Prenatal diagnosis of the spinal muscular atrophy type I using genetic information from archival slides and paraffin-embedded tissues

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Spinal muscular atrophy (SMA) type I is a common severe autosomal recessive inherited neuromuscular disorder that has been mapped to chromosome 5q11.2-13.3. The survival motor neuron (SMN) gene, a candidate gene, is known to be deleted in 96% of patients with SMA type I. Presently, PCR and single strand conformation polymorphism (PCR-SSCP) analyses have been made possible for application to both archival slides and paraffin-embedded tissues. Archival materials represent valuable DNA resources for genetic diagnosis. We applied these methods for the identification of SMN gene of SMA type I in archival specimens for the prenatal diagnosis. In this study, we performed the prenatal diagnosis with chorionic villus sampling (CVS) cells on two women who had experienced neonatal death of SMA type I. DNA extraction was done from archival slide and tissue materials and PEP-PCR was performed using CVS cells. In order to identify common deletion region of SMN and neuronal apoptosis-inhibitory protein (NAIP) genes, cold PCR-SSCP and PCR-restriction site assay were carried out. Case 1 had deletions of the exons 7 and 8, and case 2 had exon 7 only on the telomeric SMN gene. Both cases were found to be normal on NAIP gene. These results were the same for both CVS and archival biopsied specimens. In both cases, the fetuses were, therefore, predicted to be at very high risk of being affected and the pregnancy were terminated. These data clearly demonstrate that archival slide and paraffin-embedded tissues can be a valuable source of DNA when the prenatal genetic diagnosis is needed in case any source for genetic analysis is not readily available due to previous death of the fetus or neonate.

Keywords: Survival motor neuron gene, neuronal apoptosis inhibitory protein gene, archival slides and paraffin-embedded tissues

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder in children which is characterized by degeneration of the anterior horn cells of the spinal cord, leading to progressive muscle weakness (Roberts *et al.*, 1970; Pearn, 1973, 1978; Czeizel and Hamula, 1989; Rajcan-Separovic *et al.*, 1996). SMA is a fatal disorder which is classified into three types depending on clinical severity and onset age of muscle weakness (Dubowitz, 1978; Brooke, 1986). SMA type I, which has an onset age within 3 months after birth, is a fast degenerative muscular disease which leads to weakness and hypotonia of the muscle resulting in certain death within two years by respiratory failure. Infants with type II are able to sit by themselves but can't walk without help and generally live about 4 years. Type III patients have onset age in two years after birth characterized by proximal muscle weakness. Frequency of SMA is about 1 in 10,000 live births with a carrier frequency of 1/40 - 1/60 (Pearn, 1973; Spiegler *et al.*, 1990).

Recently, it has been the mapped 5q13 of the chromosome which is responsible for SMA, and two candidate genes have been reported, the survival motor neuron (SMN) (Lefebvre *et al.*, 1995) and neuromal apoptosis inhibitory protein (NAIP) (Roy *et al.*, 1995). SMN, found in

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the candidate region of 5q11.2-q11.3, has been a highly homologous copy of telSMN and cenSMN. Homozygous deletion of exons 7 and 8 in the telSMN one found in 90-98% of SMA patients. On the other hand, only 4-5% of homozygote deletion in cenSMN could be found in the carrier and control group. This shows homozygous deletion of cenSMN does not have significant clinical consequences (Roy *et al.*, 1995).

We were able to distinguish telSMN from cenSMN by restriction-site analysis or single strand conformation polymorphism (SSCP) which could detect the difference of two base pair (Lefebvre *et al.*, 1995; Chang *et al.*, 1995; van der Steege *et al.*, 1995). The deletion of exons 7 and 8 in the telSMN region was found in 98% of SMA type I and 90% in SMA type II and III. After the PCR of the exon 8, because the cenSMN had a recognition site by restriction enzyme *Dde*I, we could cut the PCR products with *Dde*I and distinguish SMN from telSMN gene (Lefevbre *et al.*, 1995). The deletion of exons 5 and 6 in the NAIP gene can be found in 60% of SMA type I and 15% in the SMA type II and III (Roy *et al.*, 1995). In this study, we performed 2 pregnant women who had experienced one or two neonatal deaths of SMA type I.

In order to confirm their history, we requested the archival or paraffin embedded tissue slides which result from standard procedure of neonatal death so that we could obtain genetic information through genetic analysis by PCR-restriction site assay and SSCP concerning SMN of the neonates. With the genetic information, we performed prenatal diagnosis through chorionic villi sampling on 3 pregnant women to determine whether fetuses are affected or not.

