Original Article

MOLECULAR DIAGNOSIS IN IRANIAN PATIENTS WITH SPINAL MUSCULAR ATROPHY

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BACKGROUND—*Spinal muscular atrophies (SMA) are a group of heterogeneous disorders characterized by the degeneration of the lower motor neurons of the ventral horns of the spinal cord. They are inherited by an autosomal recessive pattern, and because of the high rate of consanguinity in the Iranian population the incidence of these diseases is very high in this region. The precise frequency has not been determined. We set up molecular tests for the diagnosis of affected patients, carrier detection, and prenatal diagnosis for high risk pregnancies.*

MATERIALS AND METHODS—*We investigated the presence or absence of the survival motor neuron (SMN) gene in 47 Iranian families, including 60 patients by polymerase chain reaction amplification of exons 7 and 8 in affected individuals and parents of patients for carrier detection. In addition, prenatal testing was performed on 15 pregnant mothers.*

RESULTS—*Mutation detection in the 22 live patients showed that in the 21 cases, both alleles were deleted. In 1 case only one of the mutations was detected, therefore the other must have been a point mutation. In 34 families, both of the parents were carriers, that is, they carried only one copy of the normal SMN gene. In 9 of the couples only one mutation was detected, therefore in the other one, it should have been a point mutation that was not diagnosed. Molecular testing of 15 fetuses by prenatal diagnostic procedures showed that 4 of the fetuses were normal, 3 fetuses were affected and carried both of the mutations, five fetuses were carriers (they carried one of the mutations), and the other 2 were carriers or healthy, but only one case might have been carrier or affected.*

CONCLUSION—*SMA is a very common disease in the Iranian population, due to the high frequency of consanguineous marriages. Preventive measures by genetic counseling, carrier detection, and prenatal diagnosis are helpful in the prevention of recurrences in the future pregnancies.*

Keywords: ***SMA; Werdnig-Hofmann disease; molecular analysis; prenatal diagnosis; genetic counseling; carrier detection.***

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INTRODUCTION

The childhood spinal muscular atrophies (SMA) are a group of heterogeneous disorders characterized by degeneration of anterior horn cells of the spinal cord, resulting in progressive symmetric limb and trunk paralysis. Most types are inherited by autosomal recessive pattern, but autosomal dominant and X-linked recessive types have also been reported. According to the age at onset and severity of the disease, patients with spinal muscular atrophies can be subdivided into types I, II, and III. The childhood onset autosomal recessive form affects between one in 6,000 and one in 10,000 live births and is the second most common lethal disorder in caucasians after cystic fibrosis. Type I (Werdnig-Hoffmann disease) is the most severe, with onset in utero, reduced fetal movements, hypotonia, wasting, fasciculation, areflexia, weak cry, and paradoxical breathing. Affected children have normal intelligence but are unable to sit, have feeding difficulties, and die from respiratory failure or aspiration pneumonia before the age 2 (Figure 1).[1] Type II is of intermediate severity. The onset is usually after 3 or 6 months of age. Patients have hypotonia and delayed motor milestones. They are able to sit, but can not stand or walk unaided. Survival depends on the degree of respiratory muscle involvement and usually expands till age 4.

