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ORIGINAL INVESTIGATION

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Molecular analysis of survival motor neuron (SMN) and neuronal apoptosis inhibitory protein (NAIP) genes of spinal muscular atrophy patients and their parents

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Abstract We have assayed deletions of two candidate genes for spinal muscular atrophy (SMA), the survival motor neuron (SMN) and neuronal apoptosis inhibitory protein (NAIP) genes, in 101 patients from 86 Chinese SMA families. Deletions of exons 7 and 8 of the telomeric SMN gene were detected in 100%, 78.6%, 96.6%, and 16.7%, in type I, II, III, and adult-onset SMA patients, respectively. Deletion of exon 7 only was found in eight type II and one type III patient. One type II patient did not have a deletion of either exon 7 or 8. The prevalence of deletions of exons 5 and 6 of the NAIP gene were 22.5% and 2.4% in type I and II SMA patients, respectively. We also examined four polymorphisms of SMN genes and found that there were only two, SMN-2 and ^{C}BCD541-2, in Chinese subjects. In our study, analysis of the ratio of the telomeric to centromeric portion (T/C ratio) of the SMN gene after enzyme digestion was performed to differentiate carriers, normals, and SMA patients. We found the T/C ratio of exon 7 of the SMN gene differed significantly among the three groups, and may be used for carrier analysis. An asymptomatic individual with homozygous deletion of exons 7 and 8 of the SMN gene showed no difference in microsatellite markers in the SMA-related 5q11.2–5q13.3. In conclusion, SMN deletion in clinically presumed child-onset SMA should be considered as confirmation of the diagnosis. However, adult-onset SMA, a heterogeneous disease with phenotypical similarities to child-onset SMA, may be caused by SMN or other gene(s).

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Introduction

Proximal spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by degeneration of anterior horn cells of the spinal cord. The clinical diagnosis of the disease is based on progressive symmetric weakness and atrophy of the proximal muscles (Dubowitz 1978; Pearn 1982). Affected individuals are usually classified into three groups according to the age of onset and progression of the disease. Children with type I (acute) SMA are the most severely affected. They usually have symptoms of SMA before 6 months of age, and rarely live beyond the age of 2 years. Type II and type III (chronic) SMA are milder forms, and the onset of symptoms varies between 6 months and 17 years of age (Brooke 1985; Dubowitz 1964; Kugelberg and Welander 1956; Pearn 1973; Pearn et al. 1978; Wohlfart et al. 1955).

SMA occurs in 1 in 10,000 live births and has an estimated carrier frequency of 1:40 (Pearn 1973, 1978; Roberts et al. 1970). All three forms of autosomal recessive proximal SMA have been mapped to 5q11.2-q13.3 by linkage analysis (Brzustowicz et al. 1990; Gilliam et al. 1990; Melki et al. 1990a, b, 1994; Wang et al. 1995; Wirth et al. 1994). Two candidate genes for SMA have been cloned from this region. The telomeric survival motor neuron (SMN) gene was identified by Lefebvre et al. This gene was either deleted or interrupted in 226 of their 229 SMA patients. The few patients retaining this gene (n = 3) carried a point mutation or short deletions in the consensus splice sites of introns 6 and 7. The neuronal apoptosis inhibitory protein (NAIP) gene was cloned by Roy et al. The first two coding exons were deleted in approximately 50% of type I SMA patients in their series (Lefebvre et al. 1995; Roy et al. 1995).

In this study, we present a molecular analysis of the SMA-determining gene, the survival motor neuron gene (SMN, exons 7 and 8), and candidate gene, the neuronal apoptosis protein gene (NAIP, exons 5, 6, and 13), in 101 Chinese patients and their families.

Patients and methods

Patients, blood samples, and fetal tissues

We examined blood samples from 101 patients with SMA in 86 families, their family members (including parents and siblings of the SMA patients), and 300 normal individuals. Of the affected patients, 18 cases from 17 families were diagnosed as type I SMA, 42 cases from 35 families were diagnosed as type II SMA, 29 cases from 22 families were diagnosed as type III SMA, and 12 cases from 12 families were adult-onset SMA patients who developed symptoms after 25 years of age. All patients fulfilled the diagnostic criteria of proximal SMA as defined by the International SMA Consortium (Munsat 1991; Munsat and Davies 1992). DNA was extracted from white blood cells by standard methods (Chiou et al. 1993). Forty-eight patients from 42 families had been previously studied (Chang et al. 1995).

