Article

# Preimplantation genetic diagnosis of X-linked retinoschisis

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## Abstract

The aim of this study was to perform preimplantation genetic diagnosis (PGD) for X-linked retinoschisis using multiple displacement amplification (MDA) for whole genome amplification and linked markers to the *RS1* gene. The study evaluates the ability of MDA to amplify the whole genome directly from a single blastomere. MDA products were used for polymerase chain reaction analysis of two polymorphic markers flanking the *RS1* gene and a new X/Y marker, X22, to sex embryos in an X-linked retinoschisis PGD programme. Two couples in whom the wives were carriers of the *RS1* gene mutation (599 G→A), previously identified in their families, were subjected to two PGD cycles each. The main outcome measure was the ability to analyse single blastomeres for X-linked retinoschisis using MDA. As a result, the development of an MDA–PGD protocol for X-linked retinoschisis allowed for the diagnosis of 20 embryos in the four PGD cycles performed. These were biopsied on day 3 of culture and analysed. Eight embryos were affected males and two embryos were female carriers. In summary, three healthy female and four healthy male embryos, and a female carrier embryo, were transferred 48 h after biopsy. One single pregnancy was achieved. This report shows that the MDA technique is useful for overcoming the problem of insufficient genomic DNA in PGD. It also allows the simultaneous amplification of different targets to perform diagnosis of any known gene defect and sexing test by standard methods and conditions.

***Keywords:*** *multiple displacement amplification, preimplantation genetic diagnosis, X-linked retinoschisis*

## Introduction

Preimplantation genetic diagnosis (PGD) allows selection of unaffected IVF embryos in order to achieve pregnancies in couples at risk for transmitting a genetic disorder (Verlinsky and Kuliev, 2003), including single gene defects, sex-linked conditions or chromosome rearrangements (Munné *et al.*, 2004). These couples face a reproductive risk, and PGD is a diagnostic tool that represents an alternative to prenatal diagnosis. The scope of PGD has also been extended to screening for chromosomal aneuploidy in infertile couples (Wells *et al.*, 2002).

Despite the significant advantages provided by PGD, there are still technical limitations. For single gene disorders, the development and validation of labour-intensive highly sensitive amplification strategies are required, often using nested or fluorescent polymerase chain reaction (PCR) methods for specific diagnosis of gene defects single cell. The fifth report of the European Society of Human Reproduction and Embryology (ESHRE) PGD consortium (Harper *et al.*, 2006) lists over 100 monogenic diseases for which PGD has been applied. 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 methods.

Multiple displacement amplification (MDA) is an isothermal whole genome amplification technique based on the used of Φ29 DNA polymerase and random primers. The Φ29 polymerase combines high processivity with strand displacement ability, leading to the synthesis of DNA fragments >10 kb and favouring uniform representation of sequences (Paez *et al.,* 2004). MDA is a technique used in the amplification of very low DNA quantities in clinical samples (Dean *et al.,* 2002). Sequence representation in the amplified DNA assessed by multiple SNP (single nucleotide polymorphism) analysis is equivalent to genomic DNA, and amplification is superior to PCR-based methods (Lovmar *et al.,* 2003).

X-linked juvenile retinoschisis (RS) is a recessively inherited vitreo–retinal degeneration characterized by macular pathology and intraretinal splitting of the retina. The *RS1* gene has been localized to Xp22.2. The method of choice for X-linked RS PGD is sexing with selection of female embryos. The advantage of relying on sexing only comes from its non-specificity and suitability for all couples at risk of X-linked conditions. However, 50% of those female embryos will be carriers, who later in life will have a 50% chance of transmitting their defective X chromosome to the next generation, while 50% of the discarded male embryos will be unaffected. This not only represents an ethical problem, but also reduces the pool of embryos suitable for transfer.

For families with an identified X-linked recessive disease-causing mutation, non-specific diagnosis by sex identification can be considered as a sub-standard method (Gigarel *et al.,* 2004), rather it should be treated as a monogenic disease specific molecular diagnosis should be performed. Moreover, in order to avoid misdiagnosis due to the fact that polymorphic markers segregate with sexual chromosomes and allele drop-out (ADO), gender determination could be included. Until now, no specific PGD for RS has been performed.

