

Correlation between deletion patterns of SMN and NAIP genes and the clinical features of spinal muscular atrophy in Indian patients

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Background: Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder involving degeneration of anterior horn cells of spinal cord resulting in progressive muscle weakness and atrophy. **Aims:** The molecular analysis of two marker genes for spinal muscular atrophy (SMA) i.e., the survival motor neuron gene (SMN) and the neuronal apoptosis inhibitory protein gene (NAIP) was conducted in 39 Indian patients with clinical symptoms of SMA. Out of these, 28 showed homozygous deletions and the phenotypic features of these SMA patients were compared with the corresponding genotypes. **Settings:** A tertiary care teaching Hospital. **Design:** This is a prospective hospital based study. **Materials and Methods:** Polymerase chain reaction (PCR) combined with restriction fragment length polymorphism (RFLP) was used to detect the deletion of exon 7 and exon 8 of SMN1 gene, as well as multiplex PCR for exon 5 and 13 of NAIP gene. **Results:** Exons 7 and 8 of SMN and NAIP (exon 5) were homozygously deleted in 73% of SMA I and 27% of SMA II patients. SMN exon 7 and 8 deletions without NAIP deletions were seen in 27% of type I SMA and 46% of SMA type II patients. Two patients of type III SMA showed single deletion of SMN exon 7 along with 27% of SMA type II patients. **Conclusion:** With the advent of molecular biology techniques, SMN gene deletion studies have become the first line of investigation for confirmation of a clinical diagnosis of SMA. The findings of homozygous deletions of exons 7 and/or 8 of SMN1 gene confirms the diagnosis of SMA, even in patients with atypical clinical features. Deletions of NAIP gene were mainly seen in severely affected patients, hence is useful for predicting the prognosis.

Key words: Neuronal apoptotic inhibitory protein gene, spinal muscular atrophy, survival motor neuron gene

Introduction

Spinal muscular atrophies (SMA) are a clinically and genetically heterogeneous group of disorders characterized by primary degeneration of anterior horn cells of the spinal cord and brain stem nuclei causing progressive muscle weakness.^[1]

SMA is one of the most common lethal autosomal recessive disorder, with the childhood onset form of SMA between 1 in 6000 and 1 in 10,000 live births.^[2,3] The three types of SMAs present with diverse symptoms and differ in age of onset, mode of inheritance, distribution of muscle weakness and progression of symptoms.^[2]

SMA of all types is associated with homozygous mutations in the Survival Motor Neuron gene (SMN1 gene) located at the telomeric end of chromosome region 5q13.^[4,5] A large inverted repeat at the centromeric region of 5q13 consists of homologous gene SMN2. SMN1 and SMN2 differ in exons 7, 8 and 6 by only one base pair each and in five introns.^[2,6]

The presence of a C->T transition at codon 280 within exon 7 of SMN2 causes disruption of an exonic splice enhancer.^[7] This leads to abnormal splicing which produces unstable truncated protein.^[8] SMN2 does produce a small amount of functional full-length protein but the majority of the full-length protein is produced by SMN1.^[9]

Another gene, the neuronal apoptosis inhibitory protein gene (NAIP), located on the same chromosomal region of 5q13, was found to be deleted in SMA patients.^[1,10] The NAIP has been postulated to have neuroprotective effect and acts as a negative regulator of motor neuron apoptosis.^[10] It was suggested that the absence of the full-length NAIP gene is necessary but not sufficient to produce SMA.^[10]

The purpose of this study was to confirm the clinical diagnosis of SMA patients and to correlate the frequency of deletions within SMN and NAIP genes. The study mainly concentrates on the

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pattern of gene deletions seen in three different types of SMA patients and their correlation with the clinical severity. It does not attempt to determine the prevalence of deletions in clinically suspected SMA patients.

Materials and Methods

A total of 49 blood samples received between the period of September 2004 and December 2005, all from the Indian population, were studied for SMN1 and NAIP gene deletions. The 39 patients included in the study were strongly suspected of SMA after a full clinical evaluation. Ten samples from non-SMA patients were also run as controls.

