Unusual molecular findings in autosomal recessive spinal muscular atrophy

Gert Matthijs, Els Schollen, Eric Legius, Koen Devriendt, Nathalie Goemans, Hülya Kayserili, Memnune Yüksel Apäk, Jean-Jacques Cassiman

Abstract

All three types of autosomal recessive spinal muscular atrophy map to chromosome 5q11.2-q13.3 and are associated with deletions or mutations of the SMN (survival motor neurone) gene. The availability of a test to distinguish between the SMN gene and its nearly identical centromeric copy ^cBCD541 allows molecular diagnosis. We have analysed patients from 24 Belgian and 34 Turkish families for the presence or absence of a deletion in the SMN gene. A homozygous deletion in the SMN gene was seen in 90% of unrelated SMA patients. A non-radioactive SSCP assay allows for a semiquantitative analysis of the copy number of the centromeric and SMN genes. Hence, direct carrier detection has become feasible under certain conditions. We observed a phenotypically normal male, father of an SMA type I patient, presenting with only a single copy of the SMN gene and lacking both copies of the ^cBCD541 gene. This illustrates that a reduction of the total number of SMN and ^cBCD541 genes to a single SMN copy is compatible with normal life. In another SMA type I family, there is evidence for a de novo deletion of the centromeric gene in a normal sib. This observation illustrates the susceptibility of the SMA locus to de novo deletions and re-arrangements.

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Spinal muscular atrophy (SMA) is a lethal autosomal recessive disease common in white populations (1 in 6000 newborns).¹ SMA is characterised by degeneration of the anterior horn cells of the spinal cord, leading to symmetrical paralysis of voluntary muscles with muscular atrophy. Three different clinical syndromes can be defined on the basis of severity. The acute form of SMA, SMA type I (Werdnig-Hoffmann disease) starts with hypotonia and weakness in utero or within the first few months of life. These children are never able to sit unsupported. The majority of these children die within the first three years of life because of recurrent respiratory infections. In the intermediate type of SMA (Dubowitz disease or SMA type II) onset is usually between 3 and 15 months of age. These children learn to sit without support but never manage to stand or walk unaided. They survive beyond 4 years of

age until adolescence or later. The onset of the chronic form of SMA, SMA type III (Kugelberg-Welander disease) is after the age of 2 years and is characterised by proximal muscle weakness, predominantly of the legs. These patients manage to stand and walk unsupported, but have problems with running, jumping, and climbing. Their walking distance is limited. The long term survival is usually good, depending on respiratory function.

All three types map to chromosome region 5q11.2-q13.3. Recently, Lefebvre *et al*² and Roy *et al*³ have described the genomic structure of the SMA locus, although both groups identified a different gene. The region is characterised by an inverted duplication of approximately 500 kb. Patients with SMA have deletions in the telomeric repeating unit. Lefebvre *et al*² identified a gene in this region, which is called SMN (Survival Motor Neurone, ^tBCD541), and contains eight exons extending over approximately 20 kb. There is an almost identical copy of this gene in the centromeric repeating unit (^cBCD541). In the series of Lefebvre *et al*,² the vast majority (98.6%) of patients have a deletion in the SMN gene, whereas point mutations have been identified in the remaining three of 229 patients, indicating that mutations in this gene may indeed cause SMA. In the same region, Roy *et al*³ identified the NAIP gene (Neuronal Apoptosis Inhibitory Protein) with 16 exons spanning 60 kb. They showed that 45% of SMA type I and 18% of SMA type II and III patients have a partial or complete homozygous deletion of the NAIP gene.³ However, further analysis showed that other loci are probably involved in the pathogenesis. In summary, it is hypothesised that mutations in the SMN gene are the major determinant of the SMA phenotype, whereas the extent of the deletions, which may include the NAIP gene, may correlate with the severity of the disease.^{2,3}

We report on the presence or absence of a deletion in the SMN gene in 58 SMA patients, using a non-radioactive SSCP (single stranded conformation polymorphism) assay. This test discriminates between the centromeric gene ^cBCD541 and telomeric SMN gene and allows for the quantification of the different copies. In some cases it can be used for carrier testing. Together with the overall data, two unusual cases are described.