MATERIALS AND METHODS

Sample preparations of the subjects

In this study, we performed the prenatal diagnosis on two pregnant women who had experienced two neonatal death of SMA type I. Two couples showed a normal clinically and phenotypically (Fig. I). To determine whether or not the patient was a true SMA, we analyzed genetic diagnosis using archival slides and paraffin embedded tissues which was preserved during the autopsy. After we obtained genetic results from archival slides and paraffin embedded tissues, for the prenatal diagnosis, we applied PEP-PCR using 1-2 pieces of the fetal cells through chorionic villi sampling during 10-11th weeks of gestation.

Extraction of DNA from archived slides and paraffin embedded tissues

DNAs from two neonatal death samples were extracted perfectly using soaking the archived slides and paraffin embedding tissues in xylene during 2-3 h (Grant *et al.*, 1995; Sago *et al.*, 1996) Cellular materials in archived slides was hydrated by soaking 3×5 min in H_2O. Then 100 µl lysis buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin, 0.5% Tween 20; Sago *et al.*, 1996) was added to the portion of each slide surface adjacent to the frosting and the liquid was dispersed and sealed to the slide with a 25mm × 55mm rectangular

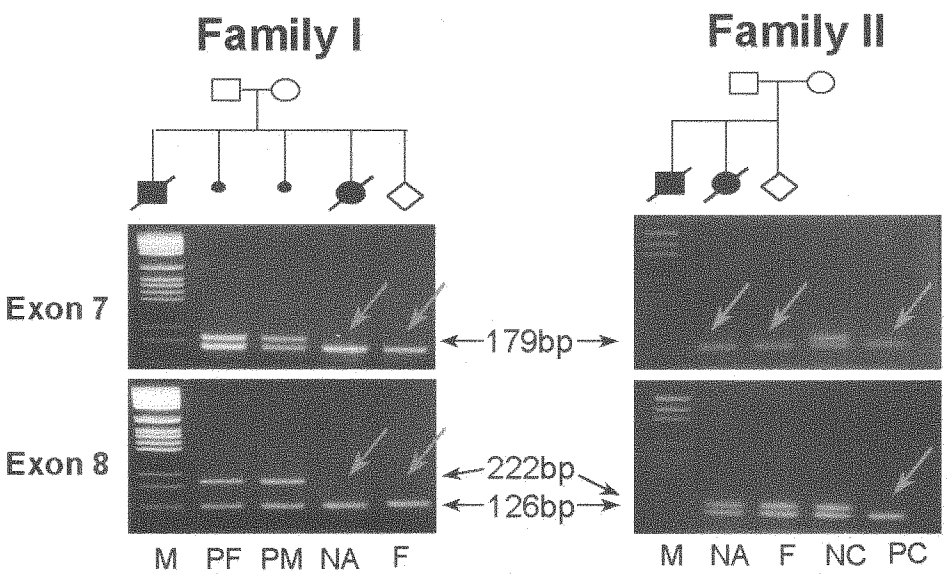

Fig. 1. Prenatal diagnosis of families I and II through CVS. Results of PCR-restriction site assay of the SMN gene. The DraI and DdeI have two recognition site bands on the exons 7 and 8, of which the upper is the telSMN gene, the lower being the cenSMN gene. The arrow marks are deletion bands; the fetus from family I was deleted of exons 7 and 8 of the telSMN gene. The fetus from family II was deleted of exon 7 only of the telSMN gene. M: pGEM marker, NA: archived cells from neonatal death, PF: father, PM: mother, F: fetus, NC:normal control and PC: patient control.

piece of Parafilm M at 56˚C for 15 min. After adding an additional 200 µl water to each slide, the softened cellular material was carefully scraped off the surface using a new individual single edge surgical carbon steel razor blade for each slide on clean bench. Suspended cellular material was collected into a 0.6 ml effendorf microcentrifuge tube. And paraffin embedded tissues were put into the 0.5 ml effendorf tube and adding an 500 µl xylene for 10 min at room temperature. After incubation, the tissues were collected by centrifugation at 12,000 rpm for 5 min. After discarding the supernatant, the cell pellets were washed by 100% ethanol. The cellular suspension of archival slides and paraffin embedded tissues were collected by centrifugation at 12,000 rpm for 5 min. After removing and discarding the supernatant, the cell pellets were resuspended in 200 µg/ml proteinase K in 50 µl lysis buffer by vortexing and incubating at 56˚C for 45 min. Subsequently the proteinase K was in-activated at 95˚C for 10 min. The samples were used immediately for PCR or were stored at -80˚C. For the prenatal diagnosis, we performed PEP-PCR using 1-2 pieces of biopsied chorionic villi cells.

