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# Deletion analysis of *SMN1* and *NAIP* genes in southern Chinese children with spinal muscular atrophy*

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**Abstract:** Spinal muscular atrophy (SMA) is a disorder characterized by degeneration of lower motor neurons and occasionally bulbar motor neurons leading to progressive limb and trunk paralysis as well as muscular atrophy. Three types of SMA are recognized depending on the age of onset, the maximum muscular activity achieved, and survivorship: SMA1, SMA2, and SMA3. The survival of motor neuron (*SMN*) gene has been identified as an SMA determining gene, whereas the neuronal apoptosis inhibitory protein (*NAIP*) gene is considered to be a modifying factor of the severity of SMA. The main objective of this study was to analyze the deletion of *SMN1* and *NAIP* genes in southern Chinese children with SMA. Here, polymerase chain reaction (PCR) combined with restriction fragment length polymorphism (RFLP) was performed to detect the deletion of both exon 7 and exon 8 of *SMN1* and exon 5 of *NAIP* in 62 southern Chinese children with strongly suspected clinical symptoms of SMA. All the 32 SMA1 patients and 76% (13/17) of SMA2 patients showed homozygous deletions for exon 7 and exon 8, and all the 13 SMA3 patients showed single deletion of *SMN1* exon 7 along with 24% (4/17) of SMA2 patients. Eleven out of 32 (34%) SMA1 patients showed *NAIP* deletion, and none of SMA2 and SMA3 patients was found to have *NAIP* deletion. The findings of homozygous deletions of exon 7 and/or exon 8 of *SMN1* gene confirmed the diagnosis of SMA, and suggested that the deletion of *SMN1* exon 7 is a major cause of SMA in southern Chinese children, and that the *NAIP* gene may be a modifying factor for disease severity of SMA1. The molecular diagnosis system based on PCR-RFLP analysis can conveniently be applied in the clinical testing, genetic counseling, prenatal diagnosis and preimplantation genetic diagnosis of SMA.

**Key words:** Spinal muscular atrophy (SMA), Survival motor neuron (*SMN*) gene, Neuronal apoptosis inhibitory protein (*NAIP*) gene, Mutation

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## INTRODUCTION

Spinal muscular atrophy (SMA) is a clinically and genetically heterogeneous group of neuromuscular disorders characterized by progressive muscle weakness because of degeneration and loss of the anterior horn cells of the spinal cord and the brain stem nuclei. SMA is the second most common lethal autosomal recessive disorder after cystic fibrosis (CF) in Caucasian populations with an overall incidence of 1 in 6000 live births and a carrier frequency of approximately 1 in 50 (Frugier *et al*., 2002; Ogino and Wilson, 2004; Darras and Kang, 2007). The onset of weakness ranges from birth to adolescence or young adulthood. Diagnostic criteria vary by age of onset. Three types of SMA are recognized depending on the

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age of onset, the maximum muscular activity achieved, and survivorship: SMA1 (OMIM: 253300), SMA2 (OMIM: 253550) and SMA3 (OMIM: 253400).

Among all the candidate genes, the survival of motor neuron (*SMN*) is believed to be the primary SMA disease-causing gene (Lefebvre *et al*., 1995; Battaglia *et al*., 1997; Hsieh-Li *et al*., 2000; Andreassi *et al*., 2004; Azzouz *et al*., 2004; Schmutz *et al*., 2004; Trülzsch *et al*., 2004; Burnett and Sumner, 2008; Girardet *et al*., 2008). In humans, *SMN* is contained in a 500-kb sequence on chromosome 5q12.2-q13.3 which consists of 9 exons, and is present in two copies: a telomeric one (*SMN1*, or *SMNt*) and a centromeric one (*SMN2*, or *SMNc*). *SMN1* gene has a highly homologous copy with *SMN2*. This copy is present in 90%~95% of normal controls and hampers detection of deletions and mutations within the *SMN1* gene (Frugier *et al*., 2002; Ogino and Wilson, 2004). The coding sequence of *SMN2* exon 7 differs from that of *SMN1* by a single nucleotide (840C<T), which alters a restriction enzyme site and allows one to easily distinguish *SMN1* from *SMN2* using a polymerase chain reaction (PCR)-based assay. The finding of homozygous deletions of exon 7 and/or exon 8 of *SMN1* patients with consistent clinical features is generally considered to be diagnostic of SMA (Lefebvre *et al*., 1995; Chang *et al*., 1995; Chen K.L. *et al*., 1999; Chen W.J. *et al*., 2007; Tsai *et al*., 2001; Su *et al*., 2005; Watihayati *et al*., 2007). The neuronal apoptosis inhibitory protein (*NAIP*) gene located on 5q12.2-q13.3 has been hypothesized to be an SMA modifying gene because of its deletion in approximately two-thirds SMA1 chromosomes and its homology with baculoviral apoptosis inhibitory proteins (Roy *et al*., 1995; Gotz *et al*., 2000).

