Preimplantation genetic diagnosis for achondroplasia: genetics and gynaecological limits and difficulties

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BACKGROUND: We report the first attempts at preimplantation genetic diagnosis (PGD) and IVF and their accompanying difficulties for achondroplasia (ACH) patients. METHODS: A PGD test was developed using fluorescent single cell PCR on lymphoblasts from patients and controls and from blastomeres from surplus IVF embryos. A specific digestion control based on the use of two fluorochromes was elaborated. Ovarian stimulation and oocyte retrieval were carried out using conventional protocols. RESULTS: We performed 88 single cell tests for which amplification was obtained in 86 (97.7%) single lymphoblasts. Allele drop out (ADO) was observed in two out of 53 (3.7%) heterozygous lymphoblasts. If we combine the results from the blastomere testing from surplus embryos with those from PGD cycles and re-analysis after PGD, we obtained a PCR signal in 84% of cases of which 91% were correctly diagnosed at the G380 locus. A total of six cycles were performed resulting in three embryo transfers. We observed difficulties in ovarian stimulation and oocyte retrieval with affected female patients. No pregnancy was obtained. CONCLUSION: A PGD test for ACH is now available at our centre but our initial practice raises questions on the feasibility of such a test, specially with affected female patients.

Key words: achondroplasia/IVF/ovarian stimulation and puncture/preimplantation genetic diagnosis(PGD)

Introduction

Achondroplasia (ACH, MIM100800) is the most common genetic form of short-limbed dwarfism, occurring at a frequency between 1 in 15,000 and 26,000 (Rousseau et al., 1994; Shiang et al., 1994). Classical features include proximal shortening of the extremities, genu varum, trident hand, limitation of elbow extension, exaggerated lumbar lordosis, megalencephaly and characteristic faces with frontal bossing and midface hypoplasia (Jones, 1997). Associated medical problems, which include delayed motor development, cervicomедullary compression due to a narrow foramen magnum and various orthopaedic-neurological problems, are highly variable among ACH patients. There is no mental retardation associated with ACH. Patients have an increased mortality risk from birth to early childhood and later between the fourth and fifth decades (Hecht et al., 1987). No fertility problems have been reported associated with ACH.

ACH is inherited as an autosomal dominant trait with 100% penetrance, 90% of cases are sporadic with an increased risk correlated with paternal age at the time of conception suggesting a de-novo and paternal origin of the mutation (Wilkin et al., 1998). Rare familial cases (10%) have been described. Homozygosity of the ACH alleles lead to a more severe phenotype with, in most cases embryonal lethality (Pauli et al., 1983; Stanescu et al., 1990). ACH is due to a unique amino acid substitution of a glycine to an arginine at position 380 (G380R) in the transmembrane domain of the fibroblast growth factor receptor 3 (FGFR3) (Rousseau et al., 1994; Shiang et al., 1994). This missense mutation at position 1138 is due to a G to A transition in 97% of the cases and a G to C transversion for the rest of the cases (Bellus et al., 1995).

After receiving a request from a couple, we developed a preimplantation genetic diagnosis (PGD) for ACH. As for prenatal diagnosis (Bellus et al., 1994), the detection of the mutation is based on restriction fragment length polymorphism (RFLP). Indeed, the transition G to A creates a SfiI restriction site and the transversion G to C creates different unique sites including a MspI site (Shiang et al., 1994; Bellus et al., 1995). Therefore, a unique PCR allows for the detection of the two types of mutations described in ACH. A specific digestion control was elaborated to ensure the reliability of the test.

During the PGD cycles we have been confronted with specific problems in ovarian stimulation and oocyte retrieval which we describe here. Since we have been unable to find any
report concerning attempted IVF in patients with ACH, this article represents the first description of PGD and IVF attempts for ACH. In addition we discuss the ethical and obstetrical considerations which should be taken into account with these couples.

