Molecular diagnosis of spinal muscular atrophy

Ki-Sun Lee, Hee-Yu Hwang, Key-Hyoung Lee, Moon-Sung Park, Si-Houn Hahn[1] and Chang-Ho Hong

Spinal muscular atrophy (SMA) is the second most common fatal disease of childhood with autosomal dominant mode of inheritance, and in its less severe form the third most common neuromuscular disease of childhood after Duchenne muscular dystrophy. The genetic defect was found to be on the long arm of chromosome 5 (5q11.2-q13.3) where many genes and microsatellite markers were missing. One of the most important genes is the Survival Motor Neuron (SMN) gene which is homozygously missing in 90% of SMA patients. Another important gene, the Neuronal Apoptosis Inhibitory Protein (NAIP) gene was found to be defective in 67% of SMA type I patients. Studies so far suggest SMA occurs when the genes on the long arm of chromosome 5 are mutated or deleted. Recently our hospital encountered 2 SMA patients of type I and II respectively. These patients both had homozygously defective SMN genes but intact NAIP genes. We are reporting these cases with bibliographic review and discussion. Korean SMA patients presumably have defects in SMN genes similar to those found in Caucasian patients, although the siginificance of NAIP genes remains to be established. SMN gene defects can be easily diagnosed using PCR and restriction enzymes, and this method could be applied towards convenient prenatal diagnosis and towards screening for family members at risk.

Keywords: Spinal muscular atrophy, Survival motor neuron (SMN) gene, Neuronal apoptosis inhibitory protein (NAIP) gene

METHOD

SMN gene is known to have a highly homogeneous copy gene in the centromeric region (Fig. 1). This gene is intact in 95.5% of normal subjects, and could complicate the interpretation of the test results. The two genes differ by only 5 base-pairs, 2 of which exist on exon 7 and 8. In exon 8, this single base-pair variation creates a cleavage site on the copy gene for DdeI restriction enzyme. The authors examined the banding patterns after PCR and restriction enzyme treatment. Exon 8 of SMN copy gene has a cleavage site for DdeI whereas SMN^{tel} gene does not, so the banding pattern can easily differentiate the genes. In exon 7 no specific restriction site existed, so we employed mismatch priming method to create a restriction site on the copy gene for DraI. The primers used to amplify exon 8 were 5'-GTAATAACCAAATGCAATG TGAA-3' and 5'-CTACAACACCCTTCTCACAG-3'. The PCR product was cleaved using DdeI and was electrophoresed on 4% metaphor agarose gel. The primers for exon 7 were intron 6 primer 5'-AGACTATCAACTTAATTTCTGATCA-3' and mismatch primer 5'-CCTTCCTTCTTTTTGATTTTGTTT-3'. PCR was carried out 35 times at 94°C for 1 min, 57°C for 1 min, 72°C for 2 min. The amplified product was cleaved using DraI and was electrophoresed on 4% metaphor agarose gel.

NAIP gene presence can be tested by amplifying exon 5 using PCR. The primers used were 5'-ATATAGGTAAACA GGACAAGG-3' and 5'-TGGGGAACCATTTGGCATG-3'. PCR was carried out 30 times, at 94°C for 1 min, 57°C for 1 min, 72°C for 2 min.

RESULTS

The experiment design involved 2 patients and 3 normal subjects. Normal subjects had 3 bands for exon 8 after cleavage; one for the SMN^{tel} gene, and two for the fragments of the copy gene. However, the patients displayed only 2 small bands for the fragments of the copy gene. Fig. 2A shows these results, demonstrating that both patient 1 and 2 have SMN^{tel} gene defects. The banding patterns for exon 7 draw similar conclusions. Normal subjects have one 187bp and other smaller PCR products, whereas the patients have

