

Molecular Analysis of the *SMNI* and *NAIP* Genes in Iranian Patients with Spinal Muscular Atrophy

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Abstract

Introduction: Childhood-onset proximal spinal muscular atrophies (SMAs) are an autosomal recessive, clinically heterogeneous group of neuropathies characterised by the selective degeneration of anterior horn cells. SMA has an estimated incidence of 1 in 10,000 live births. The causative genes are survival motor neuron (SMN) gene and neuronal apoptosis inhibitory protein (NAIP) gene. Deletions of the telomeric copy of SMN gene (*SMNI*) have been reported in 88.5% to 95% of SMA cases, whereas the deletion rate for NAIP gene (*NAIP*) is between 20% and 50% depending on the disease severity. The main objective of this study was to genetically characterise the childhood onset of SMA in Iran. **Materials and Methods:** Molecular analysis was performed on a total of 75 patients with a clinical diagnosis of SMA. In addition to common PCR analysis for *SMNI* exons 7 and 8, we analysed *NAIP* exons 4 and 5, along with exon 13, as a internal control, by bi-plex PCR. **Results:** The homozygous-deletion frequency rate for the telomeric copy of *SMN* exons 7 and 8 in all types of SMA was 97%. Moreover, exons 5 and 6 of *NAIP* gene were deleted in approximately 83% of all SMA types. Three deletion haplotypes were constructed by using *SMN* and *NAIP* genotypes. Haplotype A, in which both genes are deleted, was seen in approximately 83% of SMA types I and II but not type III. It was also found predominantly in phenotypically severe group with an early age of onset (i.e., less than 6-month-old). We also report 34 of our prenatal diagnosis. **Conclusions:** To our knowledge, the present study is the first one giving detailed information on *SMN* and *NAIP* deletion rates in Iranian SMA patients. Our results show that the frequency of *SMNI* homozygous deletions in Iran is in agreement with previous studies in other countries. The molecular analysis of SMA-related gene deletion/s will be a useful tool for pre- and postnatal diagnostic.

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Key words: Deletion analysis, Iran, *NAIP* gene, Prenatal diagnosis, Spinal muscular atrophy, *SMN* gene

Introduction

Proximal spinal muscular atrophy (SMA) is one of the most common autosomal recessive disorders. It is characterised by degeneration of the anterior horn cells of the spinal cord, resulting in symmetrical limb muscle atrophy and weakness. SMA has an estimated incidence of 1 in 10,000 live births.¹ Its severe form is the most common genetic disorder lethal to infants, whereas its milder form is the second most common paediatric neuromuscular disorder after Duchenne muscular dystrophy. The clinical picture of SMA is quite variable and childhood SMA has been classified into 3 types on the basis of the age of onset and clinical course.^{2,3} Type I, Werdnig-Hoffmann disease,

is the most acute and severe, with an onset before the age of 6 months and death usually occurring before the age of 2. Affected children are unable to sit without support. Type II (the intermediate, chronic form) has an onset before the age of 18 months, and death after 2 years. Affected children are unable to stand or walk without aid. Type III, Kugelberg-Welander disease, is the mild form of SMA, with an onset after 18 months of age. These patients learn to walk unaided for long periods in their lives. An adult-onset form is also known, termed type IV by Pearn et al.⁴ In the past, a clinical diagnosis of SMA was confirmed by muscle biopsy and sometimes electromyography (EMG). Typically, muscle biopsy shows degeneration of muscle fibres without

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inflammation, fibrosis or histochemical abnormality. These procedures are invasive, time consuming, and could give non-conclusive results in very young infants. All 3 clinical types of childhood SMA have been mapped by linkage analysis to 5q13⁵⁻⁸ with 2 SMA-related genes in the SMA critical region.^{9,10} This region is duplicated on the long arm of chromosome 5, resulting in 2 copies of survival motor neuron (*SMN*) and neuronal apoptosis inhibitory protein (*NAIP*) genes in the human genome. The 2 copies of *SMN* gene, a telomeric copy (*SMN2*) and a centromeric copy (*SMN1*), are nearly identical. The *SMN1* gene has been recognised to be responsible for SMA because of homozygous deletions, sequence conversions, or intragenic mutations in *SMN1* result in childhood onset of SMA.⁹⁻¹¹ The *NAIP* gene deletions, in exons 5 and 6, tend to be found in cases with more severity in phenotype, and therefore seem to affect the disease severity.¹²

