Genetics and Molecular Biology, 22, 4, 487-492 (1999)

CLINICAL AND MOLECULAR ANALYSIS OF SPINAL MUSCULAR ATROPHY IN BRAZILIAN PATIENTS

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ABSTRACT

Spinal muscular atrophy (SMA), the second most common lethal autosomal recessive disorder, has an incidence of 1:10,000 newborns. SMA is divided into acute (Werdnig-Hoffmann disease, type I), intermediate (type II) and juvenile forms (Kugelberg-Welander disease, type III). The gene of all three forms of SMA maps to chromosome 5q 11.2-13.3. Two candidate genes, the survival motor neuron (SMN) gene and the neuronal apoptosis inhibitory protein (NAIP) gene, have been identified; SMN is deleted in most SMA patients. We studied both genes in 87 Brazilian SMA patients (20 type I, 14 type II and 53 type III) from 74 unrelated families, by using PCR and single strand conformation polymorphism (SSCP). Deletions of exons 7 and/or 8 of the SMN gene were found in 69% of the families: 16/20 in type I, 9/12 in type II and 26/42 in type III. Among 51 families with deletions, 44 had both exons deleted while seven had deletions only of exon 7. Deletions of exon 5 of the NAIP gene were found in 7/20 of type I, 2/12 of type II and 1/42 of type III patients. No deletion of SMN and NAIP genes was found in 112 parents, 26 unaffected sibs and 104 normal controls. No correlation between deletions of one or both genes and phenotype severity was found.

INTRODUCTION

Spinal muscular atrophies (SMA) include a group of motor neuron disorders characterized by degeneration of spinal cord anterior horn cells leading to muscular wasting and atrophy. Childhood-onset SMA is the most common autosomal recessive genetic disorder after cystic fibrosis, with an estimated incidence of 1:10,000 newborns and a carrier frequency of about 1/40-1/60 individuals (Pearn, 1980). Affected patients are classified into three groups according to age at onset, developmental milestones and phenotype severity (International SMA Consortium, 1992).

Type I SMA or Werdnig-Hoffmann disease is the most severe form and starts at birth or by six months of age. Patients are never able to sit unaided, and respiratory involvement is frequently responsible for their death, which usually occurs before two years of age.

In type II SMA, the intermediate form, affected children are able to sit unassisted and may be able to walk for a short distance. Usually they survive beyond age 10, depending on the degree of respiratory muscle weakness.

Type III SMA or Kugelberg-Welander is the mildest form. Its onset may occur in the first, second or even third decade. Its course is highly variable, but patients are always able to walk unassisted.

The gene for all three forms of SMA was mapped to chomosome 5q 11.2- 13.3 by linkage analysis (Brzustowicz *et al*., 1990; Melki *et al*., 1990; Gilliam *et al*., 1990). In 1995, two candidate genes were mapped in the SMA region (Lefebvre *et al*., 1995; Roy *et al*., 1995): the survival motor neuron (SMN) gene (present in two copies - SMN which is telomeric and cBCD54 which is centromeric) and the neuronal apoptosis inhibitory protein gene (NAIP). Both SMN copies transcribe 1.7-kb mRNAs which code identical amino acid sequences and span ~20 kb at the genomic level. Single base exchanges in exons 7 and 8 allow one to distinguish centromeric (cenSMN) from telomeric (telSMN) SMN copies through single strand conformation polymorphism (SSCP) analysis. Molecular studies have shown that a vast majority of SMA patients have homozygous deletions of exons 7 and 8 on the SMN telomeric copy (Lefebvre *et al*., 1995), independent of the severity of the disease.

On the other hand, the reported frequency of deletions in the NAIP gene, which has 16 exons spanning 60 kb of genomic DNA, varies in different populations from 67.9 to 0% (Chang *et al*., 1995; Rodrigues *et al*., 1996; Velasco *et al*., 1996) and is apparently higher in type I patients than types II and III (Cobben *et al*., 1995; Hahnen *et al*., 1995; Roy *et al*., 1995; Velasco *et al*., 1996).

No correlation between phenotype and deletions in the SMN gene has been reported (Aubry *et al*., 1995; Bussaglia *et al*., 1995; Chang *et al*., 1995; Kant *et al*., 1995). However, analysis of deletions in both NAIP and SMN genes in a large number of SMA patients suggests that more extensive deletions may be associated with greater disease severity (Rodrigues *et al*., 1995; Roy *et al*., 1995; Burlet *et al*., 1996).

Here we present clinical findings and deletion frequencies of SMN and NAIP genes in 87 Brazilian SMA patients using PCR and SSCP techniques.

