First preimplantation genetic diagnosis of hereditary retinoblastoma using informative microsatellite markers

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Retinoblastoma is a malignant intra-ocular tumour of developing retina initiated by inactivation of both alleles of the retinoblastoma susceptibility (RB1) gene. This paper reports the first clinical experience of preimplantation genetic diagnosis (PGD) for hereditary retinoblastoma using two highly polymorphic microsatellite markers RB1.20 and D13S284, located within and close to the RB1 gene respectively. Duplex PCRs were tested on more than 300 single lymphocytes from heterozygous individuals at both loci, in order to test the accuracy and reliability of the single-cell protocol. This procedure requires a nested PCR and the analysis of fluorescently labelled PCR products on an automatic DNA sequencer. Amplification efficiency and allele drop-out rates ranged from 96.7 to 98.4%, and 3.7 to 5.4% respectively. This test was found to be accurate and reliable enough to be applied to the study of human blastomeres. Subsequently, this approach was used in a PGD treatment cycle for a couple who already had a child affected with hereditary retinoblastoma and found to be informative for both microsatellite markers.

Key words: D13S284/preimplantation genetic diagnosis/RB1.20/retinoblastoma

Introduction

Retinoblastoma (RB1; OMIM accession number: 180200) is the most common malignant intra-ocular tumour in childhood with an average incidence of one case in every 15 000–20 000 live births (Vogel, 1979). Because retinoblastoma originate from immature retina cells that are fully differentiated by age 2–3 years, most of the cases are diagnosed before the age of 5. Retinoblastoma occurs in non-hereditary (~60% of the patients) and hereditary forms (~40%) and is initiated by inactivation of both alleles of the retinoblastoma gene (RB1) (Knudson, 1971) located on chromosome band 13q14 (Sparkes et al., 1983). In non-hereditary retinoblastoma, the two RB1 gene mutations arise in the same retina cell and patients are always unilaterally affected. Hereditary predisposition to retinoblastoma results from a germline mutation either transmitted from an affected parent or that occurred de novo in parental germline cells (mainly during spermatogenesis) or during embryonal development. The predisposing germline mutation is transmitted as a highly penetrating autosomal dominant trait (~90%), resulting in a ~45% risk for offspring of patients with hereditary retinoblastoma. During early childhood, a second mutation is somatically acquired in a retina cell initiating the development of a tumour focus. The onset of tumours occurs earlier than in non-hereditary patients, since a single somatic event on the residual RB1 normal allele is sufficient for development of retinoblastoma. Patients may be affected with bilateral or unilateral multifocal retinoblastoma or remain unaffected (non-penetrant carriers). In addition, carriers of a RB1 gene mutation are at increased risk for the development of secondary nonocular tumours including osteosarcomas, bladder, prostate, lung and breast carcinomas, as well as soft tissue sarcomas (Draper et al., 1986). The risk is much greater in patients who received irradiation therapy (Eng et al., 1993). The development of a retinoblastoma can be explained by the activity of the nuclear phosphoprotein pRB encoded by the RB1 gene that plays a major role in cell proliferation control through negative regulation of G1/S progression (Goodrich et al., 1991). In the absence of intact RB1 protein, unscheduled cell proliferation occurs.

Mutation detection in the RB1 gene is complicated and time-consuming because the gene is ~180 kb long, is composed of 27 exons grouped in three domains separated by two large introns, and because of the heterogeneity of the causative molecular events ranging from point mutations to large deletions (Lohmann, 1999). More than 360 mutations spread all over the gene have been reported to the RB1 gene mutation database (http://www.d-lohmann.de/Rb). In the familial form of retinoblastoma (~1/4 of hereditary cases), segregation of the disease-causing mutation can be followed indirectly by linkage with polymorphic DNA markers. This allows a risk prediction for all relatives in a family (Alonso et al., 2001) and may also be used during prenatal diagnosis to identify the fetuses carriers of the mutant RB1 haplotype (Onadim et al., 1992).