Materials and methods

Single cell testing

Two Epstein–Barr-transformed lymphoblast cell lines were used to perform single cell analysis: one from an affected female asking for PGD and heterozygous for the mutation G380R in FGFR3 gene, transition G to A (HC62) and one from a control homozygous for the normal allele (N/N, cell line GM04540A; Coriell Cell Repositories, Camden, USA). Tubing procedure was performed as previously described (Moutou and Viville, 1999).

Supernumerary IVF embryos not suitable for freezing and thus scheduled for destruction and PGD embryos were incubated for 10 min in an embryo biopsy medium, calcium/magnesium-free (EBM, Medicunt-France Lyon France) prior to biopsy. They were then biopsied as previously described (Hardy and Handside, 1992) under an inverted microscope (Leica, Wetzlar, Germany) using Research Instruments micromanipulators and micropipettes (Research Instruments Ltd, Penryn, UK). Zona pellucida drilling was performed using a Zylos laser (Hamilton-Thorn, Beverly, USA) and blastomeres were gently aspirated and placed in G2.1 medium (Scandinavian Science, Gothenburg, Sweden). Each blastomere was washed three times in EBM, transferred into a reaction tube containing 2.5 μl of Lysis Buffer (LB: 200 mmol/l KOH, 50 mmol/l DTT) and frozen at −20°C until amplification by PCR.

PCR reactions

Primers used to characterize the G380R mutation have been designed for conventional diagnostic use and generate a 191bp fragment: ACH-R (CTGTCGCTTGACCGGGAAGC) and ACH-F (CCGAGGAGGGTCTGGTGGGA). Primer ACH-R was fluorescently labelled either with tet, ned or 6-fam fluorochromes (Perkin Elmer Applied Biosystems, Warrington, UK). Aliquots of 22.5 μl PCR master mix containing neutralizing buffer (NB: 900 mmol/l TrisHCl, 300 mmol/l KCl and 200 mmol/l HCl), 2 mmol/l MgCl2, 200 μmol/l each dNTP, 0.2 μmol/l each primer and 1 U per reaction AmpliTaq DNA Polymerase (Applied Biosystems, Courtaboeuf, France) were added to the reaction tubes. PCR reactions were performed using a Mastercycler Gradient PCR machine (Eppendorf, Hamburg, Germany) or a 9700 PCR system (Applied Biosystems, Courtaboeuf, France). The programme used was 5 min of initial denaturation at 96°C followed by 5 s of denaturation at 96°C for the first 10 cycles and 94°C for the remaining 35 cycles. Annealing and elongation cycles were performed for 5 s at 62°C and 5 s at 72°C, respectively. Reactions were terminated after 5 min at 72°C. A total of 10 μl PCR product were incubated at 37°C for 1 h with 2.5 U SfiI restriction enzyme (Ozyme, St Quentin en Yvelines, France) which cuts the mutated allele and generates two fragments of 61 and 130 bp. Only the 130 bp fragment was detectable since it includes ACH-R cuts the mutated allele and generates two fragments of 61 and 130 bp.

Genomic DNA from each couple requesting PGD was extracted from whole blood samples using the standard saline protocol (Rousseau et al., 1992). Analysis of the G380 locus of FGFR3 gene was performed using the PCR procedure described above, except that NB was replaced by buffer II (BII; 500 mmol/l KCL, 100 mmol/l Tris–HCl pH 8.3) and 30 cycles were sufficient for amplification.

Clinical PGDs

Three couples with dramatic history of termination of pregnancies (TOP) requested PGD for achondroplasia. In all cases the mutation G to A at position 1138 was found.

Case 1

A 30-year old affected woman, heterozygous for the G380R mutation, and her 23-year old husband had three previous TOPs after prenatal diagnosis.

Case 2

A 26-year old affected woman and her 31-year old husband had experienced one TOP and one miscarriage following prenatal diagnosis.

