Preimplantation genetic diagnosis of X-linked adrenoleukodystrophy with gender determination using multiple displacement amplification

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Objective: To evaluate the use of multiple displacement amplification (MDA) for whole genome amplification in the preimplantation genetic diagnosis (PGD) of X-linked adrenoleukodystrophy.
Design: MDA was used to amplify the whole genome directly from a single blastomere. MDA products were used for polymerase chain reaction (PCR) analysis of two polymorphic markers flanking the ABCD1 gene and a new X/Y marker, X22, to sex embryos in an X-linked adrenoleukodystrophy PGD program.
Setting: Fertility and gynecology private center in Alicante, Spain.
Patient(s): A couple in which the wife is a carrier of the ABCD1 gene mutation (676A→C) that was previously identified in her family.
Intervention(s): MDA of single blastomere and PCR tests for PGD.
Main Outcome Measure(s): The ability to analyze single blastomeres for X-linked adrenoleukodystrophy using MDA.
Result(s): The development of an MDA-PGD protocol for X-linked adrenoleukodystrophy allowed for the diagnosis of five embryos. These were biopsied on day 3 of culture and analyzed. One embryo was an affected male and one embryo was a female carrier. Three healthy female embryos were transferred 48 hours after biopsy. Unfortunately, no pregnancy was achieved.
Conclusion(s): The MDA technique is useful for overcoming the problem of insufficient genomic DNA in PGD and allows the simultaneous amplification of different targets to perform a diagnosis of any known gene defect and a sexing test by standard methods and conditions. (Fertil Steril® 2007;88:1327–33. ©2007 by American Society for Reproductive Medicine.)

Key Words: MDA, multiple displacement amplification, PGD, preimplantation genetic diagnosis, ALD, X-linked adrenoleukodystrophy

Couples with genetic disorders including single-gene defects, sex-linked conditions, or chromosome rearrangements face a reproductive risk. Preimplantation genetic diagnosis (PGD) is a diagnostic tool to avoid inheritance of genetic disease by transferring unaffected IVF embryos and represents an alternative to prenatal diagnosis (1).

PGD first consisted of the selection of female embryos for patients at risk of transmitting X-linked recessive diseases (2), and usually female embryos are transferred. Fifty percent of those female embryos are carriers, who later in life will have a 50% chance of transmitting their defective X-chromosome to the next generation. Fifty percent of the discarded male embryos are unaffected, which represents not only an ethical problem but diminishes the pool of embryos suitable for transfer.

Advances in molecular biology allow the development of specific diagnosis in a single cell for gene defects. Indeed, the fifth report of the European Society for Human Reproduction and Embryology (ESHRE) PGD Consortium (3) lists over 40 monogenic diseases for which PGD has been applied. Furthermore, for families with an identified X-linked recessive disease-causing mutation, nonspecific diagnosis by sex identification can be considered a substandard method (4), and it might be considered as a monogenic disease performing specific molecular diagnosis.

Despite the significant advantages provided by PGD, the setting up and testing of molecular diagnoses on a single cell is work intensive, difficult, expensive, and time-consuming. Labor-intensive development and validation of highly sensitive amplification strategies for single-cell diagnosis are required, usually using nested polymerase chain reaction (PCR), whole genome amplification (WGA), or fluorescent PCR methods. The main disadvantage of nested and fluorescent PCR is the difficulty in choosing primers for multiplex PCR (5). On the other hand, 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 less than 1 kb long (6). For those reasons, PGD requires a technique that would be able to amplify the single-cell DNA with a high fidelity that suits the

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diagnosis of any known single-gene disorder by the standard PCR technique.

Multiple displacement amplification (MDA) is an isothermal WGA technique based on the used of ϕ29 DNA polymerase and random primers. The ϕ29 polymerase combines high processivity with a strand displacement ability leading to the synthesis of DNA fragments >10 kb and favoring uniform representation of sequences (7). MDA is a technique that is used in the amplification of very low DNA quantities in clinical samples (6). Sequence representation in the amplified DNA assessed by multiple single-nucleotide polymorphism analysis is equivalent to genomic DNA, and amplification is superior to PCR-based methods (8).

