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ARTICLE

Premature termination mutations in exon 3 of the *SMN1* gene are associated with exon skipping and a relatively mild SMA phenotype

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Autosomal recessive spinal muscular atrophy (SMA) is a common motor neuron disease caused by absence or mutation in the survival motor neuron (*SMN1*) gene. *SNM1* and a nearly identical copy, *SMN2*, encode identical proteins, but *SMN2* only produces a little full length protein due to alternative splicing. The level of functional SMN protein and the number of *SMN2* genes correlate with the clinical phenotype ranging from severe to very mild. Here, we report on premature termination mutations in *SMN1* exon 3 (425del5 and W102X) which induce skipping of the mutated exon. The novel nonsense mutation W102X was detected in two patients with a relatively mild phenotype who had only two copies of the *SMN2* gene, a number that has previously been found associated with the severe form of SMA. We show that the shortened transcripts are translated into predicted in frame protein isoforms. Aminoglycoside treatment suppressed the nonsense mutation in cultured cells and abolished exon skipping. Fibroblasts from both patients show a high number of nuclear structures containing SMN protein (gems). These findings suggest that the protein isoform lacking the exon 3 encoded region contributes to the formation of the nuclear protein complex which may account for the milder clinical phenotype. *European Journal of Human Genetics* (2001) **9,** 113–120.

Keywords: spinal muscular atrophy; survival motor neuron gene; exon skipping; mutations

Introduction

Proximal spinal muscular atrophy (SMA) is one of the most frequent autosomal recessive diseases with an estimated incidence of 1 in 10 000 live births. It is a motor neuron disorder characterised by the degeneration of spinal cord anterior horn cells which results in weakness of proximal limb and trunk muscles. Based on the age of onset and severity of the clinical course, childhood onset SMAs can be classified into three types.[1] Type I is the most severe form with onset ranging from prenatally to 6 months of life; the children are never able to sit without support. Type II is an intermediate form with onset before the age of 18 months; the patients are unable to stand or walk. Type III is a relatively mild, chronic form with onset after the age of 18 months.

All three forms of SMA are caused by absence or mutation of the survival motor neuron (*SMN1*) gene localised to chromosome 5q13.[2] This region contains a second gene (*SMN2*) which is nearly identical to *SMN1* but can be distinguished from it by two base changes in exons 7 and 8.[2] It has recently been demonstrated that the synonymous C-to-T transition in exon 7 is responsible for alternative splicing of exon 7 of the *SMN2* transcripts.[3,4] Hence, the two genes generate different proportions of full length transcripts: *SMN1* produces primarily full-length transcripts, whereas *SMN2* produces primarily transcripts lacking exon 7 plus minor amounts of full length transcripts and

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transcripts lacking exon 5 or both exon 5 and exon 7.[2,5] It has been shown that the protein product of the alternatively spliced transcripts is less stable and functionally defective.[6] Thus, SMA is caused by reduced levels of functional protein.[7] Recently, we and others have shown that the clinical severity is influenced by the copy number of *SMN2* genes.[8–10]

The great majority of SMA patients show homozygous absence of *SMN1* caused by deletion or gene conversion.[2,11] Subtle mutations in *SMN1* are relatively rare. The 23 different mutations described so far are distributed all over the gene but are more frequently found at the 3′ end.[12] There is no strict correlation between the type of mutation and the clinical phenotype. For unrelated compound heterozygotes with identical missense or frameshift mutations the difference in phenotypic expression can often, but not always, be explained by the different number of *SMN2* genes.[13,14]

The SMN protein is part of a large protein complex and is localised in both the cytoplasm and in the nucleus. In the cytoplasm SMN plays a role in the biogenesis of spliceosomal small nuclear riboproteins (snRNPs).[15] In the nucleus SMN appears concentrated in a few dot-like structures, designated gems,[16] where it is involved in pre-mRNA splicing.[17] Immunofluorescence analysis of different cell types has shown that the number of gems is markedly reduced in SMA patients.[9,18]

The SMN protein comprises several functionally important domains. The N-terminus interacts directly with a novel protein, Gemin2, whereas the C-terminal part is involved in oligomerisation and binding of a novel DEAD box protein, Gemin3.[19] The C-terminus has also been implicated in interacting with several snRNP Sm proteins.[15] In contrast, Bühler *et al*[20] have reported that the central region of SMN is necessary and sufficient for binding Sm proteins. This central region is encoded by exon 3 and encompasses a tudor domain which is present in proteins with RNA binding function.[21]

Here, we report on patients with mutations in *SMN1* exon 3 leading to premature termination codons and skipping of the entire exon. The shortened transcripts are translated into protein isoforms which can be included into the nuclear protein complex, as suggested by the particularly high number of gems detected in fibroblasts of these patients. The production of these protein isoforms probably explains the relatively mild phenotype of these patients.