Materials and methods

SUBJECTS AND FAMILY DATA

Twenty-four families are of Belgian origin. All samples from these SMA families had pre-

Center for Human Genetics, University of Leuven, Gasthuisberg O&N6, B-3000 Leuven, Belgium

G Matthijs
E Schollen
E Legius
K Devriendt
J-J Cassiman

Department of Paediatrics, University Hospital Leuven, Gasthuisberg, B-3000 Leuven, Belgium
N Goemans

Division of Medical Genetics, Institute of Child Health, University of Istanbul, Millet Caddesi Çapa, 34390 Istanbul, Turkey
H Kayserili
M Yüksel Apäk

Correspondence to:
Dr Matthijs.

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Figure 1 SSCP analysis for the direct detection of the absence of exons 7 and 8 of the SMN gene in SMA patients. In (A), the patient in family 7 (lane 4) had a homozygous deletion of exons 7 and 8 of the SMN gene on both chromosomes (C=carrier, N=normal, S=SMA patient, *=confirmed with genetic markers). In (B), family 21, the deletion on one of the disease chromosomes does not include the telomeric exon 8, whereas in (C), in family 4, both chromosomes show deletions in the SMN gene that do not include exon 8. Tel and Cen indicate bands of the SSCP pattern that correspond to exons 7 and 8 of the telomeric (SMN) and centromeric (^CBCD541) genes respectively.

viously been referred to our centre for molecular analysis. Another 34 patients were of Turkish origin. In the Belgian series, 11 patients had been diagnosed as SMA type I, seven as SMA type II, and six as SMA type III. All patients in the Turkish group were seen by HK: 27 type I, five type II, and two type III patients.

MICROSATELLITE MARKERS

Families were typed for three dinucleotide repeats: cVS19 (D5S435), proximal to the SMA locus and two repeats in the 5' and 3' region of the MAP-1B gene (D5S112, MAP5' and MAP3' respectively) located distal to the SMA locus.⁴⁻⁶ One PCR primer of each pair was fluorescently labelled (FITC, fluorescein-isothiocyanate). All PCR products were electrophoresed on an ALF DNA Sequencer (Pharmacia) and analysed using Fragment Manager software (Pharmacia).

SMN AND NAIP DELETION DETECTION

The centromeric gene ^CBCD541 and the telomeric SMN gene differ by only five base substitutions. Lefebvre *et al*² developed a radioactive SSCP analysis which distinguishes the centromeric gene and the SMN gene in exons 7 and 8. A modified SSCP method was used in this study. Fragments encompassing exons 7 and 8 were amplified from genomic DNA using primers R111 (SMA7A), 541C960 (SMA8A), and 541C1120 (SMA8B) and primer SMA7B (5'-TCCTTAATTGAAG-GAATGTGAGCA-3'). The primers SMA7B and SMA8B were FITC labelled. DNA from peripheral leucocytes (300 ng) was amplified for 30 cycles in 50 µl of PCR mix containing 200 µmol/l dNTPs, 0.5 µmol/l each primer, and 1 U *Taq* polymerase (Perkin-Elmer). Cycling conditions were one minute at 94°C, one minute at 60°C, and one minute at 72°C. Ten to 15 µl of the PCR products were mixed with an equal volume of formamide, denatured for five minutes at 95°C, loaded onto a non-de-

naturing polyacrylamide gel (0.5 × Hydrolink MDE (J T Baker) in 0.6 × TBE (10 × TBE is 1 mol/l Tris, 0.82 mol/l boric acid, 10 mmol/l EDTA)), and electrophoresed at 4°C for 10 hours at 400 V. The gels were directly scanned on a Fluorimager (Vistra) and the signals were analysed with the Imagequant software (Molecular Dynamics).

