Article

Successful polar body-based preimplantation genetic diagnosis for achondroplasia

Gheona Altarescu obtained her MD *magna cum laude* from the Jassy School of Medicine, Romania, and completed her training in Genetics at the National Institutes of Health, USA from 1997 to 2000. Since then she has established and now runs the Preimplantation Genetic Diagnosis Clinic in the Medical Genetics Unit at the Shaare Zedek Medical Center in Jerusalem. Current clinical research interests include development of new techniques for single cell diagnosis.

Dr Gheona Altarescu

G Altarescu[1,4], P Renbaum[1], B Brooks P[2], EJ Margalioth[2], A Ben Chetrit[2], G Munter[3], E Levy-Lahad[1,4], T Eldar-Geva[2,4,5]
[1]Medical Genetics Unit, Shaare Zedek Medical Center, Jerusalem, Israel; [2]IVF Unit, ZOHAR PGD Lab, Shaare Zedek Medical Center, Jerusalem, Israel; [3]Internal Medicine Department, Shaare Zedek Medical Center, Jerusalem, Israel; [4]Hebrew University School of Medicine, Jerusalem, Israel
[5]Correspondence: IVF Unit, Shaare Zedek Medical Center, POB 3235, Jerusalem 91031, Israel; Tel: +972 2666 6055; Fax: +972 2666 6211; e-mail: gevat@szmc.org.il

Abstract

Achondroplasia, the most common form of dwarfism, is a candidate for preimplantation genetic diagnosis (PGD) because a single mutation accounts for almost all cases. Multiplex fluorescent assay including the common G380R mutation in the *FGFR3* gene and eight close polymorphic markers was developed. First and second polar bodies (PB) were used for PGD analysis. An affected woman was treated with routine long-protocol ovarian stimulation and puncture. In the first PGD cycle, out of four fertilized oocytes, PB analysis revealed two mutant oocytes, one with total amplification failure of the maternal allele and one with inconclusive results. In the second PGD cycle, 14 oocytes were retrieved following a higher FSH dose and by performing oocyte retrieval and by placing the patient in the anti-Trendelenburg position using abdominal pressure to allow all follicles to be drained. Following PB analysis, two embryos containing the wild-type *FGFR3* allele were transferred. This led to an uncomplicated pregnancy and delivery by Caesarean section at week 38 of a healthy boy, carrying the *FGFR3* wild-type maternal allele. In conclusion, oocyte retrieval, while difficult in patients with achondroplasia, can be successfully performed. PB analysis is a reliable and sensitive method for PGD for maternal achondroplasia.

Keywords: *achondroplasia,* FGFR3 *gene, multiplex fluorescent PCR, PGD, polar body biopsy, single-cell PCR*

Introduction

Achondroplasia (ACH) (OMIM:100800), the most common genetic form of dwarfism is inherited as an autosomal dominant trait with 100% penetrance. The estimated frequency of ACH is between 1:15,000 to 1:26,000 (Rousseau *et al*., 1994; Shiang *et al*., 1994). Classical features of the disease include rhizomelic shortening of the extremities, trident hand, genu varum, increased head circumference with frontal bossing and midface hypoplasia, and normal intelligence (Jones, 1932). Associated medical problems include delayed motor development, various orthopedic problems and life threatening cervicomedullary compression due to a narrow foramen magnum.

The gene responsible for ACH is fibroblast growth factor receptor 3 (*FGFR3*) located telomerically on the short arm of chromosome 4 (Shiang *et al*., 1994). In 97% of cases, ACH is due to a unique amino acid substitution of glycine to arginine at position 380 (G380R, G1138A) in the transmembrane domain of *FGFR3* (Rousseau *et al*., 1994). Only 3% of ACH cases are due to a G to C mutation in the same gene, at the same position (G1138C). More than 90% of the ACH cases are novel mutations correlated to an increased paternal age at the time of conception (Wilkin *et al*., 1998). Homozygosity for mutant *FGFR3* alleles leads to a severe phenotype with fetal lethality in most cases (Pauli *et al*., 1983; Stanescu *et al*., 1990).

