

Molecular diagnosis of spinal muscular atrophy

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Abstract

The frequency of deletions within the survival motor neurone (SMN) and neuronal apoptosis inhibitory protein (NAIP) genes in patients with spinal muscular atrophy (SMA), and the impact of this on the diagnosis and prenatal diagnosis of SMA, were investigated by molecular analysis of stored DNA and retrospective review of case notes. In type I SMA, 16 of 17 cases were homozygously deleted for exons 7 and 8 of SMN, 14 of 17 were homozygously deleted for exon 5 of NAIP, and 13 of 17 were deleted for both. In types II and III SMA, seven of nine cases were deleted for exons 7 and 8 of SMN. Deletions of SMN and NAIP occurred in four of nine cases. With one exception, the deletion genotypes of probands, affected siblings, and terminated fetuses were identical. Molecular studies are replacing conventional investigations for SMA and have a high uptake prenatally.

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The spinal muscular atrophies (SMAs) form a heterogeneous group of diseases inherited as autosomal dominant, autosomal recessive, or X linked recessive traits.^{1,2} The childhood onset autosomal recessive form affects between one in 6000 and one in 10 000 live births and is the second most common lethal autosomal recessive disorder in white people after cystic fibrosis. The carrier frequency is one in 60-80 in the UK.³

The characteristic pathology of SMA is degeneration of motor neurones in the anterior horn of the spinal cord and, in some cases, motor nuclei in the brain stem, resulting in muscular atrophy and progressive paralysis.¹ Recent studies have advanced the understanding of the possible mechanisms involved in the pathogenesis of SMA. A novel protein (SMN) is located in the cytoplasm and in novel nuclear bodies called gems. SMN is found at high concentrations in motor neurones and the spinal cord in normal subjects but at reduced concentrations in patients with SMA.⁴ SMN is associated with another novel protein (SIP1). These proteins are thought to play a role in the metabolism of small nuclear RNAs. However, their role in motor neurone degeneration remains undetermined.^{5,6}

There are three clinical subtypes of autosomal recessive SMA. Type I (Werdnig-Hoffmann disease) is the most severe, with onset in utero, with reduced fetal movements,

or in the first six months of life, with hypotonia, wasting, fasciculation, paucity of movement, and areflexia. Affected children have normal intelligence but are unable to sit unaided, have feeding difficulties, and die from respiratory failure or aspiration before the age of 2 years. Type II is of intermediate severity. Patients have hypotonia, delayed motor milestones, are able to sit unsupported, but cannot stand or walk unaided. Complications include scoliosis and feeding difficulties. Survival depends on the degree of respiratory muscle involvement but is usually greater than 4 years. Type III (Kugelberg-Welander disease) has an onset after the age of two and patients are able to walk unaided, although they do so late, fall frequently, and have a shuffling gait, foot drop, and difficulty with stairs. Slow deterioration results in scoliosis and wheel chair dependence.

The diagnosis is confirmed by muscle biopsy (showing degeneration of muscle fibres, in the absence of inflammation, fibrosis, or histochemical abnormality) and electromyography (EMG) (showing denervation, paucity of movement, and fasciculation). These procedures are invasive, time consuming, and may give inconclusive results when performed in young infants. Findings in cerebrospinal fluid, serum enzymes, and nerve conduction are normal.

In 1990, 95% of cases of all three recessive forms of SMA were found to be linked to the 5q13 region of chromosome 5.^{7,8} This enabled prenatal prediction on the basis of linkage in some families.^{9,10} Deletion events within this region were found to be associated with SMA,^{11,12} and in 1995, two candidate genes within this region were postulated: the survival motor neurone (SMN) and neuronal apoptosis inhibitory protein (NAIP) genes.¹³⁻¹⁶ This region of the genome is complex and inherently unstable, with two almost identical copies of SMN, one centromeric and one telomeric, and multiple copies of pseudogenes of NAIP¹⁷ (fig 1).

Homozygous deletions of exons 7 and 8 of the telomeric (functional) copy of SMN occur in > 95% of patients with SMA but not in normal control populations.¹⁸ Short deletions in the consensus splice sites of introns 6 and 7,

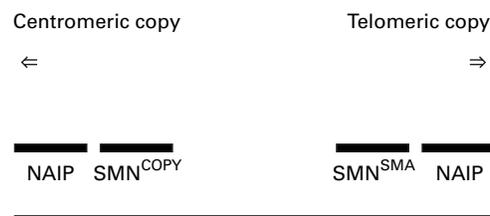


Figure 1 Map of chromosome 5q13.

