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ARTICLE

SMN protein analysis in fibroblast, amniocyte and CVS cultures from spinal muscular atrophy patients and its relevance for diagnosis

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Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by the homozygous absence of the telomeric copy of the survival motor neuron (SMNt) gene, due to deletion, gene conversion or point mutation. SMNt and its homologous centromeric copy (SMNc) encode the SMN protein, which is diffusely present in the cytoplasm and in dot-like structures, called gems, in the nucleus. We have studied the SMN protein in different cell cultures, including fibroblasts, amniocytes and CVS cells from SMA individuals and controls. By immunofluorescence analysis we found a marked reduction in the number of gems in fibroblasts, amniocytes and chorionic villus cells of all SMA patients and foetuses, independent of the type of the genetic defect. We also show that immunolocalisation of the SMN protein may be a useful tool for the characterisation of particular patients of uncertain molecular diagnosis.

Keywords: spinal muscular atrophy; survival motor neuron protein; immunofluorescence analysis; cell cultures

Introduction

Proximal spinal muscular atrophy (SMA) is an autosomal recessive motor-neuron disorder characterised by the degeneration of spinal cord anterior horn cells and weakness of limb and trunk muscles. On the basis of age of onset and severity of the clinical course, three forms of childhood onset SMA (types I, II and III),[1] and one adult onset form (type IV)[2] can be distinguished. Childhood onset SMA is the most frequent genetic cause of infant death, affecting approximately 1 in 10 000 live births. Type I (Werdnig-Hoffmann disease) is the most severe form with onset ranging from the prenatal period to age 6 months; the children are never able to sit without support and the majority have a life expectancy of less than 2 years. Type II is an intermediate form with onset before the age of 18 months; the patients are unable to stand or walk. Type III (Kugelberg-Welander disease) is a relatively mild, chronic form with onset after the age of 18 months. Adult onset SMA is mild with a variable age of onset ranging from the third to the fifth decade.[2]

All these different types of SMA are in linkage with the chromosomal region 5q13 which harbours several genes.[3–5] One of these, designated survival motor neuron (*SMN*) gene, was identified as the SMA determining gene.[6] This gene is present in two highly

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homologous copies, one telomeric (*SMNt*) and one centromeric (*SMNc*). Molecular studies have demonstrated that *SMNt*, but not *SMNc*, is absent in about 95% of SMA patients due either to deletion or gene conversion into *SMNc*.[6–12] Several missense or frameshift mutations in *SMNt* have also been reported providing further evidence that *SMNt* is responsible for SMA.[6,13–17]

SMNt differs from *SMNc* at only two nucleotides in the coding region in exons 7 and 8, respectively, which do not alter the amminoacid sequence.[6] However, the two genes generate different proportions of alternatively spliced isoforms: about 90% of transcripts from the *SMNt* gene are full length, whilst the *SMNc* gene produces predominantly transcripts which lack exon 7 and, to a less amount, transcripts that lack exon 5 or both exons 5 and 7 as well as full length transcripts.[6,18]

Both *SMNt* and *SMNc* are expressed in most if not all tissues;[6,19–21] (own unpublished data 1996). Immunocytochemical studies of HeLa cells using monoclonal antibodies against SMN have shown that the protein is localised in both the cytoplasm and in the nucleus.[22] Whereas in the cytoplasm the SMN protein shows a diffuse localisation, in the nucleus the protein appears concentrated in a few dot-like structures termed gems.[22] These gems are closely associated with coiled bodies which are nuclear organelles thought to be involved in the assembly and processing of snRNP complexes.[23] SMN was subsequently found to be a subunit of a large protein complex containing a novel protein, termed SIP1 (for SMN interacting proteins) and spliceosomal snRNP proteins.[24] Evidence has been given that this complex is involved in the biogenesis and function of spliceosomal snRNPs.[25]

We have analysed the SMN protein in different cell cultures from SMA patients with various clinical severity and controls and report here on a significant reduction in SMN protein in the cells from all SMA patients independent of the type of mutation.