Preimplantation genetic diagnosis (PGD) has been developed for couples at high risk of transmitting a genetic disorder to their offspring (Handyside et al., 1990). One or two blastomeres are biopsied from embryos at about the 6–10 cell stage after IVF and used for genetic diagnosis, after which embryos shown to be free of the disease under investigation are transferred to the maternal uterus to initiate pregnancy. This procedure avoids an eventual therapeutic abortion following genetic prenatal diagnosis by chorionic villus sampling or amniocentesis in the first and second trimesters of gestation.
We describe the development and standardization of a multiplex PCR strategy for PGD of hereditary retinoblastoma using two informative microsatellite markers—RB1.20 and D13S284—located within and close to the RB1 gene respectively. These markers are characterized by high heterozygosity rates (89 and 87% respectively) (Alonso et al., 2001) and the recombination fraction between them has been estimated to be 0.022 (Girardet et al., 1999). To our knowledge, this article presents the first experience of PGD for hereditary retinoblastoma predisposition. Although a single-cell protocol allowing the detection of a specific RB1 gene mutation together with an intragenic restriction fragment length polymorphism (RFLP) and an independent highly polymorphic marker was developed for hereditary retinoblastoma (Sütterlin et al., 1999), no PGD clinical case was reported using this strategy.

Materials and methods

Genetic background of the PGD patients

A 34-year-old patient and her husband (38 years old) were referred to our centre for PGD. Their family pedigree is shown in Figure 1. The grandfather (I.1) who was affected with bilateral retinoblastoma was eunucleated of one eye and was treated with radiotherapy; his son (II.1) was non-affected (non-penetrant carrier), and his granddaughter (III.1) developed unilateral retinoblastoma and received irradiation therapy. The couple requested PGD after three terminations of pregnancies on the basis of prenatal diagnosis in the second trimester, revealing four affected fetuses.

The predisposing RB1 mutation in this family was not identified and an indirect diagnosis was performed. Two markers within and flanking the RB1 gene were studied in order to avoid the risk of misdiagnosis due either to meiotic recombination or to allele drop-out (ADO) of the allele segregating with the mutation: RB1.20, a tetranucleotide repeat located in intron 20 of the RB1 gene (Yandell and Dryja, 1989) and D13S284, an extragenic dinucleotide repeat closely linked to the RB1 gene (Dib et al., 1996). Familial segregation analysis of polymorphic markers (D13S284, RB1.20, Rbi.4, Rbi.2, D13S164, D13S155) was previously performed in the Department of Genetics (Necker Hospital, Paris, France), showing that individuals I.1, II.1 and III.1 shared a common haplotype.

After the patients were counselled on IVF and PGD and the possible outcomes, they consented to undergo an IVF treatment cycle. This study was approved by the institutional ethical committee and by the local multidisciplinary network licensed for prenatal diagnosis, in accordance with French law.

Lymphocyte preparation

Peripheral blood samples (10 ml) were collected in heparin tubes from both parents (II.1 and II.2) and their affected child (III.1). Whole blood (6 ml) was layered over 6 ml of Histopaque-1077 (Sigma, Saint-Quentin Fallavier, France) and centrifuged at 1000 g for 20 min. The layer of mononuclear leukocytes (predominantly lymphocytes) was collected and washed three times with phosphate-buffered saline without calcium and magnesium (Sigma). The cells were resuspended in 90% Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine (Invitrogen, Cergy Pontoise, France), 10% fetal calf serum (Eurobio, Les Ulis, France) and centrifuged at 1000 g until further processing. For transfer of single cells, an aliquot of the cell suspension was thawed and 1 µl was added to 4 ml of RPMI 1640 with L-glutamine supplemented with 10% fetal calf serum (Eurobio, Les Ulis, France) in a culture plate. Individual lymphocytes were isolated with a mouth-controlled glass micropipet under an inverted microscope, in order to develop a duplex single-cell PCR assay. Single cells were put into microtubes containing 3 µl of lysis buffer (200 mmol/l KOH, 50 mmol/l dithiothreitol) (Cui et al., 1989).

Ovarian stimulation, ICSI procedure, embryo culture and biopsy

The patient was stimulated with a combination of GnRH agonist (Decapeptyl PL 3; Ipsen, Paris, France) and recombinant FSH (Puregon; Organon, Paris, France). Ovarian response was evaluated by serum estradiol levels and daily ultrasound examination to observe follicle development. Retrieval of oocytes occurred 36 h after hCG administration and was performed under ultrasound guidance. Oocytes were then fertilized using ICSI in order to reduce the risk of sperm DNA contamination of biopsied blastomeres. Sequential media SM1/SM2 (Medicult, Jyllinge, Denmark) were used for embryo culture. Fertilization was evaluated 16–18 h following the sperm injection procedure, then the embryo quality was assessed 2 and 3 days post-microinjection. According to the percentage of anucleated fragments and to the number of blastomeres, the embryos were subdivided into grades A, B, C and D. On day 3, grade A, B and C embryos were biopsied under a microscope using a laser to punch holes in the zona pellucida (De Vos and Van Steirteghem, 2001) through which one (embryos < 6 cells) or two (embryos > 7 cells) blastomeres were gently

Figure 1. RB1.20 and D13S284 analysis of the studied family with hereditary retinoblastoma. The family is informative for both microsatellite markers. Unilaterally (half-filled symbol) and bilaterally (filled symbol) affected individuals are shown. Dot in figure indicates a carrier without retinoblastoma. RB N, normal allele; RB M, mutant allele.

