Preimplantation genetic diagnosis of a large pericentric inversion of chromosome 5

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We report the first established pregnancy using preimplantation genetic diagnosis in order to avoid chromosomal imbalance in the progeny of a woman carrying a large inversion of chromosome 5. This is also the first time where it has been possible to study the distribution of balanced and unbalanced gametes in a female inversion carrier. In total, 23 embryos were biopsied in two separate treatments and analysed by fluorescent in-situ hybridization. Of these, 10 were unbalanced, nine were balanced and for four the analysis was inconclusive. The diagnostic procedure was performed within 3.5 h. This allowed the biopsied embryos to be transferred the same day as the biopsy was taken (day 3). Two embryos were transferred each time, and in the second treatment a twin pregnancy with two chromosomally balanced fetuses was established. Healthy twins were delivered at 34 weeks of gestation.

Key words: chromosome 5/crè du chat syndrome/fluorescent in-situ hybridization/pericentric inversion/pre-implantation genetic diagnosis

Introduction

Carriers of balanced chromosomal abnormalities are at high risk of having children with severe handicap. Pericentric inversions are chromosomal rearrangements where the carriers are at risk of having unbalanced offspring due to meiotic crossing-over. Four types of gametes may arise in an inversion carrier. None or an even number of meiotic cross-overs within the inverted segment will give rise to a normal gamete or a gamete which is balanced but will transmit the inverted chromosome. Any odd number of cross-overs will result in gamete which is balanced but will transmit the inverted segment will give rise to a normal gamete or a gamete which is balanced but will transmit the inverted chromosome. Any odd number of cross-overs will result in gamete which is balanced but will transmit the inverted chromosome. Any odd number of cross-overs will result in gamete which is balanced but will transmit the inverted chromosome. Any odd number of cross-overs will result in gamete which is balanced but will transmit the inverted chromosome. Any odd number of cross-overs will result in gamete which is balanced but will transmit the inverted chromosome. Any odd number of cross-overs will result in gamete which is balanced but will transmit the inverted chromosome. Any odd number of cross-overs will result in gamete which is balanced but will transmit the inverted chromosome. Any odd number of cross-overs will result in gamete which is balanced but will transmit the inverted chromosome. Any odd number of cross-overs will result in gamete which is balanced but will transmit the inverted chromosome. Any odd number of cross-overs will result in gamete which is balanced but will transmit the inverted chromosome. Any odd number of cross-overs will result in gamete which is balanced but will transmit the inverted chromosome. Any odd number of cross-overs will result in gamete which is balanced but will transmit the inverted chromosome. Any odd number of cross-overs will result in gamete which is balanced but will transmit the inverted chromosome.

PGD was first reported 7 years ago and the largest number of diagnoses has been performed for aneuploidy screening of oocytes from in-vitro fertilization (IVF) patients of advanced maternal age using polar body biopsy (data presented at the Second International Symposium on Preimplantation Genetics, Chicago 1997). Whether this will improve pregnancy rates or not is debated (Ecozcue, 1996, 1997; Reubinoff and Shushan, 1996, 1997; Verlinsky and Kuliev, 1996; Munne and Cohen, 1997). When using embryo biopsy, the largest number of diagnoses has been performed for patients who carry an X-linked disease. The sex of the embryos is determined using polymerase chain reaction (PCR) or fluorescent in-situ hybridization (FISH) techniques and only female embryos are then selected for implantation (Handyside et al., 1990; Griffin et al., 1994). PCR has also been used for PGD of a number of single gene disorders (reviewed by Lissens and Sermon, 1997). So far, no established pregnancy after PGD for a carrier of an inversion has been reported.

Cri du chat, or 5p− syndrome, is a congenital disorder with clinical features including severe mental retardation, growth deficiency, microcephaly and an abnormal cry, often described as similar to the mewing of a cat, from which the syndrome derives its name. It results from the deletion of a region on the short arm of chromosome 5 within band 5p15, although the extent of the deletion can vary. It is one of the most common human deletion syndromes, with an incidence of 1:50 000 in the general population and 1:350 among severely mentally retarded (Niebuhr 1978). It is estimated that 12% of the cases are of familial origin with parental translocations accounting for the majority of these. Only 5% of the familial cases are associated with an inversion of chromosome 5. A few cases of cri du chat syndrome due to recombination aneusomy arising from a parental inversion of chromosome 5 have previously been described (Warter et al., 1973; Beemer et al., 1984; Dobbs et al., 1988; Chernos et al., 1992).