The first clinical application of PGD for single gene disorders was reported in 1990 (Strom *et al*., 1990; Verlinsky *et al*., 1990) and the first birth of a normal girl after PGD was reported in 1992

(Handyside *et al.*, 1992). Although over 5000 single gene disorders have been identified, PGD has been applied for only several hundred diseases (Verlinsky and Kuliev, 2003; Sermon *et al.*, 2007). This is due to difficulties in developing a standardized single-cell polymerase chain reaction (PCR) protocol unique for each specific mutation. The PGD protocol must allow reliable results within a short period of time (<72 h) so that embryos may be transferred within the window of implantation.

Most PGD protocols use blastomere biopsy for diagnosis of the embryos (Sermon *et al.*, 2007). PGD using polar body (PB) analysis has been shown to be an effective method for maternal autosomal dominant, X-linked and recessive disorders (Verlinsky *et al.*, 2002; Rechitsky *et al.*, 2003; Tomi *et al.*, 2006) and for genetic aneuploidy screening (Dawson *et al.*, 2006). PB-PGD has reduced allele drop out (ADO) frequency compared to blastomere PGD and reduces the need for embryo biopsy (Rechitsky *et al.*, 1998; Altarescu *et al.*, 2006). ADO, resulting from selective amplification of one allele in single-cell PCR, is the main cause of misdiagnosis in PGD (Piyamongkol *et al.*, 2003). For the majority of genes investigated (depending on their position on the chromosome), 60–70% of first polar bodies are shown to be heterozygote (contain two alleles on both chromatids) (Verlinsky and Kuliev, 2004a). Detection of two alleles in first polar body (PB1) minimizes the possibility of ADO and the diagnosis will depend on analysis of the second polar body (PB2): if second polar body has the wild-type allele, the oocyte will have the mutant allele and vice versa. Analysis of multiple informative polymorphic markers linked to the gene improves the detection of ADO, further increasing the accuracy and efficiency of the PGD diagnosis.

This study reports the case of a couple in which the female and her only daughter were affected with ACH. Since abortion was not an option in this family due to religious considerations, they presented to the study clinic for PGD. PB-PGD could be performed in this couple since the female was affected with an autosomal dominant disorder, thereby reducing the need for embryo biopsy.

Although no fertility problems have been associated with ACH, difficulties in ovarian stimulation and oocyte retrieval in affected women have been reported (Moutou *et al.*, 2003), leading these authors to raise doubts about whether it is appropriate to offer PGD to couples where the woman is affected with ACH. They concluded that it may be unreasonable to practice PGD for such couples. However, the first successful birth of a healthy child following PB-PGD for ACH is presented here.

Materials and methods

A 37-year-old woman presented at the PGD clinic at Shaare Zedek Medical Centre. Clinically she was diagnosed shortly after birth with ACH and later on she was found to carry the common G380R mutation in the *FGFR3* gene. During childhood, she underwent several surgical procedures increasing her height by 20 cm to a final height of 1.28 m. Due to religious restrictions, no prenatal diagnosis was performed in the first pregnancy and a child affected with ACH was born.

Ovarian stimulation, oocyte retrieval and embryo transfer

IVF treatment was performed using the long down-regulation protocol consisting of 0.1 mg/day decapeptyl s.c. (Ferring Ltd, Herzliya, Israel) and a daily dose of 150 IU of recombinant FSH (Gonal F; Serono, Israel) and 150 IU of human menopausal gonadotrophin (HMG; Menogon; Ferring Ltd). The starting dose was changed in the following cycle according to the patient's response. Follicular tracking was performed using both vaginal and abdominal ultrasonography to enable accurate visualization of the ovaries. Human chorionic gonadotrophin (HCG; Ovitrelle 250 µg, Serono) was administered when at least three follicles >18 mm developed. Vaginal ultrasound-guided oocyte retrieval was performed 35 h after HCG injection under general anaesthesia and oropharyngeal airway insertion. Transfer of two unaffected embryos was performed 48–72 h later.

Polar body biopsy, intracytoplasmic sperm injection (ICSI) and embryo cultures

Cumulus–oocyte–complexes (COC) were identified, washed and transferred to organ culture dishes containing equilibrated culture medium (Medicult, Denmark) and placed in an incubator with 5% CO_2. Oocytes were denuded with hyaluronidase (Sigma Aldrich, USA) 2 h after egg collection and were allowed to recover in the incubator for a further 2 h. PB1 were removed using the 'zona-slitting' technique (Verlinsky and Kuliev, 2004b).

