Preimplantation genetic diagnosis of chromosome balance in embryos from a patient with a balanced reciprocal translocation

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Duplications or deletions are present in a high percentage of the gametes produced by individuals carrying balanced translocations. Preimplantation genetic diagnosis was used to examine chromosome balance in embryos from a patient having a reciprocal translocation within the short arms of chromosomes 5 and 8 (46,XX,t(5;8)(p13;p23)). This woman has two sisters with the translocation unbalanced, resulting in a partial trisomy for chromosome 5 and partial monosomy for chromosome 8 (46,XX,-8, +der(8)t(5;8)(p13;p23)) with associated mental retardation and physical abnormalities. The patient and her husband desired to have children without the abnormal chromosome balance and wished to reduce the likelihood of spontaneous abortion or need for therapeutic abortion. Fluorescence in-situ hybridization (FISH) probes for the α-satellite region of chromosome 8 and for a region on the short arm of chromosome 5 (5p15.2) were tested initially on lymphocytes from the patient and her sisters. The hybridization signal for chromosome 5 was detected in the expected two copies for the patient and three copies for the sisters in 87% of the cells. Two hybridization signals for chromosome 8 were detected in 96% of the cells from all individuals. Additional probe testing was done using blastomeres from polyspermic embryos. The couple then proceeded with a stimulated in-vitro fertilization (IVF) cycle and biopsies were done on 13 embryos at the 7–10-cell stage using a method of zona drilling and fluid displacement. Diagnosis was possible on at least one blastomere for nine embryos. Three embryos had nuclei with three hybridization signals for chromosome 5, three had fewer than two signals for one or both chromosomes, one was mosaic, and two had two signals for each chromosome. The latter were transferred to the patient, but pregnancy was not achieved. The results demonstrate that preimplantation genetic diagnosis for patients with reciprocal translocations can be used to identify embryos having normal chromosome balance. The potential advantages and limitations of this approach are discussed.

Key words: chromosome translocation/embryo biopsy/FISH/preimplantation genetic diagnosis

Introduction

Reciprocal translocations, the interchange of chromosome segments between non-homologous chromosomes, are present in ~1 in 500 newborns (Hook and Hamerton, 1977). Reciprocal translocations are usually phenotypically harmless, since there is a balanced complement of genes. The translocations, however, are associated with the production of large numbers of gametes with unbalanced genetic complement and therefore an increased potential for one or more spontaneous abortions or phenotypically abnormal liveborn.

The production of abnormal gametes is the result of pairing and subsequent segregation of the translocation chromosomes and their normal homologues during meiosis. Gametes with complete chromosome complement are produced by alternate segregation, the translocation chromosomes and normal chromosomes passing to opposite poles (Figure 1). The other possible 2:2 segregations, adjacent I and II, with homologous centromeres passing to the opposite or same pole, respectively, produce gametes with genetic duplications and deficiencies. Unbalanced gametes can also be produced by 3:1 segregations. The relative frequency of segregation modes varies widely depending on the specific translocation (Martin and Hultén, 1993).

Preimplantation genetic diagnosis (PGD) has been used for couples known to be at risk of transmitting specific genetic disease to their offspring. Several births have been reported following transfer of biopsied human embryos (Handyside et al., 1992; Grifo et al., 1994; Liu et al., 1994; Griffin et al., 1995; Gibbons et al., 1995). Another application of PGD is the identification of specific chromosomes using fluorescence in-situ hybridization (FISH) to reduce the risk of transferring embryos with aneuploidy (Munné et al., 1993), potentially increasing the implantation rate and reducing the possibility of miscarriage or a fetus with a viable chromosomal abnormality. The use of FISH for identifying chromosome balance in embryos from patients with Robertsonian translocations has also been reported (Munné et al., 1996; Roh et al., 1996).

This report describes the development of PGD for a couple in whom the woman is a carrier of a balanced reciprocal translocation involving the short arms of chromosomes 5 and...
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Figure 1. Diagrams of chromosome pairing between chromosomes 5, 8 and the translocation derivatives during meiotic prophase and the chromosome combinations resulting from different modes of segregation. The approximate location of probe hybridization is indicated by the large circles. G = green fluorochrome-labelled probe for chromosome 8 α-satellite; R = rhodamine-labelled probe for chromosome 5 band p15.2.