Materials and Methods

Cell Cultures from Patients and Controls

Primary fibroblast lines were established from skin biopsies of four type I, three type II, one type II/III, and one type IV patient and of three non-SMA individuals with different disorders used as controls. Two of the SMA type I patients (C1, MO) are over 3 years old and survive with assisted ventilation; one patient (SR) had type I SMA associated with severe arthrogryposis; the type II/III patient (BE) has a clinical phenotype characteristic of SMA III but a histopathological pattern compatible with SMA type II. Amniocyte cultures were prepared from amniotic fluids of three SMA I predicted and four control foetuses. Cultures from chorionic villus samples (CVS) of one SMA I predicted foetus and one normal foetus were established. A myoblast culture was established from a muscle biopsy of a normal individual.

Fibroblasts, amniocytes and chorionic villus cells were cultured in BioAmf (Biological Industries, Haemek, Israel) supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin in monolayers at 37°C. Myoblasts were cultured as reported earlier.[26]

SMN Analysis

The molecular analysis was performed by SSCP analysis[6] and restriction digest assay[27] on DNA extracted from fibroblasts, amniocytes or cells from peripheral blood or chorionic villus samples (CVS). Sequence analysis of DNAs from patients MO and MB was performed using either thermo sequenase cycle sequencing kit (Amersham, Pharmacia, Uppsala, Sweden) or dye terminator cycle sequencing kit (Perkin Elmer, Foster City, CA, USA).

Antibodies to SMN

The following antibodies were used in the present study: monoclonal antibody 2B1, kindly provided by Dr G Dreyfuss; antisera 95020, a generous gift of Dr S Lefebvre; and polyclonal antibody AC1. The latter was produced as follows: SMNc cDNA was cloned into the expression vector pET28a (Novagen, Abingdon, UK) in the NheI/BamHI sites. The His6-SMN fusion protein was expressed in *E. coli*, strain BL21(DE3) following induction with 1 mM IPTG for 2.5 h at 37°C, and purified under denaturing conditions using a Ni^{2+} chelation resin (Qiagen, Hilden, Germany). Two rabbits were immunised with 250 μg of purified protein by three boosts, one every month. The antisera (AC1 and AC2) were purified using GammaBind G Sepharose (Pharmacia). The concentration of total IgG was determined using Bio-Rad protein assay.

Immunofluorescence Analysis

Cells were trypsinised with 5% trypsin (Difco, Detroit, Michigan, USA) and grown in 35 mm dishes for 24 h at 37°C with 5% CO_2 until they reached a confluence of 50–60%. The cells were rinsed three times with cold PBS and fixed for 15 min in ice-cold 100% methanol at –20°C. The plates were dried for 5 min at room temperature and then rehydrated by washing on ice twice with PBS for 5 min. Upon microscopic inspection, a suitable area of about 1 cm^2 was chosen and circled. Cells of the selected area were incubated with 100 μl of the 2B1 monoclonal antibody, diluted 1:1000 in PBS, for 1 h at 4°C in a humidity chamber. The plates were then washed twice in PBS for 5 min on ice and incubated with 100 μl of the anti-mouse IgG secondary antibody, FITC conjugated (Sigma) at a dilution of 1:64 in PBS, for 1 h at 4°C in a dark humidity chamber. After two washes for 5 min in PBS the cells were overlaid with antifade solution and covered with a coverslip. The cells were observed by fluorescence microscopy on a Zeiss Axiophot microscope under oil immersion with a 100 × objective. Digital images were taken using a CCD camera (CE 200A, Photometrics, Tucson, Arizona, USA).

Western Blot Analysis

Cells from SMA patients and controls were grown to confluence in monolayer in flasks, detached by scraping, pelletted, and rinsed in PBS. Cell pellets were collected after centrifugation and lysed on ice in a lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 100 mM sodium fluoride, 0.1% SDS) containing freshly prepared protease inhibitors (10 mM Na_3VO_4, 10 sodium pyrophosphate mM, 1 mM PMSF) for 30 min. The cellular debris was then eliminated by centrifugation and the supernatants were collected and kept frozen at –20°C. Protein concentrations were determined by Bio-Rad protein assay. For western blot analysis three volumes of protein solution containing 30 μg of protein were mixed with one volume of sample buffer (125 mM Tris-Cl containing 0.1% SDS, pH 6.8, 4.6% SDS, 30% glycerol, 10% β-marcaptoethanol, 0.16 g bromophenol blue), boiled for 5 min and electrophoresed on a 12% SDS-polyacrylamide gel in a 1% running buffer (25 mM Tris, 192 mM glycine, 3.4 mM SDS, pH 8.3) and subsequently electrotransferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) using a MilliBlot-Graphite Electroblotter I (Millipore) at 0.8 mA/cm^2 of gel area for 1 h in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). The blotting membranes were incubated in blocking solution (PBS, 5% non-fat milk, 0.2% Tween-20) for 1 h at room temperature, rinsed with PBS containing 0.1% Tween-20 and then incubated in the same solution with the primary antibody (antisera 95020 diluted 1:4000, AC1 diluted to 0.5 μg/ml), overnight at room temperature. The membranes were washed in PBS containing 0.1% Tween-20 and incubated in the same solution with the secondary antibody (anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:1000) for 1 h at room temperature. After the last washes in PBS containing 0.1% Tween-20, the immune complexes were revealed by autoradiography (Kodak X-OMAT AR) using ECL kit (Amersham). Blots were stripped and reprobed with the polyclonal anti-actin diluted 1:1000 (Sigma).

