Single intragenic microsatellite preimplantation genetic diagnosis for cystic fibrosis provides positive allele identification of all CFTR genotypes for informative couples

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This study is part of a strategy aimed at using fluorescent polymerase chain reaction (PCR) on informative genetic microsatellite markers as a diagnostic tool in preimplantation genetic diagnosis (PGD) of severe monogenic disease. Two couples, both of whom had previously had children who were compound heterozygote for severe cystic fibrosis mutations, were offered PGD using fluorescent PCR of the highly polymorphic cystic fibrosis transmembrane conductance regulator (CFTR) intragenic microsatellite marker IVS17bTA. Cleavage-stage embryo biopsy followed by PCR resulted in transfer of one unaffected carrier embryo for each couple. This approach eliminates the need for single cell multiplex PCR strategies to detect CF compound heterozygotes. It also provides a control of chromosome 7 ploidy in the blastomeres and a selection against allele dropout by positive detection of each CFTR copy of all genotypes in preimplantation embryos from genetically informative families.

Key words: allele dropout/CFTR/fluorescent PCR/polymorphic microsatellite marker/preimplantation genetic diagnosis

Introduction

Correct assignment of genotypes in polymerase chain reaction (PCR)-based preimplantation genetic diagnosis (PGD) requires absolute certainty of allele amplification. For simple autosomal recessive conditions, settling for elimination of homozygous affected embryos is generally considered an acceptable option. However, when dealing with dominant or compound heterozygous disease, positive identification of the alleles from both parents is imperative before deciding whether or not to accept an embryo for transfer. In cases of compound heterozygosity, direct multiplex amplification of alleles may produce complicated patterns of PCR products. It may then be difficult to distinguish between homozygote healthy cells or healthy carrier cells on one hand and allele dropout (ADO) in diseased cells on the other. Previously, cases of misdiagnosis in PGD for compound heterozygosity have been reported (Harper and Handyside, 1994).

Microsatellites are polymorphic loci of DNA containing a repeated nucleotide sequence. The repeat unit consists of two to seven nucleotides, and the total size of a microsatellite varies in discrete steps of repeat unit length. Microsatellites are found evenly distributed throughout the genome (Edwards et al., 1992). When microsatellite allele frequencies and the span of allele sizes are known, established genetic linkage between specific alleles and distinct repeat unit numbers of polymorphic microsatellites provide useful diagnostic tools. Couples seeking PGD often have a personal history of affected children and terminated pregnancies. Thus relevant samples from the families are usually available to establish haplotypes for genetically informative markers linked to the locus of the disease gene.

Cystic fibrosis (CF) is an autosomal recessive condition caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, and it is by far the most frequent diagnosis encountered in PCR-based PGD. Within the relatively genetically homogeneous population of Denmark, 88% of CF carriers share the common ΔF508 mutation (Schwartz et al., 1992). Other mutations are very diverse, covering the spectrum from single amino acid changes to fairly large intragenic deletions, with several hundred mutations in the European population reported by the Biomed CF Mutational Analysis Consortium (Estivill et al., 1997). The identification of each pathogenic mutation is necessary when establishing the correct diagnosis of CF patients and in genetic evaluation of CF families prior to prenatal or PGD. However, direct mutational analysis based on knowledge of CF genotypes is
not necessarily feasible on a single blastomere level or within the limited time available for PGD.

In a recent paper (Dreesen et al., 2000), an approach using four microsatellite markers flanking the CFTR gene on both sides was used to establish a single cell protocol for CF diagnosis applicable for ~87% of couples carrying CFTR mutations. Several polymorphic regions consisting of repeated dinucleotide units are also found within the human CFTR gene sequence (Zielenski et al., 1991a). One of these is IVS17bTA: a highly polymorphic microsatellite TA-repeat located in intron 17b (Zielenski et al., 1991b) reported to be informative for 89% of couples carrying different CFTR mutations (Mornet et al., 1992). At least 24 different alleles with sizes ranging from 7 to 56 TA repeat units have been reported in carriers of CF (Zielenski et al., 1991b). Due to its high degree of heterozygosity (Zielenski et al., 1991b; Mornet et al., 1992; Morrall et al., 1993), this marker has been used in initial prenatal testing for CF in cases of rare or compound heterozygous mutations (Casals et al., 1996).

