Prenatal Diagnosis of Spinal Muscular Atrophy Type I (Werdnig-Hoffmann) by DNA Deletion Analysis of Cultivated Amniocytes

Feodora Stipoljev, Jadranka Sertić, Višnja Latin, Ana Rukavina-Stavljenić, Asim Kurjak
Department of Obstetrics and Gynecology, Zagreb University School of Medicine, Sveti Duh Hospital, Zagreb; and 1Clinical Institute of Laboratory Diagnosis, Zagreb University Hospital Center, Zagreb, Croatia

Aim. Presentation of a prenatally diagnosed case of Werdnig-Hoffmann disease, the most severe type of spinal muscular atrophy.

Methods. DNA obtained from cultivated amniocytes was analyzed for deletions in the survival motor neuron gene and neuronal apoptosis inhibitory protein gene.

Results. The fetus was diagnosed as an affected homozygote for deletions in exon 7 and exon 8 of the survival motor neuron gene. No deletions of exon 5 in the neuronal apoptosis inhibitory protein gene were found.

Conclusion. Direct DNA deletion analysis of the survival motor neuron gene and neuronal apoptosis inhibitory protein gene in affected families represents a highly reliable and fast method for prenatal diagnosis of Werdnig-Hoffmann disease.

Key words: amniotic fluid; gene deletion; muscular atrophy, spinal; polymerase chain reaction; Werdnig-Hoffmann disease

Spinal muscular atrophy is the second most common lethal autosomal recessive disorder of childhood after cystic fibrosis affecting approximately 1 in 10,000 births, with carrier frequency of 1 in 50. It primarily affects the anterior horn cells of the spinal cord and motor cranial nerve nuclei, leading to progressive paralysis with atrophy (1).

The International Consortium on Spinal Muscular Atrophy has distinguished three forms of childhood-onset spinal muscular atrophy (types I, II, and III) on the basis of age and severity of the clinical course as assessed by clinical examination, muscle biopsy, and electromyography (2).

Type I is the acute spinal muscular atrophy or Werdnig-Hoffmann disease, with an onset almost immediately after birth and dramatic rapid course proceeding to death within 2-4 years (3). Type II is an intermediate spinal muscular atrophy which also has an infantile onset but proceeds more slowly. Type III is the Kugelberg-Welander disease, which has juvenile onset and is slowly progressive to chronic course (4).

All three forms of spinal muscular atrophy were linked to markers on the chromosome 5q11.2-q13.3. Spinal muscular atrophy region is highly unstable, with high degree of natural variations in the number, position, and orientation of multiple markers. The polymorphic markers in spinal muscular atrophy candidate region provided indication that large-scale deletion could be involved in spinal muscular atrophy (5,6). Recently, homozygous deletions of two presumptive genes, survival motor neuron gene (SMN) and neuronal apoptosis inhibitory protein gene (NAIP), have been identified in patients with spinal muscular atrophy.

The survival motor neuron gene exists in two almost identical forms, termed SMNt (telomeric) and SMNc (centromeric) copies. Both copies show identical sequences, except for five exchanges of a base pair at the 3' end of the gene, intron 6 to exon 8. However, deletion/mutations in the telomeric copy seem to cause spinal muscular atrophy (7). According to the type of spinal muscular atrophy, 80-90% of patients show homozygous deletions of telomeric copy of exons 7 and 8 of survival motor neuron gene, whereas homozygous deletions of centromeric copy were found in 2-3% of carriers and controls (8). Neuronal apoptosis inhibitory protein gene, which lies in region adjacent to the survival motor neuron gene, is deleted in 45% of patients with type I and in 18% of those with types II and III spinal muscular atrophy (9).

