Original Article

SMN1 Deletions Among Singaporean Patients With Spinal Muscular Atrophy

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Abstract

Introduction: Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterised by degeneration of spinal cord anterior horn cells, leading to muscular atrophy. It is the second most frequent autosomal recessive disease among Caucasian populations with a prevalence of between 1 in 6000 and 1 in 10,000 live births, and a carrier frequency of about 1 in 50. The International SMA Consortium classification defines several types of SMA depending on the age of onset and clinical severity. In the past, the diagnosis of SMA was confirmed by muscle biopsy and, sometimes, electromyography. In 1990, SMA was linked to the 5q13 region of chromosome 5. In 1995, it was found that >95% of patients with SMA have homozygous deletions of exons 7 and 8 of the survival motor neurone 1 (SMN1) gene, one of the candidate genes identified within 5q13. The purpose of our study was to determine the frequency of SMN1 deletions in patients with known SMA and the impact of this on the diagnosis of SMA. Materials and Methods: Molecular analysis was performed on stored DNA and case notes were reviewed retrospectively. Results: Twenty-two (91.7%) out of 24 patients with all types of SMA were homozygously deleted for exons 7 and/or 8 of SMN1. We also report our experience with prenatal diagnosis of SMA. Conclusions: Molecular studies can replace conventional investigations for SMA and have made the option of prenatal diagnosis possible for couples at risk.

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Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterised by degeneration of the anterior horn cells in the spinal cord and, in some cases, of the motor nuclei in the brain stem, resulting in symmetrical muscle weakness and atrophy. It is the second most common autosomal recessive disease among Caucasian populations. It has a prevalence of between 1 in 6000 and 1 in 10,000 live births,[1-3] with an estimated carrier frequency of about 1 in 50.[4]

The International SMA Consortium classification defines several types of SMA depending on the age of onset and clinical severity.[5] Type I (Werdnig-Hoffman disease) is the most severe form with onset in utero with reduced fetal movements or within the first 6 months of life. These children have normal intelligence, but are unable to sit without support and usually have feeding difficulties. The majority of children with SMA type I die from aspiration or respiratory failure within the first 2 years. Type II (Dubowitz disease) is the intermediate form with onset before the age of 18 months. Affected children have hypotonia and delayed motor milestones. They are able to sit unsupported, but are unable to stand or walk unaided. Survival depends on the extent of involvement of the respiratory muscles, but is usually >4 years. Type III (Kugelberg-Welander disease) is the chronic form with onset after the age of 18 months and a mild clinical course. Affected individuals are able to walk unaided, but attain motor milestones late and tend to fall frequently and have difficulties with climbing stairs. Type IV is the adult-onset form of SMA. The age at onset ranges from 20 to 32 years,[6] although some authors define SMA type IV as onset after 30 years.[7]

In the past, a clinical diagnosis of SMA was confirmed by muscle biopsy and, sometimes, electromyography (EMG).

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Typically, muscle biopsy shows degeneration of muscle fibres without inflammation, fibrosis or histochemical abnormality. These procedures are invasive, time-consuming and may give inconclusive results in very young infants.

In 1990, linkage studies in families of SMA patients found that 95% of all cases of SMA were linked to the 5q13 region of chromosome 5.[8-10] In 1995, two candidate genes within this region were described: the survival motor neurone (SMN) gene and neuronal apoptosis inhibitory protein gene. Each of these genes was found to be present in at least 2 copies. Later, the p44 gene (a subunit of the basal transcription factor) was identified as a third candidate gene.[11]

Among all the candidate genes, the SMN gene is believed to be the primary SMA disease-causing gene. It is present in 2 almost identical copies, 1 centromeric and 1 telomeric. Both copies contain 9 exons. They differ only in 8 nucleotides; 5 are intronic and 3 are exonic, located in exons 6, 7 and 8. The telomeric copy is the functional copy and is termed SMN1. Homozygous deletions of exons 7 and 8 of SMN1 were found to occur in >95% of patients with SMA, but not in normal control populations.[12] The patients who did not have homozygous deletions were shown to have point mutations in the SMN1 gene.[13]

The exact genotype-phenotype correlation in SMA is not fully understood. The possible role of the centromeric copy of the SMN gene (SMN2) has been investigated. It was originally thought that SMN2 was non-functional, because the absence of both copies of SMN2 in the presence of at least 1 telomeric copy (SMN1) does not produce the phenotype of SMA. Later, it was shown that SMN2 produces 20% to 30% of its transcripts as functional protein and that patients with milder SMA phenotypes generally have more copies of SMN2 than patients with a more severe phenotype. The method by which the extra copies develop has been postulated to be gene conversion or duplication. It has also been proposed that there are different functional alleles of the SMN2, some of which may modify the phenotype more than others.[4,14] Although the genotype-phenotype correlation in SMA is not fully elucidated at present, the finding of homozygous deletions of exons 7 and 8 of SMN1 in a child with consistent clinical features is considered to be diagnostic of SMA.