Material and methods

Patients

We studied three patients with clinical diagnosis of SMA including EMG and muscular biopsy. For all three patients both *Dra*I digestion of PCR products and SSCP analysis showed the presence of *SMN1* exon 7. Patient PA is a girl aged 2 years and 9 months with SMA type I. She suffered from periodic respiratory crisis but is neither intubated nor ventilated artificially, thus representing an attenuated expression of SMA I. Patient ZE is a 19-year-old young man and has a clinical phenotype typical of SMA type II. Patient DG, is a 24-year-old mother of a 2-year-old healthy girl and presently at her second pregnancy. She is a type III patient and walks mostly with support.

Primary fibroblast lines from skin biopsies and lymphoblastoid cell lines were established from patients ZE and DG.

RT – PCR

Total RNA was isolated from peripheral blood leucocytes, fibroblast and lymphoblastoid cell lines using Trizol (GIBCO BRL, Grand Island, NY, USA), according to the instructions of the manufacturer. First-strand cDNA synthesis was performed as previously described.[22] In some experiments SMN cDNA isoforms were amplified together with a 390-bp partial *MLH1* cDNA, employed as internal standard, in a multiplex PCR reaction.

Cloning of cDNAs and genomic DNA

Single strand cDNAs were amplified with 300 nM of each primer SMNpro-F (5′- TGC GCA TCC GCG GGT TTG CTA T -3′) in 5′ UTR and c770[2] in exon 7, 200 μM each dNTPs, 1 × expand HF buffer, 1.5 mM $MgCl_2$ and 2.6 U Expand High Fidelity Polymerase (Boehringer Mannheim), in a final reaction volume of 100 μl; annealing temperature was 60°C.

Genomic DNA of patients was amplified with 1 μM of each primer 2BF2 (5′- AGA GCT GAT GTC AGG TGT AT -3′) in intron 2a and EX4R (5′- GAG AGG TTA AAT GTC CCG AC -3′) in intron 4, 200 μM dNTPs, 2 mM $MgCl_2$, 1 U of Taq polymerase (Boehringer Mannheim); annealing temperature was 55°C.

Of both PCR products, 20 ng were cloned into the pCRII-TOPO cloning vector (Invitrogen), according to the manufacturer's protocol. Colonies derived from cDNAs were analysed by PCR with primers X7DRA[23] and C618[2] and digested with 6 U of *Dra*I (New England Biolabs) to select *SMN1* and *SMN2* clones. A minimum of six *SMN1* cDNA colonies and 15 genomic colonies were cultured, DNA was purified and sequenced with Dye Terminator Cycle Sequencing kit (PE Applied Biosystems) according to the manufacturer's instructions by using both vector and internal primers.

The W102X and S139S mutations were confirmed on colonies, genomic DNA and cDNA by PCR amplification, respectively, with specific primers SMN3Stop forward (5′- GGG ACA AAT GTT CTG CCA CTT -3′) and SMN3Dde reverse (5′- CAC AGA TTG GGG AAA GTA GCT C -3′): these primers introduce *Dde*I restriction sites in mutated templates only. Cycling conditions consisted of an initial denaturation step at 95°C for 10 s, 32 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 20 s and a final extension step of 3 min at 72°C, followed by overnight digestion of 10 μl of PCR products with 3 U of *Dde*I (New England Biolabs) and electrophoresis on 2% TBE 1 × agarose gel.

