Detection of Spinal Muscular Atrophy Carriers by Nested Polymerase Chain Reaction of Single Sperm Cells

YUVAL YARON,1–3 TANIA COHEN,2 NAVA MEY-RAZ,2 TAMAR SCHWARTZ,2 AMI AMIT,2,3 and MIRA MALCOV2

ABSTRACT

Spinal muscular atrophy (SMA) is an autosomal recessive disorder with a carrier frequency of ~1 in 40. Approximately 95% of patients have homozygous deletions of exon 7 and/or 8 of the SMN1 gene. Carrier testing for SMA is relatively complex and requires quantitative polymerase chain reaction (PCR) of genomic DNA to determine SMN1 copy number. The purpose of this study was to assess the feasibility of carrier testing for SMA in males, by nested PCR analysis of SMN1 deletions in single sperm cells. A nested PCR method was developed to amplify SMN1 exon 7 in single cells. Restriction enzyme digestion with DraI was used to differentiate between the highly homologous SMN1 and SMN2 genes. Single sperm cells from five known SMA carriers and six noncarriers were analyzed. Among the five carriers, a total of 132 single sperm cells were analyzed and SMN1 exon 7 deletion was detected in 68 cells (51.5%). In contrast, among the six noncarriers, a total of 136 single sperm cells were analyzed. Of these, an apparent SMN1 exon 7 deletion was detected in four sperm cells. This was interpreted as an allele dropout (ADO) rate of 2.9%. We conclude that nested PCR of SMN1 exon 7 is an accurate and reproducible method for detection of SMA male carriers with a SMN1 deletion.

1Prenatal Diagnosis Unit, Genetic Institute and 2Sara Racine In Vitro Fertilization Unit, Department of Obstetrics & Gynecology, Lis Maternity Hospital, Tel Aviv Sourasky Medical Center, Israel, affiliated with 3Sackler Faculty of Medicine, Tel Aviv University, Israel.
mosomes have two SMN1 copies in cis configuration (two copies of a gene on the same chromosome) (Feldkott et al. 2002; Ogino and Wilson 2002). This implies that approximately 3% of SMN1 deletion carriers would be misinterpreted as non-carriers because they are found to have two SMN1 copies, albeit on the same chromosome. Moreover, approximately 1.7% have point mutations, thus limiting the sensitivity of all PCR-based dosage assays to less than 95% (Feldkott et al. 2002; Ogino and Wilson 2002). The recently described multiplex mutation-dependent probe amplification may be easier to perform (Tomaszewicz et al. 2005), but would still not detect deletion carriers having two SMN1 copies in cis.

To overcome the masking of SMN1 gene deletion by amplification of normal allele, it would be theoretically advantageous to evaluate each allele separately. For point mutations or small deletions, allele separation may be achieved by PCR amplification of a DNA fragment harboring the mutation, followed by cloning of the product into a vector. With larger deletions, however, the deleted fragment cannot be amplified, and therefore cannot be cloned. An alternative approach is to use the natural process of allele separation, occurring in male meiosis. Among meiotic divisions, only one or the other allele reach the haploid sperm cell. Thus, single-cell analysis may analyze each allele separately. A similar approach has been suggested by Reubinoff et al. (1996) to assess the carrier status of males for the RhD-negative genotype, which is also caused by a large-scale deletion of the RhD locus. Using nested PCR of the RhD locus in single sperm cells, they were able to distinguish between Rh-positive individuals who are either homozygous for the RhD locus (+/+) or heterozygotes (+/-). RhD genotype was correctly assigned in all 10 patients by analyzing 8 to 17 single sperm cells for each (Reubinoff et al. 1996).

The purpose of our study was to assess the feasibility of performing carrier testing for SMA in males by single sperm multiplex nested PCR analysis of SMN1 exon 7.

MATERIALS AND METHODS

Patient selection

An adult female patient with SMA-III with a known homozygous deletion of SMN1 exon 7 served a positive control. The study group included five obligatory SMA-carrier males, ascertained by the having more than one affected offspring (child or fetus) with SMA, as confirmed by molecular analysis showing homozygous deletions of SMN1 exon 7. Six other individuals with healthy offspring served as controls.

