Birth of a healthy boy after PGD for X-linked heterotaxy syndrome

R. Bautista-Llácer · M. Pardo-Belenguer · E. García-Mengual ·
C. Sánchez-Matamoros · E. Raga · J. M. Calafell ·
M. S. Cívico · F. Fábregues · X. Vendrell

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Introduction

Heterotaxy syndrome is a clinically and genetically heterogeneous rare disorder included in the group of congenital heart diseases (CHD). The word heterotaxy comes from the Greek “heteros”, meaning “different”; and “taxis”, meaning “arrangement”. The thoracic and visceral organs (including the heart, liver, lungs, spleen, and stomach) have an abnormal placement, as they are situated randomly with respect to the left-right axis and with respect to one another [20].

The inheritance pattern of the disease is heterogeneous, as it could be autosomal recessive, autosomal dominant or X-linked. At least six different disease-causing genes have been identified [3, 13, 20] and one novel candidate has been

determined [15]. Mutations in the zinc finger transcription factor gene (*ZIC3*, MIM *300265) cause the X-linked heterotaxy syndrome (OMIM #306955) [7], which accounts for less than 5 % of sporadic heterotaxy cases. Female carriers in the reported familial cases are usually asymptomatic, although in one case they presented phenotype [20]. The estimated prevalence is unclear, ranging from 2.5/10000 to 1/15000 (from Orphanet reports series, November 2012) [14].

Affected patients present high morbidity and mortality resulting from a wide variety of congenital defects, as they typically present complex cardiovascular malformations. Clinical diagnosis and surgical treatment for patients have improved recently. However, survival rates of patients undergoing surgery are not satisfactory. In this context, preimplantation genetic diagnosis (PGD) has been proved over the years to be a valid reproductive option for couples carrying a genetic disorder who wish to have offspring free of the disease, avoiding the traumatic termination of pregnancy (TOP). Since its first application in 1990 [8], PGD has been made available for a large number of rare genetic disorders [16] and the number of cycles continuously increases year by year [9].

The aim of this paper is to show, for the first time, the successful application of PGD for X-linked heterotaxy, resulting in the birth of a healthy boy.

Capsule Preimplantation genetic diagnosis was performed for X-linked heterotaxy syndrome in a couple with three previous affected pregnancies, resulting in the birth of a healthy boy.

R. Bautista-Llácer · M. Pardo-Belenguer · E. Raga
Molecular-PGD Laboratory, Sistemas Genómicos S.L., Ronda G.
Marconi 6, 46980, Parque Tecnológico, Paterna, Valencia, Spain

E. García-Mengual · C. Sánchez-Matamoros
Reproductive Genetics Laboratory, Sistemas Genómicos S.L., Ronda
G. Marconi 6, 46980, Parque Tecnológico, Paterna, Valencia, Spain

J. M. Calafell · M. S. Cívico · F. Fábregues
Assisted Reproduction Unit, Institut Clinic of Gynecology,
Obstetrics and Neonatology (ICGON). Hospital Clinic, Villarroel
170, 08036 Barcelona, Spain

X. Vendrell
Reproductive Genetics Unit, Sistemas Genómicos S.L., Ronda G.
Marconi 6, 46980, Parque Tecnológico, Paterna, Valencia, Spain

R. Bautista-Llácer (✉)
Sistemas Genómicos S.L., Ronda G. Marconi 6, Parque Tecnológico
de Valencia, 46980 Paterna, Valencia, Spain
e-mail: rosa.bautista@sistemasgenomicos.com

Case report

Family history

The couple composed of a 35 year-old woman and a 35 year-old man attended genetic counseling after three pregnancies affected of heterotaxy syndrome, that were terminated (Fig. 1). Consanguinity was not referred. The first pregnancy was a male foetus with asplenia, persistent bilateral superior vena cava, right pulmonary isomerism, congenital cardiopathy