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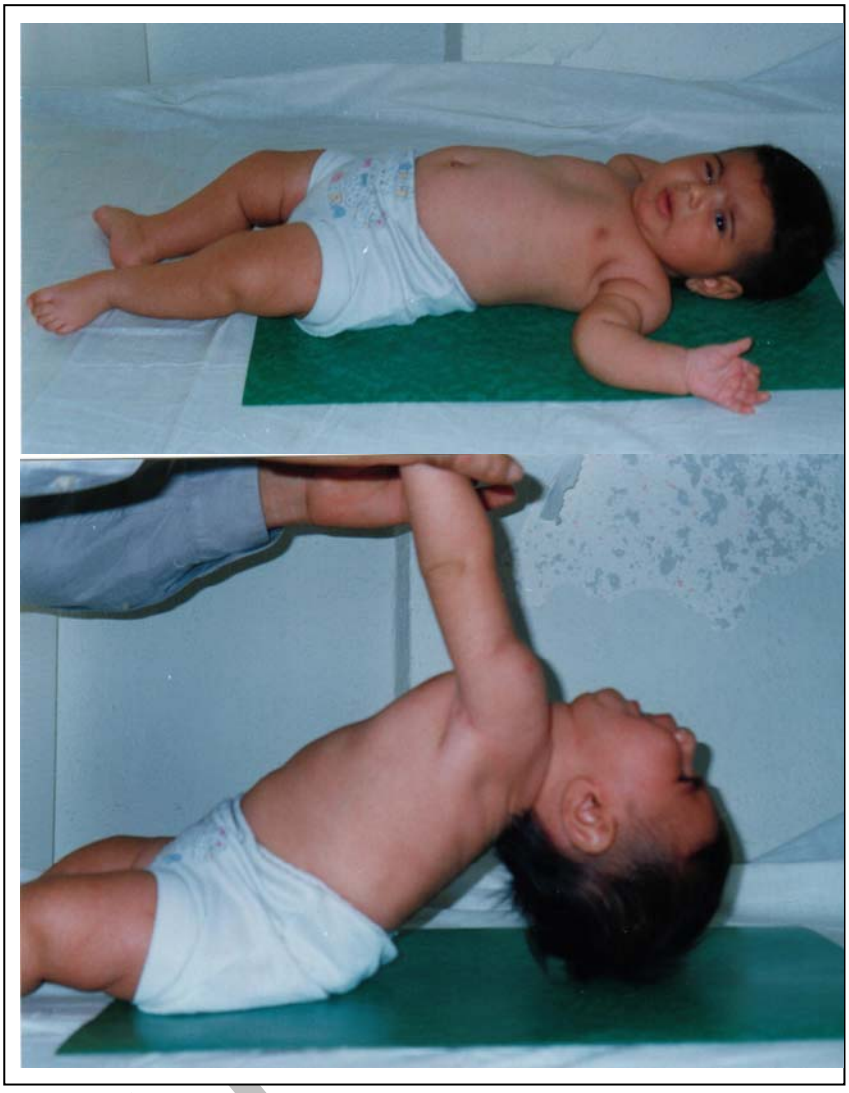

Figure 1. Infant with type I SMA. Note the mental brightness, hypotonia, and froglike position.

Type III (Kugelberg-Welander disease) has an onset after the age two and patients are able to walk unaided, but fall frequently. Slow deterioration results in scoliosis and wheel-chair dependence.[1]

Using linkage analysis, a locus was mapped to chromosome number 5q13, which was shown to be associated with spinal muscular atrophies. Recent studies[2, 3] have shown that deletions in a small sub-region of band 5q13 are associated with the clinical presentation of the disease. These findings have led to the identification of two candidate genes,[3, 4] the survival motor neuron (SMN) gene and neuronal apoptosis inhibitory protein (NAIP) gene (Figure 2). Polymerase chain reaction (PCR) based DNA methods have been developed to detect deletions in both SMN and NAIP genes. There are two SMN and NAIP genes in the locus, designated as telomeric (SMNt) and centromeric (SMNc) copy genes. These genes are almost identical except in 5 different sites including sequences within exon 7 and 8, the difference in sequence are used to distinguish telomeric copy from the centromeric one (Figure 2).

Because of higher rate of consanguinity in the Iranian population, the frequency of SMA is high among Iranians, although precise epidemiologic information is not available. In 1999, a molecular diagnostic service was established to investigate SMA patients in Iran. At present we are able to detect mutations in homozygote affected individuals, heterozygote carriers, and prenatal diagnosis for high risk pregnancies. This study investigates the deletions of the SMN genes of 60 individuals in 47 families.

MATERIALS AND METHODS

Sixty individuals from 47 families were included in the study. Families were selected according to the

international SMA consortium criteria.

