Preimplantation genetic diagnosis (PGD) for SHOX-related haploinsufficiency in conjunction with trisomy 21 detection by molecular analysis

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Received: 21 June 2010 / Accepted: 8 November 2010 / Published online: 1 December 2010
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Abstract

Purpose Development of a molecular PGD protocol for a male with an X-linked deletion in the SHOX gene region, located in the pseudoautosomal region of the X/Y chromosomes. Due to excessive recombination in this region, the deletion can be found in male offspring.

Methods We developed a 13 marker multiplex fluorescent PCR protocol: 3 markers within the deleted SHOX region, 5 flanking markers, 3 informative markers on chromosome 21 (advanced maternal age) and 2 markers for sex determination.

Results Of four embryos, two wild type males, diploid for chromosome 21 were transferred resulting in twin boys. One embryo was an affected female and another embryo was Turner. Amniocentesis confirmed the implanted embryos were males (46XY), with no recombinations.

Conclusions While many X-linked disorders can be analyzed by sexing, genes located in the pseudoautosomal regions have

high XY recombination rates, requiring multiple markers to enable an accurate diagnosis.

Keywords Preimplantation genetic diagnosis · Multiplex PCR · Blastomere · SHOX-related haploinsufficiency · Trisomy 21

Introduction

Short stature homeobox (SHOX)-related haploinsufficiency disorders range from Leri–Weill dyschondrosteosis (LWD) at the more severe end of the spectrum, to SHOX-related short stature at the mild end of the spectrum [1]. The classic clinical triad in LWD is short stature, mesomelia, and Madelung deformity [2]. The majority of children with short stature caused by SHOX-related haploinsufficiency have disproportionate short stature and/or wrist abnormalities [3]. The SHOX gene, located on both the pseudoautosomal region of the X (Xp22.3) and Y (Yp11.3) chromosomes is the only gene known to be associated with SHOX-related haploinsufficiency. SHOX is present in two identical copies in all individuals: in females one copy is present on each X-chromosome (the pseudoautosomal region of the X chromosome is not inactivated) while in males, one copy is present on the X-chromosome and one copy is present on the Y chromosome. Overall, one third of SHOX mutations are point mutations in the gene, while approximately two-thirds of individuals with SHOX-related haploinsufficiency have large-scale SHOX deletions that vary in size between 90 kb and 2.5 Mb or more [4], often located entirely outside the SHOX gene coding sequence [4]. These deletions can be detected either by fluorescent in situ hybridization (FISH) or by molecular techniques using

Capsule A PGD protocol is presented for a paternal SHOX-related Haploinsufficiency with screening for trisomy 21. High recombination rates require multiple markers for analysis.

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single nucleotide polymorphisms (SNPs) or polymorphic microsatellite markers. SHOX-related haploinsufficiency disorders are inherited in a pseudoautosomal dominant manner. In pseudoautosomal dominant inheritance, homologous genes located on Xp and Yp follow the rules of autosomal inheritance. A genetically related disorder to SHOX haploinsufficiency can be Turner syndrome. While Turner syndrome usually presents as 45X0, it can also be caused by cytogenetically visible deletions of part of Xp chromosome including the SHOX gene [5].

Preimplantation genetic diagnosis (PGD) was developed more than two decades ago for couples at high genetic risk for having affected children. PGD is performed by blastomere and/or polar body biopsy for Mendelian and chromosomal disorders [6]. Because only unaffected embryos are transferred to the uterus, PGD provides an alternative to current post conception diagnostic procedures (amniocentesis or chorionic villous sampling), which can be followed by pregnancy termination. The main causes of misdiagnosis in PGD are occurrence of allele dropout (ADO) in single cell analysis and undetected recombination events. Therefore the gold standard for molecular PGD analysis is the use of linked polymorphic markers in combination with the family specific mutation [7]. These polymorphic markers are selected in the vicinity of the analyzed gene in order to reduce the chances of undetected recombination events [8].

We present a PGD analysis in a family where the affected daughter presented with a deletion in the SHOX related region in addition to being mosaic for Turner syndrome (45X0, 31%) [9]. Since this deletion was inherited from the paternal X chromosome, and due to the autosomal dominant mode of inheritance of the SHOX gene located in the pseudoautosomal X region, theoretically all males of this couple (Y chromosome inherited from father), should be unaffected. However it has been demonstrated that crossover between the human sex chromosomes during male meiosis, in the pseudoautosomal pairing regions, has a recombination rate 20 times greater than autosomal chromosomes, allowing the possibility of an affected male (Fig. 1) [10]. Therefore in this family we adopted a strategy of combined sex selection for males together with polymorphic markers within and surrounding the deletion in the SHOX related region in order to identify possible crossover events. Furthermore, since the female in this couple was 38 years old we included markers on chromosome 21 in order to enable detection of embryos with trisomy 21.