8 (46,XX,t(5;8)(p13;p23)). The patient’s mother has the same karyotype and her two sisters each carry the modified chromosome 8 in addition to 2 normal copies of chromosome 5 (46,XX,-8, +der(8)t(5;8)(p13;p23)). This partial trisomy for the short arm of chromosome 5 produces a phenotype of mental retardation, seizures, and other physical abnormalities. Several cases of partial trisomy 5p and the associated phenotypic effects have been reported previously (Yunis et al., 1978; Zabel et al., 1978; Antonenko et al., 1984). The objective of the PGD was to reduce the risk of pregnancy with a fetus having this viable chromosome imbalance or the likelihood of spontaneous abortion due to other chromosome imbalance.

Materials and methods

Preparation of mononuclear white blood cells
Blood was drawn into tubes containing EDTA to prevent clotting. Blood (3 ml) was carefully layered over 3 ml of Ficoll (HistoPaque, density 1.077 g/ml, Sigma, St Louis, MO, USA) and centrifuged at 400 g for 30 min. Most of the plasma was aspirated and discarded, and the layer of white blood cells was collected, rinsed three times with phosphate-buffered saline (PBS), then fixed with freshly-made, ice-cold Carnoy’s fixative (methanol:acetic acid, 3:1). The fixed cells were left at 4°C for 1 h or longer, then dropped onto ethanol-cleaned slides and allowed to dry in a humidified chamber at 37°C.

Fluorescent in-situ hybridization
Slides were treated for 2 min at 70°C in 70% formamide/2× sodium chloride/sodium citrate (SSC), pH 7.0 and dehydrated through an ethanol series and air dried. A fluorochrome-labelled probe for the α-satellite of chromosome 8 (CEP8, Vysis, Downers Grove, IL, USA) was diluted 1 to 3 in buffer (Hybrisol VI; Oncor, Gaithersburg, MD, USA) and denatured 5 min at 74°C. After cooling, 1 µl was mixed with 9 µl of a digoxigenin-labelled probe specific for chromosome region 5p15.2 (Oncor). The probe mix was pipetted onto the slide, covered with a glass coverslip, sealed with rubber cement and incubated for 18 h in a humidified chamber at 37°C. The slides were washed with 50% formamide/2× SSC and treated with fluorochrome-labelled anti-digoxigenin according to the Oncor protocol, except that normal goat serum was added to a final concentration of 2% in the detection reagent to reduce non-specific background. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) and visualized using an Olympus BH-2 fluorescence microscope.

IVF and embryo culture
All human embryos were from a single in-vitro fertilization (IVF) cycle. The 35-year-old woman was treated with leuprolide acetate (Lupron®; Tap Pharmaceutical, Deerfield, IL, USA), then stimulated with human menopausal gonadotrophins (Metrodin®; Serono Laboratories, Randolph, MA, USA). A dose of 10 000 IU human chorionic gonadotrophin (HCG, Profasi®, Serono Laboratories) was given 34 h before egg retrieval. Follicles were aspirated and flushed with modified Dulbecco’s phosphate buffered saline (DPBS, Sigma) containing 20 IU/ml heparin. Oocytes and surrounding cumulus were cultured in Ham’s F-10 containing 4 mg/ml bovine serum albumin (BSA) as described elsewhere (Loutradis et al., 1993). Insemination was done 6 h later by adding 250 000 motile spermatozoa per ml culture medium. Eggs were examined 18 h post-insemination for the presence of pronuclei. Eggs with two pronuclei (PN) were transferred to Ham’s
Figure 2. (A) Fluorescence in-situ hybridization (FISH) on a lymphocyte nucleus from the balanced translocation carrier. Two red signals for chromosome 5 and two green signals for chromosome 8 are present. The close proximity of the red and green signals at the 10 o’clock position denotes the location of the translocation chromosome (der 8). (B) FISH on a lymphocyte nucleus from one of the patient’s sisters (proband). The presence of the 5p trisomy is indicated by the three red hybridization signals. (C) FISH on a lymphocyte nucleus from an individual with a normal karyotype.