Case 3

A 36-year old healthy woman and her 39-year old husband heterozygous for the mutation had experienced two TOPs after prenatal diagnosis. This couple had previously experienced ten unsuccessful intra-uterine inseminations with donor sperm and considered PGD as their last chance to have an unaffected child. For the three couples, ovarian stimulation, oocyte recovery and ICSI procedures were carried out using standard protocols.

Two different stimulation protocols were used. Either a GnRH agonist long protocol (Decapeptyl®; Ipsen-Biotech, Paris, France) where patients were first desensitized with GnRHa triptorelin and then stimulated with hMG (Menogon®; Ferring, Paris, France) and/or recombinant FSH (rhFSH, Gonad-F®; Serono, Boulogne, France or Puregon®; Organon, Saint Denis, France), or, a daily dose of GnRH antagonist protocol where patients were treated, when follicles reached a diameter of at least 14 mm, with a daily dose of 0.25 mg of Cetrorelix® (Serono, Boulogne, France) continued during the gonadotrophin treatment until the induction of ovulation.

Triggering ovulation was obtained with hCG 5000 units (Gonadotrophine chorionique ‘endogravida’ Organon) when at least 5 follicles reached at least 15 mm. Transvaginal ultrasound-guided oocyte retrieval was performed after 36 h, under general anaesthesia. Three days after ICSI, embryos were biopsied and blastomeres were collected and analysed. For each biopsied cell, a blank control was prepared from a drop of the final EBM washing medium. PCR controls containing single lymphoblast from the normal cell line (N/N) were added. At least two blastomeres were individually reanalysed from the untransferred affected embryos. Embryo transfer was performed at day 4 after ICSI. No more than two embryos were replaced. The luteal phase support comprised vaginal progesterone 200 mg two times daily (Utrogestan; Besins, Paris France).

Results

Lymphoblast and blastomere testing

We performed 88 (35 N/N and 53 HC62) single cell tests for which amplification was obtained in 86 (97.7%) single lymphoblasts. ADO was observed in two out of 53 (3.7%)
heterozygous lymphoblasts from which only the normal allele was amplified. One blank out of 27 (3.7%) was contaminated and presented a heterozygous profile. This contamination was probably due to a tubing error since this blank was prepared from a drop of the final EBM washing medium during tubing of HC62 lymphoblasts.

Twenty blastomeres from surplus embryos that were unsuitable for transfer or cryopreservation were tested. Positive amplification was observed in 17 (85%) samples. PCR products of three blastomeres were digested using SfcI and, as expected, no mutation was detected after digestion. The accuracy of the diagnosis on blastomeres from surplus embryos was impossible to evaluate since only homozygous normal embryos were available, which also prevented the evaluation of the ADO rate.

**Digestion control**

Since only mutated alleles are cut by SfcI enzyme, digestion failure could lead to transfer of an affected embryo. It was thus important to make sure that the 191 bp fragments observed are actually normal alleles and not due to digestion failure. We took advantage of the Applied Biosystem technology to set up an internal digestion control. Indeed, such an automated sequencer offers the possibility to simultaneously analyse PCR fragments labelled with different fluorochromes. So, for the PGD of ACH, we performed a first PCR on the patient’s lymphocytes using one fluorochrome (6-fam appearing in blue) and the PGD analysis with another fluorochrome (tet appearing in green or ned appearing in yellow). Then the two PCR products were mixed for the digestion. Since one allele of the PCR product from the patient’s lymphocyte carries the mutation, it must be digested. Therefore, after digestion, we expect to observe, in blue, two peaks (130 and 191 bp) corresponding to the control heterozygous lymphoblast and, in green or yellow, either one 191 bp fragment in homozygous normal embryos, or one 130 bp and one 191 bp in heterozygous affected embryos, or one 130 bp fragment in homozygous affected embryos (Figure 1). Such a control was set up with lymphoblast cells before use in PGD and turned out to be highly reliable since out of 86 tests all of them were correctly digested.

We thus concluded that our PCR is efficient and reliable and felt confident for clinical application.