The purpose of our study was to determine the frequency of SMN1 deletions in patients with known SMA. This was achieved through molecular analysis of stored DNA and retrospective review of case notes. We discuss the impact of available molecular tests on the diagnosis of SMA and on the prenatal diagnosis of SMA.

Materials and Methods

Subjects

We included in our study all patients with a clinical diagnosis of SMA whose blood samples were sent to our DNA laboratory. Informed consent for DNA analysis was obtained from the patient's parents. The study was carried out with the approval of the Hospital Ethics Committee.

The patients' case notes were reviewed retrospectively. In the case of patients who were not seen primarily in our hospital, the diagnosis was provided by the referring neurologist. We collected data on the demographic profile of the patients, the age of onset of symptoms, the need for home ventilation and age of death. The data were entered using the Statistical Package for the Social Sciences (SPSS) version 9.0.

Molecular Analysis

DNA was extracted from peripheral blood samples using a standard DNA extraction kit (Quantum Prep AquaPure Genomic DNA Blood kit; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Polymerase chain reaction (PCR) amplification of SMN exon 7 was carried out based on the method described by Wirth et al[15] with slight modifications. The primer SMN7F used was 1 base shorter than described, as the shorter sequence matched most databases. The reaction was carried out in a final volume of 25 μL reaction using 50 to 100 ng of genomic DNA, 10 nmol each of, 20 nM dNTP, 0.25 U *Taq* polymerase (Roche Applied Science, Nonnenwald 2, Penzberg, Germany) in a 1X PCR buffer with magnesium chloride. PCR was carried out using the following cycling conditions: initial denaturation at 94°C for 7 minutes; 35 cycles of 94°C for 55 seconds, 51.5°C for 55 seconds and 72°C for 1 minute; and a final extension of 72°C for 7 minutes in a Applied Biosystems GeneAmp PCR System 9600 (Foster City, CA, USA). A PCR product of 135 bp was obtained at the end of the cycles. The PCR products (12 μL) were digested with 10 u *Hinf*I (New England Biolabs Inc., Beverly, MA, USA) for 3 hours at 37°C and run on 20% polyacrylamide (PAGE) at 150 V in 1X tris-borate-EDTA (TBE) (Amresco Inc., Solon, Ohio, USA) for 1.5 hours (Fig. 1a) or on pre-cast 10% PAGE (Bio-Rad Laboratories, Inc., Hercules, CA, USA, Cat no: 161-1110) at 150 V in 1X TBE (Amresco Inc., Solon, Ohio, USA) for 30 minutes. Presence of various restriction fragments allowed the recognition of SMN2 (101 bp and 34 bp) and SMN1 (78 bp, 34 bp and 23 bp).

PCR amplification of SMN exon 8 was carried out in a 25 μL reaction volume using 50 to 100 ng of genomic DNA, 10 nmol each of SMN8F (5'-ACACCCTTCTCAC

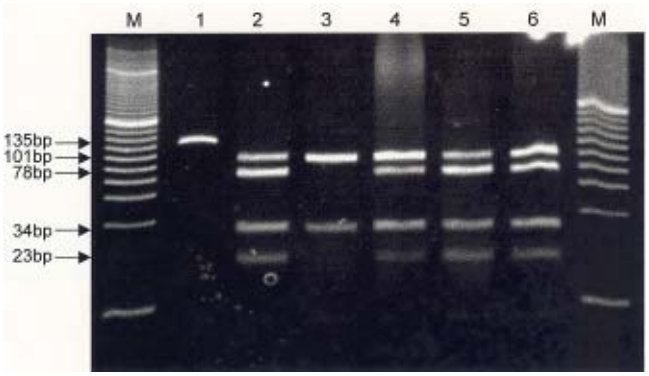

Fig. 1a.

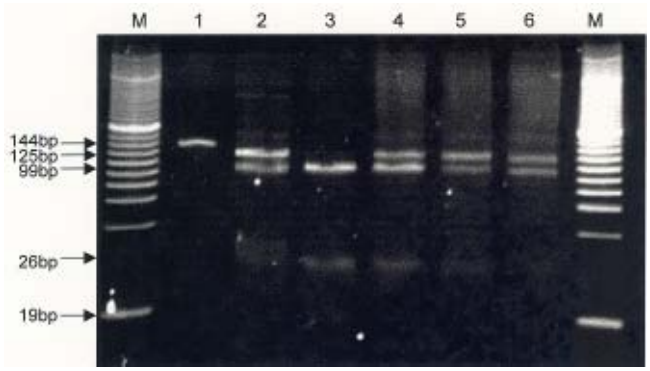

Fig. 1b.