Isolation of single lymphocytes

To establish the single-cell nested PCR protocol, we used leukocytes from the SMA patient with the SMN1 exon 7 deletion. Leukocytes were isolated by the Red Blood Cell Lysis Solution (Beit Haemek, Israel), according to the manufacturer’s instructions. Briefly, 3 ml of blood were collected, added to the above solution at a 1:2 ratio, gently mixed for 10 min, and centrifuged at 300g for 10 min. The supernatant was discarded and the pellet containing the leukocytes was re-suspended in P1 culture medium (Irvine Scientific, Santa Ana, CA), supplemented with 20% serum. The concentrated pellet was then diluted with phosphate buffered saline (PBS; Gibco, Grand Island, NY) containing 0.01% polyvinylpyrrolidone (PVP; Sigma, St. Louis, MO) to prevent clumping, and 0.1 mg/ml phenol red (Sigma) to enable visualization of the sample. Dilution was performed under an inverted microscope until single cells could be aspirated with a pulled glass micropipette. Each single cell was transferred to a sterile UV-irradiated PCR tube. DNAse inactivation was performed by heat at 65°C for 10 min.

Single sperm cell isolation

Fresh semen was allowed to liquefy at room temperature for 30 min, washed in mHTF supplemented with 5% serum (Irvine Scientific), and centrifuged at 480g for 10 min. The pellet was then left in the incubator at 37°C for 60 min to allow swim-up. The supernatant containing the motile sperm was transferred to a petri dish containing drops of PVP solution (Irvine). To prevent motility and facilitate single cells collection, sperm tails were cut off using a pulled glass micropipette, washed, and diluted in PBS containing 0.01% PVP and 0.1 mg/ml phenol red, until single sperm could be aspirated. Morphologically normal sperm were aspirated using an intracytoplasmic sperm injection (ICSI) micropipette (Cook, Queensland, Australia) attached to an IM-6 (Narishige, Tokyo, Japan) piezoejector under 200× magnification of a Diaphot inverted microscope (Nikon, Tokyo, Japan) equipped with micromanipulators (Narishige). Each single sperm was transferred to a sterile UV-irradiated PCR tube. DNAse inactivation was performed by heat at 65°C for 10 min.

Cell lysis

Single leukocytes were lysed by adding 3 μl of alkaline lysis buffer (200 mM NaOH, 50 mM dithiothreitol) and incubated at 65°C for 10 min. Lysis of sperm cells was performed by three cycles of snap-freezing in liquid nitrogen followed by heating at 96°C for 5 min, and kept in −20°C until use. To lyse the sperm cell completely and to free the DNA from the protamines, 3 μl of alkaline lysis buffer (200 mM NaOH, 150 mM dithiothreitol) are then added and incubated at 65°C for 20 min.

Prevention of contamination

To eliminate contamination of the PCR components, lysis buffer was UV-irradiated for 1 hr. In addition, the PCR mixture minus Taq polymerase was incubated with 0.5 unit AluI for 2 hr at 37°C. Because the expected product has an AluI restriction site, digestion would obliterate all contaminating DNA templates. This was followed by inactivation of the restriction enzyme at 65°C for 30 min.

PCR primer design

To amplify the SMN gene in single sperm cells, a two-step nested PCR approach was used. The protocol was initially designed for preimplantation genetic diagnosis (PGD) of SMA (Malcov et al. 2004). The same inner and outer primers of exons 7 amplify both SMN1 and SMN2 genes, which are then distinguished by restriction enzyme analysis. Thus the reaction has a “built-in” internal control to evaluate amplification efficiency. The inner reverse primer for exon 7 incorporates a mismatch such that following the simultaneous amplification of SMN1 and SMN2, a novel DraI restriction site is created in the SMN2 gene, but not in the SMN1 gene (van der Steege et al. 1995).
After enzymatic digestion with DraI, the SMN1 PCR product of 240 bp remains intact, while the SMN2 PCR product is cleaved to create two fragments of 28 bp and 212 bp (Fig. 1).

**First round—Outer PCR**

For the first-round PCR, the following are added to the reaction tube containing the single cell in alkaline lysis buffer: 2 µl of 10X PCR buffer (OptiBuffer™, Bioline, Randolph, MA), 1 µl MgCl₂ (50 mM), 2 µl of 5X Specificity Enhancer (Bioline), 0.5 µl Tricine 1 M, 1 µl of dNTP mixture stock (5 mM), 1 µl of dimethyl sulfoxide (DMSO), 0.5 µl gelatin (1% w/v), 0.5 µl of each outer primer (13 µM) (Table 1). H₂O is added for a final volume of 20 µl. The mixture is denatured at 96°C for 8 min and temperature is then decreased to 75°C. At this stage, 5 µl of enzyme mix containing 0.5 µl 10X PCR buffer (OptiBuffer™, Bioline), 0.25 µl MgCl₂ (50 mM), 0.25 µl Taq polymerase (Bio-X-Act, 4 U/µl) and 3.85 µl H₂O, is added.

