

Molecular Analysis of Survival Motor Neuron (SMN) and Neuronal Apoptosis Inhibitory Protein (NAIP) Genes in a Chinese Type III Spinal Muscular Atrophy (SMA) Family

Guey-Jen Lee-Chen¹*, Kuo-Hsuan Chang², and Yi-Wen Chin¹

¹ Department of Biology, National Taiwan Normal University
Taipei, Taiwan 117, Republic of China

² Department of Medicine, Taipei Medical College
Taipei, Taiwan 105, Republic of China

ABSTRACT

In this report we present the genetic studies in a Chinese type III spinal muscular atrophy (SMA) family. The survival motor neuron (SMN) gene exons 7 and 8 and neuronal apoptosis inhibitory protein (NAIP) gene exon 5 were amplified by sequence-specific oligonucleotide primer and polymerase chain reaction (PCR). The SMN gene deletion was detected by restriction endonuclease digestion and single strand conformation polymorphism (SSCP) analysis of PCR-amplified products. The deletion in NAIP gene was detected directly by agarose gel electrophoresis of multiplex PCR-amplified products. All three affected siblings are homozygous for telomeric SMN gene deletion, whereas no NAIP gene deletion was found. Both parents, maternal uncle, and paternal grandmother of affected sibs showed heterozygosity for telomeric SMN deletions. Two unaffected sibs are non-carriers for SMN deletions.

Key words: spinal muscular atrophy, SMN, NAIP

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disorder with an overall incidence of 1 in 10,000 live births and a carrier frequency of 1/50 (Pearl, 1980). SMA is characterized by loss of α -motor neurons in the spinal cord, leading to progressive muscular weakness and atrophy. The affected individuals are classified into three groups on the basis of their clinical severity and the age of onset (Dubowitz, 1978). Type I is the most severe with clinical onset before age of 6 months. These children are never able to sit unsupported and the majority of them die from respiratory failure within the first three years of life. In the intermediate type II, onset is usually before age of 15 months. These children are able to sit without support but never manage to stand or walk unaided. They survive beyond 4 years of age until adolescence or later, depending on the degree of respiratory muscle

involvement. The mild type III has onset of muscle weakness that occurs between 18 months to 18 years. These patients manage to stand and walk unsupported, although their walking distance is limited. The long term survival is good, again dependent mainly on respiratory function, and the affected person may have a near-normal life span.

On the basis of linkage studies, all three types of SMA map to chromosome 5q13 (Brzustowicz *et al.*, 1990; Gilliam *et al.*, 1990; Melki *et al.*, 1990a, 1990b). Studies of microsatellite markers which show linkage disequilibrium with the disease indicate a high frequency of null alleles and deletions in SMA patients (DiDonato *et al.*, 1994; Melki *et al.*, 1994; Daniels *et al.*, 1995). Recently, the genomic structure of the SMA locus was reported (Figure 1A) (for review, see Lewin, 1995). The presence of multiple copy repetitive sequences, pseudogenes, and retrotransposon-like sequences underlies the instability of this chromosomal region (Theodosiou *et al.*, 1994; Francis *et al.*, 1995).

* Corresponding author: Guey-Jen Lee-Chen. FAX: 886-2-9312904; E-mail: biofv047@see.ntnu.edu.tw

