Autosomal dominant polycystic kidney disease (ADPKD) is primarily characterized by renal cysts and progression to renal failure. It is a genetically heterogeneous disease, with mutations in the \textit{PKD1} gene accounting for the majority of cases. Direct mutation detection for \textit{PKD1}-linked ADPKD or type 1 is complicated by the large size and complex genomic structure of \textit{PKD1}. This paper describes a microsatellite marker-based assay for PGD in couples at risk of transmitting ADPKD type 1. During PGD, genetic analysis is carried out on single blastomeres biopsied from preimplantation embryos obtained after IVF, and only embryos unaffected by the disease under investigation are selected for transfer. Single-cell genetic analysis relied on a fluorescent duplex-PCR of linked polymorphic markers followed by fragment length determination on an automated sequencer. The co-amplification of the intragenic KG8 and the extragenic D16S291 marker at the single-cell level was evaluated in pre-clinical tests on lymphoblasts and research blastomeres. The developed assay proved to be efficient (96.1% amplification) and accurate (1.4% allele drop-out and 4.3% contamination), and can be applied in all informative ADPKD type 1 couples. From five clinical cycles carried out for three couples, two pregnancies ensued, resulting in the birth of two healthy children.

Key words: dominant polycystic kidney disease/PGD

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a common genetic disorder with a worldwide incidence of 1 in 1000 births (Dalgaard, 1957; review by Boucher and Sandford, 2004). It is typically a late-onset disease primarily characterized by multiple, large fluid-filled cysts in one or both kidneys and a gradual loss of renal function, which leads to renal failure and end-stage renal disease (ESRD). Other symptoms are liver cysts, hypertension, cardiac valve anomalies and intracranial aneurysms (Gabow, 1993). Clinical management of the patients aims at control of symptoms and prevention of complications, but progression to renal failure cannot be stopped. ESRD patients are offered repeated dialysis or kidney transplantation.

ADPKD is genetically heterogeneous and two major genes have been identified. Two types of ADPKD with almost identical phenotypes have been distinguished depending on the mutated gene. ADPKD type 1 (80–85% of ADPKD cases), caused by mutations in the \textit{PKD1} gene located on chromosome 16p13.3, has been associated with more severe clinical symptoms and an earlier onset of renal failure than type 2 (10–15% of ADPKD families), which is caused by mutations in the \textit{PKD2} gene on chromosome 4q21–23 (European Polycystic Kidney Disease consortium, 1994; Burn et al., 1995; Hughes et al., 1995; Mochizuki et al., 1996). The genetic diagnosis of the disease often relies on indirect linkage analysis. Direct mutation analysis is difficult because of the large size of \textit{PKD1} and \textit{PKD2} (46 exons spread over 47 kb DNA and 14.1 kb mRNA; 15 exons spread over 70 kb DNA and 5.1 kb mRNA, respectively) and the absence of mutational hot-spots. Testing in case of \textit{PKD1} is further complicated by the presence, on the same chromosome, of several transcribed sequences that are highly homologous to the 5' part (exon 1–33) of the gene (Bouba et al., 2001). The \textit{PKD1} mutation rate is high in both germline and somatic cells. So far, more than 250 unique mutations, often nonsense and frame-shift mutations, have been found, but no clear genotype–phenotype correlation could be deduced (Rosetti et al., 2001). Prenatal diagnosis can be offered in theory, if the mutation has been identified in an affected family member or if linkage has been established in the family. In practice, it has only rarely been performed (Bellone et al., 1991, Turco et al., 1992), and a survey of ADPKD families has suggested that only 4–8% of family members would terminate a pregnancy for this late-onset disease (Hodgkinson et al., 1990). PGD may be an option for ADPKD families. PGD is an early form of prenatal diagnosis in which genetic testing is carried out on blastomeres from 3-day-old embryos obtained after IVF (Sermon et al., 2004). Only embryos unaffected by the disease under investigation are transferred back to the mother. This technique represents a valuable alternative to prenatal diagnosis and gives couples that have a high risk of transmitting a genetic disease, the advantage of being able to circumvent a termination of pregnancy (TOP). PCR is used in case of monogenic diseases and a prerequisite to any development of a single-cell PCR is that the causal mutation has been identified or that linkage to polymorphic markers in or near the gene has been clearly established. Our centre received three PGD requests from ADPKD patients for whom linkage to \textit{PKD1} had been
ascertained and one request for which a PKD1 mutation had been found. A panel of four markers (KG8, D16S291, D16S663 and D16S665) was tested at the time of the first PGD request. Two markers, the intragenic KG8 located in the 3' untranslated region and the extragenic D16S291 located nearly 154 kb downstream of the gene, were selected on the basis of their characteristics and the informativity results of the first couple. KG8 is a CA-repeat with eight possible alleles and 50% heterozygosity (Snarey et al., 1994). D16S291 is a CA-repeat with 80% heterozygosity and nine alleles (Thompson et al., 1992; Peral et al., 1994). The efficiency and accuracy of the single-cell duplex-PCR KG8-D16S291 was evaluated pre-clinically before it was applied during PGD. Another three couples at risk of transmitting ADPKD to their children had a PGD work-up with informativity and segregation testing.

