

Preimplantation genetic diagnosis of pericentric inversions

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Inversions are structural chromosome abnormalities that may be associated with infertility, multiple miscarriage and chromosomally unbalanced offspring. Preimplantation genetic diagnosis (PGD) with subtelomeric probes was used to select for transfer only those embryos that were normal or balanced for three pericentric inversions. In contrast to previous protocols the present procedure allows the detection of unbalanced embryos that might arise from U-recombination in the inverted region. Additionally, aneuploidy screening was carried out in two cases by a second round of fluorescent *in situ* hybridization (FISH) with centromeric probes. Of the three couples that underwent the procedure one became pregnant twice. The first pregnancy delivered a healthy and chromosomally normal baby and the second pregnancy is ongoing with triplets. Copyright © 2001 John Wiley & Sons, Ltd.

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INTRODUCTION

Pericentric inversions are among the most frequent chromosomal rearrangements in humans, with a frequency of 1–2% of the population (Nielsen and Psiloin, 1975). In most cases, heterochromatic regions are implicated and these inversions are usually considered polymorphisms without phenotypical consequences, as for example 1qh, 9qh and 16qh. However, carriers of inversions not involving heterochromatic regions are at risk of having unbalanced offspring due to meiotic crossing-over; the most common of these inversions being those involving chromosome 2 (Gardner and Sutherland, 1996). It has been reported that pericentric inversion of chromosome 2 causes repeated abortions, congenital anomalies and mental retardation (Pallota *et al.*, 1983; Kleckowska *et al.*, 1987). Carriers of inversions involving chromosome 4 are also at risk of having unbalanced offspring with Wolf-Hirschhorn syndrome (Hirsch and Baldinger, 1993; Villa *et al.*, 1995; Ogle *et al.*, 1996). This is a rare chromosomal abnormality caused by loss of material from the distal aspect of the short arm of chromosome 4. Characteristics include marked prenatal and postnatal growth retardation with psychomotor delay, profound mental deficiency, distinctive faces, and midline defects (Thomson, 1998).

The risk of unbalanced progeny is caused by the occurrence of an odd number of meiotic crossovers between a normal chromatid and an inverted chromatid. If this happens, four different gametes are produced: normal gametes, carrier of the inversion gametes, gametes with duplication of the region distal to the inversion on the p arm of the chromosome and a deletion of the region distal to the inversion on the q arm of the chromosome, and gametes with

duplication of the region distal to the inversion on the q arm of the chromosome and a deletion of the region distal to the inversion on the p arm of the chromosome. The risk of unbalanced progeny for inversion carriers occurring through a recombinant is estimated at 5% for males and 10% for females (Sutherland *et al.*, 1976).

An estimated 4% of the offspring from paracentric inversion carriers are unbalanced possibly as a result of U-loop recombination; this incidence could be higher in the early stages of development (Pettenati *et al.*, 1995). The frequency of U-loop recombination has not been determined for pericentric inversion, but it would produce an acentric fragment and a dicentric chromosome. Because the acentric fragment lacks a centromere, it could have misorientation or be lost in subsequent divisions. The dicentric chromosome would produce a chromosomal bridge in the first or the second division of the meiotic process which could stop the division in the gametogenesis process or could prevent McClintock's classical breakage–fusion–bridge cycle from starting. If this did occur, gametes with broken chromosomes (with only one telomere) would occur. Therefore only diagnosis protocols that include breakpoint-spanning probes (Cassel *et al.*, 1997) or two telomeres and one centromere probe would detect such events.

Preimplantation genetic diagnosis (PGD) for structural abnormalities using fluorescent *in situ* hybridization (FISH) has so far been applied in the diagnosis of (i) translocations (Conn *et al.*, 1998, 1999; Fung *et al.*, 1998; Munné *et al.*, 1998a,b,c, 2000; Pierce *et al.*, 1998; Weier *et al.*, 1999; Van Assche *et al.*, 1999; Verlinsky and Evsikov, 1999; Willadsen *et al.*, 1999), (ii) inversions (Cassel *et al.*, 1997; Iwarsson *et al.*, 1998) and (iii) deletions (Iwarsson *et al.*, 1998).

