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# SURVIVAL MOTOR NEURON GENE TRANSCRIPT ANALYSIS IN MUSCLES FROM SPINAL MUSCULAR ATROPHY PATIENTS

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**Summary:** We have identified and characterized four different mRNA isoforms of the survival motor neuron (SMN) gene from skeletal muscle of 9 SMA patients and 15 unaffected controls.These isoforms appear to be generated by combinatorial splicing of both exons 5 and 7 of the SMN telomeric and centromeric gene copies. The full-size and the truncated SMN-1b isoforms of the telomeric SMN copy are significantly reduced in muscle of SMA patients, irrespective of the disease types. Our results suggest that multiple RNA splicing is operative in the two SMN-related genes and that SMN-related polypeptides may be active in the muscle. 

The survival motor neuron (SMN) is a protein encoded by a duplicated gene in the 5q13 region [1]. Each gene encompasses approximately 20 Kb, with five nucleotide differences only in their complete transcribed sequence [1,2]. Both SMN copies are transcribed in a variety of human tissues including heart, brain, liver, muscle, lung kidney, pancreas and spinal cord [1]. The two genes can be

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distinguished at the RNA level, since the centromeric element (SMNc) undergoes an alternative splicing of exon 7, resulting in a truncated transcript lacking this exon and a putative protein with a different C-terminal end. On the contrary, the telomeric copy (SMNt) is expressed only in the full-length form, which encodes for a 294 amino acid putative protein [1]. SMNt is lacking or interrupted in about 95% of patients with spinal muscular atrophy (SMA) providing strong evidence that this gene is a SMA determining gene [1]. SMA is a motor neuron disease which affects about 1 in 10,000 births. Based on age of onset and life-span, three forms of SMA can be distinguished [3]. Type I SMA has an onset before birth or in early infancy and it is characterized by severe, generalized muscle weakness and hypotonia. Death from respiratory failure usually occurs within the first two years. Type II patients are able to sit, although they cannot stand or walk unaided, and survive beyond age of 4 years. Type III patients have proximal muscle weakness, beginning after the age of 2 years with a good prognosis mainly depending on the degree of respiratory embarrassment [3]. The frequency of SMN deletions is similar in the severely affected patients and in those presenting the milder variants of the disease [1,4]. Since no obvious correlation between phenotype and genotype can be determined on the basis of SMN deletion analysis, it is possible that genomic rearrangements of the SMA region influence the SMN isoform pattern in target tissues.

We report on the effect of SMN gene deletion onto the transcriptional level in muscle of 9 patients affected by different SMA types.

## Materials and Methods

**Tissue specimens.** Biopsies were taken from the peroneus brevis muscle of 9 patients (4 males, 5 females) affected by chronic proximal SMA, and selected on the basis of clinical, electrophysiological, and histoenzymological criteria [5]. Two patients were classified as SMA type I, 3 as SMA type II, and 4 as SMA type III. The mean age at the time of biopsy was 5.6 years (range 1.5-25 years). The biopsies were frozen in deep-cooled isopenthane and stored at -80°C. One part of each biopsy was serially sectioned for immunocytochemical and biochemical analysis, and the remaining used for nucleic acid extraction. Control fragments of *peroneus brevis* muscles were taken from 2 patients affected by myotonic dystrophy.
Diagnosis of SMA was confirmed also by direct SMN gene analysis [1,4].
All SMA patients were homozygous for deletion of the telomeric SMN exons 7 and 8. Control individuals showed the presence of centromeric and telomeric exons. Informed consent to use the muscle specimens for this study was obtained from the patients' parents.

**RNA extraction and cDNA synthesis.** Total RNA was extracted from 100-300 mg of biopsied muscle by the acid guanidium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi [6]. Ten micrograms of total RNA was electrophoresed on 1% agarose/formaldehyde, and concentration determined by OD 260 nm. About 8 μg of total RNA was converted to cDNA using a first-strand cDNA synthesis kit (CLONTECH Laboratories,Inc. USA) following the manufacturer's protocol. Each cDNA synthesis included a control transcription of one sample without RNA.