Detection of SMN1 and NAIP gene deletions by PCR-RFLP method

Genomic DNA was extracted using sigma NA 2020 kit from peripheral blood (collected in EDTA bulbs). Polymerase chain reaction (PCR) amplification of SMN exons 7 and 8 and NAIP exons 5 and 13 was carried out in a total volume of 50 µl using Hot Star Taq Master Mix (QIAGEN) for SMN (7 and 8) gene and QIAGEN multiplex PCR kit for NAIP exons 5 and 13. The Hot Star Taq used in all the PCR reactions was activated at 95°C for 15 mins before the cycles commenced.

SMN gene

Exon 7 was amplified with intron 6 primer R111 (2 µM) and mismatch primer X7-Dra (2 µM) in 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C.^[6,11] PCR products (25 µl) along with 1x enzyme buffer SM (25 µl) were subsequently digested to completeness with restriction enzyme Dra I (2 units) (Sigma) [Table 1].

By using a mismatch primer (X7-Dra) on the 3' end of exon 7, a G to A change is introduced at positive 875 of the cDNA. This change creates a Dra I site (TTTAAA). The third T nucleotide of the sequence is present only in the SMN 2 gene and is a C in the SMN 1 gene, thus the enzyme cuts only the SMN 2 amplified PCR product.^[6,11] Hence, after digestion two bands are seen, one at 188 bp (indicating SMN 1) and the other at 176 bp (representing SMN 2) as gene amplified products.

For exon 8, DNA was amplified with primers 541C960 and 541C1120 (2 µM each), with the same PCR conditions, except for an annealing temperature of 59°C instead of 55°C.^[6,11] PCR products (25 µl) were subsequently digested with restriction enzyme Dde I (2 units).

The nucleotide in exon 8 which differs between SMN 1 and SMN 2 genes is G>A. The A in the SMN 2 gene (located at position 1155 of the cDNA) creates a Dde I site which will digest the amplified DNA into two products of 122 bp and 78 bp.^[6,11]

The exon 8 of SMN1 gene (200 bp) does not contain any Dde I, site hence it will not be cut and will remain as 200 bp.^[12]

NAIP exon 5

For NAIP, exon 5 and 13 (exon 13 taken as positive control), a multiplex reaction was carried out for 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min using primers 1864 and 1863 for exon 5 and primers 1258 and 1343 for exon 13.^[10,12]

PCR products from both SMN gene products and NAIP were run on 2% agarose high fidelity gel (Sigma) stained with ethidium bromide in 1x TBE buffer and visualised under UV transilluminator and gel documentation system (Vilber Lourmat - France).

SMA was classically subdivided into three types based on age of onset and clinical severity. The standard ISMAC (International SMA consortium) classification for SMA given in Table 1 was used for this study.^[13]

Results

Molecular analysis using PCR-RFLP assay, on 39 clinically suspected cases of SMA revealed 28 patients with homozygous deletions in SMN1 or/and NAIP genes [Table 2]. Out of these 28 SMA patients showing deletions, the maximum number (54%) were of Type II followed by Type I (39%) and only 7% were Type III. The classification into different types was based on the ISMAC.

SMA I: Eleven SMA I patients showed homozygous deletions for SMN1 gene and/or NAIP gene [Table 2]. Their ages ranged between 1.5 months to 2 years. Ten patients were between 1.5 months to 10 months; only one patient was age two years, who died shortly. All these patients were unable to sit or hold head and were floppy infants. EMG was possible only in four of the 11 patients and showed AHC (anterior horn cell disease).

The deletion analysis for SMN1 and NAIP genes of these patients showed 73% to have homozygous deletions of both SMN1 gene (exons 7 and 8) and NAIP gene (exon 5) (Figure 1 lane 4c, 7c and 10c), while 27% showed deletions of exons 7 and 8 of only the SMN1 gene. None of the patients of SMA type I revealed a deletion of only exon 7 of the SMN gene. It was observed in these 11 patients that a severe phenotype is manifested mainly when deletions occur in both exon 7 and exon 8 of SMN and/or NAIP gene [Table 2].

