Preimplantation genetic diagnosis of a reciprocal translocation t(3;11)(q27.3;q24.3) in siblings

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Preimplantation genetic diagnosis (PGD) was performed in two couples to avoid chromosomally unbalanced progeny in a family in which a brother and a sister carry an identical maternally inherited balanced translocation t(3;11)(q27.3;q24.3). Embryos were biopsied 3 days after fertilization and blastomeres were analysed by fluorescent in-situ hybridization (FISH). Embryos were classified as unbalanced or normal/balanced. In the first case, the male carrier and his wife underwent one IVF/PGD treatment cycle. In all, 18 embryos were analysed. Of those, 15 revealed an unbalanced karyotype. For one embryo, results were not conclusive, from one embryo results were contradictory and one embryo was classified as normal/balanced and subsequently transferred. A singleton pregnancy was achieved. The PGD analysis was confirmed at 16 weeks gestation by amniocentesis. At term, a healthy girl with a balanced karyotype was born. Pregnancy and delivery were without complications. In the second case, the female carrier and her husband underwent two IVF/PGD treatment cycles. During the first cycle, three embryos were analysed. One embryo revealed an unbalanced karyotype and two embryos were designated a normal/balanced karyotype and transferred but no pregnancy was achieved. During the second PGD cycle three embryos were analysed. Of those, none appeared suitable for transfer. The couple decided not to undergo further treatment. Our results indicate that for individuals carrying a reciprocal translocation PGD is a feasible approach to obtain embryos with a normal chromosome balance and to avoid both spontaneous and induced abortion.

Key words: fluorescent in-situ hybridization/preimplantation genetic diagnosis/single cell diagnosis/translocation

Introduction

Preimplantation genetic diagnosis (PGD) offers selective transfer of unaffected embryos to couples at high risk of transmitting inheritable disease to their children. So far, PGD using fluorescent in-situ hybridization (FISH) has mainly been used for sex determination in case of X-linked disease (Coonen et al., 1996a,b; Harper 1996; Staessen et al., 1996; Delhanty 1997). Furthermore, FISH is used for preconception or preimplantation aneuploidy screening of oocytes or embryos retrieved during the course of an IVF cycle in case of elevated maternal age (Munné et al., 1995a,b; Verlinsky et al., 1996). More recently, FISH has been used to detect unbalanced segregation in preimplantation embryos due to parental translocations (Conn et al., 1998; Munné et al., 1998a,b; Pierce et al., 1998; Van Assche et al., 1999).

Structural chromosomal aberrations play an important role in the majority of cytogenetic abnormalities with a high recurrence risk. Couples in whom one of the partners carries a balanced translocation are known to be at risk of having an affected pregnancy. The actual risk depends on the chromosomes involved in the translocation, the position of the breakpoints and the sex of the translocation carrier (Goldman and Hulten, 1992, 1993). Moreover, the likelihood of unbalanced progeny due to a parental reciprocal translocation depends on both the proportion of chromosomally unbalanced gametes that is being produced and the phenotypic effects of this particular imbalance upon the zygote (Goldman and Hulten, 1992, 1993).

General information on the latter is usually available from empirical data but details on the segregational properties and gamete output of translocations are more elusive (Jalbert et al., 1980; Martin and Hulten, 1993). Apart from the increased risk of producing genetically abnormal offspring, translocation carriers may have an increased risk of spontaneous abortions. Instead of natural conception, using PGD to select for normal or balanced embryos might be a preferable way to achieve an ongoing pregnancy or to avoid recurrent abortions following prenatal diagnosis in case of an unbalanced fetus.

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Materials and methods

At the time of PGD treatment, the proband was a 33 year old male carrying a reciprocal translocation t(3;11)(q27.3;q24.3). Cytogenetic pedigree studies revealed that his sister, mother and maternal grandmother were also carriers of the same translocation. Both brother and sister with their respective partners attended our clinic for PGD treatment.

Case 1

The proband’s wife had at least seven early spontaneous miscarriages, probably representing non-viable recombinants. During the fifth pregnancy, an amniocentesis was performed at 16 weeks gestation. Cytogenetic analysis showed an unbalanced karyotype: 46,XX,–11,+der(11),t(3;11)(q27.3;q24.3), resulting in partial monosomy 11q and partial trisomy 3q. A termination of pregnancy followed on request of the parents. A stillborn girl with multiple congenital anomalies was delivered.

