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Gene Deletion Patterns in Spinal Muscular Atrophy Patients with Different Clinical Phenotypes

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Key Words

Deletion · NAIP gene · Polymerase chain reaction · Spinal muscular atrophy · SMN gene

Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by degeneration of lower motor neurons. We have assayed deletions in two candidate genes, the survival motor neuron (SMN) and neuronal apoptosis inhibitory protein (NAIP) genes, in 108 samples, of which 46 were from SMA patients, and 62 were from unaffected subjects. The SMA patients included 3 from Bahrain, 9 from South Africa, 2 from India, 5 from Oman, 1 from Saudi Arabia, and 26 from Kuwait. SMN gene exons 7 and 8 were deleted in all type I SMA patients. NAIP gene exons 5 and 6 were deleted in 22 of 23 type I SMA patients. SMN gene exon 7 was deleted in all type II SMA patients while exon 8 was deleted in 19 of 21 type II patients. In 1 type II SMA patient, both centromeric and telomeric copies of SMN exon 8 were deleted. NAIP gene exons 5 and 6 were deleted in only 1 type II SMA patient. In 1 of the 2 type III SMA patients, SMN gene exons 7 and 8 were deleted with no deletion in the NAIP gene, while in the second patient, deletions were detected in both SMN and NAIP genes. None of the 62 unaffected subjects had deletions in either the SMN or NAIP gene. The incidence of biallelic polymorphism in SMN gene exon 7 (*Bsm*AI) was found to be similar (97%) to that (98%) reported in a Spanish population but was significantly different from that reported from Taiwan (0%). The incidence of a second polymorphism in SMN gene exon 8 (presence of the sequence ATGGCCT) was markedly different in our population (97%) and those reported from Spain (50%) and Taiwan (0%).

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Proximal spinal muscular atrophy (SMA) is a common autosomal recessive neuropathy. The most significant clinical finding in SMA patients is proximal, symmetrical limb and trunk muscle weakness, which is due to loss of α motor neuron cells in the spinal cord. SMA is clinically heterogeneous and is classified into three types (I–III), based on age at onset and disease severity [14]. Type I SMA is the most severe form and children generally die before 2 years of age. The gene responsible for SMA has been located within the complex genomic region on chromosome 5q11.2-q13.3 which contains a 500-kb inverted duplication [13, 16, 17]. Within this inverted duplication

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lies the SMA-determining gene, the survival motor neuron (SMN) gene. Consequently the SMN gene is present in two copies, designated the SMNc (centromeric) and SMNt (telomeric) genes. Mutations in SMNt are thought to be responsible for the SMA phenotype [13, 22]. Also located within the SMA region is the neuronal apoptosis inhibitory protein (NAIP) gene which by itself is not an SMA-determining gene but deletions in this gene have been found in 45% of cases of SMA type I [16]. In addition to the intact NAIP gene, several NAIP pseudogenes have been detected, which apparently arise independently of the inverted duplication. Exons 5 and 6 of the NAIP gene are unique and are used to distinguish the intact copy of NAIP from pseudogenes. SMNc and SMNt are separated by approximately 1 Mb of DNA and are virtually identical. They span a 20-kb region and consist of nine exons [3]. There are at least two differences in the transcribed sequences of SMNc and SMNt [2, 10, 13]. Several groups have shown that at least SMNt exon 7 is deleted in the majority (87–100%) of SMA patients [6, 9, 11, 13, 18, 21, 24, 26]. Also, a rare point mutation and/or small deletion have been identified in the SMNt gene, which provides convincing evidence that SMNt is indeed the SMA-determining gene [1, 4, 15, 27]. Van der Steege et al. [25] identified a gene conversion event which changed the sequence of the SMNt gene into that of an SMNc gene in some SMA patients in which the SMN exon 7 was deleted but exon 8 was retained. Furthermore, they demonstrated the presence of a chimeric gene with the fusion of exon 7 of the SMNc with exon 8 of the SMNt gene and the absence of a normal SMNt gene. We have investigated the pattern of exon deletion in SMN and NAIP genes in 46 SMA patients from different ethnic backgrounds with different clinical presentations.