Fig. 1 Genealogical tree of the family requesting preimplantation genetic diagnosis for X-linked heterotaxy syndrome. The *arrow* indicates the consultant patient. The *asterisk* indicates the family members involved in the informativity study. The grandmother was studied and did not carry the mutation. The woman's sisters, father and daughter had not been studied at the time of consultation

with dextrocardia and transposition of great arteries. The second pregnancy was a healthy girl, but she had not been studied at the time of consultation. The third pregnancy was a male foetus with congenital cardiopathy and lateralization defects. After clinical diagnosis of both foetuses, as they were affected males and the second pregnancy an asymptomatic girl, a recessive X-linked heterotaxy syndrome was suspected. The study of the *ZIC3* gene on foetal remains revealed the mutation c.842_843delAT (p.His281ArgfsX62) in exon 1 in hemizygosis. This mutation creates a premature stop-codon either leading to nonsense-mediated decay of aberrant RNA or a truncated *ZIC3* protein, eliminating all important Zinc-Finger (ZF) protein domains. ZF2 and ZF3 domains are responsible for transportation of the *ZIC3* protein to the nucleus, where it acts as a transactivation regulator. Therefore, elimination of these domains will eliminate this transport. This mutation had not been previously described in any database as pathogenic. Nevertheless, due to its deleterious effect and highly severe clinical phenotype, it was determined as the disease-causing mutation.

The woman was studied and the same mutation was identified in heterozygosis, proving to be an asymptomatic carrier of X-linked heterotaxy syndrome. Her mother, who did not refer any abortions due to lateralization defects, was also studied, but she did not carry the mutation. According to literature, hemizygous males carrying a mutation in the *ZIC3* gene always show clinical symptoms [20]. For this reason, it was supposed that it could be a *de novo* mutation, as the father did not show any clinical symptoms. However, as he was not studied at the time of consultation, genetic testing was recommended, in order to ascertain if there was a genetic risk for the sisters. Incomplete penetrance in hemizygous males has been reported previously [12].

A fourth spontaneous male pregnancy underwent prenatal diagnosis of chorionic villus sampling, confirming the presence of the mutation c.842_843delAT (p.His281Arg fsX62) in exon 1 in hemizygosis. This pregnancy was also terminated.

The complete PGD process was explained to the patients and their queries were answered. The couple signed an informed consent form for PGD testing.

Materials and methods

Preclinical studies and lymphocyte validation were performed prior to the in vitro fertilization (IVF) cycle, following recommended international guidelines on PGD [10], and the norm UNE-EN ISO 9001:2000 [11, 18]. Blood samples in EDTA were requested from both members of the couple. Also, DNA sample from one of the foetus and buccal swabs from the healthy daughter were provided. DNA extraction from blood and buccal swabs was carried out using the QIAcube System (QIAGEN, Germany) following the manufacturer's instructions. PCR was first performed on genomic DNA to confirm the mutation and to determine the informative short tandem repeat (STR) markers, then on 1 ng of diluted genomic DNA in a heminested multiplex PCR with the informative STR markers and finally on single lymphocytes collected from both parents with the same PCR conditions that worked for 1 ng of genomic DNA. Oligonucleotides were tested in order to discard possible human DNA contamination.

Fifteen STR markers closely linked to the *ZIC3* gene were tested and the *AMELX* gene was used in order to know the number of expected alleles. The molecular analysis protocol was the same for lymphocytes (preclinical set-up) and blastomeres (clinical PGD cycle). Single cells were isolated and lysed as previously described [5]. PCR amplification was performed using the outer and inner oligonucleotide primers listed in Table 1. First round multiplex PCR containing the external primers was carried out in a total volume of 25 µL containing 200 µM dNTP, 1.5 mM MgCl₂, 1X PCR buffer, 1 unit Go-Taq Hot Start polymerase (Promega, U.S.A.) and 0.4 µM each of the outer forward and reverse primers. One microlitre from the first round PCR was used as template for separate second round PCR reactions with fluorescently

Table 1 Primer sequences used for preimplantation genetic diagnosis of X-linked heterotaxy

