Single-sperm analysis for haplotype construction of de-novo paternal mutations: application to PGD for neurofibromatosis type 1

G. Altarescu1, B. Brooks2, Y. Kaplan1, T. Eldar-Geva2, E. J. Margalioth2, E. Levy-Lahad1,3 and P. Renbaum1,4

1Medical Genetics Unit, 2IVF Unit, Zohar PGD Laboratory Shaare Zedek Medical Center and 3Hebrew University-Hadassah Medical School, Jerusalem, Israel
4To whom correspondence should be addressed at: Medical Genetics Unit, Shaare Zedek Medical Center, Jerusalem 91031, Israel. E-mail: renbaum@szmc.org.il

BACKGROUND: Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder caused by mutations in the neurofibromin gene. Approximately, 50% of cases are caused by de-novo mutations. Even when the NF1 mutation is known, accuracy of PGD is highly enhanced by simultaneous analysis of linked markers. In a childless couple referred to PGD, the male carried a de-novo mutation, precluding the possibility of typing relatives to establish the mutation-associated haplotype. We developed a single-sperm haplotype analysis strategy to establish the haplotype linked to the NF1 mutation. METHODS: Spermatozoa from freshly ejaculated semen were used as a substrate for multiplex PCR on single sperm. RESULTS: In addition to the NF1 mutation, six informative polymorphic markers flanking the NF1 gene (D17S1294, D17S1849, D17S841, D17S975, NF1TG2 and NF1AC5) were linked to individual alleles in single sperm from the affected male. CONCLUSIONS: Single-sperm analysis established the haplotypes of both mutant and wild-type NF1 alleles and enabled the implementation of a PGD protocol using polymorphic marker analysis. This method is generally applicable to PGD for any disease in which the haplotype of paternal mutations cannot be determined by typing relatives.

Key words: single-sperm PCR/NF1/PGD/polymorphic markers

Introduction

Neurofibromatosis type 1 (NF1, von Recklinghausen syndrome) has an estimated birth incidence of about 1 in 3500 individuals worldwide and is caused by autosomal dominant mutations in the neurofibromin gene (Hart, 2005; Ward and Gutmann, 2005). NF1 is traditionally regarded as a neurocutaneous disorder (phakomatosis), but disease manifestations occur in many other organ systems as well (Rose, 2004; Hart, 2005; Korf, 2005; Ward and Gutmann, 2005). The clinical picture varies from mild to severe, even in patients carrying the same NF1 mutation, and there is significant intrafamilial variability (Carey and Viskochil, 1999; Viskochil, 2002). Although the only consistent features of this disorder are cafe-au-lait spots and fibromatous skin tumours, several other organs are often found to be affected with NF1-related tumours (Rose, 2004; Page and Franklin, 2004). There is also a predisposition towards malignant transformation of the benign tumours associated with NF1 in an estimated 3–15% of cases (Hart, 2005; Hasiotou et al., 2005; Kim et al., 2005; Takazawa et al., 2005).

The neurofibromin gene, NF1, is located on the long arm of chromosome 17. It is a particularly large gene, extending over 359 kb and including 58 exons. Over 500 different mutations have been identified in the NF1 gene, the majority of which are family specific (Carroll and Stonecypher, 2005). Many types of NF1 mutations have been reported, including large and small deletions and insertions, missense, non-sense, splicing mutations and chromosomal aberrations (Carroll and Stonecypher, 2004). Although protein-truncation testing (PTT) and sequencing can detect up to 95% of mutations in clinically diagnosed NF1 patients, diagnostic criteria remain clinical, and mutation analysis is not commonly performed owing to its high cost. In families with more than one affected relative, linkage analysis provides a much simpler and cheaper means of molecular diagnosis. However, over 50% of NF1 mutations occur de novo, precluding conventional linkage analysis in over half of NF1 cases.

PGD for NF1 has been reported by two different centres. In the first instance, maternal mutations were tested by sequential PCR of polar bodies 1 and 2 in conjunction with three linked polymorphic markers in introns 19A, 27A and exon. Two unrelated couples underwent two cycles each resulting in a pregnancy in each couple (Verlinsky et al., 2002). In the second report, blastomeres were analysed for the NF1 mutation in...
conjunction with two polymorphic markers (D17S1800 and D17S841) resulting in an unaffected male (Spits et al., 2005).

