Mutation analysis in spinal muscular atrophy using allele-specific polymerase chain reaction

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Polymerase chain reaction (PCR), followed by restriction digestion is universally used for molecular diagnosis of spinal muscular atrophy (SMA). In the present study, we have used a modified strategy based on amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) to develop a rapid and reliable method for mutation detection and prenatal diagnosis in SMA patients. The telomeric (SMN1) and centromeric (SMN2) copies of exon 7 of the survival motor neuron (SMN) gene were amplified by ARMS-PCR, using primers specific to SMN1 and SMN2 nucleotide sequence with the exonic mismatch G (for SMN1) and A (for SMN2) at the 3' end. The PCR products were analyzed on agarose gels. All the patients who had homozygous deletion of exon 7 of SMN1 gene by conventional PCR-restriction fragment length polymorphism (PCR-RFLP) method showed the same deletion status by ARMS-PCR. This procedure showed a 100% concordance between PCR-RFLP and ARMS-PCR methods for the detection of SMN1/SMN2 status in patients with SMA. An artifact due to incomplete digestion is not a problem while using ARMS-PCR. The modified protocol is specific, rapid and highly reliable for use in prenatal diagnosis as well.

Keywords: spinal muscular atrophy, allele-specific amplification, DNA diagnosis, prenatal diagnosis, SMN gene, mutation analysis, polymerase chain reaction.

Proximal spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder, characterized by the degeneration of α-motor neurons of anterior horn cells of the spinal cord. SMA comprises a clinically and genetically heterogeneous group of diseases with an estimated incidence of 1/10,000 live births\(^1\) and a carrier frequency of 1/50\(^2\). All three forms of childhood SMA have been mapped to chromosome 5q11.2-q13.3 containing a large inverted duplication consisting of at least four genes, each of which is present in a telomeric (t) and a centromeric (c) copy\(^3\). Survival motor neuron gene (SMN1 or SMN2) is the most important gene associated with SMA. It is believed that homozygous deletion in SMN1 causes the disease in 90-95% of classical SMA patients\(^3\). Molecular diagnosis of patients with suspected SMA and prenatal diagnosis in families with a history or suspicion of SMA are based on the detection of homozygous deletion of exons 7 and 8 of SMN1 gene\(^4\). The conventional protocol involves polymerase chain reaction (PCR), followed by restriction digestion\(^4,5\), which is time-consuming and prone to diagnostic error due to incomplete digestion. Recently Moutou et al\(^6\) developed an allele-specific amplification method of fluorescent PCR for the pre-implantation genetic diagnosis (PGD) of SMA from a single cell. Here we report a strategy with appropriate modifications, which does not involve the use of fluorescent dyes. The PCR conditions were also modified which enabled the use of this method for rapid and reliable mutation detection as well as prenatal diagnosis of SMA.

Materials and Methods
Consecutive SMA patients from January 2000-June 2002 diagnosed in the Genetics and Neurology outpatient department (OPD) of the Institute, on the basis of inclusion and exclusion criteria as per International SMA Consortium\(^2\) have been included in the study. The patients belonged to three types of SMA (types I-III). The blood (5 ml) was collected in ethylene diamine tetra acetic acid (EDTA) from 35 patients and 20 controls. Controls had no symptoms of any muscle disease and were apparently healthy. DNA was extracted from blood by the standard phenol-chloroform method\(^3\). Chorionic villi sampling was done under ultrasonography by expert clinicians from

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Note

Abbreviations: SMA, spinal muscular atrophy; SMN, survival motor neuron; ARMS-PCR, amplification refractory mutation system-polymerase chain reaction; RFLP, restriction fragment length polymorphism; CVS, chorionic villi sample; PGD, pre-implantation genetic diagnosis; OPD, out-patient department; EDTA, ethylene diamine tetra acetic acid; dNTP, deoxyribose nucleotide triphosphate.
women who came for prenatal diagnosis. DNA from 4 chorionic villi samples (CVS) was obtained, using DNA extraction kit (Qiagen, Germany).

Molecular analysis of SMN gene by PCR-RFLP

Polymerase chain reaction was used to amplify exons 7 and 8 of SMN gene with an initial denaturation at 96°C for 7 min followed by 35 cycles (94°C, 62°C and 72°C for 1 min each). The primer sequences for exons 7 and 8 were taken from Chang et al. PCR products of exons 7 and 8 were digested with Dra I and Dde I, respectively. The digested products were analysed on 10% PAGE.

