Strategies and outcomes of PGD of familial adenomatous polyposis

C. Moutou1, N. Gardes1, J.-C. Nicod1 and S. Viville1,2,3

1 Service de Biologie de la Reproduction, SIHCUS-CMCO, CHU de Strasbourg, Schiltigheim Cedex and 2 Institut de Genétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Illkirch Cedex, CU de Strasbourg, France
3 To whom correspondence should be addressed at: Institut de Genétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, BP163 1, rue Laurent Fries, F-67400 Illkirch Cedex, CU de Strasbourg, France. E-mail: viville@igbmc.u-strasbg.fr

Owing to adult onset of hereditary cancer, prenat al diagnosis (PND) raises numerous ethical issues on the acceptability to terminate an affected pregnancy (TOP). PND for these disorders is often considered as unacceptable by couples as well as geneticists and legal or ethical authorities, but preimplantation genetic diagnosis (PGD), even if subject to controversy, seems to be a more acceptable option. Therefore, many couples, who do not want to transmit their cancer to their children, consider PGD as their only reproductive option. This article describes our experience of PGD for familial adenomatous polyposis (FAP). Twelve couples were referred between 2000 and 2005. We developed PGD tests to detect the mutation alone, but we rapidly set up multiplex PCR combining mutation detection and indirect diagnosis. Finally, we set up duplex and triplex indirect diagnoses to be able to offer a PGD, whatever mutation was involved in familial cases. PGD strategies were based on (i) a new double allele-specific PCR approach (D-ARMS) allowing the detection of the wild-type and mutated allele; (ii) PCR fragments sizing and (iii) restriction length polymorphisms. For the 12 referrals, we developed eight tests, and 11 cycles have been performed for four couples, resulting in eight embryo transfers and five pregnancies, with the birth of one healthy boy and two ongoing pregnancies. We are now able to propose PGD to most couples at risk of transmitting FAP to their offspring, whether the mutation is familial or occurred de novo.

Key words: familial adenomatous polyposis (FAP)/adenomatous polyposis coli gene (APC)/mutation-dependent strategy/PGD

Introduction

Fifteen years after the first birth after preimplantation genetic diagnosis (PGD) (Handyside et al., 1990), the progress in single-cell PCR and mutation detection has led to a considerable increase in the number of available diagnoses. If PGD was initially proposed to couples at risk of having a child affected by a severe genetic disorder (such as cystic fibrosis, myotonic dystrophy and spinal muscular atrophy), late-onset diseases such as Huntington’s disease and, more recently, inherited cancers have become a new class of indications. Owing to the adult onset of hereditary cancer, prenatal diagnosis (PND) raises numerous ethical issues on the acceptability to terminate an affected pregnancy (TOP). This is the reason why PND for these disorders is often considered as unacceptable by couples as well as geneticists and legal or ethical authorities. PGD for hereditary cancer, even if subject to controversy, seems to be a more acceptable option because it does not lead to TOP but consists of selecting embryos free from the mutation. Therefore, many couples, who do not want to transmit their cancer predisposition to their children, consider PGD as their only reproductive option. In 1998, Ao et al. (1998) reported the first PGD attempts for familial adenomatous polyposis (FAP). Since then, PGD is available for several hereditary cancers such as Li-Fraumeni syndrome (Verlinsky et al., 2001), neurofibromatosis type 1 and 2 (Abou-Sleiman et al., 2002; Verlinsky et al., 2002; Spits et al., 2005; Fiorentino et al., 2006), Von Hippel–Lindau syndrome (Rechisky et al., 2002), retinoblastoma (Rechisky et al., 2002; Fiorentino et al., 2003; Girardet et al., 2003; Xu et al., 2004) and BRCA1 (Sermon et al., 2005; Harper et al., 2006). A list of PGD Consortium centres offering PGD for inherited cancers is given on the ESHRE PGD Consortium website www.eshre.com.