Western blot quantification was performed by scanning the autoradiographs with a computerised densitometer using the program Phoreticx 1D (Phoreticx International). SMN-actin ratios were determined and normalised to controls.

RT-PCR

Total RNA was isolated from fibroblast cultures using Trizol (Gibco BRL, Grand Island, NY, USA). First-strand cDNA synthesis from total RNA was performed as previously described.[14] SMN cDNA isoforms were amplified together with a 390 bp partial MLH1 cDNA, employed as internal standard, in a multiplex PCR reaction. For each primer 25 pmol were used. Primers of SMN were in exon 4 (5'-CAAGCCCAAATCTGCTCCATGGA-3') and in exon 8 (5'-ACTGCCTCACCACCGTGCTGG-3') and primers of MLH1 were forward (5'-TCCCAAAGAAGGACTTGCT) and reverse (5'-GGAGGCAGAATGTGTGAGCG-3'). Forward primers were end-labelled with gamma (^{32}P) ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) and purified on Chromaspin columns-10 (Clontech, Palo Alto, CA, USA). Annealing temperature was 60°C and PCR was run for 18–20 cycles. PCR products were separated on a 4% denaturing polyacrylamide gel and revealed by autoradiography (Biomax MR-1, Kodak, Rochester, NY, USA) after 8 days of exposure.

Results

Western Blot Analysis

Overexpression of the His6-tagged SMN protein in *E. coli* is shown in Figure 1A. The protein was extracted from the insoluble fraction under denaturing conditions, purified and used for immunisation. The obtained antisera (AC1) detected the purified 38 kD SMN protein (Figure 1B) and a protein of the expected size in HeLa cell extracts, identical to that detected by the previously established antisera 95020[21] (not shown). Both AC1 and 95020 were used for western blot analysis.

Figure 2A shows a western blot of amniocytes. The level of the SMN protein was found markedly reduced in the cells from two foetuses predicted to be affected by SMA type I (lanes 1 and 2) being only 12.7% and 16.1%, respectively, of that detected in amniocytes from a control foetus (lane 4). Amniocytes from a foetus predicted to be a carriers of SMA type III had 46% protein level compared with the control.

Reduced amounts of the SMN protein were also found in fibroblasts from SMA patients. However, especially for patients with chronic SMA (type II, III, and IV), these reductions were less significant, or even marginal, compared with controls (Figure 2B), similar to previously reported data.[20,21]

Immunofluorescence Analysis

To investigate whether SMN protein analysis by immunocytochemistry could be more effective in detecting variations in protein expression in SMA patients

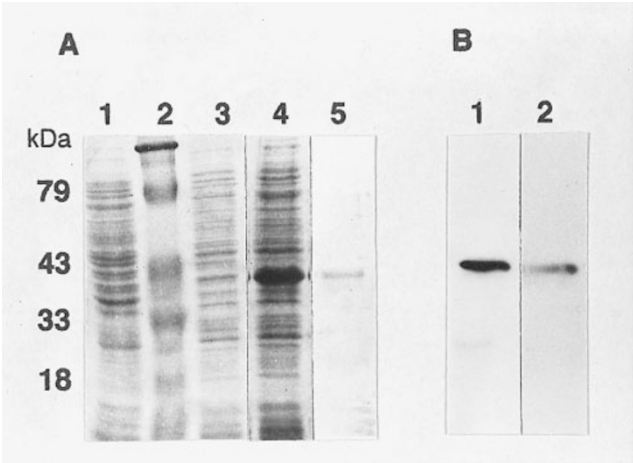

Figure 1 **A***) SDS-PAGE. Untransfected* E. coli *cells (1), molecular weight markers (2), transfected, uninduced (3), and IPTG induced* E. coli *cells (4), purified protein (5).* **B***) Immunoblot. The purified protein is detected by anti-SMN antibody 95020 (1) and antibody AC1 (2)*

