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Deletion of *SMN* and *NAIP* Genes in Korean Patients with Spinal Muscular Atrophy

Childhood-onset proximal spinal muscular atrophies (SMAs) are an autosomal recessive, clinically heterogeneous group of neuronopathies characterized by selective degeneration of anterior horn cells. The causative genes to be reported are survival motor neuron (*SMN*) and neuronal apoptosis inhibitory protein (*NAIP*) genes. The deletion of telomeric copy of *SMN* (*SMN*^{T}) gene was observed in over 95% of SMAs. The deletion rate of NAIP gene is 20-50% according to disease severity. The objective of this article is to genetically characterize the childhood-onset spinal muscular atrophy in Koreans. Five Korean families (14 constituents containing 5 probands) with SMA were included in this study. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were used for the deletion analysis of *SMN*^{T}. Multiplex PCR method was used for *NAIP* analysis. Four probands showed deletion of *SMN*^{T} gene. Deletion of *SMN*^{C} (centromeric *SMN*) gene was found in one proband who did not show the deletion of *SMN*^{T} gene and in the father of one proband who showed the deletion of *SMN*^{T} gene. The deletion of *NAIP* gene was not found among all the studied individuals. The extent of deletion in Koreans was smaller than that in other studied population. PCR-RFLP deletion analysis can be applied to diagnose SMA and make a prenatal diagnosis.

Key Words: *Muscular Atrophy; Neuromuscular Manifestations; Gene Deletion*

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INTRODUCTION

Childhood-onset proximal spinal muscular atrophies (SMAs) are autosomal recessive diseases characterized by degeneration of the anterior horn cells of the spinal cord, resulting in symmetrical limb muscle atrophy and weakness. SMA is, in its severe form, the most common genetic disorder lethal to infants and, in its milder forms, the second most common pediatric neuromuscular disorder after Duchenne muscular dystrophy. SMA affects between 1 in 6,000 and 1 in 10,000 live births (1) with a carrier frequency from 1 in 40 to 1 in 60.

Based on the age at the time of onset and severity, SMA is classified into three types (2). Type I SMA (Werdnig-Hoffmann) is acute and fatal with an onset in the first 6 months and death before 2 years of age. These children never raise their heads or sit unattended, and they usually die from infection secondary to respiratory insufficiency. Type II SMA is less severe with clinical signs appearing before 18 months of age; an affected patient is able to sit unaided, but never walks, and death usually follows at about 2 years of age. Type III SMA (Kugelberg-Welander) is the mildest form of SMA with an onset age of after 18 months; the patients learn to walk unaided for long periods in their lives.

Until recently, the most useful diagnostic tools for SMA were electromyography (EMG) and muscle biopsy. The diagnostic criteria were based on the consensus attained at the international SMA consortium meeting held in Bonn, June 1992 (Table 1). However, with an advance in molecular genetics, the diagnosis of SMA is possible on the basis of positional cloning of the causative genes. Two candidate genes that may have a direct involvement in the pathogenesis of SMA are mapped to chromosome 5q11.2-q13.3 (3-7).

The SMA locus containing two inverted copies of a 500 kb element was characterized by an abundance of low-copy repeat DNA sequences. In the telomeric 500 kb region, two candidate genes, the neuronal apoptosis inhibitor protein (*NAIP*) gene and survival motor neuron (*SMN*) gene, separated by 15.5 kb, are positioned in a tail-to-tail (5'→3':3'→5') orientation (8) (Fig. 1A).

SMN gene, spanning 28 kb genomic region with 1.7 kb transcript consists of 8 exons, is associated with all

Table 1. Diagnostic criteria for spinal muscular atrophy (International SMA Consortium, 1992)

Inclusion criteria	Exclusion criteria
Symmetrical muscle weakness of trunk and limbs	Involvement of extraocular muscles, diaphragm and myocardium
Proximal muscles > distal	Marked facial weakness
Lower limbs > upper limbs	CNS dysfunction
Fasciculation of tongue, tremor of hands	Athrogryposis
Neurogenic changes in EMG and muscle biopsy	CK activity > 10 times normal
	Reduction of motor NCV < 70% of lower normal limit or abnormal sensory nerve action potentials

CNS, central nerve system; EMG, electromyography; NCV, nerve conduction velocity; CK, creatine kinase