Southern blot analysis
Approximately 8 μg of genomic DNA of patients were digested with *Eco*RI and migrated on a 0.8% TBE 1 × agarose gel. Probes and hybridisation protocol were previously described.[8]

Study of exon skipping
cDNAs from leukocytes of patients of the present study, 11 SMA patients of various clinical phenotype, and 22 unaffected controls were amplified by standard PCR (35 cycles) reaction with 1.5 mM $MgCl_2$. Primer pairs used were SMNPro-F/C770 and/or SMNPro-F/EX4R2 (5′- TCC ATG GAG CAG ATT TGG GCT TG -3′) in exon 4.

cDNAs from fibroblast and lymphoblastoid cells of patients and controls were studied by radioactive PCR. Three separate low-number-cycle (22 cycles) radioactive PCRs were performed with primers SMNPro-F and C770 as described above except that 0.1 μl α-dCTP (NEN Life Science Products) was added. PCR products were electrophoresed in a 4% acrylamide TBE 0.6 × Urea 6 M gel for 3 h at 35 W. Gels were exposed overnight on a Cyclone Storage Phosphor System (Packard Instrument Company) and densitometric analysis was performed (OptiQuant software). Average percentage of −3/+7 over the total of −3/+7 plus +3/+7 isoform PCR products for each sample were calculated.

Western blot analysis
Western blot analysis was performed as described elsewhere[18] with the exception that the commercial anti-SMN was used as primary antibody.

FISH
Fluorescence *in situ* hybridisation (FISH) was performed on interphase nuclei preparations as previously described.[8]

Quantitative PCR
SMN1 copy-number assay was performed as described by Wirth *et al*[14] with the exception that radioactive PCR was used and that acrylamide gels were exposed on the Cyclone Storage Phosphor System.

Analysis of *SMN2* isoforms
cDNAs of patients and controls were amplified using primers SMNPro-F and C1120[2], 124 nt downstream the *Dde*I restriction site in *SMN2*; cycling conditions consisted of an initial denaturation step at 94°C 10 s, followed by 35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 2 min and a final extension step of 3 min at 72°C. Ten μl of PCR were digested overnight with 3 U of *Dde*I and electrophoresed on a 2% TBE 1 × agarose gel. Band intensity was investigated by direct densitometric analysis of the gels using Gel Doc 2000 System (BIO-RAD). Percentage of *SMN2* full length (fl) was deduced from the ratio between *SMN2* fl and the total of *SMN2* fl plus −7 *SMN2* isoforms. Average values were obtained from three different PCR experiments.

Immunofluorescence analysis
Immunofluorescence studies of the SMN protein were performed on fibroblasts of patients and controls as previously described.[18] The protein was detected by using the commercial anti-SMN monoclonal antibody (Transduction Laboratories) and the 2B1 antibody,[16] kindly provided by Dr G Dreyfuss. Each experiment was performed three times.

Aminoglycoside treatment
In preliminary tests fibroblasts and lymphoblastoid cells were incubated with medium (BioAmf and RPMI, respectively) supplemented with increasing concentrations (50, 100 and 200 μg/ml) of Geneticin G-418 sulphate (GIBCO BRL) as previously described.[24] At the concentration of 200 μg/ml fibroblasts died within 48 h, whereas at 100 μg/ml or less cells were viable for at least 1 week. For transcript analysis, RNA was extracted from lymphoblastoid cells and fibroblasts treated for 48 h with 50 μg/ml and 100 μg/ml G-418, respectively. For protein analysis, cells were treated for 72 h with above indicated concentrations of G-418.

Results
Mutations and exon 3 skipping
Standard SSCP analysis of SMN exon 7 from the type II (ZE) and the type III (DG) patient revealed an abnormal pattern of migration. Sequencing of exon 7 and adjacent intron junctions showed an A-G transition 96 nt upstream from the exon 7 start site for both patients which has been described before as a rare polymorphism.[25] Since *SMN1* exon 7 specific bands were absent, we postulated that the two patients are compound heterozygotes and that the A-G base substitution is associated with a mutation elsewhere in the *SMN1* gene.