Roy *et al*³ described a PCR assay for the detection of homozygous NAIP deletions by amplification of exon 5 of the gene. Two primer sets were used, one for exon 5 and one for exon 13, the latter as an amplification control.³

Results

MOLECULAR DIAGNOSIS

In 34 of 38 type I patients, in 11 of 12 type II patients, and in seven of eight type III patients, a homozygous deletion of exon 7 of the SMN gene was detected (an example of a type I family is given in fig 1A). Of these, two unrelated patients with SMA type I, one patient with SMA type II and one patient with SMA type III, were found to have a single copy of exon 8 of SMN (fig 1B, a type II family). These patients are thus compound heterozygotes for a deletion including exon 8 and a smaller deletion of exon 7 but not exon 8. In a single type I patient, both copies of exon 8 of the SMN gene were present, whereas both copies of exon 7 of the SMN gene were missing (fig 1C). This patient is thus homozygous for the smaller deletion. Therefore, of the 104 deletion chromosomes observed in these patients, 98 were also lacking exon 8 (94%).

In the six remaining, unrelated patients, four type I, one type II, and one type III patients, at least one copy of the exons 7 and 8 of the SMN gene was present on SSCP analysis. A Belgian type I patient's presentation was clinically and histopathologically indistinguishable from Werdnig-Hoffmann disease. The diagnosis of Kugelberg-Welander type in the remaining Belgian patient has been confirmed by several specialists in the field, including Dr V Dubowitz in London. The clinical diagnosis of

Figure 2 PCR analysis for the direct detection of the homozygous absence of exon 5 of NAIP. The amplified fragments are 435 bp and 241 bp for exon 5 and exon 13 respectively. Lanes 1, 2, and 3: probands of families 7, 21, and 4 respectively (see fig 1). Lanes 4 to 8: members of family 11 (same order as in fig 4A). Lanes 9 to 12: family 12 (same order as in fig 4B).

Figure 3 Analysis of the copy number of the centromeric and telomeric genes by SSCP. A panel of samples was selected from members of different families under study. The different combinations of centromeric and telomeric genes are indicated under each lane. The genotypes were quantified and confirmed by analysis of the corresponding families. (Cen and Tel as in fig 1.) The weak band at Cen in lanes 7 and 8 is because of the incomplete separation of the alleles in the SSCP for exon 7.

SMA type I was confirmed in three patients of Turkish origin. Two of these patients are the product of first cousin marriages. However, analysis of the CVS and MAP-1B markers did not show homozygosity, which might suggest but does not prove that the disease is not linked to chromosome 5q in these patients. Diagnostic features in a Turkish type II patient include a slowing nerve conduction velocity on EMG, which may take it beyond classical SMA. These cases will be further investigated at the molecular level by sequence analysis.

In summary, SSCP analysis of the SMN gene showed a homozygous deletion in 52 of 58 cases (90%).

The homozygous deletion excluding exon 8 in family 4 (fig 1C) has been further documented by looking at NAIP.³ PCR amplification showed the presence of at least one copy of exon 5 of NAIP in the patient in family 4. On the other hand, there is evidence for a homozygous deletion of exon 5 of NAIP in the patient in family 7 (fig 1A). Exon 5 of the NAIP gene could also be amplified in the proband of family 21 (fig 1B). The results are shown in fig 2.

CARRIER TESTING AND PRENATAL DIAGNOSIS

The use of a single, fluorescently labelled primer in the PCR resulted in simple SSCP patterns which could easily be interpreted (compared to the radioactive methods used by Lefebvre *et al*² and Rodrigues *et al*^{7,8}). The SSCP results could be used in a quantitative way. The gel system reproducibly generated single bands for the exon 8 fragments of the telomeric and centromeric genes respectively,

and the intensities of the bands corresponded to the number of alleles (fig 3). Carriers of a deletion of the SMN gene with two copies of the centromeric gene (2Cen/1Tel, being SMA carriers) and normal subjects lacking an exon of the centromeric gene (1Cen/2Tel, being neutral in terms of SMA) could easily be identified. However, it was not possible to distinguish between carriers of an SMN deletion that also lacked a copy of an exon of the centromeric gene (1Cen/1Tel) and normal (2Cen/2Tel) subjects. Also, the distinction between SMA patients with 2Cen/0Tel or 1Cen/0Tel could not be made based on SSCP results alone (see below), nor could people lacking both copies of the exons of the centromeric gene be directly typed as 0Cen/1Tel or 0Cen/2Tel (see below). Addition of an internal standard during PCR did not improve the test (not shown). The SSCP pattern obtained for exon 7 was more complex, and the reproducibility of the pattern in a routine setting was not as good, but it could be interpreted in most cases. A reference set of samples with different combinations of deletions was included in every run (fig 3), and was used for comparison. Densitometric scans of the bands were performed routinely (not shown).