Scanning of the digested products of the SMN gene

The principles and methods of the polymerase chain reaction (PCR) used to amplify exons 7 and 8 of the SMN genes were similar to those used by Van der Steege et al. (1995) and our previous study (Chang et al. 1992). However, the downstream primer was much longer (5′-TAAGGATGTGAGCACCTTCCTTCCTCTTTTTTGATTTTGTTT-3′) to make the digested product easier to be recognize.

The PCR products were digested by restriction enzymes *Dra*I and *Dde*I, and then electrophoresed on 3.5% agarose gel (Metaphor, FMC, Rockland, Me, USA). The intensity of the digested fragments was scanned and analyzed by the Collage image analysis system (Fotodyne, Hartland, Wi., USA) to calculate the ratio of digested to undigested parts. Analysis of variance (ANOVA) followed by the Newman-Keuls test was used for statistical analysis of significance in differences.

PCR of exons 5, 6, and 13 of the NAIP gene

The PCR conditions and primers used to amplify the exons 5 and 13 or exons 6 and 13 were identical to those used in the study of Roy et al. (1995). The PCR products were visualized on ethidium bromide-stained 3% agarose gel after electrophoresis.

Microsatellite markers

Five microsatellite markers (D5S435, Ag1-Ca, D5S1413, D5S1414 and MAP-1B) were used to amplify polymorphisms of the SMA linkage area. The PCR conditions were as described previously (DiDonato et al. 1994; Soares et al. 1993; Wang et al. 1995).

Detection of four polymorphisms in SMN genes

The PCR products of exon 7 (mentioned above) were used and were digested with *Bsm*AI to reveal the SMN-$2^{Bsm\ AI}$ polymorphism (Velasco et al. 1996). The same PCR products of the above-mentioned exon 8 were digested with *Hae*III or *Alu*I to reveal the polymorphisms "GC" or "AG". For detection of the polymorphism "G^{tel}" or "A^{cen}" at the 44 bp upstream of the 3′ end of intron 6, we used a mutagenic primer: 5′-GGAAGTTAAAAAAAATAGCTATGTAGA-3′ which has a mutagenic base G at the 5th base from the end (underlined). After PCR, the mutagenic base in combination with "G^{tel}" creates an "*Acc*I" enzyme cutting site. For detection of SMN-$2^{\Delta AT}$, the downstream primer was 5′-CAGTCTTTTACAGATGGTTTTTCAGAA-3′, which had a mutagenic base at the 3rd from last base. This mutagenic base in combination with TCT at the non-SMN-$2^{\Delta AT}$ allele creates an *Mbo*II cutting site. The upstream primer was 5′-AGGAAGTGGAATGGGTAACTC-3′.

Results

Deletion patterns in SMA type I–IV patients

The PCR products of exon 7 of the SMN gene digested by restriction enzyme *Dra*I are shown in Fig. 1 (exon 7 part). Normal individuals and carriers of SMA should have 188-bp, 149-bp, and 39-bp fragments. Since the 39-bp band was not visible on 3.5% agarose gel, only the 188-bp and 149-bp bands were found. In SMA patients, there was only a 149-bp band after complete digestion of PCR products by *Dra*I. The undigested part belonged to the telomeric SMN gene and the digested part to the centromeric SMN gene. The SMA patients only had the centromeric SMN gene. The PCR products of exon 8 digested by *Dde*I are also shown in Fig. 1 (exon 8 part). In normal subjects and carriers, there were three fragments (187 bp, 123 bp,