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Table 1 Results of DNA deletion analysis

| | Type I | Types II and III | All types |
|-------------------|--------------|------------------|---------------|
| SMN exons 7 and 8 | 16/17 (94%)* | 7/9 (78%) | 23/26 (88.5%) |
| NAIP exon 5 | 14/17 (82%) | 4/9 (44%) | 18/26 (69%) |
| Both | 13/17 (76%) | 4/9 (44%) | 17/26 (65%) |

*Remaining patient was deleted for exon 8 of SMN and exon 5 of NAIP.

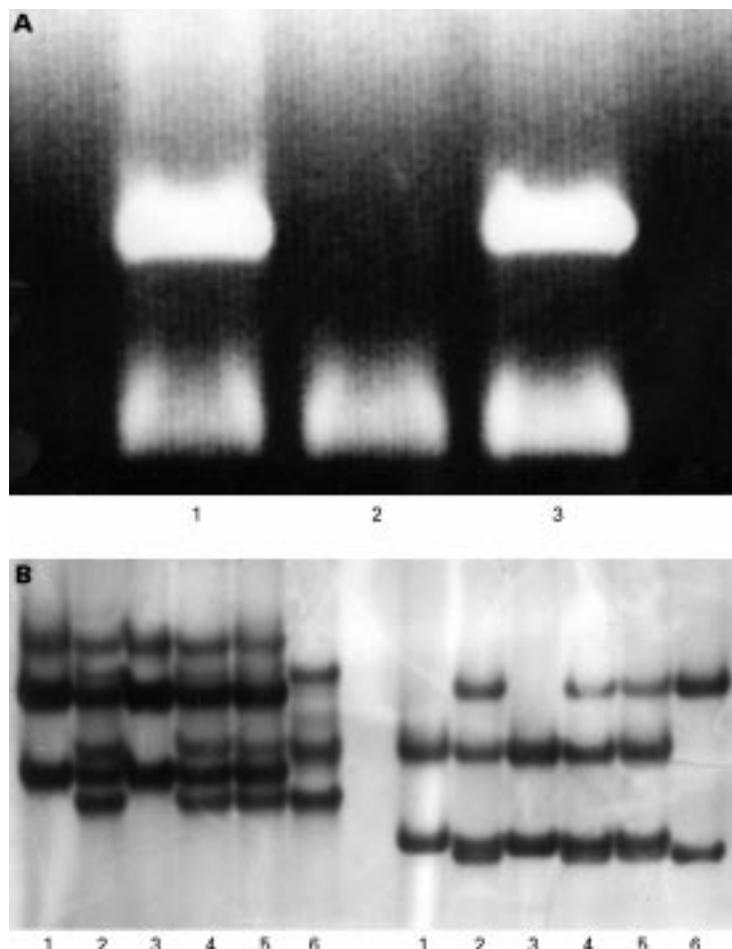


Figure 2 (A) Agarose gel showing polymerase chain reaction (PCR) analysis of neuronal apoptosis inhibitory protein (NAIP) genes. Lane 1, normal control, both exons 5 and 13 amplify. Lanes 2 and 3, both patient samples. Lane 2, homozygous deletion of exon 5, normal exon 13 supporting a diagnosis of spinal muscular atrophy (SMA); lane 3, no deletion of exon 5. (B) Single strand conformation dependent polymorphism analysis (SSCP) analysis of survival motor neurone (SMN) exons 7 and 8. Lane 1, control sample homozygously deleted for SMN functional gene; lane 2, normal control; lane 3, patient homozygously deleted for exons 7 and 8 of the functional SMN gene, confirming the diagnosis of SMA; lanes 4 and 5, normal pattern, excluding the diagnosis of SMA; lane 6, control sample homozygously deleted for the non-functional SMN gene.