ICSI was then performed on each mature egg. Injected oocytes were transferred to 25 µl droplets of Global medium (Life Global Media, IVFonline, Guelph, Ontario, Canada) under mineral oil. Approximately 18 h post injection, the oocytes were assessed for fertilization by observing the presence of two pronuclei (2PN), and the PB2 was removed using the same procedure as for the PB1 removal. Fertilized oocytes were then placed in fresh 25-µl droplets of Global medium under oil and cultured for a further 24–48 h. Polar bodies were each carefully transferred to a separate 0.5 ml tube containing 5 µl of proteinase K lysis buffer (Thornhill *et al.*, 2001). A sample of culture medium (media blank) from each droplet that contained a biopsied PB was analysed to verify the absence of maternal cellular genetic material or DNA in the culture medium. In addition, a no template control (NTC, reaction blank) was used to monitor the absence of external contamination in each PCR reaction.

Molecular analysis

Genetic testing for G380R and haplotype analysis was performed with DNA extracted from peripheral blood cells using high-salt precipitation (Miller *et al.*, 1988). Biopsied PB1 and PB2 were transferred to a tube containing 5 µl of proteinase K lysis buffer (Blake *et al.*, 1999) and incubated at 45°C for 15 min, followed by inactivation at 94°C for 15 min. Eight microsatellite polymorphic markers were detected flanking the *FGFR3* gene (**Table 1**). The G380R mutation resides between markers Ach-AT and Ach-TG. A multiplex PCR reaction was prepared containing 0.2 µmol/l dNTP, 10% dimethylsulphoxide, 0.1 µmol/l primer from each of five primer pairs (four polymorphic genetic markers in addition to the G380R) with 1.25 U *Taq* polymerase in reaction

Table 1. Flanking polymorphic makers: primer sequences for hemi-nested polymerase chain reactions.

Forward primer	*Hemi-nested primer*	*Reverse primer*
D4S114 F	**D4S114** F	**D4S114** R
AATTTTGTGTTGCTGTGGTG	TATGGCTCACCTCTCATCTCTGTG	CTGTCATAAGAGGGCCCAGTACTC
D4S3038 F	**D4S3038** F	**D4S3038** R
GAAGACCAGCATTCGG	CACGGTCTGTGATGGTTTAG	GGTTTAATACACAGTAATTGTTCA
G380R F	**G380R** F	**G380R** R
AGGAGCTGGTGGAGGCTGA	GTGTGTATGCAGGCATCCTCATGTAC	GGAGATCTTGTGCACGGTGG
ACH-TA F	**ACH-TA** R	**ACH-TA** R
ATTATAGGCAAGAGCCACCA	GAGCGAGACCCTGTCTAAAA	CTATGATTTTGCACCACTGC
ACH-TG F	**ACH-TG** F	**ACH-TG** R
TTGTATAGGACCCATTGCAG	CACGATGGGCTTTGACATT	CAGGCCTGAACTTTATCCAA
ACH-AT F	**ACH-AT** F	**ACH-AT** R
CTAGGCTCACCACAACCTCT	TGGGATTACGGGTGTGTG	ACAGGCAGATCACTTGAGG
ACH-CTTT F	**ACH-CTTT** F	**ACH-CTTT** R
TTAGCCAGGATGGTCTTGAT	CCCGGTCTCTTTCTTTCTTTCT	GATCATGCCACTGCACTC
ACH-AT2 F	**ACH-AT2** F	**ACH-AT2** R
TTCAATTAATTTATTATGCCATTCA	TTGTTTTATAAGAGGAATTTGTCG	AAAAATCACAAAAGACAAAGAAGA
ACHAC F	**ACH-AC** F	**ACH-AC** R
CCGTCACTCACTCATCTCTG	TCCTGGGATTGAGTTGTCT	GCACCTCACCTTTTCCAAC

Flanking polymorphic markers are in bold. F = forward primer, R = reverse primer.

buffer supplied by the manufacturer (JMR801, UK) in a 50 µl volume. The reaction was thermocycled for 30 cycles using a touch-down protocol: 20 s denaturation step at 95°C, 1 min annealing at 62–50°C and 30 s elongation at 72°C.