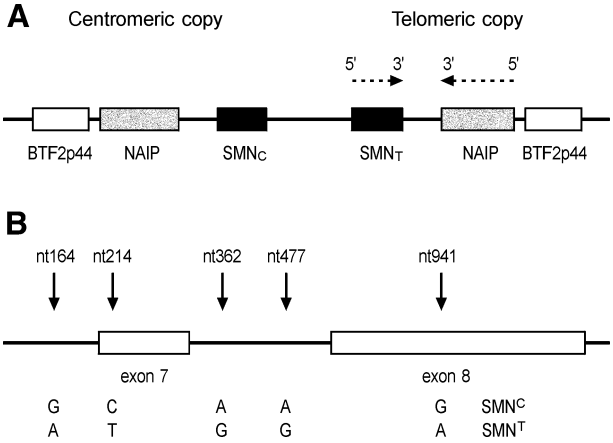

Fig. 1. A: Schematic genomic map of SMA critical region on 5q13.1. B: Exon 7 and exon 8 of survival motor neuron gene show five sequence differences between the centromeric (*SMN*^{C}) and telomeric (*SMN*^{T}) copies (9).

three types of SMA. There are two *SMN* genes, designated telomeric (*SMN*^{T}) and centromeric (*SMN*^{C}) copy genes. These genes are almost identical except in 5 different sites including sequences within exon 7 and 8, which are used to distinguish telomeric copy from centromeric one. These differences do not affect the sequence of the coded protein.

Single strand conformation polymorphism (SSCP) analysis shows an absence of the telomeric copy of SMN exon 7 and exon 8 in 93% of SMA patients, while exon 7 deletion only is found in 5.6% of all SMA patients. The absence of exon 7 may be the result of either a deletion or a sequence-conversion event between the *SMN*^{C} and *SMN*^{T} gene. Point mutations of *SMN*^{T} gene are also identified in some SMA patients (1.3%) who have at least one intact *SMN*^{T} (9).

The other candidate, *NAIP* gene, was identified in the same region. The *NAIP* gene, spanning 56 kb of genomic sequence with 6.1 kb transcript, consists of 17 exons and is present in multiple and variable copy numbers on chromosome 5; however, exons 5 and 6 appear to be unique to the intact *NAIP* gene which is present in telomeric part (10). Deletion of intact *NAIP* gene is associated with SMA. Homozygous deletion of *NAIP* exon 5, or exons 5 and 6 was more commonly observed in the severe and acute form of SMA than mild or chronic forms (11, 12). Deletion of *NAIP* is found in approximately 45% of type I SMA and 18% of types II and III SMA. The estimated frequencies of exons 5 and 6 deleted chromosomes were 67% in type I SMA and 42% in type II and type III SMAs. The deletion of *NAIP* gene tends to be found in more severe cases, therefore it seems to modify the disease severity rather than to have a direct effect on the disease (13, 14).

According to a recent revision of original *NAIP* cDNA, three fundamental changes were identified: loss of exon 1, addition of a novel exon between original exon 14 and 15, and replacement of exon 17 with a new exon. As a consequence, a full-length human *NAIP* cDNA comprised of 17 exons and original nomenclature exon 5 and 6 have to be replaced by newly named exon 4 and 5, respectively (8).

The objective of this article is to genetically characterize the childhood-onset spinal muscular atrophy in Koreans. The alterations of *SMN* gene and *NAIP* gene were analyzed using PCR based exon deletion test.

MATERIALS AND METHODS

Subjects

Five Korean probands with childhood onset SMA and their 9 parents were included in this study. Diagnosis and classification of childhood onset SMA were made by electromyography and muscle biopsy as well as neurological examination. Clinical examinations of the probands were consistent with a diagnosis of spinal muscular atrophy: symmetrical weakness more pronounced in proximal than distal parts. Neurologic disability was limited to the lower motor neurons with no evidence of sensory or intellectual impairment. Muscle biopsy showed fiber group atrophy indicative of denervation and reinnervation. Electromyography confirmed denervation. The pa-

Table 2. Clinical characteristics of five probands with childhood-onset spinal muscular atrophy

Proband ID number*	SMA type	Onset	Sex	Age/ Death†	CK/LDH (U/L)	Motor development	EMG/NCV	Muscle biopsy	Additional clinical feature
21	Type I	5 m	Female	16 mo	135/282	Roll over: 6 mo. Sit balance:14 mo.	r/o congenital myopathy	c/w SMA	Atlantoaxial subluxation, scoliosis
31	Type I	3 m	Male	7 mo	70/241	Head control; sit by assistance	c/w MND	c/w SMA	Tongue fasciculation
41	Type I	birth	Male	5 mo†	177/302	-	Sensorimotor polyneuropathy	c/w SMA	
51	Type II	12 m	Female	16 mo	179/328	Sitting balance; stand with aid	ant. horn cell disease	c/w SMA	Mainly lower extremity weakness
61	Type III	6 yr	Female	17 yr	230/239	Walking	c/w MND	c/w SMA	Finger resting & intentional tremor Calf muscle pseudohypertrophy