FAP is an autosomal dominant disorder (MIM175100) characterized by the occurrence of multiple polyps of the colon during the second decade of life. These polyps most often proliferate throughout the colon and finally degenerate in colon cancer early in adult life. Additional extracolonic manifestations can be observed in affected patients. FAP is caused by mutations in the adenomatous polyposis coli (APC) tumour suppressor gene (Groden et al., 1991), located on chromosome 5 (5q21-q22). The gene has 15 exons, and 70% of the mutations are located in exon 15, the most common being a 5-bp deletion at codon 1309 (Beroud and Soussi, 1996). To reduce morbidity and mortality, mutation carriers are under close surveillance from childhood with annual colonoscopy from the age of 10, followed by gastroduodenal fibroscopy every 3 years from the age of 20, prophylactic subtotal colectomy when polyps start to proliferate, followed by annual endoscopy of the remaining rectum and upper endoscopy to detect extracolonic manifestations, particularly desmoids, adenomas and cancer of the stomach. As this intensive surveillance cannot completely prevent cancer in mutation carriers, PND or PGD can be a solution for couples at risk of having an affected child.

This article describes our experience of PGD for FAP.

Materials and methods

Patients

Between 2000 and 2005, 12 couples asked for PGD for FAP. Data on patient’s reproductive history, reason for referral, as well as the centre’s and the
patient’s decision to go forward were those collected by the FileMaker Pro 6™ (FP6) database that we designed for the ESHRE PGD Consortium (Sermon et al., 2005).

Genomic DNA from the couples and from relatives when available was extracted from whole blood samples using the standard saline protocol (Rousseau et al., 1992). Mutations were documented using medical reports and confirmed in-house on genomic DNA from the affected parent-to-be. In order to choose the microsatellite to be combined with mutation detection in the multiplex PCR, informativity testing was performed on 200 ng DNA. For linkage analysis, we tested both partners plus at least one affected relative (affected child or parent) of the mutation carrier.

PGD requests were accepted by the local multidisciplinary council for PND, as required by the French law. Patient ovarian stimulation, oocyte recovery, ICSI and biopsy procedures were carried out as previously described (Moutou et al., 2002). Two blastomeres were analysed per embryo. At day 4, one or two unaffected embryos were transferred according to their kinetics and morphology, the woman’s age and to the rank of transfer. Untransferred healthy embryos were incubated until day 5 and were then cryopreserved if they reached the blastocyst stage.

**Single-cell testing**

Epstein–Barr transformed lymphoblast cell lines and isolated single lymphocytes from affected patients were used to setup single-cell PCRs. Tubing of single cells was performed as previously described (Moutou and Viville, 1999). Supernumerary IVF embryos not suitable for transfer or for freezing and thus scheduled for destruction were used for final validation of the single-cell PCRs.

Embryos were incubated for 10 min in a Ca- and Mg-free embryo biopsy medium (EBM, MedicIn Vitro, Lyon, France) before biopsy. They were then biopsied under an inverted microscope (Leica, Wetzlar, Germany) using Research Instrument micromanipulators and micropipettes (Research Instruments Ltd, Penryn, UK); zona pellucida drilling was performed using a Zylors laser (Hamilton-Thorn, Beverly, MA, USA). Blastomeres were gently aspirated and placed in G2.1 medium (Scandinavian Science, Gothenburg, Sweden). Each blastomere was washed three times in EBM, transferred into a reaction tube containing 2.5 μl of lysis buffer [LB: 200 mM KOH, 50 mM dithiothreitol (DTT)] and frozen at −20°C until PCR amplification. Contamination controls consisted of one blank for each blastomere by adding the EBM remaining in the capillary after cell tubing to the LB and of two reaction mix blanks with LB only.

Cells were lysed in 2.5 μl of LB at 65°C during 10 min and kept at 4°C until the PCR.

**PCR**

All PCRs were performed in a single round. Primers are given in Table I. Reaction mixes were added to the templates in a final volume of 25 μl. Forward primers were labelled either with 6-Fam, hex or red fluorochromes (Applied Biosystems, Warrington, UK). A minimum of 60 lymphoblasts or lymphocytes were used for each validation. PCR products were analysed on an ABI3100 automated sequencer with Genescan Rox-500 internal size standard (Applied Biosystems) in each sample. PCR efficiency was calculated on the totality of the cells, whereas specificity and allele drop-out (ADO) rate were calculated from samples showing amplification for at least one locus.

