Preimplantation genetic diagnosis for an insertional translocation carrier

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BACKGROUND: While preimplantation genetic diagnosis (PGD) is well established for carriers of reciprocal terminal translocations, reports on PGD for insertional translocation carriers are lacking. Here, we report on the PGD of an insertional translocation carrier with karyotype 46,XX,ins(14;2)(q21;q31q35). Due to the possibility of crossovers within the inserted region, rather than a single probe, four probes are required for proper embryo selection. METHODS: Probes were generated for PGD using fluorescence in situ hybridization and two PGD cycles. RESULTS: Analysis of 10 embryos revealed four embryos to be normal diploid. Two embryos were consistent with 3:1 segregation of the theoretical quadrivalent and one was consistent with 2:2 or 1:1 segregation. Furthermore, one embryo was mosaic abnormal and one remained without diagnosis. CONCLUSIONS: With increased acceptance of PGD, it is likely that more carriers of complex translocations will enter PGD programmes. The present results suggest that a careful genetic work-up of complex translocations is essential for proper embryo selection. While theoretical modelling may predict that quadrivalents will form during the meiosis of insertional translocations, experimental proof for the occurrence of quadrivalents is still lacking and more research on the meiotic process of both female and male insertional translocation carriers is warranted.

Key words: insertional translocation/meiosis/preimplantation genetic diagnosis (PGD)/quadrivalents/reciprocal translocation

Introduction
Balanced translocations occur in 0.2% of the neonatal population. Among infertile couples and patients with recurrent abortions, however, balanced translocations occur at higher frequency. Balanced translocations were found in 0.6% of infertile couples, 3.2% of couples that failed >10 IVF cycles and 9.2% among fertile couples experiencing three or more consecutive first trimester abortions (Stern et al., 1999).

Preimplantation genetic diagnosis (PGD) can be offered to carriers of balanced translocations to reduce the risk of the unbalanced transmission of aberrant chromosomes. PGD can thus reduce the risk of chromosomally abnormal offspring, reduce the frequency of miscarriages and, as a result, increase the baby take home rate. In 2001, the ESHRE consortium reported 51 PGD cycles for carriers of Robertsonian translocations and 96 PGD cycles for carriers of balanced reciprocal translocations (ESHRE PGD Consortium Steering Committee, 2002). The large majority of reciprocal translocations are terminal translocations. It is well established that reciprocal translocations form quadrivalents during meiosis which, in turn, lead to a higher incidence of chromosomal anomalies (Goldman and Hulten, 1993; Martin and Hulten, 1993; Gersen and Keagle, 1999). Not only can the 2:2 segregants be unbalanced for the translocation chromosomes, but also a higher incidence of 3:1 segregants resulting in aneuploidy for the abnormal chromosomes is observed. PGD of such translocations is performed by fluorescence in situ hybridization (FISH), using a variety of approaches (Munne et al., 1998; Scriven et al., 1998). The most common approach is by performing interphase FISH on blastomeres. One, time-consuming, approach has been to develop specific probes spanning the breakpoints of each translocation (Munne et al., 1998; Weier et al., 1999). Whilst it may be ideal to use a different enumerator probe for each of the four translocation segments (both translocated segments and both centric segments), suitable probes labelled with appropriate fluorochromes or haptns may not be readily available. However, only enumerator probes for three of the four segments are required to detect all the possible segregation products with chromosome imbalance (Scriven et al., 1998). The use of three enumerator probes, one for each translocated segment (typically subtelomere probes) and one of the centric segments (typically a centromere probe), is recommended for single-cell biopsy because there are then two probes informative for imbalance consistent with adjacent–1 segregation and, providing the centromere probe has been selected appropriately, for potentially viable imbalance associated with 3:1 segregation (Munne et al., 1998, 2000; Pierce et al., 1998; Van Assche et al., 1999). However, successful strategies in clinical practice have also used enumeration probes for both
centric segments and one translocated segment in conjunction with 2-cell embryo biopsy (Simopoulou et al., 2003). A small minority of translocations, however, are not terminal but interstitial. Both intra- and interchromosomal insertional translocations have been reported (for a review see Van Hemel and Eussen, 2000). In the former case, only a single chromosome is involved. In the latter, an intrachromosomal fragment from one chromosome is inserted into another chromosome, thus generating one chromosome with an intrachromosomal deletion and another chromosome with an insertion. PGD for an intrachromosomal insertional translocation has been reported (Simopoulou et al., 2003). However, PGD for interchromosomal insertional translocations has, to our knowledge, not yet been reported. Here we demonstrate that in the case of an interchromosomal insertional translocation, a minimum of four probes are needed to identify all possible unbalanced events and we apply PGD on a patient with an insertion of chromosome (2)(q31 → q35) in chromosome 14q.