In order to anticipate on the possible chromosome combinations encountered in pre-implantation embryos, the proband’s spermatozoa were analysed with the same combination of probes used for PGD (Martini et al., 1998). Of the spermatozoa, ~45% were found to originate from an alternate segregation mode, thus having a normal or balanced chromosomal constitution.

Case 2

The proband’s younger sister had at least six early spontaneous abortions. Her third pregnancy ended at 24 weeks gestation with the birth of an hydropic fetus (Willekens et al., 1994). Analysis of abortion material revealed an unbalanced karyotype: 46,XX,–11,+der(11),t(3;11)(q27.3;q24.3). For obvious ethical reasons, no oocytes were collected prior to the PGD treatment to study their genetic content with respect to chromosomes 3 and 11.

The reproductive history of the mother and maternal grandmother was unremarkable, with no miscarriages or stillbirths known.

IVF and biopsy procedures

The stimulation protocol used has been described previously (Land et al., 1996). IVF and embryo culture were performed as described earlier (Dumoulin et al., 1999). Following ovarian stimulation, follicles were aspirated and fertilization was evaluated 22 h after insemination. Developmental stage and morphology of all embryos were recorded once daily. Embryos were biopsied on day 3 after insemination. Developmental stage and morphology of all embryos were recorded once daily. Embryos were biopsied on day 3 after insemination.

Blastomere spreading

After biopsy, blastomeres were washed in culture medium and subsequently spread on Super Starfrost Plus® slides (Maenzel Glaeser, Braunschweig, Germany) using a solution of 0.01 N HCl/0.1% Tween20 (Coonen et al., 1994a). Slides were left to air-dry, washed in phosphate-buffered saline (PBS) and further treated for FISH analysis (see FISH procedure).

Lymphocyte and fibroblast preparation

Interphase nuclei from methanol–acetic acid (3:1) fixed preparations of lysed peripheral blood cells obtained from both translocation carriers and their healthy partners were used to test the efficiency and specificity of DNA probes, probe labelling and FISH procedure and to check for possible probe polymorphisms. Furthermore, ethanol-fixed fibroblasts obtained from the proband’s unbalanced aborted child were used as test material. Lymphocytes of a healthy individual were used as a control.

Spare embryos

Spare embryos used for probe efficiency testing were donated by couples undergoing routine IVF for infertility treatment. All had given written informed consent for their embryos to be used for research purposes. This study was approved by the Research Ethics Committees of the Academic Hospital Maastricht and the Maastricht University.

DNA probes

Three different DNA probes were used to study the translocation of interest: (i) CEP 11 probe (Vysis, Inc., Downers Grove, IL, USA), specific for repetitive sequences within the centromeric region of chromosome 11, labelled with Spectrum Orange® and Spectrum Green®; (ii) D11S-477 cosmide probe (insert size ~40 kb), specific for a unique sequence located at position 11q25 (Tokino et al., 1991) (remapped-personal observation) and labelled by nick-translation with digoxigenin-12-dUTP (Boehringer Mannheim, Mannheim, Germany); (iii) TYAC162 YAC probe (insert size ~250 kb), specific for a unique sequence located at position 3q29 (Vocero-Akbani et al., 1996) and ALU–polymerase chain reaction (PCR) labelled with biotin-11-dUTP (Enzo Diagnostic, New York, USA).

FISH procedure

The FISH pre-treatment procedure was performed as described previously (Coonen et al., 1994a,b) with minor modifications. In summary, after blastomere spreading and washing of slides in PBS, slides were incubated for 10 min at 37°C with pepsin (Sigma, St Louis, MO, USA; 100 μg/ml in 0.01 N HCl) to increase the accessibility of nuclei for hybridization to the probes. After incubation, slides were rinsed in PBS and nuclei were fixed by incubation in 1% paraformaldehyde/0.1 mol/l PBS (pH 7.3) for 5 min at 4°C. Finally, slides were rinsed once more in PBS, dehydrated through an ascending ethanol series and air-dried.

Multi-target FISH was performed using the probe cocktail described under DNA probes. This probe mixture allowed discrimination between normal/balanced interphase nuclei and unbalanced nuclei (Martini et al., 1998).

