Molecular Analysis and Prenatal Prediction of Spinal Muscular Atrophy in Chinese Patients by the Combination of Restriction Fragment Length Polymorphism Analysis, Denaturing High-Performance Liquid Chromatography, and Linkage Analysis

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Background: The difficulties and incurability of spinal muscular atrophy (SMA) highlight the importance of prenatal diagnosis in families with SMA. However, the system applied in prenatal screening is far from perfect.

Objectives: To optimize the molecular assays and establish a relatively perfect system for prenatal screening.

Design, Setting, and Patients: A total of 87 patients and 132 parents from 77 families with SMA were screened for SMN1 mutations. Prenatal prediction was performed for 11 fetuses from 10 families with SMA. All of the samples to be tested were from the Department of Neurology, First Affiliated Hospital, Fujian Medical University, Fuzhou, China.

Main Outcome Measures: All of the 87 patients and their parents were screened for SMN1 deletion by restriction fragment length polymorphism analysis and denaturing high-performance liquid chromatography (DHPLC). For those patients without the SMN1 deletion, the SMN1 copy numbers were detected by real-time fluorescence quantitative polymerase chain reaction and the subtle mutations of SMN were screened by direct sequencing. Prenatal prediction was performed by restriction fragment length polymorphism analysis, DHPLC, and linkage analysis for 11 fetuses. Furthermore, the SMN1 copy numbers and detected carriers of SMA were found by DHPLC and real-time fluorescence quantitative polymerase chain reaction in 14 parents and the fetuses without the SMN1 deletion. Results in aborted fetuses and born babies were reconfirmed by restriction fragment length polymorphism analysis and DHPLC. The born babies were followed up and physically examined twice a year.

Results: The frequency of the SMN1 deletion we detected was 93.3% (72 of 77 patients). No subtle mutations were detected in the other 5 families. Four fetuses had the SMN1 deletion and were aborted. The other 7 fetuses, 4 carriers and 3 normal individuals, were born under suggestion by the physician. Fourteen parents were carriers. The reconfirmation of results in the aborted fetuses and born babies was completely consistent with prenatal prediction. The 7 born babies were followed up until recently and all were normal.

Conclusions: The molecular diagnosis system based on restriction fragment length polymorphism analysis, DHPLC, and linkage analysis is an efficient and accurate method that is well suited for routine use in clinical laboratories.

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SPINAL MUSCULAR ATROPHY (SMA) is an autosomal recessive hereditary disease characterized by degeneration of α-motor neurons in the anterior horn of the spinal cord, leading to paralysis and atrophy of proximal muscles. It has an estimated incidence of 1 in 10,000 newborns and a carrier frequency of about 2%. Patients are classified into 3 groups according to age at onset and disease progression. Type I is the most severe form, with onset before age 6 months and a life span rarely longer than 2 years. Type II is of intermediate severity, and type III is the mildest form.

The survival motor neuron gene (SMN) is a disease-causing gene comprising 2 nearly identical copies, SMN1 and SMN2. SMN1 and SMN2 differ by 5 nucleotides. More than 90% of patients with SMA have the SMN1 deletion, which can be used for diagnosis. In patients retaining SMN1, subtle mutations of SMN1 have been identified. The SMN2 deletion does not cause SMA, and about 5% of normal individuals have the SMN2 deletion. However, the conversion of SMN1 to SMN2 may increase the SMN2 copy number, which has been shown to be associated with a milder SMA phenotype. Therefore, SMN1 is...
the critical factor for SMA and SMN2 appears to modify disease severity in a dose-dependent manner.