In this study, we try to confirm the clinical diagnosis of southern Chinese SMA patients and to correlate the frequency of deletions within *SMN* and *NAIP* genes with SMA.

## SUBJECTS AND METHODS

### Study population

A total of 62 unrelated southern Chinese children (aged 1 month to 11 years) with strongly suspected SMA from the Affiliated Children's Hospital, School of Medicine, Zhejiang University, China, between the period of January 2000 and September 2007, and 100 healthy controls (>24 years of age) were investigated. Thirty-two children had a diagnosis of SMA1, 17 had SMA2, and the remaining 13 were diagnosed with SMA3. All of the patients fulfilled the diagnostic criteria on the basis of clinical, electrophysiological, and/or histological examination. The clinical manifestations included reduced muscle tension, decreased or absent tendon reflexes, different degrees of muscle atrophy. Levels of serum creatine phosphokinase (CPK) were found to be characteristically normal. Electromyography (EMG) revealed spontaneous discharge activity in resting muscles, increased amplitude, and prolonged duration of motor unit potentials during voluntary efforts. The muscle biopsies of some patients showed different degrees of atrophy in muscle fibers. The family history is in accordance with autosomal recessive inheritance (Table 1). Eleven patients had been studied previously (Yu *et al*., 2001).

This study was approved by the ethics committee for the protection of human subjects of Zhejiang University School of Medicine, and informed consent was obtained from all individuals.

### Extraction of genomic DNA and PCR

Peripheral blood samples were collected and genomic DNA was extracted using the standard protocol. The primers used for the amplification were as follows (van der Steege *et al*., 1995; Roy *et al*., 1995): exon 7 of *SMN*: 5′-AGACTATCAACTTAATTTCTGATCA-3′ (forward), 5′-CCTTCCTTCTTTTTGATTTTGTTT-3′ (reverse); exon 8 of *SMN*: 5′-GTAATAACCAAATGCAATGTGAA-3′ (forward), 5′-CTACAACACCCTTCTCACAG-3′ (reverse); exon 5 of *NAIP*: 5′-CTCTCAGCCTGCTCTTCAGAT-3′ (forward), 5′-AAAGCCTCTGACGAGAGGATC-3′ (reverse); exon 13 of *NAIP*: 5′-ATGCTTGGATCTCTAGAATGG-3′ (forward), 5′-CCAGCTCCTAGAGAAAGAAGGA-3′ (reverse).

PCR was performed on an ABI-2720 Thermal Cycler (Applied Biosystems, Foster City, California, USA) with a heated lid. Each reaction was carried out in a 50-μl volume containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 μmol/L each primer, 200 μmol/L each dNTPs, approximately 200 ng genomic DNA, and 1 U

TaKaRa Ex Taq polymerase (TaKaRa, Shiga, Japan) (He *et al*., 2004). For *SMN* gene, amplification consisted of an initial denaturation step at 94 °C for 3 min, then 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 7 min. One normal sample as a negative control and one sample from a patient known to have exon 7 and exon 8 deletion (courtesy by Dr. Ke-lian Chen at University of Pennsylvania Medical Center) as a positive control were included in each PCR run. PCR amplification produced 190-bp and 187-bp fragments, respectively. For *NAIP* gene, a multiplex PCR was carried out, the annealing temperature was 60 °C, and the 435-bp (exon 5) and the 241-bp (exon 13) bands appeared (exon 13 taken as positive control).