We here report PGD performed on a female carrier of a large pericentric inversion of chromosome 5, 46,XX,inv(5) (p14q35) using a short FISH protocol. We were also able to
Four types of gametes may arise in an inversion 5 carrier. The p-arm and the q-arm of chromosome 5 are indicated with pink and blue respectively. (I) Normal gamete. (II) Gamete which is balanced but will transmit the inverted chromosome. (III) Gamete with a duplication of 5q together with a deletion of 5p. (IV) Gamete with a duplication of 5p together with a deletion of 5q.

Figure 3. Fluorescent in-situ hybridization analysis using a single copy probe from chromosome 5p15.2 (green), and a centromere-specific probe from chromosome 17 (red). (a) Interphase and metaphase preparations from lymphocytes of the carrier. Two green signals and two red signals indicate a balanced karyotype. (b) Interphase and metaphase preparations from lymphocytes of the child with cri du chat syndrome. Only one green signal together with two red signals indicate imbalance and a 5p– karyotype.
study the distribution of balanced and unbalanced gametes in this female inversion carrier.

Material and methods

The spare preimplantation embryos that were used for probe efficiency test were donated from couples undergoing routine IVF procedures. Written consent was obtained from the couples in order to confirm that the spare embryos could be used for research purposes. The use of spare embryos for probe efficiency testing and PGD to detect chromosomal imbalance was approved by the Research Ethics Committee of the Karolinska Institute.

Proband

The proband was a 29 year old woman carrying a large pericentric inversion of chromosome 5, 46,XX,inv(5)(p14q35). Her first child was diagnosed with cri du chat syndrome due to a deletion of the short arm of chromosome 5, caused by recombination of the mother’s inversion during meiosis. After the child with cri du chat syndrome was born, our proband had two spontaneous abortions during the first trimester, which may represent non-viable recombinants.

IVF, biopsy procedure and embryo transfer

The patient was down-regulated with buserelin 1200 mg/day intranasally (Supre巡航®; Hoechst, Frankfurt, Germany) and stimulated with recombinant FSH (Gonal F®; Ares Serono, Geneva, Switzerland). When several follicles were >18 mm and oestradiol had risen for at least 7 days, 10 000 IU human chorionic gonadotrophin (HCG, Profasi®; Ares Serono) was given s.c., and ultrasound-guided transvaginal follicular aspiration was performed 37 h later, using a 1.4 mm outer diameter needle (Swemedlab International, Billdal, Sweden). All oocytes were cultured in a serum-free IVF medium and a control probe. The normal and the balanced embryos were labelled centromere-specific probe from chromosome 17 as a control probe. The normal and the balanced embryos were aspirated by gentle suction with the same micropipette. Two blastomeres were removed from each embryo. After the biopsy procedure, the embryos were kept under culture conditions until the results of the genetic analysis were finally evaluated. Ultrasound-guided transfer was performed late in the evening of day 3.

Blastomere spreading

After biopsy, blastomeres were washed in phosphate-buffered saline (PBS) for 2 min and transferred into a drop of spreading solution on a poly-L-lysine (Sigma, St Louis, MO, USA)-coated slide. The blastomere was observed under an inverted microscope until the nucleus was free of cytoplasm. The slides were then washed in PBS and dehydrated through an alcohol series.

DNA probes

A single copy probe LSI D5S23 (Vysis, Downers Groove, IL, USA), specific for chromosome band 5p15.2 and directly labelled with Spectrum Green dUTP, was used to identify the distal part of 5p. The chromosome 17 centromere-specific probe D17Z1 (ATCC, Manassas, VA, USA), labelled by nick translation with rhodamine-4-dUTP (Amersham International, Little Chalfont, Bucks, UK), was used as an internal control. The efficiency of the probes was tested using chromosome slides from lymphocyte preparations of healthy males and signals were counted in 200 nuclei. Two signals from the chromosome 5p-specific probe were present in 96.5% of the cells and two signals from the chromosome 17 centromere-specific probe were present in 98% of the cells. Efficiency tests were also performed using lymphocyte preparations of the carrier woman, her first child and spare preimplantation embryos.