PCR and Restriction - site Assay

For the SMN gene, we applied PCR for exons 7 and 8, then the PCR products were treated with *Dra*I and *Dde*I restriction enzyme respectively. After electrophoresis, we confirmed the deletion on the 0.8% agarose gel. For the NAIP gene, after the multiplex-PCR for exons 5 and 13, direct confirmation of exon deletion was made.

Single Strand Conformation Polymorphism

SSCP was also applied the exon 7 and the exon 8 for the analysis of the SMN gene, after the PCR. Loading dye was added to PCR products with a ratio of 1:1, and it was denatured at 95˚C for 10 min. Denatured mixtures were electrophoresed on the 12% nondenaturing gel for 4-4.5 h under 3 watts of current at 4˚C wet chamber. After the electrophoresis, the gel was stained with ethidium bromide for 15 min, then we observed the deletion bands under the UV transilluminator.

RESULTS

We confirmed the success of PCR amplification of exon 7 and exon 8 by observing the single band treated the products with *Dra*I and *Dde*I respectively. Generally, the *Dra*I and *Dde*I have two recognition site bands on the exon 7 and 8, of which upper is the telSMN gene, the lower being the cenSMN gene. For the family I, one from samples of previous neonatal death we observed the concordance of deletion of both exon 7 and exon 8 for telSMN gene in the fetus and in the previous neonates, samples of which came from the archival slides and paraffin embedded tissues. For the family II, we only observed the deletion of exon 7 (Fig. 1).

As for exon 5 and exon 13 of the NAIP gene were found to be normal in all cases (Fig. 2). In the analysis with SSCP, the upper telomeric band for exon 7 and exon 8 could not be observed for both fetus and neonates in family I, thereby we could confirm the deletion of both exons 7 and 8. For the family II, upper telomeric band could not be observed only for exon 7. These results were consistent with results from restriction-site assay (Fig. 3).

DISCUSSION

The two cases in our experiment all showed deletion in the telSMN gene but not the NAIP gene. Generally, for

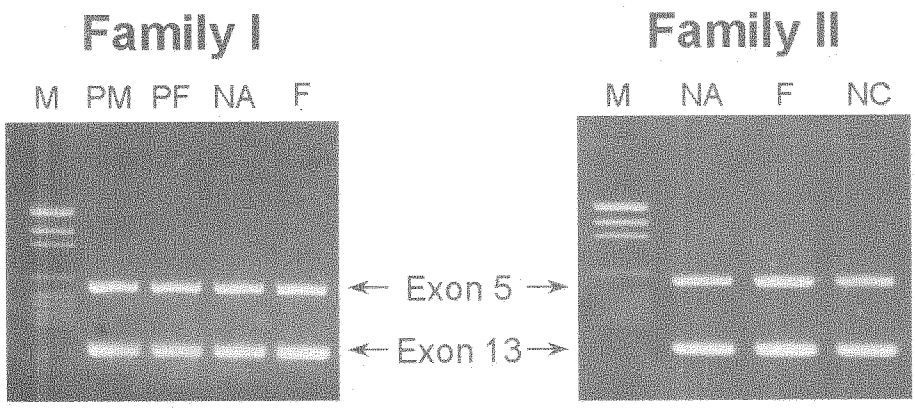

Fig. 2. Multiplex-PCR results for exons 5 and 13 of NAIP gene from archived and fetal cells by PEP-PCR. Fetuses and neonatal archived cells from families I and II were found to have normal bands of exons 5 and 13 of the NAIP gene. M: pGEM marker, PM: mother, PF: father, NA: archived cells from neonatal death, F: fetus and NC:normal control.