PCR reaction begins with a single denaturation step of 98°C for 2 min, followed by 10 cycles of denaturation at 96°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. This is followed by six more cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 1 min, and extension at 72°C for 2 min. Final extension is performed at 72°C for 8 min.

**Second round—Inner PCR**

One microliter of the first-round PCR product is aliquoted to a fresh tube for the inner (nested) PCR. The following are added to the tube: 2 µl of 10X PCR buffer (OptiBuffer™, Bioline), 1 µl MgCl₂ (50 mM), 2 µl of 5X Specificity Enhancer (Bioline), 1 µl of dNTP mixture stock (5 mM), 1 µl of DMSO, 1.5 µl of each inner primer (13 µM). H₂O is added for a final volume of 20 µl. The mixtures are denatured at 96°C for 8 min and temperature is then decreased to 75°C. At this stage, 5 µl of enzyme mix containing 0.5 µl 10X PCR buffer (OptiBuffer™, Bioline), 0.25 µl MgCl₂ (50 mM), 0.4 µl Taq polymerase (Bio-X-Act, 4 U/µl) and 3.85 µl H₂O, is added.

PCR begins with a single denaturation step of 98°C for 2 min, followed by 14 cycles of denaturation at 96°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 2 min. This is followed by 20 more cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 1 min and extension at 72°C for 2 min. Final extension is performed at 72°C for 8 min.

**Restriction enzyme digestion**

A 10-µl aliquot of the secondary PCR product is digested with 20 units DraI, and incubated at 37°C for 2 hr. The products of enzymatic restriction digestion are resolved by electrophoresis on a 3% agarose gel stained with ethidium bromide. Products are visualized under UV light.

**Ethics**

The study was approved by the Tel Aviv Medical Center Internal Review Board (Helsinki Committee), and by the National High Committee for Genetic Research. Informed consent was obtained from all subjects.

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**Table 1. Primer Sets Used for Multiplex Nested PDR of SMN1 Exon 7 in Single Sperm Cells**

<table>
<thead>
<tr>
<th>Exon 7 primer set</th>
<th>SMN7 primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 7 outer forward</td>
<td>SMN7-O-F</td>
<td>TGC AGC CTA ATA ATT GTT TTC TTT GGG</td>
</tr>
<tr>
<td>Exon 7 outer reverse</td>
<td>SMN7-O-R</td>
<td>AGA TTC ACT TAT ATA ATG CTG GCA GA</td>
</tr>
<tr>
<td>Exon 7 inner forward</td>
<td>SMN7-I-F</td>
<td>TGC AGC CTA ATA ATT GTT TTC TTT GGG</td>
</tr>
<tr>
<td>Exon 7 inner reverse</td>
<td>X7-DraI</td>
<td>GCA CCT TCC TTC TTG ATT TTG TTT</td>
</tr>
</tbody>
</table>

*Same primer used.*
Statistical analysis

Statistical analysis was performed using SPSS for Windows (SPSS Inc., Chicago, IL). To compare the incidence of non-amplification of SMN1 exon 7 between SMA carriers and non-carriers, the nonparametric Mann-Whitney test was used. A p value < 0.01 was considered as statistically significant.

RESULTS

Single-cell nested PCR in single leukocytes

The single-cell nested PCR protocol was first developed on single leukocytes obtained from a SMA type III patient with a known SMN1 exon 7 deletion (Fig. 2). Single leukocytes from healthy individuals served as controls. Primer sets for exon 7 were designed to allow distinguishing between the respective SMN1 and SMN2 copies, after enzymatic digestion with DraI. The absence of noncleaved fragments reflects homozygous deletion of the SMN1 exon 7 or possibly, allele drop-out (ADO). After establishment of the single-cell nested PCR protocol, 200 single leukocytes from the SMA patient and 200 single leukocytes from normal controls were evaluated. SMN1 exon 7 was amplified in 99% of normal single leukocytes and in none of the SMA-affected leukocytes. Exon 7 of SMN2 was amplified in 100% of both normal and SMA-affected leukocytes. There were no false-negative results and no contamination was detected in all wash-drop blanks tested.