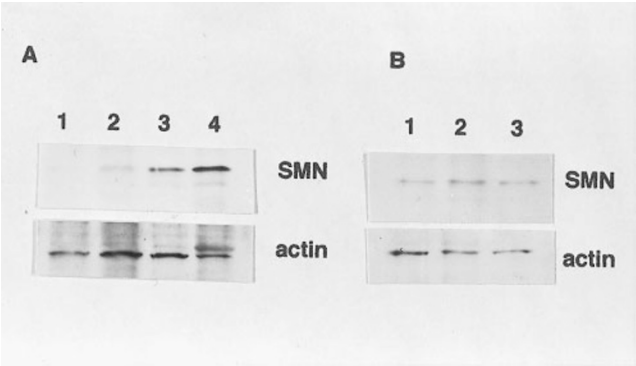

Figure 2 *Western blot analysis of the SMN protein and actin used as control.* **A***) SMN expression detected in amniocytes from foetuses by the antibody 95020. Lanes 1–4: type I (12.7%), type I (16.1%), carrier type III (46%), and control (100%), respectively.* **B***) SMN detected in fibroblasts by the antibody AC1. Lanes 1–3: type II (52%), type IV (95.7%), and control (100%)*

compared with western blot analysis, we have performed immunolocalisation studies in fibroblast, amniocyte, and CVS cultures. We used the monoclonal antibody 2B1 which has previously been shown to detect SMN in the cytoplasm and in dot-like nuclear structures (gems) in a variety of cells.[22]

We first studied primary fibroblast lines, established from SMA patients with various clinical severity and non-SMA individuals affected by different disorders used as controls (Figure 3A–D, Table 1). In control fibroblasts we found the presence of one or more gems in 60–70% of nuclei and a number of gems/100 nuclei ranging from 87 to 163. In contrast, fibroblasts lines from 7 SMA patients with various clinical phenotypes showed a marked reduction in both the number of nuclei with gems and the number of gems/100 nuclei. We also noted for most, but not all, patients an increase in the number of discrete dots, smaller than gems, in the cytoplasm compared with the controls (Figure 3C). The reduction of the nuclear signal was most evident in the cells from a patient with SMA type I who showed gems only in 4.8% of nuclei and 6.5 gems/100 nuclei. Fibroblasts from patients with chronic SMA, including three type II, one type II/III and one type IV patients, respectively, showed similar reduced numbers of gems, ranging from 9.6% to 16.5% of nuclei (Table 1).

We included in the immunocytochemical study fibroblast lines from three patients with uncertain molecular diagnosis based on DraI restriction digest assay which showed the presence of SMNt exons 7 and 8. Two of these patients (MO, MB) had muscle biopsies, electromyography and clinical findings consistent with typical SMN type I. SSCP analysis of all nine exons of SMN did not show any band shifts for both of them. Furthermore, no point mutation could be detected by sequencing, using the manual procedure, of the SMN coding region and the exon–intron boundaries of patient MO. However, immunolocalisation studies showed a marked reduction in the number of gems, (about 10–12%) for both patients. In addition, a peculiar immunostaining was observed in the perinuclear region which was particularly evident in some of the fibroblasts from patient MB (Figure 3D). These findings prompted us to perform additional mutation screening by using automated sequence analysis which demonstrated the presence of a point mutation in both patients in exon 6 at codon 272 (TAT–TGT) resulting in a tyrosine–cysteine substitution (Y–C). Molecular studies of the parents of the two unrelated patients indicated that the mutation was of maternal (MO) and paternal (MB) origin, respectively.

The third patient who was positive for SMNt exons 7 and 8 had a muscle biopsy compatible with SMA type I, as well as additional clinical manifestations including severe arthrogryposis. His fibroblasts showed a number of nuclei with gems (51%) close to that of the controls, a finding which suggests a diagnosis of a variant form of SMA I, not associated with SMN protein defects.