Sequencing of genomic DNA of all exons did not show any apparent difference with respect to the wild-type sequence. However, by RT–PCR of the SMN mRNA, shortened transcripts were found for both patients (Figure 1). Sequence analysis of these shortened cDNAs showed a deletion of exon 3 (−3) which does not lead to a shift in the translational reading frame. No mutation at the exon 3 splice sites could be detected. Also, deletion of exon 3 in the genomic DNA was excluded by Southern blot analysis (data not shown). We therefore cloned and sequenced RT–PCR products comprising the complete coding region. A G-A transition at base 305 of the coding sequence was detected in several clones from both patients (Figure 2a). This previously unreported mutation alters a tryptophan residue to a translational termination codon (W102X) in exon 3 of the *SMN1* gene. The presence of this base substitution was confirmed by cloning and sequencing genomic DNA and by digestion of amplified genomic DNA and cDNA with *Dde*I by using a specific primer which creates the restriction enzyme site in the mutated DNA. A second mutation was detected in *SMN1*

exon 3 of patient DG, which is a synonymous third base substitution TCC-TCT (S139S) (Figure 2b).

Sequence analysis of the DNA of patient PA (attenuated SMA I) revealed a 5-bp deletion (425del5) in exon 3 of *SMN1* in the genomic DNA and cloned cDNAs (Figure 2c). The same mutation, which predicts a premature stop codon four nucleotides downstream, was described before for an unrelated very severely affected patient.[22] Shortened transcripts lacking exon 3 were found in RT–PCR products also from this patient's mRNA (Figure 1).

The finding of skipping of exon 3 in a proportion of transcripts from all three patients with mutations in the same exon has led us to postulate that exon skipping was induced by the mutations. To investigate whether the transcripts lacking exon 3 originated from the mutated *SMN1* gene we have analysed 15 cloned cDNAs from leukocyte mRNA of patient ZE by *Dra*I restriction digestion and in part by sequence analysis. Six clones contained *SMN1* transcripts, of which four had the W102X mutation and two lacked exon 3, whereas nine clones had *SMN2* transcripts, which had retained a normal exon 3. Moreover, the PCR product obtained by re-amplification of a gel-extracted -3 cDNA from patient DG with X7Dra primer was found resistent to *Dra*I enzyme digestion, providing further evidence that the shortened transcripts are derived from the *SMN1* gene. RT–PCR of mRNA from peripheral blood leukocytes or lymphoblastoid cells of 22 controls and 11 patients of SMA types I-III did not show isoforms lacking exon 3. These findings suggest that skipping of exon 3 in a proportion of *SMN1* transcripts from our patients is induced by the mutations which are distant from the splice junction consensus sequences.

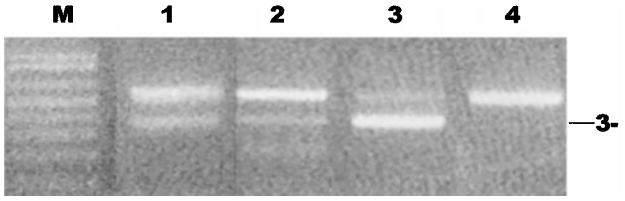

Figure 1 Detection of −3 isoforms. Agarose gel showing cDNA amplification products from SMN exons 1–4 obtained from peripheral blood leukocyte RNA. Transcripts lacking exon 3 were observed for patients PA (lane 1), ZE (lane 2) and DG (lane 3) but not for a control (lane 4). M: DNA size marker.

Transcripts lacking exon 3 were also detected in mRNA from fibroblasts and lymphoblastoid cells from patients ZE and DG. The extent of exon skipping was 39 and 44% (−3/total transcripts; see Material and methods) in fibroblast mRNA of patients ZE and DG, respectively, but was less in lymphoblastoid cells (23%) of patient DG.

Shortened protein isoforms detected by Western blot

To investigate whether the transcripts lacking exon 3 are translated into protein isoforms we performed Western blot analysis on extracts of fibroblasts and/or lymphoblastoid cells from patients ZE and DG. The anti-SMN antibody detected a 38-kDa SMN protein, which should comprise full length and −7 *SMN2* gene products, and two shortened SMN protein isoforms (Figure 3). The molecular mass of approximately 31 kDa of the smallest isoform corresponds to that