The diagnoses were confirmed by clinical symptoms, electromyographic evaluation (EMG), and muscle biopsy. Analysis of the cerebrospinal fluid, serum enzymes, and nerve conduction velocity were normal. EDTA-anticoagulated peripheral blood samples were obtained from the subjects. Leukocytes were isolated using 5% dextran solution. Salting out method was used for genomic DNA extraction. The deletion of the exons 7 and 8 of the SMN gene from patients, their parents, and amniocytes were examined, using PCR amplification and restriction enzyme digestion. Oligonucleotide primers that were used for PCR amplification are shown in Table 1.

Exon 7 was amplified using primers RIII and SMA (Table 1) under the following conditions: initial denaturation 94°C for 2 min; annealing temperature of 55°C for 1 minute; then extension temperature of 72°C for 1 minute. The procedure was continued for 35 cycles. The PCR products were then digested with 20 units of DraI (New England Biolabs, Beverly, MA, USA) enzyme overnight. The digested material was then electrophoresed on polyacrylamide gel 8%, followed by silver staining (Table 2).

Exon 8 was amplified using primers 541c960 and 541c1120 (Table 1). The PCR conditions were 30 cycles with 94°C denaturation for 1 minute; 60°C annealing for 1 minute; and 72°C extension for 1 minute. The PCR product of exon 8 was digested with 20 units of DdeI (Boehringer Mannheim, Germany) enzyme overnight. These digested products were also visualized by electrophoresis on polyacrylamide gel 8%, followed by silver staining (Table 2).

The rate for consanguineous marriage in this study was approximately 66%. Common types of consanguinity are shown in Table 3.

RESULTS

The most common type in our patients was type I. Thirty families with 41 affected cases belonged to this group, followed by type II with 11 families. The frequency of different types is summarized in Table 4.

In 22 affected patients, mutations (deletion of exon 7and 8) were analysed. In 21 cases, mutations were detected in both alleles. In 1 case, deletion was detected only in one of the alleles, but the other allele would have been a point mutation (and should be studied by sequencing in the next step).

In 34 families, both of the parents were carriers of the SMN gene mutation. In 9 couples, mutation in only one of the alleles was detected. In Figures 3A and 3B, the product of PCR amplification, restriction enzyme digestion, and electrophoresis on polyacrylamide gel, 8% (with silver staining of the 2 affected families) are depicted (Figures 3A and 3B).

Prenatal diagnoses were carried out in 15 pregnancies by amniocentesis. Four fetuses were normal. They carried none of the mutations. Three fetuses were affected and carried both of the mutations. Five fetuses were carriers. They carried one of the mutations. Two fetuses were healthy or carriers, and only 1 fetus was a carrier or affected.

Table 1. Oligonucleotide primers for PCR analysis of SMN gene (exon 7 and 8).

Amplified exon	Direction	Primers sequence	PCR product (bp)
Exon 7	Upstream Downstream	5'-CTA TCA ACT TAA TTT CTG ATC A-3' 5'-CCT TCC TTC TTT TTG ATT TTG TT*T-3'	188
Exon 8	Upstream Downstream	5'-GTA ATA ACC AAA TGC AAT GTG AA-3' 5'-CTA CAA CAC CCT TCT CAC AG -3'	188

Table 2. Conditions for PCR amplification, DNA digestion, electrophoresis, and staining.

Denaturation Temp	Annealing Temp	Extension Temp	No. of cycles	Digesting enzyme	Electrophoresis staining
94°C/2 min Exon 7	55°C /1 min	72°C /1 min	35	DraI/20 unit	Polyacrylamide 8% silver staining
94°C /1 min Exon 8	60°C /1 min	72°C /1 min	35	DdeI/20 unit	Polyacrylamide 8% silver staining