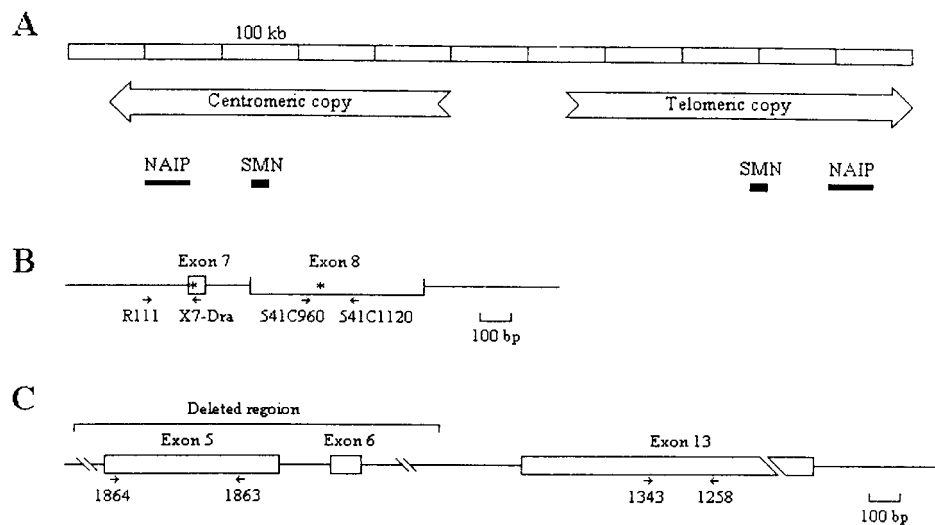


Figure 1. (A) Diagrammatic representation of an inverted duplication of chromosome region 5q13. (B) Gene structure containing exons 7 and 8 of SMN. Exon is represented by opened box and intron by thin line. The nucleotide change between the two copies of SMN gene is depicted by asterisk. The location of primer for PCR amplification is depicted by arrow. (C) Gene structure containing exons 5, 6 and 13 of NAIP. Exon, intron and location of PCR primer are depicted as above. The deleted region containing exons 5 and 6 of pseudogene copy of NAIP is also indicated.

Two candidate genes, the survival motor neurone (SMN) gene and the neuronal apoptosis inhibitory protein (NAIP) gene, were isolated from the region.

The SMN gene contains 8 exons extending over approximately 20 kb (Lefebvre *et al.*, 1995). Two copies of the SMN gene, centromeric and telomeric have been identified. They are virtually identical at their genomic sequence (only five nucleotide changes along 20 kb) and both are transcribed (Lefebvre *et al.*, 1995). The vast majority (98.6 %) of patients have a deletion in the telomeric copy of SMN gene, whereas point mutations have been identified in the remaining 3 of 229 patients (Lefebvre *et al.*, 1995). Mutation in the telomeric SMN causing SMA is further supported by a number of other studies reported (Brahe *et al.*, 1995a; Bussaglia *et al.*, 1995; Chang *et al.*, 1995; Cobben *et al.*, 1995; Matthijs *et al.*, 1996; Rodrigues *et al.*, 1995b, 1996; Velasco *et al.*, 1996; Zappata *et al.*, 1996).

The NAIP gene lies adjacent to the SMN gene and contains 16 exons spanning 60 kb (Roy *et al.*, 1995). The two first coding exons (exons 5 and 6) of NAIP were deleted in 45 % of type I patients and 18 % of types II and III (Roy *et al.*, 1995). Studies of Spanish SMA patients showed that NAIP exon 5 was deleted in 67.9 % type I patients and 16.2 % types II and III (Velasco *et al.*, 1996). Thus, loss of NAIP is not sufficient to cause the disease. It was hypothesised that mutations in the telomeric SMN gene are the major determinant of the SMA phenotype, whereas the extent

of the deletions, which may include the NAIP gene, may correlate with the severity of the disease (Lefebvre *et al.*, 1995; Roy *et al.*, 1995).

We used a PCR-based DNA test to investigate a Chinese SMA III family for the presence of deletions at both loci. The results revealed that exons 7 and 8 of the telomeric SMN gene are deleted in all three affected sibs. No deletions were detected in the NAIP gene within the family.

Materials and Methods

Subjects and family

A family with three affected sibs were referred to our laboratory for molecular analysis. Clinically, the affected sibs in the family are classified as SMA type III as defined by the International SMA Consortium (Munsat and Davies, 1992). The proposita (III-1 of Figure 2) is 27 years old. Her 19 years old brother (III-5 of Figure 2) had onset of symptoms at the age of five years. Both her 23 and 21 years old sisters (III-3 and III-4 of Figure 2) had onset of symptoms at the age of eight years. All three affected sibs are wheel-chair-bound now.