Materials and methods

Case report

The couple presented with one normal boy and two severely mentally retarded boys. Karyotyping of all family members was performed by G-banded metaphase spreads of cultured lymphocytes using conventional methods. The mentally retarded boys were shown to carry an abnormal karyotype with trisomy for the inserted segment 2q31 → 2q35. Revision of the translocation carriers’ karyotype pinpointed the breakpoint in 2q31 rather than 2q32 as published previously (Lukusa et al., 1999). The karyotype of the mother was 46,XX,ins(14;2)(q21;q31q35) (Figure 1), showing that the abnormal chromosome in the boys was the result of the transmission of a rearranged maternal chromosome. The patient thus carries an insertional translocation of part of the long arm of chromosome 2 (2q31 → 2q35) inserted in the long arm of chromosome 14q21. This chromosomal segment corresponds to 1.46% haploid autosomal length (HAL). Prenatal diagnosis during a fourth pregnancy was performed by combining Spectrum orange, Spectrum green, Spectrum red (Vysis, Abbott laboratories, IL) and diethylaminocoumarin-5-dUTP (DEAC) (Perkin Elmer Life Sciences). Two clones of the same chromosomal regions were mixed to obtain strong fluorescent signals. A probe within the translocated region was obtained by using a non-contact, 1.48 μm diode laser system (Fertilase®, MTG, Germany) coupled to the inverted microscope. Two blastomeres were gently aspirated from each embryo. The embryos were immediately transferred to fresh medium. Following biopsy, a single blastomere was washed twice with culture medium to get rid of possible oil remnants. Next a 2 μL drop of 0.01 mol/l HCl with 0.1% Tween-20 was placed on a Superfrost plus microscope slide (LaboNord). The cell was transferred to this solution using a small glass pipette. Following lysis of the cell, the nucleus was allowed to dry and became fixed on the glass slide. Finally slides were washed in 1 × phosphate-buffered saline (PBS) for 5 min and dehydrated by sequential washing in 70, 90 and 100% ethanol, respectively. Fixed nuclei were pre-treated for 20 min with 0.5 mg pepsin/ml (Sigma) in 0.01 mol/l HCl at 37°C followed by a 3 min wash in 1 × PBS. Post-fixation was performed by incubating the slides for 10 min in a 1% formaldehyde solution with 0.05 mol/l MgCl2 and 1 × PBS at 4°C. Slides subsequently were washed in 1 × PBS and dehydrated by ethanol series.

Fluorescence in situ hybridization

BAC/PAC DNA was isolated by Nucleobond AX (Machery-Nagel, Düren, Germany) and the DNA was directly labelled by the Random Prime Labeling System (Invitrogen, Carlsbad, CA). Different fluorochromes used were Spectrum orange, Spectrum green, Spectrum red (Vysis, Abbott laboratories, IL) and diethylaminocoumarin-5-dUTP (DEAC) (Perkin Elmer Life Sciences). Two clones of the same chromosomal regions were mixed to obtain strong fluorescent signals. A probe within the translocated region was obtained by using a non-contact, 1.48 μm diode laser system (Fertilase®, MTG, Germany) coupled to the inverted microscope. Two blastomeres were gently aspirated from each embryo. The embryos were immediately transferred to fresh medium. Following biopsy, a single blastomere was washed twice with culture medium to get rid of possible oil remnants. Next a 2 μL drop of 0.01 mol/l HCl with 0.1% Tween-20 was placed on a Superfrost plus microscope slide (LaboNord). The cell was transferred to this solution using a small glass pipette. Following lysis of the cell, the nucleus was allowed to dry and became fixed on the glass slide. Finally slides were washed in 1 × phosphate-buffered saline (PBS) for 5 min and dehydrated by sequential washing in 70, 90 and 100% ethanol, respectively. Fixed nuclei were pre-treated for 20 min with 0.5 mg pepsin/ml (Sigma) in 0.01 mol/l HCl at 37°C followed by a 3 min wash in 1 × PBS. Post-fixation was performed by incubating the slides for 10 min in a 1% formaldehyde solution with 0.05 mol/l MgCl2 and 1 × PBS at 4°C. Slides subsequently were washed in 1 × PBS and dehydrated by ethanol series.

