Real-time reverse linkage using polar body analysis for preimplantation genetic diagnosis in female carriers of de novo mutations

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BACKGROUND: Single cell diagnosis for preimplantation genetic diagnosis (PGD) requires simultaneous analysis of multiple linked polymorphic markers in addition to mutation analysis in order to reduce misdiagnosis. This type of analysis requires building family haplotypes spanning at least two generations. We present three childless couples in whom the female was a de novo mutation carrier in the Duchenne Muscular Dystrophy (DMD), incontinentia pigmenti (IKBKG) or Neurofibromatosis type 2 (NF2) genes, precluding linkage prior to the PGD cycle. We constructed haplotypes based on linked polymorphic markers in these families and performed concurrent diagnosis enabling embryo transfer from the first PGD cycle.

METHODS: Informative markers flanking the DMD, IKBKG and NF2 genes were used to construct non-linked haplotypes. Polar bodies 1 (PB1) and 2 (PB2) were biopsied and analyzed to determine allelic association between the mutation and markers in multiplex PCR reactions.

RESULTS: For each family, the first PGD cycle allowed the establishment of linked haplotypes based on homozygous PB1 and PB2 analysis; however, no embryos were available for transfer. Subsequent cycles, when performed, confirmed this linkage. A mutation-free child was born to the family affected with DMD and an ongoing pregnancy (32 weeks) was achieved with the carrier of the IKBKG deletion.

CONCLUSIONS: PB analysis for reverse linkage in real-time coupled with the PGD cycle is a powerful tool for diagnosis and linkage between markers and de novo mutations for maternal autosomal dominant or X-linked disorders. Simultaneous amplification of multiple informative markers in conjunction with the mutation allows the building of familial haplotypes and accurate PGD analysis.

Key words: preimplantation genetic diagnosis / polar bodies / single cell multiplex PCR / de novo mutations / reverse linkage

Introduction

Preimplantation genetic diagnosis (PGD) was developed for couples at high genetic risk for having affected children. PGD is performed by blastomere, blastocyst and/or polar body (PB) biopsy for Mendelian and chromosomal disorders (Handyside et al., 1990). Although PB biopsy requires more skill, and is more time-consuming and costly, it has been used successfully in a few centers worldwide for females affected with autosomal dominant disorders or carriers of recessive or X-linked disorders (Verlinsky et al., 2004; Altarescu et al., 2008a, b).

The first clinical application of PGD for single-gene disorders was reported in 1990 (Handyside et al., 1990), and although close to 3000 single-gene disorders have been identified (Online Mendelian Inheritance in Man http://www.ncbi.nlm.nih.gov/omim/), PGD has been applied for only several hundred (Lissens et al., 1996; Sermon et al., 2007). This is due to the need to develop unique protocols for each specific mutation in each family. Although in theory, PGD could be accomplished using mutation analysis alone, due to allele drop out (ADO) this would be accompanied by a high error rate which could reach 20% (Verlinsky et al., 2002). Therefore, PGD protocols include several linked polymorphic microsatellite markers.
flanking the disease gene in order to minimize misdiagnosis due to ADO (Verlinsky et al., 2002; Sanchez-Garcia et al., 2005). The use of polymorphic microsatellite or single-nucleotide polymorphism markers allows PGD accuracy rates approaching 100% (Renbaum et al., 2007). Prior to the PGD cycle, parental haplotypes are prepared on genomic DNA from immediate family members and relatives to establish linkage between the mutation and informative microsatellite markers (Altarescu et al., 2008a, b). However, de novo mutations in childless couples undergoing PGD preclude the possibility of linking the mutation to polymorphic markers beforehand.