The main goal of this article is to optimize the molecular assays and to offer physicians an accurate SMA diagnosis alternative. To date, 3 assays have been described for detection of the SMN1 deletion, and they are all polymerase chain reaction (PCR) based. Single-stranded conformation polymorphism analysis is a troublesome and time-consuming procedure. Although restriction enzyme digestion analysis (restriction fragment length polymorphism [RFLP] analysis) is a rapid, clear, and reliable method, it is unable to detect the subtle mutations of SMN1 and incomplete digestion may lead to false-negative results. Denaturing high-performance liquid chromatography (DHPLC) is a new technology for mutation screening that has many advantages, such as rapidity, accuracy, and high throughput. Since the locus for SMA was assigned to chromosome 5q11.2-q13.3 in 1990, prenatal diagnosis by linkage analysis had been applied to families with SMA. Single-stranded conformation polymorphism and RFLP analyses have also been reported to apply SMA prenatal diagnosis. However, to our knowledge, there has been no report of the use of DHPLC in prenatal diagnosis so far. Here we describe the results of a molecular analysis in 87 Chinese patients with SMA and prenatal prediction in 11 fetuses by the combination of RFLP analysis, DHPLC, and linkage analysis.

METHODS

SUBJECTS

A total of 77 unrelated Chinese families with SMA, including 87 patients and 132 parents, recruited by our department were screened for the SMN1 deletion from September 10, 1998, to May 28, 2005. All of the patients fulfilled the diagnostic criteria; 19 patients had type I SMA, 53 had type II, and the remaining 15 had type III. Genomic DNA was isolated from peripheral blood lymphocytes by standard methods. Prenatal prediction was performed on request for 11 fetuses from 10 families in which the proband had the SMN1 deletion. Skilled obstetricians were selected to perform the operations to avoid contamination of maternal blood or tissue. With the aid of B-scan ultrasonography, 30 to 50 mL of amniotic fluid was extracted from the pregnant mother at the gestational age of 16 to 20 weeks, it was centrifuged at 2000 rpm for 10 minutes, and the sediment was collected for genomic DNA extraction using the GFX blood isolation kit (Amersham Biscience, Buckinghamshire, England). Informed consent was obtained from each patient or legal guardian of those younger than 18 years, and the protocol was approved by the ethical committee of First Affiliated Hospital, Fuzhou, China.

MOLECULAR ANALYSIS

OF PATIENTS WITH SMA

All of the subjects were screened for the SMN1 deletion by RFLP analysis and DHPLC. The detailed procedures have been described previously. For those patients without the SMN1 deletion, the SMN1 copy number was detected by real-time fluorescence quantitative PCR as previously described, and the patients with 1 SMN1 copy were screened for the subtle mutations of SMN by direct DNA sequencing. The entire coding region (exons 1-7) including the intron-exon boundaries of SMN was amplified using the primers listed in Table 1. The DNA sequencing was performed on a 3730 DNA automatic sequencer (Applied Biosystems, Foster City, Calif) and the procedure was performed in accordance with the previous study. Sequences obtained by sample sequencing were compared with the genomic DNA sequence of SMN (Ensembl gene identification number ENSG00000197979), and nucleotide changes were numbered corresponding to their position in the SMN messenger RNA (GenBank accession number NM_000344).

Table 1. Oligonucleotide of Primers and Conditions of Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Primers</th>
<th>Directionality</th>
<th>Sequence</th>
<th>Size, bp</th>
<th>Annealing Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>1F</td>
<td>5’-ACGTAAGCTTGCCTGCCAGA-3’</td>
<td>281</td>
<td>60</td>
</tr>
<tr>
<td>Exon 2.1</td>
<td>1R</td>
<td>5’-AGTGAAGCTGATGACTGAC-3’</td>
<td>309</td>
<td>58</td>
</tr>
<tr>
<td>Exon 2.2</td>
<td>2.1F</td>
<td>5’-GTGACTTGGATACTACA-3’</td>
<td>342</td>
<td>59</td>
</tr>
<tr>
<td>Exon 3</td>
<td>3F</td>
<td>5’-GCAAAGTATATGTG-3’</td>
<td>351</td>
<td>60</td>
</tr>
<tr>
<td>Exon 4</td>
<td>4F</td>
<td>5’-CTAGAGCAAGACATACTTG-3’</td>
<td>338</td>
<td>60</td>
</tr>
<tr>
<td>Exon 5</td>
<td>5F</td>
<td>5’TGGATCTGTGACTCAGG-3’</td>
<td>316</td>
<td>60</td>
</tr>
<tr>
<td>Exon 6</td>
<td>6F</td>
<td>5’-AGTAGAGATTATAAGCA-3’</td>
<td>325</td>
<td>60</td>
</tr>
<tr>
<td>Exon 7</td>
<td>7F</td>
<td>5’-AGACTACACTTACTTCTG-3’</td>
<td>270</td>
<td>60</td>
</tr>
<tr>
<td>β-Actin</td>
<td>F</td>
<td>5’-TAGAGCAAGACATACTTG-3’</td>
<td>313</td>
<td>57</td>
</tr>
<tr>
<td>SMN</td>
<td>F</td>
<td>5’-AGACTACACTTACTTCTG-3’</td>
<td>450</td>
<td>57</td>
</tr>
</tbody>
</table>