The main objective of this study was to determine the frequency of *SMN* and *NAIP* deletions among Iranian patients diagnosed with SMA. The alterations in *SMN* and *NAIP* were analysed using a PCR-based exon-deletion test, and as a result, prenatal diagnosis and genetic counselling have become feasible in Iran for the first time.

Materials and Methods

Subjects

A total of 75 Iranian patients were diagnosed according to the criteria set by the international SMA consortium.¹³ There were 70 patients with type I SMA, 3 with type II and 2 with type III. Clinical examinations of the probands were indicative of SMA: symmetrical weakness more pronounced in proximal rather than distal parts; neurological disability was limited to the lower motor neurons with no evidence of sensory or intellectual impairment; muscle biopsy showed fibre group atrophy indicative of denervation and re-innervation; electromyography confirmed denervation. The patients' characteristics and individual identification numbers can be provided by the corresponding author upon request.

Consanguinity was defined by each family having a first cousin. The consanguinity rate within one generation was 97% (73/75), as shown in Table 1. Besides the SMA

patients, a total of 251 samples including 34 chorion villus sampling (CVS) of the pregnant women, 150 parents, 25 unaffected siblings and 42 unrelated control individuals were also tested.

Extraction of Genomic DNA from Blood Samples

EDTA-anticoagulated peripheral blood samples from the patients were obtained after informed consent and the genomic DNA was extracted using standard procedures.¹⁴ DNA quantification was carried out using a UV-spectrophotometer at 260-nm wavelength absorption.

Analysis of *SMN1* Exons 7 and 8 Deletions

The *SMN1* gene differs from *SMN2* by 5 nucleotide changes, 2 of which occur in exons 7 and 8. The presence of exons 7 and 8 for either *SMN1* or *SMN2* were determined using polymerase chain reaction (PCR) followed by restriction enzyme analysis. PCR products of exon 8 from *SMN1* and *SMN2* were readily distinguishable by the presence of the recognition site for *DdeI* (Roche, Germany), which is absent in *SMN1* but present in *SMN2*. For exon 7, a mismatched downstream oligonucleotide primer, directly adjacent to the variant site that contains the restriction site to create *DraI* (Roche, Germany) site in the PCR product of *SMN2* exon 7.¹⁵ PCR was carried out using 100 ng of genomic DNA as a template in a 50 µL reaction mixture containing 1.25 units of Taq DNA polymerase (Roche, Germany), 200 µL dNTPs, 10 pmoles of each upstream and downstream primer and PCR reaction buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3). The amplification for exon 7 of *SMN* gene with an initial denaturation at 95°C for 5 min was followed by 35 cycles (95°C, 55°C, and 72°C for 1 min each) and ended by an extension at 72°C for 5 min. Exon 8 DNA was amplified with the same PCR conditions except for an annealing of 59°C with Primus X6 (MWG-Biotech, Germany). Amplified products were electrophoresed on 1% agarose gel for further analysis. PCR products were digested with *DraI* for exon 7 and *DdeI* for exon 8. PCR products and their digested products were electrophoresed on 3% agarose gel.

