Preimplantation genetic diagnosis of DiGeorge syndrome

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We report the first case of preimplantation genetic diagnosis used in order to avoid chromosomal imbalance in the progeny of a woman mildly affected by DiGeorge syndrome and carrier of a microdeletion of chromosome 22q11.2. In total, seven embryos were biopsied in three separate treatments and analysed by fluorescent in-situ hybridization (FISH). Of these, four were carrying the deletion, two were normal and in one the analysis was inconclusive. The diagnostic procedure was performed within 5 h. This allowed the biopsied embryos to be transferred the same day as the biopsy was taken (day 3). Two embryos were transferred in the third treatment, but no pregnancy was established. Patients with a 22q11 microdeletion, who have a 50% risk of transmitting the deletion to their offspring, can now be offered preimplantation genetic diagnosis using FISH for the detection of a 22q11 deletion.

Key words: chromosome 22/deletion/DiGeorge syndrome/fluorescent in-situ hybridization/preimplantation genetic diagnosis

Introduction

DiGeorge syndrome (DGS) is a developmental defect where abnormal cephalic neural crest cell migration into the derivatives of the pharyngeal arches and pouches is responsible for the phenotype (Kirby and Bockman, 1984). There is a wide variation in DGS phenotype. Severely affected patients usually present in the neonatal period with hypocalcaemia due to aplasia or hypoplasia of the parathyroid gland, immune deficiency resulting from thymus gland aplasia or hypoplasia and a variety of cardiac malformations, in particular affecting the outflow tract. A common feature of DGS is also a dysmorphic face including low set ears, telecanthus with short palpebral fissures, a square nasal tip, a short philtrum and a relatively small mouth with thin lips. It has been proposed that DGS should be seen as the severe end of the clinical spectrum of some syndromes (i.e. DGS, velocardiofacial syndrome, conotruncal anomaly face syndrome and cases of isolated congenital heart malformations) embraced by the acronym CATCH 22 syndrome; Cardiac defects, Abnormal facies, Thymic hypoplasia, Cleft palate and Hypocalcaemia resulting from 22q11 deletions (Wilson et al., 1993).

The minimal DiGeorge critical region (MDGCR) is a 250 kb region of chromosome 22q11.2 (Li et al., 1994; Gong et al., 1997; Jaquez et al., 1997). Nevertheless, most patients share a large deletion which spans ~1.5 Mb within 22q11.2 (Driscoll et al., 1992; Wilson et al., 1992; Desmaze et al., 1993; Budarf et al., 1995). A deletion of this region is found in 83% of DGS patients (Driscoll et al., 1993). Several reports have described familial DGS, inherited as an autosomal dominant trait, and a review of the literature indicates that an average of 25% of the DGS patients show familial transmission of deletions (Leana-Cox et al., 1996). There is wide phenotypic variation also within members of the same family who presumably have inherited the same deletion (Driscoll et al., 1993; McLean et al., 1993).

Preimplantation genetic diagnosis (PGD) was first reported in 1990 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). 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 are determined using polymerase chain reaction (PCR) or fluorescent in-situ hybridization (FISH) 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). Recently, PGD has been used to avoid unbalanced transmission of parental balanced translocations (Y.Verlinsky, personal communication) and we have previously reported PGD for a carrier of a large pericentric inversion of chromosome 5 (Iwarsson et al., 1998). Other attempts to detect chromosomal abnormalities in preimplantation embryos have been described, though not used clinically yet (Pellestor et al., 1996; Cassel et al., 1997). So far, no established pregnancy after PGD for a carrier of a microdeletion has been reported.
We here report PGD performed on a female carrier of a microdeletion on chromosome 22q11, by using a short FISH protocol.

Materials and methods

The spare preimplantation embryos that were used for probe efficiency tests 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 Karolinska Institutet.

Proband

The proband was a 31-year-old woman with the typical facial appearance of DGS but otherwise no clinical symptoms. She was diagnosed after the birth of a severely affected child with tetralogy of Fallot and hypoplasia of the thymus gland. The child died at 4 months of age. A deletion on chromosome 22q11.2 was found in the mother and her child who inherited the same deletion but was more severely affected.