SUBJECTS AND METHODS

Eighty-seven patients (46 males and 41 females) from 74 unrelated families, with a characteristic SMA clini-

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cal picture, were screened for deletions in SMN and NAIP genes. With the exception of two families (one African-Brazilian and the other of Caucasoid-oriental descent), all patients were Caucasoid.

Sixty-three patients (72%) were sporadic cases, and 24 patients (28%) were familial cases with two or three affected sibs. Parental consanguinity was observed in 20% of the 74 families studied. All patients were submitted to neurological and clinical examinations and detailed information including age at onset, motor development as well as respiratory complications were recorded.

Affected patients were classified into three subgroups according to the criteria of the International SMA Consortium (1992) as follows: 20 were grouped as type I, 14 as type II and 53 as type III SMA. Complementary exams included serum creatine kinase (CK) analysis and electromyography. Some patients with an uncertain diagnosis were also submitted to muscle biopsy. In addition to the 87 affected patients, 112 parents, 26 unaffected sibs and 104 control individuals were tested for SMN and NAIP genes.

DNA analysis

Blood was drawn from affected patients and relatives after informed consent. For DNA analysis, DNA was extracted from whole blood according to the method of Miller *et al.* (1988).

SMN gene analysis

SMN exons 7 and 8 were studied by SSCP analysis after PCR amplification of genomic DNA using specific oligonucleotide primers described by Lefebvre *et al*. (1995).

Amplification conditions, which were repeated for 30 cycles, were as follows: denaturation at 94°C, annealing at 58°C for 30 s and extension at 72°C for 2 min.

PCR products were mixed with an equal volume of formamide (95% formamide, 20 mmol/l EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured for 5 min at 95°C, loaded onto a gel (Phast Gel Homogeneous 20) and electrophoresed at 15°C, during 350 AVH for exon 7 and 300 AVH for exon 8 in a PhastSystem apparatus.

NAIP gene analysis

NAIP gene analysis was performed by PCR amplification of exons 5 and 13 (which was used as a positive PCR control for exon 5) using specific oligonucleotide primers as described by Roy *et al*. (1995). PCR products were visualized by ethidium bromide stained on 2% agarose gels and scored for the presence or absence of exon 5 using exon 13 as a positive control.

RESULTS

Clinical and genealogical findings

In the SMA type I group, the age at onset varied from birth to 6 months, with an average of two months. The main symptom was severe hypotonia with poor limb mobility. None of the children were able to sit. Only two patients could hold up their heads, but only for a short period of time.

Eight patients (40%) died between 17 days and 8 months of age due to respiratory complications. The 12 patients who were still alive were all maintained by permanent mechanical ventilation, which was started from birth to 15 months. The oldest patient, who is currently 11 years old, has been under mechanical ventilation since 5 months of age.

In the SMA type II group, patient age of the 14 cases varied from 1 to 16 years (average 4.4 years). Only one patient died of pneumonia at age 6. In the SMA type III group, the clinical course varied greatly: 18 patients (43%) were confined to a wheelchair at ages ranging from 2.5 to 16 years; the remaining patients, aged from 2 to 57 years (mean 20.6 years), were still able to walk, but half of then needed support.

Fasciculation of the tongue was observed in 16/20 of SMA I patients, 6/14 of SMA II patients and 7/53 of SMA III patients. Moderate or severe ciphoscoliosis was observed in 50% of the SMA II and SMA III patients at 4 to 57 years old.

Except in four SMA I cases, no abnormal fetal movements were reported by the probands' mothers during gestation of the affected fetus. One-third of the patients were born from the mother's first pregnancy. Parental age varied from 15 to 38 years (mean of 26.6 years) for mothers and from 18 to 49 years (mean of 29.0 years) for fathers, which did not differ from the general population for both sexes.

In all 13 familial cases, affected sibs belonged to the same generation. There were no cases of affected patients in two generations. Thus, autosomal dominant transmission was excluded. None of the SMA I cases were familial.

There was concordance in SMA type classification among sibs in 11 families. The affected sibs were classified into the same category: three SMA type II and eight SMA type III. The age at onset was similar among affected sibs, varying by one or two years. In one family, although both patients were classified as SMA type III, the difference in onset was 10 years. Motor ability was similar among all affected sibs, including the age of wheelchair confinement or capacity to walk with or without help. In two families, one of the sibs was affected by SMA type II and the other by SMA type III.

Molecular analysis

Deletions of exon 7, exon 8 or both in the SMN gene were observed in 69% of the families with the fol-

lowing distribution: 16/20 of SMA type I, 9/12 of SMA II and 26/42 of SMA III. Among the 51 families with deletions, 44 had both exons deleted while seven had deletions only of exon 7: one SMA I and six SMA III. None of the patients had deletions of only exon 8 (Table I).