Patients and Methods

Forty-six families were included in this study with at least one child fulfilling the diagnostic criteria for SMA. Type I SMA patients had onset before the age of 6 months, with symmetrical proximal muscle weakness, in the absence of extraocular, diaphragmatic, or cardiac weakness, sensory disturbance, arthrogryposis or CNS dysfunction [14]. They were never able to sit without support. The serum creatine kinase activity was normal, and EMG and or muscle biopsy showed evidence of denervation (with >70% normal nerve conduction velocities). Type II SMA patients had onset before the age of 18 months and were able to sit but were unable to walk or stand without assistance. Otherwise the same criteria for inclusion/exclusion as for type I applied. Type III SMA patients were also chosen according to the criteria suggested by the International SMA Consortium [14].

This study was approved by the ethics committee for the protection of human subjects at the Kuwait University, Faculty of Medicine. Blood samples were collected from probands and unaffected healthy subjects (after obtaining informed consent) in EDTA tubes, and DNA was extracted using a standard procedure [19]. SMN gene exon 7 was amplified using primers R111 [13] and X7-Dra [24]. SMN gene exon 8 was amplified using primers 541C960 and 541C1120 under the conditions described earlier [24]. The PCR products from SMN gene exons 7 and 8 were subsequently digested to completion with the restriction enzymes *Dra*I and *Dde*I, respectively. The products were analyzed on 2% agarose gels. The SMNt gene and its highly homologous centromeric copy (SMNc) differ by only 2 bp, one each in exons 7 and 8. PCR products of exon 8 from the two genes were distinguished by the presence of the restriction enzyme *Dde*I site only in the SMNc gene and its absence from the SMNt gene. Therefore, in a healthy individual with an intact SMNt (exon 8 is expected to be present), *Dde*I would not cleave the exon 8 PCR product because the site is absent in the intact gene. For exon 7, no such difference in the restriction site for any known enzyme exists. van der Steege et al. [24] developed an oligonucleotide allowing specific priming directly adjacent to the variant site and introduced a mismatch such that a restriction site for *Dra*I was created in the PCR product of exon 7 of the SMNc gene but not in the PCR product of the SMNt gene, thus making it possible to distinguish them clearly. NAIP gene exons 5, 6 and 13 were amplified as described previously [16]. A second PCR-RFLP method [10] was used to reconfirm the deletion pattern in the type II SMA patient in which both SMNt and SMNc genes were deleted.

Two polymorphisms in the SMNt gene, a *Bsm*AI (an A → G transition in exon 7) and *Hae*III (presence of the sequence ATGGCCT, having an *Hae*III site in exon 8) were analyzed by cleaving the PCR products of exon 7 with the restriction enzyme, *Bsm*AI, and that of exon 8 with *Hae*III in buffer supplied by the manufacturer. The cleavage products were analyzed on 2% agarose gels after ethidium bromide staining. Both these polymorphisms are biallelic. In the case of *Bsm*AI, the absence of a restriction enzyme site indicates the presence of allele 1, and its presence shows the presence of allele 2. However, in the case of *Hae*III polymorphism in exon 8 of the SMN gene, the presence of an enzyme cleavage site is associated with allele 1, while its absence shows the presence of allele 2 [6]. The *Bsm*AI polymorphism was screened in 59 subjects (30 SMA patients and 29 unaffected controls) and the *Hae*III polymorphism was analyzed in 62 subjects (30 SMA cases). Allelic frequencies were expressed as a percent of the number of alleles present of the total number of chromosomes analyzed.

Results

The patterns of cleavage obtained from PCR-RFLP analysis of SMN gene exons 7 and 8 and from NAIP gene exons 5 and 13 are shown in figures 1 and 2. The deletion analysis of SMN and NAIP genes was carried out on 108 samples, of which 46 were from SMA patients (23 type I, 21 type II and 2 type III), and 62 were from unaffected subjects. The SMA patients included 3 from Bahrain, 9 from South Africa, 2 from India, 5 from Oman, 1 from