*The first number indicates the family number and the second number (1) designates the proband.
†Expired proband was designated by the age of death.
EMG, electromyography; NCV, nerve conduction velocity; MND, motor neuron disease; r/o, rule out; c/w, compatible with

tients' characteristics and individual identification numbers are listed in Table 2.

Extraction of genomic DNA from blood samples

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 (15). The quantitation of DNA was done spectrophotometrically at 260 nm.

Analysis of *SMN*^{T} gene deletions

Among the five nucleotide variations distinguishing centromeric (*SMN*^{C}) from telomeric *SMN* gene (*SMN*^{T}), two exonal variation sites around exon 7 and exon 8 were selected (Fig. 2B). The presence of exons 7 and 8 of the *SMN*^{C} and *SMN*^{T} copy genes was determined using DNA

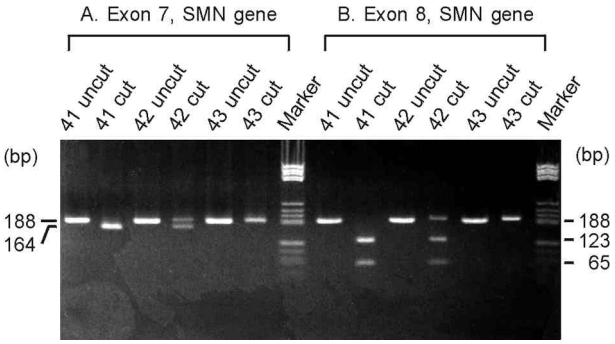

Fig. 2. Deletion analysis of *SMN* gene. Exons 7 and 8 of *SMN* gene were amplified and cut by *Dra* I and *Dde* I, respectively. Lane number is the case number (Table 2). Uncut and cut means the PCR products before and after the restriction enzyme digestion. 42 (normal) showed the products of both *SMN*^{C} and *SMN*^{T} copies. 41 and 43 had the deletion of *SMN*^{T} and *SMN*^{C} copy, respectively.

based polymerase chain reaction and restriction enzyme digestion. PCR products of exon 8 of the *SMN*^{T} gene and the *SMN*^{C} gene were readily distinguishable by the presence of the recognition site for enzyme *Dde*I, of which the site is absent in *SMN*^{T}. For exon 7, a mismatched downstream oligonucleotide primer directly adjacent to the variant site that contains the restriction site to create *Dra*I site in the PCR product of *SMN*^{C} exon 7 (Table 3) (16).

Polymerase chain reaction was carried out using 200 ng of genomic DNA in a 50 μL reaction mixture containing 1.25 units of Taq DNA polymerase (Boerhinger Mannheim, Germany), 200 μM dNTPs, 20 pmoles of each upstream and downstream primer and PCR reaction buffer (10 mM Tris-HCl; 1.5 mM $MgCl_2$; 50 mM KCl; pH 8.3). For exon 8, a cycle consisted of 60 second at 95℃ (denaturation), 60 second at 59℃ (annealing), and 60 second at 72℃ (extension) was repeated 35 times with an initial 5 min denaturation at 95℃ and 7 min final extension at 72℃ with GeneAmp PCR 9600 (Perkin-Elmer Cetus, U.S.A.). Exon 7 DNA was amplified with the same PCR condition except for an annealing temperature of 55℃. Amplified products were electrophoresed on 1% agarose gel to confirm the PCR products.

PCR products were subsequently digested with *Dra*I (New England Biolabs, Beverly, MA, U.S.A.) for exon 7 and *Dde*I (Boerhinger Mannheim, Germany) for exon 8. PCR products and their digested products were electrophoresed on 3% agarose gel.