Department of Pediatrics, Ajou University School of Medicine, Suwon 442-749, Korea
Correspondence: Si-Houn Hahn, Department of Pediatrics, Ajou University School of Medicine, San 5, Wonchon-dong, Paldal-ku, Suwon 442-749, Korea
Tel: 82-331-219-5166, Fax: 82-331-219-5169

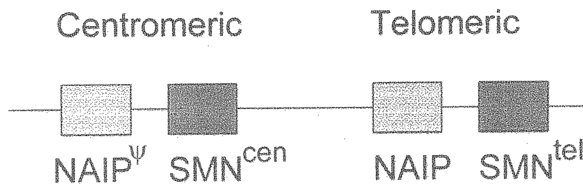

Fig. 1. Candidate genes for SMA lie in an inverted duplication spanning 500kb on 5q13.

only the smaller PCR pruducts (Fig. 2B). We concluded that the copy gene was intact but the SMN^{tel} gene was defective in the patients.

PCR of exon 5 of NAIP gene amplified the genes in both the patient and normal control groups, showing that NAIP gene was not defective in the patients.

DISCUSSION

SMN is a clinically and genetically heterogeneous group of diseases caused by loss or degeneration of anterior horn cells and brainstem nuclei, and resulting in symmetric muscular weakness. Sensory neurons and upper motor neurons are not affected. We do not as yet know the exact mechanism by which the disease develops and no definitive treatment is available, so genetic counselling and determination of prognosis is clinically very important. Classification of SMA is based on age of onset, severity, main lesions, and mode of inheritance, although the lack of biochemical and genetic data raises much doubt as to its validity. Recent developments in molecular biology has enabled us to accurately diagnose this disease by studying SMN genes on chromosome 5.

SMA has two modes of inheritance, AD and AR, but AD form is very rare and clinical distinction is often difficult. The most severe type I cases have an incidence rate of 1 child in 10,000-25,000, whereas type II and III each strike about 1 child in 25,000-83,000. Assuming that all AR form of SMA have an incidence rate of 1 in 10,000, this disease is a relatively common disease with a carrier rate of 1 in 50. AR form of SMA is a disease claiming many lives in early childhood and genetic counselling is essential. Diagnostic criteria for SMA, which was laid out at the 1990 International SMA consortium, include age of onset, severity (course, age of death), muscle weakening pattern, other accompanied symptoms, and mode of inheritance (Table 1). Classification of SMA is shown in table 2. Genetic diagnosis would be helpful in patients with anterior horn cell diseases that do not meet the criteria for typical SMA. Prenatal diagosis would also be useful considering the severity of the disease, and if

Table 1. Diagnostic Criteria of Proximal Spinal Muscular Atrophy (International SMA Consortium)

Inclusion Criteria	Exclusion Criteria
Symmetric muscle weakness of trunk and limbs	Involvement of extraocular muscles, diaphargm and myocardium
Proximal muscle > distal	Marked facial weakness
Lower limbs > upper limbs	CNS dysfunction
Fasciculations of tongue, tremor of hands	Arthrogryposis
Neurogenic changes in EMG and muscle biopsy	CK activity > 10 times the upper normal limit
	Reduction of motor nerve conduction velocities <70% of lower normal limit or abnormal sensory nerve action potentials