Analysis of *NAIP* Exons 5 and 6 Deletions

Simultaneous amplification of *SMN* and *NAIP* genes

Table 1. Gene Deletion Frequencies of the SMA Patients and Correlation Between *SMN* and *NAIP* Deletions with the Consanguinity Rates

SMA type	<i>SMN</i> : Deletion of exons 7 and 8	<i>NAIP</i> : Deletion of exons 5 and 6
(t = 75, fc = 73, n = 2)	(t = 73, fc = 71, n = 2)	(t = 62, fc = 62, n = 0)
Type I (fc = 68, n = 2)	fc = 68 (97%), n = 2 (3%)	fc = 61 (87%), n = 0 (0%)
Type II (fc = 3, n = 0)	fc = 2 (67%), n = 0 (0%)	fc = 1 (33%), n = 0 (0%)
Type III (fc = 2, n = 0)	Fc = 1 (50%), n = 0 (0%)	0 (0%), n = 0 (0%)

fc: number of the patients from first cousin married parents; n: number of the patients from non-related parents; *NAIP*: neuronal apoptosis inhibitory protein; SMA: spinal muscular atrophy; *SMN*: survival motor neuron gene; t: total

has been reported.^{16,17} For detection of *NAIP* gene, exons 5 and 6 were amplified. Each reaction was a bi-plex PCR with the primers for exon 13 as a positive control for PCR amplification.¹⁵ The telomeric *NAIP* can be distinguished from its centromeric pseudogene counterpart by PCR analysis for the presence or absence of exon 5, which exists only within the functional telomeric gene. The PCR conditions were the same as those used for *SMN* except for the annealing temperature (58°C) and cycle number (30).¹⁰

Prenatal Diagnosis

In this study, CVS of 34 pregnant women were obtained by either transcervical or transabdominal placental biopsy at 10 to 12 weeks of gestation. The CVS was carefully dissected from maternal deciduas under an inverted microscope and DNA was extracted by a standard procedure. The genomic DNA from each fetus was collected by CVS.^{18,19} DNA samples from affected children are stored in our lab that also provides the positive control for comparison with the respective fetus. For accurate diagnosis, 1 positive DNA (affected sibling of the fetus) and 1 negative control DNA (unrelated healthy individual) were amplified along with the fetal DNA. Polymorphism of the hypervariable 3' region of the apolipoprotein B (apoB) gene was examined in all cases to rule out the possibility of maternal DNA contamination.²⁰

Results

SMA Types I-III

The homozygous-deletion frequency rate of telomeric copy of exons 7 and 8 in *SMN* gene with all types of SMA was 97%. Moreover, *NAIP* exons 5 and 6 were deleted in approximately 87% of type I SMA and 33% of type II but not in type III. Haplotype A, which has the deletions of both genes involved, was observed in approximately 83% of type I and II SMA but not in type III. This haplotype was also found predominantly within the patients with severe phenotypes and an onset less than 6 month of age. The SMA population we collected was analysed for the absence

of either *SMN1* exon 7 (Fig. 1) or exon 8 (not shown) and *NAIP* exons (Fig. 2) in 75 SMA patients. According to clinical criteria, 70 type I (Werdnig-Hoffmann disease) patients, 3 type II (intermediate chronic form) patients, and 2 type III patients (Kugelberg-Welander disease) were characterised. We found an overall deletion frequency rate of 97% for *SMN* exons 7 and 8. Homozygous deletions of both exons 7 and 8 in *SMN1* were seen in 100% of type I SMA, 66% of type II, and 50% of type III patients. None of the patients had a homozygous deletion of exon 7 without a deletion of exon 8 for *SMN1* gene. We also found an overall mutation frequency rate of 83% for the *NAIP* gene in exons 5 and 6, of which 87% were for type I and 33% for type II, but we found no mutation for type III. No homozygous deletions were seen in the parents. Table 1 shows a summary of the homozygous deletions of *SMN* and *NAIP* for the 75 SMA patients.

Haplotype A, B, and C

By using the genotypes of *SMN* and *NAIP*, 3 deletion haplotypes were classified. Table 2 shows the percentages of the 3 haplotypes in our study group. Haplotype A was seen in 87% of type I and 33% of type II patients, but it was not present in type III. Haplotype B is the most common haplotype that is observed in 100% of type I individuals. It is also observed in 66% of type II and 50% of type III patients. Finally, haplotype C appears to be the least common among the 3 haplotypes. It is seen in 33% of type II and 50% of type III, but it is not seen among type I SMA patients.