**Restriction enzyme digestion for *SMN* and gel electrophoresis**

*SMN* PCR products (4 μl) were subsequently digested with restriction enzyme *Dra* I or *Dde* I (1 U) (New England Biolabs, Beverly, Massachusetts, USA), and the resultant bands were visualized in 4% (w/v) agarose gel stained with ethidium bromide under UV light. For *Dra* I digestion of exon 7, *SMN2* was cut into 170-bp and 20-bp fragments, and *SMN1* remained as a 190-bp fragment. For exon 8, after *Dde* I digestion, *SMN2* had 119-bp and 68-bp fragments, while *SMN1* had a 187-bp band.

## RESULTS

Thirty-two SMA1 children with age ranged from 1 month to 18 months were unable to sit or hold up their heads and were floppy toddler. They were found to have homozygous deletions for *SMN1* gene (exon 7 and exon 8), while 11 of 32 (34%) had a deletion in the *NAIP* gene (exon 5).

Seventeen SMA2 patients, from 2 to 3 years old, were unable to walk. The majority of them showed wasting and areflexia of the lower limbs. Thirteen (76%) had deletions in both exon 7 and exon 8 of *SMN1*, while 4 (24%) had deletions only in exon 7 of the *SMN1* gene.

All the 13 SMA3 patients aged from 3.5 to 11 years had slowly progressive weakness of the lower limbs. These patients revealed *SMN1* gene deletion of exon 7 only.

None of SMA2 and SMA3 patients lacked *NAIP* exon 5.

In 100 normal controls, only 2 individuals deleted exon 7 of *SMN1*.

The results are summarized in Table 1.

## DISCUSSION

According to the survey of Chung *et al*.(2003), SMA was the second most frequent inherited neuromuscular disease in southern Chinese children. The prevalence of SMA in Hong Kong, a major city in southern China with a population of around 6.7 million, is $18.7\times10^{-6}$ (about 1 in 53000 children). The carrier rate for deletional SMA amongst the general population in Hong Kong is 1.6%, which is close to the 2% quoted in western countries (Chan *et al*., 2004). The clinical manifestation of SMA is similar to many other neuromuscular diseases. It is difficult to identify SMA only from patients' clinical symptoms and physical signs. So far, SMA is diagnosed primarily through a blood DNA testing including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), single-strand conformation polymorphism (SSCP), denatured high-performance liquid chromatography (DHPLC), real-time quantitative PCR, and multiplex ligation-dependent probe

**Table 1 Genotype-phenotype correlation of 62 southern Chinese SMA patients**

| SMA type | Age | Patient number | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Gender | | Family history | | EMG* | Muscle biopsy (+)** | *SMN1* deletion | | *NAIP*-E 5 deletion | Deletion of both *SMN1*-E 7 & E 8 and *NAIP*-E 5 |
| | | M | F | Pos. | Neg. | | | E 7 & E 8 | E 7 only | | |
| SMA1 | 1~18 months | 17 | 15 | 12 | 20 | 32 | 7 | 32 (100%) | 0 (0%) | 11 (34%) | 11 (34%) |
| SMA2 | 2~3 years | 11 | 6 | 4 | 13 | 17 | 4 | 13 (76%) | 4 (24%) | 0 (0%) | 0 (0%) |
| SMA3 | 3.5~11 years | 8 | 5 | 3 | 10 | 13 | 5 | 0 (0%) | 13 (100%) | 0 (0%) | 0 (0%) |
| Total | | 36 | 26 | 19 | 43 | 62 | 16 | 45 (73%) | 17 (27%) | 11 (18%) | 11 (18%) |