Stimulation and embryo biopsy

Two PGD/IVF cycles were performed. Therefore, the patient was treated with HMG (Menopur®, Ferring, Germany); the follicular response was monitored by regular gynaecological ultrasound measurements and peripheral blood measurements for estradiol; ultrasound-guided oocyte aspiration was carried out 36 h after i.m. injection of 10 000 IU of HCG (Fregyn®. Organon, Oss, The Netherlands). During the first and second cycle, 10 and 17 oocytes, respectively, were retrieved and fertilized with conventional IVF, resulting in seven and 10 fertilized oocytes. All embryos were cultured in sequential media (Medicult, Lucron, Denmark). On day 3 after oocyte aspiration, all embryos with ≥6 blastomeres were biopsied using a non-contact, 1.48 μm diode laser system (Fertilase®, MTG, Germany) coupled to the inverted microscope. Two blastomeres were gently aspirated from each embryo. The embryos were immediately transferred to fresh medium. Following biopsy, a single blastomere was washed twice with culture medium to get rid of possible oil remnants. Next a 2 μL drop of 0.01 mol/l HCl with 0.1% Tween-20 was placed on a Superfrost plus microscope slide (LaboNord). The cell was transferred to this solution using a small glass pipette. Following lysis of the cell, the nucleus was allowed to dry and became fixed on the glass slide. Finally slides were washed in 1 × phosphate-buffered saline (PBS) for 5 min and dehydrated by sequential washing in 70, 90 and 100% ethanol, respectively. Fixed nuclei were pre-treated for 20 min with 0.5 mg pepsin/ml (Sigma) in 0.01 mol/l HCl at 37°C followed by a 3 min wash in 1 × PBS. Post-fixation was performed by incubating the slides for 10 min in a 1% formaldehyde solution with 0.05 mol/l MgCl2 and 1 × PBS at 4°C. Slides subsequently were washed in 1 × PBS and dehydrated by ethanol series.

Figure 1. A partial ideogram and karyotype showing the normal and aberrant chromosomes 2 and 14.
orange-labelled PAC 278P6 and PAC 146O2 (2q12.13) (Nothwang et al., 1998) and combining DEAC-labelled RP11-367B19 and RP11-680O16 (2q37) (CHORI, Oakland, CA). For the detection of chromosome 14, the telomeric clone CTC-820M16 was labelled with Spectrum red.

To prepare the final probe mixture, the four different probes were combined and dissolved in hybridization mix containing 50% formamide, 2× SSC and 10% SDS. Then 1 μl of probe was applied to the slide, covered with a coverslip (diameter of 10 mm) and sealed with rubber cement. Nuclei and probe were denatured simultaneously on a hot plate at 75°C for 5 min. Hybridization was allowed to take place overnight in a humid chamber at 37°C. After hybridization, excess or non-specific bound probe was removed by subsequent washes in 0.4× SSC/0.3% NP-40 (73°C for 2 min), 2× SSC/0.1% NP-40 (room temperature for 1 min) and 2× SSC (room temperature for 1 min) followed by dehydration through ethanol series. After drying, the slides were mounted in Vectashield anti-fading medium (Vector Laboratories, Peterborough, UK) containing 2.5 ng/ml 4′,6-diamidino-2-phenylindole (DAPI; Boehringer Ingelheim GmbH, Germany). Nuclei were examined using an Axioplan 2 microscope (Zeiss NV, Zaventem).

The quality of the probe mixture was tested on nuclei derived from stimulated blood lymphocytes from both parents. In 100 nuclei, the number of signals for each of the four probes was counted. Individual probe analysis showed 98, 99, 97 and 95% efficiencies for probes for 2q12, 2q33, 2q37 and 14qter, respectively. In combination, two signals were detected for all four probes in 91% of the nuclei.