Analysis of *NAIP* gene deletions

For the detection of deletion in *NAIP* gene, exon 4 and exon 5 were amplified. Each reaction was multiplexed with the primers for exon 12 as a positive control for PCR amplification. Oligonucleotide primers are

Table 3. Oligonucleotide primers for PCR analysis of SMN and NAIP genes

Gene	Amplified exon	Direction	Primer sequence	PCR product (bp)
SMN	Exon 7	Upstream	5-CTA TCA ACT TAA TTT CTG ATC A-3'	188
		Downstream	5'-CCT TCC TTC TTT TTG ATT TTG TT*T-3'	
	Exon 8	Upstream	5-GTA ATA ACC AAA TGC AAT GTG AA-3'	188
		Downstream	5'-CTA CAA CAC CCT TCT CAC AG -3'	
NAIP	Exon 4	Upstream	5-AAA GCC TCT GAC GAG AGG ATC-3'	436
		Downstream	5-CTC TCA GCC TGC TCT TCA GAT-3'	
	Exon 5	Upstream	5-TGC CAC TGC CAG GCA ATC TAA-3'	128
		Downstream	5-CAT TTG GCA TGT TCC TTC CAA G-3'	
	Exon 12	Upstream	5-CCA GCT CCT AGA GAA AGA AGG A-3'	242
		Downstream	5-ATG CTT GGA TCT CTA GAA TGG -3'	

*mispriming sequence to create *Dra* I site

shown in Table 3. The PCR conditions were same as those used for *SMN* gene except using the annealing temperature of 60℃ and cycle number of 25 (10).

RESULTS

The SMA population we collected was analyzed for the absence of SMN^T exon 7 and exon 8 (Fig. 2) and the absence of intact *NAIP* exon 4 and 5 (Fig. 3). We detected the homozygous deletion of exon 7 and exon 8 in SMN^T gene in four probands out of five childhood onset SMA families. There were no patients who had homozygous deletion of exon 7 without deletion of exon 8 in SMN^T gene. Proband 21, who did not show SMN^T deletion, showed homozygous deletion of SMN^C gene. The father of proband 41 also showed deletion of SMN^C gene. All five SMA probands and their 9 family members studied showed no homozygous deletion of exon 4 or exon 5 in *NAIP* gene, so they all seemed to have intact *NAIP* gene (Table 4).

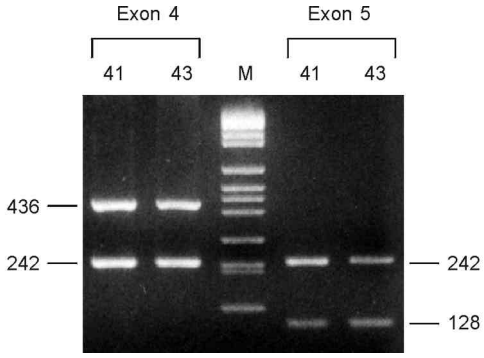

Fig. 3. Results of PCR amplification of *NAIP* gene. Exons 4 and 5 of *NAIP* gene were amplified with exon 12 which was the internal control. 436 bp, 128 bp, and 242 bp were PCR products of exons 4, 5 and 12, respectively. Lane number is the case number (Table 2). *NAIP* genes were not deleted in all of the cases.

Table 4. Results of SMN^T, SMN^C and *NAIP* gene analysis in the patients with spinal muscular atrophy and their family members

ID number*	SMN^T		SMN^C		*NAIP*	
	Exon 7	Exon 8	Exon 7	Exon 8	Exon 4	Exon 5
21	+	+	−	−	+	+
22	+	+	+	+	+	+
23	+	+	+	+	+	+
31	−	−	+	+	+	+
32	+	+	+	+	+	+
41	−	−	+	+	+	+
42	+	+	+	+	+	+
43	+	+	−	−	+	+
51	−	−	+	+	+	+
52	+	+	+	+	+	+
53	+	+	+	+	+	+
61	−	−	+	+	+	+
62	+	+	+	+	+	+
63	+	+	+	+	+	+

*ID number is in accordance with the number in Table 2.
The second digit (1,2,3) indicates the proband, the mother and the father, respectively.
(+) and (−) denote presence or absence of exon

DISCUSSION

Since Lefebvre (9) reported that the telomeric *SMN* gene (SMN^T) was either lacking or interrupted in 98.6% of SMA patients and the remaining 1.4% of SMA patients carried either point mutation or short deletions, many articles were published on *SMN* gene and *NAIP* gene analysis in different countries. The frequencies of SMN^T exon 7 and/or exon 8 deletion are above 90% in SMA type I and above 75% in types II & III SMA patients. As to *NAIP* gene deletion, approximately 50% deletion frequency was reported in type I SMA patients and 20% in the milder forms. Considering the sequence around the *SMN* gene and *NAIP* gene, the severity of the clinical disease tends to correlate with a larger deletion range.