Materials and methods

Patient work-up

Relevant clinical information on the four couples requesting PGD for ADPKD is given in Table I. In 1993, the second year of our PCR-PGD programme, our centre received a PGD request for ADPKD from a young couple in whom the male partner was affected. A family study, based on the variable number of tandem repeats marker D16S85 (Pignatelli et al., 1992), showed that the disease was linked to the PKD1 locus and allowed to offer prenatal diagnosis using Southern blot analysis. However, it was not possible to optimize this test at the single-cell level for PGD because of the large size of the DNA fragments. Therefore, the couple opted for prenatal diagnosis.

As the male had extreme oligoasthenoteratozoospermia, the couple needed ICSI treatment. Of the four ICSI cycles without PGD, one miscarriage at 6 weeks and one ongoing pregnancy ensued. The couple declined a therapeutic abortion after chorion villus sampling showing an affected fetus, and an affected boy was born. The child presented an early onset of renal cysts for 1 year. As more microsatellite markers linked to PKD1 were found, the family linkage analysis was repeated and two markers were selected for development of a PCR-based assay for PGD.

In the second couple the male partner had inherited ADPKD from his mother, who had undergone kidney transplantation at the age of 54. The patient himself had mild symptoms. This couple opted for PGD because of concurrent infertility problems and their objection to abortion. In the third couple, the patient was the male partner. The female partner had already had two spontaneous abortions, which had been traumatic experiences for the couple. Since they objected to a TOP, they requested PGD. The applicability of the duplex KG8–D16S291 was checked and the phase could be established for the wife. In the case of the husband, his mother's DNA sample of the duplex KG8–D16S291 was checked and the phase could be established for the male partner. Since they objected to a TOP, they requested PGD. The applicability of the duplex KG8–D16S291 was evaluated pre-clinically before it was applied during PGD. Another three couples at risk of transmitting ADPKD to their children had a PGD work-up with informativity and segregation testing.

ICSJ and cleavage-stage embryo biopsy

ICSJ was chosen as the fertilization method in order to avoid contamination by supernumerary sperm cells (Lissens and Sermon, 1997) and fertilization failure (Staessen et al., 1999). Ovarian stimulation, retrieval of the oocytes and subsequent removal of cumulus cells was performed according to previously described protocols (Ubaldi et al., 1995; Platteau et al., 2002; Joris et al., 2003). The ICSI procedure was performed on metaphase II oocytes as described by Devroey and Van Steirteghem (2004). Fertilization was examined 16–22 h after injection, and embryo cleavage and quality were evaluated in the morning of day 2 and day 3. Embryo culture was performed in sequential media (GI–GII VitroLife, Gothenburg, Sweden). Only embryos with at least six cells in the morning of day 3 were biopsied using laser beam drilling (Fertilase, Octax, Herborn, Germany). Two clearly nucleated blastomeres were gently aspirated through the hole (Joris et al., 2003).

Cell lysis and PCR procedure

The amplification reactions were carried out using the Expand High Fidelity Kit (Roche Diagnostics, Vilvoorde, Belgium) with the following primer sets: (1) PKD1 5'-CTCCACGGGTGGAAGGTG-3' and PKD2 5'-ACAGCAGAGCTCCGAG-3' for KG8 (GDB accession number: 377657) and (2) PKD3 5'-CCAGGCTAGTGTGTTCTCTTAA-3' and PKD4 5'-TATCCGGGATACGGGATACC-3' for D16S291 (GDB accession number: 18088). The forward primers PKD1 and PKD3 were labelled with FAM indocarbocyanine (Eurogentec, Seraing, Belgium). Immediately prior to PCR, cells were lysed by incubation at 65°C for 10 min and then PCR reaction mixes were added to a final volume of 25 μL. The PCR reaction mixes contained 0.6 μM of PKD1/PKD2 primers, 0.3 μM of PKD3/PKD4 primers, 0.3 μM of PKD1/PKD3 primers, and 0.2 μM of PKD2/PKD4 primers. The PCR reaction mixes contained 0.6 μM of PKD1/PKD2 primers, 0.3 μM of PKD3/PKD4 primers, and 0.2 μM of PKD2/PKD4 primers. The PCR reaction mixes contained 0.6 μM of PKD1/PKD2 primers, 0.3 μM of PKD3/PKD4 primers, and 0.2 μM of PKD2/PKD4 primers.
primers, 200 μM dNTPs, 1X Expand High Fidelity buffer (Roche), 5% dimethylsulphoxide, 20 mM Tricine pH 4.95 for neutralization of the ALB buffer and 1.4U Expand High Fidelity polymerase (Roche Diagnostics, Mannheim, Germany). The PCR programme was as follows: 5 min at 95°C, then 50 cycles of 30 s at 95°C, 30 s at 68°C, 30 s at 72°C and a final extension of 10 min at 72°C. The fluorescent-labelled PCR products were analysed on an Automated Laser Fluorescence Express DNA Sequencer (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) as previously described (De Vos et al., 2003).