DNA preparation

Peripheral blood (5-10 ml) from subjects were collected into tubes containing EDTA and lysed in

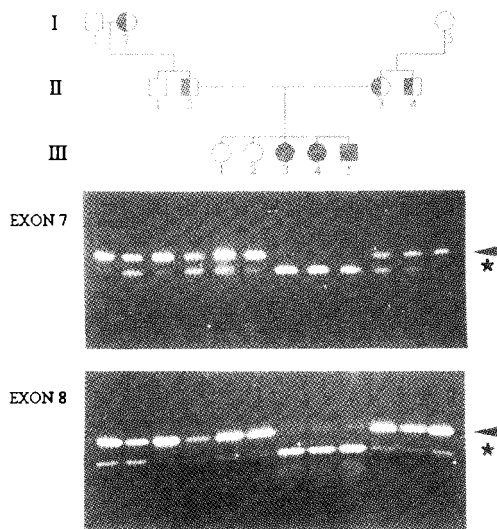


Figure 2. Photographs of restriction endonuclease analysis of PCR-amplified exons 7 and 8 of the telomeric SMN gene and its centromeric copy. PCR products were digested to completion with *DraI* (upper panel) or *DdeI* (lower panel) and resolved in agarose gel. PCR products amplified from the telomeric copy are indicated by arrows, whereas those amplified from the centromeric copy indicated by stars. Pedigree of the type III SMA family is shown above the photographs. Affected individuals are indicated by filled symbols. Carriers for deletion of the telomeric SMN gene are indicated by half-filled symbols.

buffer containing 0.32 M sucrose - 10 mM Tris pH 7.5 - 5 mM MgCl₂ - 1 % Triton X-100. Nuclei were collected by centrifugation and digested in buffer containing 50 µg/ml proteinase K - 10 mM Tris pH 7.8 - 5 mM EDTA - 0.5 % SDS. Genomic DNA was then isolated and quantitated using standard procedures (Sambrook *et al.*, 1989).

PCR and restriction endonuclease analysis of SMN exons 7 and 8

Amplification of the SMN gene was carried out by using exon 7 primers (R111 and X7-Dra) and exon 8 primers (541C960 and 541C1120) (Figure 1B) as previously described (van der Steege *et al.*, 1995; Lefebvre *et al.*, 1995). Samples of *DraI* digested exon 7 product were loaded on to a 2.0 % agarose minigel containing ethidium bromide and electrophoresed at 100 V for 60 min to resolve 184 bp (telomeric copy) and 160 bp (centromeric copy) fragments. Samples of *DdeI* digested exon 8 product were separated in a 1.8 % agarose mini gel to resolve 187 bp (telomeric copy) and 121 bp (centromeric copy) fragments.

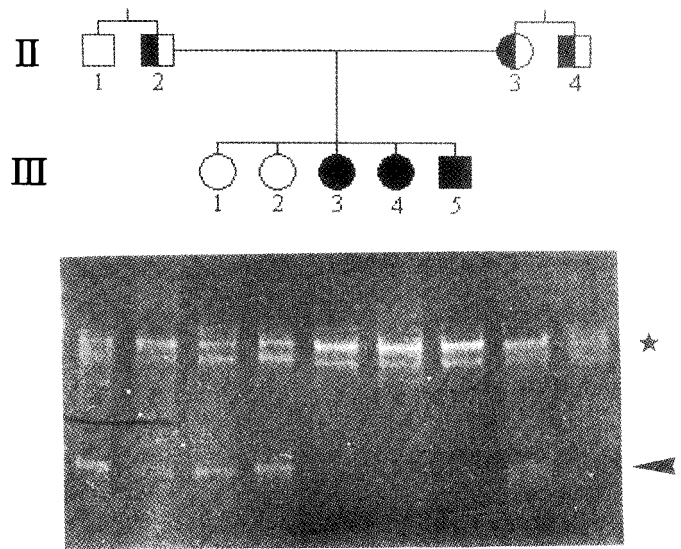


Figure 3. Photograph of SSCP analysis of PCR-amplified exon 7 of the telomeric SMN gene and its centromeric copy. PCR products amplified from the telomeric copy is indicated by an arrow, whereas those amplified from the centromeric copy indicated by star.

