Preimplantation genetic diagnosis (PGD) represents an alternative to prenatal diagnosis and allows selection of unaffected IVF embryos to establish pregnancies in couples at risk of transmitting a genetic disorder (1). Although PGD was offered initially to couples at risk of having children with monogenic diseases, numerical and structural chromosomal abnormalities can also be diagnosed by this method (2). The scope of PGD has also been extended to chromosomal aneuploidy screening in infertile couples (3).

Despite the significant advantages provided by PGD there are still technical limitations. For single-gene disorders, the development and validation of highly sensitive amplification strategies are required, often using nested polymerase chain reaction (PCR), whole-genome amplification (WGA), or fluorescent PCR methods. The main disadvantages of WGA are the generation of nonspecific amplification artifacts, incomplete coverage of loci, inefficiency of microsatellite amplification, and the generation of DNA <1 kb long (4). On the other hand, the main disadvantage of nested and fluorescent PCR is the difficulty in choosing primers for multiplex PCR (5). For that reason, there is a need for a technique that would be able to amplify the single-cell DNA with a high fidelity that suits the diagnosis of any known single-gene disorder by standard PCR technique.

Whole-genome amplification by isothermal multiple displacement amplification (MDA) is based on the use of \( \phi 29 \) DNA polymerase and random primers. The \( \phi 29 \) polymerase combines high processivity with a strand displacement ability leading to the synthesis of DNA fragments \( >10 \) kb and favoring uniform representation of sequences (6). Multiple displacement amplification is a technique used in the amplification of very low DNA quantities in clinical samples (4). Sequence representation in the amplified DNA assessed by multiple single nucleotide polymorphism analysis is equiv-
Multiple displacement amplification in PGD

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alent to genomic DNA and amplification is superior to PCR-based methods (7).

Marfan syndrome is an autosomal dominant disorder of the connective tissues. The underlying defect in Marfan syndrome is due to a defective fibrillin gene (FBN-1) on chromosome 15q15-21 (8). The first method for PGD of Marfan syndrome, reverse transcriptase–polymerase chain reaction (RT-PCR), was used to show the presence of paternal mRNA carrying the disease-causing mutation in preimplantation embryos (9). A panel of highly informative intragenic microsatellite polymorphisms are widely interspersed in FBN-1 and these linked markers have been used for the development of PGD for Marfan syndrome (10, 11).

In this report, we investigate the use of MDA from single cells as a universal first step for PGD of single defects. Moreover, MDA was used to amplify single blastomere DNA, which was then used in the Marfan syndrome–PGD program.

MATERIALS AND METHODS

Lysis of Single Cells

Lymphocytes from a female carrier of Huntington expansion and heterozygous for the mts-2, mts-4, X22, and Fragile X loci were separated from blood by centrifugation over Ficoll, washed and resuspended in phosphate-buffered saline. Single cells were collected and transferred to 0.2-mL PCR tubes containing 0.5 μL of alkaline lysis buffer. The samples were kept at −80°C for 30 minutes. Cells were lysed by incubation at 65°C for 10 minutes (11). Lysis was then stopped by adding 0.5 μL of neutralization buffer (11).

Multiple Displacement Amplification Protocol

Cell lysates were used directly for MDA. The WGA by isothermal MDA was achieved using bacteriophage φ29 DNA polymerase, exonuclease-resistant phosphorothioate-modified random hexamer oligonucleotide primers, and reaction buffer according to the manufacturer’s instructions (Amersham Biosciences, Little Chalfont, United Kingdom) in a 20-μL reaction at 30°C (16 hours). The reaction was terminated by incubation at 65°C for 10 minutes to inactivate the enzyme and the amplified DNA stored at −20°C.