The survival motor neuron gene has a few mismatches in exons 7 and 8 compared with its centromeric homologue, the cBCD541 gene. The nucleotide difference has been used in polymerase chain reaction (PCR)/single strand conformation polymorphism, SSCP (6), and PCR/restriction fragment length polymorphism, RFLP (10) for identification of homozygous deletions of exons 7 and 8. The telomeric neuronal apoptosis inhibitory protein gene can be distinguished from its centro-meric
pseudogene counterpart by PCR test for the presence or absence of exon 5, which only exists within telomeric functional gene. Determining SMNc/SMNt dose ratio by densitometry of SSCP bands, Velasco et al (11) proposed that spinal muscular atrophy phenotype can be modified by the presence of different numbers of survival motor neuron centromeric copies. It was found that parents of type II and type III patients carried more copies of SMNc than were carried by parents of type I patients. In a further study, solid-phase minisequencing was used to obtained SMNt/SMNc ratio of gene copies (12). To date, the protein product of the survival motor neuron gene has no known function. The underlying biochemical defect remains unknown.

Clinical indications for the DNA testing include: infants and children with delayed gross motor milestones in the setting of normal fine skills and intellectual development and the absence of spasticity, sibling of known affected individuals, infants with arthrogryposis congenita multiplex, adults with slowly progressive weakness, and children with atypical syndromes involving the motor neuron but with additional problems such as mental retardation or congenital heart disease (4). Prenatal diagnosis is most frequently requested by families with spinal muscular atrophy type I. There is general consensus that deletions of survival motor neuron gene are the highest in patients with spinal muscular atrophy type I. This makes the deletion analysis a particulary useful tool for prenatal diagnosis and genetic counseling, extendable to those families in which DNA of affected individuals is not available.

We analyzed DNA obtained from cultivated amniocytes for homozygote deletions in exons 7 and 8 of survival motor neuron gene and in exon 5 of neuronal apoptosis inhibitory protein gene.

Case Report
A 32-year-old woman was referred at 16 weeks of gestation for prenatal diagnosis. The couple had two children who died at the age of 3 and 4 months as a result of Werdnig-Hoffmann disease and one 8-year-old unaffected child (daughter). Pedigree of the family is shown in Figure 1. Family history was negative regarding hereditary diseases and spontaneous miscarriages. No consanguinity was detected in the family. Amniocentesis was performed at 17 weeks of gestation. Prenatal diagnosis was done by direct DNA deletion analysis from cultivated amniocytes.

Material and Methods

PCR/RFLP Analysis of Exons 7 and 8 in Survival Motor Neuron Gene
DNA was extracted from cultured fibroblasts using a previously described protocol (13). PCR/RFLP analysis of survival motor neuron gene exons 7 and 8 was carried out as described by van der Steege et al (10). Genomic DNA was amplified using specific oligonucleotide primers (MWG Biotech, Ebersberg, Germany) for exon 7 (primer R111; mismatch primer X7-Dra) and exon 8 (541C1120; 541C960). Polymerase chain reaction product was digested with restriction enzyme DraI (Boehringer Mannheim, Germany) and visualized in a gel, giving specific pattern for spinal muscular atrophy patients. Polymerase chain reaction product of exon 8 of the survival motor neuron gene and the copy gene can readily be distinguished since the copy gene contains a recognition site for the restriction enzyme Ddel and that site is absent in exon 8. For exon 7 no such difference in restriction site for any known enzyme existed (6). Van der Steege et al (10) developed an oligonucleotide allowing specific priming directly adjacent to the variant site and introduced mismatch creating a restriction site for Dral in polymerase chain reaction product of exon 7 of the copy gene but not in polymerase chain reaction product of the survival motor neuron gene. The amplified DNA was digested with restriction enzymes and separated by gel electrophoresis. The gel containing the separated polymerase chain reaction products was stained with ethidium bromide. This allowed the detection of deletions in exons 7 and/or 8 of survival motor neuron gene and indicated a positive results and a spinal muscular atrophy phenotype. Negative results were indicated by the presence of normal polymerase chain reaction product for exons 7 and 8 of survival motor neuron gene.

PCR Analysis of Exons 5 and 13 in Neuronal Apoptosis Inhibitory Protein Gene
Deletion analysis of the neuronal apoptosis inhibitory protein gene was determined by polymerase chain reaction amplification of exon 5 using primers 1863 and 1864 (MWG Biotech). The reactions were multiplexed with exon 13 (primers 1258 and 1343) as positive polymerase chain reaction control for exon 5 which is present in the full-length gene. Homozygous deletion of neuronal apoptosis inhibitory protein gene exon 5 indicates positive result (14).