Nested PCR in single sperm cells

Single sperm cells were then analyzed in 5 obligatory SMA carriers and in 6 normal controls. In 15% of test tubes analyzed, no PCR amplification signals of any kind were noted. The non-amplification rate was as low as 0% in fresh high-quality sperm samples, and as high as 45% in frozen samples of low-quality sperm. Tubes that demonstrated no amplification were not taken into account. Table 2 describes the results of the single cell nested PCR. Among the 5 SMA-carrier males, a total of 132 single sperm cells were successfully analyzed (median = 26; range, 19–35). Of these, SMN1 exon 7 was undetected in 68 cells (51.5%). The percentage of single sperm demonstrating the deletion in these carrier males ranged from 31.6% to 74.1%. Among the 6 noncarrier males, a total of 136 single sperm cells were analyzed (median = 20; range, 15–43). Of these, SMN1 exon 7 was undetected in 4 single sperm cells (2.9%). The percentage of single cells without SMN1 exon 7 amplification ranged between 0% and 5.9% among the noncarriers, and in the none of the cases was this demonstrated in more than one sperm cell. The lack of SMN1 exon 7 amplification in these cases was interpreted as allele drop-out (ADO). The difference of SMN1 nonamplification between carriers and non-carriers was statistically significant (p = 0.004, Mann-Whitney test).

Interestingly, one of the SMN1 carrier males (case 4) was found to be a compound heterozygote with a SMN2 deletion on the other allele. The results of this patient are described in Fig-

TABLE 2. ANALYSIS OF SMN1 EXON 7 IN SINGLE SPERM OF SMA CARRIERS AND NONCARRIER MALES

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patient</th>
<th>Single sperm cells with SMN1 exon 7 deletion</th>
<th>Single sperm cells with SMN1 exon 7 amplification</th>
<th>% deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncarriers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>43</td>
<td>2.3%</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>23</td>
<td>4.3%</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1</td>
<td>16</td>
<td>0.0%</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>17</td>
<td>5.9%</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>1</td>
<td>15</td>
<td>0.0%</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Total</td>
<td>4</td>
<td>16</td>
<td>136</td>
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<td>SMA carriers</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>27</td>
<td></td>
<td>74.1%</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>26</td>
<td></td>
<td>42.3%</td>
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<tr>
<td>3</td>
<td>11</td>
<td>25</td>
<td></td>
<td>44.0%</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>35</td>
<td></td>
<td>57.1%</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>19</td>
<td></td>
<td>31.6%</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>132</td>
<td></td>
<td>51.5%</td>
</tr>
</tbody>
</table>

SMA, spinal muscular atrophy.
and 7) or the 212-bp band, which corresponds to the intact SMN2 allele. In contrast, the noncarrier (lanes 9–11) demonstrates both 240 bp and 212 bp bands, corresponding to SMN1 and SMN2, respectively.

FIG. 3. Analysis of SMN in single sperm cells in a carrier who is a compound heterozygous for both SMN1 and SMN2 deletion (lanes 1–8) and in a noncarrier (lanes 9–11). Lanes 4 and 8 represent nonamplification. Note that in the compound heterozygote, individual sperm cells demonstrate either the 240-bp band, which corresponds to the intact SMN1 allele (lanes 2, 5, and 7) or the 212-bp band, which corresponds to the intact SMN2 allele (lanes 1, 3, and 6).

ure 3. Note that successfully analyzed sperm cells demonstrate either the upper band (corresponding to the undigested SMN2 copy) or the lower band (corresponding to the digested SMN1 copy). The fact that in this case there were no samples demonstrating both SMN1 and SMN2 suggests that each tube does indeed contained only a single sperm cell.

DISCUSSION

In this study we describe the molecular analysis of SMN1 gene deletions in single human sperm for the purpose of diagnosing SMA carriers. This test may be an alternative in males to carrier-detection by quantitative PCR. While the quantitative assay has been shown to be efficient in detecting SMA carriers, it is inherently limited by the fact that only 95% of deletion carriers are detected. This is because some SMA carriers have two SMN1 copies in tandem on the same chromosome, and a deletion on the other (McAndrew et al. 1997; Wirth et al. 1999; Feldkotter et al. 2002). Feldkotter et al. (2002) noted that in 20 of 834 (2.4%) healthy chromosomes, two SMN1 copies are found in tandem. They estimated that 4.8% of all deletion carriers would be misinterpreted as non-carriers. Because an additional 1.7% of SMA carriers have a nondeletion mutation, they concluded that the sensitivity of dosage-based carrier testing would be, at best, 93.5% (Feldkotter et al. 2002).