Polymerase Chain Reaction Analysis

One microliter of MDA product was used for PCR analysis. The PCR for Huntington expansion screening was carried out using the TaKaRa LA Taq kit (Takara Bio Inc.). The primers included HU4 (5’-GGCGGCTGAGGAAGCTGAGGA-3’), labeled at 5’ with 6-FAM, whereas the reverse primer (5’-TAATGAGAGTTGGAAAGAAA-3’) was unlabeled. The PCR product (2 μL) was mixed with 2 μL of loading buffer and loaded on an ABI PRISM 3100 sequencer. The results were processed using the GeneScan Analysis software.

To use linkage analysis in Marfan syndrome–PGD, two intragenic markers (mts-2 and mts-4) were amplified using MDA amplification products. The primers used were described by Pereira et al. (14), the forward primers were labeled at 5’ with 6-FAM. A reaction mix, total volume of 25 μL, contained 100 pmol of each primer, 200 mM of dNTPs, 1X buffer provided by the manufacturer, and 1 U of DNA polymerase provided by the TaKaRa La Taq kit (Takara Bio Inc.). Polymerase chain reaction was performed as follows: 5 minutes at 96°C, 10 cycles of 30 seconds at 96°C, 30 seconds at 58°C, and 30 seconds at 72°C, followed by 31 cycles for 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C, and 5 minutes extension at 72°C. Two microliters of the PCR product was mixed with 2 μL of loading buffer denatured by boiling for 5 minutes and loaded on the ABI PRISM 3100 sequencer. The results were processed using the GeneScan Analysis software.

For sex-linked diseases, identification the sex of the embryos allows transfer of unaffected embryos to the patient. Sexing of human DNA by PCR-based methodology can be accomplished by amplifying X–Y homologous genes. To assess sex status of embryos a new X/Y chromosome marker, X22 (15), was detected by fluorescent PCR. The forward primer (5’-TAATGAGAGTTGGAAAGAAA-3’) was 5’ labeled with 6-FAM, whereas the reverse primer (5’-CCCATTGTGTCTACTTGAGA-3’) was unlabeled. The PCR amplification was performed for 25 cycles at the fol-
lowing temperatures: 95°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1 minute. The amplification products were sized using an ABI 3100 DNA sequencer and GeneScan software.

Patient Description and Informativity Test

The 30-year-old husband has Marfan syndrome. He had an aneurysm of the aorta for which he had undergone surgery and he showed ocular signs of Marfan syndrome. He carried a novel causative mutation 2446 T→C in exon 20 of the FBN-1 gene. This mutation results in the amino acid substitution of cysteine for an arginine at position Cys816Arg. His wife is 27 years old and did not present with any type of clinical alteration of interest. The affected husband was heterozygous at the mts-2 and mts-4 locus. He showed an allele of 140 bp and allele of 161 bp for mts-2. Moreover, he carried two alleles of 109 bp and 106 bp for the mts-4 locus. The 140-bp and 109-bp alleles cosegregate with Marfan syndrome in this family. The wife was heterozygous for both alleles. She carried 153-bp and 157-bp alleles for the mts-2 locus and 106-bp and 115-bp alleles for the mts-4 locus. This study had the approval of the Instituto Bernabeu Review Board.