Results

Informativity and segregation testing

During work-up, the couples were tested for informativity of the intragenic KG8 and the extragenic D16S291 marker. PCR for these markers was also carried out on family members’ genomic DNA in order to derive the couples’ haplotypes and to distinguish affected from unaffected haplotypes in the patient. The results of the informativity and segregation testing are summarized in the pedigrees shown in Figure 1.

The first couple was informative for KG8 and half-informative for D16S291. Informativity means that the chromosomes carrying the mutation can be identified in all potential genotypes, whereas for half-informative couples the identification is possible only for half of the potential genotypes.

The second couple was only informative for the extragenic marker. The recombination rate between PKD1 and this marker is estimated at 2% (Viribay et al., 1994). The couple was counselled about the misdiagnosis risk due to recombination. The development of another duplex using D16S291 and another informative-linked marker would not reduce this risk as all informative markers, in this couple, are proximal to the gene.

The third and fourth couples were informative for both markers and segregation was established for affected and unaffected partners.

Single-cell duplex PCR

A fluorescent duplex-PCR of markers linked to PKD1 was set up for indirect diagnosis of ADPKD type 1 in preimplantation embryos. Co-amplification of both the intragenic KG8 and the extragenic D16S291 markers was performed on genomic DNA of the parents, and further optimization of PCR reaction conditions was carried out on single cells. Fragments were analysed on an ALF Express automated sequencer. The KG8 PCR fragments were in the 110–116 bp range, while those of the D16S291 marker provided a minimum length of 154 bp. Owing to this difference in fragment length and the use of primers with similar annealing temperatures, both markers could be amplified in a single-round duplex-PCR. A representative ALF Express pattern for marker D16S291 is shown in Figure 2. The efficiency and accuracy of the assay were evaluated on lymphoblasts that were heterozygous for both markers and on research blastomeres at a later stage. The research blastomeres had been sampled from four embryos of the same couple and were heterozygous for both markers. The evaluation of amplification, contamination and allele drop-out (ADO) rates yielded similar results for both markers (Table II). The overall amplification efficiency was high with 74 single cells amplified out of 77 (96.1%). Only two of the 70 blanks

Figure 1. Pedigrees of ADPKD families. Only information for PGD work-up is shown. Black squares and circles indicate the affected family members and diagonal lines indicate members who have died. Haplotyping results of microsatellite markers linked to PKD1 are numbered according to the allele scored. Numbers in bold represent markers linked to affected alleles. Parentheses indicate that the phase was not established whereas square brackets indicate that the phase was deduced from family member’s haplotypes. Arrows between loci indicate recombinant haplotypes. (A) For couple 1, linkage with PKD1 was first established by analysis of D16S85 in 1994. The first child (10) of the couple (5, 6) was shown to be affected during prenatal diagnosis using this marker. Further analysis with KG8 and D16S291 was carried out in 1999 during the work-up for PGD. (B) Linkage analysis for couple 2 (6, 7) was carried out using the intragenic KG8 and four extragenic markers. (C) Couple 3 (10, 11); a previous analysis of OT2, KG8, SM6 and CW4 was extended with the analysis of D16S291 in duplex with KG8. (D) Linkage analysis for couple 4 (3, 4) using the intragenic KG8 and the extragenic D16S291.
Figure 2. Automated sequencer analysis patterns of the D16S291 marker during the second clinical cycle for couple 2. The x-axis indicates the fragment size in base pairs (bp). Lane 5 shows the 50–250 bp fragments of the 50 bp ladder standard. The internal standard of 200 bp is marked with a solid circle. Lane 1 shows the alleles of the female partner (5 and 4). Lane 2 shows the healthy allele (3) and the affected allele (2) of the affected male partner. An affected embryo with alleles 4 and 2 is shown in lanes 3 and 4 (blastomere 1 in lane 3 and blastomere 2 in lane 4). The healthy allele from the mother (4) has dropped out in the first blastomere. Lanes 6 and 7 indicate unaffected alleles (5 and 3) in two blastomeres from the same embryo. Lanes 8 and 9 show alleles 4 and 2 in two blastomeres of an affected embryo. Lanes 10 and 11 represent again the profile of an unaffected embryo with alleles 5 and 3 in the two cells analysed.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Amplification (%)</th>
<th>ADO (%)</th>
<th>Contamination (%)</th>
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<tr>
<td></td>
<td>KG8</td>
<td>D16S291</td>
<td>KG8</td>
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<tr>
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<td>57/59 (96.6)</td>
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<td>Research blastomeres</td>
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<td>17/18 (94.4)</td>
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<td>74/77 (96.1)</td>
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were contaminated (4.3%) with KG8 and none with D16S291, whereas ADO, i.e. the random failure of amplification of one of the two alleles in a heterozygous cell, occurred in one of the 74 amplified cells (1.4%) for the D16S291 marker and in none for KG8.