Temp = temperature

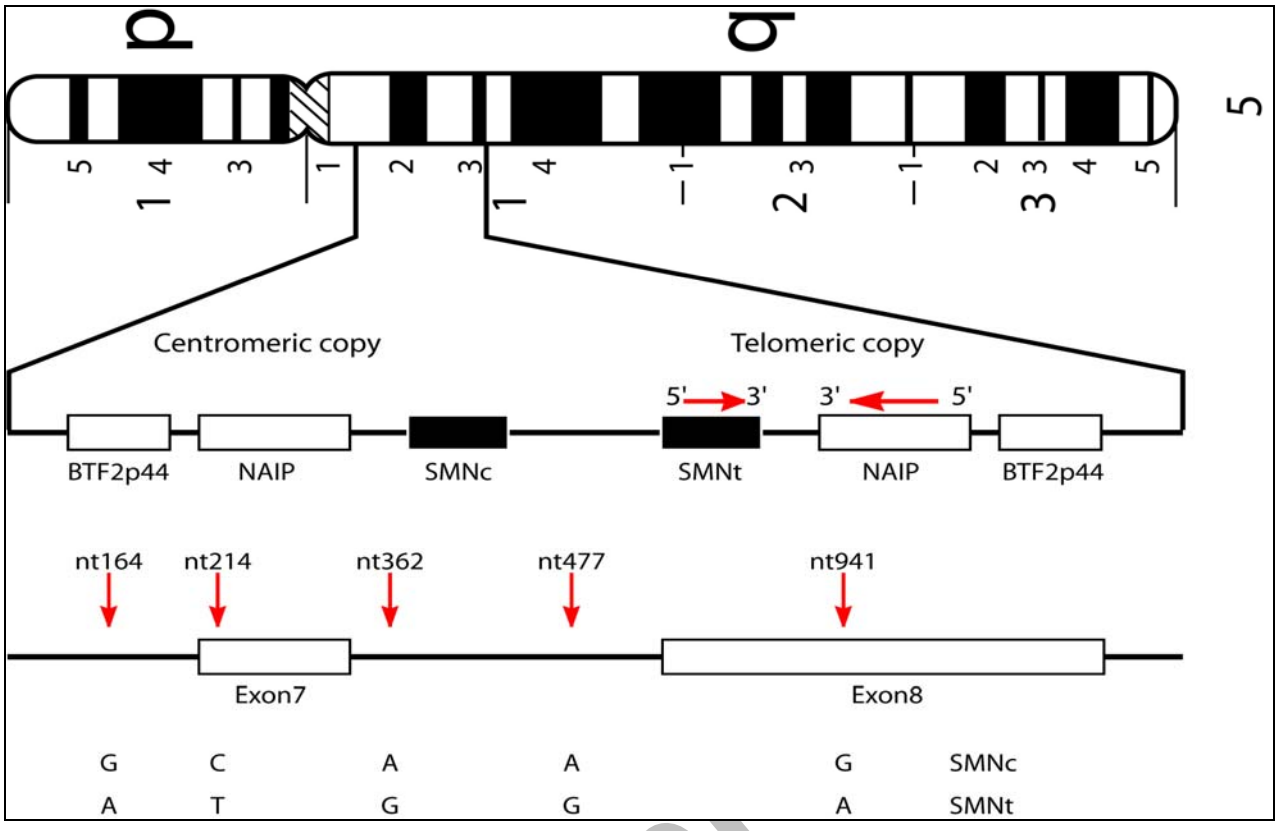

Figure 2. Gene locus of genes responsible for SMAs. Note to the structural differences of SMNt and SMNc.