IVF Cycle and Intracytoplasmic Sperm Injection Procedure

Intracytoplasmic sperm injection (ICSI) was carried out 4 hours after oocyte retrieval on a heated stage (model MATS-U505R30; Tokai Hit Thermoplate, Japan) at 37°C, mounted on an inverted microscope (Nikon Eclipse TE2000, Japan) equipped with Hoffmann modulation optics and Narishige (Narishige, Japan) micromanipulation system. Microinjection was performed according to Van Steirteghem et al. (16). After taking oral contraceptives for 1 month, a long luteal protocol was used to stimulate follicular development, which includes pituitary desensitization with leuprolide acetate (LA) agonist (Ginecrin Depot; Ross-Abbott, Madrid, Spain) and ovarian stimulation with a combination of recombinant human FSH (Gonal F; Serono, London, United Kingdom) and hMG (HMG-Lepori; Farma-Lepori, Barcelona, Spain). Ovarian response was monitored by transvaginal ultrasound and E2 concentration. Ovulation was induced with 250 μg of recombinant hCG (Ovitrelle; Serono). Oocytes were aspirated 36 hours after hCG administration by a transvaginal ultrasound-guided needle aspiration under sedation. Surrounding oocyte cumulus and corona radiata cells were removed by a brief exposure to 80 IU/mL of hyaluronidase (Hyase; Vitrolife, Göteborg, Sweden) followed by gentle pipetting. Only metaphase II oocytes were injected and then incubated individually in 30-μL droplets of G1.3 medium (Vitrolife AB, Kungsbacka, Sweden) covered with sterile equilibrated mineral oil (Ovool; Vitrolife) at 37°C in an atmosphere of 6% CO2. Fertilization was assessed 16–18 hours after ICSI. Further development was evaluated in the morning of day 2 and again at day 3, when embryos were evaluated before biopsy.

Blastomere Biopsy of Cleavage Embryos

The embryos were biopsied in the morning of day 3. A noncontact, 200-mW diode laser system (Saturn, Research Instruments LTD, Cornwall, United Kingdom) coupled to an inverted microscope was used to deliver two to four laser pulses of 5.274 millisecondes to the zona pellucida (ZP), creating a funnel-shaped hole. One clearly nucleated blastomere was then gently aspirated through the hole. In one embryo, compaction had already started, resulting in difficulties with the biopsy. This embryo was incubated for a few minutes in Ca2+-Mg2+-free medium (G-PGD, Vitrolife AB), after which a biopsy could be carried out easily.

Lysis and Polymerase Chain Reaction Analysis of Embryos

Single blastomeres were collected and transferred to 0.2-mL PCR tubes containing 0.5 μL of alkaline lysis buffer. Lysis of the single blastomere, MDA amplification, and PCR analysis were performed as described for single lymphocytes.

RESULTS

Evaluation of the Multiple Displacement Amplification Technique

Multiple displacement amplification was successful in 4/4 single lymphocytes. The determination of the quality of the amplified DNA by agarose gel electrophoresis showed all
MDA-amplified fragments to be larger than 2 kb, with a majority larger than 20 kb (Fig. 1). Amplification rate, calculated from spectrophotometrical DNA measurements, was an average of $6.5 \times 10^6$-fold (the amount of the MDA product was $23 \pm 3 \mu g$).

To detect the homogeneity of the amplification and the value in PGD, MDA products were used for the amplification of five different loci involved in gene disorders. The PCR amplification from the single lymphocyte DNA was detected in 60/60 reactions (Table 1). Allele sizes matched those amplified from genomic DNA. At heterozygous loci, preferential amplification of alleles was reported and allele drop-out (ADO) was detected in 6/60 (10%). In Figure 2 we show the result of the five loci amplification analysis from a single lymphocyte. The image obtained is similar to genomic DNA (data not show).

**Clinical Marfan Preimplantation Genetic Diagnosis Program**

To perform the PGD linkage analysis for Marfan syndrome, the couple had to be tested. The healthy chromosome 15 of the male partner had to be identified using at least one intragenic marker (mts-2 and mts-4), and the sequence tagged sites had to be different in length from the female partner. Segregation studies of the family were performed and showed that the FBN-1 intragenic markers mts-2 and mts-4 were informative (Fig. 3).

Twenty-two cumulus oocyte complexes were retrieved, 13 metaphase II oocytes were injected, and 9 of them showed fertilization. In the morning of day 3, seven of the nine embryos had developed normally and could be biopsied.

The PCR reactions for the diagnosis of seven biopsed embryos were performed in triplicate. The results obtained were consistent in the three PCR reactions. Table 2 shows the allele sizes amplified from the seven embryos. The amplification efficiency for mts-2 and mts-4 was 5/7 and 6/7, respectively. In addition, no ADO was reported. Four embryos were determined to carry the nonaffected haplotype, two embryos were affected, and one could not be diagnosed. Two healthy embryos were transferred and the other two were blocked and discarded. A singleton pregnancy resulted in the birth of a healthy boy. A prenatal diagnosis was offered to the couple but they decline it and no prenatal diagnosis has been performed.