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

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

# Materials and methods

## Lysis of single cells

Lymphocytes from a female heterozygous for the CA6, TAAA2 and X22 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 –80ºC for at least 30 min. Cells were lysed by incubation at 65ºC for 10 min (Sermon *et al.,* 1999). Lysis was then stopped by adding 0.5 µl of neutralization buffer (Sermon *et al.,* 1999).

## MDA protocol

Cells lysates were used directly for MDA. Whole genome amplification (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 instructions (Amersham Biosciences, UK), in a 20 µl reaction at 30ºC (16 h). The reaction was terminated by incubation at 65ºC for 10 min to inactivate the enzyme, and the amplified DNA was stored at –20ºC.

## PCR analysis

In order to use linkage analysis in X-linked retinoschisis PGD, two extragenic polymorphic markers (CA6 and TAAA2), flanking the *RS1* gene, were amplified using 1 µl of MDA products. The location of the markers according to the *RS1* gene position and the sequence of the primers used was described by Huopaniemi *et al.* (1999) The forward primers were labelled at 5′ with 6-FAM. PCR for the markers was carried out using the TaKaRa LA Taq kit (Takara Bio Inc., Shiga, Japan). The reaction mix was in a total volume of 25 µl containing 100 pmol of each primer, 200 mmol/l dNTPs, 1 × buffer provided by the manufacturer and 1 IU of DNA polymerase provided by the TaKaRa La Taq kit. PCR was performed as follows: 5 min at 95ºC, 35 cycles of 45 s at 95ºC, 45 s at 55ºC (for CA6 marker) or 60ºC (for TAAA2 marker), and 45 s at 72ºC followed 5 min extension at 72ºC. An aliquot of 2 µl of the PCR product was mixed with 2 µl loading buffer, denatured by boiling for 5 min, and loaded on the ABI PRISM 3100 DNA Sequencer (Applied Biosystems, USA). The results were processed using GeneScan Analysis software. (Applied Biosystems).

Sexing of human DNA by PCR based methodology can be accomplished by amplifying X–Y homologous genes (Cirigliano *et al.*, 1999). To assess the sex status of embryos, a new X/Y chromosome marker, X22 (Lledo *et al.,* 2007), was detected by fluorescent PCR. The primers used were described by Cirigliano *et al.* (1999); the forward primer was 5' labelled with 6-FAM, while the reverse primer was unlabelled. PCR amplification was performed for 25 cycles at the following temperatures: 95ºC for 45 s, 55ºC for 45 s, and 72ºC for 1 min. The amplification products were sized using an ABI PRISM 3100 DNA sequencer and Genescan software.

## Patients' description and informativity test

Two couples were studied, in whom the female partner carried the X-linked retinoschisis gene. Both had the same causative mutation, 599 G→A in the *RS1* gene. The male partners did not show any type of clinical alteration of interest. In family 1, the carrier female was homozygous at the CA6 and heterozygous at the TAAA2 locus. She showed an allele of 211 bp and allele of 207 bp for TAAA2. The 211 bp allele cosegregated with X-linked RS in this family. The male partner carried a 211 bp allele for the locus. In family 2, the carrier female was heterozygous at the CA6 and the TAAA2 locus. She showed an allele of 211 bp and allele of 208 bp for TAAA2. Moreover, she carried two

alleles of 184 bp and 180 bp for CA6 locus. The 211 bp and 184 bp alleles cosegregated with X-linked RS in this family. The male partner carried a 211 bp allele for the TAAA2 locus and a 184 bp allele for the CA6 locus. In order to identify the sex chromosomes, X22 informativity tests were performed. The female partner from family 1 was heterozygous for the X/Y marker and carried 223 bp and 228 bp alleles for this locus. The male partner carried two alleles of 194 bp and 199 bp. It was possible to identify the Y-chromosome from the male partner from family 1 by the 199 bp allele. The female partner from family 2 was heterozygous for the X/Y marker and carried a 213 bp and 228 bp alleles for this locus. The male partner carried two alleles of 228 bp and 199 bp. It was possible to identify the Y-chromosome from the male partner from family 2 by the 199 bp allele. This study had the approval of the Instituto Bernabeu Review Board.