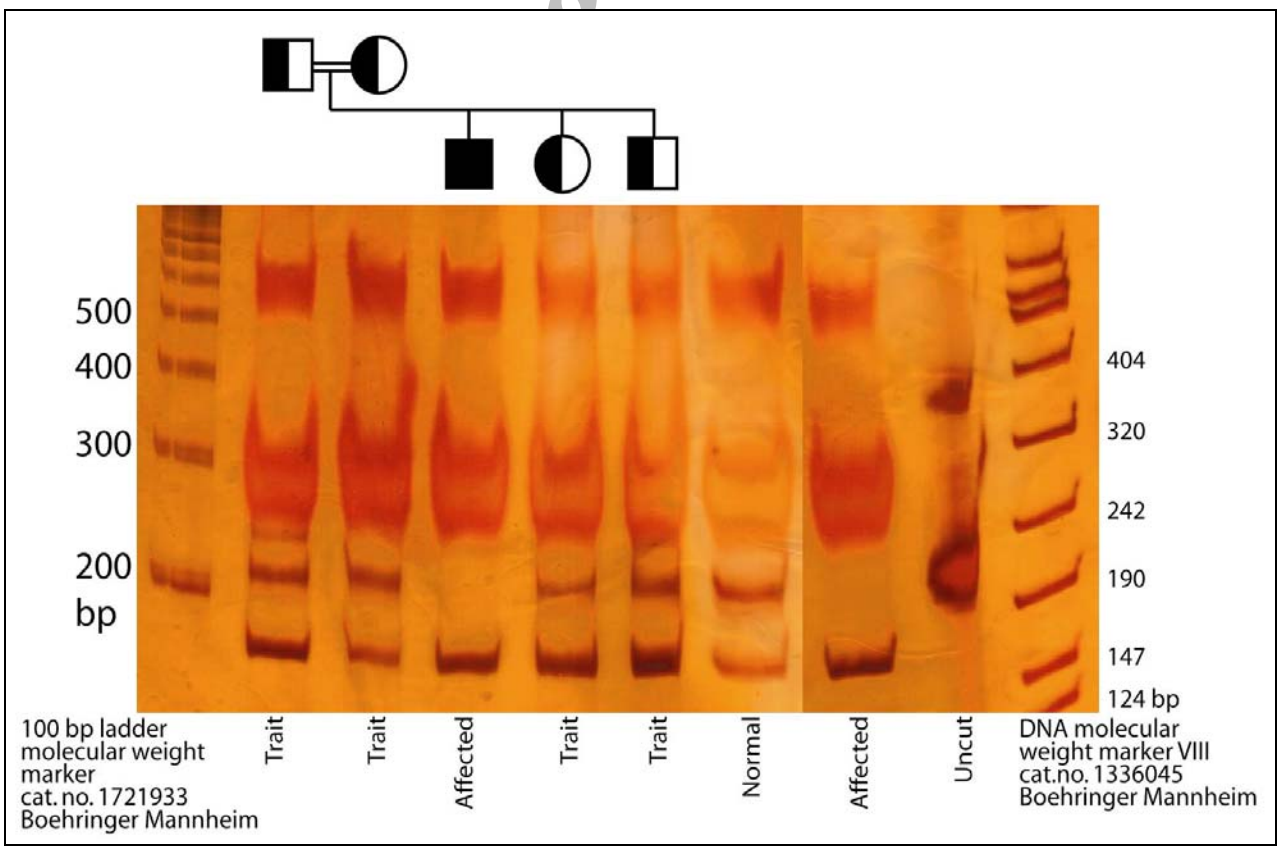

Figure 3A. Note the deletion of the exon 7 of SMNt in the affected families. Note that the band in the 190 bp region is deleted in the affected boy of the family in comparison to the affected control in column 8. In the carriers the same band's density is half the normal.