FISH analysis

After spreading and dehydrazation the slides were treated with pepsin (0.1 mg/ml) in 0.01 M HCl for 20 min at 37°C as described by Harper et al. (1994). The nuclear DNA was denatured in 70% formamid/2× standard saline citrate (SSC) at 75°C for 3 min followed by dehydrazation in 70, 85 and 100% ethanol at –20°C for 4 min each. The chromosome 17 centromere-specific probe was added to the hybridization mixture provided with the LSI D5S23 probe to a final concentration of 1–2 ng/µl and then denatured at 75°C for 5 min. The probe mixture was added to the slide under a coverslip and hybridization was performed in a moist chamber at 37°C for 2 h. The slides were then washed for 2 min in 0.4×SSC/0.3% Nonidet P-40 (NP-40) at 72°C, followed by a second wash in 2×SSC/0.1% NP-40 at room temperature for 40 s. After dehydrazation, the slides were mounted in glycerol containing 2.3% DABCO [1,4-diazabicyclo-(2,2,2) octane] as antifade and DAPI (4,6-diamino-2-phenyl-indole) 0.5 µg/ml to counterstain the nuclei. The signals were analysed using a Zeiss Axioskop fluorescence microscope equipped with a cooled CCD camera (SenSys; Photometrics Ltd, Tucson, AZ, USA) controlled by a Power Macintosh computer. Gray scale images were captured, pseudocoloured and merged using the SmartCapture software (Vysis).

Results

We used FISH and a directly labelled single copy probe from 5p15.2, in order to detect chromosomal imbalance within the critical region on the short arm of chromosome 5. In order to detect aneuploidy and hybridization failure we used a directly labelled centromere-specific probe from chromosome 17 as a control probe. The normal and the balanced embryos were then detected as two 5p signals and two chromosome 17 signals in each nucleus (Figure 2a,b). Embryos with imbalance of the chromosome 5p critical region were detected as one or three 5p signals, respectively, together with two chromosome 17 signals (Figure 2c,d). The balanced karyotype of the carrier showed two 5p signals (green) in each interfase/metaphase (Figure 3a). In the interfases/metaphases of the child with cri du chat syndrome, only one 5p signal was detectable, which validates the use of this probe to diagnose 5p imbalance (Figure 3b).

Two PGD treatments were performed. Both times, we were able to transfer two embryos, which we consider to be the maximum number in order to avoid multiple pregnancies. There was a great difference in the number of balanced embryos during the first and second treatment cycle (Table I). In the first treatment cycle, 29 oocytes were retrieved, which after fertilization resulted in 12 normally fertilized embryos.
Table I. Number and status of normally fertilized embryos

<table>
<thead>
<tr>
<th>Treatment cycle</th>
<th>No. of oocytes</th>
<th>No. of normally fertilized embryos</th>
<th>No. biopsied</th>
<th>Balanced</th>
<th>Unbalanced</th>
<th>Diagnosis not possible</th>
<th>No. transferred</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>18</td>
<td>12</td>
<td>3</td>
<td>8</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>17</td>
<td>11</td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

*Normally fertilized with two pronuclei visible 16–18 h post-insemination.

*Six showed abnormal number of control probe signals.

*One showed abnormal number of control probe signals.

Table II. Individual blastomere data from biopsied embryos, treatments 1 and 2

<table>
<thead>
<tr>
<th>Embryo No.</th>
<th>Blastomere 1</th>
<th>Blastomere 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>1/3</td>
<td>b</td>
</tr>
<tr>
<td>I-4</td>
<td>1/3</td>
<td>c</td>
</tr>
<tr>
<td>I-6</td>
<td>3/2</td>
<td>3/2</td>
</tr>
<tr>
<td>I-7</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>I-9</td>
<td>1/2</td>
<td>e</td>
</tr>
<tr>
<td>I-10</td>
<td>4/3</td>
<td>a</td>
</tr>
<tr>
<td>I-12</td>
<td>4/4</td>
<td></td>
</tr>
<tr>
<td>I-15*</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>I-20</td>
<td>1/3</td>
<td></td>
</tr>
<tr>
<td>I-22*</td>
<td>2/2</td>
<td>f</td>
</tr>
<tr>
<td>I-28</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>I-29</td>
<td>g</td>
<td></td>
</tr>
</tbody>
</table>

**First treatment**

**Second treatment**

<table>
<thead>
<tr>
<th>Embryo No.</th>
<th>Blastomere 1</th>
<th>Blastomere 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>I-2</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>I-3</td>
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<td>d</td>
<td>d</td>
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<tr>
<td>I-5</td>
<td>3/1</td>
<td>3/2</td>
</tr>
<tr>
<td>I-6</td>
<td>c</td>
<td>b</td>
</tr>
<tr>
<td>I-9</td>
<td>h</td>
<td></td>
</tr>
<tr>
<td>I-10</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>I-11</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>I-16</td>
<td>c</td>
<td>2/1</td>
</tr>
<tr>
<td>I-18*</td>
<td>2/2</td>
<td>2/2</td>
</tr>
</tbody>
</table>

Balanced embryos are shown in bold type. Transferred embryos are indicated with an asterisk.

*Green represents chromosome 5p; red the centromere of chromosome 17.