## IVF cycle and ICSI procedure

Intracytoplasmic sperm injection (ICSI) was carried out 4 h after oocyte retrieval on a heated stage (Tokai Hit Thermoplate, Model MATS-U505R30, Japan) at 37ºC, mounted on an inverted microscope (Nikon Eclipse TE200, Japan) equipped with Hoffmann modulation optics and Narishige (Japan) micromanipulation system. Microinjection was performed according to the method of Van Steirteghem *et al.* (1995). After 1 month's use of oral contraceptives, a long luteal protocol was used to stimulate follicular development; this includes pituitary desensitization with leuprolide acetate agonist (Ginecrin Depot; Ross-Abbott, Madrid, Spain) and ovarian stimulation with a combination of recombinant human FSH (Gonal F; Serono, London, UK) and human menopausal gonadotrophin (HMG-Lepori; Farma-Lepori, Barcelona, Spain). Ovarian response was monitored by transvaginal ultrasound and oestradiol concentration. Ovulation was induced with 250 mcg of recombinant human chorionic gonadotrophin (HCG; Ovitrelle; Serono, London, UK). Oocytes were aspirated 36 h after HCG administration by transvaginal ultrasound-guided needle aspiration under sedation. Surrounding oocyte cumulus and corona radiata cells were removed by 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 $\mu$l droplets of G1.3 medium (Vitrolife AB, Kungsbacka, Sweden) covered with sterile equilibrated mineral oil (Ovoil; Vitrolife, Göteborg, Sweden) at 37ºC in an atmosphere of 6% $CO_2$. Fertilization was assessed 16–18 h after ICSI. Further development was evaluated on 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 on the morning of day 3. A non-contact, 200 mW diode laser system (Saturn, Research Instruments Ltd, Cornwall, UK) coupled to an inverted microscope was used to deliver two to four laser pulses of 5.274 ms to the zona pellucida, creating a funnel-shaped hole. One clearly nucleated blastomere was then gently aspirated through the hole. Several embryos showed early compactation, resulting in difficulties with the biopsy. These embryos were incubated for a few minutes in $Ca^{2+}$–$Mg^{2+}$ free medium (G-PGD; Vitrolife AB, Kungsbacka, Sweden), after which biopsy could be carried out easily.

## Lysis and PCR analysis of embryos

Four PGD cycles were performed, two for each family. Single blastomeres were collected and transferred to 0.2 ml PCR tubes containing 0.5 $\mu$l of alkaline lysis buffer. Lysis of the single blastomere, MDA amplification and PCR analysis were performed as described for single lymphocytes.

# Results

## Single lymphoblast test

MDA was successful in 10/10 single lymphocytes. The quality and quantity of the amplified DNA matched with previous studies (Lledo *et al.*, 2006). A total of 30 PCR analyses for each polymorphic marker were performed. The amplification efficiency obtained during the preclinical test on single lymphoblasts were 100% (90/90) for all markers, and the ADO rate was 6.7% (2/30) for TAAA2 marker, and no ADO was detected in the amplification of X22 and CA6 alleles. They all fell within the limits set in the ESHRE PGD Consortium's guidelines (Thornhill *et al.*, 2005): amplification efficiencies >90% and ADO rates <10%. None of the blanks showed contamination.

## Clinical X-linked retinoschisis PGD cycles

In order to be able to perform the PGD linkage analysis for X-linked RS, the two couples had to be informative, i.e. the healthy chromosome X of the female partner had to have been identified using at least one marker (CA6 and TAAA2), and the X22 had to identify the Y chromosome from the male partner to sex the embryos. Segregation studies of the families were performed, and showed that the markers TAAA2 and X22 were informative for family 1 and CA6, TAAA2 and X22 were informative for family 2 (**Figure 1**).

The details of the clinical cycles are shown in **Table 1**. A total of 59 cumulus–oocyte complexes were retrieved, and 52 metaphase II oocytes were injected, of which 23 showed fertilization. In the morning of day 3, 20 out of the 23 embryos had developed normally and could be biopsied.