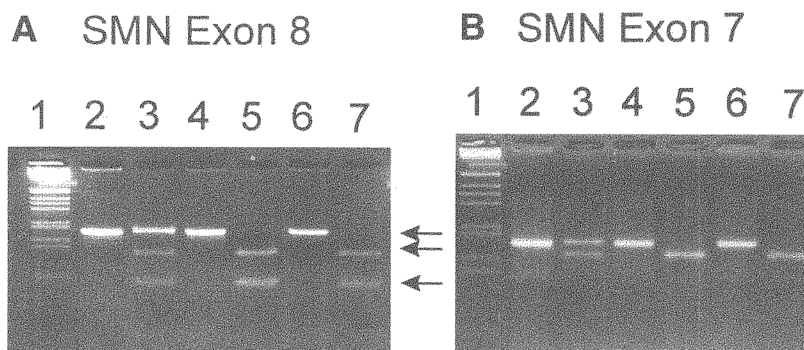

Fig. 2. Demonstration of SMN gene deletions in SMA patients by presence or absence of specific PCR products. **A.** Exon 8 amplification. Lane 1, standard marker; lane 2, control individual; lane 3, control individual digested with *DdeI*; lane 4, patient-1; lane 5, patient-1 digested with *DdeI*; lane 6, patient-2; lane 7, patient-2 digested with *DdeI*. **B.** Exon 7 amplification. Lane 1, standard marker; lane 2, control individual; lane 3, control individual digested with *DraI*; lane 4, patient-1; lane 5, patient-1 digested with *DraI*; lane 6, patient-2; lane 7, patient-2 digested with *DraI*.

Table 2. Classification of Proximal Spinal Muscular Atrophy

SMA Type	Principal Synonyms	Definition	Genetics
I	Werdnig-Hoffmann disease Acute infantile SMA	Sitting not achieved Onset usually within the first 6 months Death > 90% by 10 years	Autosomal recessive
II	Chronic childhood SMA Arrested Werdnig-Hoffmann disease Intermediate SMA	Unaided sitting possible, walking not achieved Onset usually in the first year of life Survival > 90% by 10 years	Autosomal recessive
III	Kugelberg-Welander disease Juvenile SMA	Walking without aids achieved IIIa:Onset < 3yr;IIIb:Onset > 3yr Mild course, life span not markedly reduced	Autosomal recessive in at least 80% Autosomal dominant mutations in-20%(?)
IV	Adult SMA	Onset > 30years Variable severity, normal life span	Autosomal dominant in-70% Autosomal recessive in-30%

SMN gene defect is found, linkage analysis guarantees 90% accuracy of diagnosis.

Since Austrian neurologist Guido Werdnig first reported SMA cases in 1891, and 2 years later German neurologist Johann Hoffmann reported 4 more cases, research has shown that the genetic defect is on the long arm of chromosome 5, where many microsatellite markers and genes were defective. The most siginificant of these defects were homozygous SMN and NAIP deletion.

95% of type I SMA, the severest form, had defects in exon 7 and 8. 94% and 82% of type II, III respectively had the defects, and type IV patients have also been reported to have the defects. NAIP gene defects were found in 46-67%, 17-18%, 7% of type I, II, III respectively. However, SMN gene defects are not found in at least 5% of patients clinically diagnosed as definite SMA type I, and in 10-20% of patients diagnosed as type III patients. Very few of these patients showed point mutations and it is suspected that others genes are also associated with the disease.

SMN gene is repeated in inverted position along 500kb area on 5q13 (Fig. 1), and mostly exist as 2 identical copies. SMN gene is close to the telomere (SMN^{tel}) whereas the copy gene is in the centromeric region. The two genes differ by only 5 base-pairs, resulting in no structural change in the resulting protein. 90% of patients have defects in exon 7 and 8 of SMN^{tel}, but 2-3% of normal person also display these defects, so the true SMA gene remains elusive. It is clear that single gene defect cannot compeletely account for this disease. The exact function of SMN^{tel} gene also need to be investigated.

NAIP gene is located in close proximity to the SMN gene, and is repeated in the 5q13 region. NAIP gene defects can easily be determined by PCR of exon 5. 46-67% and 18% of type I and type II/III patients had these defects respectively. A recent experiment suggests that NAIP inhibits motor neuron apoptosis by acting as a negative regulator, and any defect contributes to the SMA phenotype. In addition NAIP has some homogeneity with baculoviral gene product. This viral inhibitor of apoptosis protein(IAP) prevents infected insect cells from killing themselves, and act as a first line of defense against viral attacks. The discovery gave rise to a siginificant amount in interest in the apoptosis mechanism in SMA patients. A very baffling question concerns many asymptomatic parents of SMA patients who had NAIP gene defects. These parents are thought to be protected by "pseudogenes". NAIP gene defect frequency is likely to be higher than reported because truncated copies of the gene exist (Table 3).