a volume of 1.5 µl from each reaction was used as a template with a hemi-nested primer that was fluorescently labelled at the 5 end with 6-carboxyfluorescein (6-FAM; MWG, Germany), HEX (MWG), or NED (Applied Biosystems) and with one outside primer for an additional 35 cycles for each of the five individual PCR reactions. The primers for both PCR reactions are presented in **Table 1**. The familial mutation was determined by enzymatic digestion with the enzyme BsrGI (New England Biolabs, MA, USA). Reaction products were diluted and run on an ABI Prism 3100 Avant automated sequencer, and analysed using Genotyper® software, ABI). Reactions that showed ADO or had media blank contamination were not included in the molecular analysis of the corresponding PB. Only samples that were informative for a minimum of three polymorphic markers flanking the gene in addition to the G380R mutation were considered for diagnosis. Stringent precautions to avoid any source of contamination, as recommended by the European Society for Human Reproduction and Embryology (ESHRE) PGD consortium, were used during all steps (Thornhill *et al.*, 2005).

Results

The patient's day-3 FSH was 3.9 IU/l and oestradiol 47 pmol/l. Prolactin and thyroid stimulating hormone were within the normal range. Diagnostic hysteroscopy revealed a normal uterine cavity. She had a typical abnormally high pelvic positioning of the ovaries, causing difficulties in their ultrasonographic visualization using both vaginal and abdominal probes.

Prior to performing the first PGD cycle, 50 single fibroblasts from an unrelated individual were isolated and used to test amplification efficiency. All eight polymorphic markers were successfully amplified in single fibroblasts with an efficiency of 92%.

Four informative polymorphic markers (mapping in the order: D4S3038, Ach-AT, Ach-TG, Ach-AC), less than 2 Mb from the *FGFR3* gene, were identified, fluorescently labelled and used to create a haplotype map for this family (**Figure 1**). The other four polymorphic markers were not informative in this family.

The results of two cycles of PB-PGD analysis for ACH in this family are presented in **Table 2**. The patient responded well to the ovarian stimulation. In the first cycle, 15 follicles with a diameter >12 mm were seen on the last day of ovarian stimulation. However, difficulties in ovarian visualization and follicular puncture resulted in less than 50% recovery of oocytes during the first cycle (**Table 2**). Of the seven retrieved oocytes, six were mature (metaphase II oocytes) and PB1 were successfully biopsied from all of them (**Table 2**). Four oocytes underwent fertilization and four PB2 were successfully biopsied. Only those PB1 from oocytes that were fertilized and extruded PB2 were analysed. Two PB1 were homozygous wild type; one was mutant and one showed total amplification failure. Both oocytes that extruded a wild-type PB1 showed a mutant PB2, indicating that the oocytes were mutant. One oocyte, for which PB1 was mutant, extruded a wild-type PB2 indicating that the oocyte was wild type. However, only two markers, on the same side of the mutation amplified, therefore ADO could not be ruled out. The oocyte that showed total amplification failure of PB1 had inconclusive results in PB2. The embryo derived from this oocyte with inconclusive PB results and the wild-type embryo underwent blastomere biopsy. Blastomere analysis confirmed that one embryo was wild type, while the

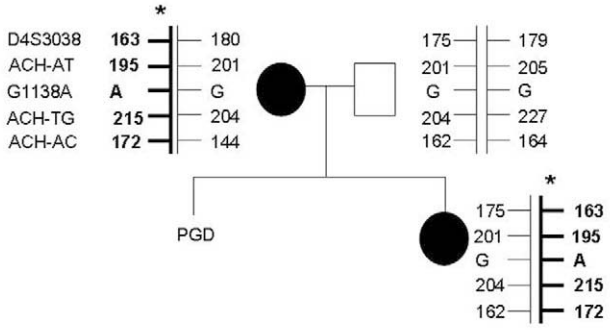

Figure 1. Family haplotype map based on genomic DNA from the couple and affected child. Numbers represent length of the alleles in base pairs. PGD = preimplantation diagnosis.

other one showed again inconclusive results. Both these two embryos arrested at 6–8 cells, and thus in this cycle there were no embryos available for transfer.

