN^T gene in two patients shown to harbor one $\Delta 7SMN^T$ allele. The clustering of numerous missense mutations in exon 6 suggests that this exon codes for a domain which is critical for SMN function. Taken together, dosage analysis of SMN^T exon 7 is the next step in the differential diagnosis of 5q SMA patients who are not homozygous for a $\Delta 7SMN^T$ allele. Having established the presence of a $\Delta 7SMN^T$ allele, SSCA or other mutation detection tests can be used to identify a subtle mutation in the SMN^T gene. When consanguinity, genetic drift or founder effect are suspected, linkage analysis can establish whether a given patient may be homozygous for a subtle mutation in the SMN^T gene. Establishing an accurate diagnosis of 5q SMA is crucial to appropriate genetic counseling. Furthermore, a better estimate of the extent of genetic heterogeneity in the spinal muscular atrophies becomes possible.

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