Results

Genetics of a balanced insertional translocation

Little experimental evidence exists on the meiotic behaviour of insertional translocations, but a theoretical analysis shows that at least 26 possible segregants can result from the meiotic process. If the inserted segment is relatively small, the involved chromosomes will pair independently and form bivalents. The inserted segment will loop out and a normal segregation will result in four different gametes (Figure 2). Non-disjunction would lead to segmental nullisomic or disomic gametes. However, the incidence of such non-disjunction events is likely to be similar to the incidence of

Figure 2. Rearranged chromosomes can form bivalents during meiosis. The grey line indicates chromosome 14 while the black line indicates chromosome 2. (A) Schematic representation of the diental meiotic pairing of the rearranged chromosomes. (B) Possible gametes resulting from 2:2 segregation.

Figure 3. Rearranged chromosomes may form a quadrivalent during meiosis. A crossover can occur within the translocated segment. The grey line indicates chromosome 14 while the black line indicates chromosome 2. (A) Schematic representation of quadrivalent homologous pairing and, within the box, the products resulting from a crossover within the translocated segment. (B–D) The possible segregants following, respectively, 3:1 segregation without crossover in the translocated segment, and 2:2 and 3:1 segregation with crossover in the translocated segment.
non-disjunction in normal chromosomes. Therefore, we do not include this possibility in the genetic work-up.

If, however, the inserted fragment is large (e.g. >1.5% HAL), a quadrivalent with a loop might be formed allowing complete pairing of the chromosomes involved in the rearrangement (Figure 3) (Van Hemel and Eussen, 2000; Gardner and Sutherland, 2004). A crossover can thus occur within the insertion loop and the recombinant chromosomes will result in unbalanced gametes. Six possible segregants can result from the 2:2 segregation (Figure 3C) and eight possible segregants can result from the 3:1 segregation (Figure 3D). The 3:1 segregation without crossing over in the translocated segment can produce another eight segregants (Figure 3B).

The genetic analysis shows that at least four different probes are required to identify all possible unbalanced embryos (Figure 4). Besides one locus-specific probe within the translocated region, two terminal probes and one centromeric probe also are necessary.

### Results of PGD analysis

In two cycles, 10 embryos were analysed for four different chromosomal regions (2q12.13, 2q33, 2q37 and telomere 14q) (Table I). During the first cycle, all four embryos were

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normal diploid for the investigated regions (Figure 5B). During the second cycle, five embryos were found to be unbalanced and for one embryo no diagnosis could be made. Embryo 2-E1 shows in a single cell three, two, two and three signals for regions 2q12, 2q33, 2q37 and 14qter, respectively (Figure 5C). Embryo 2-E2 shows a single blastomere with two, two, one and two signals in the respective regions. Both these observations fit with a 3:1 segregation of the quadrivalent in which a crossover took place. Embryo 2-E3, a single blastomere, presented with a single signal in 2q33 and two signals for the other regions which can be explained by a 2:2 segregation of the quadrivalent or by the segregation of a bivalent with transmission of the unbalanced chromosome. Finally, embryo 2-E4 was mosaic and 2-E8 presented with signals that are not represented in the spotograms of Figure 2. All results are shown in Table II. Unfortunately, the remaining cells from the embryos were not available for further confirmatory analysis.

In the first cycle, one embryo with normal chromosomal diagnosis was transferred at the morula stage on day 5. The other three embryos were frozen. No pregnancy was obtained in the first cycle. In the second cycle, no embryo was transferred since no chromosomally normal embryo was available. After thawing of the three frozen embryos, the patient also received an early blastocyst in a thawing cycle. Unfortunately, no pregnancy was obtained.

**Discussion**

A theoretical total of 10 2:2 segregants and 16 3:1 segregants can result from an insertional translocation. We demonstrate that, if the breakpoint sites are unknown, four different
probes are needed to identify all abnormal segregants unequivocally. We have used this approach to determine the chromosome composition of the blastomeres from embryos derived from an interstitial translocation carrier. Another approach to detect all abnormal segregants could be to generate breakpoint-spanning probes, one on each derivative chromosome. However, such an approach requires the identification of the rearrangement sites, which is a time-consuming approach.