Recently, we showed that in male carriers of new mutations, analysis of the haploid content of single sperm allowed the setting of allelic phase and establishment of linked haplotypes (Altarescu et al., 2006). This method is applicable in establishing linked haplotypes of any paternal mutation when linkage cannot be performed due to the absence of other affected relatives. While this analysis of single sperm is performed before the couple begins the PGD cycle, single female haploid cells are not available prior to oocyte retrieval. A similar strategy has been reported using PBs; however, this required a full IVF cycle for the purpose of haplotype construction with no intention to return embryos (De Vos et al., 2003). We present here a protocol for real-time haplotype analysis in women with de novo mutations using PB1 and PB2 during the first clinical PGD cycle with full intention to transfer wild-type embryos showing allelic linkage. PB1 is the result of meiosis I, and can be either homozygous (no crossovers between the two alleles) or heterozygous (when crossing-over occurs). Homozygous PB1s contain either the normal or mutant allele, while the other allele remains in the oocyte. PB2 is the result of meiosis II and contains only one allele. Since only a single allele is observed for homozygous PB1s and all PB2s, linkage between the mutation and markers can be performed in a similar fashion to single-sperm linkage, and followed immediately by possible transfer of unaffected embryos. Whereas blastomere biopsy is performed by most labs for routine PGD analysis, for female de novo mutation carriers accurate blastomere analysis by reverse linkage is only possible for male embryos with X-linked disorders. In autosomal dominant and recessive disorders along with female embryos from X-linked disorders, mutation analysis in blastomeres is always only partially informative and ADO can never be completely disregarded.

We describe the PGD strategy, including PB haplotype construction, in three childless couples who presented to our PGD unit, in whom the females were carriers of de novo mutations. To our knowledge, this is the first report of a robust methodology of reverse linkage in real time, performed concurrently with the actual PGD cycle, for new mutations in females requiring PGD, and it is applicable for almost all known new mutations.

### Materials and Methods

#### Patients

In couple #1, the female was a carrier of a missense c.1055T>G mutation in the Duchenne Muscular Dystrophy (DMD) gene (OMIM *300377). In couple #2, the female was a de novo carrier of the common deletion g.(exon10−exon10)del in the IKBKG gene (OMIM *300248) causing incontinentia pigmenti (OMIM #308300) in males; in couple #3, the female was affected with Neurofibromatosis type 2 (OMIM #101000) and diagnosed with a four base pair deletion c.1334–1337delAGAG in the NF2 gene (OMIM *607379) (Table I).

#### Ovarian stimulation and oocyte retrieval

IVF treatment was performed using the long down-regulation protocol consisting of 0.1 mg/day decaprot© s.c. (Ferring Ltd, HerZliya, Israel) and a daily dose of 150 IU of recombinant FSH (Gonal F; Serono, Israel) and 150 IU of HMG (Menogon; Ferring Ltd). HCG 10 000 IU (Chorigon; Teva, Petach-Tiqua, Israel) was given when at least 3 follicles >18 mm developed. Vaginal ultrasound-guided ovum pick-up was performed 36 h after HCG injection.

#### PB biopsy, intracytoplasmic sperm injection and embryo cultures

Cumulus–oocyte complexes (COCs) were identified, washed and transferred to organ culture dishes containing equilibrated culture medium (Medicult, Denmark) and placed in an incubator with 5% CO2. Oocytes were denuded with hyaluronidase (Sigma-Aldrich, USA) 2 h after collection and were allowed to recover in the incubator for a further 2 h. The first polar bodies (PB1) were removed using the ‘zona-slitting’ technique (Verlinsky et al., 2005). Intracytoplasmic sperm injection was then performed on each mature oocyte. Injected oocytes were transferred to 20 µl droplets of Global medium (Lifeglobal, Ingenta US) under mineral oil. Approximately 18 h post-injection, the oocytes were assessed for fertilization by assessing the presence of two pronuclei (2PN) and the second polar body (PB2) was removed using the same procedure as for the first polar body removal. Fertilized oocytes were then placed in fresh 20 µl droplets of P1 medium under oil and cultured for a further 48 h. PBs were each carefully transferred to a separate 0.5 ml tube containing 5 µl of proteinase K lysis buffer (Thornhill et al., 2001). A sample of culture medium (media blank) from each droplet that contained a biopsied PB was analyzed to verify the absence of maternal cellular genetic material or DNA in the culture medium. In addition, a No Template Control (NTC, reaction blank) was used to monitor the absence of external contamination in each PCR reaction.