Deletions of exon 5 of the NAIP gene were found in 7/20 of SMA type I, two families of SMA type II and only one family of SMA type III. All of them also had deletions of exon 7 and 8 in the SMN gene. No patient had deletion in NAIP without a deletion in SMN.

All affected sibs in 13 familial cases were concordant for molecular findings, including two families in which two brothers had different clinical courses. In one of these families, the older brother was still ambulant at age 19 while the youngest, aged 16, was never able to walk or stand unsupported despite early and constant physiotherapy (Figure 1). No SMN or NAIP gene deletions were found in the 112 parents, 26 unaffected SMA patient sibs and 104 control individuals.

Table I - Analysis of SMN gene in patients.

	Deletion of exon 7	Deletion of exon 8	Deletion of exons 7 and 8	Total of deletion
SMA I	1/20	-	15/20	16/20
SMA II	-	-	9/12	9/12
SMA III	6/42	-	20/42	26/42

DISCUSSION

Clinical classification of SMA is helpful in providing medical care and prognostic relevance to families, but it is based on subjective and arbitrary parameters which may still be controversial and subject to errors.

Hausmanowa-Petrusewicz *et al.* (1985) and Russman

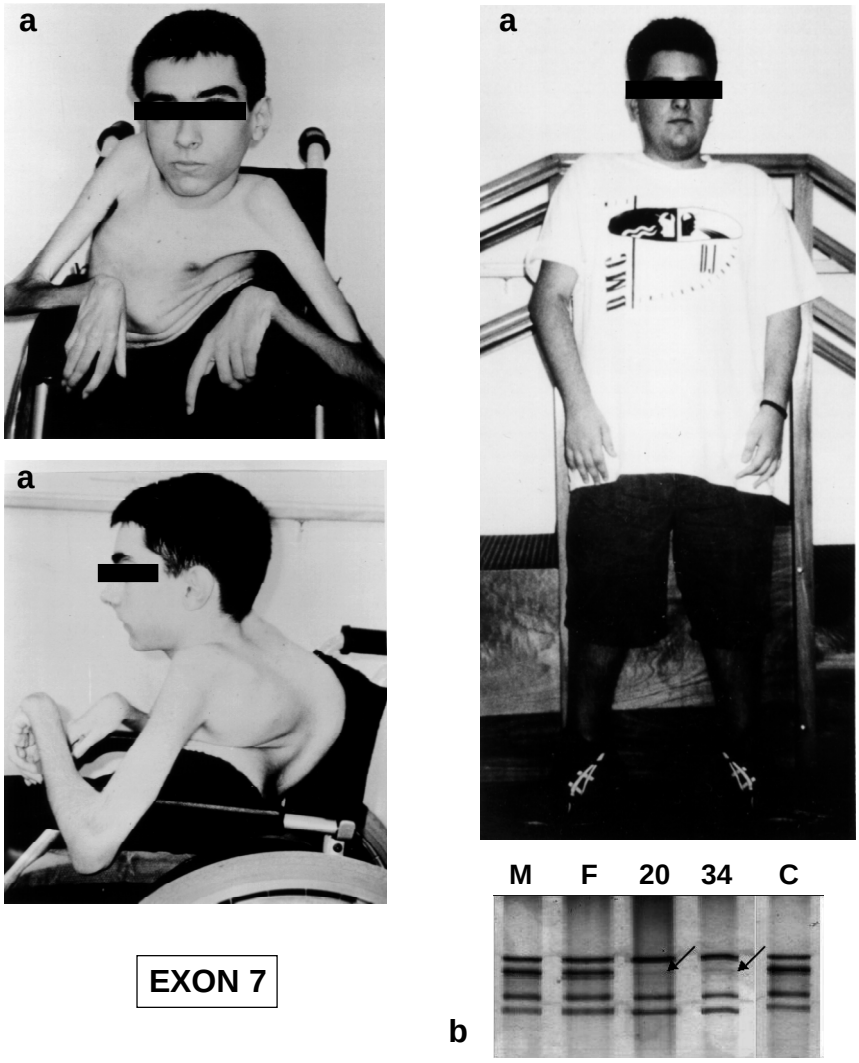

Figure 1 - a, Brothers affected with SMA types II and III. b, Molecular analysis of exon 7 of SMN gene. M - Mother, F - father, cases 20 and 34, and C - control.

et al. (1992) also experienced the same difficulties based on the current criterion. Zerres and Rudnik-Schonebörn (1995), in a retrospective study of 445 SMA patients, found 106 cases (24%) that could not be classified and suggested subdividing type III SMA into two other groups, resulting in a total of four SMA types.