**Amplification and characterization of the RT-PCR products.** After reverse transcription of total RNA, the single-stranded cDNAs were amplified by PCR using 20

pmol each of forward (SMN1A, 5' GGA GGA TTC CGT GCT GTT CC 3') and reverse (SMNZ2A, 5' TGT CAT TTA GTG CTG CTC TAT GCC 3') primers. For exon skipping detection and sequencing of PCR products, additional primers were synthesized within exon 4 (SMN1B, 5' TAA CAT CAA GCC CAA ATC TGC 3') and exon 8 (SMN8B, 5' CTA CAA CAC CCT TCT CAC AG 3'). PCR conditions included an initial denaturation of 3 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, 1.5 min at 72°C and a final extension of 5 min at 72°C. PCR reactions were performed in a final volume of 50 µl containing 50 mM Tris-HCl pH 9.0 (25°C), 50 mM KCl, 7 mM MgCl2, 200 µM dNTPs, 0.2 mg/ml BSA, 16 mM $(NH_4)_2SO_4$ and 1 Unit of SuperTaq (HT Biotechnology LTD, U.K.). To evaluate the PCR results, 15 µl of the reaction mixture were withdrawn and electrophoresed in a 1.8% agarose gel. Specific PCR products were digested with Dde I restriction enzyme (New England Biolabs, USA) according to manufacturer's protocol. Quantitative analysis was performed using an image analyzer (BIOIMAGE, Millipore, USA). Sequencing of single PCR products was performed by asymmetric PCR [7] using an asymmetric ratio ranging from 50:1 - 100:1 of primers SMN1A and SMN8B. The ssDNAs were sequenced using a Sequenase DNA sequencing kit (U.S. Biochemical, USA) and primers SMN1A, SMNZ2A, SMN8B and SMN1B. The reaction products were electrophoresed on 6% acrylamide gels containing 8M urea. Sequences were ordered and the predicted amino acid sequence was deduced using a TRANSL software (PC/GENE IntelliGenetics Inc., USA). Predicted biochemical properties of single peptides produced by mRNA isoforms obtained were evaluated using FLEXPRO, CHARGPRO,GGBSM, PROSITE softwares (PC/GENE IntelliGenetics Inc., USA).

## Results

Four different PCR products were observed on agarose gel electrophoresis from normal muscle (Fig. 1). Two main bands of 877 bp (SMN) and 823 bp (SMN-1a) were observed, as well as two minor bands of 781 bp (SMN-1b) and 727 bp (SMN-1c). The PCR products were not due to contaminating genomic DNA because similar PCR reactions did not amplify any product in these samples in the absence of reverse transcriptase. No PCR product was obtained in the blank containing water control only. Sequence analysis of the different isoforms demonstrated that they derived from the full-length cDNA of the SMN gene by alternative splicing of separate exons (Fig.2B). With respect to the SMN full length cDNA, SMN-1a excludes exon 7, SMN-

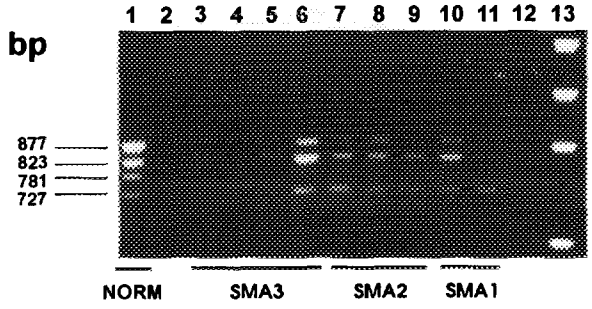


Figure 1. Analysis of PCR products of SMN mRNA, using SMN1A and SMNZ2A primers. Lanes 2 and 12: negative control, lane 13: ϕx174/Hae III DNA marker.