Adrenoleukodystrophy (ALD) is an X-linked recessive disorder that is secondary to a mutation in the ABCD1 gene (in the terminal segment of the long arm of the X, i.e., Xq28) and results in peroxisomal beta oxidation defect and the accumulation of the saturated very long chain fatty acids in all tissues of the body. The manifestations of the disorder occur primarily in the adrenal cortex, the myelin of the central nervous system, and the Leydig cells of the testes. The choice method for X-linked ALD PGD is sexing. The advantage of relying on sexing only comes from its nonspecificity and suitability for all couples at risk for X-linked conditions. Indirect diagnosis without gender determination has been used (4), but to avoid misdiagnosis due to the fact that polymorphic markers segregate with sexual chromosomes and allele dropout (ADO), a gender determination could be included.

We describe for the first time haplotype and gender determination using MDA for PGD of X-linked ALD that enables the selection of both male and female unaffected embryos. This approach offers an alternative to sexing, which is frequently used for X-linked disorders and which results in the discarding of all male embryos, including the 50% that would have been normal, thus increasing the chance of pregnancy and avoiding the loss of healthy male embryos.

The aim of this work was to increase the reliability of PGD for X-linked ALD and to improve our ability to respond in a fast and safe way because of the ability to obtain enough quality DNA by MDA from a single cell for multiple PCR analyses.

MATERIALS AND METHODS

Lysis of Single Cells

Lymphocytes from a female heterozygous for the DXS1073, DXS9901, and X22 loci were separated from blood by centrifugation over Ficoll, washed, and resuspended in PBS. 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 at least 30 minutes. Cells were lysed by incubation at 65°C for 10 minutes (9). Lysis was then stopped by adding 0.5 μL of neutralization buffer (9).

MDA Protocol

Cells lysates were used directly for MDA. 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, UK) 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.

PCR Analysis

To use linkage analysis in the PGD of X-linked ALD, two extragenic polymorphic markers (DXS1073 and DXS9901), which flank the ABCD1 gene, were amplified using 1 μL of MDA products. The primers used were described by Gigarel et al. (4), and the forward primers were labeled at 5' with 6-FAM. PCR for the markers was carried out using the TaKaRa LA Taq kit (Takara Bio, Shiga, Japan). A reaction mix in a total volume of 25 μL containing 100 pmol of each primer, 200 mM dNTPs, 1× buffer was provided by the manufacturer, and 1 U of DNA polymerase was provided by the TaKaRa LA Taq kit. PCR was performed as follows: 5 minutes at 95°C, 35 cycles of 45 seconds at 95°C, 45 seconds at 55°C, and 45 seconds at 72°C followed by 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.

Sexing of human DNA by PCR-based methodology can be accomplished by amplifying X-Y homologous genes. To assess the sex status of embryos, a new X/Y chromosome marker, X22 (10), was detected by fluorescent PCR. The forward primer (5'-TAATGAGAGTTGGAAAGAAA-3') was 5' labeled with 6-FAM, while the reverse primer (5'-CCCATTGTTGCTACTTGAGA-3') was unlabeled. PCR amplification was performed for 25 cycles at the following 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 29-year-old wife carried a causative mutation 676 A→C in the ABCD1 gene that causes X-linked ALD. Her husband was 33 years old, and he did not present any type of clinical alteration of interest. The affected wife was heterozygous at the DXS1073 and DXS9901 loci. She showed an allele of 126 bp and an allele of 128 bp for DXS1073. Moreover, she carried two alleles of 141 bp and 136 bp for the DXSS9901 locus. The 126-bp and 141-bp alleles cosegregate with X-linked ALD in this family. The husband carried a 126-bp allele for the DXS1073 locus and a 136-bp allele for the DXS9901 locus. To identify the sex chromosomes, an X22 informativity test was performed. The wife was homozygous

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