The PCR reactions for the diagnosis of the 20 biopsied embryos were performed in triplicate. The results obtained were consistent in the three PCR reactions. **Table 2** shows the results of the PCR analysis. The amplification efficiencies for CA6, TAAA2 and X22 were 100, 90 and 100% respectively. In addition, ADO rates in TAAA2 and X22 were 5 and 8.3% respectively. **Figure 2** shows the electropherograms obtained from the clinical PGD cycles for X-linked RS using single blastomeres. The diagnosis of the 20 embryos biopsied in the four PGD cycles performed was as follows: eight affected male, two female carrier, five healthy male and four healthy female embryos. One embryo could not be diagnosed. In summary, three healthy female and four healthy male embryos, and a female carrier embryo, were transferred 48 h after biopsy. The embryos transferred in each PGD cycles are shown in **Table 1**. One healthy male embryo that was not transferred in the fresh cycle was cryopreserved. In all cycles, embryos suitable for transfer were available. The second cycle carried out for couple 1 resulted in an ongoing

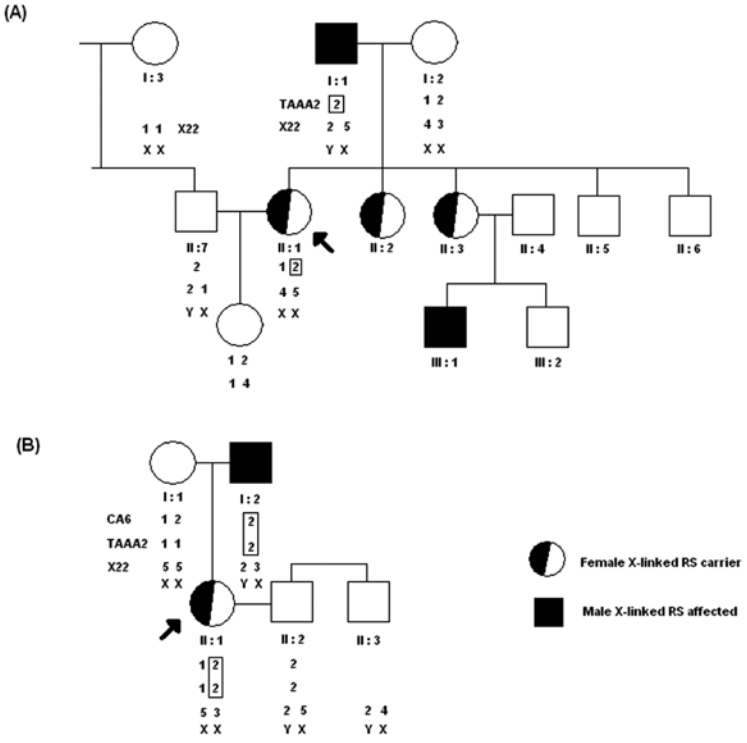


**Figure 1.** Pedigree of families: (**A**) family 1, (**B**) family 2. Arrow indicates the proband of family. Haplotype segregating with X-linked retinoschisis (RS) is marked with a box.

**Table 1.** Results of clinical cycles.

| *Couple* | | *1* | | *2* | |
|---|---|---|---|---|---|
| Cycle | | 1 | 2 | 1 | 2 |
| COC | | 12 | 17 | 17 | 13 |
| ICSI | | 12 | 17 | 13 | 10 |
| 2PN | | 2 | 6 | 6 | 9 |
| Biopsied | | 2 | 5 | 5 | 8 |
| XX | Healthy | – | 2 | 1 | 1 |
| | Carrier | 1 | – | – | 1 |
| XY | Healthy | – | 2 | 1 | 2 |
| | Affected | 1 | 1 | 3 | 3 |
| No diagnosis | | – | – | – | 1 |
| Embryo transferred | | 1 | 3 | 2 | 2 |
| PGD outcome | | No pregnancy | Ongoing pregnancy healthy boy | No pregnancy | No pregnancy |

COC = cumulus–oocyte complex; ICSI = Intracytoplasmic sperm injection; 2PN = presence of two pronuclei.