Alternate segregation of quadrivalents during gametogenesis in the translocation carrier would lead to the production of normal or balanced gametes and, after fertilization of a normal gamete from the healthy partner, give rise to normal or balanced embryos. On a single segregation, the production of normal or balanced gametes and, after fertilization of a normal gamete from the healthy partner, give rise to normal or balanced embryos.
Preimplantation genetic diagnosis of a reciprocal translocation

Figure 1. (A) Schematic drawing of the chromosomes involved in the translocation (3;11)(q27.3;q24.3) and the DNA probes used. (B) Multi-target fluorescent in-situ hybridization (FISH) on peripheral blood cells of a healthy individual, showing two hybridization signals for the CEP 11 probe (orange), TYAC162 probe (green) and D11S-477 probe (red).

Figure 2. Multi-target fluorescent in-situ hybridization (FISH) on human blastomeres from preimplantation genetic diagnosis (PGD) case 1 (a–c) and PGD case 2 (d), showing a normal/balanced karyotype (a) and an unbalanced karyotype (b–d) respectively. (a) Normal/balanced karyotype, reflected by two hybridization signals for the CEP 11 probe (orange), the TYAC162 probe (green) and the D11S-477 probe (red). (b) Unbalanced karyotype, reflected by two hybridization signals for the CEP 11 probe (orange), two signals for the TYAC162 probe (green) and one for the D11S-477 probe (red). (c) Unbalanced karyotype, reflected by two hybridization signals for the CEP 11 probe (orange), three signals for the TYAC162 probe (green) and one for the D11S-477 probe (red). (d) Unbalanced karyotype, reflected by two hybridization signals for the CEP 11 probe (orange), three signals for the TYAC162 probe (green) and none for the D11S-477 probe (red).

ascending ethanol series and air-dried. Slides were kept at room temperature until further use.

The probe hybridization mixture containing 60% FA/2×SSC/10% dextran sulphate, 10 ng/µl TYAC162-digoxigenin, 20 ng/µl D11S-477-biotin, 1 ng/µl CEP 11- Spectrum Orange®, 1 ng/µl CEP 11- Spectrum Green®, 2 µg/µl salmon testis DNA and 1 µg/µl human Cot DNA (Gibco BRL, Life Technologies, Breda, The Netherlands) was heat-denatured at 70°C for 5 min and pre-annealed at 37°C for 20 min. Blastomere slides were then pre-warmed and 10 µl of hybridization solution was applied under a coverslip (18×18 mm). Slides were placed in a moist chamber and hybridization was allowed to take place overnight.

Post-hybridization washes were carried out twice for 5 min at 42°C with 2× SSC/0.05% Tween20 followed by two washes, for 5 min each, at 60°C with 0.1× SSC and finally twice for 5 min with 4× SSC/0.05% Tween20 at room temperature.

Preceding the detection of indirectly-labelled probes, slides were incubated for 15 min in 5% non-fat dry milk (NFDM)/4× SSC at room temperature. The biotin-labelled probe was detected by 20 min incubation with avidin–fluorescent isothiocyanate (FITC) (Vector
**Table I.** Fluorescent in-situ hybridization (FISH) results of biopsied blastomeres and non-transferred preimplantation embryos from preimplantation genetic diagnosis (PGD) case 1

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Blastomere</th>
<th>FISH results (nr signals)</th>
<th>Blastomere karyotype</th>
<th>Embryo transfer</th>
<th>Embryo culture day 6</th>
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<td>na</td>
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<td>no</td>
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</tr>
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</table>

na = not analysable.

*a* Multinucleated blastomere; *b* In case of conflicting FISH results, the embryo was considered not suitable for transfer.

**Results**

**FISH results on lymphocytes and fibroblasts**

Extensive testing of probes was performed previously (Martini *et al*., 1998). Of the 600 control and carrier lymphocytes that were tested, 97% displayed the expected number of signals. Using the conditions as described above, the hybridization efficiency of the centromeric, cosmid and YAC probe was optimal while aspecific binding of the probes was limited. Lymphocytes of both translocation carriers and their partners were used as test material. FISH results revealed clear signal patterns, allowing a reliable diagnosis (Figure 1b). No hybridization polymorphisms were detected. FISH analysis of the fetal fibroblasts clearly showed the unbalanced karyotype.