**Clinical PGDs**

The results of PGD cycles are summarized in Table I. The three female patients had a complete evaluation: day 3 basal hormonal level, hysteroscopy and ultrasonography. The two affected women presented an increased estradiol (E2) level (75 pg/ml for case 1 and 84 pg/ml for case 2) predicting a poor ovarian response. It is important to note that for both affected women we noticed an abnormally high pelvic positioning of the ovaries. This rendered the visualization by ultrasonography, the follow up of the follicular growth and the puncture of the ovaries, very difficult. Even under general anaesthesia, which was used for all the described cycles, and using support for the abdominal area, the punctures remained difficult and partial.

The unaffected woman had normal biological results. Semen parameters were normal for all three male partners.

**Case 1**

A first cycle was cancelled due to poor response after ovarian stimulation of the affected woman. After a GnRH agonist triptorelin long protocol and stimulation with 200 IU rhFSH, after 9 days of treatment only one follicle >15 mm was observed.

In a second cycle, a daily dose of GnRH antagonist protocol with 75 IU of hMG and 100 IU of rhFSH allowed the growth of five follicles of >15 mm with an E2 level at 1950 pg/ml. Only one cumulus-oocyte complex (COC) was retrieved, injected by ICSI and generated a single two pronuclei (2PN) embryo. On day 3, the embryo had eight cells and two blastomeres were biopsied. For both blastomeres, one 191 bp peak corresponding to the normal allele was obtained after digestion whereas the

![Figure 1. Electrophoregram of single cell PCR of the G380 locus in the FGFR3 gene. Size standard Genescan Rox 500 is shown by empty peaks, digestion control of a heterozygous lymphoblast is in grey, and the cell tested is in black (labelled with ned for ABI3100 automated sequencer). The first profile was obtained from HC62 cell line (heterozygous G380R/N) and the second one from the normal control (cell line GM04540A, N/N).](https://academic.oup.com/humrep/article-abstract/18/3/509/626079/511)
digestion controls were as expected. PCR controls and blank controls were correct. We thus concluded that the embryo was unaffected and transferred it on day 4, but no pregnancy ensued.

A third cycle was started with the same stimulation protocol. At day 12, six follicles of >15 mm were observed with an E2 level of 2760 pg/ml. Only two COC were retrieved, one injected and one 2PN embryo was obtained. Embryo development was blocked at the 4-cell stage and no biopsy could be performed.

Case 2
A GnRH agonist triptorelin long protocol was established for the affected woman. On the day of hCG injection, 11 follicles >16 mm were obtained with an E2 level of 2960 pg/ml. During ovarian puncture the patient experienced breathing difficulties using the face mask; these were easily corrected by modifying the position of the patient and inserting an oropharyngeal airway. Seven COC were retrieved, four were injected and three 2PN embryos were obtained. On day 3, two blastomeres of each embryo were successfully removed. Amplification failure occurred for embryos 1 and 4 whereas embryo 2 was diagnosed as unaffected and was transferred on day 4, but no pregnancy ensued. All controls gave expected results. Two blastomeres from embryo 1 were re-analysed and showed that this embryo was affected. The re-analysis of three blastomeres of embryo 4 revealed that it was affected. The woman is now spontaneously pregnant with an unaffected fetus (see Note added at proof).

Case 3
After these previous failed attempts and in spite of the fact that the woman was not affected, we chose a daily dose of GnRH antagonist protocol with 150 units of rhFSH. At the day of hCG injection we obtained an E2 level at 1552 pg/ml with nine follicles >15 mm. Twelve COC were obtained, 10 were inseminated by ICSI, seven of them exhibited 2PN. Three days later, six embryos were suitable for biopsy and two blastomeres were analysed for each embryo. Two embryos were unaffected and transferred but the patient did not become pregnant. The four other embryos were diagnosed as affected and the results were confirmed during the re-analysis.