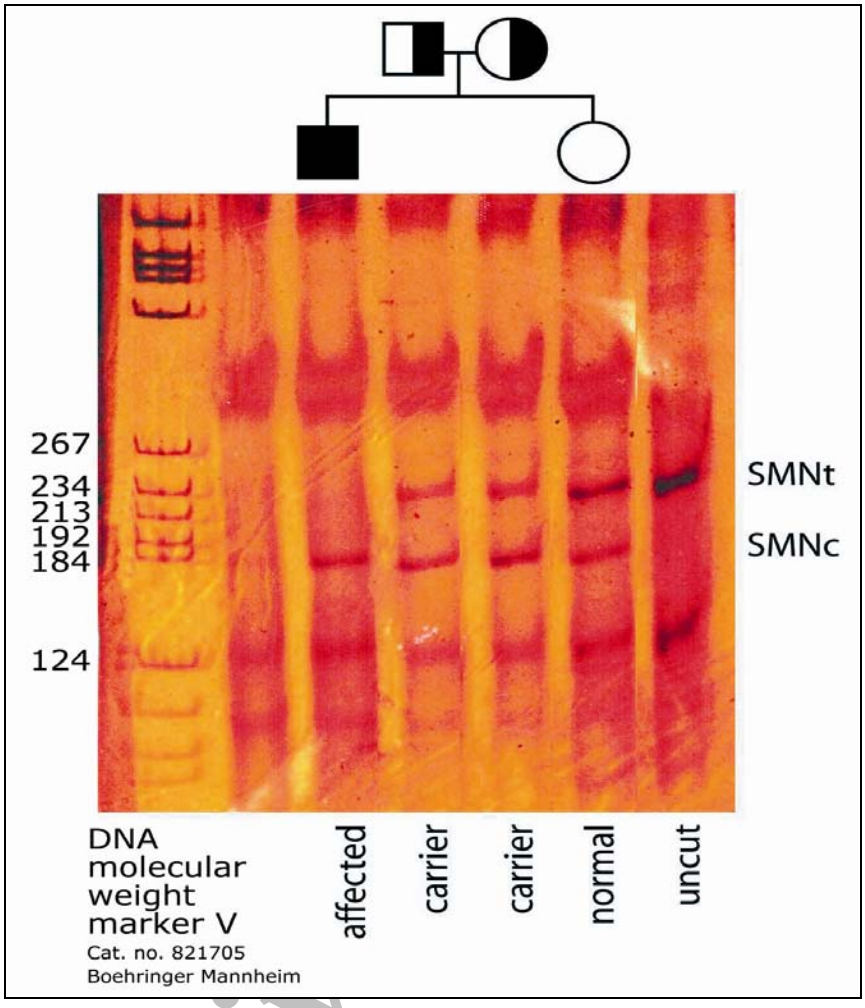

Figure 3B. Note the deletion of xon 8 in the affected individual and heterozygote parents in the family. Comparing the normal in the affected individual, the band in the region of 234 bp is deleted. In the parents the density of this band is half the normal.

Table 3. Frequency of the types of consanguinity in the Iranian families with SMA disease.

Degree of relationship	No. of families
3rd degree	22 families
4th degree	6 families
3rd degree once removed	1 family
distant relatives	2 families
not relatives	16 families

Table 4. Frequency of SMA types in this study.

SMA type	No. of families	No. of cases
Type I	30	41
Type II	11	13
Type III	1	1
Type I or II	5	5

DISCUSSION

Iran is a large country with many different ethnic groups. The consanguinity rate is very high and in some regions is more than 50%. So, genetic disorders are prevalent in this society, and for this reason, the incidence of homozygosity for SMA alleles is also high. At present the precise prevalence rate of SMA in the Iranian population is not known, however these disorders are one of the primary causes of morbidity and mortality in the infancy and childhood in Iran.

Van der Steege et al[5] have reported homozygous deletions of SMNt gene in 98.6% (226 of 229) of Dutch patients with SMA. Cobben et al[6] have found the homozygous deletion of the SMN gene in 93% of their patients. In our study, we detected homozygous deletion of the SMN gene in 95.4% (21 of 22) of the affected patients.

Presently, the molecular diagnosis for SMA is a part of routine molecular diagnostic testing in Iran. The accuracy rate of the test is near 100%. [7 – 9] Now we can diagnose the suspected patients accurately. Carrier detection of high risk parents or prenatal diagnosis of affected fetuses is a simple and reliable test in our hands. Because there is no effective treatment at the present for these diseases and the recurrence rate is 25% for high risk families, the only option for prevention is genetic counseling followed by prenatal diagnosis in each pregnancy.

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