Immunolocalisation studies were also performed in several cultures of amniocytes (Figure 3E and 3F, Table 2). Amniocytes from normal foetuses had 84–109 gems/100 nuclei, whilst amniocytes from three foetuses, predicted to have SMA type I, showed a marked reduction in the number of gems (about 5–28 gems/100 nuclei). Similar to fibroblast cultures, we noted an increase in cytoplasmic staining in the cells from SMA foetuses. In a prenatal diagnosis of a foetus (MB-F), a sib of patient MB carrying the Y272C mutation, we found a number of gems in the normal range in the amniocytes. The molecular analysis revealed that the foetus has inherited the same paternal mutation but a maternal haplotype of microsatellite marker alleles different from that present in his affected sib. These data suggested that the foetus is a healthy carrier which is in agreement with the immunocytochemical data.

We also analysed the SMN protein in CVS cells from two foetuses, one predicted to be affected by SMA I and the other to be normal (Figure 3G). We detected gems in 60.3% of nuclei in the cells of the normal foetus, whilst only two nuclei with one gem each were found in more than 100 cells from the SMA I foetus.

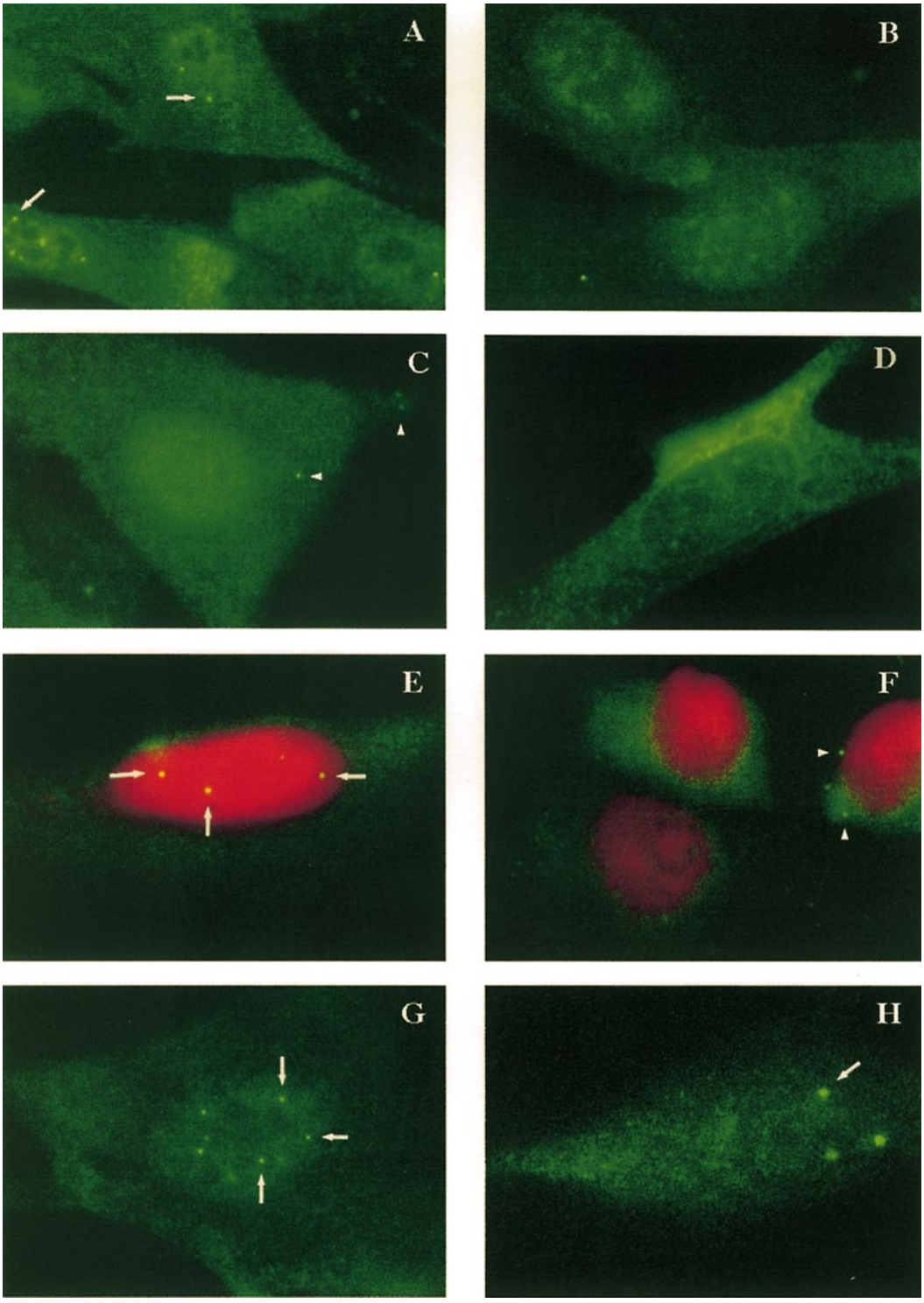

Figure 3 *Immunolocalisation of the SMN protein using the monoclonal antibody 2B1. Arrows indicate gems in the nuclei, arrow heads indicate dot-like cytoplasmic signals. Control fibroblasts (***A***), fibroblasts from a type I SMA patient (***B***) and from a type II patient (***C***); fibroblasts from patient MB with the Y272C mutation showing perinuclear staining (***D***); amniocytes from a normal (***E***) and an SMA I predicted foetus (***F***), the nuclei are counterstained in red with propidium iodide; CVS cells from a normal foetus (***G***); myoblasts from a normal individual (***H***)*