For the R283X mutation detection, 10 μl of PCR product was digested at 37°C for 1 h in the presence of 5 U NalIII, 1.25 μl NEB buffer4 and 0.13 μl bovine serum albumin (BSA) (Ozyme, Saint-Quentin en Yvelines, France).

Indirect diagnoses were set up using four highly informative microsatellites flanking APC gene: D5S2027, D5S1965, D5S346 or D5S421. These markers were chosen using the UCSC Genome Bioinformatics site (www.genome.ucsc.edu; March 2006) in view of their vicinity to APC gene and of their high informativity (Table I). The tests for the R168X mutation remained unfinished because the couple was turned down for PGD before the end of the setup as the woman had unfavourable hormone concentrations.

**Results**

**Patients**

Between March 2000 and April 2005, 12 couples were referred for FAP-PGD. A description of the couples is given in Table II. Average female age at intake was 29.4 years, which is younger than the average female age in our PGD centre (31.2 years, based on 369 referrals). Previous pregnancies were reported for four women: one ending in a spontaneous abortion, one untested boy was born after ICSI in an infertile couple, one unaffected woman previously had a son with another partner and finally one woman underwent two TOPs after PND and one termination without PND. The other couples had had no previous pregnancy. One was infertile, and the seven others did not want to transmit their cancer to their children but objected to TOP and considered PGD as their only reproductive option.

Half of the mutation carriers were males; half were females. The mutation was found in exon 15 of the APC gene in eight carriers, four of them carrying the recurrent 5-bp deletions at codon 1309. A family history of FAP was reported in 10 families, and two mutations occurred de novo.

**PCR amplification**

We initially set up simplex PCR tests to detect the mutation responsible for APC in the family. In order to increase PCR accuracy and to avoid misdiagnosis due to ADO or amplification failure, we rapidly decided to combine direct diagnosis with microsatellite analysis by duplex PCR. Five different regions of the APC gene were amplified for mutation detection. This enabled us to detect two 5-bp deletions in exon 15 (codons 1062 and 1309) and substitutions in exon 4 (R168X), in exon 8 (R283X) and in exon 15 (Q1378X). Two simplex PCRs were firstly set up for couples 1 and 3. We then went on to develop four duplex PCRs co-amplifying the mutation locus and a microsatellite according to informativity test results for each couple. Since 2005, for inherited mutations undetectable with the available single-cell tests, we developed a triplex PCR combining D53S2027, D5S1965 and D5S421. A total of nine single-step-PCR protocol setups were started of which eight were fully optimized (Table III).

**Diagnostic strategies**

Different strategies were used according to the type of mutation involved (Table III).

1. A double allele-specific amplification (D-ARMS) was developed to detect the Q1378X substitution (couple 7). For this purpose, we used a set of three primers: a common forward primer that was fluorescently labelled, one primer specific for the normal allele (reverse N) and one specific for the mutated allele (reverse M). An additional mismatch was introduced four or five bases upstream of the 3′ end of the specific primers to increase annealing specificity. A five-A-tail was added at the 5′ end of primer N to enable sizing discrimination between normal and mutated alleles (Figure 1). Moreover, the allele-specific amplification protocol was adapted to amplify both normal and mutated alleles in the same PCR by using a common forward primer and two specific reverse primers in ratios that favoured the amplification of the mutation.

2. Migration and direct sizing of PCR products were used to detect the 5-bp deletions at codons 1062 (couple 1) and 1309 (couples 4, 8 and 10).