In total, we analysed 10 embryos that reached the 6-cell stage at day 3. Two cells were biopsied. For six out of 10 embryos, only one nucleus was present and available for analysis. Four embryos appeared balanced for the chromosome aberration and five embryos were unbalanced. One embryo was without diagnosis. From the unbalanced embryos, two (embryos 2-E1 and 2-E2) were consistent with the fertilization of a 3:1 segregant, one (embryo E-E3) was consistent with the fertilization of the meiotic product of a bivalent or a 2:2 segregant, one is mosaic (embryo 2-E4) and one (embryo 2-E8) was not consistent with any of the potential segregants of our scheme (Table II). Embryo 2-E4 is a mosaic embryo with one cell with three signals for 2q33 and two signals for 14qter and a second blastomere presenting with two signals for 2q33 and a single signal for 14qter. The simplest explanation would be that the embryo contained the der(14) which is lost by a non-disjunction event in the other cell. Embryo 2-E8 presented with two similar cells containing a single signal for both 2q33 and 2qter. Such a chromosomal constitution cannot be explained by non-disjunction events, but may reflect the presence of a terminal 2q deletion. Equally, we cannot exclude that the abnormal blastomeres with a chromosome constitution consistent with 3:1 segregation are actually more complex karyotypes resulting from additional aneuploidies for chromosomes 2 and 14 in the sperm or oocyte and/or, considering that the combined probe efficiency is 91%, that some apparent imbalances are actually due to technical artefacts. In addition, the two affected boys and single fetus carrying the der(14) chromosome provide additional insights in the meiotic process. Their karyogram is most probably the result of a bivalent or quadrivalent 2:2 segregation during maternal meiosis with transmission of the der(14), but without crossovers in the translocated segment.

While the meiotic intermediates and the resulting segregants of the meiosis of terminal translocation carrier is well documented (Goldman and Hulten, 1993; Martin and Hulten, 1993), little is known about the meiotic intermediates of insertion translocations. It is generally assumed that such chromosomes can form quadrivalents (Gersen and Keagle, 1999). However, no direct visualization of such quadrivalents during meiosis has been documented. Goldman et al. (1992) have investigated both the meiotic process and the sperm composition in a male insertion translocation carrier. Electron microscopic investigations of the meiotic pairing did not show the expected ‘hairpin’ loops in any of the 54 pachytene spermatocytes investigated, nor was any quadrivalent visible. Upon sperm karyotyping, approximately half were balanced and half were unbalanced, indicating a normal transmission ratio. Based on current knowledge of the meiotic process, it is not obvious that such quadrivalents will form. The meiotic homologue pairing appears to start at the telomeres during the so-called bouquet phase (Scherthan, 2001). Subsequently, the two homologues synapse along their lengths. While it is obvious that such a process will result in quadrivalents for terminal translocations, the pairing of insertion translocations may not be necessary for the meiotic process to proceed. However, recently it was shown that telomere-independent pairing of chromosomes does occur. Artificial ring chromosomes—which do not carry any telomeres—can pair during meiosis and cross over (Voet et al., 2003). Although quadrivalents have not been visualized, evidence that recombination does occur is provided by the recombinaton pattern in families transmitting insertion translocations. Three different case reports describe insertion translocation carriers with offspring carrying unbalanced chromosomes apparently as a result of recombination within an insertion translocation (Jalbert et al., 1975; Romain et al., 1990; Boyd et al., 1995). The ability of quadrivalents to form is likely to be dependent on the size of the translocated segment. In an overview of all reported cases of interchromosomal insertions, it was noted that in all three case reports of likely recombination, the segmental translocation is relatively large with a HAL > 1.5% (Van Hemel and Eussen, 2000) Also in the present case, the HAL is ~ 1.5%.

Whether or not quadrivalents are formed and, if so, whether size constraints of the insertion on the formation of quadrivalents exist and whether there is an increased incidence of 3:1 segregations are more than theoretical questions for the PGD community. With the increased acceptance of PGD, it is likely that more carriers of complex translocations will enter PGD programmes. The results presented here suggest that quadrivalents do form and, hence, considerably more care should be taken and a larger effort to generate the relevant FISH probes is needed. If there would be no increased incidence of 3:1 segregation of the quadrivalents, three probes would be sufficient to detect all possible segregation products. If, however, 3:1 segregation without crossover in the insertion translocated segment is also taken into account, four probes are required to detect all possible segregants. Therefore, more research to understand the behaviour of such rare complex chromosomes during meiosis and the outcome of meiosis is warranted.

References


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