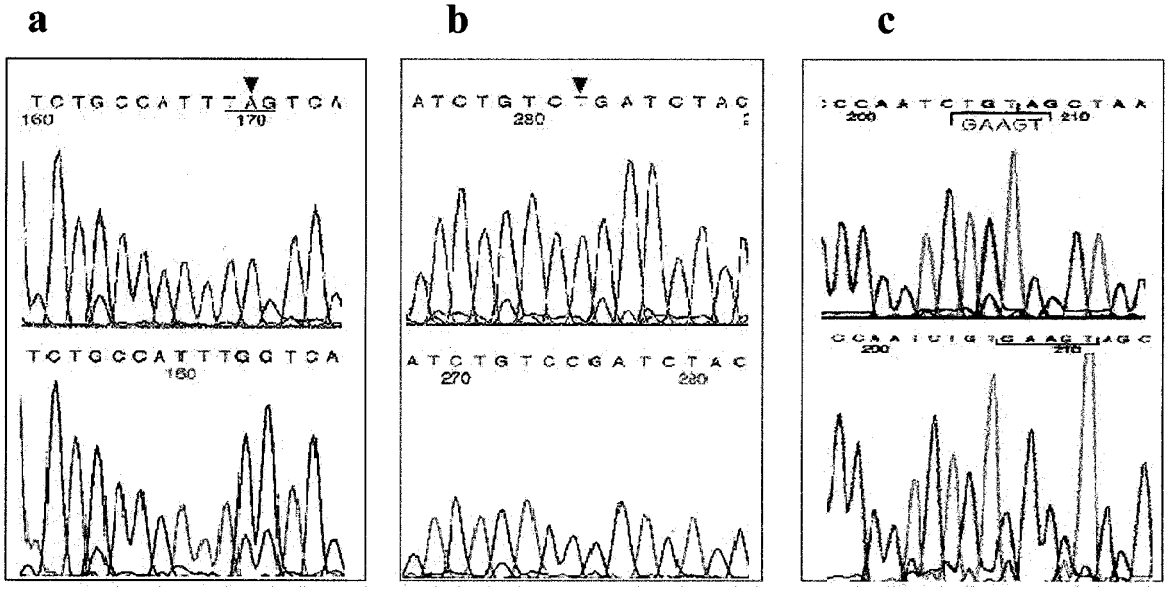

Figure 2 Mutations in *SMN* exon 3. Partial sequences of cloned genomic DNA showing the mutations (arrows) in the top panel compared to the wild-type sequence in the lower panel. (**a**) G-A transition at base 305 which alters a tryptophan residue to a translational termination codon (W102X) detected in patients ZE and DG. (**b**) Silent C-T substitution at codon 139 present in patient DG. (**c**) Deletion of 5 bp (472del) found in DNA of patient PA.

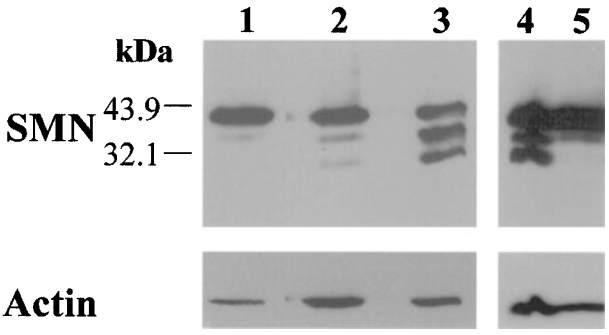

Figure 3 SMN protein isoforms. Western blot of SMN and actin in extracts from fibroblasts (lanes 1 – 3) and lymphoblastoid cells (lanes 4 and 5). The anti-SMN antibody detects an SMN protein of approximately 38 kDa and two smaller isoforms in the extracts of patients ZE (lane 3) and DG (lane 4) which are absent or negligible in the extracts of a SMA typeI/II patient (lane 2) and controls (lanes 1 and 5).

expected for an SMN protein lacking the 67 aminoacids (7.48 kDa) of exon 3. The origin of the isoform of intermediate molecular weight is unknown.

SMN gene copy analysis

To establish whether the diverse clinical severity of the patients could be ascribed to a difference in the *SMN2* copy number we performed FISH analysis on interphase nuclei. In the preparations of all three patients we found in G_1 phase nuclei one and two SMN signals, respectively, close to a chromosome 5q12 reference signal, indicating the presence of one *SMN* gene on one chromosome 5 and two *SMN* genes on the other chromosome 5 (Figure 4). These data indicate that all three patients had a total of three *SMN* genes. FISH analysis does not allow distinction between *SMN1* and *SMN2*. However, based on the above described SSCP data, showing absence of an intact *SMN1* exon 7 for patients SE and DG and that of quantitative PCR which provided evidence for one single *SMN1* gene (*SMN1* value 0.35) for patient PA, we could infer that the patients had one mutated *SMN1* gene and two copies of *SMN2* and therefore are compound heterozygotes with one *SMN1* allele deleted.