**Table 2.** Results of the biopsied embryos PCR reactions from the clinical PGD cycles for X-linked RS.

| | *TAAA2* | *CA6* | *X22* |
|---|---|---|---|
| Amplification efficiency % | 90 (54/60) | 100 (39/39) | 100.0 (60/60) |
| Allele drop out % | 5 (3/60) | 0 (0/39) | 8.3 (5/60) |

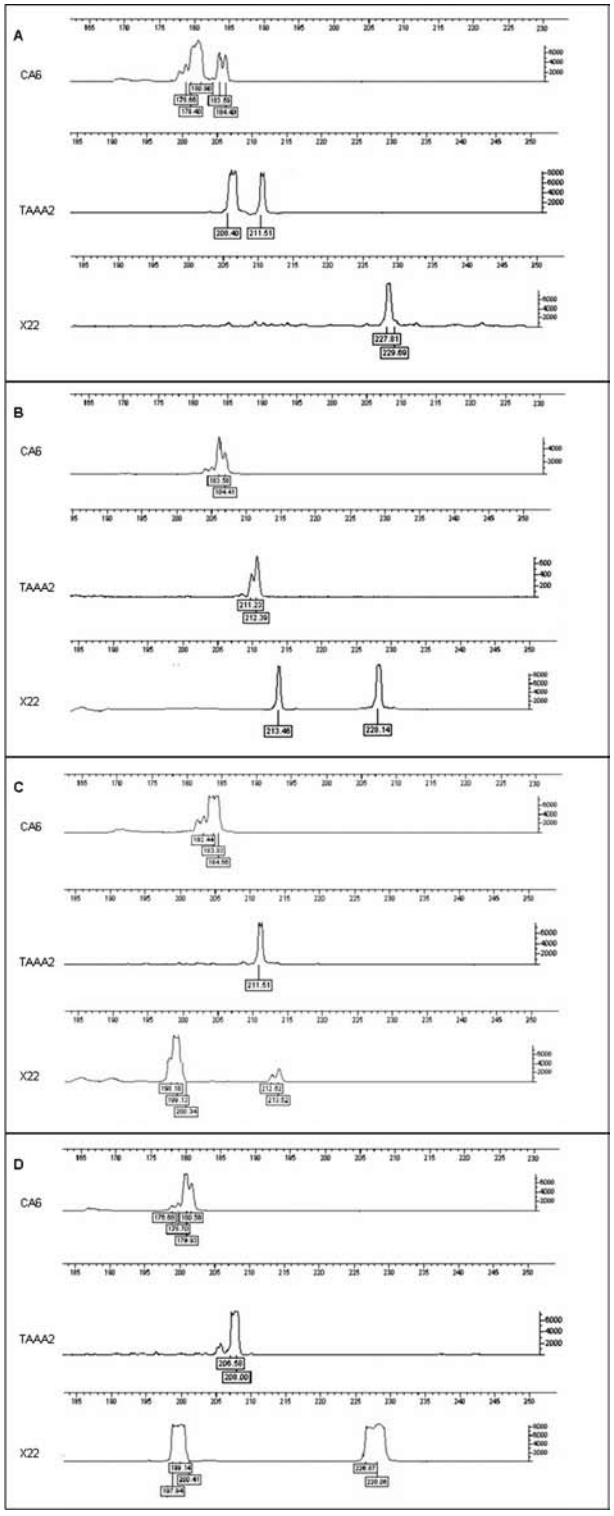


**Figure 2.** Electropherograms of the amplification from multiple displacement amplification (MDA) products in family 2 undergoing clinical preimplantation genetic diagnosis for X-linked retinoschisis in a single blastomere. The results of each marker from the same blastomere are shown: (**A**) healthy female embryo; (**B**) carried female embryo; (**C**) affected male embryo (**D**) healthy male embryo.

pregnancy of a healthy boy, confirmed by prenatal diagnosis.

## Discussion

Setting up new diagnoses for PGD requires spending a lengthy period designing and optimizing single cell PCR protocols. PCR conditions have to be optimized to minimize ADO and amplification failure. The fifth report of the ESHRE PGD consortium (Harper *et al.,* 2006) lists over 140 monogenic diseases for which PGD has been applied, a small number compared with the thousands of different genetic abnormalities that have been diagnosed by prenatal diagnosis. For that reason, for recessive X-linked diseases, sexing is the method of choice. Unfortunately, this approach has several disadvantages: half of the male embryos could be healthy. This means a decreased pregnancy rate and a matter of ethical concern. Moreover, the replacement of carrier female embryos cannot be avoided, thus the transmission of the disease to the offspring has not definitely been prevented.