In the second cycle, a higher FSH dose (300 IU FSH and 150 IU of HMG daily) was used for ovarian stimulation. During the oocyte retrieval procedure, the patient was put in the anti-Trendelenburg position and was prepared for intubation under fibroscopy in case of breathing difficulties. In addition, the patient was prepared for trans-rectal ovarian manipulation and abdominal oocyte retrieval, should it be necessary. However, using moderate abdominal pressure to mobilize the ovaries as close as possible to the vaginal fornixes, with close observation of the patient by the anaesthetist, 14 oocytes were retrieved vaginally. Eleven of them were mature and underwent successful PB1 biopsy. Of these eight PB1 were heterozygotes, one homozygous wild type, one homozygous mutant and one showed total amplification failure. Following ICSI, seven eggs fertilized, of which five were derived from heterozygotes PB1, one from a wild-type PB1 (indicating a mutant PB2 and mutant oocyte), and one from a mutant PB1 (having a corresponding wild-type PB2 and wild-type embryo). Of the five oocytes that extruded heterozygotes PB1, three produced wild-type PB2 indicating mutant oocytes, one produced mutant PB2 indicating wild-type oocyte and one oocyte showed total amplification failure in both PB1 and PB2. At the end of analysis, two healthy embryos were obtained. An example of the results of hemi-nested fluorescent PCR reactions for four markers in both PB1 and PB2 are shown in **Figure 2** (**a**) and (**b**).

The couple requested the transfer of both wild-type embryos, as they were prepared to perform fetal reduction if a twin pregnancy resulted. Two 8-cell embryos that carried the wild-type *FGFR3* allele were transferred to the patient, resulting in a singleton uncomplicated pregnancy and a delivery of a healthy boy (weight 2670 g; length 52 cm; 75% percentile) by an elective Caesarean section at week 38. The couple did not agree to prenatal confirmation diagnosis due to religious consideration but post-natal analysis showed that the child inherited the wild-type maternal *FGFR3* allele.

ADO analysis

A total of 43 fibroblasts showed amplification of all four markers, two fibroblasts showed ADO of the marker D4S3038, one fibroblast showed ADO of the marker ACH-AC, and no markers amplified in the remaining four samples. The two informative markers flanking the mutation, showed no ADO in either reaction in all samples.

Total ADO was observed in nine out of 40 informative reactions in the second cycle. Four out of the nine ADO events were all in the same sample of PB1. The ADO rate in the remaining 11 samples was 12%.

Table 2. Summary of two cycles of polar body-based analysis for achondroplasia.

Parameter	*Cycle 1*	*Cycle 2*
Follicles >12 mm	15	16
COC	7	14
Mature oocytes	6	11
ICSI	6	11
PB1 biopsied	6	11
PB1 diagnosed	3[a]	10
Oocytes fertilized	4	7
PB2 biopsied	4	7
PB2 diagnosed	4	6
Embryos diagnosed	3	6
Wild type embryos	0	2
Embryos transferred	0	2
Gestational sacs	0	1
Live born	0	Singleton

COC = cumulus–oocyte complex; ICSI = intracytoplasmic sperm injection.
[a]Only PB1 from oocytes that were fertilized and extruded PB2 were analysed.