Both prenatal and preimplantation diagnoses of NF1 rely on previous determination of the specific mutation, or in familial cases, the linked haplotype. In PGD, genetic analysis is performed on single cells: the first and second polar bodies or embryonic blastomeres. Molecular testing is done by single-cell PCR, which is prone to allele dropout (ADO), i.e. failure of amplification of one of the alleles. Failure of amplification of the mutant allele can result in an erroneous diagnosis in which a mutant embryo is mistaken for a wild-type embryo. This critical problem can be minimized by co-amplifying linked, polymorphic markers because it is unlikely that ADO will occur in all reactions. In blastomeres, simultaneous testing of linked markers reduces the rate of misdiagnosis from 20–30% to 3% (Verlinsky and Kuliev, 2000). Analysis of two blastomeres from each embryo has also been used for this purpose but may result in lower implantation and pregnancy rates (Cohen, 2005).

To allow the use of linked markers for PGD in men with de-novo mutations, we have developed a strategy to determine haplotypes from single sperm. We report a couple with a de-novo NF1 mutation in a mildly affected male, who presented to our clinic for PGD. Linkage analysis was not possible because this couple had neither affected relatives nor previous pregnancies. To establish linkage between the new NF1 mutation and polymorphic markers, we isolated and analysed single sperm (Huang et al., 1995; Zhang et al., 1995; Brohede et al., 2004), using single-cell PCR, for both the new NF1 mutation and NF1-linked markers.

Materials and methods

**Single-sperm isolation, IVF and blastomere biopsy procedure**

Spermatozoa were prepared from freshly ejaculated semen using discontinuous gradient centrifugation (Isolate, Irvine Scientific, Santa Ana, CA, USA). Separated sperm were washed in culture medium (Flusing, Medi-Cult, Copenhagen, Denmark), and small numbers of sperm were pipetted into a 2.5-μl droplet of polyvinylpyrrolidone (PVP, Medi-Cult) under mineral oil. Single spermatozoa were then transferred to individual 0.5-μl droplets of P1 culture medium (Irvine Scientific) under mineral oil. Droplets containing single-sperm cells were aspirated using finely drawn Pasteur pipettes and were transferred to PCR tubes containing 5 μl of alkaline lysis buffer (Thornhill et al., 2001).

IVF treatment was performed by pituitary down-regulation with GnR analogue, followed by controlled ovarian hyperstimulation with rFSH (Merviel et al., 2004), Vaginal ultrasound-guided oocyte retrieval was performed 34 h after HCG injection. Cumulus-oocyte complexes (COCs) were identified, washed and transferred to organ culture dishes containing equilibrated culture medium (Medi-Cult) and placed in an incubator with 5% CO₂. Oocytes were fed with hyaluronidase (Sigma-Aldrich, USA), and ICSI was performed on each mature egg (M2). Injected oocytes were transferred to P1 medium (Medi-Cult) under mineral oil. Fertilized oocytes were cultivated in P1 medium for a further 48 h. Blastomere biopsy was performed using mechanical zona slitting (Verlinsky and Kuliev, 2005). Blastomeres were carefully transferred to separate 0.5-ml tubes containing 5 μl of proteinase K lysis buffer (Thornhill et al., 2001), and embryos were transferred to fresh culture medium for blastocyst development (Irvine Scientific). A sample of culture medium from the same droplet that contained each biopsied blastomere was used as a ‘no template control’ (NTC).

**Molecular analysis**

A couple presented to our clinic for PGD with NF1, where the mildly affected male was known to carry a de-novo NF1-splicing mutation, 6365(-2)A>G. The couple was childless and had no previous pregnancies, the woman was 25 years old. The male was previously shown to harbour a de-novo NF1 splice mutation upstream of exon 34, 3465(-2)A>G. In addition to the new NF1 mutation, polymorphic markers flanking the NF1 gene were amplified in a single multiplex PCR for each individual sperm. The reactions contained 1 U SuperTaq polymerase (JMR, Kent, UK), 0.32 mM dNTPs, 10% DMSO, 0.1 μM each forward and reverse primers (Table I), in a buffer supplied by the manufacturer containing 1.5 mM MgCl₂ and was cycled using a touchdown protocol (Verlinsky et al., 2004). Aliquots (1.5 μl) from the multiplex reaction were used as templates for individual hemi-nested PCR using a nested primer (0.28 μM) 5’ fluorescently labelled with 6-FAM, HEX (MWG-Biotech AG, Ebersberg, Germany) or NED (Applied Biosystems, CA, USA) with one outside primer (0.28 μM) for each marker (Table I). Each nested PCR was amplified with 0.6 U coloured Taq polymerase (SILEKS, Moscow, Russia) and contained 0.24 mM dNTPs, 6% DMSO, with the buffer described above, for 30 cycles. Amplification products were diluted and run on an ABI Prism 3100 Avant automated capillary sequencer and analysed using GeneScan or Genotyper software (Applied Biosystems, Foster City, CA, USA), relevant peaks were distinguished from stutter peaks according to standard procedures (User bulletin, ABI Prism linkage mapping set, November 1997), and peak amplitude thresholds were set at 50 pts. Analysis of the 6365(-2)A>G NF1 mutation was performed using a hemi-nested primer designed with two mismatches which create a BsrGI (MBI Fermentas, Vilna, Lithuania) restriction site on the wild-type sequence. Following PCR, the amplified product was digested with BsrGI, electrophoresed on a 4% Nusieve gel (Cambrex, Rockland, ME, USA) and visualized by staining with ethidium bromide.

