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GENETICS

Preimplantation diagnosis for β-thalassemia combined with HLA matching: first "savior sibling" is born after embryo selection in Brazil

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Introduction

β-Thalassemia is a common hereditary hemoglobinopathy disorder that affects many organs and represents the most common monogenetic disorder worldwide [8, 11]. Diverse phenotypes exist within the β-thalassemia syndromes, ranging from the β-thalassemia minor to the β-thalassemia major [3]. β-Thalassemia major is characterized by reduced synthesis of the hemoglobin subunit beta (hemoglobin beta chain) that results in severe anemia and hepatosplenomegaly [3, 11].

Affected children require regular blood transfusions to sustain life and, without treatment, have shortened life expectancy [14]. Treatment with a regular transfusion program and chelation therapy allows for normal growth and development and extends life expectancy into the third to fifth decade [1].

Bone marrow transplantation (BMT) remains the only definitive cure currently available for patients with β-thalassemia [3]. The success of haematopoietic stem cell (HSC) transplantation will depend on the ability of the human leukocyte antigen (HLA) types of the donor and recipient to match. When the search for compatible donors in bone marrow living donor banks and databases is exhausted, the only way to save the life of a child may be to use preimplantation genetic diagnostic (PGD) to facilitate the birth of a child who can be a matching tissue donor, the so called "savior siblings" [13].

Currently, the selection of a compatible sibling to assist a transplant is an acceptable application of PGD, through HLA haplotyping. The ESHRE PGD consortium reported that in the last decade there were 225 cycles of HLA typing along with exclusion of a specific disorder. The majority (75 %) were for β-haemoglobinopathy [4]. Other indications include Fanconi anaemia, Gaucher disease, adrenoleukodystrophy and osteopetrosis.

According to the ESHRE PGD Consortium "when all other clinical options have been exhausted, PGD is acceptable for couples who already have a child affected with a malignant disorder or a genetic disorder, if the affected child is likely to be cured or life expectancy is substantially prolonged by stem cell transplantation with cord blood from a HLA-matched sibling" [5].

This article presents the first Brazilian clinical experience demonstrating feasibility of combined PGD and HLA matching for β-thalassemia major, designed to preselect for transfer only those unaffected embryos that are HLA antigen compatible with a sibling needing cord blood transplantation.

Capsule Preimplantation embryos can be selected according to their genetic status as well as a possible HLA matching with the affected sibling.

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Material and methods

Patient history

A 34-year-old female patient, carrier of a genetic heterozygous IVSI-1 G→A mutation for β-thalassemia, and in otherwise good health, was enrolled for ICSI. Her husband was a compound heterozygote carrying Cd39 C→T for β-globin gene (HBB). Their affected 5-year-old daughter had both mutations, which often required blood transfusion therapy. The couple requested PGD for β-thalassemia major, together with HLA antigen test of embryos, in order to have an unaffected child who could be a compatible cord blood donor for their affected daughter.

Sample collection

Two hundred μL of peripheral blood samples with EDTA (BD Vacutainer® Plus Plastic K2EDTA Tubes) was collected from the couple for DNA extraction. From their affected daughter, the sample was collect by a buccal swab test in order to avoid data differences due to frequent blood transfusions. Genomic DNA was isolated with automated QIAcube system, according to QIAamp DNA Blood Mini Kit protocol manufacturer's instruction (Qiagen, Valencia, CA, USA). Samples were eluted in 100 μL of elution buffer.

Selection of microsatellites

In order to verify the most informative polymorphic markers and short tandem repeat (STR) haplotype of the family, an analysis model was constructed. This model identified the segregation of each parental allele, both in relation to genetic markers of the disease, as well as markers of the human leukocyte antigen (HLA) complex. Twenty-nine microsatellites STRs (Table 1) covering the HLA region were tested [2]. The genetic markers were equally spaced and had some alleles per locus with a high heterozygosity. Firstly, β-thalassemia was identified with the confirmation of the mutations in family members, by bidirectional sequencing of HBB gene and linkage studies, which analyzed five polymorphic STR markers (D11S1760; D11S1997; D11S4146; D11S4148; e D11S4149).

Standardization of Preimplantation Genetic Diagnosis (PGD)

To identify the most informative STR markers for disease and HLA complex, each marker was individually tested in a Singleplex PCR. An STR marker was considered informative when both parents were heterozygous (two different combination of alleles presented for the locus), which allowed to determine the segregation of each allele in the family. Selected markers (D11S1997 and D11S4146, both related to disease; and D6S291; D6S2447; DRA-CA; LH-1; 82–1; MIB; and D6S276, related to HLA complex) were amplified in a multiplex PCR (Fig. 1).