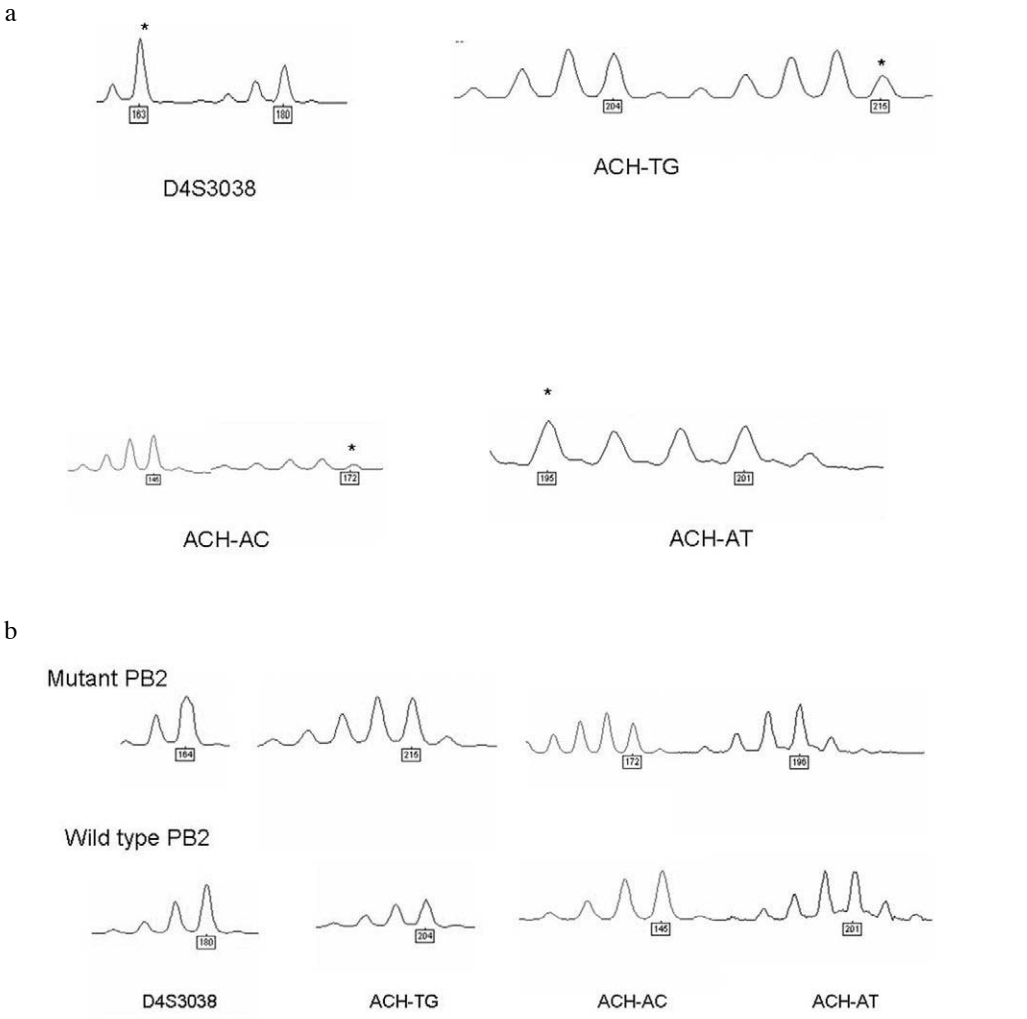

Figure 2. (**a**) GeneScan analysis of a heterozygous first polar body: electrophoretogram from PB1 DNA samples showing four informative markers analysed on an Applied Biosystems 3100 Avant analyser and GeneScan software and two examples of heterozygous PB1. Blue traces, 6-carboxyfluorescein (6-FAM)-labelled primers; green traces, HEX-labelled primers; black traces, NED-labelled primers. Asterisks (*) mark the affected alleles. X axis: base pairs; Y axis: fluorescence intensity. (**b**) GeneScan analysis of second polar bodies: electrophoretograms for mutant (upper) and wild-type (bottom) PB2 from oocytes that had extruded heterozygous PB1 (four markers shown). Blue traces, 6-FAM-labelled primers; green traces, HEX-labelled primers; black traces, NED-labelled primers. X axis: base pairs; Y axis: fluorescence intensity.

Discussion

This report presents the first successful case of PGD for achondroplasia and the results of two cycles performed for an affected proband. Previous failures have been attributed mainly to difficulties in ovarian stimulation and oocyte retrieval (Moutou *et al.*, 2003). These authors concluded that their experience raised questions about the feasibility of PGD for ACH, especially with affected female patients, and opined that it might be unreasonable or inappropriate to offer PGD for these couples.

In the first PB-PGD cycle, only six metaphase II oocytes were retrieved, mainly due to technical difficulties in the transvaginal ovarian puncture, similar to the difficulties described by Moutou and co-authors (2003) due to the very high pelvic localization of the ovaries. In the second treatment cycle, twice as many oocytes were retrieved due to meticulous special preparations: (i) high-dose FSH for ovarian stimulation was used, assuming that follicular accessibility would be partial; (ii) both vaginal and abdominal ultrasonography were used to overcome the difficulties related to the elevated pelvic position of the ovaries and to enable optimal follicular tracking; (iii) a different body position, the anti-Trendelenburg position, which raises the chest and head and lowers the pelvis of the patient, was used for the oocyte retrieval procedure; (iv) general anaesthesia was used and oropharyngeal airway was inserted, as recommended for ACH patients (Monedero *et al.*, 1997) – these techniques facilitated the approach of the ovaries to the vaginal fornixes and relieved

the pressure from the respiratory system and this enabled harmless manual abdominal pressure for ovarian mobilization to the pelvis and easier and more complete follicular aspiration; and (v) the patient was prepared for abdominal oocyte retrieval, should it be indicated. The anaesthetist was also advised of this option, although, in the end, it was not necessary.