During the second cycle five COC were obtained, four were injected, four of them exhibited 2PN. Three days later two embryos were biopsied and two blastomeres were analysed for each embryo, one embryo was unaffected and transferred. No pregnancy was obtained. Since the affected embryo died between day 3 and 4, no re-analysis was performed to confirm its status.

If we combine the results from the blastomere testing with those from PGD cycles and re-analysis after PGD, we have analysed a total of 60 blastomeres in which 55 had a visible nucleus. A PCR signal was obtained in 46 of 55 (84%) and 42 were correctly diagnosed at the G380 locus (91%). ADO was observed in four out of 20 heterozygous blastomeres (20%).

Discussion
We present here the set up of a PGD for achondroplasia and the results of six cycles performed for three couples. The test developed can be offered to any ACH patient. Indeed, only two mutations have been described both involving a substitution at the same position (G380R) of the transmembrane domain of the FGFR3. These substitutions introduce either SfcI or MspI restriction sites. Therefore the test, as for the prenatal diagnosis (Bellus et al., 1994), is based on RFLP with the mutated fragment being digested. Since only the mutated fragment is digested we had to be very cautious in the development of a digestion control. Indeed, a digestion failure may lead to the transfer of an affected embryo. We took advantage of the possibility of performing a multicolour fluorescent PCR to set up a reliable internal digestion control. For this purpose, we first carry out a PCR on the patient’s lymphocytes using one fluorochrome and the analysis of the embryos’ blastomeres.

<table>
<thead>
<tr>
<th>Case</th>
<th>Embryo</th>
<th>Embryo grade at day 3</th>
<th>No of cells analysed</th>
<th>Result</th>
<th>Diagnosis</th>
<th>Transfer</th>
<th>Confirmatory results (no of cells)</th>
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<tr>
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<td>1</td>
<td>8</td>
<td>2</td>
<td>Failed/191 bp</td>
<td>Unaffected</td>
<td>Yes</td>
<td>±</td>
</tr>
<tr>
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<td>1*</td>
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<tr>
<td>2*</td>
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<td>Unaffected</td>
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<td>–</td>
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<tr>
<td>4</td>
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<td>2</td>
<td>2</td>
<td>Failed/Failed</td>
<td>No diagnosis</td>
<td>No</td>
<td>Affected (3d)</td>
</tr>
<tr>
<td>3 Cycle1</td>
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<td>5</td>
<td>2</td>
<td>130–191 bp/Failed</td>
<td>Affected</td>
<td>No</td>
<td>Affected (5f)</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>2</td>
<td>2</td>
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<td>Affected</td>
<td>No</td>
<td>Affected (2e)</td>
</tr>
<tr>
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<td>6</td>
<td>2</td>
<td>2</td>
<td>191bp/191 bp</td>
<td>Unaffected</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
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<td>6</td>
<td>2</td>
<td>2</td>
<td>130–191 bp/130 bp ADO</td>
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<td>No</td>
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<tr>
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<td>191 bp/191 bp</td>
<td>Unaffected</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td>3 Cycle2</td>
<td>2</td>
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<td>2</td>
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<td>–</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>Failed/130–191 bp</td>
<td>Affected</td>
<td>No</td>
<td>±</td>
</tr>
</tbody>
</table>

*fragmented embryo
*no visible nucleus
*One affected, one amplification failure
*Two affected, one amplification failure
*Three affected, two ADO (-191bp)
*One affected, one ADO (130-bp)
using another fluorochrome. Both fluorescent PCR fragments were mixed together for digestion and analysis. Therefore, we can judge the quality of the digestion by analysing the patient’s lymphocytes fragment. If it is digested we can consider that there was no problem at that stage of the analysis. Such a procedure is possible only with automatic sequencers accepting multiple fluorochromes. We are still confronted with the risk of ADO which, as for any dominant disease, can lead to the transfer of affected embryos. This is precisely why biopsies of two cells per embryo is recommended.