This, together with advances in molecular biology, has led to a situation in which, if the mutation in a given family is known or can be analysed through linked markers (Renwick *et al.,* 2006), couples who wish to undergo PGD are likely to prefer a specific DNA diagnosis rather than simple embryo sexing (Sermon, 2002). Accordingly, the disease becomes a monogenic one with a requirement for gender determination because one must discern between a male with only one X chromosome and an ADO female.

PCR was the first technique developed for the analysis of DNA from single cells (Handyside *et al.,* 1990). Single-cell PCR is a demanding labour-intensive technique. If it is necessary to amplify different targets, the technique of multiplex PCR is generally used. However, this approach is very time consuming, as primers have to be designed to amplify the mutation and/or linked markers in the same reaction. PCR conditions have to be optimized for that and to reduce the risk of ADO and amplification failure. ADO was found to be affected by amplicon size, amount of DNA degradation, freezing and thawing, the PCR programme, and the number of cells simultaneously amplified. In order to improve the accuracy of PGD for single gene disorders, a low ADO rate may be achieved by the design of specific PGD protocols (Piyamongkol *et al.,* 2003). In agreement, there is a need for a technique that can produce enough DNA from single cells with high fidelity, and that suits the diagnosis of any known single gene defects using standard PCR methods avoiding specific optimization steps. MDA is a promising tool that allows whole genome single cell amplification. This reaction, catalysed by Φ29 polymerase with random hexamer primers, readily amplifies linear, human genomic DNA in a cascading, strand displacement manner (Lizardi, 2000). This new tool has the potential to significantly expand the role of PGD in the diagnosis of single gene disorders, since it produces sufficient DNA with sequence representation, assessed by multiple SNP analysis, equivalent to genomic DNA from a single cell to allow for multiple PCR analyses using standard procedures diagnosing a wide spectrum of single gene defects (Lovmar *et al.*, 2003) without the time-consuming design of specific PCR protocols.

Until now, X-linked RS has been diagnosed at single cell level by sexing using fluorescence in-situ hybridization, and no other method has been reported previously. This study has described the first efficient and reliable method for the molecular diagnosis of X-linked RS with gender determination at the single cell level, using standard PCR procedures. The availability of sufficient DNA, thanks to the MDA reaction, allows one to perform replicate reactions for one blastomere, and different target amplification can be performed, resulting in a very accurate diagnosis. Moreover, a gender determination can be achieved by including only an X/Y specific marker.

The amplification efficiency and accuracy in blastomeres seems to be similar to that in lymphocytes. It was not possible to diagnose one embryo in one PGD cycle for X-linked RS. Despite the use of two markers and gender determination, in some situations it is still difficult to establish the correct diagnosis. This happens mainly when the same allele is shared by both parents, because an ADO phenomenon could be occurring. To overcome this problem, more informative markers should be used.

The approach used in this report for the PGD is linkage analysis, which should be applicable to this and other diseases for which a direct test is not available or mutation undetectable (Renwick *et al.*, 2006). Haplotype analysis identifies family high risk markers associated with the inherited single gene disorder; a series of linked markers within or close to the mutated gene are tested. For X-linked disorders with the addition of gender determination, complete embryo genotyping could be achieved, because one must distinguish between a male with only one X chromosome and an ADO result. Using MDA, enough DNA could be obtained, thereby avoiding use of the multiplex optimization protocol for the detection of markers for the disease specific diagnosis and gender determination. The method reported for the diagnosis of X-linked RS should be applicable to most patients carrying X-linked RS, as long as they are informative for the markers. In order to perform gender determination, a new X/Y marker, X22, was used that avoids misdiagnosis caused by ADO, and this could be a better approach to sexing embryos because the absence of a Y-specific product of amelogenin could lead to misdiagnosis through ADO (Lledo *et al.*, 2007).

This study describes for the first time a PGD protocol for X-linked RS. MDA was used as a first step, as it produces enough DNA from a single cell to allow for multiple sort tandem repeat analyses, including an X/Y marker gender determination, and gonosome aneuploidies could be established, avoiding a multiplex optimization protocol for the detection of markers. The method reported for the diagnosis of X-linked RS should be applicable to most patients carrying RS, as long as they are informative for the markers.