Abbreviations: bp, base pairs; F, forward; R, reverse.
PRENATAL PREDICTION USING PCR–RFLP ANALYSIS, DHPLC, AND LINKAGE ANALYSIS

On September 10, 1998, and October 5, 2003, we performed prenatal prediction analysis for 2 fetuses by RFLP analysis and linkage analysis. The 2 fetuses were analyzed retrospectively by DHPLC at a later time. From October 16, 2003, the other 9 fetuses were screened for carrying SMA by detecting the SMN1 deletion and their parents were further analyzed by linkage analysis to exclude maternal contamination. Three short tandem repeat markers, D5S435, DSF14951, and D5S351, were analyzed in the fetuses together with their parents and probands. The PCR amplification and detection of short tandem repeat polymorphisms with denaturing polyacrylamide gel electrophoresis were in accordance with previous studies.9,16 The results in the aborted fetuses and born babies were reconfirmed by RFLP analysis and DHPLC. The born babies were followed up and physically examined twice a year.

CARRIER DETECTION OF FETUSES WITHOUT SMN1 DELETION

Fetuses without the SMN1 deletion and their parents were further screened for carrying SMA by detecting the SMN1 copy number using DHPLC as follows. A DNA sample with 2 SMN1 and 0 SMN2 copies (a gift from Brunhilde Wirth, PhD, Clinic University, Bonn, Germany) was used as a standard control. The standard control and the unknown DNA samples were quantified by a DU-600 spectrophotometer (Beckman Coulter, Inc, Fullerton, Calif), and the measurement was repeated 3 times. β-Actin was used as an internal control to minimize error. The multiplex PCR was used to amplify SMN1, SMN2, and β-actin simultaneously. The primers used for the multiplex PCR and the annealing temperatures are listed in Table 1. We ensured that an equal amount of DNA (200 ng) was used in each PCR. The PCR products were loaded into the WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Inc, Omaha, Neb) and the amplitudes of the SMN1, SMN2, and β-actin peaks were measured. The SMN1 and SMN2 copy numbers of the unknown samples in comparison with the standard control were calculated by the following equation: \[ \frac{(\text{peak height of SMN1 in unknown sample/peak height of β-actin in unknown sample})}{(\text{peak height of SMN1 in standard control/peak height of β-actin in standard control})} \times 2 \]. In addition, the results were confirmed by real-time fluorescence quantitative PCR as in the previous studies.9,16

RESULTS

MOLECULAR ANALYSIS OF PATIENTS WITH SMA

After screening by RFLP analysis and DHPLC, 82 patients from 72 families had the SMN1 deletion; thus, the frequency was 93.5% (72 of 77 patients). For the other 5 patients, 1 had the SMN2 deletion and 4 had neither the SMN1 deletion nor the SMN2 deletion. The results of DHPLC were consistent with those of RFLP analysis (Figure 1). Three peaks were detected by DHPLC in the 4 patients possessing both SMN1 and SMN2. They represented an SMN1-SMN2 heteroduplex, an SMN2 homoduplex, and an SMN1 homoduplex. In 82 patients, only the SMN2 homoduplex peak was detected, indicating the SMN1 deletion. In 1 patient, only the SMN1 homoduplex peak was detected, indicating the SMN2 deletion.