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Table I. Amplification and allele drop-out (ADO) rates obtained after amplification of RB1.20 and D13S284 microsatellites on single lymphocytes

<table>
<thead>
<tr>
<th>Microsatellite marker</th>
<th>Amplification (%)</th>
<th>ADO* (%)</th>
</tr>
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<tbody>
<tr>
<td>RB1.20</td>
<td>295/305 (96.7)</td>
<td>16/295 (5.4)</td>
</tr>
<tr>
<td>D13S284</td>
<td>500/505 (98.4)</td>
<td>11/300 (3.7)</td>
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*Based on the number of cells that gave a positive amplification signal.

Table II. Preimplantation genetic diagnosis results of family with hereditary retinoblastoma

<table>
<thead>
<tr>
<th>Embryo grade before biopsy (no. of cells)</th>
<th>E2</th>
<th>E3</th>
<th>E4</th>
<th>E5</th>
<th>E7</th>
<th>E8</th>
<th>E9</th>
<th>E10</th>
<th>E11</th>
<th>E13</th>
<th>E15</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB1.20 results</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>N</td>
<td>N</td>
<td>A</td>
<td>A</td>
<td>N</td>
<td>A</td>
<td>N</td>
</tr>
<tr>
<td>D13S284 results</td>
<td>A</td>
<td>F</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>F</td>
<td>N</td>
<td>F</td>
<td>N</td>
</tr>
<tr>
<td>Outcomes</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>ADO pat.</td>
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N = normal; A = affected; F = amplification failure; ADO pat. = allele drop out of the paternal allele; AR = ambiguous results; T = transferred; NT = not transferred.

Aspirated. Embryos were then immediately transferred back into fresh SM2 medium. Unavailable unaffected embryos were replaced in the evening of the same day. Individual nucleated blastomeres which had been biopsied as part of the clinical case were washed and collected in microtubes containing the lysis buffer (200 mmol/l KCl, 50 mmol/l dithiothreitol), as for single lymphocytes. An ~2-μl aliquot of culture medium of each embryo was transferred to a separate microtube containing lysis buffer and further processed to check contamination with exogenous DNA. All tubes were kept refrigerated until they could be placed in a thermal cycler for the lysis incubation.

Lysis procedure and single-cell PCR

Lysis was performed by incubation at 65°C for 10 min, then first-round PCRs were either immediately performed (blastomeres) or tubes were kept at ~20°C until further processing (lymphocytes).

For the PGD clinical case, a number of single lymphocytes from the family members were included as positive controls alongside the studied blastomeres. Several negative controls (microtubes containing identical reaction mixes without added DNA) were run simultaneously with sample tubes to check for the absence of contamination.

DNA amplification in single cells was performed by nested PCR using primers as already described (Girardet et al., 1997, 1999). The first-round PCR contained (in a total volume of 30 μl): 3 μl of lysis buffer, 3 μl of neutralization buffer (900 mmol/l Tris–HCl pH 8.3, 300 mmol/l KCl, 200 mmol/l HCl), 3 μl of potassium-free amplification buffer [100 mmol/l Tris–HCl pH 8.3, 15 mmol/l MgCl₂, 0.1% (w/v) gelatin], 2 mmol/l dNTPs, 7.5 and 4 pmol of nested primers for RB1.20 and D13S284 markers respectively, and 1 U Taq DNA polymerase. The PCR cycling conditions for RB1.20 were 4 min at 96°C, then 25 cycles of 20 s at 94°C, 20 s at 62°C and 40 s at 72°C. For D13S284, the PCR programme was 4 min at 96°C, then 32 cycles of 20 s at 94°C, 20 s at 58°C and 40 s at 72°C. For both reactions, the final elongation step was 10 min at 72°C. The internal forward primer for each set was 5’-end labelled with 6-carboxyfluorescein for fragment analysis.

Analysis of PCR products

PCR efficiency was checked by running 10 μl of the second-round products on 1.5% agarose gels prestained with ethidium bromide. For all successfully amplified products, a mixture of 1 μl of fluorescently labelled samples, 2 μl of deionized formamide, 0.5 μl of loading buffer and 0.5 μl of TAMRA-labelled size standard GS500 (Applied Biosystems) was prepared, denatured for 3 min at 90°C, cooled immediately on ice, then loaded on a 4% polyacrylamide gel. Electrophoresis was performed in an ABI PRISM 377 DNA sequencer (Applied Biosystems) at 51°C and 2000 V for 2 h. After the run was complete, results were analysed using the Genescan 3.1.2 software supplied by Applied Biosystems.