We have treated three couples; for two of them the females were affected, for the third one the male was affected. We did not encounter any specific difficulties with the latter couple for which we performed two cycles. For the two other couples, we have been confronted with problems concerning the ovarian stimulation and ovarian puncture. Both of them, despite their relatively young age and an absence of sub-fertility antecedents showed an elevated estradiol level (75 pg/ml and 84 pg/ml respectively). Furthermore, for the first couple, three stimulations were attempted, modifying the protocol each time, without any improvement. For the second couple, only one cycle was tried allowing the retrieval of seven cumuli, but only four mature oocytes. In both cases we have noticed a very high pelvic localization of the ovaries rendering the follow-up of the follicular growth and the transvaginal ovary puncture extremely difficult even under general anaesthesia. In agreement with the first couple, considering the poor ovarian response, the difficulty of the ovarian puncture and the anaesthetic risk in ACH patients (Monedero et al., 1997), we decided to stop the procedure. The couple is thinking of adopting a child. The wife of the second couple is spontaneously pregnant with an unaffected fetus.

This preliminary experience raises doubts about whether it is appropriate to offer PGD to couples where the woman is affected. Indeed, if, for an unknown reason, women with ACH are difficult to treat either in terms of ovarian stimulation or puncture, it may turn out to be unreasonable to practice PGD for these couples. Indeed, because of the elevated pelvic position of the ovaries the follow-up of the follicular growth by ultrasonography and the puncture were extremely difficult and remain partial even if practised under general anaesthesia. Because of the poor responses and the partial COC recovery, the number of oocytes and then of embryos is insufficient for PGD practice. Furthermore, in ACH patients general anaesthesia presents difficulties of ventilation with a face mask or of intubation due to stiffness of the cervical spine suggesting that an intubation under fibroscopy would be preferable (Monedero et al., 1997). Conversely there should be no problem to take care of couples where the male is affected.

Another difficulty which may be confronted, is the number of embryos that can be reasonably transferred in women affected with ACH. Here again, we have been unable to find any report in the literature on twin pregnancies in ACH patients. How many embryos can, reasonably, be transferred without taking the risk to compromise the health of the patient? Is an ACH patient physically able to sustain a twin pregnancy under conditions good enough to ensure birth at an acceptable term? The only reports on pregnancy in ACH patients concerns single pregnancies and the need to carry out a Caesarean section or the difficulties of performing either an epidural or a general anaesthesia (Monedero et al., 1997; Morrow and Black, 1998).

We expect that other centres will offer PGD for this condition and help us to find answers to all the questions raised by our initial experience of PGD and IVF for ACH.

It is interesting to note that, in the last data collection exercise, the European Society of Human Reproduction and Embryology PGD consortium (Committee, 2002) has reported the refusal of one couple who were asking for a PGD for ACH on ethical grounds. No explanations were given concerning the reasons for refusing PGD in this case. Since prenatal diagnosis is proposed in France we do not see any reason to refuse them PGD. However, it is worth noticing that, for the moment, we have been confronted only by couples in which only one member is affected by ACH and from whom the demand was to have a child with a normal phenotype, all of them having experienced at least one TOP for this condition. It is important to note that part of the demand is motivated by other people’s perception of ACH. Indeed, the three ACH patients, in addition to their handicap, have suffered from social difficulties ranging from climbing stairs to social segregation. As mentioned by Braude, ACH, as for some genetic blindness or deafness, can lead to the patient requesting transfer of affected embryos in order to have a child like themselves (Braude, 2001). For the moment we have not been confronted with such a demand but the French legislation is clear on the subject (Viville et al., 2001). PGD is allowed in order to avoid the transmission of a genetic disorder of a particular gravity that is incurable at the moment of the diagnosis. It is thus out of the question to provide PGD for such requests that are not responding to medical needs but to a social selection since, in this situation, the couple does not consider the condition as a disease.

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Note added at proof
The patient delivered a healthy boy.

References
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