One of the main problems with PGD of structural chromosome abnormalities is that different breakpoints are involved in each abnormality. Several approaches have been used, of which two can be

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usefully applied to PGD of inversions. One has been the use of breakpoint spanning probes specific for each translocation which allow the differentiation of normal, balanced and unbalanced embryos (Munné *et al.*, 1998b; Weier *et al.*, 1999), however this method is very expensive and time consuming. An alternative is the use of probes distal to the breakpoints or telomeric probes, preferably in combination with centromeric or proximal probes (Conn *et al.*, 1998, 1999; Munné *et al.*, 1998b, 2000; Van Assche *et al.*, 1999). Although this alternative does not permit the differentiation between normal and balanced products, it does identify unbalanced nuclei, whichever recombination type (X or U) has occurred in the inverted region. In both approaches, the use of a centromeric probe allows the differentiation of unbalanced from illogical or nonsense results produced by FISH errors (Munné *et al.*, 2000). Here is presented a novel FISH approach for inversions, using two subtelomeric probes and a centromeric one for the PGD for the products of three inversions: 46,XX,inv(2)(p21q13); 46,XY,inv(2)(p11.2q13) and XY,inv(4)(p16q21), for a total of five PGD cycles.

PATIENTS AND METHODS

During Day 3 of development, one cell per embryo was biopsied by opening a hole in the zona pellucida using acid Tyrode's solution, followed by careful cell aspiration, as described previously (Grifo, 1992). All blastomeres were fixed individually following a previously described protocol (Munné *et al.*, 1996). Embryo biopsy and fixation of biopsied cells were performed at The New England Clinic of Reproductive Medicine for Patient 1 and at the Colorado Center for Reproductive Medicine for Patient 3. FISH analysis for all patients was done at The Institute for Reproductive Medicine and Science of Saint Barnabas Medical Center. These protocols were in accordance with guidelines approved by the Internal Review Board of Saint Barnabas Medical Center, Internal Review Board of Colorado Center for Reproductive Medicine, and The New England Clinic of Reproductive Medicine through TUFTS Medical Center, respectively, and included individual written informed consent.

Case 1 involved a 33-year-old female carrier of a 46,XX,inv(2)(p21q13) pericentric inversion. The couple experienced three spontaneous first trimester abortions from natural conception. No products of conception were sent for karyotypic analysis. Subsequent to the third spontaneous abortion, the patient underwent a recurrent miscarriage work-up including karyotypic analysis on the patient and her husband. Her husband was found to be karyotypically normal, while she was found to carry the inversion. The husband was also found to be normospermic on a subsequent semen analysis.

Case 2 was a 31-year-old male carrier of a 46,XY,inv(2)(p11.2q13) pericentric inversion. The patient was found to be teratospermic on a subsequent

semen analysis. Because the female had primary amenorrhoea, the couple also opted for oocyte donation with a karyotypically normal 23-year-old female donor.

Case 3 was a 42-year-old male carrier of a 46,XY,inv(4)(p16q21) pericentric inversion. The couple experienced one spontaneous first trimester abortion from natural conception. No products of conception were sent for karyotypic analysis. The patient was found to be normospermic on a subsequent semen analysis. The female was 40.8 years old with a normal 46,XX karyotype.

FISH procedures

In cases 2 and 3, fixed cells were analyzed by two rounds of FISH procedures, the first to identify the products of inversion, the second to detect numerical chromosome abnormalities for chromosome 18 in case 1 and for chromosomes 13, 16, 18, 21 and 22 in cases 2 and 3.

For case 1, in the first cycle a single round of FISH procedure was performed following the procedure recommended by Cytocell (Adderbury, UK) with some modifications. Ten microliters of hybridization solution (Cytocell) were mixed and supplemented with 0.5 µl specific centromere 2 probe, labeled in Spectrum Aqua (prepared at Vysis, Downers Grove, IL, USA) and 0.5 µl specific centromere 18 probe, labeled in Spectrum Blue (prepared at Vysis) used here as a ploidy control. The final mixture was added on a dry coverslip supplied by Cytocell which already had telomere probes attached specific for the two arms of chromosome 2, p arm labeled with FITC and q arm with Cy3. The coverslip, with the centromere-supplemented hybridization solution, was flipped over the slide containing the fixed cells to be analyzed, such that the hybridization solution and the attached telomere probes faced the cells. The slides were placed for 5 min at 37°C, denatured at 75°C for 5 min, and left overnight to hybridize. Next morning the slides were washed in 0.4 × saline sodium citrate (SSC) for 2 min at 72°C, and the signals were subsequently demonstrated with the antibodies supplied by Cytocell as specified in their protocol without modification, counterstained with DAPI/antifade (Oncor, FL, USA) and observed with a fluorescent microscope (Olympus BX70). For the second cycle of this patient, another single round of FISH procedure was performed with two TelVysion TM probes for the regions 2ptel and 2qtel labeled in Spectrum Green and in Spectrum Orange, respectively (Vysis) and one centromeric probe for chromosome 2 labeled in Spectrum Aqua (prepared at Vysis). Ten microliters of the first hybridization solution were applied to the class slides containing fixed blastomeres and covered with a regular 18 × 18 mm coverslip. The slides were placed for 5 min on a slide warmer preheated to 73°C, sealed with rubber cement, and placed in a dark moist chamber at 37°C for 3 h. After the hybridization, the slides were washed individually at 71°C in a