**Analysis not possible due to:** a lysed blastomere during biopsy, b lost nucleus during spreading, c biopsy failure, d fluorescent in-situ hybridization failure, e damaged nucleus, f autofluorescent debris on the nucleus, g two haploid nuclear fragments.

with good morphology. Biopsy of two cells per embryo was performed, and FISH analysis showed that only three out of 11 analysed embryos displayed a normal number of both 5p and chromosome 17 signals (Table II). Two embryos were transferred, but no pregnancy was established.

The second treatment cycle yielded 18 oocytes. Eleven embryos were normally fertilized and of good morphology. This time six out of eight analysed embryos showed a normal signal pattern (Table II) and two of them were transferred. Raised HCG concentrations were detected 13 days after transfer, indicating implantation, and ultrasonography confirmed the presence of two fetal hearts 2 weeks later. Amniocentesis was performed at 15 weeks and cytogenetic analysis confirmed two balanced inversion carrier karyotypes. Healthy twins, one male and one female, were delivered at 34 weeks of gestation.

**Discussion**

To the best of our knowledge, this is the first report of an established pregnancy using PGD in order to avoid chromosomal imbalance in the progeny of an inversion carrier. PGD using FISH has so far been applied for sex selection (Griffin et al., 1994) as well as for aneuploidy detection using polar body analysis (Munné et al., 1995; Verlinsky et al., 1996). Recently, FISH has also been used for the detection of unbalanced transmission of parental balanced translocations, which has resulted in ~10 babies born (Y. Verlinsky, personal communication). One of the problems with structural chromosomal aberrations and PGD is the fact that most chromosomal abnormalities are unique for each patient, with patient-specific breakpoints, which makes it necessary to find unique probes for every case. Another problem is that locus-specific probes, for technical reasons, often demand overnight hybridization, which is in conflict with the desire to transfer the embryos the same day as the biopsy. We were able to reduce the hybridisation time to 2 h by treating the slides with pepsin prior to hybridisation along with the use of directly labelled probes which allowed us to transfer the embryos on the same day as the biopsy was taken.

Moreover, this is the first time it has been possible to study the distribution of balanced and unbalanced gametes in a female inversion carrier. Chromosome analysis of spermatozoa has previously been performed in a few cases of male pericentric inversion heterozygotes (Balkan et al., 1983; Martin 1991, 1993; Jenderny et al., 1992; Navarro et al., 1993; Martin et al., 1994; Colls et al., 1997). As it is more likely that crossing-over will occur within a large rather than a small inverted segment, the risk of producing a significant number of recombinant spermatozoa increases if the size of the inverted segment constitutes >30% of the chromosome (Martin et al. 1994). The inverted segment in the particular inversion described here comprises ~85% of chromosome 5. Therefore, the likelihood that one or more interstitial cross-overs will occur during meiosis is quite high, explaining why only half of the analysed embryos were balanced. The conflicting results in the first and second treatments indicates that the distribution of balanced and unbalanced gametes may vary in the same carrier.

The presumed occurrence of a high proportion of unbalanced embryos in carriers of large pericentric inversions makes PGD
very useful, as it will increase the number of pregnancies with normal/balanced fetuses.

Cassel et al. (1997) described an attempt to develop patient-specific probes for PGD of structural chromosomal abnormalities, though these have not yet been used clinically. By using breakpoint spanning probes they were able to differentiate between normal and balanced embryos from a carrier of a balanced structural chromosomal abnormality. With our diagnostic approach we were not able to differentiate between normal and balanced embryos, which in this case was not desired as they both will result in a normal phenotype. The probes used were already available and we did not spend more time finding breakpoint spanning probes as the gain was questionable. Moreover, the YAC clones used by Cassel et al. demanded overnight hybridization and signal amplification, which did not suit our desire to transfer the embryos the same day as the biopsy was taken.

The possibility of PGD will be of great importance in families with inherited chromosomal abnormalities where the risk of having children with severe disease can be up to 50% and where the pregnancies often end in a miscarriage. These families are at present offered prenatal diagnosis, if possible, and they usually choose to terminate the pregnancy if the fetus is unbalanced. By using PGD the couple can start a pregnancy with the assurance that the disease will not be transmitted to the child and the need to terminate the pregnancy is thereby avoided.

Acknowledgements
We would like to acknowledge Sirpa Lundman for technical assistance and Dr Joyce Harper for valuable advice. This work was supported by the Swedish Medical Research Council. E.L. is a Swedish Medical Association scholar.

References


Received on November 19, 1997; accepted on May 13, 1998

PGD of chromosome 5 inversion