M: male; F: female; Pos.: positive; Neg.: negative; E: exon; EMG: electromyography. *EMG shows neurogenic abnormalities; **Muscle biopsies of skeletal muscle show different changes of denervation with small groups of atrophic muscle fibers associated with markedly hypertrophied fibers. Most patients (46 cases) refused to take this procedure

amplification (MLPA). MLPA detects the presence or absence of the *SMN1* gene that is present in normal individuals, in conjunction with a suggestive history and physical examination (Huang *et al*., 2007). PCR-RFLP method was constructed by van der Steege *et al*.(1995) in 1995, which can diagnose SMA quickly and has been applied by clinicians for many years (van der Steege *et al*., 1995; Watihayati *et al*., 2007). For exon 7, there is no known difference in restriction site. Therefore, a specific oligonucleotide prime directly adjacent to the variant site was introduced, which allows a mismatch such that a restriction site for *Dra* I is created in the PCR product of exon 7 of the *SMN2*, i.e., *Dra* I will cleave *SMN2* exon 7 specifically. And *SMN1*/*2* exon 8 homozygous absence can be distinguished by a restriction enzyme (*Dde* I) digestion and gel electrophoresis. *SMN2* is cut with *Dde* I while the *SMN1* is not.

Here we concentrated on the alterations in these 2 exons and *NAIP* exon 5 in 62 southern Chinese children in Zhejiang Province, who were strongly suspected to have SMA. It was observed that the majority of patients with more *SMN1* gene deletions resulted in a severe phenotype. Our results of exon 7 deletion were consistent with the studies from Taiwan, Hong Kong, and Fujian Province, China (Chang *et al*., 1995; 1997; Tsai *et al*., 2001; Wong and Chan, 2001; Su *et al*., 2005; Chen W.J. *et al*., 2007). Thus, deletion of the *SMN1* exon 7 is a major cause of SMA in southern China.

Several investigations suggested the possibility of *NAIP* involvement in the development of SMA (Roy *et al*., 1995; Gotz *et al*., 2000; Watihayati *et al*., 2007). All the patients enrolled in this study showed the deletion of the *SMN1* gene, so we eliminated the clinical bias and looked at only the frequency of *NAIP* deletion among these SMA patients lacking *SMN1*. After *SMN1* deletion was confirmed in these patients, *NAIP* deletion was analyzed. In our study, 34% SMA1 patients were found to lack *NAIP* exon 5. Therefore, the *NAIP* gene deletion seems to affect disease severity. In fact, 24 SMA1 patients have died later, and we had no further details of 8 patients because of missing follow-up.

The PCR-RFLP test used in this study is fast, sensitive, and inexpensive, and forgoes the need for invasive diagnostic procedure like a muscle biopsy from the patients (mostly children). Also, it is particularly applicable for prenatal diagnosis and preimplantation genetic diagnosis (PGD). The limitation of this method is that smaller rearrangements or point mutations of *SMN* gene can also result in a large series of SMA patients (Wirth, 2000; Tsai *et al*., 2001; Su *et al*., 2005). Further analyses revealed that *SMN2* copy number has been well established as a modifying factor of clinical severity. The absence of *SMN* gene in SMA1 is associated with gene dosage effect, whereas no gene dosage effect was detected in SMA2 or SMA3 (Lefebvre *et al*., 1995; Campbell *et al*., 1997; Frugier *et al*., 2002; Yamashita *et al*., 2004). These observations raised the hypothesis of a gene deletion event in SMA1 and a gene conversion event in SMA2 or SMA3, which would result in an increased number of *SMN2* copies (Lefebvre *et al*., 1995; Campbell *et al*., 1997; Talbot *et al*., 1997; Frugier *et al*., 2002; Yamashita *et al*., 2004). Therefore, the copy numbers of *SMN1* and *SMN2* genes should be determined by the point mutation and gene dosage analysis. In addition, PCR-based assay for determining the presence or absence of *SMN1* is not quantitative, and therefore, cannot identify SMA carriers. The genomic complexity of the *SMN* region and its high degree of variability hamper the ability to directly screen the SMA carriers. Thus the comprehensive SMA tests including SMA deletion analysis, linkage analysis, and SMA heterozygosity detection should be the most complete evaluation of the clinical diagnosis or suspicion of SMA (Chan *et al*., 2004; Dastur *et al*., 2006; Chen W.J. *et al*., 2007).

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