SMA II: Fifteen patients showing homozygous deletions were classified as SMA II, which is the largest group in our study (54%). Age of patients ranged from one year to 20 years. All patients could not walk and 93% of them showed wasting and areflexia of the lower limbs. Tongue fasciculations were seen in three of the patients, out of which two were sibs (brother and sister). EMG was done in only five of the 15 patients and showed AHC (anterior horn cell) disorder.

Table 1: Classification of spinal muscular atrophies.^[13]

Designation	Symptom onset	Course	Survival
Type I (severe) Werdnig-Hoffmann	0-6 months	Never sits	<2 years
Type II (intermediate- chronic)	6-18 months	Never stands	>2 years
Type III (mild) Kugelberg - Welander	>12 months	Stands alone	Adult

Table 2: Genotype phenotype correlation of 28 spinal muscular atrophy patients

TOTAL: 28	SMA I (11)	SMA II (15)	SMA III (2)
Deletion of SMN 1 - Ex 7 and 8; NAIP-Ex 5	8/11 6 males/2 females Age range: 1.5 mts-2 yrs Could not sit or hold head: 8/8 Floppy infant: 8/8 Areflexia: 7/8 Prox. and dist. weakness in all 4 limbs: 8/8 Respiratory discomfort: 8/8 EMG- AHC: 2/8 Age of death 2 mts-2 yrs: 6/8	4/15 3 males/1 female Age range: 3- 20 yrs Could not walk: 4/4 Delayed milestones: ¼ Areflexia of lower limbs: 3/4 Wasting of lower limbs: 3/4 Tongue fasciculation: 2/4 EMG- AHC: 2/3	
Deletion of SMN 1 - Ex 7 and 8	3/11 3 males/0 females Age range: 2 mts-11 mts Could not sit or hold head: 3/3 Floppy infant: 3/3 Areflexia: 3/3 Prox. and dist. weakness in all 4 limbs: 3/3 Respiratory discomfort: 3/3 EMG- AHC: 2/3 Age of death 2-3 mts: 2/3	7/15 2 males/5 females Age range: 2 yrs - 5 yrs Could not walk: 7/7 Areflexia of lower limbs: 7/7 Wasting of lower limbs: 7/7 Tongue fasciculation: 1/7 EMG- AHC: 2/7	
Deletion of SMN 1 - Ex 7		4/15 3 males/1 female Age range: 1 yr - 9 yrs Could not walk: 4/4 Delayed milestones: 4/4 Areflexia of lower limbs: 4/4 Wasting of lower limbs: 4/4 EMG- AHC: ¼	2/2 1 male / 1 female Age range: 14-24 yrs Could walk: 2/2 Proximal leg weakness: 2/2 Slowly progressive: 2/2

*AHC-Anterior horn cell, SMA-Spinal muscular atrophies

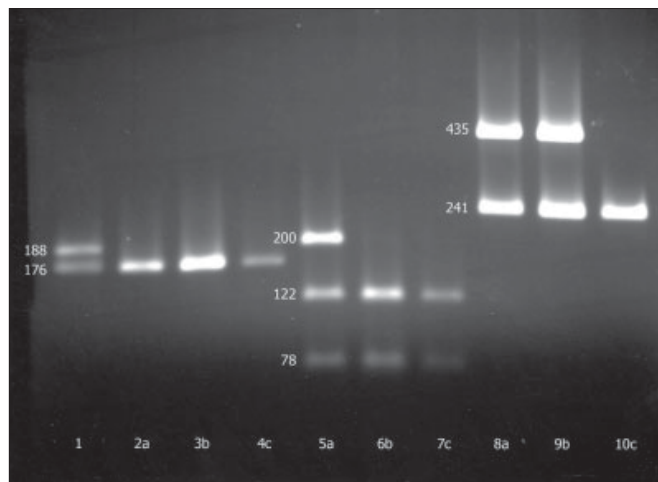


Figure 1: Agarose gel electrophoresis of PCR amplified products of SMA patients for SMN exon 7 (after Dra I digestion) (row 1 to 4), exon 8 (after Dde I digestion) (row 5 to 7) and NAIP exon 5 and 13 (row 8 to 10). Lane 1: SMN exon 7 control showing no deletion of exon 7 of SMN 1. Patient 'a': lanes (2, 5 and 8) showing only SMN1 exon 7 deletion (2a). Patient 'b': lanes (3, 6 and 9) showing exons 7 and 8 of SMN 1 gene deleted. Patient 'c': lanes (4, 7 and 10) showing deletions of SMN1 exon 7 and 8 and NAIP exon 5