Prenatal Diagnosis

The CVSs that we used for prenatal diagnosis were analysed for the absence of *SMN1* exons 7 and 8, as well as *NAIP* exons 5 and 6 in 34 SMA type I pregnant women with affected children. All pregnant women have at least 1 affected child, and 5 women possess 2 affected children. All affected children of type I were diagnosed by muscle biopsy, EMG, and molecular analysis. Homozygous deletions of *SMN1* exons 7 and 8 were detected in 21% of

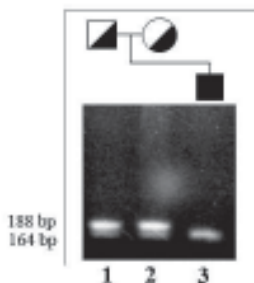


Fig. 1. PCR-RFLP of *SMN* gene exon 7 using *Dra I*. Lane 1: father; lane 2: mother; and lane 3: affected baby. *Dra I* digested the *SMN* pseudogenes (*SMN2*, 164 bp) amplified by PCR, but not the functional *SMN* gene (*SMN1*, 188 bp). No band for functional *SMN* gene exon 7 was seen in the affected baby. Carriers of SMA should have 188 bp (*SMN1*) and 164 bp (*SMN2*) bands after complete digestion of PCR products by *Dra I*.

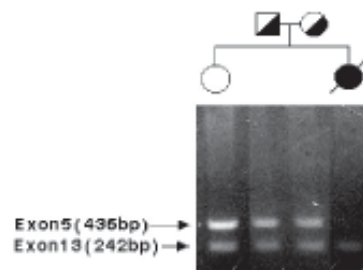


Fig. 2. Biplex-PCR amplification of *NAIP* gene exons 5 and 13 (exon 13 was used as a control). Lane 1: healthy patient's sister; lane 2: patient's father; lane 3: patient's mother; and lane 4: the affected child (she died after this study). The affected child had a deletion in both exons 5 and 6, as well as exon 6 (not shown).

Table 2. SMA Patients Haplotypes

	Haplotypes (%)		
	A	B	C
SMA type I (n = 70)	87	100	0
SMA type II (n = 3)	33	66	33
SMA type III (n = 2)	0	50	50

SMA: spinal muscular atrophy

Haplotype A has deletions in *SMN* exons 7 and 8, as well as *NAIP* exons 5 and 6. Haplotype B has deletions in *SMN* exons 7 and 8. Haplotype C has no abnormalities in the examined regions

the CVS. We were unable to distinguish between samples with heterozygous deletions (i.e., carriers) and normal fetuses because the gene dosage test, a standard practice in such cases, has not been set up in our laboratory yet. None of the CVS had a homozygous deletion for *SMN1* exon 7 without an exon 8 deletion. We also found an overall deletion frequency rate of 71% for *NAIP* exons 4 and 5.

Discussion

Although the role of *SMN1* and *SMN2* genes in the pathogenesis of SMA is still uncertain, the deletion of *SMN1* is the most important clue for diagnosing SMA. Transcripts of the two 20-kb genes differ only at 2 nucleotides in the terminal exons 7 and 8, but these differences do not change the sequence of the coded protein. The majority of the SMA patients are characterised by a homozygous deletions of exons 7 and 8 for *SMN1*.^{9,17-19,21} Deletion of the *SMN* in the absence of *NAIP* is sufficient to give the SMA phenotype. Patients with additional deletions are more likely to belong to a more severe phenotype. In the present study, Iranian SMA patients were analysed for alterations in the *SMN1* and *NAIP* genes. Our results show homozygous deletion of *SMN1* gene exons 7 and 8 in 97% of SMA patients, which is in agreement with the majority of previous studies.^{9,17,22-24} The exception is a report by Nguyen et al²⁵ on the Vietnamese SMA patients claiming a 41% to 50% deletion frequency for *SMN1*. However, their difference in the *SMN1* deletion frequency rates with ours study appears to be a result of the small sample size used by Nguyen and colleagues. The frequency of homozygous *SMN1* deletion was found in 100% of type I patients, which is similar to some reports on adult-onset SMA.²⁶ The frequency of homozygous *SMN1* deletion in types II and III patients were 66% and 50% respectively. All *NAIP*-deleted patients also lacked the *SMN* gene. We observed a strong correlation between telomeric *NAIP* deletion and the severity of SMA. The overall frequency of *NAIP* exons 5 and 6 in our study was 83%. Deletions of the *NAIP* gene were seen more frequently in type I patients (87%) compared with type II (33%) and type III (0%), which is in accordance with