Ratio of full length to total *SMN2* transcripts

We performed PCR amplification of cDNA spanning exons 1 to 8 and subsequent *Dde*I enzyme digestion which separates the *SMN2* transcripts from the uncut *SMN1* transcripts (Figure 5). Densitometric analysis of PCR products from three independent amplifications showed an average percentage of full length over total *SMN2* transcripts of 41.2 and 31.2% for patients DG and ZE, respectively. Thus, the two *SMN2* genes of the patient with the milder phenotype produce a higher relative amount of full length transcripts.

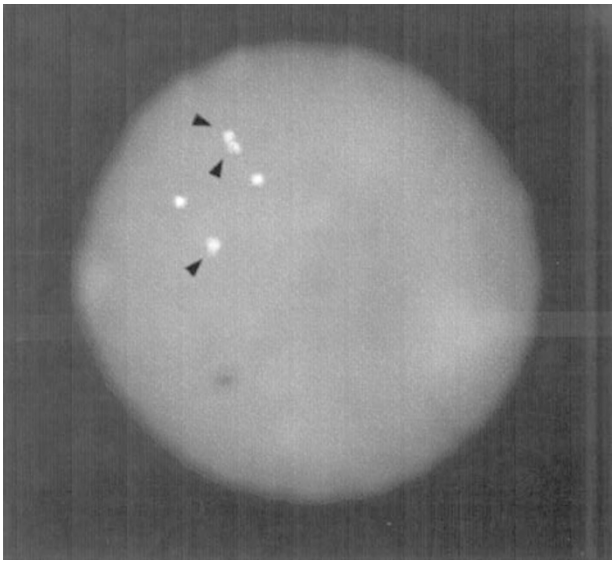

Figure 4 Representative example of FISH analysis. FISH on a G_1 interphase nucleus of patient DG. Arrows indicate the SMN signals, the signals close to SMN derive from a chromosome 5q12 reference probe.

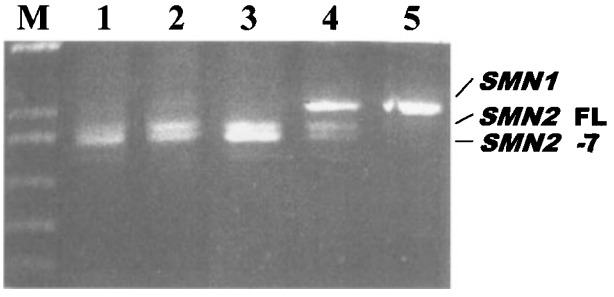

Figure 5 *SMN2* full length and −7 transcript analysis in lymphoblastoid cells. PCR of cDNA spanning exon 1 to 8 were digested with *Dde*I to distinguish *SMN1* from *SMN2* products. Bands corresponding to full length (FL) and −7 *SMN2* transcripts of patient DG (lane 2) were of similar intensity, whereas −7 *SMN2* products appear more abundant for ZE (lane 1) and a control SMA type II patient (lane 3). *SMN1* products of patients DG and ZE are barely visible, likely due to degradation of the mutated transcripts. Lane 4: normal control, lane 5: control homozygously deleted for the *SMN2* gene, M: DNA size marker.

Analysis of gems in fibroblasts

Immunofluorescence analysis of fibroblasts from patients ZE and DG was performed to determine the number of nuclei containing gems. A significantly higher number of gems was found in the nuclei from both patients compared to that of a type II patient with homozygous absence of *SMN1* (−7/−7). In particular, gems were found in approximately 43 and 60% of nuclei from the type II and type III patient, respectively, compared to only 13% of nuclei with gems from the control SMA

patient (CR) and to 71% from a normal control (FA) (Table 1).