Forty-six percent of type II patients showed both exons 7 and 8 of SMN1 gene deletion, (Figure 1 lane 3b, 6b and 9b) while about 27% showed deletion of only exon 7 of SMN gene. NAIP gene (exon 5) along with SMN1 (exon 7 and 8) was deleted in 27% of SMA II patients. Two sets of siblings (brother and sister) with SMA type II were studied.

One pair of sibs aged 12 and 20 years showed deletions in both SMN1 (exons 7 and 8) and NAIP (exon 5). Both brother and

sister first manifested with symptoms at age six months. Both had an insidious onset of weakness of the limbs and trunk and over the next few years regressed in their motor milestones. They were unable to stand. On clinical examination they showed tongue fasciculation, areflexia and marked proximal muscle weakness in both upper and lower limbs. Their EMG confirmed anterior horn cell disease. Their elder sister (25 years) was normal and showed no gene deletions.

The other pair of sibs also brother and sister aged two years and five years respectively showed deletions in both exon 7 and 8 of SMN1 gene only.

SMA III: Only two patients from a group of 28, aged 14 years (male) and 24 years (female) were of SMA type III. Both these patients had slowly progressive weakness of lower limbs. One of the patients, a 24 year-old female, initially showed difficulty in climbing stairs at the age of eight years. She had a very slowly progressive proximal leg weakness and was unable to do heavy work although still able to walk and climb unaided. Her EMG indicated AHC disease (EMG was not done on the other patient). Both these patients showed SMN1 gene deletion of exon 7 only (Figure 1 lane 2a, 5a and 8a).

The remaining 11 who failed to show any deletion for SMN1 gene ranged from age 14 days to 38 years. Six out of 11 were adults between the ages of 10 to 38 years with late onset of symptoms. Three patients whose age ranged between 14 days to 7 months were born as "floppy infants" and had a differential diagnosis between SMA I, congenital myopathy or hypotonic cerebral palsy. One, 1 year-old patient showed strong clinical features of SMA but failed to show any deletion in either SMN1

or NAIP genes. The remaining patient aged 1.5 years showed clinical symptoms such as difficulty in walking, areflexia but failed to show any deletion. Further molecular studies on these patients were not possible hence it was difficult to calculate the total frequency of deletions seen in this group of patients.

Discussion

Among all the candidate genes, SMN1 is believed to be the primary SMA disease-causing gene. The finding of homozygous deletions of exons 7 and/or 8 of SMN1 patients with consistent clinical features is generally considered to be diagnostic of SMA.^[14]

Our study concentrates on genotype phenotype correlation of patients showing homozygous deletions in SMN1 gene or both SMN1 and NAIP gene. The extent of deletions i.e., SMN1 gene exons 7 and 8 and NAIP gene exon 5 seen in our 28 patients were studied along with the clinical history and symptoms [Table 2]. Though there was an overlap in deletions seen and the severity and type of SMA, on the whole it was observed that majority of patients showing more deletions resulted in a severe phenotype. This was apparent in our 11 type I patients where 73% showed SMN1 gene exon 7 and 8 as well as NAIP gene deletion. While 27% of SMA II patients had all three deletions, no patients of SMA III (mild phenotype) showed all three or even two deletions [Table 2].