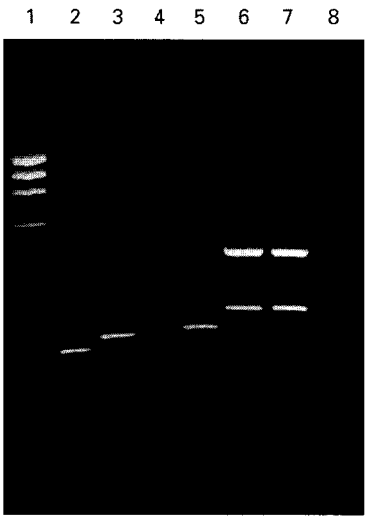


Fig. 1. Detection of deletions in SMN and NAIP gene exons. Lane 1: *Hae*III cut φX174 M_r markers; lane 2: SMN gene exon 7 PCR products cleaved with *Dra*I from a type I SMA patient; lane 3: SMN gene exon 7 PCR products cleaved with *Dra*I from an unaffected subject; lane 4: SMN gene exon 8 PCR products cleaved with *Dde*I from a type I SMA patient (SMNt copy, the 187-bp upper band is deleted, the lower 123-bp band from the SMNc gene is present); lane 5: SMN gene exon 8 products cleaved with *Dde*I from an unaffected individual; lanes 6 and 7: NAIP gene PCR products from a type II SMA patient with both exons 5 (upper) and 13 detected; lane 8: no sample. The products resulting from PCR (NAIP gene) and PCR-RFLP analysis (SMN gene) were analyzed on 2% agarose gel and visualized by ethidium bromide staining.



Fig. 2. Detection of deletions in the SMN gene in type II SMA patients. Lane 1: SMN gene exon 7 PCR products cleaved with *Dra*I from an unaffected subject; lanes 2–4: SMN gene exon 7 PCR products cleaved with *Dra*I from type II SMA patients; lane 5: SMN gene exon 8 PCR products cleaved with *Dde*I from an unaffected subject; lanes 6–8: SMN gene exon 8 PCR products cleaved with *Dde*I from type II SMA patients (both SMNt and SMNc genes are deleted in the type II SMA patient in lane 7). The products resulting from PCR-RFLP analysis were analyzed on 2% agarose gel and visualized by ethidium bromide staining.

Table 1. Incidence of SMN and NAIP gene deletions in the three different clinical types of SMA patients (n = 46)

Clinical phenotype	Cases	SMN gene		NAIP gene		
		exon 7	exon 8	exon 5	exon 6	exon 13
Type I	23	23/23	23/23	22/23	22/23	1/23
Type II	21	21/21	19/21[a]	1/21	1/21	0/21
Type III	2	2/2	2/2	1/2	1/2	0
Unaffected	62	0	0	0	0	0

[a] In one of the SMA type II patients, both the centromeric and telomeric copies of exon 8 were deleted.

Saudi Arabia and 26 from Kuwait. The data on the deletion pattern of the SMN and NAIP genes from SMA patients belonging to the three clinical forms is presented in table 1. Briefly, both exons 7 and 8 of the SMN gene were deleted in all 23 of 23 type I patients, and NAIP gene exons 5 and 6 were deleted in 22 of 23 type I patients. In type II SMA patients, SMN gene exon 7 was deleted in all (21 of 21) patients. However, SMN exon 8 was deleted in only 19 of 21 type II patients. In 1 type II SMA patient, both centromeric and telomeric copies of SMN exon 8 were deleted (fig. 2, lane 7). In this type II patient, the loss of both SMN gene copies was reconfirmed using a different set of primers and the PCR-RFLP method [10] followed by direct DNA sequencing using a biotinylated

Table 2. Incidence of *Bsm*AI polymorphism in exon 7 of the SMN gene and *Hae*III polymorphism in SMN gene exon 8

Polymorphism of SMN gene	Chromosomes analyzed	Genotype for	
		allele 1	allele 2
From Kuwait			
*Bsm*AI (exon 7)	118	115/118 (97)	3/118 (2.5)
*Hae*III (exon 8)	124	120/124 (97)	4/124 (3.0)
From Spain [26]			
*Bsm*AI, %		98	2
*Hae*III, %		50	50
From Taiwan [6]			
*Bsm*AI, %		0	0
*Hae*III, %		0	0

Figures in parentheses represent percentage.

reverse primer as described earlier [10]. These analyses reconfirmed the earlier findings which showed the loss of both SMNt and SMNc gene copies in this type II SMA patient. NAIP gene exons 5, 6 and 13 were intact in 20 of 21 type II SMA patients and in 1 of 21 type II patients both exons 5 and 6 of the NAIP gene were deleted. SMN gene exons 7 and 8 were deleted in both type III SMA patients, but NAIP gene deletions were detected only in one 2.5-year-old child. None of the 62 unaffected subjects had deletions in either the SMN or NAIP genes.

The incidences of two polymorphisms in SMN gene exon 7 (*Bsm*AI) and in exon 8 (*Hae*III) are presented in table 2. Allele 1 of *Bsm*AI polymorphism was detected in 115 of 118 (97%) of the chromosomes, while allele 2 was detected in 3 of 118 (2.5%). Allele 1 of *Hae*III polymorphism of SMN gene exon 8 was detected in 120 of 124 (97%) chromosomes, and allele 2 was found in 4 of 124 (3%) chromosomes (table 2).