Table 1 Polymorphic markers and short tandem repeat (STR) haplotype for β-globin gene (HBB) and human leukocyte antigen (HLA)

Gene	Chromosomal region	STR Locus	Informative STRs
HBB	11p15.5	D11S4146	D11S4146
		D11S4148	
		D11S4149	
		D11S1997	D11S1997
		D11S1760	
HLA	6p21.3	D6S426	
		D6S291	D6S291
		D6S439	
		Ring3CA	
		TAP-CA	
		G51152	
		D6S2447	D6S2447
		DQCAR	
		DQCAR-II	
		DRA-CA	DRA-CA
		LH-1	LH-1
		D3A	
		D6S273	
		9N-2	
		82-1	82-1
		TNFa	
		TNFb	
		62	
		MICA	
		MIB	MIB
		HLABC-CA	
		HLAC-CA	
		D6S1624	
		D6S265	
		D6S510	
		D6S248	
		MOGCA	
		D6S105	
		D6S276	D6S276

PGD validation—single cell test

In order to validate the reliability of the protocol developed for PGD and evaluate the rate of allelic loss [12], multiplex PCR for simultaneous amplification of the markers linked to the mutation and the HLA complex was tested in 30 paternal and 30 maternal lymphocytes.

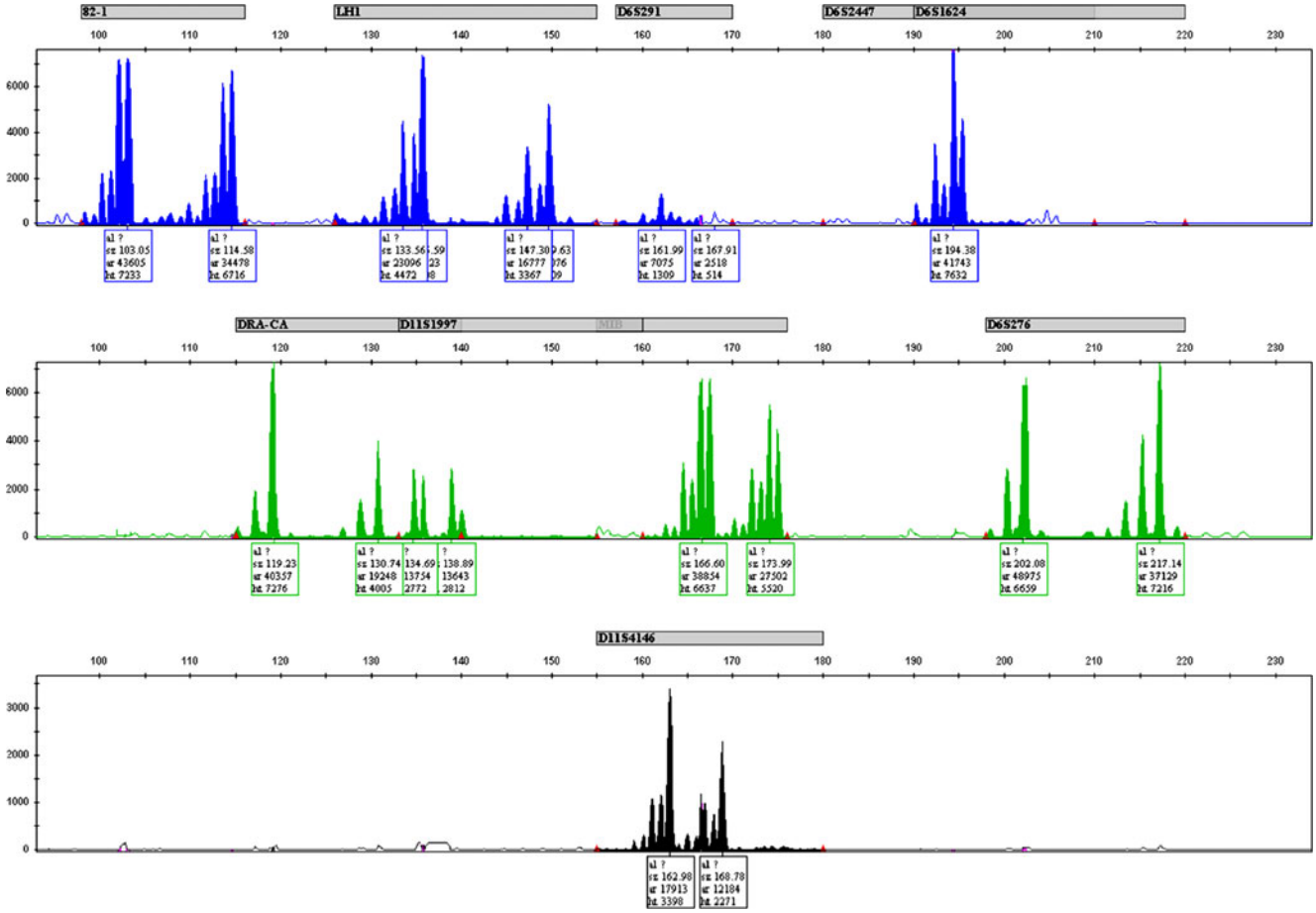

Fig. 1 PGD Standardization results related to STR markers for β-thalassemia and HLA complex