Five patients without the SMN1 deletion were further detected by real-time fluorescence quantitative PCR. Two patients had 2 SMN1 copies and the other 3 had 1 SMN1 copy. Thus, direct DNA sequencing was used to detect the subtle mutations of SMN in these 3 patients, but no mutations were found in the coding sequence or splice site. One hundred thirty parents showed neither SMN1 nor SMN2 deletion, and the other 2 showed SMN2 deletion.
PRENATAL PREDICTION OF SMA IN FETUSES AND FOLLOW-UP STUDIES

After prenatal prediction of SMA in 11 fetuses by RFLP analysis and DHPLC, 4 were found to have the SMN1 deletion and were aborted. The other 7 showed neither SMN1 nor SMN2 deletion and were further analyzed by linkage analysis. The results were consistent with those of RFLP analysis and DHPLC and showed no maternal contamination (Figure 2). Also, we screened for carriers of SMA among these 7 fetuses and their parents. In comparison with the peak height of the standard control (2 SMN1 copies), 4 fetuses and all of the 14 parents had only 1 SMN1 copy, indicating that they were SMA carriers. The other 3 fetuses had 2 SMN1 copies, indicating that they were normal (Figure 3). The result of the real-time fluorescence quantitative PCR was consistent with those results. Thus, these 7 fetuses were born under suggestion by the physician. Results in the aborted fetuses and born babies were reconfirmed and were com-

Figure 2. Prenatal prediction of the fetus (II:2; fetus 7 in Table 2). A, The family pedigree. Squares indicate males; circle, female; diamond, sex unknown; half-filled symbols, carriers of spinal muscular atrophy (SMA); filled symbol, individual with SMA; open symbol, SMA status unknown; arrow, proband. The prediction was made by a combination of restriction fragment length polymorphism (RFLP) analysis (B), denaturing high-performance liquid chromatography (C), and linkage analysis (D). B, The RFLP analysis showed that the proband deleted exon 7 (E7) and exon 8 (E8) of the SMN1 gene. The fetus and the parents did not have the SMN1 deletion. PCR indicates polymerase chain reaction; bp, base pairs. C, Denaturing high-performance liquid chromatography showed that the proband deleted SMN1 and the fetus had 1 SMN1 copy, just as the parents did, which indicated that the fetus and parents were carriers of SMA. D, Linkage analysis showed that the fetus inherited the SMA chromosomal haplotype from the father and the normal chromosomal haplotype from the mother; thus, the fetus was a carrier of SMA. Also, linkage analysis excluded maternal contamination.
The best approach to SMA is prenatal diagnosis because SMA is incurable. Therefore, it is necessary to ascertain the molecular defect of the proband prior to performing prenatal prediction in a family with SMA. Ninety percent to 98% of patients with SMA have homozygous SMN1 deletions, and subtle mutations of SMN1 are responsible for a few cases. At present, 52 different subtle mutations have been published (http://www.hgmd.org/). According to Hardy-Weinberg equilibrium, 99.7% of patients with SMA have at least 1 deleted SMN1 copy. Thus, patients with SMA without the SMN1 deletion detected by RFLP analysis actually had 1 deleted SMN1 copy and carried 1 subtle mutation on the other SMN1 copy.\(^1\) Identification of subtle mutations in the SMN1 gene is hampered by the presence of SMN2, which can be solved by cloning reverse-transcriptase–PCR products of patients. All of these make it possible to confirm the clinical diagnosis of SMA or SMA-like cases by molecular analysis.

Our main goal is to establish a relatively perfect diagnosis system that is accurate, is rapid, and can be used as a routine method to diagnose patients with SMA and help their families. Therefore, we conducted molecular analysis for each patient with suspected SMA as follows. First, we detected the SMN1 deletion by RFLP analysis and DHPLC. Second, we quantified the SMN1 copy number of patients without the SMN1 deletion. Third, we sequenced the SMN coding region of the patient having only 1 SMN1 copy. Finally, if the patients carried subtle mutations of SMN, we cloned reverse-transcriptase–PCR products of patients to discriminate whether the mutation lies in the SMN1 or SMN2 gene.