We present an efficient and sensitive method of combined SHOX haploinsufficiency and trisomy 21 detection using molecular based PGD. One cycle resulted in the transfer of 2 wild type males yielding a twin pregnancy. Subsequent amniocentesis confirmed that both embryos were 46XY and received the wild type paternal Y allele.

Fig. 1 Diagram showing possible patterns of inheritance in a couple with an affected male carrying a deletion near the SHOX gene on the pseudoautosomal region of chromosome X. Asterisks indicate presence of the deletion

Material and methods

IVF treatment was performed by a standard long protocol involving pituitary down regulation with GnRH analog, followed by controlled ovarian hyperstimulation with five ampoules per day of gonadotrophins (Menogon, Ferring, Germany). Vaginal ultrasound-guided ovum pick-up was performed 36 h after hCG injection. Oocytes were identified, washed and transferred to organ culture dishes containing equilibrated culture medium and placed in an hypoxic incubator (5% O₂ with 5% CO₂). Oocytes were denuded with hyaluronidase (Sigma–Aldrich, USA) 2 h after oocyte collection. Intracytoplasmic sperm injection (ICSI) was then performed on each mature oocyte. Oocytes were then placed in 20 µl droplets of G1 Vitrolife, (Goteborg, Sweden) under oil and cultured for a further 48 h. Blastomere biopsy was performed on day 3 at the 6–8 cell embryos using laser assisted biopsy. Each blastomere was separately transferred to a 0.5 ml tube containing 5 µl of proteinase K lysis buffer [11]. The biopsied embryos were further cultured in G2 Vitrolife medium till their transfer to the uterine cavity on day 4.

A sample of culture medium (media blank) from each droplet that contained a biopsied blastomere was analyzed 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 absence of external contamination in each PCR reaction.

Molecular analysis

Prior to the PGD cycle genetic testing for the SHOX deletion and haplotype analysis were performed on DNA extracted

from peripheral blood cells from the parents and daughter, using a high salt precipitation method [12]. We developed a multiplex fluorescent PCR protocol that included 13 markers: 3 markers within the deleted region of the SHOX locus (rs6644384, rs6644385, DXYS10096), 5 informative flanking markers (SHOX-AC, chrX:490164-491019; SHOX-GA, chrX:516193-517420; SHOX-AGGA, chrX:684139-685372; DXYS10085; SHOX-TTTC, chrX:816751-817727, UCSC genome browser, March 2006) [13] in conjunction with 3 informative markers on chromosome 21: D21S1899, D21S267, D21S1411 along with SRY and Amelogenin96 for sex determination.

Blastomere analysis was carried out following Proteinase K digestion and inactivation at 94°C for 15 min followed by PCR in a 50 µl multiplex reaction containing 0.2 µM dNTPs, 10% DMSO, 0.1 µM each primer, 1.25U Taq polymerase in a buffer supplied by the manufacturer (JMR801, UK). The reaction was thermocycled for 30 cycles using a program of 20 s at 95°C, 1 min. at 62°C to 50°C and 30 s at 72°C. Primer sequences are listed in Table 1. From each multiplex reaction, 1.5 µl was used as a template with a hemi-nested primer 5' fluorescently labeled with 6-FAM, HEX or TAMRA (Metabion, GmbH), with the reverse unlabeled primer for an additional 35 cycle PCR reaction for each individual marker. Reaction products were diluted and run on an ABI Prism 3130xl automated sequencer, and analyzed using GeneMapper software (ABI). Single cell sequencing was performed for each of

the two intragenic SNPs in all analyzed blastomeres. Following the multiplex and hemi-nested reactions, PCR products were purified and sequenced with BigDye Terminator v1.1 cycle sequencing (ABI) and electrophoresed on an ABI Prism 3130xl automated sequencer.

Since PGD is a clinical medical procedure IRB for PGD is not required in our institution.

Results

A couple with a daughter affected with SHOX related haploinsufficiency and mosaic Turner's presented to our PGD clinic. Her original analysis performed in another center demonstrated a paternally derived deletion including the markers rs6644384, rs6644385, DXYS10096 in the vicinity, but not including, the SHOX gene coding region [9].