**Results**

Nine highly polymorphic markers flanking the NF1 gene were selected for haplotype analysis (Figure 1), and an assay to identify the 6365(-2)A>G mutation was designed (Figure 2A). The male was found to be heterozygous for six of the nine polymorphic markers (D17S1294, D17S1849, D17S841, D17S975, NF-TG2 and NF-AC5, see legend to Figure 1), and an assay to identify the 6365(-2)A>G mutation was designed (Figure 2A). The male was found to be heterozygous for six of the nine polymorphic markers (D17S1294, D17S1849, D17S841, D17S975, NF-TG2 and NF-AC5, see legend to Figure 1), and all the six of these informative markers were used in this case. The proband, his wife and the proband’s parents were each haplotype with these markers, and this allowed the identification of the individual alleles of the proband. However, no allele could be linked to the NF1 mutation because it was a new mutation in the proband.

We used a multiplex PCR, followed by individual hemi-nested reactions, to establish linkage between the NF1 de-novo mutation and the polymorphic markers on 14 isolated single sperm. Digestion with BsrGI for the 6365(-2)A>G mutation identified eight mutant and five wild-type sperm (data for five single sperm are summarized in Table II). Sperm 1, 2 and 3 carried the mutant allele, whereas sperm 4 and 6 were wild type. No amplification was observed in sperm 5 in any reaction (data not shown). Simultaneous analysis of the six informative polymorphic markers from each of these sperm (Figure 3)
Single-sperm analysis for NF1 haplotypes in PGD

allowed the mutant and wild-type alleles to be linked to individual haplotypes (Table II). Construction of the patient’s haplotype enabled complete haplotype determination in this family (Figure 4).

The couple has since undergone three cycles of PGD using all six informative polymorphic markers in addition to mutation testing. Before the first cycle, the PGD protocol was tested using all the six markers and the familial mutation in a multiplex PCR in single fibroblasts from an unrelated individual. Amplification was observed in all reactions. Rates of ADO in 73 single fibroblasts informative for four different NF1 markers were measured and ranged from 1 to 10%, whereas actual ADO observed for 10 blastomeres in the PGD cases showed 0 to 3 ADO events per marker (Table III). Only in one nested PCR was an amplified product found in the medium NTC (blank) for that particular oocyte, disqualifying that reaction. Each of the three PGD cycles resulted in the transfer of one to two wild-type embryos (Table IV). A chemical pregnancy was achieved in cycle number 2.

Discussion

An individual affected with NF1 has a 50% chance of giving birth to an affected child in each pregnancy. Couples can elect to have prenatal diagnosis for NF1 by either chorionic villus sampling (CVS) or amniocentesis. Couples at risk can also undergo PGD, preempting the need for pregnancy termination in the case of an affected embryo. PGD is an especially attractive option for NF1, not only because of the high recurrence risk (50%) but also because disease severity is unpredictable. This may lead to ethical dilemmas in terminating a pregnancy which could result in a minimally affected child.
We report using single-sperm analysis for haplotype determination using a multiplex, single-cell PCR for PGD. Although in theory PGD for this couple could be accomplished using mutation analysis alone, this would be accompanied by an unacceptably high error rate due to ADO. Even the low ADO rates observed here are significantly high for families electing to undergo PGD to avoid pregnancy termination. A second possibility would be to biopsy an additional blastomere for confirmation; however, this may give rise to a reduced pregnancy rate (Cohen, 2005). By looking at ADO in fibroblasts, we found that while certain markers produced only 1% ADO, others gave up to 10%. Blastomere analysis yielded ADO rates of 0–3 events per 10 reactions; however, it should be noted that only 10 blastomeres were fully analysed (Table III).