The carrier status of the parents was tested in 13 cases in which homozygous deletions of exon 8 of the SMN gene had been found in the affected sib (13 pairs of parents tested). In four patients in four different families, the SSCP results indicated equal doses of exon 8 of the centromeric and telomeric genes (subjects typed as 1Cen/1Tel or 2Cen/2Tel). However, by determining the deletion status of sibs in these families and by following the segregation of the dinucleotide repeat alleles, these subjects were typed as 1Cen/1Tel (the families were analysed as those shown in fig 4). Thus, no evidence for de novo deletions in any of these patients was withheld. The SSCP method has also been used in five new cases for prenatal diagnosis, in combination with flanking markers. The results of the SSCP analysis were consistent with the results of the MAP-1B and cVS19 markers (results not shown). Similarly, the SSCP assay confirmed the results of eight previous prenatal diagnoses with linked markers in six families. In one case, a weak signal from the SMN gene was observed with material from an affected fetus. Quantification of the SSCP results and analysis of the flanking marker alleles confirmed that the signal was the result of contamination with maternal DNA and that the pattern should not be interpreted as that of an unaffected carrier (data not shown).

Two familial cases are described in further detail because of interesting observations.

A PHENOTYPICALLY NORMAL PARENT WITH A SINGLE COPY OF THE SMN GENE AND A HOMOZYGOUS DELETION OF THE CENTROMERIC C_{BCD541} GENE

The proband in family 11 was a girl who died of SMA type I. The family had been seeking genetic counselling and prenatal testing for a second pregnancy. Genetic marker analysis

Figure 4 Family structure and genotype of families with (A) a parent with a single copy of the SMN gene and a homozygous deletion of the *centromeric* gene (family 11) and (B) a de novo deletion of the centromeric gene (family 12). The SSCP results are shown (Cen and Tel as in fig 1) and the presence and absence of the centromeric and telomeric genes are drawn schematically for each patient. The legend to the icon representing the chromosomes is given in (C). Open boxes represent deletions in the gene (absence of the corresponding exon), filled boxes represent the presence of the gene. Above the pedigree, the haplotypes for the different chromosomes are shown: marker cVS19 is located proximal and markers MAP5' and MAP3' are distal to the SMA locus.

showed that the fetus had identical haplotypes to the dead patient and the pregnancy was terminated. Marker analysis of a third pregnancy showed a fetus with a carrier phenotype. In this case, the presence of at least one copy of the SMN gene was shown by SSCP analysis (fig 4A). SSCP analysis also showed that the father lacked the centromeric *centromeric* gene on both chromosomes. Since he is a carrier of an SMN deletion, he can have only one normal copy of the SMN gene. The mother was a normal carrier, that is, two centromeric genes and one normal copy of the SMN gene were present as evidenced by SSCP analysis of exons 7 and 8. The fetus in the third pregnancy was typed as either 1Cen/1Tel, in which case he was a carrier, or 2Cen/2Tel (see above for the limitations of this assay). Because analysis with flanking markers had shown that this fetus had indeed received a haplotype associated with the disease from his father and a normal chromosome from his mother, only the 1Cen/1Tel genotype is plausible for the fetus in the third pregnancy. The fetus received the normal centromeric and telomeric copies from the mother, and a chromosome deleted in the SMN and *centromeric* genes from the father. As a consequence, the genotype of the proband and the fetus of the second pregnancy is 1Cen/0Tel, which is compatible with the SSCP results. Thus, the father, who is phenotypically normal, is probably 0Cen/1Tel and passed an "empty" chromosome to all sibs in this family (fig 4A). Owing to the limitations of the SSCP assay, we cannot exclude that the father has two SMN genes on his normal chromosome, or the normal SMN gene on one chromosome and a centromeric to telomeric gene conversion on the other chromosome. The first possibility

cannot be checked because the normal chromosome has not been passed to the children. The latter possibility is not compatible with the SSCP data obtained for the children, unless two independent de novo deletion events are invoked. Only interphase FISH could indicate the number of SMN genes in the father's genome, but is not available yet.