#### Molecular analysis

Molecular verification of the specific mutation in each of the three genes (the c.1055T>G mutation in the DMD gene, the deletion of exons 4–10 in the IKBKG gene and the four base pair deletion in the NF2 gene)

### Table I Demographic data of the presented families

<table>
<thead>
<tr>
<th>Family no.</th>
<th>Disease</th>
<th>Gene</th>
<th>Mutation</th>
<th>Age of proband</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family no. 1</td>
<td>Duchene Muscular Dystrophy</td>
<td>DMD</td>
<td>c.1055T&gt;G</td>
<td>27</td>
</tr>
<tr>
<td>Family no. 2</td>
<td>Incontinentia Pigmenti</td>
<td>IKBKG</td>
<td>Del exons 4–10</td>
<td>31</td>
</tr>
<tr>
<td>Family no. 3</td>
<td>Neurofibromatosis type 2</td>
<td>NF2</td>
<td>c1334–1337del AGAG</td>
<td>22</td>
</tr>
</tbody>
</table>
along with microsatellite marker analysis was performed with DNA extracted from peripheral blood cells using high salt precipitation (Miller et al., 1998). For the IKBKG deletion, we designed PCR primers which do not detect pseudogenetic sequences located adjacent to the IKBKG gene (forward primer: CCCCCGCCCCGCTCCCTGTA, nested primer: CTGAAAGGAGGAGGAGGAGCG and reverse primer: CTCGGAGAACAGGAAACAGCA). This assay (used on both genomic DNA and single cells) yields an amplification product (1.2 kb) only in the presence of the 13 kb deletion. Biopsied PB1 and PB2 were transferred (individually) to a tube containing 5 µl of Proteinase K lysis buffer and incubated at 45°C for 15 min, followed by inactivation at 94°C for 15 min. Eight microsatellite informative polymorphic markers were identified flanking the DMD gene (DMD-FTTC1 chrX:29689901, DMD-CT1 chrX:29783616, DXS1214, DXS1036, DXS1219, DXS1238, DMD-AT1 chrX:32810547 and DMD-FTTC2 chrX:34154716), four informative markers flanking the IKBKG gene: (DXS8103, IP-TG6 chrX:150686405, DXS8087 and DXS1108) and six informative markers flanking the NF2 gene (D22S1150, NF2-CA1 chr22:28092423, D22S299, D22S268, D22S528 and D22S176) (UCSC genome browser March 2006) (Karolchik et al., 2003) were used for linkage in each of the three diseases. A multiplex PCR reaction for use with single cells was prepared containing 0.2 µM dNTPs, 10% DMSO, 0.1 µM primers (specific for each marker and mutation) with 1.25 U Taq polymerase in reaction buffer supplied by the manufacturer (MRB801, UK) in a 50 µl volume. The reaction was thermocycled for 30 cycles using a touch down protocol: 20 s denaturation step at 95°C, 1 min annealing at 62–50°C and 30 s elongation at 72°C. As a template, 1.5 µl from each reaction was used with a hemi-nested primer 5’ fluorescently labeled with 6-FAM, HEX or TAMRA (Metabion, Germany) with one outside primer for an additional 35 cycles for each of the six individual PCR reactions. Single cell sequencing was performed for the c.1055T>G mutation in the DMD gene. Following the multiplex and hemi-nested reactions, PCR products were purified and sequenced with BigDye Terminator v1.1 cycle sequencing (ABI) and electrophoresed on an ABI Prism 3100xl automated sequencer. Reaction products were diluted and run on an ABI Prism 3100xl Avant automated sequencer, and analyzed using GeneMapper software, ABI). Reactions which showed ADO or had media blank contamination were not included in the molecular analysis. Only samples which were informative for a minimum of two polymorphic markers flanking the gene in addition to the specific mutation were considered for diagnosis. Measures to prevent and to detect contamination as previously recommended by the ESHRE PGD consortium were used during all steps (Thornhill et al., 2005).