Prior to the first PGD cycle, 17 polymorphic markers surrounding the deleted region were identified, and eight were found to be informative: 3 in the deleted region (rs6644384, rs6644385, DXYS10096) and five in the surrounding region (SHOX-AC, SHOX-GA, SHOX-AGGA, DXYS10085, SHOX-TTTC). These markers were labeled and used to build a haplotype map (Fig. 2). Markers for SRY and Amelogenin were included in the assay for sex determination. In addition, since the proband was 38 years old, 3 informative markers on chromosome 21 were used for trisomy 21 detection (Fig. 2). This protocol was validated prior to performing the

Table 1 Polymorphic makers: primer sequences for hemi-nested PCR reactions

Forward primer	Nested primer ^a	Reverse primer
SHOX-AC TGCACCTGTAATCCCAGCTA	SHOX-AC CTGGGTGACAGAGGGAGACT	SHOX-AC CCACAAACACG GACACAGAA
SHOX-GA TTTATTTCAGGGGTGCTGCT	SHOX-GA CTCCAAATGGCTTCTTCCAG	SHOX-GA TCTCTCTGGTCG GCAATCTT
SHOX-AGGA AGAAAGAAAAAGAAATTAAG	SHOX-AGGA CAAGCAAGACAGAAAGTT	SHOX-AGGA TCTCTCTGGT CGGCAATCTT
rs6644384 AGGAAAAGACGTCCCTCCTG	rs6644384 CTGGCCCTTTCTGTTTCTGT	rs6644384 GGCCAGCAGTC TCAACTCTT
rs6644385 GGCCTCCTGGTAAGAGTTGA	rs6644385 CTGGCCCTTTCTGTTTCTGT	rs6644385 CCATCTATACGG GGGCTTG
DXYS10096 ACACCTGCAATTTGGGTCTC	DXYS10096 TTTAACAAACCGCATTCTCCAA	DXYS10096 GTGGTGGAGC TTGCAGTGA
SHOX-TTTC GCTGGGATTACAAGCGTGAG	SHOX-TTTC GCTTGCAACGTTGATCCAG	SHOX-TTTC CCTGGGCAA CAGGAGTAAAA
D21S1899 TTGATATTTGCAATCTCATGTTC	D21S1899 ACTGCATTATTCACAATAGCGAAG	D21S1899 CATGCTACAGG TTCATACACAGAG
D21S267 TGAGATGAGAGGATCCCTTG	D21S267 CCTCCAACCTGGGTGA	D21S267 ATGGATCTGGAT TTCTATCTTC
D21S1411 GGGTGGATAGAGAGATAGAACG	D21S1411 ATGATGAATGCATAGATGGATG	D21S1411 AATGTGTGTCC TTCCAGGC

^a Nested primers are in the forward orientation

Fig. 2 Parental haplotypes for the pseudoautosomal regions including the deletion in the vicinity of the SHOX gene and three informative markers on chromosome 21. Numbers represent length of amplification products in base pairs. Asterisks indicate allele containing the deletion

first PGD cycle with 50 single fibroblasts from an unrelated individual and used to test amplification efficiency and ADO rates. The overall amplification efficiency was 90–94% (in all markers), ADO rate in fibroblasts was an average of 13% while in blastomeres the overall ADO rate was later shown to be an average of 7%.

After hormonal stimulation, 5 oocytes were retrieved, four were fertilized and one blastomere from each embryo was biopsied and analyzed. One embryo was found to be a female carrying the deleted paternal X SHOX allele, one embryo was

diagnosed as Turner (XO) and two embryos were males. Both male embryos demonstrated the presence of the maternal X allele and a wild type Y allele. One of these two embryos showed recombination between the X and Y chromosomes for the distal marker SHOX-AC (outside the deleted region). The results of two SNPs, (rs6644384, rs6644385) and the polymorphic marker DXYS10096 for the two wild type male embryos are presented in Fig. 3. All four embryos were diagnosed as diploid for chromosome 21.

The two male wild type embryos were transferred to the proband resulting in a twin pregnancy. Amniocentesis performed at week 17 confirmed that both embryos were males (46XY), with wild type Y chromosomes containing no detectable recombinations in the SHOX related deleted region.