and point mutations, have been found in affected individuals who do not have homozygous deletions of exons 7 and 8 of SMN, confirming that SMN has a role in the aetiology of SMA.¹⁶⁻¹⁹ The exact role of deletions of SMN is not clear, as illustrated by the following observations: unaffected parents and siblings have homozygous deletions of exons 7 and 8 of SMN in 1% of cases²⁰; parents of children with types II and III SMA may have more than two copies of the centromeric copy of SMN and it has been postulated that this might have an effect on the phenotype²¹; 4% of normal people are homozygously deleted for the centromeric copy of SMN, so it is not known whether this copy of the gene has any function.¹⁷⁻¹⁸ Deletions of exon 5 of NAIP

occur in 67% of patients with type I SMA, 42% of those with types II and III, and 2-3% of controls.¹⁴ The role of this gene in the aetiology of SMA remains obscure, although it is thought that larger deletions involving SMN and NAIP are associated with increased disease severity.²²⁻²³ Deletion analysis of SMN has been used for prenatal diagnosis and has largely replaced linkage analysis. As the deletion in the proband arises de novo in 1% of patients with SMA, errors could be made in prenatal predictions based only on linkage analysis in subsequent pregnancies.²⁴⁻²⁶

The purpose of our study was to confirm the frequency of deletions within SMN and NAIP in patients with known SMA, to establish the pattern of deletions in affected siblings, to confirm that fetuses terminated on the basis of linkage had deletions within these genes, and to review how this information can aid patients and clinicians in diagnosing SMA prenatally and in childhood.

Methods

SUBJECTS

We included in our study all patients referred to our department before August 1996, in whom the diagnosis of SMA was confirmed clinically, their affected siblings, and those fetuses terminated following prenatal diagnosis. The patients came from a variety of different ethnic origins.

MOLECULAR ANALYSIS

DNA was extracted from peripheral blood lymphocytes on an Applied Biosystems 380A DNA extractor or from stored Guthrie cards by cutting a 3 mm² fragment of card with a sterile scalpel, boiling the fragment in 100 µl of 50 mM sodium hydroxide for 20 minutes, then neutralising with 15 µl of 1.0 M Tris, pH 8.0.²⁷

Polymerase chain reaction (PCR) amplification of SMN exons 7 and 8, and NAIP exons 5 and 13 was carried out in 10 µl volumes using 25 ng of genomic DNA, 5 pmol each of forward and reverse primers, 750 µmol/l of each dNTP, 0.2 U Taq polymerase (Gibco BRL, Paisley, UK) in a PCR buffer comprising 3.7 mM MgCl₂. PCR was carried out on a Techne PHC-2 using the following cycling parameters: initial denaturation at 94°C for three minutes; 30 cycles of 94°C for one minute, 60°C for one minute, and 72°C for one minute; and a final synthesis of 72°C for 10 minutes. Owing to the low yield of DNA from Guthrie cards, PCR was extended to 35 cycles. PCR products from NAIP were loaded on to 2% agarose gels stained with ethidium bromide, run in 1× Tris borate EDTA (TBE) buffer and visualised under UV light. Exon 13 of NAIP was analysed as a control.

Single strand conformation dependent polymorphism analysis (SSCP) was used to investigate exons 7 and 8 of SMN. SSCP is a simple, sensitive technique in which DNA is amplified by PCR then samples are denatured and loaded on to a non-denaturing polyacrylamide gel. Single stranded DNA has a tendency to fold to form complex structures with different electrophoretic properties depending on their

Table 2 Outcome of conventional investigations

| | Type I | | Types II and III | |
|--------------------------|--------------------|-----------------|--------------------|----------------|
| | Biopsy (n = 11) | EMG (n = 13) | Biopsy (n = 10) | EMG (n = 9) |
| Diagnostic | 7 | 9 | 9 | 6 |
| Atypical, but compatible | 4 | 1 | 0 | 0 |
| Atypical | 0 | 2 | 1 | 0 |
| Normal | 0 | 1 | 0 | 3 |

Table 3 Outcome of pregnancies

| | Number of pregnancies | Number of tests | Number of affected children born | Number of terminations |
|----------------------------------|-----------------------|-----------------|----------------------------------|------------------------|
| <i>Type I (n = 21)</i> | | | | |
| Prelinkage era (-1990) | 3 | 0 | 2 | 0 |
| Linkage era (1990-95) | 10 | 7* | 3† | 3* |
| Deletion era (1995-) | 8 | 8 | 0 | 1 |
| <i>Types II and III (n = 13)</i> | | | | |
| Prelinkage era (-1990) | 8 | 0 | 2 | 0 |
| Linkage era (1990-95) | 5 | 1* | 1† | 0 |
| Deletion era (1995-) | 0 | 0 | 0 | 0 |

*Linkage results subsequently confirmed by deletion testing; †no prenatal test performed.

confirmation, which in turn depends on the DNA sequence. In this case, deleted and normal exons 7 and 8 of the telomeric and centromeric copies of SMN have different mobilities and are, therefore, easily distinguished in affected patients and controls. PCR products were run on 8% polyacrylamide gels in 10× TBE buffer then visualised by silver staining.²⁸

Case notes were reviewed retrospectively.