Nonisotopic SSCP analysis of SMN exons 7 and 8

Ten microliters of the PCR products were mixed with an equal volume of formamide, denatured for 10 minutes at 95 °C, loaded onto a nondenaturing polyacrylamide gel (0.5 × HydroLink MDE (J.T.Baker) in 0.6 × TBE). The acrylamide minigel was run at 4 °C for 2.5 hours at 250 V on the Novex Xcell II (Novex). The gels were stained with ethidium bromide (0.5 µg/ml) for 20 min, destained for 15 min, and then visualized and photographed under UV light.

PCR analysis of NAIP exon 5

Deletion of NAIP gene was analyzed by using exon 5 primers (1863 and 1864) and exon 13 primers (1258 and 1343) (Figure 1C) in a multiplex PCR reaction as described (Roy *et al.*, 1995). Samples were loaded on to a 1.4 % agarose minigel containing ethidium bromide and electrophoresis at 100 V for 60 min to resolve 435 bp (exon 5) and 241 bp (exon 13) fragments.

Results

Restriction endonuclease analysis of deletions in the SMN gene

The two copies of SMN gene differ in their exons by only two base pairs, one in exon 7 and the other in exon 8, thus making distinction of the telomeric copy

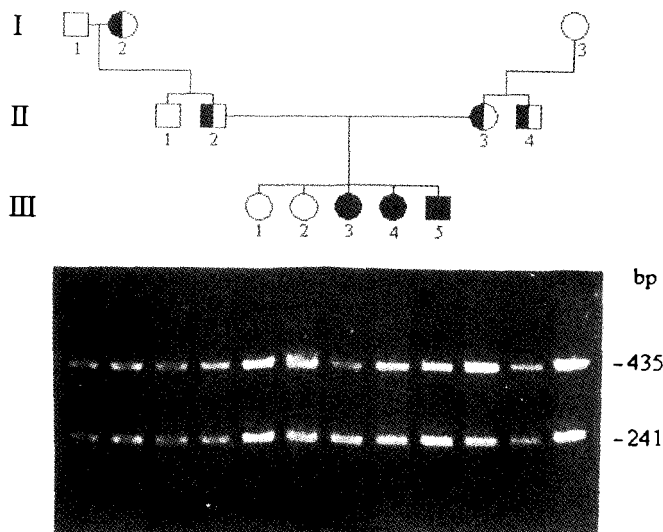


Figure 4. Photograph of 1.4 % agarose gel electrophoresis of multiplex PCR amplification of NAIP exon 5 (435 bp) and exon 13 (241 bp).

from centromeric copy by restriction endonuclease digestion (van der Steege *et al.*, 1995). With specific mismatch primer adjacent to the variant site (Figure 1B, X7-Dra), a restriction site for *Dra*I was created in the PCR product of exon 7 of the centromeric copy, but not in the PCR product of the telomeric copy. PCR products of exon 8 of two genes can readily be distinguished since the centromeric copy contains a recognition site for the restriction enzyme *Dde*I, which is absent in exon 8 of the telomeric copy. The resulting gel patterns after PCR amplification, enzyme digestion, and electrophoresis are shown in Figure 2. In three affected sibs (III-3, III-4, III-5), a homozygous deletion of exons 7 and 8 of the telomeric copy (indicated by arrows) was detected. Both parents (II-2 and II-3), maternal uncle (II-4) and paternal grandmother (I-2) were shown to be carriers of a deletion of the telomeric copy.

SSCP analysis of deletions in the SMN gene

Due to possible incomplete restriction enzyme digestion of PCR-amplified products, the non-carrier status of unaffected sibs (III-1 and III-2) in the family was further confirmed by single strand conformation polymorphism (SSCP) analysis (Lefebvre *et al.*, 1995) (Figure 3). The results from SSCP could determine the copy number of the SMN gene and thereby allow for carrier testing in some cases (Matthijs *et al.*, 1996). In three affected sibs, a homozygous deletion of exon 7 of the telomeric SMN gene (indicated by an arrow) was detected. Carriers (II-2, II-3 and II-4) and non-carrier (III-1 and III-2) in the family could be identified. A similar result from exon 8 by SSCP analysis was

obtained (data not shown).