IVF, biopsy procedure and embryo transfer

The patient was down-regulated with buserelin 1200 mg/day intra-nasally (Suprepur®, Hoechst, Frankfurt, Germany) and stimulated with recombinant follicle stimulating hormone (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 done 37 h later, using a 1.4 mm outer diameter needle (Swemedlab, Billdal, Sweden). All oocytes were incubated in medium (IVF-50; Scandinavian IVF Sciences AB, Gothenburg, Sweden) droplets under oil (Ovoil-150; Scandinavian IVF Sciences AB). IVF was performed in 50 µl medium droplets under oil containing 20 000 spermatozoa.

Fertilization was evaluated 18–20 h after insemination. For intracytoplasmic sperm injection (ICSI), cumulus cells were removed by hyaluronidase treatment (Sigma, St Louis, MO, USA). The procedure was performed in 10 µl medium droplets under oil using a Nikon–Narishige micromanipulation system. On day 2 of culture, IVF and ICSI embryos were transferred to 10 µl droplets of S2 medium (Scandinavian IVF Sciences AB) under oil.

Biopsies were performed on 6–10-cell embryos, early (before noon) on the third day after oocyte retrieval, using a single needle approach (J.Inzunza et al., unpublished). Only normally fertilized embryos of good morphology were used for biopsy. Briefly, a hole in the zona was drilled by gently blowing acidified Tyrode’s solution (pH 2.5) using borosilicate glass capillaries (6C100T-15; Clark Electromedical Instruments, Reading, Berks, UK) single-pulled and forged to an inner diameter of 35–45 µm using a MF-9 Microforge (Narishige, Japan). The 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 in culture until the results of the genetic analysis were finally evaluated. Ultrasound-guided uterine 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)-coated slide. The blastomere was observed under an inverted microscope until the nucleus was free of cytoplasm. The slides were left to air dry, then washed in PBS and dehydrated through an alcohol series.

DNA probes

Four cosmids specific for chromosome 22q11.2 (sc11.1B, DO832, CO 568 and c443) were kindly provided by Dr Peter Scambler, labelled with Spectrum Orange-dUTP (Vysis, Downers Grove, IL, USA) by nick translation and used to identify the DGCR. The chromosome 17 centromere-specific probe D17Z1 (ATCC), labelled with fluorescein-12-dUTP by nick translation (Boehringer Mannheim), was used as an internal control. A probe efficiency test was performed using chromosome slides from lymphocyte preparations of healthy males and signals were counted in 200 nuclei. Two signals from the DGCR probe were present in 93% of the cells and two signals from the chromosome 17 centromere-specific probe were present in 96% of the cells. The probes were also tested using lymphocyte preparations of the carrier woman, her first child and spare preimplantation embryos.

FISH analysis

After spreading and dehyrdration 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% formamide/2× saline sodium citrate (SSC) at 75°C for 3 min followed by dehydration in 70, 80 and 100% ethanol at -20°C for 4 min each. The four cosmids were mixed and hybridized in 60% formamide, 2×SSC, 50 mM phosphate buffer pH 7.0 at a probe concentration of 1–2 ng/µl each. In addition, 2–3 µg Cot-1 (DNA (Life Technologies, Paisley, UK) was added to the probe mixture. At this stage the probe was stored at -20°C. On the day of the analysis the probe mixture was thawed and denatured at 75°C for 3 min and prehybridized at 37°C for 1 h. The chromosome 17 centromere-specific probe was added to the hybridization mixture to a final concentration of 1–2 ng/µl and the probe mixture was then added to the slide under a coverslip and hybridization was performed in a moist chamber at 42°C for 3.5 h. The slides were then washed for 2 min in 0.1×SSC/0.3% NP-40 at 72°C, followed by a second wash in 2×SSC/0.1% NP-40 at room temperature for 40 s. After dehydration, 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 as counterstain of the nuclei. The signals were analysed using a Zeiss Axioskop fluorescence microscope equipped with a cooled CCD camera (Photometrics SenSys, Tucson, AZ, USA) controlled by Power Macintosh computer. Gray scale images were captured, pseudocoloured and merged using the SmartCapture software (Vysis).

Results

We used FISH and four directly labelled cosmid probes from 22q11.2 to detect the deletion within the DGCR on the long arm of chromosome 22. 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 (balanced) embryos were then detected as two 22q signals and two chromosome 17 signals in each nucleus (Figure 1a). Embryos with imbalance of the DGCR were detected as only one 22q signal, together with two chromosome 17 signals (Figure 1b). Both the mildly affected carrier woman and the severely affected child showed only one 22q signal (red) in...
Three PGD treatments were performed, summarized in Table I. In the first treatment cycle only six oocytes were retrieved which resulted in only two biopsied embryos and none of them were normal (Figure 2a). The second treatment cycle was similar to the first one regarding the outcome. Seven oocytes were retrieved and only one embryo biopsied. The FISH result in this embryo was unclear due to hybridization failure and it was not transferred. The third treatment cycle yielded 15 oocytes but only six of them were normally fertilized and four embryos were biopsied (Table II). Two of these embryos were normal, showing two red and two green FISH signals and they were transferred, but no pregnancy was established (Figure 2b).