Clinical cycles

Five clinical cycles were carried out for three couples. A sixth cycle, PGD1 for the fourth couple, is planned in the near future. The details of the clinical cycles are summarized in Table III. During PGD1 for the first couple, seven embryos were analysed: three of them were shown to be unaffected, three were affected and one remained without diagnosis as both the biopsied cells gave a haploid genotype. During re-analysis, this abnormal haploid genotype was confirmed together with the genotypes of the three affected embryos and one unaffected non-transferred embryo unsuitable for freezing. Two unaffected embryos were transferred on day five, resulting in a singleton pregnancy and the birth of a healthy boy at term. The couple consequently declined a control prenatal diagnosis.

The second couple was only informative for the extragenic D16S291 marker. During their first cycle with nine oocytes at the start, only two embryos were available for analysis. One embryo remained without diagnosis as only maternal alleles were detected in both the blastomeres. Post-PGD analysis again showed only the maternal haplotypes. The second embryo showed amplification failure in one blastomere and an unaffected genotype in the other. This embryo was transferred but the patient did not become pregnant. Of the nine embryos available for biopsy in the second cycle, seven were shown to be unaffected and two affected. Two unaffected embryos were transferred on day five and another two frozen on day six. Re-analysis of the remaining embryos (one was lost during transfer to the PCR tube) confirmed their diagnosis and indicated that ADO had occurred in one of the blastomeres during PGD. This cycle resulted in a singleton pregnancy. The unaffected genotype of the fetus was confirmed by amniocentesis using an assay with three linked microsatellite markers. Meanwhile, a healthy girl was born.

The third couple was fully informative for both markers. In their first cycle, four embryos were diagnosed as affected, whereas the fifth embryo was without diagnosis because of amplification failure in both the blastomeres. The affected genotypes were confirmed during post-PGD analysis and the fifth embryo without diagnosis was shown to be affected as well. In their second cycle, there was again no embryo transfer as the single embryo biopsied was diagnosed as affected. Re-analysis confirmed the embryo to be affected and indicated ADO during PGD.

In total, 48 blastomeres from 24 embryos were analysed in five clinical cycles. The overall amplification rate during PGD was 93.8% (45/48). Contamination was absent and ADO was detected in two of 41 heterozygous amplified cells (4.9%). Three embryos of 24 (12.5%) remained without diagnosis, two with presumably an abnormal chromosomal constitution and one because of a double amplification failure. In two cycles, embryo transfer was not possible as there were no unaffected embryos. Embryo transfer was carried out in three cycles and resulted in a healthy baby for two couples.

Discussion

This report describes a single-cell PCR assay for diagnosis of ADPKD type 1 in preimplantation embryos. Direct mutation analysis of *PKD1*, the gene involved in type 1, is rather difficult because of the size and complexity of the gene. Analysis on genomic DNA often uses long-range PCR, but this is not (yet) feasible at the single-cell level. Therefore, an indirect pedigree-based linkage analysis