Treatment with aminoglycoside antibiotics suppresses the stop codon

We tested whether treatment with the aminoglycoside G-418 of fibroblasts and lymphoblastoid cells from the patients with the W102X mutation could suppress the premature termination codon. RT–PCR of mRNA from cultures treated with G-418 for 48 h showed a decrease in the level of −3 transcripts and an increase in full-length transcripts (Figure 6). Moreover, Western blot analysis showed absence of the 31-kDa protein isoform in the extracts of treated cell cultures suggesting that G-418 suppresses the premature stop mutation which also results in the suppression of exon 3 skipping (Figure 7). This finding provides further evidence that skipping of exon 3 is induced by the mutation. Suppression of exon skipping appeared to be time and dose dependent as indicated by the presence of a small proportion of −3 transcripts in lymphoblastoid mRNA after 48 h but absence of the corresponding protein isoforms after 72 h, whereas treatment with 100 μg/ml was sufficient to suppress shortened transcripts in fibroblasts within 48 h. Higher doses of G-418 were toxic leading to cell death within 3 days.

Discussion

We report on three patients with mutations in *SMN1* exon 3 which lead to skipping of exon 3 in a proportion of transcripts with conservation of the open reading frame. Several genes have been reported in which mutations producing premature termination codons are associated with skipping of the exon containing the mutation (reviewed by Valentine[26]). Whereas alternative splicing involving mainly exons 7 and 5 is a common phenomena for the *SMN2* transcripts, to our knowledge this is the first observation of skipping of mutated exons in *SMN1* transcripts.

All three patients of the present study have in common a mutation in *SMN1* exon 3 and the presence of two *SMN2* genes. The most severely affected patient (PA) has a 5-bp deletion (425del5). The same frameshift mutation has been described in a severe case of SMA type I who survived for 42 days.[22] Based on haplotype analysis we had concluded that this patient had only one single *SMN2* gene which we have recently confirmed by semiquantitative PCR analysis.[27] To our knowledge, only one other patient with a single *SMN2* gene has been reported before. This infant had an unusually severe phenotype with reduced foetal movements from 30 weeks of gestation and survived for only 4 days.[10] The present patient, also a type I, is almost 3 years old and not intubated and, therefore, may be assigned to the less severe end of the type I phenotypic spectrum.[28] The relatively mild phenotype of this patient compared to the previous one with the same mutation, may likely be ascribed to the presence of one

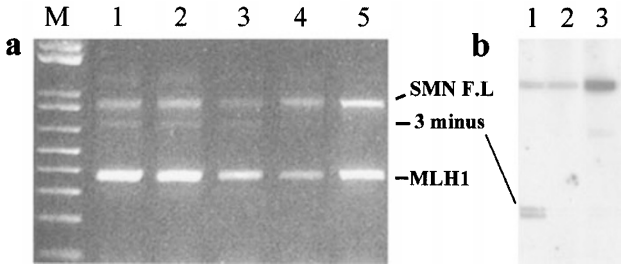

Figure 6 Reduction of −3 transcript isoforms after aminoglycoside treatment. (**a**) PCR of SMN cDNAs (exons 1–7) and partial MLH1, used as external standard, of mRNA from untreated (lane 1) and G-418 (50 μg/ml) treated lymphoblastoid cells (lane 2) and untreated (lane 3) and treated fibroblasts (100 μg/ml) from patient DG (lane 4). Lane 5: control. (**b**) Low-number-cycle radioactive PCR of cDNAs of untreated (lane 1), treated fibroblasts (100 μg) from patient DG (lane 2) and control (lane 3).

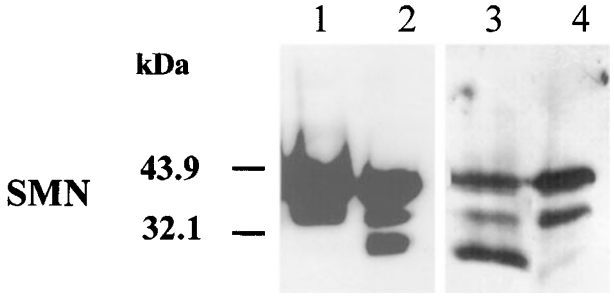

Figure 7 Western blot of SMN protein isoforms extracted from G-418 treated (50 μg/ml) lymphoblastoid cells of patient DG (lane 1) or untreated cells (lane 2) and from fibroblasts incubated with G-418 (100 μg/ml) from patient ZE (lane 4) or control cells (lane 3). Treatment with G-418 resulted in disappearance of the shortened protein isoform for both cell types.