Results

We report the use of PB analysis for reverse linkage of de novo mutations in females while simultaneously performing preimplantation diagnosis. In each couple, informative markers located within a 2 Mb region of the gene were identified using genomic DNA prior to the PGD cycle. During the first cycle, homozygote PB1 and all PB2 were used to link the informative genetic markers to the de novo mutations in all cases. After linkage was established, we used either PB or blastomere analysis for subsequent cycles. Prior to the PGD cycles, the multiplex PCR assay for each family was tested using 50 single fibroblasts. The amplification efficiencies for the protocols were between 94% and 97% and ADO rates were between 4% and 9%.

PGD for the de novo c.1055T>G female mutation in the DMD gene (X-linked)

Eight informative microsatellite markers flanking the DMD mutation were identified and used in the PGD protocol. In the first cycle, four PB1s were successfully biopsied, whereas only two oocytes were fertilized. On the basis of one homozygous wild-type PB1 and one mutant PB2, all eight markers were linked to the mutation and a tentative haplotype of the family was set. However, neither embryo reached the 6-cell stage; therefore, no embryo transfer could be performed. Three subsequent PGD cycles (two PB cycles and one blastomere cycle) confirmed this haplotype and resulted in transferable embryos (Table II). The last cycle resulted in the birth of a healthy baby girl bearing the wild-type maternal allele. An example of haplotype construction in real time by PB analysis PGD is presented in Fig. 1.

PGD for the de novo female deletion of exons 4–10 in the IKBKG gene (X-linked)

In family #2, four informative markers which did not amplify pseudogenic IKBKG sequences were identified and used for PGD. The first cycle yielded 10 PB1, of which only two PB1 were homozygous. One showed the presence of the deletion together with all four microsatellite markers allowing linkage between the markers and the deletion for a mutant PB1. The second homozygous PB1 showed the opposite allele of all four markers whereas the deletion was not detected implying a wild-type PB1. Of the remaining PB1, six were heterozygous and two showed unclear results. Although all the markers amplified, the deletion was not detected in any of the PB2 samples (suggesting wild-type PB2), and blastomere biopsy was performed in the four embryos that reached 6–8 cell stage. The IKBKG deletion (and corresponding markers) was detected in three of the four embryos, whereas the remaining embryo showed no clear result. These three mutant blastomeres confirmed the linkage that was established in the PB1 samples. In the second cycle, blastomere

<table>
<thead>
<tr>
<th>Cycle</th>
<th>MII</th>
<th>Fertilized</th>
<th>PB1 WT/Mut/Het</th>
<th>PB2 WT/Mut</th>
<th>Oocyte WT/Mut</th>
<th>Blast WT/Mut</th>
<th>Transferred</th>
<th>Children</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>2</td>
<td>1/3Rec/—</td>
<td>1/1</td>
<td>—/1</td>
<td>—</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>3</td>
<td>1/—/2</td>
<td>—/3</td>
<td>2/1</td>
<td>2</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td>4/4</td>
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<td>0</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>10</td>
<td>1/1/7</td>
<td>5/4</td>
<td>4/4</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

WT, wild type; Mut, mutant; Het, heterozygote; Rec, recombinant.
biopsy was performed and one out of eight embryos was detected as a wild-type male and this was transferred to the couple resulting in an ongoing 32-week pregnancy.

**PGD for a de novo c.1334–1337del AGAG in the NF2 gene (autosomal dominant)**

In family #3, six informative microsatellite markers were identified flanking the NF2 gene mutation and used in the multiplex assay. One cycle was performed and all five PB1 analyzed were found to be heterozygous. Three oocytes fertilized and of these, two PB2 contained the wild-type allele (resulting in mutant oocytes) and one PB2 showed total amplification failure. While no transfer could be performed in this cycle, linkage between all six markers and the mutation was established.