In this study, the frequency of the SMN1 deletion was 93.5% (72 of 77 patients), which confirms results in previous studies.\(^3\) The 5 patients without the SMN1 deletion were further analyzed by SMN1 copy number using real-time fluorescence quantitative PCR, which was first described by Feldkotter et al.\(^7\) Because the possibility of patients having subtle mutations in 2 SMN1 copies is rare, the 2 patients with 2 SMN1 copies were regarded as not having SMA and were not investigated further. The other 3 patients having only 1 SMN1 copy were screened for subtle mutations by direct sequencing, but no muta-
tions were identified. This might be explained by the following reasons. First, it is possible that some mutations lie within the unsequenced regions. Second, it is also possible that there exist some other genes, such as the neuronal apoptosis inhibitory protein gene (NAIP) or the basal transcription factor p44 subunit gene (BTF2P44), that are responsible for the disease. Finally, some clinically diagnosed patients might not genuinely have SMA. In these 5 families, we advised them to refrain from prenatal diagnosis.

For 72 families with the SMN1 deletion, detailed data were registered on request for prenatal prediction in ongoing or future pregnancies. Analysis by RFLP is a necessary tool for prenatal analysis of SMA, but incomplete digestion will lead to false-negative results. Denaturing high-performance liquid chromatography is a novel method that is very sensitive to DNA sequence variation. So far, to our knowledge, there is no report of application of DHPLC in SMA prenatal diagnosis. Here we performed prenatal diagnosis by the combination of RFLP analysis and DHPLC. Although linkage analysis is very troublesome, to be absolutely secure, we performed linkage analysis for 7 born babies. The main objective of linkage analysis here was to exclude the possibility of maternal contamination; it is unnecessary to perform linkage analysis on fetuses with the SMN1 deletion.

Notably, DHPLC has shown its superiority in detecting carriers of SMA. In previous studies about prenatal prediction by RFLP analysis, the carriers of SMA could not be identified. According to the hereditary rule of SMA, the frequency of carriers in fetuses with parents carrying SMA is 50%, which is significantly higher than that in normal individuals. When these carriers get married, it is highly likely that their offspring will have SMA. Identification of the carrier's genotype is necessary for the fetus and other members of families with SMA. The results of the present study indicate that DHPLC can quantify the SMN1 copy number accurately and differentiate carriers of SMA except for 4% of carriers who have 2 SMN1 copies in the same chromosome. Su et al. also quantified the SMN1 and SMN2 copy numbers by calculating the SMN1-SMN2 ratio with DHPLC and using CYBB and KRIT1 as internal controls. Here we used a DNA sample with 2 SMN1 and 0 SMN2 copies as the standard control, making SMN1 quantification more direct and reliable. The results of prenatal diagnosis were reconfirmed by analyzing DNA samples from the aborted fetuses or born babies. The follow-up study of the 7 born children also indicated the reliability of our prenatal prediction.

Taken together, the combination of RFLP analysis, DHPLC, and linkage analysis makes the molecular diagnosis and prenatal prediction of SMA more accurate and provides the family with appropriate, accurate information necessary to make a decision about early pregnancy termination. Restriction fragment length polymorphism analysis and DHPLC are used for the molecular diagnosis of patients and fetuses, and DHPLC is also a reliable and rapid method of detecting carriers of SMA. Linkage analysis acts as a tool to exclude the possibility of maternal contamination and makes the prenatal diagnosis absolutely secure. The diagnosis system established here can be used as a routine diagnosis method in clinics.

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REFERENCES


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**Announcement**

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For details about this new policy, and for information on how the ICMJE defines a clinical trial, see the editorial by DeAngelis et al in the January issue of Archives of Dermatology (2005;141:76-77). Also see the Instructions to Authors on our Web site: www.archneurol.com.