The biopsy of the first and second PB, rather than two blastomeres, combined with the analysis of several polymorphic markers in addition to the mutation were also very important factors in the success of the method. Moutou and co-workers (2003) used an assay for PGD in ACH in which only the *FGFR3* gene was analysed. The detection of the G380R mutation is based on restriction fragment length polymorphism with the mutated fragment being digested. Since only the mutant fragment is digested, digestion failure or partial digestion, when no markers in conjunction with the mutation are used, can lead to transfer of an affected embryo. This is precisely why biopsy of two rather than one blastomere per embryo was recommended, which may reduce the pregnancy rate (Cohen and Munné, 2005).

PGD should be performed only when several polymorphic markers can be amplified simultaneously with the mutation in order to diminish the chances of ADO (Bick and Lau, 2006). The system reported here is universal and includes eight microsatellite markers (**Table 1**) that can be used in conjunction with the G380R for blastomere- or PB-PGD for ACH. The assay using highly polymorphic markers close to the G380R mutation was developed primarily to reduce misdiagnosis due to ADO in single-cell PCR.

In addition, mutation analysis in blastomeres rather than in PB for autosomal dominant disorders can be misleading, since this analysis is only partially informative in some families (the need for four different alleles in blastomeres as opposed to only two in polar bodies). Therefore, in female autosomal-dominant or X-linked disorders, usage of PB-PGD increases the informativity of the assay for many couples, reducing the chances of misdiagnosis. In this couple, only four out of the eight microsatellite markers in the vicinity of the *FGFR3* gene (**Table 1**) were found to be informative for PB analysis and only three were fully informative (four different alleles) for blastomere analysis.

Verlinsky and Kuliev (2000) have shown that the use of three or more polymorphic markers reduce the error rate due to ADO from as high as 27% (in blastomeres for single amplicons) to almost 0%. Furthermore it has been shown that ADO rates were significantly lower in PB versus blastomere PGD (Rechitsky *et al.*, 1998; Altarescu *et al.*, 2006).

The couple requested both wild-type embryos to be transferred and agreed to perform fetal reduction should the pregnancy continue as bigeminy beyond the 12th week. Although their faith would not permit termination of pregnancy for fetal anomaly, it would permit termination if the mother's health was at risk. This cycle led to a successful singleton pregnancy and the birth of a healthy boy. No report on multiple pregnancies in ACH women has been found in the medical literature. The patient, similar to other ACH pregnant women, had suffered respiratory difficulties during the last weeks of her first pregnancy and had an elective Caesarean section in week 38. It would be reasonable to assume that a twin pregnancy would precipitate and worsen these difficulties, possibly enhancing the need to terminate the pregnancy prematurely.

In conclusion, although PGD for ACH is difficult to perform for an affected proband, due to technical problems related to oocyte retrieval and difficulties in achieving accurate genetic diagnosis of blastomeres, PB-PGD is a reliable and successful method to be used for this disease.

Acknowledgements

The authors would like to thank Rabbi David and Mrs Anita Fuld for their generous and ongoing support.

References

Altarescu G, Eldar-Geva T, Brooks B *et al*. 2006 Polar body vs. blastomere biopsy for PGD. PB or not PB? *Proceedings of the 56th Annual Meeting of the American Society of Human Genetics*, 9–13 October 2006, New Orleans, Louisiana, **53**.

Bick DP, Lau EC 2006 Preimplantation genetic diagnosis. *Pediatric Clinics of North America* **53**, 559–577.

Blake D, Tan SL, Ao A 1999 Assessment of multiplex fluorescent PCR for screening single cells for trisomy 21 and single gene defects. *Molecular Human Reproduction* **5**, 1166–1175.

Cohen J, Munné S 2005 Comment 2 on Staessen et al. (2004). Two-cell biopsy and PGD pregnancy outcome *Human Reproduction* **20**, 2363–2364.

Dawson A, Griesinger G, Diedrich K 2006 Screening oocytes by polar body biopsy. *Reproductive BioMedicine Online* **13**, 104–109.