NAIP deletion alone, without SMN I being deleted, was not seen in any of our patients including the parents of the two siblings studied. This is contrary to the results of Japanese patients where two unaffected mothers of patients with NAIP and SMN1 deletions showed deletions of only NAIP gene.^[15] Also no differences were noted in the age of onset/duration of survival in type I patients, regardless of whether the NAIP gene was deleted or not [Table 2]. In fact one of our patients lacking NAIP was two years old (who died shortly), which is consistent with a report by Taylor *et al.*^[16] Another report from Japan showed that, in type I patients lacking NAIP gene, respiratory function deterioration is more rapid than those retaining NAIP gene.^[17] However, this was not the case in our patients of type I SMA, where all 11 patients regardless of NAIP deletion showed respiratory dysfunction [Table 2]. In spite of this it was seen that a greater percent showed deletions in both genes i.e., 73% of our type I patients showed NAIP gene deletions along with SMN1 exons 7 and 8, with a resultant more severe phenotype.

NAIP was deleted (along with SMN1 exons 7 and 8) in four of the 15 patients with SMA II, though the age range of these patients was between 3-20 years [Table 2]. It should be noted that the two older patients i.e., 12 and 20 years (showing all three deletions) were siblings who manifested the symptoms at the age of 6 months, but came for deletion studies at age 12 and 20 years respectively. Though the survival age of one of the patients is 20 years, it is not unusual as 70% of SMA II patients are found to survive till the age of 25 years.^[18] The remaining type II patients were below the age of five years, except for one nine year-old showing SMN1

exon 7 deletion only.

The total frequency of deletions seen in our 39 patients was not calculated as further molecular conformation (i.e., Point mutations, gene copy number, sequencing etc.) of the remaining 11 patients who did not show deletions, was not available. Though five patients were strongly suggestive of SMA on clinical examination and EMG, it was not possible to determine with molecular genetic studies whether these patients had some other type of mutation e.g., point mutation or if any other gene was deleted. Another group from India, have shown a lower gene deletion frequency in the SMN and NAIP genes in Indian patients and have suggested that these may be mutations in other genes or a population variation. This will need further study.^[19]

EMG studies could not be carried out in all the patients (only seven out of 28 patients) as when the patient was clinically suspected of SMA, the deletion studies were done first, which often made the EMG redundant. Also, EMG studies are difficult in infants.

The role of NAIP gene in the pathogenesis of SMA is still not very clear. However, our study of 28 patients does indicate that NAIP gene can be a predictive marker of SMA prognosis as reported elsewhere.^[10,15,17,20-22] Several studies suggest that NAIP gene exerts effects on differentiation and survival of neuronal cells including the motor neurons.^[17] Further studies will be necessary to clarify its exact functional role. On the other hand, SMN2 copy number has been well established as a modifying factor of clinical severity. A higher number of SMN2 copies are observed in type II or type III SMA patients, than in type I patients.^[23-25] Hence, copy number of SMN2 gene compensates for the lack of SMN1 gene thereby influencing the severity of SMA.

Campbell *et al* reported the presence of NAIP gene and an increase of SMN2 copy number in type II or III patients.^[24] Hence, they concluded that deletion of NAIP gene is often accompanied by a decrease in total copy number of SMN genes in type I SMA and the presence of NAIP gene by an increase of SMN2 copy number or retention of total copy number of SMN genes in type II or III patients.^[24]

The PCR based test used in this study is a rapid sensitive cost effective and spares the patient (mostly children) the need for invasive diagnostic procedure such as a muscle biopsy. DNA testing has become the primary investigation for confirmation of clinical diagnosis of SMA. The only limitations of this method are in about 5% of cases where no deletions are seen in a patient highly suggestive of SMA. In such cases, further studies on gene dosage analysis to determine the copy number of SMN1 gene can be done.^[14] This analysis is also particularly applicable for prenatal diagnosis. As SMA is an autosomal recessive disorder, the recurrence risk is 25% in every pregnancy.^[14]

PCR based assay for determining the presence or absence of SMN1 is not quantitative and therefore cannot identify SMA carriers, for which quantitative PCR is mandatory.^[25,26]

Comprehensive SMA testing including SMA deletion analysis,

linkage analysis and SMA carrier testing offers the most complete evaluation of SMA patients and their families.

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