Table II. Results of single-sperm analysis for an NF1 mutation and linked markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Genomic DNA</th>
<th>Sperm 1</th>
<th>Sperm 2</th>
<th>Sperm 3</th>
<th>Sperm 4</th>
<th>Sperm 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>BsrGI digest G/A</td>
<td>G (mutant)</td>
<td>G (mutant)</td>
<td>G (mutant)</td>
<td>G (mutant)</td>
<td>A (wild type)</td>
<td>A (wild type)</td>
</tr>
<tr>
<td>D17S1849</td>
<td>232/234</td>
<td>No result</td>
<td>232</td>
<td>232</td>
<td>234</td>
<td>234</td>
</tr>
<tr>
<td>TG2</td>
<td>137/135</td>
<td>137</td>
<td>137</td>
<td>137</td>
<td>135</td>
<td>135</td>
</tr>
<tr>
<td>D17S841</td>
<td>176/174</td>
<td>176</td>
<td>176</td>
<td>176</td>
<td>174</td>
<td>174</td>
</tr>
<tr>
<td>D17S1294</td>
<td>186/182</td>
<td>186</td>
<td>186</td>
<td>186</td>
<td>182</td>
<td>182</td>
</tr>
<tr>
<td>D17S975</td>
<td>242/246</td>
<td>242</td>
<td>242</td>
<td>242</td>
<td>246</td>
<td>246</td>
</tr>
<tr>
<td>AC5</td>
<td>272/266</td>
<td>272</td>
<td>272</td>
<td>272</td>
<td>No result</td>
<td>266</td>
</tr>
</tbody>
</table>

Mutant haplotypes are shown in bold-face type, wild-type haplotypes are shown in italics. No amplification products were obtained for sperm 5 in any reaction.

*Numbers represent marker allele length in base pairs.

Figure 3. Haplotype determination of single sperm. Capillary electrophoretograms of amplified fluorescently labelled linked polymorphic markers using paternal genomic DNA and isolated single sperm. Sperm 1 harbours the mutant allele, whereas sperm 6 harbours the wild-type allele (Figure 2B). Asterisks (*) indicate mutant alleles, and paternal alleles are listed under each marker.

Table III. Results of three cycles of PGD for NF1 in this study

<table>
<thead>
<tr>
<th>Cycle</th>
<th>COCs</th>
<th>M2</th>
<th>Fertilized</th>
<th>Embryos developed to six cells</th>
<th>Embryos diagnosed</th>
<th>Wild-type embryos</th>
<th>Mutant embryos</th>
<th>Embryos transferred</th>
<th>HCG positive (rising), 2 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>8</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>16</td>
<td>13</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td>22</td>
<td>19</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>No</td>
</tr>
</tbody>
</table>

Table IV. ADO for six markers and the familial mutation in the NF1 gene in single fibroblasts and blastomeres

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fibroblasts</th>
<th>Blastomeres</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17S1294</td>
<td>6%</td>
<td>1</td>
</tr>
<tr>
<td>D17S841</td>
<td>1%</td>
<td>3</td>
</tr>
<tr>
<td>D17S975</td>
<td>9%</td>
<td>2</td>
</tr>
<tr>
<td>TG2</td>
<td>10%</td>
<td>1</td>
</tr>
<tr>
<td>D17S1849</td>
<td>Not informative</td>
<td>2</td>
</tr>
<tr>
<td>AC5</td>
<td>Not informative</td>
<td>1</td>
</tr>
<tr>
<td>NF1 6356(-2)A&gt;G</td>
<td>Not informative</td>
<td>0</td>
</tr>
</tbody>
</table>

*Of 73 single fibroblasts from an unrelated individual.

**Of 10 blastomeres from three cycles of PGD.
informative and polymorphic markers, allows for the disqualification of these reactions and is essential to achieve maximal diagnostic accuracy.

NF1 is particularly prone to de-novo mutations perhaps because of its large size of almost 360 kb. De novo mutations present a challenge for PGD because of the difficulty in establishing the mutant haplotype when there are no additional affected relatives. By determining the haplotype of individual sperm, we were able to set phase and establish the haplotypes of an affected male with a new NF1 mutation. A similar strategy was recently reported for PGD of Currarino syndrome, although in that case, haplotypes could also have been confirmed on the basis of linkage in the family (Verlinsky et al., 2005). We used six polymorphic markers which span almost 5 Mb, a large region known to contain a number of recombination hotspots (HapMap Phase I data, release 16a). Recombination is regularly observed in both polar bodies and blastomeres in PGD, although we have not yet observed any recombination events in the NF1 region in these family members or any sperm or blastomere in this family. Usage of markers on both sides of the gene, and awareness of the proximities of each marker, in addition to recombination frequencies in the region allowed accurate identification of the mutant and wild-type alleles. This method is generally applicable for establishing the haplotype of any paternal mutation when linkage cannot be performed because of lack or unavailability of other affected relatives. For PGD, this method enables the use of linked markers in these cases, thus greatly enhancing diagnostic accuracy.

Acknowledgements

We thank Rabbi David and Mrs. Anita Fuld for their generous and ongoing support and Ms. Hadassa Hartman for her valuable editorial assistance. This project was approved by the IRB of the Shaare Zedek Medical Center.

References


Submitted on October 2, 2005; resubmitted on December 29, 2005, February 7, 2006; accepted on February 10, 2006.