Further studies should reveal which of the two genes are directly related to this disease. Other studies suggest the possibility of multiple copy genes instead of one for SMN and NAIP. NAIP gene does not have a poly-A tail at 3'-end and it is possible that this affects the transcription of SMN genes. At this stage, both genes are very important in understanding SMA. It is also hypothesized that SMN gene defect

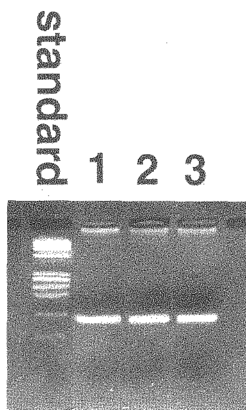

Fig. 3. Demonstration of NAIP gene in SMA patients. Exon 5 was amplified. Lane 1, standard marker; lane 2, control individual; lane 3, patient-1; lane 4, patient-2.

Table 3. Two candidate genes for spinal muscular atrophy: pros and cons

Gene	Pro	Con
SMN	- 93% of pts have no detectable copy - Partial deletion in some pts	- Normal siblings with no detectable copy - A second copy of SMN exists, is expressed in tissues examined, and has no discernible influence on manifestation or disease
	- Some pts have disabling point mutations within coding region	- No correlation between grades of disease severity and all-or-nothing deletion mutation - Other 5q-related markers frequently deleted in SMA chromosomes - Intragenic mutations cluster within 5% of gene
NAIP	- Full-length form deleted in 45% of type I SMA, 18% of type 2 SMA - Biological plausibility in partial homology to insect virus - Apoptosis-inhibiting protein	- Some asymptomatic parents known with no detectable full-length copy - Disabling mutations not yet found in all patients - Region of gene related to insect virus apoptosis-inhibiting protein lacks its apparent functional DNA binding region

is the central etiologic factor, and NAIP gene is associated with the severity of the disease. One study showed 58% of patients had homozygous genetic defects which were also found in their parents. Part of this gene had the same base-pair sequence as exon 7 and introns of NAIP gene. These results appear to support the role of NAIP in the pathogenesis of SMA.

Exact molecular biological mechanism of SMA can only be determined by studying the biological functions of SMN and NAIP genes. The studies will not only shed light on the biology of neurons and the development motor neurons, but also provide ideas on new approaches to treating this and similar diseases.

The authors confirmed SMN gene defects in 2 SMA patients. Both these patients did not have NAIP gene defects. The method employed by the authors is a relatively simple procedure and is easily applicable to prenatal and clinical diagnosis of SMA patients, replacing invasive methods such as muscle biopsy. Although this was not a large-scale research, our studies suggest that Korean SMA patients probably have SMN gene defects similar to those of their Caucasian couterparts.

REFERENCES

Brzustowicz, L. M., Lehner, T., Castilla, L. H. *et al.* (1990) Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2-13.3. *Nature* **344:** 540-541

Burglen, L., Spiegel, R., Ignatius Cobben, J., Landrieu, P., Lefebvre, S., Munnich, A. and Melki, J. (1995) SMN gene deletion in variant of infantile spinal muscular atrophy. *Lancet* **346:** 316-317

Bussaglia, E., Clermont, O., Tizzano, E., Lefebvre, S., Burglen, L., Craud, C, Urtizberea, JA, Colomer, J, Munnich, A, Baiget, M, Melki, J. (1995) A frame-shift deletion in the survival motor neuron gene in Spanish spinal musclar atrophy patients. *Nature Genetics* **11:** 335-337

Chang, J. G., Jong, Y. J., Huang, J. M., Wang, W. S., Yang, T. Y., Chang, C. P., Chen, Y. J., Lin, S. P. (1995) Molecular basis of spinal muscular atrophy in Chinese. *Am J Hum Genet.* **57:** 1503-1505