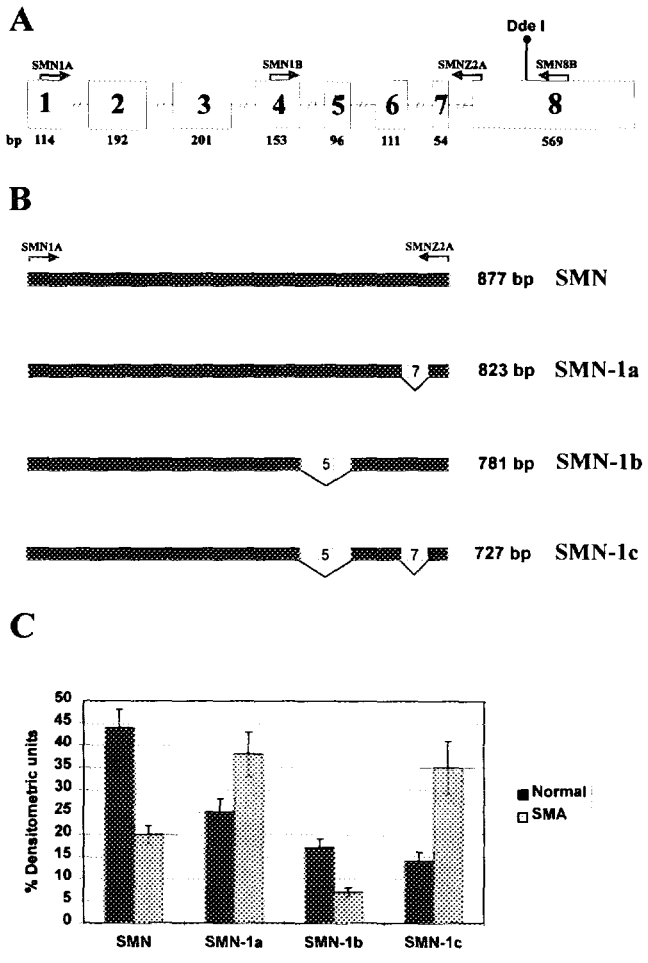


Figure 2. A) SMN gene structure and primer position.
B) Schematic representation of SMN cDNA isoforms.
C) Relative amounts of SMN transcripts, as observed by densitometric analysis.

1b excludes exon 5, while SMN-1c excludes both exons 5 and 7 (Fig.2B). Quantitative analysis of the SMN-related cDNAs showed that the percentages of the 877 bp, 823 bp, 781 bp, and 727 bp bands were 44%, 25%, 17%, and 14% respectively (Fig.2C). The corresponding amount of these isoforms in muscle of SMA patients was 20%, 38%, 7%, and 35%, (Fig.2C). Sequence and restriction enzyme analysis demonstrated that SMN-1a and SMN-1c isoforms derive from the SMNc cDNA, while SMN-1b derives mainly from SMNt and only partly from SMNc full length cDNAs. This latter isoform in conjunction with the full length isoform, is reduced of about 70% in the SMA patients homozygously deleted in the SMNt gene (Fig.1). No differences were observed between SMA types carrying the homozygous SMNt deletion, suggesting that no compensatory effect or de novo mRNA synthesis or splicing events within the

SMN genes are related to the different SMA phenotypes. These results indicate that SMNt normally undergoes exon 5 alternative splicing to produce transcripts lacking this exon, and suggest that the SMNc form in the muscle is subjected to an alternative splicing which excludes exon 5 and 7, in addition to the described exon 7-lacking isoform [1]. The deletions in the SMN-related cDNAs did not result in any frame shift. The SMN-1a, SMN-1b, and SMN-1c polypeptides consist of 282, 262, 250 amino acids. All these predicted polypeptides differ from the SMN polypeptides by structural changes, including: (1) the new C-terminal amino acid sequence (SMN-1a, SMN-1c); (2) the number of amino acid residues which confer a coil conformation (SMN-1a, SMN-1b, SMN-1c); (3) the number of amino acids in helical conformation (SMN-1a, SMN-1c); (4) the loss of one potential N-myristoylation site (SMN-1b, SMN-1c).