In the present study, clinical classification of SMA patients into three groups based on the criteria of the International SMA Consortium was possible for most affected patients. However, in 29 cases of the present study (33%) age at onset and/or death and motor milestones achieved (ability to walk independently) did not clearly distinguish one of the three subgroups. For example, 12 patients with SMA type I survived past age two, though they required continuous mechanical ventilation. In 17 patients, age at onset was before 18 months, which is characteristic of type II, but walking abilities were compatible with SMA type III.

In the present investigation, the proportion of deletions in the SMN gene was 80% for SMA I, 75% for SMA II and 61% for SMA III. Taking the three subgroups together, the total frequency of deletions (68%) was lower than those found in several other previously reported population studies (Chang *et al*., 1995; Lefebvre *et al*., 1995; Wirth *et al*., 1995; Brahe *et al*., 1996).

Indeed, a frequency of more than 90% deletions in exons 7 and 8 of the SMN gene, in particular for SMA I and SMA II, has been found in investigations made in the United States (Wang *et al*., 1995), Germany (Wirth *et al*., 1995), Italy (Brahe *et al*., 1996), Spain (Bussaglia *et al*., 1995) and China (Chang *et al*., 1995). However, a lower proportion of deletions (ranging from 57 to 84%), comparable with ours, was also found in other studies in Poland (Brzustowicz *et al*., 1995), Canada (Aubry *et al*., 1995) and the United States (Kant *et al*., 1995).

Deletions involving both exons 7 and 8 (86%) were much more frequent than deletions of only exon 7 (13%) or only exon 8. This observation agrees with previous investigations (Bussaglia *et al*., 1995; Chang *et al*., 1995; Lefebvre *et al*., 1995; Rodrigues *et al*., 1996).

In addition, there was no correlation between deletions in the SMN gene and phenotype severity in accordance with previous reports in the literature (Chang *et al*., 1995; Lefebvre *et al*., 1995; Brahe and Bertini, 1996). On the other hand, deletions in exon 5 of NAIP, which is specific for the functional NAIP gene, showed wide frequency variation (0 to 67%) in different population studies (Chang *et al*., 1995; Rodrigues *et al*., 1995; Roy *et al*., 1995; Velasco *et al*., 1996). Several investigators reported a higher frequency of NAIP deletions in SMA type I than in the other two forms of SMA (Velasco *et al*., 1996; Al Rajeh *et al*., 1998; Jordanova *et al*., 1998).

In the study of Burlet *et al*. (1996), the frequency of deletions in the NAIP gene was 66% for SMA I, 6.5% for SMA II and 13% for SMA III. In the report of Rodrigues *et al*. (1996), these frequencies were 66, 10 and 12%, respectively. On the other hand, Chang *et al*. (1995) did not mention any deletions in the NAIP gene in a study of 48 Chinese patients (11 type I, 25 type II and 12 type III).

In the present sample, a higher frequency of deletions in the NAIP gene were also found for SMA I (35%) than SMA II (16%) and SMA III (2%). The observation that deletions involving both SMN and NAIP genes are more frequently observed in SMA I than SMA II or SMA III suggests a correlation between the extent of the deletion and severity of the phenotype (Burlet *et al*., 1996; Rodrigues *et al*., 1996).

However, the fact that a great proportion of severely affected patients presented no deletions in the NAIP gene supports the hypothesis that in addition to the extent of the deletion, other factors may regulate the severity of the clinical course. Furthermore, the fact that the same pattern of deletions (involving SMN or NAIP genes) was found among affected sibs who had different phenotypes (SMA II and SMA III) also supports the hypothesis that there are other still unknown mechanisms that influence phenotype expression.

The relationship between the number of gene copies and the disease phenotype was suggested. An increased cenSMN gene copy number, which can occur as a result of gene conversion events from telSMN to cenSMN, has been observed in patients of types II and III (Burghes, 1997; Campbell *et al.* 1997; DiDonato *et al.* 1997; McAndrew *et al*. 1997). On the other hand, as pointed out by Rodrigues *et al*. (1996), the assays of exon deletions used in the present phenotypic analysis were not sensitive enough to differentiate between in frame and out of frame deletions, nor could they detect the precise extent of the deletions; therefore, evaluation of other genes in the region might be disrupted. McAndrew et al. (1997) developed a quantitative PCR assay for the determination of the gene copy number of telSMN and cenSMN, and it became an important method for diagnosis of SMA carriers and detection of SMA compound heterozygotes (patients lacking one copy of telSMN and having another mutation in their other copy).