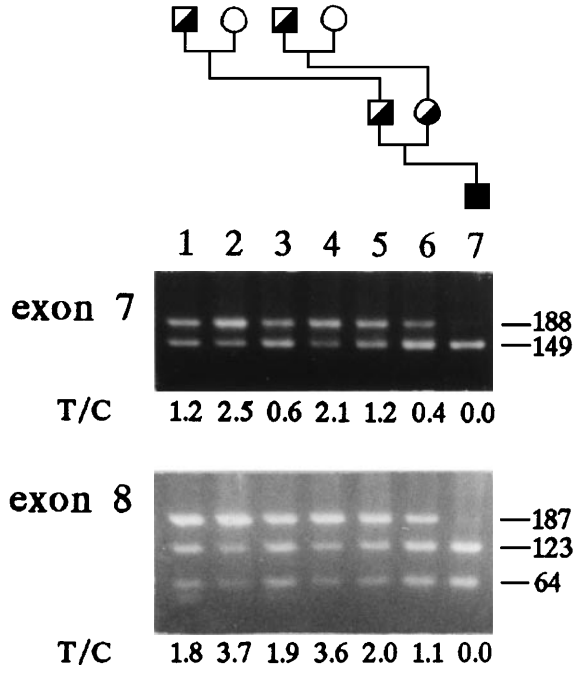

Fig. 1 Analysis of exons 7 and 8 in a typical deletional-type SMA family. For exon 7, the 188-bp fragment is from the telomeric SMN gene and the 149-bp fragment belongs to the centromeric SMN gene. For exon 8, the 187-bp fragment is from the telomeric gene, while the 123-bp and 64-bp fragments belong to the centromeric SMN gene. In this pedigree analysis, the T/C (telomeric part/centromeric part) ratio of exon 7 was from 0.4 to 1.2 in carriers, close to zero in SMA patients, and greater than 2.0 in normal persons. The T/C ratio of exon 8 was from 1.1 to 2.0 in carriers, close to zero in SMA patients, and greater than 3.0 in normal persons

Table 1 The results of molecular analysis of SMN and NAIP genes in Chinese SMA patients

Clinical diagnosis Phenotype	Gene deletion Case no.	SMN gene		NAIP gene	
		Exon 7	Exon 8	Exon 5	Exon 6
Childhood SMA type I	18	18 (100)	18 (100)	4 (22.2)	4 (22.2)
Childhood SMA type II	42	41 (97.6)	33 (78.6)	1 (2.4)	1 (2.4)
Childhood SMA type III	29	29 (100)	28 (96.6)	0 (0)	0 (0)
Adult-onset SMA	12	2 (16.7)	2 (16.7)	0 (0)	0 (0)
Total (%)	101	89 (88.1)	81 (80.2)	5 (5.6)	5 (5.6)

and 64 bp). In the SMA patients, there were two fragments (123 bp and 64 bp).

Of the 89 patients with childhood-onset SMA from 74 families, all but 1 had deletion of the telomeric exon 7, and 79 had deletion of the telomeric exon 8. The exon 7 non-deletion case also showed non-deletion of exon 8 of the telomeric SMN gene (Table 1). Of the 12 cases of adult-onset SMA from 12 families, only 2 had deletion of the telomeric exons 7 and 8, the others having a "normal" pattern.

In the analysis of deletion of the NAIP gene, 4 of 18 patients with type I, one of 42 type II, and none of type III or adult-onset SMA patients had deletion of exons 5 and 6 of NAIP gene (Table 1).

SMN gene exons 7 and 8 polymorphisms

We examined the four polymorphisms in the regions from intron 6 to exon 8 and found that the SMN gene in Chinese subjects was identical to the SMN-2 reported by Velasco et al. (1996). The copy SMN gene was ^{c}BCD541-2. SMN-2$^{\Phi AT}$. ^{c}BCD541-1, SMN^{BsmA1}, and SMN-1 were not found in this study.