In our study, four probands in five SMA families were found to have deletion of exons 7 and 8 in SMN^T gene. The overall frequency of SMN^T deletion in our study was 80% (4/5). The proband 21 showed SMN^C gene deletion, but it was also reported even in normal population (5.4%) (17). Thus, the manifestation of the SMA phenotype in the proband 21 (type I SMA) cannot be explained with the presence of SMN^C deletion, meaning further studies including point mutation analysis on this subject should be necessary.

According to the *NAIP* gene study, there was no proband who showed the deletion of intact *NAIP* gene irrespective of the disease types. Therefore, the maximum deletion ranges observed in Korean patients seemed to be SMN^T gene only and were not to extend to *NAIP* gene. The absence of *NAIP* gene deletion means that the deletion ranges in Korean SMA patients are smaller compared with those of other reports, which authors suggested the association of deletion extents with disease severity. Although deducing a specific conclusion was difficult in our study due to the small number of participants, relatively small deletion ranges may be one of the characteristics observed among the different SMA types in Koreans.

The association between SMA disease severity and deletion in the intact *NAIP* gene, coupled with *NAIP* gene function as an inhibitor of apoptosis (18), suggests that NAIP gene may play a role in modifying the disease severity. But the mutations in the intact *NAIP* gene alone may not be sufficient for the development of a clinical phenotype. Complete or partial loss of SMN^T gene function results in the selective degeneration of α -motor neuron cells in SMA. One potential pathogenic model is that the absence of SMN^T gene is presented as the primary neurotoxic insult, with the deletion of *NAIP* gene leading to an attenuated apoptotic resistance, exacerbating motor neuron attrition. Another explanation is that the *SMN* gene encodes a new protein, which appears to be involved in RNA metabolism. Therefore SMA is the result from the decreased dosage of the new protein. This hypothesis suggests that motor neurons are highly sensitive to even a slight decrease in the SMN protein level (19).

Many authors reported that the lack of genotype-phenotype correlation might be explained by the presence of a nearby disease-modifying gene that could get lost in the process of deletions associated with type I SMA. Some disease modifying gene must be tightly linked to *SMN* gene. Thus, a rigorous definition of how far deletions extend on type I SMA chromosomes is central to understanding the disease pathogenesis. The most frequently observed genotype reported on SMA type I chromosome is the deletion of *NAIP* gene as well as *SMN* gene, which resulted in a minimal deletion size of 60 kb (8). In this minimal deletion region, a critical disease modifying gene may be present according to this hypothesis. Another explanation for the lack of genotype-phenotype correlation is that the number of *SMN* gene copy is associated with the disease severity. For example, in mild cases such as in type III patients, the presence of a centromeric exon 7 linked to a telomeric derived exon 8 suggests that the different phenotypes of SMAs would depend on the copy number of SMN^C gene, compensating the lack of SMN^T gene on each chromosome. But in our study, SMN^T exon 7 deleted patient without exon 8 deletion was not found; thus, confirming chimeric gene effect was not possible.

Although the role of SMN^C and SMN^T genes in the pathogenesis of SMA is still uncertain, so far the deletion of SMN^T is the most important clue to diagnose SMA. Transcripts of the two 20 kb genes differ at only 2 nucleotides in the terminal exons 7 and 8, but these differences do not affect the sequence of the coded protein. Several methods, including SSCP and PCR-RFLP, have been used to discriminate SMN^C and SMN^T. The disadvantages of SSCP are the misleading problem of empty band in non-amplification case due to primer site mutation and incorporation of radioactive material.

The PCR and restriction fragment length polymorphism (PCR-RFLP) method are clear and unambiguous, and their results can be obtained within two days. However, gene conversion event and point mutation of SMN^T gene cannot be detected with this method. But the mutation frequency in SMA is small (<5%), so PCR-RFLP method for the detection of SMN^T gene deletion can be used with more than 95% sensitivity for diagnostic purpose (19).

In many reports, gene dosage test is used to detect the carriers of *SMN* gene deletion, who seem to have half the number of *SMN* gene compared to a normal in-

dividual, and distinguish between a non-SMA phenocopy patient (who has normal gene dosage) and a compound heterozygote SMA patient with absent SMN^T gene on one chromosome and an unknown alteration in SMN^T gene on the other chromosome (20).

In this study, we evaluate the deletion of *SMN* gene and *NAIP* gene with recently advanced molecular genetic techniques in Korean families with childhood onset SMA. This PCR based deletion study will play a major role in the accurate diagnosis and prenatal genetic counselling of childhood onset SMA in the future.

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