**DISCUSSION**

Polymerase chain reaction was the first technique developed for the analysis of DNA from single cells to diagnose monogenic diseases in embryos. Preimplantation genetic diagnosis, in general, and single-cell PCR, in particular, are labor-intensive techniques. As a result, unfortunately, only the most common monogenic diseases have been studied extensively. Testing new diagnoses with PGD is very time consuming, as primers have to be designed to amplify the mutation or linked markers. The PCR conditions have to be optimized to reduce the risk of ADO and amplification failure. The third report of the European Society of Human Reproduction and Embryology (ESHRE) PGD consortium (17) lists $>40$ monogenic diseases for which PGD had been applied, a small number compared to the hundreds of genetic abnormalities that have been diagnosed by prenatal diagnosis.

Isothermal rolling circle amplification with random hexamer primers and $\phi 29$ polymerase was recently described for circular DNA (18). Surprisingly, these reagents will also readily amplify linear, human genomic DNA in a cascading, strand displacement reaction termed MDA (19). The current report shows that MDA yields $6.5 \times 10^6$-fold amplification of DNA from a single cell, confirming the previous observation (4). This new tool has the potential to significantly expand the role of PGD in the diagnosis of single-gene disorders.

The MDA product analysis showed significant consistency in the quality and efficiency of MDA amplification, in agreement with previous description of MDA use in WGA (6).

We have illustrated an efficient and reliable method for the diagnosis of Marfan syndrome at the single-cell level, using standard PCR procedures. Because efficient and accurate detection of affected alleles depends primarily on the PCR amplification condition, the product detection methods used in the test, the availability of enough DNA to replicate for one blastomere and different target amplification, result

**TABLE 1**

Allele sizes amplified from single lymphocytes by MDA.

<table>
<thead>
<tr>
<th>Locus</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>Amplification efficiency</th>
<th>ADO</th>
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<tbody>
<tr>
<td>X-22</td>
<td>223-207</td>
<td>223-207</td>
<td>223-207</td>
<td>223-207</td>
<td>12/12</td>
<td>No</td>
</tr>
<tr>
<td>mts-2</td>
<td>132-149</td>
<td>132-149</td>
<td>132-149</td>
<td>132</td>
<td>12/12</td>
<td>Yes</td>
</tr>
<tr>
<td>mts-4</td>
<td>117-121</td>
<td>117-121</td>
<td>117-121</td>
<td>117-121</td>
<td>12/12</td>
<td>No</td>
</tr>
<tr>
<td>HD</td>
<td>165-226</td>
<td>165-226</td>
<td>165</td>
<td>165</td>
<td>12/12</td>
<td>Yes</td>
</tr>
<tr>
<td>Fragile X</td>
<td>248-234</td>
<td>248-234</td>
<td>248-234</td>
<td>248-234</td>
<td>12/12</td>
<td>No</td>
</tr>
</tbody>
</table>

Lledó, Multiple displacement amplification in PGD. Fertil Steril 2006.
in a very accurate diagnosis. The MDA test would become a universal first step in PGD.

The amplification efficiency and accuracy in blastomeres seems to be similar to that in lymphocytes. We detected three amplification failure in three cases performed in PGD–Marfan syndrome. Two amplification failures belong to the same blastomere. The use of two markers allowed us to identify that the possible cause, which would be an incomplete cell lysis or degradation of the DNA that prevents MDA, because no amplification were reported in both markers from the same blastomere.

Assessment of ADO is critical to evaluate any new technique for PGD and has important implications in sexing, as dropout of the Y signal could lead to the transfer of a male embryo with an X-linked disorder. Because X22 is present on both X and Y chromosomes in the pseudoautosomal region PAR2 and highly polymorphic, samples for heterozygous partners were expected to show two peaks, presumably different in length from each other. The low rate of ADO reported using MDA, mainly in X22 amplification, and the use of this new highly polymorphic X22 marker might overcome the problem of testing the gender of the embryo.