Amplification refractory mutation system (ARMS) for SMN gene

In amplification refractory mutation system (ARMS)-PCR for exon 7 of SMN gene, the forward primer used was similar to that used by Chang et al., while the reverse primer was designed so as to be specific to the SMN1 and SMN2 nucleotide sequence with an exonic mismatch G (for SMN1) and A (for SMN2) at its 3’ end: 5’TCTTTCTTTTTGATTTTGTCTGA 3’. The primers for connexin 26-gene, which gave a product of 360bp were included as internal control. The forward and reverse primers of Connexin 26-gene were 5’ CCCACCTTCCCCCTCTCCAGGGCATAATGGG 3’ and 5’ GGGCCTCAGTCCCAAATGGCTAAGAGGTG 3’, respectively. ARMS-PCR was performed in a 25 µl reaction mixture in two separate tubes containing buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatine, 0.005% Tween-20, 0.005% Nonidet, NP-40), 200 µM each dNTPs, 25 pmol each of exon 7 primers, 10 pmol each of control primers (Invitrogen, USA), 1 U of Taq DNA polymerase (MBI Fermentas, Lithuania) and 150 ng DNA in a thermocycler (DNA engine, M J Research, USA). After 5 min initial denaturation at 95°C, PCR was carried out for 38 cycles (denaturation at 95°C, 30 sec; annealing at 59°C, 45 sec and elongation at 72°C for 1 min) followed by a final extension for 6 min at 72°C. The PCR products were analysed on a 2% agarose gel, visualised in UV and photographed by Alpha Imager™ 1220 Documentation and Analysis System (Alpha Innotech Corporation, USA).

Results and Discussion

The exon 7 amplified product digested with Dra I gave three products (188, 149 and 39 bp); the 39bp product runs out of the gel (Fig. 1A, lane 4). While exon 8 digested with Dde I gave three products (187, 123 and 64bp) (Fig. 1B, lane 2) in all controls showing the presence of both SMN1 and SMN2 copies of the gene. The absence of 188 bp and 187 bp bands showed SMN1 deletion in SMA patients (Fig. 1A and B, lanes 2 and 4, respectively). Out of 35 suspected cases of SMA, 17 (~50%) showed deletion of SMN1 gene. We have observed that in types I and II SMA, exon 7 of SMN1 was deleted in 67% of suspected patients, while only 40% of type III SMA patients harboured homozygous deletion of SMN1 gene. None of these patients showed SMN2 deletion. Out of four DNA samples from chorionic villi, only one sample showed the deletion of exon 7 of SMN1 gene. Allele-specific amplification resulted in two bands of 185 and 360bp, corresponding to SMN1/SMN2 and internal control, respectively (Fig. 2, lanes 5 and 6). In patients with SMN1 deletion, 185bp band was absent...
as seen in lane 7 (Fig. 2), while 185bp band corresponding to SMN2 gene was present as observed in lane 8 (Fig. 2). The presence of 360bp band for connexin-26 gene ruled out any kind of PCR failure in all the reactions. The centromeric (SMN2) deletion in a patient reported earlier by us\textsuperscript{10} was also confirmed by ARMS-PCR (Fig. 2, lane 2). DNA from CVS subjected to ARMS-PCR showed bands both in SMN1 and SMN2 lanes (Fig. 2, lanes 3 and 4), thereby suggesting that there was no homozygous deletion of SMN1 or SMN2 gene in the foetus under diagnosis.

This strategy has already been used in pre-implantation genetic diagnosis of SMA using fluorescent PCR, followed by analysis on an automated sequencer\textsuperscript{6}. The protocol mentioned here requires only PCR, followed by gel analysis, which can be completed within 5 hr and it is the first application in SMA diagnosis. The main problem of SMA diagnosis is the presence of two copies of SMN gene, the telomeric (SMN1) and its homologue, the centromeric (SMN2) copy. ARMS-PCR of SMN1 and SMN2 in separate tubes enables easy and clear visualization on gels, thus reducing any ambiguity in interpreting results. This accurate and sensitive method can, therefore, be used to detect the deletion of SMN1 copy. This is especially useful because time and reliability are the most important criteria during prenatal diagnosis. The 100% concordance between PCR-RFLP and ARMS-PCR methods was validated in large number of normal controls, suspected SMA patients, as well as foetal samples. The use of allele-specific amplification method has an added advantage over PCR-RFLP where, the chances of digestion failure or partial digestion, may lead to a wrong diagnosis. The low frequency of the SMN1 gene deletion in our Type I patients as compared to other reports\textsuperscript{1,3} can be attributed to the presence of point mutations or other genetic alterations that might be more prevalent in our patients.

In conclusion, we present here a rapid and highly reliable method for mutation analysis based on ARMS strategy for screening of patients suspected to have SMA. This procedure is accurate and less time-consuming and can be used successfully in providing prenatal diagnosis of SMA.

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References