Name	Heterozygosity (%)	Gene distance (Mb)	Primer sequences
<i>ZIC3</i> -E1	N/A	N/A	<i>Fout</i> : 5' CAGCCTATCAAGCAGGAGC 3' <i>R</i> : 5'CTTCGCCTTGAAAGACTTGC 3' <i>Fin</i> : 5' 6FAM-CCAAGAAGAGCTGCGACCG 3'
DXS691 ^a	uk	1.25	<i>Fout</i> : 5'GCTATTGGCTGTGTAATGGA 3' <i>R</i> : 5'GTTACACTCTTTCAGCCAGC 3' <i>Fin</i> : 5'6FAM-TATGGGTAGGTTTGGGTTG 3'
DXS8094 ^a	0.77	0.41	<i>Fout</i> : 5'GAGAAAACATCCAGCACAAATG 3' <i>R</i> : 5'GAGTCACTGTCTCTTAGGGTC 3' <i>Fin</i> : 5' 6FAM-GCAATGTCACCATTAAACAGTTTG 3'
DXS1211 ^a	0.64	1.65	<i>Fout</i> : 5'CATCTCCAGAGCGGTAGGAT 3' <i>R</i> : 5'GCAAGTCTGTCCAAGACCT 3' <i>Fin</i> : 5' 6FAM-CATTCCCCTCCAATCTGGCAG 3'
DXS1062 ^a	0.75	0.64	<i>Fout</i> : 5'GAGATGTGTGACCTTGAGCAC 3' <i>R</i> : 5'GTTGCCTGTAAAGCACTTTG 3' <i>Fin</i> : 5' 6FAM-AGTAGCACCCACCTAATAG 3'
<i>AMELX</i>	N/A	N/A	<i>Fout</i> : 5'ACCTCATCCTGGGCACCCTGG 3' <i>R</i> : 5'ATCAGAGCTTAACTGGGAAGCTG 3' <i>Fin</i> : 5'NED-CCCTGGGCTCTGTAAAGAATAGTG 3'

Fout forward outer primer; *Fin* forward inner primer; *R* reverse primer; *N/A* not applicable; *uk* unknown

^a Rosen, N., V. Chalifa-Caspi, O. Shmueli, A. Adato, M. Lapidot, J. Stampnitzky, M. Safran, and D. Lancet (2003). GeneLoc: Exon-based integration of human genome maps. *Bioinformatics* 19(S1)

URL: <http://genecards.weizmann.ac.il/geneloc>

labelled inner forward and reverse primers in a total volume of 10 µL. PCR reactions were undertaken using a TProffessional standard Thermocycler (Biometra, Germany).

Seventeen thermal cycles were performed for the outer primers and 30 for the inner primers at 95 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min. The cycling was preceded by a 5 min initial denaturation step at 95 °C and ended with an additional elongation step of 7 min at 72 °C. Amplified inner products were electrophoresed in an automated genetic analyser ABI 3730xl (Applied Biosystems, USA). Results were analysed with the Genemapper v3.7 software (Applied Biosystems, USA).

Respecting the IVF cycle, the patient received a routine superovulation procedure based on the long protocol of down-regulation with gonadotrophin-releasing hormone agonist and ovarian stimulation with recombinant follicle stimulant hormone for 12 days [2]. Twenty nine mature oocytes were obtained, and 24 two-pronucleus zygotes were produced after intracytoplasmic sperm injection. In order to avoid ovarian hyperstimulation syndrome, all embryos were cryopreserved

at pronuclear stage and PGD was cancelled. In the next menstrual cycle, endometrial preparation was done with oestrogen and progesterone administered in a sequential regime. Frozen-thawed embryo transfer was planned when the endometrium was 9 mm thick. After thawing, 23 zygotes survived and 15 cleaving embryos were available for biopsy on day +3. Biopsy procedures were performed according to previously described protocols [1].

Results

Eleven STR markers resulted informative. Therefore, the two closest markers upstream (DXS691 and DXS8094) and downstream (DXS1062 and DXS1211) of the gene were chosen. The order with respect to the *ZIC3* gene is shown in Fig. 2. Once the diagnosis strategy to detect the c.842_843delAT mutation in the *ZIC3* gene was established, validation of the technique was carried out with 25 single isolated lymphocytes from each parent. Amplification failure and allelic drop out (ADO- the random non-amplification of

Fig. 2 Location of the short tandem repeat markers tested in the preclinical work-up with respect to the *ZIC3* gene on chromosome Xq26.3. The two closest informative markers upstream and downstream to the gene

(DXS691, DXS8094, DXS1062 and DXS1211) were selected to support the diagnosis in the PGD cycle. Distance to the gene is stated in megabases (Mb)

one of the alleles in a heterozygous sample) rates for each PCR amplicon were estimated. ADO rates were 0 % (0/25) for c.842_843delAT mutation and 0 % (0/25) for each the STR markers and the *AMELX* gene. PCR efficiency for each amplicon was 100 % (50/50) excepting DXS691, where it was 99.98 % (49/50).