Deletions of exons 7 or 8 of the SMN gene and exon 5 of the NAIP gene in asymptomatic relatives of affected SMA patients have been reported in a small proportion of cases (Cobben *et al*., 1995; Hahnen *et al*., 1995; Wang *et al*., 1996), a finding for which there is still no explanation. In the present sample, no homozygous deletion in exons 7 or 8 of the SMN gene nor exon 5 of NAIP gene was found in any healthy relative of affected patients or in the control population. Therefore, although the possibility of misdiagnosis should be considered, particularly for prenatal diagnosis of "at-risk" fetuses, it is apparently a relatively rare event.

Patients with no detectable deletion in exons 7 and 8 of the SMN gene could be due to deletions or point mutations in the promoter or intronic sequences. It is relatively rare that other intragenic telSMN mutations have

been identified, providing strong evidence that the SMN gene is the primary SMA-determining gene (Bussaglia *et al*., 1995; Lefebvre *et al*., 1995; Brahe *et al*., 1996; Parsons *et al*., 1996, 1998; Wang *et al*., 1996; Hahnen *et al*., 1997; McAndrew *et al*., 1997). The function of SMN protein is not fully understood, though a marked reduction of SMN protein levels has been found in SMA patients, particularly in type I (Coovert *et al*., 1997; Lefebvre *et al*., 1997).

Another possibility could be genetic heterogeneity, that is, another gene (or genes) that cause a similar phenotype but is more prevalent in the Brazilian population. The study of affected families large enough for linkage analysis as well as mutations screening to identify new genes responsible for neuromuscular disorders will be very important to resolve this issue.

In summary, although many questions remain to be elucidated, analysis of the SMN gene in affected families is undoubtedly extremely important for clinical diagnosis and prevention of new cases in "at-risk" families through prenatal diagnosis. However, the lack of correlation between deletion type and clinical severity can lead to difficult situations in genetic counseling. For example, the parents of the two affected sibs in the family illustrated in Figure 1 decided to have a second child (who is severely affected), because the phenotype of the first son was mild. In another situation, a couple was referred to genetic counseling, because their first child had died at six months of age with a diagnosis of SMA. After being informed that the recurrence risk was 25%, the couple, who were against abortion, stated that they would risk another pregnancy only if we could reassure them that if they had another affected child, he would also die during the first year of life.

Comprehension of the mechanisms involved in determining SMA severity and the function of the SMN protein would be extremely helpful to solve these types of Tissues.

ACKNOWLEDGMENTS

We are extremely grateful to FAPESP, CNPq, FINEP, PRONEX and IAEA for financial support. The collaboration of Marta Canovas, Dr. Mariz Vainzof, Dr. Rita C.M. Pavanello and Constancia Urbani is gratefully acknowledged. We would also like to thank all the families who collaborated with us in this study. Publication supported by FAPESP.

RESUMO

As amiotrofias espinhais progressivas (SMAs) constituem as doenças degenerativas de origem genética letais mais comuns do sistema nervoso central e mais freqüentes dentre as doenças autossômicas recessivas após a mucoviscidose. A incidência estimada das SMAs é de aproximadamente 1:10.000 nativivos. Clinicamente, as SMAs são classificadas em mais grave (doença de Werdnig-Hoffmann, tipo I), intermediária (tipo II) e tardia e benigna (doença de Kugelberg-Welander, tipo III). O gene para os três tipos de SMAs foi mapeado no cromossomo 5 q11.2-13.3. Foram identificados dois genes candidatos na mesma região: SMN (sobrevida do neurônio motor) e NAIP (proteína inibidora de apoptose neuronal). Estudamos ambos genes em 87 pacientes brasileiros (20 tipo I,14 tipo II e 53 tipo III) pertencentes a 74 famílias, utilizando as técnicas de PCR e SSCP. Foi encontrada deleção nos exons 7 e/ou 8 do gene SMN em 69% das famílias: 16/20 na tipo I, 9/12 na tipo II e 26/42 na tipo III. Dentre as 51 famílias com deleção, 44 tiveram deleção nos exons 7 e 8 enquanto 7 tiveram deleção somente no exon 7. Deleção no exon 5 do gene NAIP foi encontrada em 7/20 na tipo I, 2/12 na tipo II e 1/42 na tipo III. Não foi encontrada deleção nos genes SMN e NAIP nos 112 progenitores, 26 irmandades assintomáticas e 104 controles normais. Não houve correlação entre deleção de um ou ambos genes com a gravidade do quadro clínico.

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(Received May 5, 1998)