Discussion

We present a PGD protocol and single successful cycle for SHOX-related haploinsufficiency in combination with molecular trisomy 21 detection. This cycle was performed for a family in which an affected offspring carried an X chromosome paternally inherited deletion in the downstream SHOX gene region. Short stature homeobox (SHOX)-related haploinsufficiency disorders range from the mild to severe skeletal phenotypes [14]. In addition to the SHOX related deletion, in this case, the daughter was found to be mosaic for Turner syndrome. Although partial deletion in one Xp chromosome, is genetically related to the SHOX haploinsufficiency syndrome, the deletions are cytogenetically visible [15], larger than the deletion identified in this couple.

Fig. 3 Results from embryos 3 and 4, compared with the genomic DNA of the affected daughter. **a** Sequences of two SNPs, rs6644384 and rs6644385. **b** Chromatogram of the polymorphic microsatellite marker DXYS10096 (numbers represent length of amplification products in base pairs). Both alleles can be observed from all three markers in the two embryos indicating the presence of the wild type allele

For X linked disorders sex selection could be a sufficient method for PGD analysis to achieve an unaffected child [6]. The SHOX gene is located in the pseudoautosomal regions of the X and Y chromosomes, and mutations in the gene region are inherited in a pseudoautosomal dominant manner with a 50% chance of passing a SHOX related mutation to the next generation. In this family the affected daughter carried a deletion inherited from the paternal X chromosome, therefore, all males of this couple should inherit the Y chromosome and be unaffected. However the rates of recombination between the X and Y chromosomes in the pseudoautosomal region have been shown to be much higher than for the rest of the genome [16]. Therefore sex selection alone for PGD is not suitable for mutations residing in the pseudoautosomal regions. While this case could be performed by either FISH or molecular PGD, we opted to use a PCR approach based on our extended experience using molecular techniques with multiple markers.

Single cell PCR may lead to misdiagnosis due to ADO [17] or undetected crossovers between the wild type and mutant alleles [8]. Polymorphic markers are therefore used to increase the accuracy and efficiency of diagnosis. ADO is defined as failure of (or preferential) amplification of one allele and is the primary cause of misdiagnosis in PGD [18]. Linked polymorphic markers help overcome misdiagnosis by reducing the error rate due to ADO from as high as 27% (in blastomeres for single amplicons) to almost 0% when 3 or more markers are used [19]. Crossover events between markers and a specific mutation cannot be completely ruled out even for very closely linked markers [8], and for this reason, it is imperative that the PGD protocol include marker analysis on both sides of the mutation. This situation is even more acute in the pseudoautosomal region of the sex chromosomes, and we therefore chose an approach that included sexing for males together with multiple markers both in and flanking the deletion in order to ensure that no crossover events occurred.

Out of 4 embryos two males inherited the non-deleted SHOX locus. One of these 2 embryos showed distal recombinations between 2 markers between X and Y chromosomes. Since both maternal and paternal markers in the SHOX region were present this assured the presence of the wild type SHOX region. These two wild type embryos were transferred to the proband resulting in the birth of healthy twin boys. We observed one female embryo in which markers for only a single X allele were detected, suggesting a possible Turner karyotype.

Preimplantation Genetic Screening (PGS) performed by FISH analysis of one or two blastomeres is controversial regarding increased pregnancy rates [19, 20]). However, since we were already performing molecular PGD for a monogenic disorder, due to increased maternal age, we

included in our assay 3 fully informative markers (4 different alleles) located on chromosome 21. This strategy allowed us to test for chromosome 21 aneuploidy derived from meiosis I. All four analyzed blastomeres showed diploidy for chromosome 21.

In conclusion, while most X-linked disorders can be analyzed by sexing, genes located in the pseudoautosomal regions have high recombination rates, requiring multiple region specific markers to enable an accurate diagnosis. Molecular analysis of aneuploidy is a convenient and useful tool which can be used in conjunction with single gene testing.

Disclosure of interests Nothing to disclose

Contribution to Authorship Gheona Altarescu was the principal investigator and responsible with the design of the methods, genetic counseling and analysis.

Orit Reish performed the original genetic diagnosis in the family.

E. Kasterstein, D. Komarovsky, A. Komsy, O. Bern, D. Strassburger performed all the embryo biopsy.

Paul Renbaum performed genetic analysis and manuscript preparation/editing

Ephrat Levy-Lahad- advice for molecular strategy manuscript editing
Raphael Ron El was also principle investigator and responsible of all the IVF management.

Ethics Approval The PGD is performed in our medical center as routine medical procedure and is not experimental therefore IRB was not required.

Funding Funding was obtained from the family seeking PGD.

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