Table 1 Summary of SMN analysis by immunocytofluorescence in fibroblasts

Individual	*Phenotype*	*SMNt*	*Total no. cells*	*Nuclei with gems*	*Gems/100 nuclei*	*Cells with cyto-plasmic signal*
TA	Normal	Exon 7+	553	384 (72.1%)	163.0	26 (4.9%)
SC	Normal	Exon 7+	522	315 (60.3%)	137.0	5 (1.0%)
PI	Normal	Exon 7+	154	92 (59.7%)	87.0	2 (1.3%)
C1	SMA I	Exon 7–	168	8 (4.8%)	6.5	71 (42.3%)
W1	SMA II	Exon 7–	760	96 (12.6%)	13.7	78 (14.1%)
SP	SMA II	Exon 7–	266	44 (16.5%)	21.4	52 (19.5%)
SS	SMA II	Exon 7–	125	12 (9.6%)	30.4	3 (2.4%)
BE	SMA II/III	Exon 7–	434	47 (10.8%)	13.6	90 (20.7%)
IT	SMA IV	Exon 7–	242	40 (16.5%)	31.0	49 (20.3%)
MO	SMA I	Y272C	338	40 (11.8%)	18.0	19 (5.6%)
MB	SMA I	Y272C	374	38 (10.1%)	16.6	10 (2.7%)
SR	SMA I[a]	Exon 7+	584	300 (51.3%)	107.3	16 (2.7%)

[a]SMA I associated with severe arthrogryposis.

Table 2 Summary of SMN analysis by immunocytofluorescence in aminocytes and chronic villus cells

Foetus	*Phenotype*	*SMNt*	*Total no. cells*	*Nuclei with gems*	*Gems/100 nuclei*	*Cells with cyto-plasmic signal*
N1	Normal	Exon 7+	226	170 (75.2%)	178.7	3 (1.3%)
N2	Normal	Exon 7+	187	112 (59.9%)	109.1	7 (3.7%)
N3	Normal	Exon 7+	103	46 (44.7%)	97.1	0 (0.0%)
N4	Normal	Exon 7+	409	166 (40.6%)	83.9	8 (2.0%)
TR	SMA I	Exon 7–	215	41 (19.1%)	27.9	23 (10.7%)
CE	SMA I	Exon 7–	205	10 (4.8%)	4.9	39 (19.0%)
MA	SMA I	Exon 7–	154	21 (13.6%)	23.4	54 (35.1%)
MB-F	Carrier	Exon 7+	213	159 (74.5%)	175.0	17 (2.0%)
VI	Normal villus	Exon 7+	209	129 (60.3%)	145.4	4 (1.9%)
MI	SMA I villus	Exon 7–	103	2 (1.9%)	1.9	2 (1.9%)

Lastly, immunolocalisation performed on one culture of normal human myoblasts clearly showed the presence of 1 to 5 gems in the majority of nuclei (Figure 3H).