Fig. 1. Separation of restriction fragments of PCR product for (a) exon 7 with *Hin*fI and (b) exon 8 with *Dde*I. Electrophoresis was carried out on 20% PAGE gel at 150 V for 1.5 hours. M: 20 bp marker; Lane 1: uncut PCR product; Lanes 2, 4, 5 and 6: samples with restriction fragments representing exons 7 and 8 of both SMN1 and SMN2 genes; Lane 3: sample showing homozygous deletion of SMN1 gene indicated by absence of 78 bp and 23 bp restriction fragments in (a) exon 7 and 125 bp fragment in (b) exon 8.

AGCTCAGAAAATTA-3') and SMN8R (5'-TTTGAAA AACCATCTGTAAAAGACTG-3'), 20 nM dNTP, 0.25 U *Taq* polymerase (Roche Applied Science, Nonnenwald 2, Penzberg, Germany) in a 1X PCR buffer with magnesium chloride. PCR was carried out in an Applied Biosystems GeneAmp PCR System 9600 (Foster City, CA, USA) using the same cycling conditions described above. The eighth base from 3' end of forward primer SMN8F was changed from T to G to create a *Dde*I site (c/tnag). This served as a control digest for Exon 8 in SMN1 gene, which carries no *Dde*I site in the region amplified. Enzyme digest using 10 u *Dde*I (New England Biolabs Inc., Beverly, MA, USA) was then carried out for 12 μL PCR product of 144 bp for 3 hours at 37°C and run on 20% (Fig. 1b) or 10% PAGE as above. Presence of various restriction fragments allowed the recognition of SMN2 (99 bp, 26 bp and 19 bp) and SMN1 (125 bp and 19 bp).

Results

SMA Type I

There were 8 unrelated patients with SMA type I, of whom 5 were male and 3 were female. There was parental consanguinity in 1 of the families. Five patients were Chinese, 2 were Malays and 1 was Indian. The dates of births of the patients ranged from 1992 to 2002.

The age of onset ranged from birth to 6 months, with a mean of 2.86 months. Two of the children received home ventilation, commencing at 4 months and 6 months, respectively. Six of the 8 children have died, with the age at death ranging from 4 to 6 months (mean, 4.8 months). One patient was lost to follow-up and the remaining patient is 20 months old and on home ventilation.

Seven (87.5%) out of the 8 patients with SMA type I were homozygously deleted for SMN1. Six of them were homozygously deleted for exons 7 and 8, while 1 was homozygously deleted for exon 7 only. The remaining patient did not have homozygous deletions of exons 7 or 8 of SMN1. The last patient was diagnosed with SMA based on muscle biopsy findings.

SMA Type II

There were 13 unrelated patients with SMA type II. Of these, 7 were male and 6 were female. Eleven patients were Chinese, 1 was Malay and 1 was of Indonesian origin. There was no parental consanguinity in any of these families. The children were born between 1994 and 2003.

The age of onset of symptoms ranged from 2 to 36 months, with a mean of 10.85 months. Ten of the patients are being followed up at our hospital. Four of them are on home ventilation. The age at which this was commenced ranged from 1 to 4 years. Two of the children have died at the ages of 2 years 9 months and 6 years 5 months, respectively; both these children were on home ventilation. The remaining 8 children range in age from 10 months to 9 years, among whom the 2 oldest are on home ventilation.

Twelve (92.3%) out of the 13 children with SMA type II were homozygously deleted for exons 7 and 8 of SMN1.

SMA Type III

There were 3 unrelated patients with SMA type III, 2 Chinese and the other of Indonesian descent. Two were male and 1 was female. The age of onset of symptoms was 3 years for 2 patients and 22 years for the third patient. These patients are not managed primarily at our hospital and the diagnoses were provided by the referring doctors.

All 3 patients were found to have homozygous deletions of exons 7 and 8 of SMN1.

Table 1: Frequency of Deletions of SMN1

	Homozygous deletions of exons 7 and/or 8 of SMN1*	No homozygous deletions of exons 7 or 8 of SMN1
SMA type I (n = 8)	7 (87.5%)	1 (12.5%)
SMA type II (n = 13)	12 (92.3%)	1 (7.7%)
SMA type III (n = 3)	3 (100.0%)	0
All cases of SMA (n = 24)	22 (91.7%)	2 (8.3%)

SMA: Spinal muscular atrophy; SMN1: survival motor neuron 1

* Of the 22 patients, 21 patients had homozygous deletions of both exons 7 and 8 of SMN1. One patient with SMA type I had homozygous deletions of exon 7 but not of exon 8 of SMN1.