Figure 3. (A) Fluorescence in-situ hybridization (FISH) on a blastomere nucleus. Two red signals (at 1 and 2 o’clock) and two green signals indicate normal chromosome balance for chromosomes 5 and 8. The large orange spot at the 4 o’clock position is autofluorescence. (B) FISH on a nucleus from a blastocyst-stage embryo. See text for details.

Table I. Fluorescent in-situ hybridization (FISH) detection of chromosomes 5 and 8 in lymphocyte nuclei

<table>
<thead>
<tr>
<th>Lymphocyte from</th>
<th>Total no. nuclei examined</th>
<th>Expected signals</th>
<th>Percentage of nuclei with expected no. signals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C5</td>
<td>C8</td>
</tr>
<tr>
<td>Patient</td>
<td>100</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>(balanced translocation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sister #1</td>
<td>120</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>(5p trisomy)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sister #2</td>
<td>93</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>(5p trisomy)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>312</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>(normal karyotype)</td>
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C5 = chromosome 5; C8 = chromosome 8.
According to the method described above, embryo biopsy was done using a method of zona drilling and fluid displacement previously tested on mouse embryos (Pierce et al., 1997). Embryos at the 7–10-cell stage were placed in paraffin oil-covered 100 µl drops of calcium-free modified DPBS (Sigma, cat. no. D 5652, made without the calcium supplement) containing 4 mg/ml BSA and 0.1 mM EDTA. The embryo was immobilized against a holding pipette and an opening was made in the zona pellucida using a stream of acidified Tyrode’s solution, pH 2.5, from a micropipette with a tip diameter of ~3 µm. The flow was controlled by a Narashige IM-6 microinjector. The pipette was then rinsed and filled with the DPBS solution and inserted through the zona pellucida. Fluid was expelled from the pipette until one or two blastomeres were forced through the opening in the zona pellucida. All manipulations were carried out on a heated microscope stage to maintain a temperature of 37°C. Pipette movements were controlled using Narashige hydraulic micromanipulators. Biopsied embryos were returned to culture and isolated blastomeres were incubated for 2 min in 1% sodium citrate, then fixed on slides using freshly-made Carnoy’s fluid. After a microinjection with a holding pipette and an opening was made in the zona pellucida. Fluid was expelled from the pipette until one or two blastomeres were forced through the opening in the zona pellucida. All manipulations were carried out on a heated microscope stage to maintain a temperature of 37°C. Pipette movements were controlled using Narashige hydraulic micromanipulators. Biopsied embryos were returned to culture and isolated blastomeres were incubated for 2 min in 1% sodium citrate, then fixed on slides using freshly-made Carnoy’s fixative. Slides were dried for 1 h at 65°C and FISH was carried out according to the method described above.

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**Results**

In order to estimate the accuracy of the FISH technique with the probes used in this study, hybridization signals were examined on lymphocyte nuclei from the patient, her sisters, and control individuals with normal karyotypes, including the patient’s husband (Figure 2). The expected number of hybridization signals for chromosome 5 (C5) were detected in 85% of the patient’s lymphocytes, 89 and 87% of her two sister’s lymphocytes, and 95% of control lymphocytes (Table I). The expected two hybridization signals for chromosome 8 (C8) were detected in at least 95% of lymphocytes in each case. The expected signals for both chromosomes were found in 87% of all lymphocytes (range 82–92%).

One of the priorities of the lymphocyte study was to estimate the probability of detecting only two signals for C5 in a nucleus that had three copies of the region detected by the probe, since that type of error could lead to a pregnancy with an affected fetus. Of the lymphocyte nuclei from the patient’s sisters, 10% had only two detectable signals for C5.

Tests were also made on nuclei in abnormal embryos (from 1PN or 3PN eggs, or 2PN eggs arrested during cleavage or showing multinucleate blastomeres) obtained from unrelated IVF cycles. A total of 27 nuclei from 12 embryos were examined following hybridization with the probes for C5 and C8. A total of 18 nuclei had at least one signal for each chromosome, four nuclei had only C5 signals, two nuclei had only C8 signals, and three nuclei had no detectable hybridization signals. It was not possible to quantify the accuracy of chromosome detection, as these embryos are often chromosomal mosaics.