Analysis of deletions in the NAIP gene

A multiplex PCR reaction to amplify exon 5 and exon 13 was conducted. Exon 13 is present in both functional and pseudogene copies of NAIP and therefore can be used as an amplification control for exon 5 which is present only in the functional NAIP gene. Exon 5 (435 bp) and exon 13 (241 bp) were detected on a 1.4 % agarose gel in all family members tested (Figure 4). Thus no exon 5 deletion was found in the affected sibs

Discussion

Two different genes (NAIP and SMN) have been recently reported to be associated with the SMA disease (Lefebvre *et al.*, 1995; Roy *et al.*, 1995). The SMN gene encodes a protein of 294 amino acids. Both the telomeric copy and the centromeric copy are widely expressed and their predicted amino acid sequence is identical. Moreover, exon 7 of the centromeric copy is shown alternatively spliced (Lefebvre *et al.*, 1995). NAIP is expressed in motor neurons and is homologous with baculovirus proteins that inhibit virally induced insect cell apoptosis (Roy *et al.*, 1995). NAIP has been shown to have an anti-apoptotic effect in mammalian cells (Liston *et al.*, 1996). The motor neuron depletion observed in SMA suggests that a genetic defect in NAIP may result in a pathologic persistence or reactivation of normally occurring apoptosis.

The incidence of deletions of both telomeric SMN and NAIP genes was higher in type I SMA in comparison with types II and III (Cobben *et al.*, 1995; Rodrigues *et al.*, 1996; Velasco *et al.*, 1996). Thus patients suffering from the milder forms of the disease showed smaller deletions predominantly in the telomeric SMN but not in NAIP. High frequency of deletions in the telomeric SMN makes an important tool for SMA diagnosis. In our study, the three mild type III affected sibs had deletions of telomeric SMN exons 7 and 8 (Figures 2 and 3), but no deletion of NAIP exon 5 was detected (Figure 4).

Two deletion assays were used in this study to examine the SMN gene in the affected family. The restriction enzyme digestion analysis is based on the presence or absence of *Dra*I or *Dde*I restriction site in exon 7 or 8 that allows distinction of the telomeric SMN gene from its centromeric copy. The SSCP analysis is based on the alternatively folded conformation assumed by the mutated single strand DNA, as indicated by a single base difference in both exons 7 and 8 of the two genes. By restriction enzyme digestion and SSCP

analysis, we have identified carriers in the family. Although assays of exon deletions are not sensitive enough to detect the precise extent of the deletions or to differentiate between inframe or out of frame deletions, the methods has allowed us to confirm directly the clinical diagnosis of SMA in patients. It has also been used for presymptomatic diagnosis (Brahe *et al.*, 1995b) and prenatal diagnosis (Rodrigues *et al.*, 1995a) in families at risk of SMA.

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台灣一個第三型脊椎性肌肉萎縮症(Type III SMA)家族致病基因的分子研究

李桂楨¹ 張國軒² 秦義雯¹

¹國立臺灣師範大學生物學系

²私立臺北醫學院醫學系

摘要

本研究中，我們檢視了台灣一個第三型脊椎性肌肉萎縮症 (type III spinal muscular atrophy) 家族致病基因的分​​子構造。我們以 DNA 序列專一的寡核苷酸引子 (sequence-specific oligonucleotide primer) 及聚合酵素鏈反應 (polymerase chain reaction) 放大 survival motor neuron (SMN) 基因的表現子 7 和 8 及 neuronal apoptosis inhibitory protein (NAIP) 基因的第五表現子。SMN 基因的缺失可藉限制酵素切割 (restriction endonuclease digestion) 及單股核酸構形多型性 (single strand conformation polymorphism) 分析聚合酵素鏈反應放大的產物而檢測之。NAIP 基因的缺失則可直接以瓊脂膠體電泳 (agarose gel electrophoresis) 檢測之。三位患者的 telomeric SMN 基因皆為同型合子 (homozygous) 的缺失，而 NAIP 基因則無缺失。患者的雙親、舅舅及祖母皆為異型合子 (heterozygous) 的 telomeric SMN 基因缺失，其二位正常的姊姊則不帶有 SMN 基因缺失。

關鍵詞：脊椎性肌肉萎縮症、SMN、NAIP