## Discussion

The SMN gene codes for a small protein of 294 amino acids without homologies to other known proteins. No functional role for this protein has been established so far (J. Melki, personal communication). SMN gene product is expected to be absent in SMA patients since the vast majority of them has a deletion of at least the 3' end of the gene (and possibly of the entire gene), and a small number have a truncation of the gene from the 5' end. A limited number of patients have different mutations received from heterozygous parents [1; J. Melki, personal communication]. However, in absence of allelic heterogeneity, it is difficult to explain the different SMA phenotypes on the basis of a unique molecular defect [2]. It has been suggested that the extension of deletion to adjacent loci could account for the differences in phenotypic expression of this disease. This explanation is supported by demonstration that SMA types I and II generally are associated with gross deletions compared to type III SMAs [1, 4; 8]. However, definitive proof that differential deletions are causative for the clinical heterogeneity of SMAs will require further studies. As an alternative hypothesis, it has been suggested that a different expression of the SMN genes influence the SMA phenotype [2,9]. The relative level of expression of the two SMN genes has been not firmly established. Lefebvre et al. [1], have demonstrated the simultaneous expression of both SMN gene copies in lymphoblastoid cell lines. Only transcripts from SMNc were found in SMA patients, while control individuals expressed both RNA transcripts. These studies also revealed that SMNc transcripts differently from SMNt transcripts, may normally undergo exon 7 alternative splicing to produce transcripts lacking this exon. This has suggested that the truncated transcripts are specific for the centromeric gene [1 ].

In this report, we have investigated and characterized different mRNA isoforms transcribed from the SMN genes in muscle of SMA patients and normal controls. We have confirmed the presence of SMN-1a transcripts, corresponding to the previously described isoform, and found two new mRNA isoforms derived from both SMN genes (SMN-1b and SMN-1c). SMN-1b is mainly derived from SMNt and in part from SMNc,

while the SMN-1c is derived from the SMNc gene. The present data suggests that SMN in muscle is expressed in four different forms, as a result of alternative splicing of the pre-mRNA transcribed from the two SMN genes. The full length forms and SMN-1b are significantly reduced in muscle of all 9 SMA patients included in this study. By contrast, the amount of truncated SMNc transcripts was more abundant compared to that of the full length RNA in SMA patients. The ratio between different SMN isoforms was similar in all SMA types examined, excluding that specific alterations or compensatory effects in the SMN level expression are pathogenetically related to phenotypic diversity. The present results support and extend the hypothesis that the disease is caused by a reduced dosage of the SMNt products [1]. This is confirmed by evidence of deletion of the SMNc gene in about 10% of unaffected population However, it is still unclear if and how the SMNc-related products interact or contribute to the SMN expression in different tissues. According to Lewin [2], it is possible that a functional difference does exist between the two gene copies. Mutually exclusive splicing is frequently observed in muscle-specific transcripts which produce protein diversity [10]. It is possible that the polypeptide isoforms generated via alternative splicing cassette exons have important implications on the structure or function of the SMN protein in the skeletal muscle. The novel SMN-1b isoform described in this study, differ from the full length type in coil and helical conformations as well as in myristoylated sites, which have important role in juxtapositioning proteins to particular membranes [11]. We have also shown that this isoform is present in combination with the full length in the central nervous system, including the anterior columns, thalamus, dorsal root ganglia and geniculate nuclei (unpublished results). This suggests that the absence of this new SMNt-related isoform does contribute to the SMA phenotype.

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