3. Two diagnoses were based on a restriction digest to detect the single-nucleotide substitution R283X (couple 3) and R168X (couple 5). The workup for the R168X mutation was stopped before testing the patient’s lymphocytes because the woman’s FSH was too elevated for IVF. To provide an internal digestion control, PCR primers were chosen to generate a fragment including a common restriction site in all amplicons in addition to the one created by the mutation. The primer close to the mutation was fluorescently labelled (Figure 2). Unaffected cells showed one peak corresponding to the common site, and affected cells showed two peaks: the common one for the normal allele and a smaller one for the mutated allele. Complete digestion was confirmed by total removal of undigested PCR fragments (Figure 2).
We improved these three strategies by adding the co-amplification of an informative polymorphic microsatellite, allowing a double, direct and indirect, diagnosis (see Table III). Even for our D-ARMS protocol, a successful discrimination of normal and mutated alleles was obtained in co-amplification with D5S346 microsatellite, which was informative for couple 7 (Figure 1).

4. For familial mutations, different from the mutations for which the single-cell PCRs were previously set up, and in view of increasing the number of couples to whom we can offer FAP-PGD, we set up a multiplex PCR combining microsatellites of the APC region. We first combined D5S2027, D5S1965 and D5S421 markers because couples 9 and 12 were informative at these loci (Table III).
Once PCR conditions were optimized, 60–114 single cells were tested per PCR protocol with an amplification rate ranging from 95 to 100% (Table III). The amplification rate varied between 81 and 99%, and 94% of the cells with amplification showed a complete genotype (i.e. a correct genotype for each locus tested), whereas a conclusive result could be reached in 99% (95–100%) of these cells. A total of 20 out of 493 heterozygous cells showed ADO for at least one locus, meaning a 4% rate (0–14%) when calculated on a per-cell basis. The results in terms of amplification efficiency (i.e. cells with a signal and peak height, specificity and ADO rates) were similar between lymphocytes and lymphoblasts. The number of heterozygous cells used to estimate ADO rate was different from the number of amplified cells because simplex PCRs were performed using heterozygous affected cells and normal homozygous ones. A global contamination rate of 3% was observed in 233 blank controls, mostly due to one test where 3 out of 20 blanks showed carry-over contamination. Tests on blastomeres from supernumerary embryos showed that protocols were feasible on blastomeres. However, it was not possible to test affected blastomeres before PGD, and the expected genotypes were unknown, so we could not estimate accuracy of the tests on blastomeres (data not shown).

**PGD cycles**

Couple 2 dropped out because they found the time for setup too long, couple 5 was stopped because of unfavourable female hormonal concentrations and couple 8 decided to try a spontaneous pregnancy.
followed by PND. Cycles are scheduled in the coming months for couples 6, 9, 10, 11 and 12. Table IV summarizes PGD cycles that were performed for couples 1, 3, 4 and 7. Eleven cycles were started, 10 reached the stage of embryo biopsy and eight had an embryo transfer procedure, with a mean number of 1.6 embryos per transfer. A total of 85 blastomeres from 44 embryos were analysed, and 73 (86%) blastomeres from 40 embryos gave a result. Two unaffected embryos were cryopreserved for couple 3. During the PGD cycles, 2 out of 85 blank controls showed a single-peak contamination, probably due to carry-over contamination. A positive HCG was obtained for 5 cycles (2 for couple 1, one for couples 3, 4 and 7). Unfortunately, couple 1 had two biochemical pregnancies. They discontinued the PGD procedure, and the woman is now spontaneously pregnant. The couple declined to undergo PND for this ongoing pregnancy. Three singleton clinical pregnancies were obtained: for couple 3, who delivered a healthy boy, and couples 4 and 7, whose pregnancies are ongoing. No prenatal or post-natal testing was performed to confirm PGD results. However, a total of 13 untransferred embryos were reanalysed, and all PGD results were confirmed, which indirectly shows that PGD tests were accurate.

Discussion

Twelve couples were referred for PGD of FAP between March 2002 and April 2005. The reason for PGD and the patients’ reproductive history was obviously different from other referrals and typical for late-onset diseases. Indeed, objection to TOP of FAP fetuses is the first indication (75 versus 29% for all referrals), and only one couple experienced TOP before requesting PGD (versus 29% in the total population). This is partly due to the younger mean age of patients in the FAP group (on average 1.8 years younger than in our PGD population) but also because most of the couples at risk (7/12) do not wish to transmit the disease to their offspring but do not consider PND as acceptable and thus choose to remain childless. PGD seems to be the only acceptable solution for these couples. We noticed a similar trend for Huntington’s disease (Moutou et al., 2004), another late-onset dominant disorder, which also raises numerous ethical issues.