Transcript Analysis

The *SMNc* gene produces only 20–30% of full length transcripts;[6,18,20] the rest is a mixture of isoforms which lack exon 7 (7 minus), exon 5 (5 minus) or both (5/7 minus) due to alternative splicing. We investigated the levels of the different transcripts in a number of fibroblast lines. Table 3 shows the isoform levels relative to a standard external to SMN (MLH1 partial cDNA) and the percentages of the different SMN isoforms compared with the total amount of transcripts. All four mRNA isoforms, described by Gennarelli *et al*,[18] were detected (Figure 4). Reduced total amounts of SMN transcripts, normalised to the standard, were detected in three out of four patients with absence of the *SMNt*

Table 3 Transcript analysis of SMN

	C1	*SS*	*W1*	*IT*	*MB*	*MO*	*SR*	*SC*
SMA type	I	II	II	IV	I (Y272C)	I (Y272C)	I variant	Control
Full length	0.10 (24)	0.11 (24)	0.20 (21)	0.10 (24)	0.30 (48)	0.41 (45)	0.62 (63)	0.49 (65)
7 minus	0.19 (47)	0.22 (48)	0.57 (59)	0.21 (50)	0.22 (36)	0.38 (41)	0.16 (16)	0.15 (20)
5 minus	0.05 (12)	0.05 (11)	0.06 (6)	0.03 (7)	0.05 (8)	0.06 (6)	0.13 (13)	0.08 (10)
5/7 minus	0.07 (17)	0.08 (17)	0.14 (14)	0.08 (19)	0.05 (8)	0.07 (8)	0.07 (7)	0.04 (5)

Numbers in parenthesis indicate the percentage of the respective transcripts relative to the total amount of transcripts.

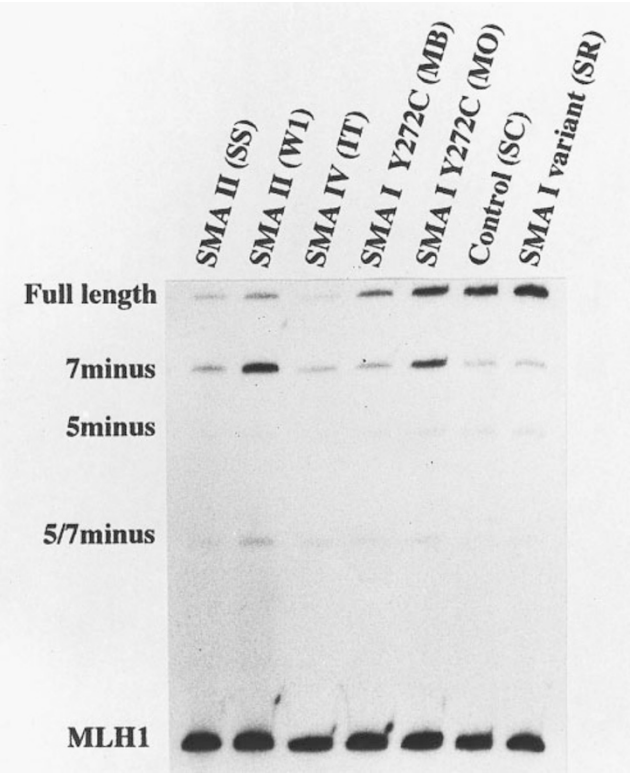

Figure 4 *Autoradiogram showing the different SMN transcripts and MLH1 partial cDNA obtained by RT-PCR of fibroblast RNA*

gene. SMA patients with no *SMNt* gene had on average 22.5% and 51% of the full length and 7 minus isoforms, respectively. In contrast, carriers of the Y272C mutation had on average 46% and 38% of full length and 7 minus isoforms, reflecting the presence of one, albeit mutated, *SMNt* gene. Both the control and one SMA I variant had three to four times more full length transcripts than the 7 minus isoforms. We found no significant differences in the levels of the isoforms 5 minus and 5/7 minus in SMA and non SMA individuals.