Analysis of carriers of SMA by genomic DNA analysis

Although there are at least four types of disease allele for the SMN gene, most of them can be digested by *Dra*I and can be used to develop a method for carrier detection. The ratio of the telomeric SMN gene of exon 7 to the centromeric SMN gene was analyzed. The PCR products were digested to two almost equal parts in carriers, whereas only partial digestion was noted in normal persons, and complete digestion in SMA patients. Ninety-six SMA carriers and 300 normal individuals were analyzed. In SMA carriers, the T/C ratio was about 1.0 (mean = 1.30, SD = 0.63). In normal subjects, the T/C ratio was around 2.0 or higher (mean = 3.88, SD = 2.00). Hence, these two groups were differentiated on the basis of the undigested/digested ratio ($P < 0.0001$) (Fig. 2A). Similar results were obtained in the analysis of T/C ratios of exon 8 for normal controls and carriers of SMA ($P < 0.0001$; in normal controls, mean = 3.63, SD = 1.66; in carriers, mean = 2.19, SD = 3.99). However, we used the T/C ratio of exon 7 as an indicator of the carrier state of SMA rather than that of exon 8 since there were more cases with non-deletional SMN genes in exon 8 (Fig. 2B). In our series, 4

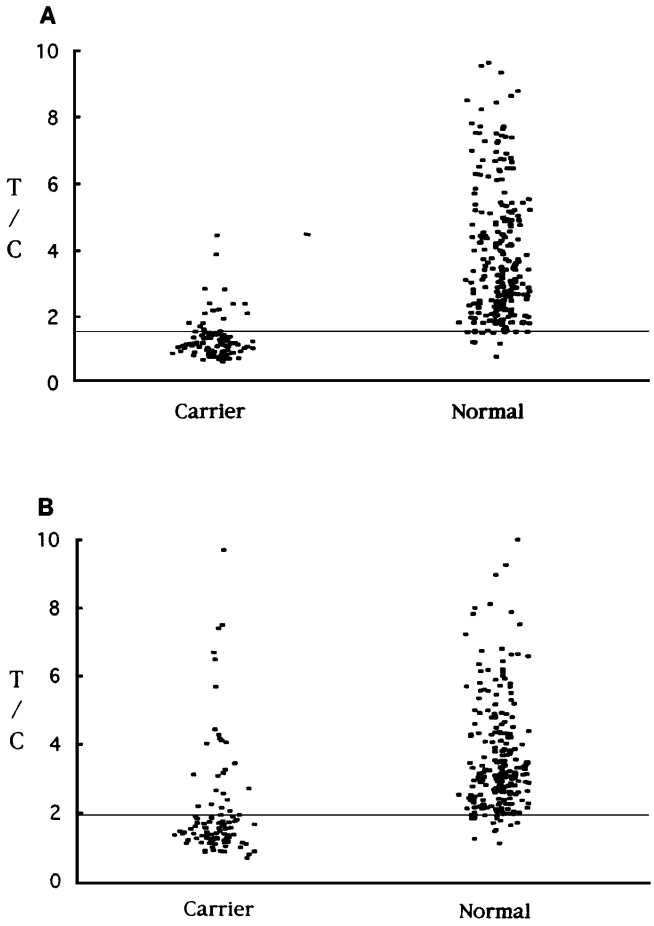

Fig. 2 **A** Distribution of the T/C ratios of exon 7 of normal persons and carriers. Normal persons and carriers can be differentiated easily on the basis of T/C ratios ($P < 0.0001$). Fifteen cases of homozygotes without a centromeric allele are not included. **B** Distribution of the T/C ratios of exon 8 of normal persons and carriers. There are significant differences between these two groups ($P < 0.0001$). The case with a non-deletional exon 8 is not included

of the 300 clinically normal persons had a T/C ratio around 1.0, so we estimated the carrier frequency at about 1%–2% in this population. In 15 of the 300 normal persons (5%), only the telomeric SMN genes were found. Such a high incidence of lack of the centromeric SMN gene may affect the accuracy of our method for detecting SMA carriers; for example the combination of one allele having no centromeric SMN gene and the other allele having no telomeric SMN gene may affect prediction of the carrier state. However, in our series, we detect 132

cases of carriers in 148 parents of child-onset SMA families, with an estimated accuracy of 90%. Meanwhile, we are analyzing more family members to overcome this problem. For example, we found the father of an affected fetus showed a normal pattern. However, his paternal grandmother was a carrier.

In our series, we found a family with affected and unaffected siblings showing deletion of exons 7 and 8. We also analyzed exons 5 and 6 of the NAIP gene in the siblings and found that both of them had an intact NAIP gene. We further studied five microsatellite markers (D5S435, Ag1-Ca, D5S1413, D5S1414, and MAP-1B). Both of the sibling showed an identical haplotype for all five markers.