**PGD results case 1**

The proband and his wife underwent one IVF/PGD treatment cycle. Of the 20 oocytes that were retrieved, 19 showed signs of normal fertilization (2PN). In all, 18 embryos were biopsied and all biopsied blastomeres (n = 28) were analysed by means of FISH. On the basis of the FISH results, 15 embryos were thought to have an unbalanced karyotype, in one case FISH results were not conclusive (Table I, embryo 14), from one
embryo results were contradictory (Table I, embryo 10) and one embryo was found to have a normal/balanced karyotype (Table I, embryo 13) (Table I; Figure 2a,b,c). The supposedly normal/balanced embryo was transferred. Raised HCG concentrations were detected 14 days after transfer and ultrasonography revealed the presence of a single fetus with heart activity at 7 weeks gestation. Amniocentesis was performed at 16 weeks gestation and cytogenetic analysis revealed a balanced karyotype. The balanced fetal karyotype was confirmed by means of FISH, using whole chromosome paints for the chromosomes 3 and 11 (Cambio) (Figure 3a,b). A healthy girl with a balanced karyotype was born at term. Pregnancy and delivery were without complications.

Embryos that were not transferred were kept in culture until day 6 post-fertilization and analysed using FISH (Table I). Results of the primary analyses were confirmed in nine cases initially classified as unbalanced. FISH analysis showed a uniform unbalanced pattern in four embryos (44%), three embryos were mosaics (33%) and two embryos (22%) showed a chaotic chromosome pattern. In six cases, no material could be analysed due to in-vitro degradation (Table II, embryo 3). The embryo with the non-conclusive results no material could be analysed due to in-vitro degradation was not transferred and remained in culture until day 6 post-fertilization and was analysed by FISH. The karyotype of the two embryos initially classified as unbalanced was confirmed. From the embryo with the non-conclusive results no material could be analysed due to in-vitro degradation (Table II, embryo 3).

The one embryo that was not transferred remained in culture until day 6 post-fertilization and was analysed by FISH. Results of the FISH analysis confirmed the unbalanced genetic constitution of the embryo (Table II, embryo 3).

During the third PGD cycle, nine oocytes were retrieved of which eight showed signs of normal fertilization. Three embryos were biopsied and all biopsied blastomeres (n = 5) were analysed by means of FISH. On the basis of FISH results, two embryos were concluded to have an unbalanced karyotype and in the third embryo FISH results were not conclusive (Table II). No embryo transfer could be performed and the couple decided not to undergo further treatment. All three embryos remained in culture until day 6 post-fertilization and were analysed by means of FISH. The karyotype of the two embryos initially classified as unbalanced was confirmed. From the embryo with the non-conclusive results no material could be analysed due to in-vitro degradation (Table II, embryo 3).

**Discussion**

Structural aberrations are less frequently involved in chromosomal abnormalities than numerical abnormalities. However, structural aberrations mainly run in families. On the one hand this lays a heavy burden on the family. On the other hand the presence of unbalanced offspring can be detected and the birth of another unbalanced child prevented by prenatal diagnosis and selective abortion. It is the purpose of this paper to show that PGD is a realistic alternative. Furthermore, if the translocation results in recurrent abortion, hyperstimulation, IVF and PGD are alternatives to achieve an ongoing pregnancy.

**PGD case 1**

On the basis of FISH analysis of sperm cells it was expected that less than half of the oocytes would be fertilized by genetically normal/balanced spermatozoa, resulting in genetically normal/balanced embryos respectively. However, the majority of embryos analysed during PGD treatment (15 out of 18) was found to have an unbalanced genetic content. The fact that it was not possible to relate the genetic content of the spermatozoa to their fertilizing ability, might account for
the remarkably different percentages of abnormalities found between the spermatozoa and the preimplantation embryos. On the basis of sperm cell analyses there seemed to be a preference for adjacent I and, to a lesser extend, adjacent II segregation (Martini et al., 1998). This phenomenon was not observed in the preimplantation embryos.

The high percentage of unbalanced embryos indicates the usefulness of PGD, as it will increase the chance of obtaining an embryo with a normal/balanced karyotype, ultimately leading to a pregnancy and birth of a child with a normal/balanced karyotype. On the basis of the DNA probes used in this study, one cannot differentiate between embryos with a balanced or a normal genetic constitution. However, in the vast majority of translocation cases either karyotype would result in a normal phenotype.

**PGD case 2**

In both treatment cycles, the number of embryos obtained was quite low. However, FISH results show that the distribution of balanced/normal and unbalanced embryos varied (but was not statistically different) from that observed in PGD case 1. This might indicate that the sex of the translocation carrier is a factor influencing the gamete output. As holds true for PGD case 1, no preference for either adjacent I or adjacent II segregation was observed. All fully informative blastomeres seemed to have resulted from a 3:1 segregation mode.