Discussion
To the best of our knowledge, this is the first report of using PGD in order to avoid chromosomal imbalance in the progeny of a microdeletion 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 (Munne et al., 1995; Verlinsky et al., 1996). Recently, FISH has also been used for the detection of unbalanced gametes in an inversion carrier (Iwarsson et al., 1998) and to avoid unbalanced transmission of parental balanced translocations, which has resulted in ~10 babies born (Y. Verlinsky, personal communication).

There are several problems with structural chromosomal aberrations and PGD. One is the fact that most chromosomal abnormalities are private, with unique breakpoints, which makes it necessary to find new locus-specific 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 hybridization time to 3.5 h by treating the slides with pepsin prior to hybridization which in combination with the use of directly labelled probes allowed us to transfer the embryos on the same each interphase/metaphase. In the interphases/metaphases of healthy controls two 22q signals in each nucleus were present.
Secondly, if the deletion is transmitted it is very difficult to predict the degree of symptoms in the future child. The choice of whether to have an abortion may therefore be difficult, and this makes PGD a more attractive alternative as it allows only unaffected embryos to be selected for transfer to the uterus and the need to terminate the pregnancy is thereby avoided.

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### References


### 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</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>6</td>
<td>4</td>
<td>2</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>0</td>
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<td>6</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

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

©One showed abnormal number of control signals.

### Table II. Individual blastomere data from biopsied embryos

<table>
<thead>
<tr>
<th>Embryo no.</th>
<th>No. of red/green signals*</th>
</tr>
</thead>
<tbody>
<tr>
<td>First treatment</td>
<td></td>
</tr>
<tr>
<td>I-3</td>
<td>1/2</td>
</tr>
<tr>
<td>I-4</td>
<td>1/2</td>
</tr>
<tr>
<td>Second treatment</td>
<td></td>
</tr>
<tr>
<td>II-4</td>
<td>†ª</td>
</tr>
<tr>
<td>Third treatment</td>
<td></td>
</tr>
<tr>
<td>III-4</td>
<td>2/2</td>
</tr>
<tr>
<td>III-9</td>
<td>1/2</td>
</tr>
<tr>
<td>III-12</td>
<td>2/2</td>
</tr>
<tr>
<td>III-13</td>
<td>1/1</td>
</tr>
</tbody>
</table>

Balanced embryos are shown in bold type. Transferred embryos are underlined.

*Results of the two separate blastomeres are given. Red represents chromosome 22q; green the centromere of chromosome 17.

†Analysis not possible due to: †a 5-cell embryo where only one blastomere was biopsied; †b 4-cell embryo where only one blastomere was biopsied; †c fluorescent in-situ hybridization failure; †d nucleus lost during spreading.

*The deletion was taken. An additional problem is illustrated by the present case where there were few oocytes after stimulation and even fewer developing embryos suitable for biopsy. This may result in a situation where there are no normal embryos to transfer, which of course is very frustrating for the patient. The possibility of a ‘no transfer’ situation should therefore be included in the information given during counselling prior to PGD.

There was a low number of biopsies performed compared to the number of normally fertilized embryos. This was due to the fact that in the three treatment cycles only 2, 1 and 4, respectively, of the normally fertilized embryos were developing normally and were of good morphology and consequently suitable for biopsy.

Patients with microdeletions of 22q11 show a wide spectrum of symptoms, from a few dysmorphic features to severe malformations and immune defects. No correlation between the size of the deletion and the severity of the symptoms has been found and there is a large variation, even within families, which may be illustrated by the present case. A possible explanation is that there are modifying factors, such as the in-utero environment, that affect fetal development (Ammann et al., 1982; Lammer et al., 1985; Wilson et al. 1993; Novak and Robinson, 1994). Alternatively, another genetic locus may be implicated. In the situation of prenatal diagnosis, the variation of the phenotype may create a serious problem. Firstly, the risk of transmitting the deletion is high (50%), and secondly, if the deletion is transmitted it is very difficult to


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