Handyside AH, Lesko JG, Tarin JJ et al. 1992 Birth of a normal girl after in-vitro fertilization and preimplantation diagnostic testing for cystic fibrosis. *New England Journal of Medicine* **327**, 905–909.

Jones EW 1932 Studies in Achondroplasia. *Journal of Anatomy* **66**, 565–577.

Miller SA, Dykes DD, Polesky HF 1988 A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* **16**, 1215.

Monedero P, Garcia-Pedrajas F, Coca I *et al*. 1997 Is management of anesthesia in achondroplastic dwarfs really a challenge? *Journal of Clinical Anesthesia* **9**, 208–212.

Moutou C, Rongieres C, Bettahar-Lebugle K *et al*. 2003 Preimplantation genetic diagnosis for achondroplasia: genetics and gynaecological limits and difficulties. *Human Reproduction* **18**, 509–514.

Pauli RM, Conroy MM, Langer LO Jr *et al*. 1983 Homozygous achondroplasia with survival beyond infancy. *American Journal of Medical Genetics* **16**, 459–473.

Piyamongkol W, Bermudez MG, Harper JC *et al*. 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.

Rechitsky S, Verlinsky O, Kuliev A et al. 2003 Preimplantation genetic diagnosis for familial dysautonomia. *Reproductive BioMedicine Online* **6**, 488–493.

Rechitsky S, Strom C, Verlinsky O *et al*. 1998 Allele dropout in polar bodies and blastomeres. *Journal of Assisted Reproduction and Genetics* **15**, 253–257.

Rousseau F, Bonaventure J, Legeai-Mallet L *et al*. 1994 Mutations in the gene encoding fibroblast growth factor receptor-3 in achondroplasia. *Nature* **371**, 252–254.

Sermon KD, Michiels A, Harton G *et al*. 2007 ESHRE PGD consortium data collection VI: cycles from January to December 2003 with pregnancy follow-up to October 2004. *Human Reproduction* **22**, 323–336.

Shiang R, Thompson LM, Zhu YZ *et al*. 1994 Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. *Cell* **78**, 335–342.

Stanescu R, Stanescu V, Maroteaux P 1990 Homozygous achondroplasia: morphologic and biochemical study of cartilage. *American Journal of Medical Genetics* **37**, 412–421.

Strom CM, Verlinsky Y, Milayeva S et al. 1990 Preconception genetic diagnosis of cystic fibrosis. *Lancet* **336**, 306–307.

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.

Thornhill AR, McGrath JA, Eady RA *et al.* 2001 A comparison of different lysis buffers to assess allele dropout from single cells for preimplantation genetic diagnosis. *Prenatal Diagnosis* **21**, 490–497.

Tomi D, Schultze-Mosgau A, Eckhold J *et al.* 2006 First pregnancy and life after preimplantation genetic diagnosis by polar body analysis for mucopolysaccharidosis type I. *Reproductive BioMedicine Online* **12**, 215–220.

Verlinsky Y, Kuliev A 2004a PGD for single gene disorders. In: *An Atlas of Preimplantation Genetic Diagnosis 2nd edition*. Taylor and Francis, London, p. 61.

Verlinsky Y, Kuliev A 2004b Micromanipulation and biopsy of polar bodies and blastomeres. In: *An Atlas of Preimplantation Genetic Diagnosis 2nd edition*. Taylor and Francis, London, pp. 15–22.

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

Verlinsky Y, Kuliev A 2000 *An Atlas of Preimplantation Genetic Diagnosis*. Taylor and Francis, London, UK, pp. 233–234.

Verlinsky Y, Rechitsky S, Verlinsky O *et al.* 2002 Polar body-based preimplantation diagnosis for X-linked disorders. *Reproductive BioMedicine Online* **4**, 38–42.

Verlinsky Y, Ginsberg N, Lifchez A *et al.* 1990 Analysis of the first polar body: preconception genetic diagnosis. *Human Reproduction* **5**, 826–829.

Wilkin DJ, Szabo JK, Cameron R *et al.* 1998 Mutations in fibroblast growth-factor receptor 3 in sporadic cases of achondroplasia occur exclusively on the paternally derived chromosome. *American Journal of Human Genetics* **63**, 711–716.

Declaration: The authors report no financial or commercial conflicts of interest.

Received 17 August 2007; refereed 5 October 2007; accepted 2 November 2007.