The aim of this study has been to establish a fluorescent PCR procedure by use of this highly polymorphic intragene microsatellite marker alone in PGD for CF. The procedure is independent of which CFTR mutations are involved and is thus applicable in cases of CF compound heterozygosity. It does not involve nested or multiplex PCR, thus reducing the risks of exogenous PCR product contamination and ADO. We also omit the cumbersome work of establishing separate single cell protocols for each mutation and diminish the chance of confusing ADO in heterozygous cells with homozygosity by always providing positive identification of each CFTR allele in embryos from genetically informative couples.

Materials and methods

Genetic background of the PGD patients

The two couples for whom PGD was performed both had children who were compound heterozygous for severe CFTR mutations. These mutations were known prior to inclusion into the PGD programme and DNA samples from both couples and their children were available for establishing CFTR/IVS17bTA haplotypes. In couple I, the mother was a carrier of the ΔF508 deletion, whereas the father carried the mutation W1282X. In addition to one child with severe CF the couple had also experienced one previous abortion on basis of a prenatal diagnosis revealing an affected fetus. In couple II, the mother carried a deletion covering at least exon 1 of the CFTR gene. The exact boundaries of this deletion are not known. The father was heterozygous for the mutation R334W. This couple had one CF child and one child who was a healthy non-carrier. Six previous pregnancies were terminated after prenatal diagnosis for CF.

Contamination control procedures

All analyses involving DNA from the patients and their families were performed outside the Fertility Clinic. PCR machines and all instruments for post-PCR analysis were also located elsewhere, and no equipment or clothing used during these procedures were allowed back into the PGD laboratory in the Fertility Clinic. Prior to each round of PGD, several aliquots of each medium used during the sperm preparation, intracytoplasmic sperm injection (ICSI) and biopsy procedures were analysed for the presence of exogenous DNA by fluorescent PCR according to the protocols followed for blastomeres. All handling of embryos was performed in a class II flow hood with the operators wearing caps, surgical masks and gloves.

Embryo culture and biopsy procedure

Cleavage stage embryos were obtained after stimulation, aspiration and ICSI treatment according to the Fertility Clinic’s standard procedures. After fertilization by ICSI, the embryos were cultured in Petri dishes (Falcon) in 30 µl droplets of IVF culture medium (Medi-Cult) overlaid with paraffin oil (Medi-Cult) for 48 h and then in 30 µl droplets of M3 medium (Medi-Cult) covered with paraffin oil until transfer early on day 4. Embryo biopsy was performed on the morning of day 3. One biopsy dish (Falcon 1006) containing one 10 µl droplet of Ca²⁺- and Mg²⁺-free biopsy medium (Medi-Cult) and two 10 µl droplets HEPEs-buffered IVF medium (Medi-Cult) covered by 4.5 ml paraffin oil was prepared in advance for each embryo. Each embryo was incubated in biopsy medium for 2 min at 37°C, then rinsed in the first HEPEs-buffered IVF medium droplet and transferred to the second droplet. Biopsies were performed under microscope with a 1480 nm laser (Labotec) to punch holes in the zona pellucida through which one or two blastomeres were biopsied. For embryos with five or more blastomeres, two cells were removed. For embryos with fewer than five blastomeres, only 1 cell was removed. The embryo was then immediately transferred back into fresh M3 medium and incubated until selection and transfer of the embryos on the following morning.

Blastomere lysis

Biopsied blastomeres were visually checked for the presence of a nucleus and immediately transferred in ~2 µl of IVF medium into PCR tubes containing 3 µl of lysis buffer consisting of 2 µl 125 µg/ml PCR grade proteinase K (Roche/Boehringer Mannheim) and 1 µl 17 µmol/l sodium dodecyl sulphate (SDS). Cell transfer was visually verified by microscopy. For each embryo, ~2 µl of the medium was transferred to a separate tube also containing lysis buffer and examined by PCR for contamination with exogenous DNA. The buffer was overlaid with two drops of light mineral oil (Sigma) and the blastomeres were lysed at 37°C for 90 min, followed by inactivation of Proteinase K by heat denaturation at 99°C for 15 min.

PCR reactions and analysis

PCR was performed immediately after cell lysis. The region encompassing IVS17bTA was amplified using primers AT17D1.2: 5’ GCT GCA TTC TAT AGG TTA TC 3’ (Zielenski et al., 1991b) and AT17R1.2-Fam: 5’ GCA AAC CTG TGT GCA TCG 3’ (Morrall and Estivill, 1992), producing fluorescent DNA fragments of 197 to ~300 bp.