Re-analysis of non-transferred embryos

Non-transferred cleavage stage embryos were removed from the zona pellucida, disaggregated into single blastomeres and collected into microtubes containing lysis buffer. They were then processed as for the rest of the single cells in order to verify the results of PGD.

Results

The couple requesting PGD was found to be fully informative for both RB1.20 and D13S284 microsatellite markers that were therefore suitable for the PGD procedure. As (i) the partners do not share common alleles and (ii) the recombination fraction between the polymorphisms is low, it was expected that the embryos obtained after IVF would have inherited one of the four possible genotypes.

Duplex PCR on single lymphocytes

Preliminary duplex PCR of RB1.20 and D13S284 microsatellites were performed on single lymphocytes from individuals heterozygous at both the microsatellite repeats in order to test the reliability of the protocol with respect to ADO and contamination. A total of 305 isolated lymphocytes was amplified, including 245 single cells from the retinoblastoma nuclear family members (II.1, II.2 and III.1).

Results of amplification efficiency and ADO rates are summarized in Table I. Amplification of the RB1.20 and D13S284 microsatellites was observed in 295 (96.7%) and 300 (98.4%) single cells respectively. Complete amplification failure was observed in five cells, probably because the single lymphocytes were not successfully transferred into the reaction microtubes. No case of contamination was detected in any of the negative control tubes. Of the cells that were successfully amplified, 5.4% were affected by ADO at the RB1.20 locus while at the D13S284 locus this was 3.7%. The ADO rates observed in these experiments were not allele-specific for any of the microsatellites studied.

Outcome of the PGD treatment

One ICSI–PGD cycle was performed for this couple. Following ovarian stimulation, 15 cumulus–oocyte complexes were collected, 13 metaphase II oocytes were sperm-injected of which 13 showed fertilization (100%). Eleven embryos were of sufficient quality (5–10 cells) for biopsy on the early morning of day 3 post-fertilization. One or two blastomeres were biopsied according to the developmental stage and morphology of the individual embryos: one cell was...
removed for analysis from embryos with fewer than six blastomeres and two cells were removed from more advanced embryos. Molecular analysis revealed five embryos to be affected (E3, E4, E5, E9, E10), one healthy embryo (E11) and four embryos with unclear results: E7 was suspected to be normal through the amplification results of the RB1.20 marker but no PCR product was obtained for D13S284, while embryos E8, E13 and E15 showed an absence of paternal alleles for both RB1.20 and D13S284 microsatellites; one embryo (E2) was without diagnosis because of amplification failure in the single blastomere analysed (Table II). The only embryo diagnosed as unaffected was transferred back to the patient on the evening of day 3 of development, but no resulting clinical pregnancy ensued.

After the clinical case, the accuracy of the genetic diagnosis was assessed by re-analysing non-transferred embryos under exactly the same PCR conditions and procedure as for clinical PGD samples. The analysis confirmed that embryos E3, E4, E5, E9 and E10 were affected. The embryo for which no results were obtained (E2) was healthy, as well as the embryo E13 that displayed ambiguous results. The embryo with unclear results that was thought to be normal (E7) was proved to be non-affected. ADO of paternal alleles was still observed for embryos E8 and E15 for which no definitive results have been obtained.

Results of RB1.20 and D13S284 PCR products generated from single blastomeres from two embryos (one affected and the healthy one that was transferred later) along with single lymphocytes from individuals II.1, II.2 and III.1 used as controls are shown in Figure 2A and B respectively.

During the clinical PGD and the re-analysis of non-transferred embryos, no incidence of contamination by extraneous DNA was observed in any of the blanks taken from the culture medium of each embryo, and no amplification product was found in any of the PCR negative tubes.