Following counselling that included discussion of the preliminary tests, the couple decided to proceed with an IVF cycle and PGD testing. In all, 20 2PN eggs were obtained from a total of 25 oocytes retrieved. Six embryos were biopsied at the 7–8-cell stage late on day 2 (~55 h post-insemination). Seven additional embryos were biopsied at the 8–10-cell stage on the morning of day 3. The remaining seven embryos were not biopsied due to slower cleavage rates, high levels of fragmentation, and/or multinucleate blastomeres.

Four of the isolated blastomeres were damaged during biopsy and one additional blastomere was lost during processing. Of the 20 remaining blastomeres, diagnosis was possible on 12 nuclei (Table II). Diagnosis was not possible in the other cases due to high background fluorescence or the absence of a nucleus (technical loss or anucleate cell). Two embryos had nuclei with two hybridization signals each for C5 and C8 (Figure 3a). Three embryos had three signals for C5 and 2 for C8, a combination that could reflect the viable chromosome imbalance. Nuclei from four embryos were missing signals for one, or both chromosomes. One of those embryos was identified as mosaic, as a second nucleus contained three signals for each chromosome.

The two embryos with the normal number of chromosome signals were transferred to the patient on day 5 at the blastocyst stage. An additional seven embryos also developed into blastocysts. The total number of cells in the blastocysts ranged from ~20–50, estimated by observations with an inverted microscope equipped with Hoffman optics.

Additional cells were biopsied on day 5 from blastocyst-stage embryos for which no diagnosis had been obtained following biopsy at the cleavage stage. Hybridization signals were observed on six nuclei from one embryo and five nuclei from a second. Three nuclei from the first embryo had two signals for each chromosome (Figure 3b), two nuclei had one C5 and two C8 signals, and one nucleus had two C5 and one C8 signals. The second embryo contained three nuclei with...
two C5 and no C8 signals, and two nuclei with one C5 and one C8 signal.

Discussion

Abnormal chromosome balance in blastomeres from pre-implantation stage embryos can be identified using FISH. Such analysis has been used to identify aneuploidy in embryos, most commonly found in embryos from older women (Munné et al., 1993, 1995). FISH has been used to obtain pregnancies for patients carrying Robertsonian translocations, analysing blastomeres (Roh et al., 1996), or the polar body from unfertilized eggs (Munné et al., 1996). The present study demonstrates that the FISH technique is also useful in identifying monosomes or trisomies of partial chromosomes that are common in embryos from patients with reciprocal translocations. By not transferring those embryos, it is possible to reduce the chances of miscarriage or pregnancy with an affected fetus.

The case presented here and previously in abstract form (Pierce et al., 1995) was the first report of PGD for a chromosome translocation and is the only reported case of PGD for a reciprocal translocation. Unfortunately, only two out of nine embryos for which a diagnosis was made were found to have the proper balance for chromosomes 5 and 8, and the transfer of those embryos did not result in pregnancy. Blastomeres from three other embryos were found to have three hybridization signals the short arm of C5 in addition to two signals for C8, the combination that is present in the nuclei from the affected sisters. Although the analysis of interphase nuclei in the present study gives limited information regarding the specific chromosomes of the embryos (e.g. normal and balanced combinations cannot be distinguished), these results are consistent with alternate and adjacent-1 modes of meiotic segregation. No evidence for adjacent-2 segregation was found. It should be noted that the results do not rule out 3:1 modes of chromosome segregation. An average of 5% of spermatozoa from men with different translocations were found to be the result of 3:1 segregation (Martin and Hultén, 1993), and a similar frequency might be expected during meiosis of oocytes. The inability to accurately diagnose 3:1 segregants was not a large concern in this case due to the expected low percentage and the inviability of resulting embryos. Surprisingly, four embryos were found to have one or more nuclei in which either C5 or C8 signals were completely missing. This was unexpected since the spermatozoa should contribute one set of chromosomes to the embryos. Although failure to detect hybridization signals is possible in some instances, results of the preliminary testing suggest that most nuclei would be accurately diagnosed. It is likely that these results were due to mitotic non-disjunction in the embryo. Further evidence for this was found in two embryos biopsied at the blastocyst stage. Different combinations of hybridization signals were found in the nuclei from each of those embryos. A high rate of mitotic non-disjunction in the embryo could be due to some unknown effect of the translocation, but is likely due to an unrelated factor. Chromosome abnormalities are not uncommon in embryos obtained from IVF, having been detected in 21–32% of embryos with normal morphology (Angell et al., 1986; Plachot et al., 1988; Jameson et al., 1994) and as high as 90% in poor quality embryos (Pellestor et al., 1994). FISH analysis of all blastomeres revealed mosaicism in 40% of embryos with normal morphology (Munné et al., 1995). The presence of mosaic embryos is a potential problem if accurate diagnoses are to be made from one or two cleavage-stage blastomeres.