The reactions were performed on DNA from single blastomeres or on controls of 50 pg genomic DNA in 50 µl total volumes with 2.5 U of AmpliTaq Gold DNA polymerase (Perkin Elmer). The PCR mix contained 0.5 µmol/l of each primer, 200 µmol/l each of dATP, dCTP, dGTP and dTTP (Roche/Boehringer-Mannheim), 1.5 mmol/l MgCl₂ and 1× AmpliTaq Gold buffer. After an initial 10 min incubation at 95°C to activate the polymerase, 40 cycles of 96°C for 20 s, 55°C for 25 s and 72°C for 35 s followed by a final 10 min hold at 72°C were performed on a Perkin Elmer 9600 or 9700 Thermocycler.

For loading onto an ABI 310 Genetic Analyzer, 1.5 µl of PCR product was mixed with 0.5 µl of Rox-labelled size standard GS500 (Perkin Elmer) and denatured in 12 µl of deionized formamide at 95°C for 2 min. The samples were then snap cooled on wet ice (0°C). Electrophoresis was performed in polymer POP4 (Applied Biosystems), 15 kV with 5 s injection times and 24 min electrophoresis...
times on a 47 cm 50 µm capillary (Applied Biosystems). After electrophoresis, fluorescence data was filtered and analysed using GeneScan software (Applied Biosystems). The PGD programme is approved by the Danish Medical Ethics Committee.

Results

Results of PCR of IVS17bTA on single blastomeres and diluted DNA

PCR analysis of amplification efficiency and allele sizes for marker IVS17bTA on blastomeres donated from IVF patients and on diluted DNA of known haplotypes showed that products differing in repeat unit numbers from 7 to 55 TA were reproducibly amplified and detected using the sample preparation and PCR protocol described. ADO rates were consistently low, but these were not calculated due to the relatively small number of single cells examined.

CF genotypes versus number of IVS17bTA repeat units for the PGD couples

Before including the couples into the PGD programme, IVS17bTA/CF haplotypes were determined to establish whether they were genetically informative. The CFTR status of each microsatellite allele was assigned using PCR by comparing the status of their children’s alleles with those of the parents. The results of this analysis are shown in Figure 1. In couple I, the healthy CFTR alleles thus segregated with IVS17bTA repeat unit numbers of 29 (maternal) and 38 (paternal), whereas the maternal ∆F508 segregated with 31 and the paternal W1282X with seven repeat units respectively. For couple II, the IVS17bTA number of repeat units for the healthy alleles were 55 (maternal), and 33 (paternal) with seven for the maternal deletion and 48 for the paternal R334W.

PCR amplification on single blastomeres or small amounts of genomic DNA and subsequent detection and accurate size determination of IVS17bTA products posed no technical problem for any alleles within the expected size range of 7 to 56 TA. The corresponding products are 197–309 bp. Their appearance after capillary electrophoresis is typical of dinucleotide microsatellites amplified using a polymerase with an intrinsic A activity, generating what is observed as a split signal. This extra signal is one nucleotide longer than the actual product. In addition, a pattern of shorter stutter bands separated from the main product in steps of two nucleotides appears. These bands, representing infidelity in in-vitro dinucleotide repeat amplification, may actually aid in distinguishing real signals from any unspecific product of PCR amplification.

Table I. Results of the first round of preimplantation genetic diagnosis (PGD) for the two compound heterozygous cystic fibrosis (CF) families

<table>
<thead>
<tr>
<th>Family I</th>
<th>Family II</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of MII oocytes retrieved</td>
<td>11</td>
</tr>
<tr>
<td>No. of oocytes injected</td>
<td>10</td>
</tr>
<tr>
<td>No. of cleaved embryos</td>
<td>4</td>
</tr>
<tr>
<td>No. of embryos biopsied</td>
<td>4</td>
</tr>
<tr>
<td>No. homozygote healthy</td>
<td>0</td>
</tr>
<tr>
<td>No. heterozygote healthy</td>
<td>2</td>
</tr>
<tr>
<td>No. compound heterozygote CF</td>
<td>1</td>
</tr>
<tr>
<td>No. trisomy 7</td>
<td>1</td>
</tr>
<tr>
<td>No. failed diagnosis</td>
<td>0</td>
</tr>
<tr>
<td>No. of embryos cleaved after biopsy</td>
<td>4</td>
</tr>
<tr>
<td>No. of embryos transferred</td>
<td>1</td>
</tr>
</tbody>
</table>

MII = metaphase II.

aSix blastomeres were analysed for this embryo, none of which produced any IVS17bTA signal.