**FIGURE 2**

Result of the amplification analysis from MDA products. (A) Amplification of the sequence tagged site mts-4; (B) amplification of the Huntington disease expansion; (C) amplification of the X22 marker; (D) amplification of the Fragile X expansion locus; and (E) amplification of the sequence tagged site mts-2.
The major disadvantage of PCR–WGA methods is the slippage of microsatellites. Results in this report showed that this phenomenon does not occur with the MDA amplified method. As a consequence, a misdiagnosis due to a discrepancy with genomic DNA does not exist, improving the success of PGD, mainly when sequence tagged site analysis is used (20).

Molecular diagnosis for Marfan syndrome has rapidly evolved since the mapping of the gene in 1990 (21) and the discovery of the FBN-1 gene. The diversity of mutations in the FBN-1 gene is high and there are no hot spots for mutations. Whether the incomplete mutation uptake is due to technical reasons or to locus heterogeneity remains an open question. The method of linkage analysis should be applicable to this and other diseases for which a direct test is not available or mutations undetectable (due to heterogeneity mutations and more complex gene), provided that: [1] linkage phase can be rigorously determined from the DNA of relatives who are known carriers; [2] sufficient polymorphism exists to allow the investigator to clearly distinguish maternal and paternal markers such as CA repeats; and [3] the linkage markers allow robust amplification from a single cell. The inclusion of two linked polymorphisms in single-cell diagnosis of dominant disorders can reduce the risk of misdiagnosis due to ADO. Moreover, the possibility of a recombination event occurring between the marker and the site of the mutation can be discounted by the use of two markers flanking the mutation site. By using the MDA test enough DNA could be yielded to avoid multiplex optimization protocol for the detection of both markers. The method we report for the diagnosis of Marfan syndrome should be applicable to most patients carrying Marfan syndrome as long as they test for the markers.

Until now, MDA has been used for the diagnosis of β-thalassaemia and cystic fibrosis (22). We describe the first MDA–PGD for Marfan syndrome. The MDA test produces enough DNA from a single cell to allow for multiple PCR analyses. At the same time, high amplification efficiency and low ADO rates are seen compared with nested multiplex PCR. Moreover, MDA may be applied to multiple genetic analyses using standard procedures, and HLA matching could be performed in a reliable manner using commercial optimized kits (22). The use of MDA as an initial step in PGD allows for DNA amplification in quantities sufficient for the diagnosis of a wide spectrum of single-gene defects using standard methods. Moreover, using comparative genome hybridization, single-cell molecular karyotyping is now possible (23).

TABLE 2

<table>
<thead>
<tr>
<th>Allele sizes amplified from the seven biopsed embryos.</th>
<th>mts-2</th>
<th>mts-4</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>165–153</td>
<td>115–106</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>—</td>
<td>Undiagnosis</td>
</tr>
<tr>
<td>3</td>
<td>140–157</td>
<td>109–106</td>
<td>Affected</td>
</tr>
<tr>
<td>4</td>
<td>161–157</td>
<td>106–106</td>
<td>Normal</td>
</tr>
<tr>
<td>5</td>
<td>161–153</td>
<td>106–114</td>
<td>Normal</td>
</tr>
<tr>
<td>6</td>
<td>161–157</td>
<td>106–106</td>
<td>Normal</td>
</tr>
<tr>
<td>7</td>
<td>—</td>
<td>109–106</td>
<td>Affected</td>
</tr>
<tr>
<td>Amplification efficiency</td>
<td>5/7</td>
<td>6/7</td>
<td>—</td>
</tr>
<tr>
<td>ADO</td>
<td>No</td>
<td>No</td>
<td>—</td>
</tr>
</tbody>
</table>

REFERENCES