Results
Isolated DNA was analyzed for deletions in exons 7 and 8 of survival motor neuron gene telomeric copy and deletion in exon 5 telomeric copy of neuronal apoptosis inhibitory protein gene. Spinal muscular atrophy DNA genotypes were determined by PCR/RFLP method amplifying exons 7 and 8 of survival motor neuron and exon 5 of neuronal apoptosis inhibitory protein gene. Telomeric copies of survival motor neuron and neuronal apoptosis inhibitory protein gene were distinguished from centromeric nonpathogenic gene homologue copy. SMN/exon 7/DraI digestion results converged in two fragments to the survival motor neuron gene and cBCD541 copy. SMN/exon 8/DraI digestion gave three fragments, the one with higher molecular weight corresponding to survival motor neuron gene, and the other two corresponding to cBCD541 copy. DNA analysis showed the presence of both homozygous survival motor neuron gene deletions of exons 7 and 8 (Fig. 2). No deletions of exon 5 in neuronal apoptosis inhibitory protein gene were found. The combination of telomeric deletions in survival motor neuron gene exons 7 and 8 only represents the deletion genotype B (7). After genetic counselling, the couple decided to terminate the pregnancy at 19 weeks of gestation.

Figure 2. Polymerase chain reaction for the spinal muscular atrophy deletion analysis. Top: Exon 7 of the survival motor neuron gene (upper band survival motor neuron gene, lower band cBCD541). Lanes 1 and 3, undigested polymerase chain reaction product. Lane 2, spinal muscular atrophy proband with a deletion of the survival motor neuron gene. Lane 4, a healthy individual with both the survival motor neuron and cBCD541 genes. Lane 5, molecular weight markers 8-587 bp. Bottom: Exon 8 of survival motor neuron gene (upper band survival motor neuron gene, two lower bands cBCD541). Lanes 1 and 3, undigested polymerase chain reaction product. Lane 2, spinal muscular atrophy proband with the a deletion of survival motor neuron gene. Lane 4, a healthy individual with both the survival motor neuron gene and cBCD541 genes.

Discussion
First prenatal diagnosis of spinal muscular atrophy type I was made by Melki et al (15) and Daniels (16), using restriction fragment length polymorphism probes linked to the spinal muscular atrophy locus. Lefebvre et al (6) described in 1995 a genome structure in the spinal muscular atrophy locus, called survival motor neurone. The gene deletions were found in 98.6% of patients with spinal muscular atrophy, whereas point mutations have been detected in remaining cases. Roy et al (14) discovered a gene encoding neuronal apoptosis inhibitory protein gene in the proximal part of spinal muscular atrophy region. They noted partial or complete homozygous deletion of the neuronal apoptosis inhibitory protein gene in 45% patients with spinal muscular atrophy type I and in 18% with type II and with type III.

During the past years, several reports of gene deletions in spinal muscular atrophy patients have been published for many different ethnic groups. Rodrigues et al (7) found deletions, which included both survival motor neuron gene and neuronal apoptosis inhibitory protein gene in 63.3% patients with type I, in 8.8% with type II, and in 12.2% with type III spinal muscular atrophy. The gene deletions were found in 77% of spinal muscular atrophy patients in the study by Sertiæ et al (17). Burlet et al (18) found high incidence of deletions involving survival motor neuron gene in 43% of the patients with Werdnig- Hoffmann disease. Deletions of survival motor neuron gene were also found in unaffected sibs of patients with spinal muscular atrophy (19). Burlet (18) suggested that other genetic mechanisms might be involved in the variable clinical expression of spinal muscular atrophy. The presence of deletions in healthy siblings of affected individuals is difficult to explain. One possibility is that some additional factors, like germline mosaicism, gene conversion, or other factors, influence the phenotype expression. Hahnem et al (20) demonstrated the presence of hybrid survival motor neuron genes which were composed of both the centromeric and telomeric copy, specially in patients with spinal muscular atrophy of Czech or Polish background.