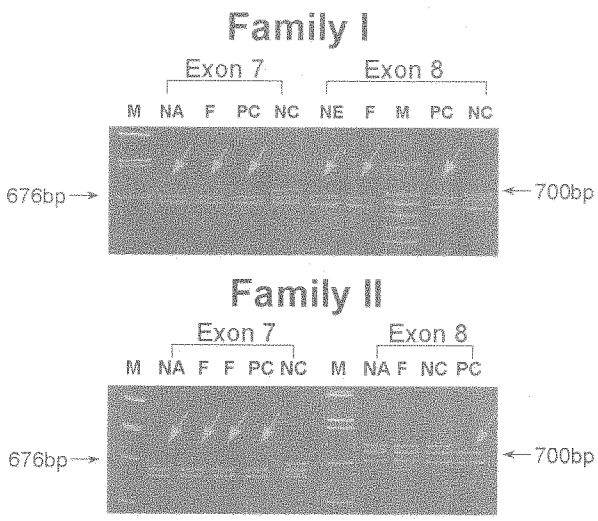

Fig. 3. Cold PCR-SSCP analysis of PCR amplified exons 7 and 8 of SMN region. Normal individual has three bands on the exons 7 and 8 respectively of the SMN gene, of which the upper most is telSMN gene, the lower two being the cenSMN gene. The arrow marks are deletion bands. Fetus and neonatal archived cells of family I had deletions of exons 7 and 8 of the telSMN gene. Fetus and neonatal archived cells of family II had deletion of exon 7 only of the telSMN gene. M: pGEM marker, NA: archived cells from neonatal death, F: fetus, PC: patient control and NC:normal control.

the SMN type I, the frequency of homozygous deletions in the NAIP gene is below 50% for Europian and north American population (Lefebvre *et al.*, 1995; Wirth *et al.*, 1995), however for other heterogeneous population, the frequency is found to be 62-66% (Burlet *et al.*, 1996; Rodrigues *et al.*, 1996). cenSMN gene has 4.4% homozygous deletion frequency in the normal population which is considered to be a normal variation (Lefebvre *et al.*, 1995). Up to now, SMN gene has been considered the most likely candidate for the SMA. However, a controversy exists due to the fact that even in the unaffected sibs of the SMA families (type II and III) 10% (4/40) have been found to have homozygous deletions of exons 7 and 8 in the SMN gene (Hahnen *et al.*, 1996).

As for why the above ones are not affected by the SMA, it is thought that there are other SMA determining gene or responsible gene, or perhaps there are yet unknown factors which lead to clinical SMA such as gene-dosage effect, incomplete penetration rate, or yet unobserved clinical variations. Aside from such controversies concerning the few exceptions, so far, genetic analyses have shown SMN and NAIP gene deletions occur in 98.9% of SMA patient group, leading us to believe that SMN and NAIP genes play a major role in the SMA (Theodosiou *et al.*, 1994; Cobben *et al.*, 1995; Gilliam, 1995; Wirth *et al.*, 1995) Accordingly, genetic analysis, SSCP and restriction-site assay, for the determination of deletions in SMN and NAIP genes are useful for diagnosis of patient and fetus.

For prenatal analysis on a mother with previous history of neonatal death from clinically SMA type I, we obtained genetic information by genetic analysis through archival slides and paraffin-embedded tissues preserved during the autopsy. To avoid over breakage of DNA from the archival slides and paraffin-embedded tissues, we need proteinase K in place of phenol, and with the obtained DNA, we were able to successfully apply the PCR. Generally, there are reports which assert that the DNA quality in comparable for fresh slides and archival slides which are about 5 years old.

However, there are also reports which assert that for older stained slides, choice of PCR primers, target DNA size and amplification condition of PCR are important factors (Sago *et al.*, 1996). Regardless of the age of the slides, instead of the in-situ PCR which only yields one or two target genes analysis, in order to obtain greater number of DNA for multi-target analysis, we have chosen to remove the cells from archival slides and paraffin-embedded slides, and applied the PEP-PCR for the amplification of the whole genome.

With the continuous development and improvements in the genetic analysis method, the archival slides have been found to be a great source for genetic analysis whereas, thus far, many have considered the archival slides as useless due to their limitations leading to costly preservation method in liquid nitrogen tank or extraction of DNA for every analysis.

However, we have shown that archival slides can be used effectively and efficiently requiring only low costs maintenance with the great benefits the archival slides could bring for the future genetic analysis scientifically and clinically.

Therefore, in cases of previous neonatal death caused by SMA type, paraffin embedding tissues and archival slide materials are very important sources of DNA for the genetic analysis and prevention of repeat death of fetus and neonates having the same parents, i.e, the following pregnancy. Previously, the wait-period for performing prenatal DNA analysis for SMA type I was about a 3-4 weeks. However, now, genetic analysis by PEP-PCR is possible within 2-3 days after fetal sampling. We believe the restriction-site assay and cold PCR-SSCP methods can be useful for the detection of deletion of SMN and

NAIP genes in the prenatal diagnosis of SMA type I.

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