Except for the recurrent 5-bp deletion at codon 1309 present in four families, a private mutation of the APC gene was present in all other couples, requiring a mutation-dependent strategy for PGD. In total, eight protocols were designed. Two simplex PCRs were initially set up for the mutations E1309X and R283X. These protocols were used for a first cycle and then improved by adding an informative marker to the mutation detection. Specific tests combining mutation detection plus one marker were also set up for three other mutations: R168X in exon 4 with marker D5S346, R283X in exon 8 with marker D5S1965 and Q1378X in exon 15 with marker D5S346. Mutations were detected using either a double allele-specific amplification or an enzymatic digestion of the PCR product. These well-known approaches were adapted to minimize misdiagnosis risks. In contrast to single-ARMS test where only one mutation-specific primer is used, D-ARMS allows for the discrimination between an unaffected embryo (no signal for the mutation but one signal for the normal allele) from an amplification failure due to poor DNA quality of the blastomere (no amplification at all). Other published techniques as single-nucleotide primer extension (or minisequencing Fiorentino et al., 2003) could be used to detect this substitution, but D-ARMS is a less time-consuming method because only one round of PCR is necessary. In our centre, we use minisequencing for small genes (i.e. VHL or HBB genes) for which we set
Table IV. Summary of PGD cycles for couples 1, 3 and 4

<table>
<thead>
<tr>
<th>Couple</th>
<th>Cycle number</th>
<th>Number of COCs</th>
<th>Number of inseminated</th>
<th>Number of 2PN</th>
<th>Number of biopsied</th>
<th>Number of analysed</th>
<th>PGD strategy</th>
<th>Blastomeres tested</th>
<th>Blastomeres with a result</th>
<th>Contaminations/number of blanks</th>
<th>Number of diagnosed</th>
<th>Number of failed</th>
<th>Number of unaffected</th>
<th>Number of transferred</th>
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<td>15</td>
<td>19</td>
<td>8</td>
<td>5</td>
<td>5</td>
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<td>10</td>
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<td>0/10</td>
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<td>4</td>
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<td>2</td>
<td>0</td>
<td>–</td>
<td>–</td>
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<td>6</td>
<td>5</td>
<td>0/6</td>
<td>3</td>
<td>0</td>
<td>1</td>
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</tr>
<tr>
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<td>1</td>
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<td>14</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>Direct + D5S1965</td>
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<tr>
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<td>7</td>
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<td>12</td>
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<td>Direct + D5S1965</td>
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<td>10</td>
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<td>2</td>
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COCs, cumulus-oocyte complex; PN, pronuclei.

Table IV. Summary of PGD cycles for couples 1, 3 and 4

COCs, cumulus-oocyte complex; PN, pronuclei.
In conclusion, we are now able to propose PGD to most couples at risk of transmitting FAP to their offspring, whether the mutation is familial or occurred de novo. Eight protocols were set up for the first 12 referrals, the first ones using simplex PCR for mutation detection. Our strategy was then turned to multiplex PCR combining either mutation detection and indirect diagnosis using microsatellites or indirect diagnosis with several microsatellites for familial cases with a private mutation. With our growing experience in PCR workups, the development of new protocols is less and less time consuming which enables us to focus on specific PGD tests for de-novo mutations for which a diagnosis is not yet available.

Acknowledgements

We would like to thank the IVF team at the SIHCUS-CMCO (Dr C. Rongières and Ms M.-P. Bailly for clinical part and Dr C. Wittener and her group IVF laboratory work) and the clinicians who referred the couples. We thank Prof. K. Sermon (Brussels) for critical reading of this article. This work was supported by funds from the Ligue Régionale contre le Cancer, from the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Hôpitaux Universitaires de Strasbourg.

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Submitted on August 11, 2006; resubmitted on October 16, 2006; accepted on October 24, 2006