The deletion analysis of the survival motor neuron gene and neuronal apoptosis inhibitory protein gene in the study of Samilchuk et al (8) on patients of Kuwaiti origin showed homozygous deletions of exons 7 and 8 of the survival motor neuron gene in all patients and of exon 5 in all patients with type I spinal muscular atrophy. Exon 5 was retained in type II patients. This finding confirmed previously reported studies that the incidence of deletions in neuronal apoptosis inhibitory protein gene was much higher in clinically more severe cases than in milder forms. Somerville et al (21) proposed the probability for spinal muscular atrophy based on the presence or absence of deletions in telomeric copy of the survival motor neuron gene. Detection of deletions in exon 7 could raise to more than 98% the probability of spinal muscular atrophy in some populations. Deletions of both neuronal apoptosis inhibitory protein exon 5 and survival motor neuron exon 7 was associated with a 5-fold increased risk of type I spinal muscular atrophy.
Campbell et al (22) have shown that gene conversion plays a major role in spinal muscular atrophy type II and type III in which the telomeric copy of survival motor neuron gene is replaced by its centromeric counterpart. An increase in survival motor neuron centromeric copy number leads to a decrease in the severity of disease because of increased level of the functional protein. Taylor et al (23) demonstrated correlation between survival motor neuron centromeric number and phenotype in patients lacking a survival motor neuron telomeric gene, indicating possible classification of patients into subgroups.

The genetic basis of spinal muscular atrophy phenotype variability is unexplained, suggesting that a loss of an additional modifying factor contributes to the severity of type I spinal muscular atrophy. Scharf et al (24) identified a novel transcript, H4F5, which lies closer to the survival motor neuron gene that any previously identified gene in the region. H4F5 is deleted in more than 90% of the type I spinal muscular atrophy chromosomes and is embedded in an intron of the survival motor neuron gene. This indicates that H4F5 is a candidate phenotypic modifier for spinal muscular atrophy. The deletion frequency in type II was between type I and controls, whereas the deletions in type III chromosomes were only slightly more frequent than in controls. Both the centromeric and telomeric copies of the survival motor neuron gene are transcribed and their predicted amino acid sequence is identical. The centromeric gene is alternatively spliced. The physiological role of these genes and transcripts and their specific role in the pathogenesis of the disease thus remain unknown (6). Direct DNA analysis of survival motor neuron and neuron apoptosis inhibitory protein deletions in affected families is confirmed to be a highly reliable and fast method of prenatal diagnosis. The availability of this method to distinguish between the survival motor neuron gene and its nearly identical centromeric copy allows precise molecular diagnosis.

Prenatal diagnosis is most frequently requested by families with spinal muscular atrophy type I. There is a general consensus among investigators that the percentage of deletions in the survival motor neuron gene is the highest in patients with spinal muscular atrophy type I. The deletion analysis of the survival motor neuron gene is particularly useful for prenatal diagnosis in cases in which DNA of an affected sib is not available. In addition, complex linkage analysis is not necessary. Several studies in which spinal muscular atrophy is associated with additional clinical manifestation, such as congenital heart defects, arthrogryposis, olivopontocerebellar hypoplasia, and congenital fractures are described in literature (4).

While the questions regarding the phenotype-genotype correlation still need to be fully clarified, there is no doubt of the importance of deletion analysis as a diagnostic tool for patients with spinal muscular atrophy. The spinal muscular atrophy region on the chromosome 5q shows some special characteristics, which should lead to caution in the molecular and prenatal diagnosis of Werdnig-Hoffmann disease.

Acknowledgment
This work was supported by the Croatian Ministry of Science and Technology project No. RH-3-01-252. We thank Senka Škaro and Karolina Petrović for their technical assistance.

References

Received: June 6, 1998
Accepted: May 11, 1999

Correspondence to:
Feodora Stipoljev
Cytogenetic Laboratory
Department of Obstetrics and Gynecology
Sveti Duh General Hospital
Sveti Duh 64
10000 Zagreb, Croatia
ana.stavljenic-rukavina@zg.tel.hr