SMA patients without SMN gene deletion carrying putative SMN gene mutation

In a 5-year-old type II SMN patient, exons 7 and 8 of the SMN gene were non-deletion, and the NAIP gene also showed no evidence of deletion of exons 5 and 6. We further analyzed the T/C ratio of this family, and found that the patients ratio was 0.4 and 0.5 for exons 7 and 8, respectively; his father's T/C ratio was 2.1 and 4.2 for exons 7 and 8, respectively; and his mother's T/C ratio was 0.5 and 1.4 for exons 7 and 8, respectively. From these results, we suggested that the patient had a deletion allele and a point mutation or small deletion another area of the SMN gene in another allele, his mother was a deletion carrier, and his father was a non-deletion carrier.

Discussion

We analyzed two SMA candidate genes, SMN and NAIP, in SMA families of mainly Chinese origin. In general, our results are in accord with the high frequency of deletions or disruptions in the SMN gene as previously reported (Bussaglia et al. 1995; Chang et al. 1995; Cobben et al. 1995; Hahnen et al. 1995; Lefebvre et al. 1995; Rodriques et al. 1995; Wirth et al. 1995). However, the deletion rate of the NAIP gene was much lower than that reported by other groups (Bussaglia et al. 1995; Cobben et al. 1995; Hahnen et al. 1995; Rodriques et al. 1995; Roy et al. 1995; Wirth et al. 1995). The difference may be attributed to different deletion types in different ethnic groups.

In order to analyze the variations of SMN genes in SMA patients, we took advantage of the nucleotide differences in exons 7 and 8 between the SMN and copy gene to design primers and run PCRs. The PCR products were further digested by restriction enzymes, and then the undigested/digested DNA fragments (T/C) ratio was analyzed. We found that the T/C ratios of exon 7 of SMA carriers (parents of SMA patients) varied between 0.5 and 1.5, and those of SMA patients were close to zero, and those of normals greater than 2.0. Similarly, in parents of SMA patients, the T/C ratios of the DNA fragments of exon 8 after restriction enzyme digestion were around 2.0, those of the SMA patients were close to zero, and those of normals greater than 3.5. In this series, positive and negative controls were used simultaneously for every reaction; moreover, exons 7 and 8 were used as a gene dosage control for each other. With this approach, we found the carrier rate in our series was 1.33% (4/300). Since there is a 5% loss of the copy gene in the general population, the estimated carrier rate of SMA will be higher than 1.33% in Chinese subject. We also used the β-globin gene as an internal control through the ratio of the SMN gene and β-globin gene. The results were not as good as an indicator (data not shown). Moreover, we examined the polymorphisms from intron 6 to exon 8 of SMN genes in these different alleles, but were unable to find differences. Our Chinese patients had only SMN-2 and ^{c}BCD541-2.

To date, homozygosity of the SMN gene deletion has not been reported in a healthy population. We and other investigators, however, have shown homozygosity for the SMN deletion of exons 7 and 8 of the SMN gene in healthy siblings of SMA patients with SMN deletions (Cobben et al. 1995; Wang et al. 1996). Within each family, these healthy siblings share with the affected ones the same 5q haplotype surrounding the SMA locus.

From a practical point of view, homozygosity of SMN deletions in unaffected persons is so rare that demonstration of homozygosity for SMN deletion in a case of clinically presumed child-onset SMA should be considered as confirmation of the diagnosis (Bussaglia et al. 1995). In adult-onset SMA patients, only 2 out of the 12 patients (17%) had deletion of exons 7 and 8 of the telomeric SMN gene. Our results differ from those of Brahe et al. (1995) and Clermont et al. (1995), but are similar to those of Zerres et al. (1995). We infer that different mutations of the SMN gene or some undetermined gene(s) may play a major role in this subgroup of patients. Further studies are needed to resolve these questions.

As to the NAIP gene, we found no deletion in exons 5 or 6, such as have been reported by others (Roy et al. 1995). The difference may be attributed to different deletion types of the candidate genes in different ethnic groups. Inversion in the NAIP gene and the SMN gene and polymorphism may also contribute to this difference.

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