Outcome of the PGD treatment

After ICSI, four and three embryos of a transferable quality (4–9 cells) were observed on day 3 for couples I and II respectively (see Table I). One cell was removed for analysis for embryos with fewer than five blastomeres; two cells were removed for the larger embryos. For the first couple four embryos were analysed, resulting in two carrier embryos, one compound heterozygote CF embryo and one embryo trisomic for chromosome 7, the latter presenting with two paternal alleles as verified in three additional cells by disaggregation of the whole embryo and PCR analysis of each remaining cell. For the second couple, three embryos were analysed and two carrier embryos were identified, whereas analysis failed for the third embryo. Results of capillary electrophoresis of PCR products generated from blood from four family members (mother, father and the two children) from family II along with a single blastomere from the embryo that was later transferred is shown in Figure 2. Disaggregation and analysis of 4 additional blastomeres from the third embryo on day 4
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Figure 2. Capillary electrophoresis of fluorescent polymerase chain reaction (PCR) products after analysis of intragenic marker IVS17bTA on DNA (four first lanes) and a single blastomere from family II. The blastomere is derived from a carrier embryo transferred after analysis. Their child with cystic fibrosis (CF) inherited alleles containing 7 and 48 TA repeats from its mother and father respectively, whereas the transferred embryo was a CF carrier with its mother’s deletion in exon 1 of the CFTR gene (TA repeat numbers of 7 and 33). Red signals are from size standard GS500-rox, whereas the IVS17bTA product is blue (Fam-labelled reverse primer). Simultaneous amplification of alleles of 7 and 55 TA in couple II produced uneven signals with an almost 60 times more intense peak for the shorter allele. Both alleles are still clearly identified. For all other observed alleles the amplification rate differences are intermediate with the shorter allele more efficiently amplified.

still produced no signal from either copy of the CFTR gene. This raises the possibility that the embryo may in fact have been nullisomic for chromosome 7. For both couples a single heterozygote carrier embryo was transferred, however none of these transfers resulted in pregnancies.

Discussion

Some 5000 monogenic diseases in man have been registered (OMIM), and their corresponding genes are rapidly being identified and characterized. As the sequencing of the human genome is progressing, an increasing number of polymorphic markers linked to disease loci are identified. Microsatellites are already widely used as genetic tools for individual identification and diagnosis, and their advantages for PGD are obvious. Combined with the sensitivity obtained with fluorescent PCR, PGD approaches involving the use of such highly polymorphic markers have become feasible.

Couples seeking PGD on the basis of recessive genetic disease have often already had an affected child, a fact that facilitates genetic haplotyping with markers linked to the disease locus prior to PGD. Although both couples in this study did have affected children, similar haplotype information may come from other family members, as may often be the case for PGD of dominant disease. Both couples included in this study produced embryos with carrier CFTR genotypes after one round of PGD for compound heterozygous CF. Positive identification of each CFTR gene copy in the biopsied blastomeres verifies segregation of one specific allele from each parent.
The presence of linked polymorphic markers is particularly valuable when dealing with minuscule amounts of DNA template of a known genetic background. Although multiplex PCR protocols may be used for direct detection of two different mutations, the analysis will be based solely on the absence or presence of the diseased allele from the parents and the intrinsic risk of ADO makes this approach undesirable. In an alternative approach (Dreesen et al., 2000) it was chosen to multiplex four highly polymorphic microsatellite markers flanking and linked to the CFTR gene. Overall, 74% blastomeres analysed were reported to have the correct number of amplified alleles and the assay is estimated to be informative in 87% of couples.

The equally high degree of heterozygosity of the microsatellite IVS17bTA (89%, Mornet et al., 1992) makes this marker a very useful tool for PGD on CF, particularly in regions where the numbers of different CFTR mutations are high. No cross-over has been reported so far within the CFTR gene that would affect its accuracy in diagnosis by altering the linkage between mutation and marker. However unlikely should such cross-over appear, it would have a harmful effect for the outcome of PGD when the marker based analysis indicates a healthy carrier embryo based on assumed haplotype for the chromosome where cross-over has occurred. Contamination of media or reagents for PCR by exogenous human DNA is a problem of constant concern in single cell analysis, as any such contamination will contribute to the reaction product at least as much as the blastomere’s chromosomes. The heterozygosity of a highly polymorphic microsatellite marker may aid in revealing contamination with exogenous DNA, since this will usually present alleles differing in size from those of the parents. Potential misdiagnosis is thus more likely to be discovered when highly polymorphic markers are used.