**Discussion**

We present here a real-time reverse linkage PGD analysis for female carriers of de novo mutations in the DMD, IKBKG and NF2 genes. This method allows simultaneous haplotype construction and PGD analysis including embryo transfer already from the first cycle. Although most protocols use blastomere biopsy for embryo diagnosis, PGD using PB analysis has been shown to be an effective method for maternal autosomal dominant, X-linked, and recessive disorders (Verlinsky et al., 2004). Homozygous PB1 represent the genetic mirror image of the oocyte; in these cases, PB2, resulting from meiosis II, confirm the allele remaining in the oocyte. However, as a result of crossover events, PB1 are often heterozygous, showing both alleles, and in these cases the complement of both alleles also remain in the oocyte until PB2 extrusion. The results of these PB2 reflect the mirror image of the allele remaining in the oocyte (if PB2 shows the wild-type allele, then the oocyte is inferred to be mutant and vice versa). Owing to ADO, which is the foremost cause of misdiagnoses in PGD, linked polymorphic markers are always used in conjunction with mutation analysis. Prior to the PGD cycle, parental haplotypes linking the mutation with informative genetic markers must be constructed from a number of affected and/or non-affected family members. For women carrying de novo mutations, the haplotype cannot be built prior to the PGD cycle; however, homozygous PB1s and all PB2 which contain only one allele can be used to link the markers to the mutation during the PGD cycle in real time. Non-transferable embryos can be reanalyzed for haplotype confirmation. This technique requires the ability to detect the mutation in single cells and can only be used if either the mutated or the wild-type sequence can be assayed. Furthermore, while pseudogenic sequences containing the mutation can be avoided in PGD by using linkage assays only (outside of the pseudogenic region), when the linkage analysis is based on mutation detection in the single-cell PGD arena, special care must be taken in order not to amplify pseudogenic sequences which could easily cause misdiagnosis.

The detection of the common deletion in the IKBKG gene, carried by the female of couple #2, causing male lethal Incontinentia pigmenti is usually performed by Southern analysis with an exon 2 probe and a genomic HindIII restriction digest. The use of the exon 2 probe is designed to avoid misinterpretation due to the IKBKG pseudogene (Bardaro et al., 2003). This is not a suitable strategy for PGD since Southern blot analysis cannot be applied to single cells. In order to detect the mutation on single cells and allow full haplotype construction, we designed a PCR assay which detects only the mutant allele, amplifying a fragment spanning the 13 kb deletion.

Since Incontinentia pigmenti is lethal in males in utero in 97% of cases (Ardelean and Pope, 2006) and the single-cell assay does not allow for the detection of the wild-type allele, we chose a conservative strategy returning only wild-type males until an offspring could confirm the
De novo mutation PGD by reverse linkage on polar bodies

De novo mutation PGD by reverse linkage on polar bodies.

Linkage analysis. In the first cycle, combined PB and blastomere analysis allow us to build a clear linked haplotype in four distinct single cells. Although no transfer was performed in this cycle, since no embryo was clearly wild-type male, a second cycle was performed which resulted in the transfer of one wild-type male embryo yielding an ongoing pregnancy.

Couple #1 carried a de novo missense mutation in the DMD gene. We used both an RLFP assay and single cell sequencing to detect the DMD c.105ST>G mutation, and all eight markers were successfully linked to the DMD familial mutation in the first cycle. No transfer was possible in the first cycle, but the second cycle resulted in transfer of two wild-type embryos with no pregnancy. On the basis of nine independent linked haplotypes in the first two cycles, we performed the third PGD cycle by blastomere biopsy based on linked markers only (since the mutation is not fully informative in blastomere analysis). The fourth cycle resulted in the birth of a healthy baby girl; subsequent mutation analysis confirmed the wild-type DMD allele. Since 30% of mutations in the DMD gene are de novo (Tuffery-Giraud et al., 2009), this method is widely applicable for DMD PGD and other diseases with high rates of new mutations.

Linked haplotypes, an absolute requirement in PGD (Thornhill et al., 2005) can neither be prepared nor confirmed prior to the PGD cycle in families with de novo female mutations. This circumstance can potentially increase the risk of misdiagnosis. At present, there are no established guidelines concerning how many single-cell linked haplotypes need to be assessed prior to embryo transfer for de novo maternal mutations. We have elected to base our decision to transfer embryos on a minimum requirement of three independent clear linkage analyses between the genetic markers and the mutation.

In conclusion, we presented PGD combined with simultaneous linkage analysis for three de novo maternal mutations resulting in two successful outcomes. Analysis of PB1 and PB2 is a powerful tool for real-time reverse linkage analysis of de novo mutations in female carriers of almost any autosomal dominant or X-linked disease.

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