DE NOVO DELETION OF THE CENTROMERIC *centromeric* GENE IN A PATERNAL MEIOSIS

In family 12, the proband was diagnosed with SMA type I at 6 months of age. Her sister was prenatally typed as normal, based on genetic markers. The results were later confirmed clinically. Direct analysis of the SMN gene by SSCP confirmed the diagnosis in the proband (absence of exons 7 and 8 of the SMN gene, fig 4B). In the sister a homozygous absence of exons 7 and 8 of the centromeric gene was found. The father is a normal carrier (2Cen/1Tel) whereas the mother was typed as 1Cen/1Tel. Analysis with cVS19 and MAP5' markers showed the segregation of the two normal chromosomes 5 in the second child, and there is no evidence for loss of a chromosome, nor for non-paternity. Because the father has two centromeric copies and the second child has none, a de novo deletion must have occurred on the paternal chromosome in the second child. The analysis also allows the phase of the alleles on the maternal chromosomes 5 to be deduced: one lacks the centromeric gene, the other the SMN gene.

Discussion

Lefebvre *et al*² showed that most cases of SMA, irrespective of their being typed as either

Werdnig-Hoffmann, Dubowitz, or Kugelberg-Welander disease, are the result of deletions in the chromosomal region 5q11.2-q13.3. These authors succeeded in pinpointing the genetic cause of the disease to the telomeric copy of a duplicated gene, the SMN gene. To show that part of this gene was deleted in 98.5% of their SMA patients, they used an SSCP method to discriminate between the SMN gene and the highly homologous, centromeric copy of that gene, ^CBCD541. This method has allowed us and others⁷ to confirm directly the diagnosis of SMA in patients.

The results of this report concerning a series of Belgian and Turkish families confirm the previous observations.² The deletion detection rate of 90% is below the 98.5% obtained by the French group.² The assay only looks at the presence or absence of two exons in the 3' end of the SMN gene. Interestingly, four of 38 SMA type I cases escaped elucidation, while one of 12 SMA type II cases, and one of eight SMA type III cases also need further investigation or re-evaluation of the clinical data. These cases will be further investigated by sequencing the SMN gene. On the other hand, a direct test is now available for the confirmation of clinical diagnosis without the need for a muscle biopsy and its use can be extended to atypical cases. Deletions in the SMN gene have already been identified in patients with infantile SMA and congenital heart defects⁹ and in a patient with the fetal hypokinesia sequence and signs of SMA.¹⁰ The use of the SSCP method in prenatal diagnosis has been previously reported.⁸ We have also successfully used the described non-radioactive SSCP method for prenatal diagnosis of SMN deletions, and the test allows diagnosis in cases in which no DNA of an affected sib is available. For families in which no evidence of a deletion was obtained, prenatal testing will have to be based on linked polymorphic markers alone, as before, and one should be aware of the fact that the disease may not necessarily be linked to chromosome 5q in such families.

A PCR method with restriction digestion, based on the polymorphic differences between the ^CBCD541 and SMN genes, has been described by van der Steege *et al.*¹¹ In our hands, the SSCP based test has proved reliable. It has the advantages of a non-radioactive method and it also allowed for a limited quantification of the different fragments and for carrier testing in some cases. However, the carrier status of the parents or sibs could only be reliably determined whenever a dose difference was observed between the fragments of the SMN gene and of the centromeric gene. In practice, the difference between two centromeric exon fragments plus two SMN exon fragments (2Cen/2Tel) and one copy of each exon (1Cen/1Tel), the latter representing an SMA carrier, could not reliably be made. Given the number of people that lack both copies of the centromeric gene (4.4%, data from Lefebvre *et al.*²), the frequency of carriers of a deletion of the ^CBCD541 gene must be high: 0.33 (based on Hardy-Weinberg equilibrium). This would simply mean that 33% of the SMA carriers also