There are potential amplification difficulties associated with the use of polymorphic dinucleotide repeat markers. Preferential amplification of one allele is frequently encountered when a limited amount of template is available, in particular when the alleles differ considerably in size. This was also observed in the IVS17bTA protocol, where the lengths of the TA repeat region may differ with >100 bp for the two alleles. When using fluorescent primers and analysing the products by high-resolution capillary electrophoresis we experienced no problems in simultaneous detection of allele sizes of 7 (197 bp) and 55 TA (307 bp) within single cells.

In the case of CF, several other intragenic markers have been reported (Zielenski et al., 1991a) and may be chosen for PGD in cases where IVS17bTA is either uninformative or the allele size differences are too large for reliable detection of all potential genotypes in DNA from a single cell.

ADO during PCR-based PGD on single blastomeres is a source of potential misdiagnosis of single gene disease with either sex-linked or dominant autosomal inheritance or in cases of recessive inheritance in compound heterozygotes (Wells and Sherlock, 1998). The frequency of failure to amplify both alleles in presumably heterozygous blastomeres been reported to be larger than that found in somatic cells (Rechitsky et al., 1998). In some cases, the basis of ADO may be of a technical nature. Earlier reports have shown that ADO is associated with misdiagnosis in heterozygote carrier embryos (Ray et al., 1998). Although affected children will not be the result when dealing with homozygous recessive conditions, it may limit the number of embryos actually considered for transfer and thus contribute to a decreased success rate after PGD.

As methods and detection thresholds have continued to improve, evidence suggests that failure to segregate chromosomes normally in female gametes may be responsible for some apparent ADO observed in human embryos (Hunt, 1998). Cleavage-stage embryos are also frequently observed to be chromosomal mosaics (Harper et al., 1995; Delhanty et al., 1997). Fluorescent in-situ hybridization was used to show that 6.5% of the nuclei in cleavage stage embryos lacked one or both copies of chromosome 7 where the CFTR gene resides (Kuo et al., 1998). In this study, we observed one embryo that was clearly trisomic for chromosome 7, having inherited both paternal alleles. In addition, one of the embryos failed to show any IVS17bTA signal in six blastomeres, indicating possible nullisomy for chromosome 7 in this particular embryo.

There is currently no established consensus concerning protocols for handling of human blastomeres for PGD (ESHRE PGD Consortium, 1999), although the methods chosen for lysis of the cells and denaturation of chromosomal DNA clearly have impact on amplification outcome and on the frequency of ADO (Gitlin et al., 1996; Ray and Handyside, 1996; El-Hashemite and Delhanty, 1997). One way to safeguard analysis outcome from this problem has been to remove two cells from each cleavage state embryo and compare the results of separate analysis of the two cells to establish the genotype of the embryo. However, not all day 3 embryos contain enough blastomeres for one to feel comfortable removing such a relatively large portion of the embryo. When using an informative linked microsatellite marker, two separate alleles are always seen when successful amplification has occurred, regardless of the embryo’s genotype. Amplification failure will thus always be detected as such, and one may, therefore, choose to rely on information obtained from one cell in cases where few blastomeres are present in an embryo at the time of biopsy. This may also open the possibility of earlier biopsy. Day 2 embryos have a looser connection between blastomeres and may thus be biopsied using gentler conditions leading to transfer on day 3. However, the relatively larger hole produced in the zona pellucida for biopsy on day 2 is found to have a negative effect on embryo development and this would have to be considered.

We believe that microsatellite analysis, such as the one we have employed in this study, will prove a valuable tool in PGD for an increasing number of single gene diseases. So far we have also used linked microsatellites in PGD for the triplet expansion disease myotonic dystrophy to avoid the possibility of misdiagnosis in a case where the parents carried identical normal alleles. The relative ease, sensitivity and robustness of fluorescent PCR of short repeat sequences make it an attractive approach in diagnosis for patients undergoing PGD.

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References


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