Results

TYPE I

There were 17 probands, six affected siblings, and four terminated fetuses. Of the 23 children, 11 were girls and 12 were boys. There was consanguinity in five of 17 families. The dates of birth of probands ranged from 1977 to 1995. The age of onset was stated in 16 children and ranged from birth to 6 months, with a mean of 2.4 months. The age at diagnosis was known in 16 children and ranged from 1 month to 7 months, with a mean of 4.1 months. In one case, where parents declined invasive investigations in 1989, the diagnosis was not confirmed until 1996, when a DNA test was performed on a stored neonatal blood spot. All 23 children died at 2 to 16 months, mean age 8.3 months.

Table 1 and fig 2 show the results of SMN and NAIP deletion testing in the 17 probands. In six families there was an affected sibling. Three were tested and found to have the same deletion genotype as their affected siblings. In two families, three pregnancies were terminated following prenatal prediction on the basis of linkage analysis. Subsequently, the fetuses were shown to have the same deletions as their affected siblings. Prenatal deletion testing identified a further fetus who had the same deletion as the affected sibling.

During life, 14 of 23 children had muscle biopsy and/or EMG performed. In three cases, neither investigation was performed: in one case, a previous sibling had a confirmed diagnosis; in one case, parents declined investigations because they were too invasive; and in

another case, the baby was born after SMN deletion testing had become possible and DNA analysis alone confirmed the clinical diagnosis. In six cases it was not clear which investigations were performed. The outcomes of these investigations are summarised in table 2.

After diagnosis in the proband, there were no further pregnancies in five families. Three of these had older, healthy children and two were recent cases (1993 and 1995). The pregnancies occurring in the remaining 12 families are summarised in table 3.

TYPES II AND III

There were 12 patients (six boys and six girls) from nine families. The parents in two families were consanguineous. The dates of birth ranged from 1969 to 1995. The mean age of onset was 12.1 months (range 7-24 months) and the mean age at diagnosis was 24.8 months (range 9-48 months). Two patients died at 9 years 5 months and 4 years 11 months.

Table 1 shows the results of SMN and NAIP deletion testing. In the two patients without deletions, the clinical diagnosis was confirmed by muscle biopsy and EMG findings. One additional patient, who was not included in this study, had late onset SMA confirmed by muscle biopsy and EMG and was found to have deletions of exons 7 and 8 of SMN on one chromosome 5 and a gene conversion event (involving a C to T transition at nucleotide 873 but not nucleotide 868-44) in exon 7 of the telomeric copy of SMN on the other chromosome 5. This transition occurred at a site critical for correct splicing of exon 7, and rendered the gene non-functional. Gene conversion events in exon 7 have been reported previously.¹⁹

There were three affected siblings from two families. All three were shown to have the same SMN deletion as the index cases. In one case, the proband was homozygously deleted for NAIP but the sibling was not, owing to recombination. No fetuses were terminated.

Muscle biopsy and/or EMG was performed in 11 of 12 cases and results are shown in table 2. One case was diagnosed by DNA analysis alone.

Three families had no further children after diagnosis of the proband. The pregnancies occurring in the remaining six families are summarised in table 3.

Discussion

The genetics of autosomal recessive SMA are complicated and the correlation between genotype and phenotype remains obscure.^{4 21-23} Despite this, in general, identification of homozygous deletions of exons 7 and 8 of SMN in a child with symptoms can be considered diagnostic and can avoid the need for invasive muscle biopsy and EMG testing.²⁶

Our series of patients included children diagnosed before DNA tests were available and in one case analysis was performed on DNA extracted with parental consent from a stored neonatal blood spot card. In 12 cases, blood samples had been taken during life and DNA

stored in anticipation of future tests becoming available.