Discussion

All our type I SMA patients showed deletions in exons 7 and 8 of the SMNt gene. NAIP exons 5 and 6 were deleted in all type I patients except for 1 in whom these two exons were intact despite a severe course and an early onset of the disease. Our data substantiate those of other groups that the majority of type I SMA cases result from large-scale deletions encompassing both the SMN and NAIP genes [12, 18, 20, 26]. Campbell et al. [5] have suggested two possibilities to explain the role of NAIP in the disease. First, it may be that as a result of its proximity, the NAIP gene is coincidently deleted along with SMNt, and that its presence or absence has no effect on the disease phenotype. Deletion of NAIP has been observed in 2% of carriers, and so can be associated with a normal phenotype [16]. Alternatively, NAIP deletion may contribute to the severity of the phenotype by generating an effect additive to that of SMNt deletion. Indeed, the deletion of NAIP is associated more often with severe SMA than with intermediate or mild forms of the disease. In 1 type I SMA patient who had an intact NAIP gene, it appears that, at least in this case, the severity of disease was unrelated to the NAIP gene deletion. However, the majority of our type I cases had deletions in exons 5 and 6 of the NAIP gene, which supports the suggestion that deletions in NAIP gene exons 5 and 6 modulate the outcome along with SMN deletions in terms of severity in SMA patients. Taylor et al. [23] have also shown that small mutations within the SMNt gene do not disrupt NAIP but may still give rise to a severe phenotype.

In type II SMA patients, SMNt exon 7 was deleted in all 21 of 21 patients, but exon 8 was intact in 2 of 21 type II patients (both of mixed race from South Africa). Previous studies have shown that in approximately 5% of SMA patients, exon 7 of SMNt was deleted but exon 8 remained intact [7, 11, 18]. It was also shown that the SMN gene in such individuals was the result of a gene conversion event, resulting in chimeric genes consisting of SMNc exon 7 linked to SMNt exon 8 [4, 5, 9, 10, 13, 24, 26]. This partial gene conversion event which does not involve the entire gene and occurs in this minority of patients has been observed in all SMA severities. However, in contrast to these previous observations, in our group, this phenomenon was noted only in type II SMA patients of South African origin and not in patients of any other nationality or severity. Although the mechanism of gene conversion is not known, it has been postulated that the presence of repeat units may facilitate alignment of nonhomologous strands, allowing sequence conversion when one repeat unit is used as a template for DNA repair following strand breakage [5]. Also, since exon 8 of the SMN gene is untranslated, a chimeric gene resulting from such a conversion event may remain functional [26]. In another type II patient, both the centromeric and telomeric copies of exon 8 were deleted indicating that in this case, the deletion was perhaps large enough to prevent the gene conversion event which could replace SMNt exon 8 with SMNc exon 8. Another interesting finding in our data was that in 1 type II SMA patient, both exons 5 and 6

of the NAIP gene were deleted along with SMN gene exons 7 and 8. Previous studies have shown that although SMN gene exons 7 and 8 are deleted in type II SMA cases, NAIP gene exon 5 remained intact [12, 20].

In the 2 Kuwaiti type III SMA patients, deletions were detected in SMN gene exons 7 and 8, but NAIP gene exons 5 and 6 were deleted in only 1 patient.

In this study, all our SMA patients had deletions in either exon 7 or 8 of their SMN gene. However, at least two previous studies have shown that type II and III mutant chromosomes occur as a result of a gene conversion event in which SMNt is converted into SMNc [5, 7, 8, 23]. In this way, patients do not possess a functional SMNt gene, even though no physical DNA deletion has occurred. Furthermore, the number of copies of SMNc has been shown to be greater in types II and III than in type I, since SMNt copies are not deleted but are converted to SMNc [5]. The higher number of SMNc copies may thus serve to ameliorate the SMA phenotype in less severe phenotypes (types II and III).

The frequency of SMN-*Bsm*AI polymorphism was found to be 97% in our population, which is similar to that (98%) reported from Spain [26]. However, in the case of *Hae*III polymorphism, our data differed from that reported from Spain and Taiwan [6, 26]. Velasco et al. [26] reported the presence of *Hae*III polymorphism (sequence ATGGCCT) in 50% of normal Spanish individuals, however in our population this polymorphism was detected in 97% of subjects. Chang et al. [6] did not detect these two polymorphisms in SMA patients or healthy subjects from Taiwan. No association was detected in our population between these two polymorphisms with SMA or disease severity.

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