The carrier testing method presented in this study is based on the individual analysis of each paternal allele, as it is represented in the single haploid sperm cell. Therefore, this test would also detect SMA carriers that have two SMN1 copies in tandem, theoretically increasing detection rates. Based on our results, and given the fact that 1.7% of SMA carriers have a nondeletion mutation, the sensitivity of this carrier test may reach approximately 98% in males. However, further prospective studies are needed to confirm that these results apply in screening of the general population.

This method would also be useful in a clinical scenario in which the father of an affected child with a homozygous deletion, is found to have two copies of SMN1. In such an event, analysis of single sperm in the father would distinguish between: (1) the presence of two copies of SMN1 in cis in the father and (2) the presence of two copies of SMN1 in trans in the father, and a de novo deletion in the child (commonly paternal in origin). Whereas the former is at a 25% risk of recurrence, the latter is associated with a very low recurrence rate.

How many single sperm cells need to be analyzed in a clinical screening test? We have shown that SMA carriers demonstrate a significantly higher number of single sperm cells with non-amplification of SMN1 than noncarriers. However, to use this as a clinical screening test, it is necessary to determine the number of cells that need to be analyzed to establish a diagnosis. This number depends upon the expected rate of SMN1 deletions in single sperm among carriers (0.5), the expected ADO rate in single sperm (0.03), and the cutoff rate of SMN1 non-amplification above which the tested individual would be defined as a carrier. Assuming a binomial distribution we chose an arbitrary cutoff of 10% SMN1 exon 7 nonamplification. Based on these parameters, 10 cells would be required to define an individual as a “carrier” and 31 cells would be required to define one as a “noncarrier,” at a significance level of \( p < 0.01 \). Thus, from a practical point of view, 31 single sperm cells would have to be analyzed successfully in each case to determine whether the individual is a carrier or not. Given the approximately 15% incidence of failed amplification, one should begin with approximately 36 single sperm cells.

We suggest that males be tested first by the single sperm analysis and if found to be carriers, have their female partners evaluated by one of the quantitative PCR assays. This method may prove particularly useful for analysis of both fresh and frozen donor sperm. The practicality of this approach is underlined in an article by Tizzano et al. (2002) who described a case of a female SMA carrier who underwent artificial insemination and achieved two successive pregnancies from two different donors. In the first pregnancy a child with SMA was born. In the second pregnancy, a fetus affected with SMA was diagnosed by prenatal molecular diagnosis (Tizzano et al. 2002). They suggested genotyping the SMN locus in sperm and oocyte donors to detect SMA carriers by quantitative analysis of the SMN1 gene, particularly if the recipient is a known or suspected carrier. They calculated that if the donor shows a single SMN1 copy, the probability of being a carrier is almost 1, with a final risk resembling that in couples with an affected child (1/4). If however, the donor had two SMN1 copies, the probability of being a carrier is approximately 1 in 1250, and the risk for an
affected child is 1 in 5000, close to the incidence in the general population.

According to the guidelines issued by the American Society for Reproductive Medicine (ASRM) carriers of autosomal recessive disorders should be excluded from the donor pool. The disorders to be screened depend on the geographical and ethnic origin of the prospective donors. Autosomal recessive disorders such as cystic fibrosis, Tay-Sachs disease, and α- and β-thalassemia are often included in the genetic studies of semen and oocyte donor protocols (Wallerstein et al. 1998). These diseases are relatively common in specific populations and severe enough to merit carrier screening. Spinal muscular atrophy is prevalent in most populations with a carrier frequency of approximately 1 in 40. The severity and frequency of this disorder clearly justify its inclusion in the genetic screening tests for all gamete donors and individuals in their reproductive period. Our method may prove particularly useful in screening stored donor sperm for SMA.

ACKNOWLEDGMENTS

We are grateful to our SMA families and particularly the carrier fathers who made this study possible. We thank Dr. Yael Villa for the statistical analysis.

ELECTRONIC DATABASE INFORMATION

Accession numbers and URLs for data in this article are as follows: Online Mendelian Inheritance in Man (OMIM), www.ncbi.nlm.nih.gov/Omim/ SMA type I, Werdnig-Hoffmann disease; [MIM 253300], SMA type II [MIM 253550], SMA type III, Kugelberg-Welander disease [MIM 253400], SMA type IV [MIM 158590]

REFERENCES


Address reprint requests to: Prof. Yuval Yaron
Prenatal Diagnosis Unit
Genetic Institute
Tel Aviv Sourasky Medical Center
6 Weizmann Street
Tel Aviv 64239
Israel

E-mail: yyaron@tasmc.health.gov.il