jar containing $0.7 \times \text{SSC}$ for 4 min. The slides were then mounted with $10 \mu\text{l}$ DAPI/antifade (Oncor) and observed with a fluorescent microscope (Olympus BX70).

For case 2, the same protocol was followed as was described in the second cycle of case 1 without modifications.

Case 3 followed a single round of FISH procedure. In this case the probes used were two TelVysion™ TM probes for the regions 4ptel and 4qtel labeled in Spectrum Green and in Spectrum Orange, respectively (Vysis) and one centromeric probe for chromosome 4 labeled in Spectrum Aqua (prepared at Vysis). Ten microliters of the first hybridization solution were applied to the glass slides containing fixed blastomeres and covered with a regular 18×18 mm coverslip. The slides were placed for 5 min on a slide warmer preheated to 73°C , sealed with rubber cement, and placed in a dark moist chamber at 37°C for 3 h. After the hybridization, the slides were washed individually at 71°C in a jar containing $0.7 \times \text{SSC}$ for 4 min. The slides were then mounted with $10 \mu\text{l}$ DAPI/antifade (Oncor) and observed with a fluorescent microscope (Olympus BX70).

For cases 2 and 3, the second hybridization used the MultiVysion™ PGT multicolor probe panel hybridization mixture (Vysis) with the probe for chromosomes 13 labeled with SpectrumRed™, 16 with SpectrumAqua™, 18 with SpectrumBlue™, 21 with SpectrumGreen™ and 22 with SpectrumGold™. After the analysis of the first set of probes, the slides were washed in $0.7 \times \text{SSC}$ at room temperature until the coverslips fell off, dipped in distilled water at 71°C for 10 s, and then dehydrated (70%, 85%, 100% ethanol, 2 min each). Ten microliters of the multicolor probe panel hybridization mixture were applied to the glass slide and covered with a regular 18×18 mm coverslip. The slides were then placed for 5 min on a slide warmer preheated to 73°C , sealed with rubber cement, and placed in a dark moist chamber at 37°C for 3 h. Subsequently the slides were washed for 30 s in $0.4 \times \text{SSC}$ at 73°C . The washed slides were then mounted with antifade II solution (Vysis) and analyzed again.

These combinations of probes did not permit differentiation between normal and balanced embryos because two signals appeared in either case for each probe. However, embryos with unbalanced chromosomes showed other signal combinations as follows: (1) one green signal, three orange signals and two aqua signals, indicating the duplication of the region distal to the inversion on the q arm of the chromosome and a deletion of the region distal to the inversion on the p arm of the chromosome or (2) three green signals, one orange signal and two aqua signals, indicating the duplication of the region distal to the inversion on the p arm of the chromosome and a deletion of the region distal to the inversion on the q arm of the chromosome.

U-recombinations cause the presence or absence of acentric fragments and broken chromosomes derived from dicentric chromosomes. In these cases, we would

expect other signal combinations as follows: (1) Presence of a broken chromosome: two aqua signals, and the absence of one telomeric signal (orange or green) or (2) presence of an acentric fragment: there are three types of fragments that could be generated depending on the telomeres involved in the acentric fragment as follows. (a) Presence of an acentric fragment with duplication of the region of the p arm and deletion of the region of the q arm: one aqua signal, three green signals and one orange signal. (b) Presence of an acentric fragment with deletion of the centromeric region of the q arm: one aqua signal, two green signals and two orange signals. (c) Presence of an acentric fragment with duplication of the region of the q arm and deletion of the region of the p arm: one aqua signal, three green signals and one orange signal. (3) Presence of a broken chromosome and an acentric fragment: any combination of (1) and (2).