We observed an overall frequency of 88.5% for deletions within the SMN gene compared with 95% in previous reports. Of 17 probands with type I SMA, 16 were homozygously deleted for exons 7 and 8 of SMN and the remaining patient was deleted for exon 8 only. Of the nine probands with type II and III SMA, seven had homozygous deletions of exons 7 and 8 of SMN. The frequency of deletions within the NAIP gene was 82% in type I and 44% in types II and III compared with previously reported frequencies of 67% and 42%, respectively.¹⁴ Discrepancies between observed and reported figures may be a result of our small sample size. The sensitivity of DNA deletion tests in confirming the diagnosis of SMA compares favourably with that of muscle biopsy (16 of 21) and EMG (15 of 22). Of four cases of SMA in our series diagnosed since 1995, only one has undergone a muscle biopsy; one was diagnosed on EMG and DNA analysis and two have been diagnosed on DNA analysis alone. The specificity of DNA testing is reflected in reported figures of < 1% of unaffected relatives with homozygous deletions of SMN and 2–3% of non-SMN chromosomes having deletions of NAIP.^{14 18}

DNA analysis is particularly useful in confirming the diagnosis of SMA in cases with atypical clinical features or atypical results on muscle biopsy or EMG. Severe neonatal SMA with diaphragmatic paralysis has been reported in two siblings discordant for DNA markers flanking the SMA locus, suggesting that this form of SMA maps outside the 5q11.2–q13.3 region.²⁹ In our series of patients, there were two babies with neonatal onset of severe SMA with early respiratory problems who died at 8 and 10 weeks. The muscle biopsy in both was not typical of SMA, but was thought to be compatible with the diagnosis when the early timing of the investigations was taken into account. Both patients had EMGs showing active denervation, consistent with SMA. Subsequent analysis of DNA in both cases revealed homozygous deletions of exons 7 and 8 of SMN, confirming the diagnosis and the validity of a prenatal test that had been performed by linkage analysis in one of the families. Both patients were also homozygously deleted for exon 5 of NAIP, which is compatible with their severe phenotype.

In general, the limitations of linkage analysis as a result of non-informativeness or recombination can be resolved by the more direct deletion testing.^{11 25} In our series, two children at risk of SMA type II had predictive tests performed by DNA linkage analysis during the first 6 months of life. In both cases prenatal testing had been declined. The first child was calculated to be at 3.5% risk of being affected. Later, he developed symptoms of SMA and the diagnosis was confirmed by open muscle biopsy at 13 months of age. Further linkage analysis using closer markers confirmed that the affected child had inherited the same SMA linked haplotypes as the older affected sibling, indicating that a recombination had occurred

Key messages

- All patients should be clinically assessed by a paediatric neurologist.
- DNA should be analysed in all cases of suspected spinal muscular atrophy.
- Counselling should be offered as well as prenatal diagnosis via linkage and deletion testing.
- Samples should be stored for future mutation testing.

between the disease locus and the original DNA markers used. Subsequent analysis confirmed homozygous deletion of exons 7 and 8 of SMN in both siblings. In the second family, the child was tested after birth and found to have inherited the same high risk chromosomes as her affected sibling. Unlike her sibling, however, she was not homozygously deleted for exons 7 and 8 of SMN. It was concluded that only one parent was a carrier for SMA and that the other parent had germinal mosaicism for the deletion or that an additional de novo deletion had arisen in the affected child.

In our series, there was a high uptake of prenatal prediction by linkage alone (eight of 15 pregnancies) and of prenatal diagnosis by both deletion testing and linkage (eight of eight pregnancies). This reflects the high level of acceptability of prenatal diagnosis and termination of affected pregnancy reported previously.¹⁰

The pattern of SMN and NAIP deletions in affected siblings and terminated fetuses was identical to that in the proband except in one case where a recombination was known to have occurred, resulting in deletion of SMN but not NAIP in the second affected sibling. Previously, consistency of the deletion has been assumed for SMN deletions but not documented.²⁵ Conversely, there are reports of siblings with identical SMN deletions who are not concordant for symptoms of SMA (types II/III).^{20 21} This is sufficiently rare that the demonstration of a homozygous deletion of SMN in the presence of symptoms of SMA can be assumed to confirm the diagnosis. In the context of prenatal diagnosis, it is not possible to confirm the phenotype of the fetus, and diagnosis relies solely on determining the presence or absence of the mutation known to cause SMA in the affected sibling. In our series, all fetuses predicted to be affected by linkage analysis have had the presence of homozygous deletions confirmed by subsequent analysis. All fetuses predicted to be unaffected by prenatal linkage or deletion testing have remained healthy after birth.

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