**FISH results**

The presence of structural aberrations in embryonic nuclei may be examined by means of conventional karyotyping, but this method is time consuming, laborious and usually inefficient. By introducing the FISH technique to detect structural aberrations in embryonic nuclei, these problems can be overcome.

Obviously, there is a need for the application of DNA probes that enable detection of changes in organization and topography of unique DNA sequences. A reliable detection of structural chromosomal aberrations at the single cell level requires strict criteria for interpretation of FISH signals (Cremer et al., 1988; Hopman et al., 1988; Matsumura et al., 1992; Selig et al., 1992). Thus, an accurate FISH analysis is accomplished by thoroughly investigating the hybridization characteristics (efficiency, signal appearance, polymorphism) of each probe before applying it in PGD. Therefore, pre-clinical testing should involve analysis of different cell types (lymphocytes, fibroblasts, blastomeres) of diverse genetic content (normal, balanced, unbalanced).

Various types of probes can be used to identify structural aberrations in interphase cells (Lichter et al., 1991; Tkachuk et al., 1991): (i) whole chromosome painting probes or region-specific probes that cover a certain chromosome area show so-called hybridization domains in interphase cells after FISH. Estimation of the number of nuclear domains can be problematic, due to domain overlap (Coonen et al., 1998); (ii) structural aberrations can also be visualized with the use of cosmid probes, which have a restricted target area, allowing high topographical resolution of FISH signals. The FISH approach described here is applicable to efficiently detect the defined structural aberration on a single cell basis. However, as in this case, the structural aberration under investigation will usually represent a chromosomal rearrangement that is unique for a limited number of individuals. Therefore, we expect that the procedures for the detection of structural rearrangements at a preimplantation stage in single blastomeres will have to be tailored to each specific diagnostic problem. In this respect, the development of a general approach, applicable to each or most of the individual cases, would greatly improve the introduction of this type of analyses in ‘routine’ PGD. It was shown that, when retrieved within a time course of 6 h, first polar bodies are suitable for PGD analysis in case of female carriers of structural abnormalities and maternally inherited aneuploidy (Munné et al., 1995b, 1998b). We have suggested that the DNA of these first polar bodies exhibits several levels of condensation, ranging from separate chromosome-like structures to an interphase-like organization (Coonen et al., 1996c). Efficient preparation of polar bodies to obtain a chromosome-like configuration of their DNA should allow PGD detection of structural aberrations when using painting probes. This procedure provides a general approach to detect maternally inherited structural aberrations at a preconception stage.

In PGD, the availability of highly specific DNA probes located at both sides of a translocation breakpoint or spanning it, is a prerequisite for the differentiation between gametes or

**Figure 3.** Fluorescent in-situ hybridization (FISH) on cultured amniocytes from preimplantation genetic diagnosis (PGD) case 1, using whole chromosome painting probes specific for the chromosomes 3 (top) and 11 (bottom), showing the balanced karyotype of the fetus.
embryos with a balanced/normal and unbalanced genotype in case of a reciprocal translocation (Tkachuk et al., 1991; Cassel et al., 1997).

Unfortunately, the nuclear organization of the interphase nucleus makes it unreliable if not impossible to differentiate between embryos with a balanced and normal genotype. Because in most cases both the balanced as well as the normal karyotype will result in a normal phenotype, the lack of differentiation between balanced and normal karyotype is not a clinical issue. It should be mentioned however, that balanced offspring might in future suffer from the same genetic and/or reproductive problems as their parents did. In view of this, the availability of gamete (polar body) or embryonic metaphase chromosomes for PGD analysis would be a step forward (Clouston, 1997). However, the efficiency and reliability of single cell culture and metaphase chromosome induction is too low for clinical application (Santalo et al., 1995; Willadsen et al., 1999). As long as the embryonic material available for PGD analysis is restricted to interphase nuclei, and the only possibility is to differentiate between embryos with a normal/ balanced and an unbalanced genotype, the chromosomal distance from the DNA probe used for PGD analysis and the translocation breakpoint is rather irrelevant. This would imply that long or short chromosome arm-specific telomere probes cover a wide range of different translocations. The availability of such probes will in the near future greatly simplify the approach to detect structural aberrations in PGD (Scriven et al., 1998).

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