PGD for a reciprocal translocation

Polar body biopsy offers a possible alternative to blastomere biopsy for cases in which the woman is the translocation carrier. By analysing polar bodies from metaphase II eggs of Robertsonian translocation carriers, it is possible to determine chromosome content within the egg and transfer only embryos derived from eggs with normal chromosome balance (Munné et al., 1996). Analysis of the first polar body offers the advantage of obtaining condensed chromosomes, but is technically difficult and, by itself, is not suitable for reciprocal translocations due to the possibility of interstitial cross-over with subsequent segregation of balanced and unbalanced sets of chromosomes during the second meiotic division. Accurate diagnosis of both polar bodies would be required to determine the chromosome balance of the egg.

PGD for patients with reciprocal translocations has not been reported prior to the current study. In fact, very little is known regarding segregation of translocation chromosomes in human oocytes. In early studies, limited information was provided through the identification of imbalance at birth (Jalbert et al., 1980) or after spontaneous abortion (Fryns et al., 1984). A better understanding of segregation in male translocation carriers has been obtained by examining human sperm chromosomes following penetration of hamster eggs (Balkan and Martin, 1983; Martin and Hultén, 1993) and by direct observation of meiotic spermatocytes in testicular biopsies (Goldman and Hultén, 1993a,b). These studies found that all modes of segregation, including alternate, adjacent-1, adjacent-2, and 3:1 were present, but at varying frequencies depending on the specific translocation. Alternate segregation, producing gametes with balanced chromosome content, was the most common mode of segregation, with a mean of 44% in 27 translocations studied (range 23–78%) (Martin and Hultén, 1993).

Limitations of the current method include the inability to distinguish between the normal karyotype and the balanced translocation. In some nuclei, juxtaposition of hybridization signals for C5 and C8 suggested the possible presence of the translocation, but it was apparent from studies on the lymphocyte interphase nuclei that the signals were not always closely juxtaposed. Also, the close proximity of the different hybridization signals, itself, may slightly increase the possibility for misdiagnosis. The expected number of hybridization signals for C5 could be detected in only 85 and 88% of the lymphocyte nuclei from the patient and the patient’s sisters respectively, compared with 95% in control lymphocytes. In some instances, the C5 signal on the translocation chromosome (der 8) may have been masked by the larger signal for the C8 α-satellite. This represents a significant error rate if a similar effect is present in blastomeres, particularly important in the possible misidentification of the viable unbalanced chromosome com-
bination. Cases such as this require thorough counselling of patients regarding possible misdiagnosis and the need for evaluating resulting pregnancies. The patients also should understand that PGD may fail to identify any embryo suitable for transfer; a significant possibility if the number of embryos is available for biopsy is small. PGD, however, represents a useful option for patients with chromosome translocations, since the risk of miscarriage or pregnancy with an affected fetus can be reduced by avoiding transfer of embryos with abnormal chromosome content. PGD is also likely to be preferred by many couples over the combination of IVF or gamete intra-Fallopian transfer (GIFT) followed by amniocyte testing and selected fetal reduction (Brambati et al., 1994). The use of additional probes with different fluorochromes, possibly on metaphase chromosomes, should increase the accuracy of PGD in future reciprocal translocation cases.

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