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<th>Embryos analysed (n)</th>
<th>Blastomeres amplified (n)</th>
<th>Blastomeres with ADO (n)</th>
<th>Unaffected embryos (n)</th>
<th>Affected embryos (n)</th>
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with microsatellite markers was used for single-cell analysis. The assay involves a duplex-PCR of the intragenic KG8 and the extragenic D16S291 marker in one round, followed by fragment analysis on an automated sequencer. The duplex-PCR was optimized on single cells to meet the standards of high amplification efficiency and low contamination and ADO rates. Contamination and ADO are major problems in single-cell analysis as they may lead to misdiagnosis. The efficiency and accuracy of the assay were high in preclinical tests as well as in clinical applications. The amplification efficiency ranged from 96.1% pre-clinically (Table II) to 93.8% in the clinical cycles (Table III), while ADO and contamination rates were as low as 1.4 and 2.9%, respectively, during pre-clinical tests and 4.9 and 0%, respectively, in the clinical cycles. Post-PGD re-analysis of 16 embryos did not reveal any diagnostic errors. Three embryos out of 24 (12.5%) remained without diagnosis. In one embryo, amplification completely failed in each of the two cells analysed. Post-PGD analysis of the whole embryo indicated that the embryo was affected. A higher incidence of non-amplification and ADO in blastomeres compared to other cell types has been observed before (Rechitsky et al., 1998; De Rycke et al., 2001; Thornhill and Snow, 2002). Effective measures can be taken against some causes of amplification failure and ADO. The visualization (microscopic guidance) of the transfer and subsequent lysis of a nucleated, morphologically normal blastomere to a PCR tube with ALB and the use of small amplicon sizes, efficient cell lysis buffers, optimal PCR conditions and sensitive detection systems all contribute to minimize amplification failure of one or two alleles (Piyamongkol et al., 2003). In the case of the two other PGD embryos remaining without diagnosis, single alleles were detected in the blastomeres during PGD as well as in the whole embryos during re-analysis. A possible explanation may be that these embryos were monosomic for chromosome 16. Using fluorescence in-situ hybridization, it has been shown that chromosome 16 is one of the most frequently involved chromosomes in aneuploidy in embryos (Abdelhadi et al., 2003; Munne et al., 2004). Problems of amplification failure and ADO as a consequence of chromosomal abnormalities or as a consequence of DNA degradation in degenerating cells from poor quality embryos cannot actually be solved, but strategies can be designed to reveal these problems at least. Co-amplification of linked markers is a useful strategy that allows the detection of both contamination and ADO and yields a substantial improvement in the accuracy of the diagnosis. This approach has also been used for other diseases (for cystic fibrosis in Dreesen et al., 2000; for X-linked adrenoleukodystrophy, X-linked hydrocephalus and X-linked incontinentia pigmenti in Gigarel et al., 2004).

Applying a marker-based duplex-PCR to two blastomeres could further reduce the risk of misdiagnosis. However, the removal of two blastomeres from a 3-day-old embryo may compromise the embryo’s implantation potential. The development of an indirect assay not only circumvented the difficulties of mutation detection in PKD1; it also reduced the most labour-intensive part of preimplantation testing, i.e. setting up and pre-clinical testing of the assays, to a minimum. The development of mutation-based protocols, if feasible, would indeed require repeated optimizations. Another important advantage of the marker-based assay is that it can be applied for all couples that are informative for the intragenic marker or preferentially for both markers. Two of the four couples were informative for both markers and one couple was informative for the intragenic marker and half-informative for the extragenic marker. Couples that are only informative for the extragenic marker, like the second couple, are counselled about the risk of misdiagnosis when recombination occurs between the PKD1 gene and the marker. The recombination rate is estimated at 2%, which would raise the risk of a real misdiagnosis, i.e. the transfer of an affected embryo that had been diagnosed as unaffected, by 1%. Recombination leading to the opposite event, i.e. the diagnosis of an unaffected embryo as affected, will not lead to embryo transfer, but will diminish the number of healthy embryos available for transfer and reduce the chances for pregnancy. Patients are advised to have a control prenatal diagnosis, which relies on flanking polymorphic markers. The second couple opted for prenatal diagnosis as they were primarily interested in karyotype analysis (the female partner was 37) and secondly, as a control for ADPKD. Three informative markers linked to PKD1 were used during prenatal testing; however, since all three markers were proximal, the risk for misdiagnosis remained unchanged.

A drawback of the marker-based approach, specifically for ADPKD, is the need for several affected family members in order to establish which of the two possible genes underlies the disease in the family. In case of diseases caused by defects in a single gene, the availability of at least one first-degree relative of the patient is sufficient to set up marker-based PGD protocols.

In conclusion, an accurate and reliable marker-based duplex-PCR was developed for the selection of preimplantation embryos that are unaffected by ADPKD type 1. Two unaffected children were born after five clinical cycles for three couples. The assay developed can be used for other couples requesting PGD for ADPKD type 1 on condition that they are informative for the used markers.

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