previously reported observations (types II and III).²⁷ The type I patients who had a *NAIP* deletion died between 1 and 4 months of age, but the death of the type I patients who had retained the telomeric *NAIP* was delayed. One of the 2 type III patients did not show a deletion for either *SMN1* or *NAIP*. Haplotype A that could be formed with *SMN* and *NAIP* deletions was seen in 87% of the type I patients. Interestingly, all type I patients with haplotype A had a very early onset of symptoms (i.e., at less than 1 month of age). Our results provide an additional dimension for the phenotype-haplotype correlation in type I SMA.

Prenatal diagnosis was performed by detection of *SMN1* and *NAIP* deletions in the fetus of 34 pregnant women with affected children (SMA I) using PCR/RFLP based on the presence or absence of particular bands. Approximately 21% of the prenatal DNA samples had homozygous deletions of exons 5 and 6 in the *SMN1* gene. Couples with affected fetuses chose to terminate the pregnancy. In other families, prenatal diagnosis showed no homozygous deletion of *SMN1* exons 7 or 8. All of the pregnancies with normal prenatal diagnosis ended up with healthy babies. The results of prenatal diagnosis are in agreement with the molecular diagnosis of the affected children. The proportion of the fetus predicted with SMA is lower than the expected (21% vs. 25%) for a recessive disorder. The results of this part of study, however, cannot be used for analysing of the transmission rate for the *SMN1*-deleted allele because of the statistically low number of studied pregnant patients.

Chorionic cells have many advantages over amniotic cells. Firstly, diagnosis can be performed as early as 10 to 11 weeks of gestation. Secondly, large amounts of cells and DNA can be obtained to carry out PCR and DNA analysis. The technique of CVS has the disadvantage of contamination with maternal cells, which may lead to false-positive prenatal diagnosis. At this point, prenatal diagnosis using chorionic villi should be performed with great care. Contamination by the maternal blood DNA was ruled out by examination of apoB marker polymorphism.²⁰

In this study, the rate of consanguineous marriages by the SMA family was about 97%, which is very high compared with other diseases. Consanguine marriages are very common among Iranians from the rural areas. In some regions, its prevalence exceeds 50% of all marriages because of social, cultural, and religious background.²⁸ In a recent study on another autosomal recessive disorder, children with β -thalassaemia, which is relatively common within the Iranian population (with an incidence of 5% to 10%), have been reported in 74% of unrelated marriages. This indicates a relatively high occurrence of affected β -globin gene compared with *SMN* deletion frequency in the population.²⁹ In the present study, we detected affected children only in 3% of unrelated marriages (Table 1). This

suggests a low frequency of affected *SMN* allele among the non-consanguineous marriages in the Iranian population. However, there is no data regarding the real incidence of *SMN* deletion in Iran. Thus, the final conclusion awaits further studies. The gene-dosage test would be useful in such cases, as well as examining the fetus for detecting the carriers of *SMN1* deletion. The carriers of *SMN1* deletion seem to have half the number of *SMN* gene compared to a normal individual. Furthermore, a dosage test, distinguishes between a non-SMA phenocopy patient (who has normal gene dosage) and a compound heterozygote SMA patient with absent *SMN1* on one chromosome and an unknown alteration in *SMN1* on the other chromosome.³⁰

The available molecular genetic tests seem to be extremely useful for SMA diagnosis in Iran. In agreement with the general consensus, our *SMN1* results show a strong correlation between genotype and phenotypic expression of the disease.

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