The telomeric probes were first tested on the fixed lymphocytes of each patient to make sure that they worked properly with the inversion and to check the efficiency of the probes. In all cases embryo transfer was performed on developmental Day 4.

RESULTS

Efficiency of the probes

Tests of probes were performed on control blood to confirm their efficiency for the characterization of the inversion. Two hundred control lymphocytes were tested for each probe mixture, and 94%, 96% and 97% of the lymphocytes showed the expected number of signals for cases 1, 2 and 3, respectively. Tests on carrier blood were also made to confirm the probes' ability to characterize the inversion. Twenty-five lymphocyte metaphases were analyzed and one metaphase captured in each case. The efficiency for the aneuploidy panel was described in our laboratory in a previous study (91%; Munné *et al.*, 1998d).

PGD results

Case 1 underwent two PGD cycles. For the first cycle 21 eggs were retrieved, 19 were injected by ICSI of which 12 fertilized. Eleven of these embryos were biopsied on Day 3 and one was discarded after arrested development. At the time of biopsy, three embryos were at the morula stage; one embryo had eight cells, four embryos had six cells, and three embryos had four cells.

FISH analysis revealed that 6/11 embryos were chromosomally normal or balanced and three were abnormal, of which one was polyploid (embryo 1) and two were unbalanced (embryos 6 and 7) (Table 1). The other two showed no nucleus after fixation (embryos 5 and 8). In accordance with the previously described logic, embryo 6 was believed to have an extra acentric fragment p, and embryo 7 was believed to have a broken chromosome 2 (without telomere 2q). Three embryos were transferred.

Table 1—PGD results for Patient 1, 46,XX,inv(2)(p21q13)

Embryo	Tel 2p	Tel 2q	Cen 2	Cen 18	Diagnosis
1	6	6	5	5	Polyploid
2	2	2	2	2	Normal/balanced
3	2	2	NR	2	Normal/balanced
4	2	2	2	2	Normal/balanced
5	—	—	—	—	No nucleus
6	4	2	2	2	Unbalanced
7	2	1	2	2	Unbalanced
8	—	—	—	—	No nucleus
9	2	2	NR	2	Normal/balanced
10	2	2	2	2	Normal/balanced
11	2	2	2	2	Normal/balanced

NR, No result.

For the second cycle 19 eggs fertilized after ICSI, and all were biopsied on Day 3. FISH analysis revealed that 8/19 embryos were chromosomally normal or balanced (Table 2). Seven were considered abnormal, and four showed no result because the lack of nucleus in the biopsied cells. Of the abnormal ones, one was considered either triploid or trisomic for chromosome 2 (embryo 2), another was considered either tetraploid or tetrasomic for chromosome 2 (embryo 9), another was considered either haploid or monosomy for chromosome 2, and four were considered unbalanced (embryos 1, 5, 13 and 18). Of the unbalanced ones, three were unbalanced because of an X-crossover in the inverted region (embryos 1, 5 and 18), and embryo number 13 could have been unbalanced because of a possible U-crossover in the inverted region and had a broken chromosome 2 (without telomere 2q). Three embryos were transferred.

Results were obtained for a total of 24 embryos for case 1. Fourteen were balanced or normal and nine were abnormal; six had abnormalities related to the inversion.

For case 2, six eggs were retrieved; all of them were

Table 2—PGD results for Patient 1, 46,XX,inv(2)(p21q13), second cycle

Embryo	Tel 2p	Tel 2q	Cen 2	Diagnosis
1	1	3	2	Unbalanced
2	3	3	3	Triploid or trisomy 2
3	—	—	—	No nucleus
4	2	2	2	Normal/balanced
5	1	3	2	Unbalanced
6	—	—	—	No nucleus
7	—	—	—	No nucleus
8	2	2	2	Normal/balanced
9	4	4	4	Tetraploid or tetrasomy 2
10	1	1	1	Haploid or monosomy 2
11	2	2	2	Normal/balanced
12	2	2	2	Normal/balanced
13	2	1	2	Unbalanced
14	2	2	2	Normal/balanced
15	2	2	2	Normal/balanced
16	2	2	2	Normal/balanced
17	—	—	—	No nucleus
18	3	1	2	Unbalanced
19	2	2	2	Normal/balanced

fertilized by IVF. Four of these embryos were biopsied on Day 3 and two were discarded after arrested development. At the time of biopsy, three embryos had seven cells and one had six cells. FISH analysis revealed that three embryos were chromosomally normal or balanced, which were all transferred (Table 3), and one was aneuploid for chromosome 16 (embryo 4). No unbalanced embryos were found.