**Discussion**

We have described an approach to PGD of hereditary retinoblastoma by use of linked polymorphic markers. This strategy, based upon indirect genetic diagnosis, has already been applied to other monogenic disorders in PGD such as fragile X syndrome (Apessos et al., 2001), cystic fibrosis (Eftedal et al., 2001) and Marfan syndrome (Loeys et al., 2002) among others. Because of the large spectrum of molecular defects identified in the RB1 gene, the development of mutation-specific PGD protocols is impracticable; therefore, this general marker-based protocol can be applied to all retinoblastoma families as long as they are informative for the markers studied. Based on the allele frequencies of RB1.20 and D13S284, ~95% of the couples requesting PGD for retinoblastoma are expected to be informative for both markers. It is well known that indirect genetic diagnosis requires families with at least two affected individuals in order to determine the mutant RB1 haplotype, but it is also useful for families with a single individual affected with sporadic hereditary retinoblastoma when loss of heterozygosity has been identified in the tumour, therefore giving the ability to perform a PGD for the patient. RB1.20 and D13S284 microsatellites were chosen on the basis of their high degree of heterozygosity (~87 and 89% respectively) and close linkage, making these markers useful tools for PGD of hereditary retinoblastoma. No cross-over has been reported so far within the RB1 gene that would affect the accuracy of the diagnosis by altering the linkage between the causative mutation and the RB1.20 marker. Moreover, both markers are easily amplified and analysed in single cells, as was already reported through the analysis of single sperm for research purposes (Girardet et al., 2000). In indirect genetic diagnosis, the analysis of intragenic markers is always more suitable than the study of markers located outside the gene. Various polymorphisms have been described within the human RB1 gene sequence, including RFLPs (Bookstein et al., 1988; Wiggs et al.,
PGD of hereditary retinoblastoma

Retinoblastoma has been generally shown to be transmitted as an autosomal dominant trait with nearly 50% of the offspring of an affected individual also being affected (mainly with multifocal bilateral tumours). In contrast, in a few families, as in the one reported in the present study, a low penetrance phenomenon is observed: most mutation carriers develop unifocal unilateral retinoblastoma, remain unaffected or present benign lesions (retinomas). These non-penetrant carriers are either individuals who possess an initial mutation but in whom, fortuitously, no sensitive retina cell loses the remaining wild-type RB1 allele, or individuals who have an initial RB1 mutation encoding a protein with a residual function (Bremner et al., 1997; Otterson et al., 1997), whereas the common type of RB1 mutations lead to premature stop codons and presumed truncated proteins which are not detectable in retinoblastoma tumours (Dunn et al., 1989). It is likely that the identification of the causative RB1 mutation would shed some light on the molecular mechanisms explaining the reduced penetrance in this family.

In families affected with hereditary retinoblastoma, prenatal diagnosis is often requested but in familial retinoblastoma with low penetrance and variable expressivity, the choice of whether or not to terminate a pregnancy may be a serious problem as it is very difficult to predict the degree of affection in the future child; for these families, in addition to the families affected with the prevalent phenotype observed in hereditary retinoblastoma (bilateral/unilateral multifocal tumours), PGD is a more attractive alternative than prenatal diagnosis, as it allows only unaffected embryos to be selected for transfer to the maternal uterus.

Although embryo selection for late-onset disorders with genetic predisposition—including inherited cancer predispositions—raises ethical discussions and concerns, clinical PGD procedures have already been performed for inherited cancer syndromes such as familial adenomatous polyposis coli (Ao et al., 1998), neurofibromatosis type 2 (Abou-Sleiman et al., 2002), and Li-Fraumeni syndrome (Verlinsky et al., 2001). In contrast to these cancers that affect adults, retinoblastoma occurs in very early childhood. Moreover, the penetrance of the disease is ~90% accounting for the high proportion of carrier individuals that develop at least one tumour. The severity of the disease as well as physical and psychological trauma related to the treatments of retinoblastoma (enucleation, radiotherapy, chemotherapy) experienced by affected children, undoubtedly influence families’ attitudes regarding reproductive options, making PGD an ethically acceptable approach.

Considering the problems encountered in the amplification techniques during the clinical application, we would like to develop a new strategy using fluorescent PCR in order to amplify RB1.20 and D13S284 microsatellites in a single round which may improve the efficiency and accuracy of the analysis, as suggested by other studies (Findlay et al., 1996; Sermon et al., 1998). Moreover, no contamination has been detected either in PGD test optimization on single lymphocytes or during the PGD cycle. Therefore, when only a few embryos, as in this study, are diagnosed as healthy following successful amplification of both RB1.20 and D13S284 microsatellites, we think it is reasonable to transfer a non-affected embryo whose diagnosis is based upon the PCR result of only one microsatellite preferably located within the retinoblastoma gene, as RB1.20.

In conclusion, we have described a general approach for PGD of hereditary retinoblastoma using two highly informative microsatellite markers, provided that the couples are informative. However, this procedure is limited to the familial form of hereditary retinoblastoma which represents 10–15% of the retinoblastoma patients.
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