Discussion

Quantitative Protein and Transcript Analysis

Previous western blot analysis of the SMN protein in whole-cell lysates from lymphoblastoid cell lines have shown a marked decrease in SMN protein level in type I SMA individuals.[21] We show here that the SMN protein level is significantly reduced also in amniocytes of SMA I predicted foetuses. In contrast, moderately reduced or normal SMN protein levels were found in fibroblasts from chronic SMA patients, similarly to the data reported earlier.[20]

We detected all four SMN isoforms in fibroblasts. As expected, low relative levels of full length transcripts were found in SMA patients with absence of *SMNt*, but not in patients with a point mutation. The small number of patients does not allow establishment of correlations between the clinical phenotypes and the relative amount of isoforms. In contrast to Gennarelli *et al*[18] who found similarly high levels of both the isoforms 7 minus and 5/7 minus in muscles of SMA patients, we detected much lower levels of the 5/7 minus isoform in fibroblasts of SMA patients and controls. This discrepancy might reflect differences in tissue expression.

Immunolocalisation Analysis

Previous immunolocalisation studies of the SMN protein in liver and spinal cord from SMA foetuses and in fibroblasts from SMA patients have demonstrated absence or reduction in the number of gems in the nuclei.[20,21] We confirm this reduction in fibroblasts from patients affected with various clinical severities of SMA and show for the first time a marked reduction in the number of gems also in the nuclei of amniocytes and CVS cells of SMA predicted foetuses. Furthermore, we observed an increase in cytoplasmic staining in the majority of fibroblast and amniocyte cultures from SMA patients which may suggest an inefficient import of certain SMN isoforms into the nuclei.

In our hands, immunofluorescence studies were more effective in detecting SMN protein reductions, especially in cell lines from SMA type II and III patients. compared with western blot analysis. This may be at least in part due to the fact that western blot analysis detects the total amount of protein (cytoplasmic and nuclear) present in the cell extracts, whilst immunolocalisation of SMN almost exclusively reveals changes in the nuclei in terms of the number of gems. Patients with milder forms of SMN may have a higher copy number of *SMNc* genes compared to type I SMA patients due to gene conversion events.[9,12,28,29] The *SMNc* genes are transcribed and translated into various isoforms of which only the full length isoform presumably can be assembled into the protein complexes detected as gems in the nucleus. Thus, whereas each extra *SMNc* gene will contribute considerably to the total SMN protein level, it will have slight effect on the number of nuclear gems.

We found gems in 4.8% and 9.6–16.5% of fibroblasts from SMA I and II patients, respectively, which is

concordant with the data reported by Coovert *et al*,[20] who found gems in 1.7–6% and 5.2–14.8% of nuclei, respectively. Surprisingly, we found a marked reduction in the number of gems also in a type IV patient who had onset of mild muscle weakness at the age of 18 years. She is at present a 39-year old practising nurse. It is unlikely that the reduction in gems in this patient is in part due to a reduced SMN expression in adults, since fibroblasts from an age-matched healthy female showed numbers of gems comparable to those of fibroblasts from children (1–3 years of age) used as controls.

We found reduction in gems independent of whether the genetic defect is a homozygous deletion of *SMNt*, a gene conversion event, as demonstrated by a higher copy number of *SMNc* genes (unpublished data 1998) or a point mutation. This suggests that immunofluorescence analysis of the SMN protein may be a useful diagnostic tool for patients with uncertain molecular diagnosis. The finding of a high number of gems in a patient positive for SMNt exon 7 will exclude the diagnosis of SMA. For example, in our patient with SMA I and arthrogryposis, normal immunostaining confirmed the diagnosis of an SMA variant, unlinked to 5q. This is not as obvious as might first appear; in fact, absence of SMNt has recently been found in about 50% of cases with SMA and either mild or severe generalised contractures.[30]

Based on our data, the finding of gems in less than 20% of cells of an SMNt exon 7+ patient will be in favour of a diagnosis of SMA. Protein analysis turned out particularly helpful for patient MO, a male who in addition to typical SMA I showed small testicles and penis and a slightly unusual face with long and flat philtrum. We initially failed to detect a mutation by standard methods in his DNA and considered the possibility of a rare X-linked form based on his gender, hypogonadism and facial appearance.[31] Instead the reduced immunostaining suggested the diagnosis of SMA I which was subsequently confirmed by the detection of the Y272C mutation. To our knowledge this missense mutation has been reported only once before.[6]

In conclusion, we have shown a marked reduction in the number of gems in various, diagnostically relevant cell types, such as fibroblasts, amniocytes and chorionic villus cells, from SMA patients or foetuses and suggest that SMN protein analysis by immunofluorescence may be an important complementary diagnostic tool for SMA patients with controversal molecular diagnosis.

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