## References

Cirigliano V, Sherlock J, Conway G *et al.* 1999 Rapid detection of chromosomes X and Y aneuploidies by quantitative fluorescent PCR. *Prenatal Diagnosis* **19**, 1099–1103.

Dean FB, Hosono S, Fang L *al.* 2002 Comprehensive human genome amplification using multiple displacement amplification. *Proceedings of the National Academy of Science of the USA* **99**, 5261–5266.

Gigarel N, Frydman N, Burlet P *et al.* 2004 Single cell co-

amplification of polymorphic markers for the indirect preimplantation genetic diagnosis of hemophilia A, X-linked adrenoleukodystrophy, X-linked hydrocephalus and incontinentia pigmenti loci on Xq28. *Human Genetics* **114**, 298–305.

Handyside AH, Kontogianni E, Hardy K *et al*. 1990 Pregnancies from biopsied human preimplantation embryos sexed by Y specific DNA amplification. *Nature* **344**, 768–770.

Harper JC, Boelaert K, Geraedts J *et al*. 2006 ESHRE PGD Consortium data collection V: cycles from January to December 2002 with pregnancy follow-up to October 2003. *Human Reproduction* **21**, 3–21.

Huopaniemi L, Rantala A, Forsius H *et al*. 1999 Three widespread founder mutations contribute to high incidence of X-linked juvenile retinoschisis in Finland. *European Journal of Human Genetics* **7**, 368–376.

Lizardi PM 2000 US Patent 6124, 120.

Lledo B, Bernabeu R, Ten J *et al*. 2007 Preimplantation genetic diagnosis of X-linked adrenoleukodystrophy with gender determination using multiple displacement amplification. *Fertility and Sterility,* in press.

Lledo B, Ten J, Galan FM *et al*. 2006 Preimplantation genetic diagnosis of Marfan syndrome using multiple displacement amplification. *Fertility and Sterility* **86**, 949–955.

Lovmar L, Fredriksson M, Liljedahl U *et al*. 2003 Quantitative evaluation by minisequencing and microarrays reveals accurate multiplexed SNP genotyping of whole genome amplified DNA. *Nucleic Acids Research* **31**, e129.

Munné S, Escudero T, Colls P *et al*. 2004 Predictability of preimplantation genetic diagnosis of aneuploidy and translocations on prospective attempts. *Reproductive BioMedicine Online* **9**, 645–651.

Paez JG, Lin M, Beroukhim R *et al*. 2004 Genome coverage and sequence fidelity of phi29 polymerase-based multiple strand displacement whole genome amplification. *Nucleic Acids Research* **32**, e71.

Piyamongkol W, Bermúdez MG, Harper JC, Wells D 2003 Detailed investigation of factors influencing amplification efficiency and allele drop-out in single cell PCR: implications for preimplantation genetic diagnosis. *Molecular Human Reproduction* **9**, 411–420.

Renwick PJ, Trussler J, Ostad-Saffari E *et al*. 2006 Proof of principle and first cases using preimplantation genetic haplotyping – a paradigm shift for embryo diagnosis. *Reproductive BioMedicine Online* **13**,110–119.

Sermon K. 2002 Current concepts in preimplantation genetic diagnosis (PGD): a molecular biologist´s view. *Human Reproduction* **8**, 11–20.

Sermon K, Lissens W, Messiaen L *et al*. 1999 Preimplantation genetic diagnosis of Marfan syndrome with the use of fluorescent polymerase chain reaction and the automated laser fluorescence DNA sequencer. *Fertility and Sterility* **71**, 163–166.

Thornhill AR, deDie-Smulders CE, Geraedts JP *et al*. 2005 ESHRE PGD Consortium 'Best practice guidelines for clinical preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS). *Human Reproduction* **20**, 35–48.

Van Steirteghem A, Joris H, Liu J *et al*. 1995 Protocol for intracytoplasmic sperm injection. *Human Reproduction Update* **1**, CD-ROM, item 3.

Verlinsky Y, Kuliev A. 2003 Current status of preimplantation genetic diagnosis for single gene disorders. *Reproductive BioMedicine Online* **7**, 145–150.

Wells D, Escudero T, Levy B *et al*. 2002 First clinical application of comparative genomic hybridization and polar body testing for preimplantation genetic diagnosis of aneuploidy. *Fertility and Sterility* **78**, 543–549.

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