Table 1 Germs in fibroblasts

Individual	*Phenotype*	*Total no. cells*	*Nuclei with gems*	*Gems/100 nuclei*
FA	Normal	668	474 (71.0%)	126.0
CR	SMA I/II (−7/−7)	105	14 (13.3%)	16.2
ZE	SMA II	469	201 (42.8%)	60.5
DG	SMA III	566	342 (60.4%)	106.7

additional *SMN2* gene. In a previous analysis of the *SMN2* copies by FISH in a limited number of patients, we found a copy number of two *SMN2* genes associated with a phenotype close to the severe end of the type I group.[8] It is possible that the clinical phenotype of the present patient, which appeared slightly less severe than may be expected based on the *SMN2* copy number alone, is also influenced to some extent by exon skipping in a proportion of *SMN1* transcripts. No cell cultures were available to investigate this hypothesis further.

The other two patients (ZE, type II and DG, type III) have a relatively mild phenotype despite the presence of the W102X mutation and only two *SMN2* copies. In our previous study, type II and type III patients were found to have three and generally four copies of *SMN2*, respectively.[8]

We postulated that skipping of the mutated exon could give rise to an incomplete protein isoform which could in part compensate for the low *SMN2* copy number. We could show that loss of exon 3 generates in-frame transcripts which are translated in shortened protein isoforms. This is indirectly supported by the disappearance of this isoform in Western blots following suppression of the nonsense codon by treatment of the cells with G-418.

The only point mutation previously identified in exon 3 is a missense mutation, E134K, found in a type I SMA patient.[29] This mutation severely reduced the ability of SMN to interact with Sm proteins.[20] However, Mohaghegh *et al*[30] have shown that the E134K mutation does not lead to loss of gem formation and is unable to exert a dominant negative effect on the formation of these structures. Consistent with these data is our finding that the fibroblasts of the patients with the W102X mutation form a high number of gems. In a previous study we found gems, on average, in 12.9% of nuclei of type II patients[18] (13.3% for the control patient in the present study). In contrast, we found gems in 43 and 60% of the nuclei of the type II and type III patient with the nonsense mutation, respectively. These results may suggest that the −3 protein isoform contributes to SMN protein complex formation in the nucleus and that the SMN tudor domain is not crucial for gem formation. Interestingly, the recently identified *Schizosaccharomyces pombe* protein Yab8p which shares homology with SMN and does not contain a tudor domain, fulfils a similar function as SMN in the nucleus but not in the cytoplasm.[31] Thus, we may hypothesize that the in-frame −3 SMN protein isoform, which may not be functional in the cytoplasm since it is presumably unable to bind Sm proteins, may be partially functional in the nucleus where it participates in the formation of the protein complex. This could consequently moderate the disease phenotype and compensate for the low *SMN2* gene copy number.

The particularly mild phenotype of the type III patient is difficult to explain. The only difference between the two patients with the W102X mutation detected at the SMN locus is the second point mutation in exon 3 in the type III patient. It is unlikely that this mutation contributes to exon skipping since no protein isoform lacking exon 3 was observed after suppression of the nonsense codon by G-418. However, the relative amount of full length *SMN2* transcripts appeared higher for the less severely affected patient, compared to that of the type II patient, suggesting reduced exon 7 exclusion in the *SMN2* transcripts of the patient with the milder phenotype.

Finally, aminoglycoside G-418 treatment suppressed the premature stop mutation in SMN in cultured cells. Similar findings have recently been reported for nonsense mutations in the dystrophin gene[32] and the cystic fibrosis transmembrane conductance regulator gene.[24] Our observation raises the prospect of a treatment for those rare SMA patients with nonsense mutations in the *SMN1* gene.

In conclusion, the disease severity of SMA is presumably determined by a combination of a variety of factors. The most important one is the number of *SMN2* gene copies. The type of subtle mutation may also be determinant. We propose here that in such rare cases with subtle mutations, skipping of the mutated exons may further modulate the phenotype. Moreover, our finding of differences in the ratio of full length to total *SMN2* transcripts supports the hypothesis of a role of modifier genes involved in regulating alternative splicing.

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