Fifteen embryos were biopsied obtaining 16 single blastomeres. Amplification failed in 11 single blastomeres. Five blastomeres from four embryos were successfully analysed. Total ADO and amplification efficiency rates were 26 % (6/23) and 77 % (23/30) respectively. Three embryos were diagnosed: two carriers and one healthy. One of the embryos presented either ADO of the *AMELX* gene or ADO of the paternal chromosome and could not be diagnosed. Two embryos (embryo no. 3 and 9) reached the blastocyst stage and embryo number 3 was transferred. A single pregnancy was achieved, resulting in a healthy birth. Prenatal diagnosis to confirm the PGD results was recommended. However, it was not performed, although the patient was exhaustively controlled by echography.

Discussion

In the last two decades, surgical approaches for the problems derived from heterotaxy syndrome have improved significantly. However, a large proportion of patients do not undergo any operation due to complex manifestations of the syndrome where surgical mortality is high and outcome uncertain [4]. Also, these approaches require aggressive neonatal interventions, careful management in every phase of the disease and frequent re-interventions. In the case presented, the couple had three TOP. As they wished to have more offspring, PGD was offered as the best reproductive alternative for them, as they did not want any more pregnancies to be terminated.

Nowadays, PGD for single-gene disorders is considered as a routine alternative to prenatal diagnosis, and several strategies have been reviewed thoroughly [17]. In all cases, preconception genetic counselling is necessary, especially in rare syndromes with genetic heterogeneity like heterotaxy. Inheritance and clinic information compiled from the couple allows orientating the genetic diagnosis, crucial aspects prior to preimplantation/prenatal decisions [6, 19].

In this case, a direct approach was decided. This is not obvious in cases of X-linked disorders. Molecular genotyping of single blastomeres supported with STR-haplotyping analyses represent an accurate option, unlike sex selection or indirect PGD based on haplotyping. In cases of X-linked disorders, when the disease-causing mutation is diagnosed, a direct genotyping approach offers clear advantages: healthy male embryos are identified, genetic status of female embryos

(strictly healthy or asymptomatic carriers) is established, and recombination events could be detected reducing the risk of misdiagnosis.

Before couples undergo PGD cycle, it is compulsory to perform a preclinical work-up. In this particular case, PGD was limited at the time of consultation as the female carried a suspected *de novo* mutation and the genetic status of her daughter was unknown. In this scenario, the maternal family was non-informative and a haplotyping strategy was inadequate because the disease-bearing haplotype could not be established *a priori* [17]. In order to overcome this limitation, a genetic study of the *ZIC3* gene was performed on foetal remains from affected pregnancies. This genetic information was confirmed on the mother, and on buccal swabs from the daughter. Thus, more than one meiosis was involved in the preclinical work-up as recommended in the ESHRE Guidelines for molecular PGD cases [10]. The daughter's genetic status (healthy or carrier) was not revealed to the family due to controversial decisions regarding genetic studies on minors.

In conclusion, a successful protocol for X-linked heterotaxy syndrome was developed, resulting in the birth of a healthy boy. In extremely rare conditions, a case by case strategy is the gold standard option for couples at risk where the disease-causing mutation is known.