lack a copy of the centromeric gene, being 1Cen/1Tel. Thus, every third "normal" SSCP result would be misinterpreted. The difference between 1Cen/0Tel and 2Cen/0Tel can also not be shown. This, however, would not affect the molecular diagnosis. The opposite result, 0Cen/1Tel versus 0Cen/2Tel, again results in an uncertain carrier determination. These limitations diminish the value of this assay for carrier detection in general and screening for SMA is not yet possible.

It is recommended to check for both exon 7 and exon 8. The exon 8 assay detected only 94% of the deletion chromosomes identified by the exon 7 assay. The existence of this rare deletion, including exon 7 but not exon 8, has also been previously reported.^{2,7} In practical terms, the combination of both assays offers more reliable genotyping. It would also detect patients in which a gene conversion may be present (see Lefebvre *et al.*²). Similarly, the availability of the closely linked genetic markers cVS19 and MAP-1B gives confirmatory information for determination of the phase and interpretation of the SSCP data in equivocal cases, and, whenever possible, a combination of both techniques is recommended for prenatal diagnosis.

There is no evidence for a de novo deletion of the SMN gene in 13 cases in which the parents have been genotyped. However, we describe a de novo deletion event in the centromeric gene in a sib of an SMA type I patient. It could be suggested that the absence of a copy of the telomeric (SMN) gene in the father has facilitated the loss of the centromeric gene on the homologous chromosome during meiosis. If so, the opposite event might occur as well: the absence of the centromeric gene might facilitate the loss of the telomeric gene on the homologous chromosome, generating a disease chromosome. This would imply an increased risk for de novo deletions in carriers of a centromeric deletion. While the ^CBCD541 deletion carrier frequency is very high,² this could explain the high frequency of SMA carriers in the population. It would be interesting to use the semiquantitative SSCP assay in families with de novo SMA cases to substantiate this hypothesis. Alternatively, the accumulation of centromeric deletions may represent the silent counterpart of random telomeric or centromeric deletions, in which case the telomeric deletion frequency in the population has been reduced because of the lethal phenotype. All together, this observation stresses the instability of this chromosomal region. Other data obtained by Lefebvre *et al.*² and by our group¹⁰ also suggest that complex rearrangements can occur on this chromosome.

Nothing is settled yet as to the size of the deletion in SMA cases. Four patients, of types I, II, and III, have been found to carry a combination of a deletion including exon 7 only on one allele, and a larger deletion, including both exon 7 and exon 8 of the SMN gene, on the other allele. Moreover, an SMA type I patient was identified, in whom exon 8 of the SMN gene was conserved on both chromosomes (fig 1C). In this patient, there is

no evidence for a homozygous deletion of NAIP. Deletions leading to the most severe phenotype may thus not include exon 8 of the SMN gene and its 3' region (see also Lefebvre *et al.*²), nor NAIP. This indicates that, if other genes play a role in the full phenotype of SMA, they probably are located centromeric to the SMN gene.

The observation of a homozygous deletion in the centromeric gene in a phenotypically normal carrier of the SMN deletion (fig 4A) shows that one functional copy of the SMN gene in the absence of a functional ^cBCD541 gene is sufficient for normal development. Lefebvre *et al.*² have shown that both the centromeric gene and the SMN gene are transcribed and their predicted amino acid sequence is identical. Moreover, the centromeric gene but not the SMN gene itself is alternatively spliced. The physiological role of these genes and transcripts and their specific role in the pathogenesis of the disease thus remain unknown.

In conclusion, direct (prenatal) molecular diagnosis of SMA has become feasible by looking at the presence or absence of a single gene on chromosome 5q. However, it has become evident that this region of chromosome 5q shows some special characteristics which should lead to caution in DNA diagnosis and prenatal diagnosis.

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