Case 3 underwent two PGD cycles. For the first cycle, 24 eggs were retrieved; 18 of them were fertilized by IVF and one of these was later discarded because it had three pronuclei. Seventeen embryos were biopsied on Day 3. At the time of the biopsy five embryos had eight cells, three embryos had seven cells, one embryo had six cells, three embryos had five cells, and five embryos had four cells. FISH analysis revealed that five embryos were chromosomally normal or balanced (Table 4), of which three were transferred. Ten were considered abnormal; one cell did not show a nucleus (embryo 9) and one was normal for all chromosomes except chromosome 4 for which there were no results (embryo 6). Of the abnormal ones, one was unbalanced (embryo 11), one was polyploid and also unbalanced (embryo 15), five were aneuploid but balanced for the inversion (embryos 3, 5, 10, 12); and three were aneuploid but showed no result for the inversion (embryos 1, 13 and 16). An X-crossover was believed to have been the cause in the unbalanced ones (embryos 11 and 15).

For the second cycle of case 3, 14 eggs were retrieved, and all of them were fertilized by IVF. Later, two showed three pronuclei and were discarded and 12 embryos were biopsied on Day 3. At the time of the biopsy, three embryos had eight cells, one embryo had seven cells, two embryos had six cells, two embryos had five cells, three embryos had four cells, and one embryo had two cells. FISH analysis revealed that four embryos were chromosomally normal or balanced, of which three were transferred (Table 5). Eight were considered abnormal. Of the abnormal ones, four were unbalanced (embryos 7, 8, 9 and 12), three were aneuploid but balanced for the inversion (embryos 3, 10 and 11), and one appeared aneuploid but was also unbalanced (embryo 2). A U-crossover could have been the cause of the unbalance in four of the embryos (embryos 7, 8, 9 and 12), while an X-crossover was the cause of unbalance in embryo 2.

Results were obtained for a total of 28 embryos for case 3. Nine were balanced or normal, one was normal for aneuploidy but did not have results for the inversion, and 18 were abnormal; only seven had abnormalities related to the inversion. Altogether, results were obtained for 55 embryos, of which 27 were balanced and 31 were abnormal; only 13 had abnormalities related to the inversion (6 by X-crossover and 7 possibly by U-crossover).

Pregnancy outcome

Three embryos were transferred in each cycle. Case 1 became pregnant twice, while cases 2 and 3 did not

Table 3—PGD results for Patient 2, 46,XX,inv(2)(p11.2q13)

Embryo	Tel 2p	Tel 2q	Cen 2	LSI 13	CEP 16	CEP 18	LSI 21	LSI 22	Diagnosis
1	2	2	2	2	2	2	2	2	Normal/balanced
2	2	2	2	2	2	2	2	2	Normal/balanced
3	2	2	2	2	2	2	2	2	Normal/balanced
4	2	2	2	2	1	2	2	2	Monosomy 16

achieve any pregnancy. The first pregnancy of case 1 delivered a chromosomally normal healthy baby, and the second pregnancy is ongoing with triplets.

DISCUSSION

As shown in the present study, telomeric probes permit the analysis of any chromosome except the five acrocentric ones and this method can be successfully applied to PGD of inversions. Also, because telomeric probes are now commercially available, there is no need for the expensive and time-consuming development of breakpoint spanning or distal probes specific for each individual case. Our previous results showed that translocation cases had on average 75% unbalanced embryos (Munné *et al.*, 2000) while the present results for inversions had less than one quarter. This could be explained by the mechanism of generation of unbalanced gametes. In translocations, the generation of unbalanced gametes depends on the frequency of the types of segregation of the chromosomes implicated: alternate, adjacent I, adjacent II, 3:1. Alternate segregation produces balanced gametes, and the other segregation types produce unbalanced gametes. Normally, the sum of the frequencies of the types, which generate unbalanced gametes, is higher than the frequency of alternate segregation; so the proportion of unbalanced gametes will be higher than 50%. In addition, crossing-over within the interstitial

segment could occur producing unbalanced gametes even with alternate segregation.