Clemont, O., Brulet, P., Lefebvre, S., Bruglen, L., Munnich, A., Melki, J. (1995) SMN gene deletions in adult-onset spinal muscular atrophy. *Lancet* **346:** 1712

Cobben, J. M., Steege, G., Brootscholten, P. *et al.* (1995) Deletions of the survival motor neuron gene in unaffected siblings of patients with spinal muscular atrophy. *Am J Human Genet* **57:** 805-808

Crawford, T. O. (1996) From enigmatic to problematic: the new molecular genetics of childhood spinal muscular atrophy. *Neurology* **46:** 335-340

Gilliam, T. C., Brzustowicz, L. M., Castilla, L. H. *et al.* (1990) Genetic homogeneity between acute and chronic forms of spinal musclar atrophy. *Nature* **345:** 823-825

Hahnen, E., Forkert, R., Marke, C., Rudnik-Schöeborn, S., Schonling, J., Zerres, K., Wirth, B. (1995) Molecular analysis of candidate genes on chromosome 5q13 in autosomal recessive spinal muscular atrophy: evidence of homozygous deletions of the SMN gene in anaffected individuals. *Hum Mol Genet* **4:** 1927-1933

Lefebre, S., Büglen, L., Revoullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., Zevaini, M. *et al.* (1995) Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* **80:** 155-165

Melki, J., Abdelhak, S., Sheth, P. *et al.* (1990a) Gene for chronic spinal muscular atrophies maps to chromosome 5q. *Nature* **344:** 767-768

Melki, J., Sheth, P., Abdelhak, S. *et al.* (1990b) Mapping of acute

(type 1) spinal muscular atrophy to chromosome 5q12-q14. *Lancet* **336:** 271-273

Melki, J., Lefebvre, S., Büglen, L. *et al* (1994) De novo and inherited deletions of the 5q13 region in spinal muscular atrophies. *Science* **264:** 1474-1477

Rodrigues, N. R., Talbot, K. and Davies, K. E. (1996) Molecular genetics of autosomal recessive spinal muscular atrophy. *Molecular Medicine* **2:** 400-404

Roy, N., Mahadevan, M. S., McLean, M., Shutler, G., Yaraghl, Z., Farahani, R., Baird, S. *et al.* (1995) The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell* **80:** 167-178

Schöeborn, S. R., Forkert, R., Hahnene, E., Wirth, B., Zerres, K. (1996) Clinical spectrum and diagnostic criteria of infantile spinal muscular atrophy: Further delineation on the basis of SMN gene deletion findings. *Neuropediatrics* **27:** 8-15

Steege, G., Grootscholten, P. M., Vlies, P., Draaijers, T. G., Osinga, J., Cobben, J. M., Scheffer, H., Buys, C. H. C. M. (1995) PCR-based DNA test to confirm clinical diagnosis of autosomal recessive spinal muscular atrophy. *Lancet* **345:** 985-986

Thompson, T. G., Didonato, C. J., Simard, L. R. *et al.* (1995) A novel cDNA detects homozygous microdeletions in greater than 50% of type 1 spinal muscular atrophy patients. *Nat Genet* **9:** 56-62

Wang, C. H., Xu, J., Carter, T. A. *et al.* (1996) Characterization of survival motor neuron (SMNT) gene deletions in asymptomatic carriers of spinal muscular atrophy. *Hum Mol Genet* **5:** 359-365

Wirth, B., Hahnen, E., Morgan, K., DiDonato, C. J., Dadze, A., Rudnik-Schöeborn, S., Simard, L. R., Zerres, K. and Brughes, A. H. M. (1995) Allelic association and deletions in autosomal recessive proximal spinal muscular atrophy: association of marker genotype with disease severity and candidate cDNA. *Hum Mol Genetics* **4:** 1273-1284