Overall Results

Out of 24 patients with all types of SMA, 22 (91.7%) were homozygously deleted for exons 7 and/or 8 of SMN1. These results are summarised in Table 1.

Prenatal Diagnosis

After diagnosis of SMA in the proband, there have been 6 pregnancies in 5 of the families. Prenatal diagnosis was utilised by the families in these pregnancies. In 3 of the families, the proband had SMA type I while in the other 2 families, the proband had SMA type II. In all 5 families, the proband was known to have homozygous deletions of exons 7 and 8 of SMN1.

In 1 of the families in which the proband had SMA type II, there were 2 subsequent pregnancies. In the first pregnancy, prenatal diagnosis showed that the fetus did not have homozygous deletions of exons 7 or 8 of SMN1. In their subsequent pregnancy, the fetus was found to have homozygous deletions of exons 7 and 8 of SMN1. The couple elected to terminate the pregnancy. In the other families, prenatal diagnosis showed that the fetus had homozygous deletions of exons 7 and 8 of SMN1 in 1 pregnancy and no homozygous deletions of exons 7 or 8 of SMN1 in the other 3 pregnancies.

Of the 4 pregnancies with normal prenatal diagnoses, all have resulted in normal live borns who remain well on follow-up.

Discussion

The molecular basis of SMA is complex. The roles of the other candidate genes and the genotype-phenotype correlations are still not fully understood. However, the finding of homozygous deletions of exons 7 and/or 8 of SMN1 in a child with consistent clinical features is generally considered to be diagnostic of SMA. This spares the child the need for invasive diagnostic procedures, such as a muscle biopsy.

The patients in our series comprised children diagnosed before molecular testing became available, as well as several who presented after DNA testing had become available. Thus, the latter group avoided having a muscle biopsy or EMG. In 2 cases, blood samples had been taken before the child's death and stored in anticipation that genetic testing would become available.

In our patients, the frequency of homozygous deletions of exons 7 and/or 8 of SMN1 in patients with all types of SMA was 91.7% (Table 1). This is similar to other reports that quote an incidence of 88.5% to 95%.[3,12]

In our institution, DNA testing has become the first-line investigation for confirmation of a clinical diagnosis of SMA. Muscle biopsy is undertaken only if no deletions of exons 7 and/or 8 of SMN1 are demonstrated in a child with clinical features that are highly suggestive of SMA.

DNA analysis is particularly applicable in the setting of prenatal diagnosis. SMA is an autosomal recessive condition; thus, couples who have had an affected child face a 1-in-4 risk of a similarly affected child in each subsequent pregnancy. Prior to DNA analysis becoming available, there was no definitive prenatal test available in Singapore. Couples either decided not to have further children or had to accept the 1-in-4 risk and simply "take their chance". Since DNA analysis became available, there has been a good uptake of prenatal diagnosis. Of the 6 cases of prenatal diagnosis performed, the fetus was found to be affected in 2 pregnancies and the couples decided on termination of pregnancy. The acceptability of prenatal diagnosis for SMA and termination of affected pregnancies among our population is similar to previous reports in other countries.[3,16] Reasons for this high level of acceptability include the incurable nature of the disease and the significant risk of recurrence.

SMA carrier testing is available clinically in a few laboratories worldwide. It is based on determining the number of SMN1 gene copies in an individual. Direct DNA testing, as we have utilised for diagnosis of SMA, cannot be used for carrier testing because it does not quantitate the number of SMN1 gene copies. Instead, a PCR-based dosage assay that allows determination of the number of SMN1 gene copies is the method of choice. Our laboratory has embarked on this as a research project, using real-time PCR. Interpretation of SMA carrier test results can be difficult for several reasons: 1) some carriers have 2 SMN1 gene copies on 1 chromosome (the so-called 2+0 genotype); 2) some carriers have 1 normal SMN1 allele and 1 SMN1 gene copy with a mutation; 3) de novo deletions occur in 2% to 3% of SMA patients, meaning that only 1 parent is a carrier. Given these potential difficulties in interpreting carrier testing, SMA carrier testing, when available, should be offered together with formal genetic counselling.[1]

Conclusions

Molecular studies can replace conventional investigations for SMA and have a particular application for prenatal diagnosis. In a patient with clinical features of SMA, SMN1 analysis should be the investigation of choice as it is non-invasive and would show homozygous deletions of exons 7 and/or 8 in at least 91.7% of cases. If no deletions are demonstrated, muscle biopsy or EMG may be considered. After the birth of an affected child, couples have a 1-in-4 recurrence risk in subsequent pregnancies. Accurate prenatal diagnosis is available via SMN1 analysis if the proband is known to have homozygous deletions of SMN1.

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