Conflict of interest None declared

References

1. Alberola TM, Bautista-Llácer R, Fernández E, Vendrell X, Pérez-Alonso M. (2009) Preimplantation genetic diagnosis of P450 oxidoreductase deficiency and Huntington Disease using three different molecular approaches simultaneously. *J Assist Reprod Genet* 26:263–271.
2. Balasch J, Vidal E, Peñarrubia J, Casamitjana R, Carmona F, Creus M, et al. Suppression of LH during ovarian stimulation: analysing threshold values and effects on ovarian response and the outcome of assisted reproduction in down-regulated women stimulated with recombinant FSH. *Hum Reprod*. 2001;16:1636–43.
3. Belmont JW, Mohapatra B, Towbin JA, Ware SM. Molecular genetics of heterotaxy syndromes. *Curr Opin Cardiol*. 2004;19:216–20.
4. Changlani DK, Kotecha M, Changlani TD, Varghese R, Kumar RS. Visceral heterotaxy in the developing world. *Heart Lung Circ*. 2012;21:598–605.
5. Cui XF, Li HH, Goradia TM, Kazazian Jr HH, Galas D, Arnheim N. Single-sperm typing: determination of genetic distance between the G gamma-globin and parathyroid hormone loci by using the polymerase chain reaction and allele-specific oligomers. *Proc Natl Acad Sci U S A*. 1989;86:9389–93.
6. D'Alessandro LC, Casey B, Siu VM. Situs inversus totalis and novel *ZIC3* mutation in a family with X-linked heterotaxy. *Congenit Heart Dis*. 2013;8:E36–40. doi:10.1111/j.1747-0803.2011.00602.x.
7. Gebbia M, Ferrero GB, Pilia G, Bassi MT, Aylsworth AS, Penman-Splitt M, et al. X-linked situs abnormalities result from mutations in *ZIC3*. *Nat Genet*. 1997;17:305–8.

8. Handyside AH, Kontogianni EH, Hardy K, Winston RM. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature*. 1990;344:768–70.
9. Harper JC, Wilton L, Traeger-Synodinos J, Goossens V, Moutou C, SenGupta SB, et al. The ESHRE PGD Consortium: 10 years of data collection. *Hum Reprod Update*. 2012;18(3):234–47.
10. Harton GL, De Rycke M, Fiorentino F, Moutou C, SenGupta S, Traeger-Synodinos J, et al. ESHRE PGD consortium best practice guidelines for amplification-based preimplantation genetic diagnosis (PGD). *Hum Reprod*. 2011;26:33–40.
11. ISO 9001. Quality management systems- requirements. Geneva: International Organization for Standardization; 2000.
12. Megarbane A, Salem N, Stephan E, Ashoush R, Lenoir D, Delague V, et al. X-linked transposition of the great arteries and incomplete penetrance among males with a nonsense mutation. *Eur J Hum Genet*. 2000;8:704–8.
13. Online Mendelian Inheritance in Man, OMIM®. Johns Hopkins University, Baltimore, MD. MIM Number: {#306955}: {12/06/2012}: World Wide Web URL: <http://omim.org/>
14. *Orphanet*: an online rare disease and orphan drug data base. Copyright, INSERM 1997. Available on <http://www.orpha.net>. Accessed 23 Jan 2013
15. Tariq M, Belmont JW, Lalani S, Smolarek T, Ware SM. SHROOM3 is a novel candidate for heterotaxy identified by whole exome sequencing. *Genome Biol*. 2011;12:R91.
16. The Preimplantation Genetic Diagnosis International Society (PGDIS). Guidelines for good practice in PGD: programme requirements and laboratory quality assurance. *Reprod BioMed Online*. 2008;16:134–47.
17. Vendrell X, Bautista-Llácer R (2012). A methodological overview on molecular preimplantation genetic diagnosis and screening: a genomic future?. *Syst Biol Reprod Med* 58(6):289–300.
18. Vendrell X, Carrero R, Alberola T, Bautista-Llácer R, García-Mengual E, Claramunt R, Pérez-Alonso M. (2009) Quality management system in PGD/PGS: now is the time. *J Assist Reprod Genet* 26:197–204.
19. Vendrell X, Bautista-Llácer R, Alberola TM, García-Mengual E, Pardo M, Urries A, Sánchez J (2011). Pregnancy after PGD for recessive dystrophic epidermolysis bullosa inversa: genetics and preimplantation genetics. *J Assist Reprod Genet* 28(9): 825–32.
20. Ware S, Peng J, Lirong Z, Fembach S, Colicos S, Casey B, et al. Identification and functional analysis of *ZIC3* mutations in heterotaxy and related congenital heart defects. *Am J Hum Genet*. 2004;74:93–105.