However in inversions, the occurrence of uneven X-crossings-over inside the inversion loop between one normal chromatid and an 'inverted' chromatid is what produces unbalanced gametes. The percentage of unbalanced gametes produced by X-recombination alone should in theory be around 50% when X-crossing-over occurs. Another source of unbalanced gametes could be U-crossing-over, the frequency of which is unknown at the gamete level, but Pettenati *et al.* (1995) have shown an incidence of chromosome imbalance of 4% in the offspring of a paracentric inversion carrier population, so U-crossings-over could also be a frequent cause of unbalanced gametes. In the present study, 7/13 unbalanced embryos may reflect this situation. Finally, the frequency of abnormal embryos produced by the pericentric inversion will depend on the size of the inversion. If the inverted segment is very small it may not form a loop during meiosis and will not produce unbalanced gametes. For instance, Patient 2 had the smallest inversion and no unbalanced embryos. However after a certain length in which the probability of a single crossing-over is maximum, larger inversions will produce less unbalanced gametes as the probability of a double crossing-over increases within the inverted fragment. The two other inversions are about three times the length of Patient 2's inversion. If haploids and other abnormalities that prevent the assessment of the

Table 4—PGD results for Patient 3, 46,XX,inv(4)(p16q21), first cycle

Embryo	Tel 4p	Tel 4q	Cen 4	LSI 13	CEP 16	CEP 18	LSI 21	LSI 22	Diagnosis
1	NR	2	2	2	2	2	2	3	Trisomy 22
2	2	2	2	2	2	2	2	2	Normal/balanced
3	2	2	2	2	2	1	2	2	Monosomy 18
4	2	2	2	2	2	2	2	2	Normal/balanced
5	2	2	2	2	2	2	3	2	Trisomy 21
6	NR	2	2	2	2	2	2	2	NR for inversion
7	2	2	2	2	2	2	2	2	Normal/balanced
8	2	2	2	2	NR	2	2	2	Normal/balanced
9	—	—	—	—	—	—	—	—	No nucleus
10	2	2	2	3	2	2	2	2	Trisomy 13
11	1	3	2	2	2	2	2	2	Unbalanced
12	2	2	2	2	3	2	2	2	Trisomy 16
13	NR	NR	2	2	2	2	3	3	Trisomy 21 and 22
14	1	1	1	2	2	2	2	1	Monosomy 4 and 22
15	2	6	4	4	4	4	4	4	Tetraploid/unbalanced
16	NR	NR	2	2	2	1	3	3	Trisomy 21 and 22
17	2	2	2	2	2	NR	2	2	Normal/balanced

NR, No result.

Table 5—PGD results for Patient 3, 46,XX,inv(4)(p16q21), second cycle

Embryo	Tel 4p	Tel 4q	Cen 4	LSI 13	CEP 16	CEP 18	LSI 21	LSI 22	Diagnosis
1	2	2	2	2	2	2	2	2	Normal/balanced
2	1	3	2	1	2	2	1	2	Unbalanced, monosomy 13 and 21
3	2	2	2	2	2	1	1	1	Monosomy 18, 21 and 22
4	2	2	2	2	2	2	2	2	Normal/balanced
5	2	2	2	2	2	2	2	2	Normal/balanced
6	2	2	2	2	2	2	2	2	Normal/balanced
7	2	1	2	2	2	2	2	2	Unbalanced
8	1	3	1	NR	2	2	2	2	Unbalanced
9	2	1	2	2	2	2	2	2	Unbalanced
10	1	1	1	2	2	1	1	2	Monosomy 4, 18 and 21
11	2	2	2	1	4	2	2	1	Tetrasomy 16, monosomy 13 and 22
12	2	1	2	2	2	2	2	2	Unbalanced

NR, No result.

inversion are not taken into account, the inversions for Patients 1 and 3 produced 26% and 33% unbalanced embryos. Iwarsson *et al.* (1998) used single telomeric probes protocol in their study of inversions, and therefore could not detect signal patterns of U-crossings-over. If single probes had been used in the present series of tests, embryo 7 in case 1 would have appeared to be normal. However, even though Iwarsson *et al.* (1998) could not detect U-crossings-over products they reported a higher rate of unbalanced embryos (7/19) than the present study (13/55). In both studies, the numbers are extremely small, and it is unreasonable to draw broad conclusions from such small figures.

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