Blood sample collection

Three mL of peripheral blood samples were collected in a tube containing heparin. Blood sample was placed in 3 mL of Histopaque - 1077 (Sigma Chemical, St Louis, MO, USA) and centrifuged at 400 g for 30 min. After centrifugation, the supernatant and plasma fractions were discarded and the remaining cell layer with mononuclear leukocytes, mostly lymphocytes, was washed three times in phosphate-buffered saline (PBS) calcium and magnesium free (Sigma Chemical, St Louis, MO, USA). For sample storage, aliquots were separated in tubes containing 0.5 mL of a solution with PBS 70 % and 30 % glycerol, cryopreserved at -80 ° C.

Isolation of lymphocytes

A cryopreserved aliquot was thawed and diluted in 3 mL of PBS. The diluted aliquot was equally distributed in 12 droplets containing 20 μL of HTF-HEPPES supplemented with 2 % HAS on a culture dish (Ultra-low-attachment culture plate, Fisher Scientific, USA). Each lymphocyte ($n \geq 80$) was individually aspirated and placed into microtubes with 3 μL of lysis solution (1 μL of sodium dodecyl sulphate 17 mM and 2 μL of proteinase K 125 mg/mL). Negative controls ($n=20$) with lysing solution and culture medium were randomly distributed among the sample tubes, in order to verify the absence of contamination.

Cell lysis

After cell isolation, reactions were carried out in a Veriti™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA) and cycling conditions were 50 °C for 30 min for activation of proteinase K, and 96 °C for 15 min to inactivate the enzyme. Afterwards, the samples were ready for PCR.

Controlled ovarian stimulation

The patient began receiving recFSH treatment (Gonal-F®, Serono, Geneve, Switzerland) daily on the third day of menstrual cycle. GnRH antagonist (Cetrotide, Serono, Geneve, Switzerland) was administered when the dominant follicle was 14 mm in mean diameter. The total dose of FSH administered was 2250 IU. Oocyte retrieval through

transvaginal ultrasonography was performed 35 h after the administration of recombinant human chorionic gonadotrophin (rhCG, Ovidrel™, Serono, Geneve, Switzerland).

Preparation of oocytes and ICSI

The 17 retrieved oocytes were maintained in culture medium (Global® for Fertilization, LifeGlobal, Connecticut, USA) supplemented with 10 % Human Synthetic Albumin (HSA, Irvine Scientific, Santa Ana, USA), which was covered with mineral oil (Ovoil™, Vitrolife, Kungsbacka, Sweden) and stored at 37 °C and 6 % CO_2 for 5 h. The surrounding cumulus cells were removed by exposure to a HEPES buffered-medium containing hyaluronidase (80 IU/mL, Irvine Scientific, Santa Ana, USA). The remaining cumulus cells were then mechanically removed by gentle pipetting with a hand-drawn Pasteur pipette. Fifteen oocytes were identified as metaphase II and two oocytes as metaphase I (MI). At the moment of injection, one MI oocyte was observed to have released the first polar body and was considered mature. ICSI was performed on 16 oocytes using the technique described by Palermo et al. [10].

Assessment of fertilization and embryo culture

Approximately 18 h after ICSI, 13 oocytes were identified as having two pronuclei and two polar bodies. The oocytes were maintained in a 50-μL drop of culture medium (Global®, LifeGlobal, Connecticut, USA) supplemented with 10 % HSA and covered with mineral oil (Ovoil™ - Vitrolife, Kungsbacka, Sweden) in a humidified atmosphere with 6 % CO_2 at 37 °C until embryo biopsy.

Preimplantation genetic diagnosis

A total of 10 embryos were biopsied. Two embryos were biopsied on the morning of day 3 (embryos presenting>5 blastomeres) and 8 embryos were biosied on day 4 (embryos presenting<5 blastomeres on day 3). One cell per embryo was biopsied by laser zona drilling using a 1.48 μm Infrared Diode Laser (Octax Laser Shot System, MTG, Bruckberg, Germany). Following the biopsies, the embryos were returned to culture until embryo transfer on day 5 of embryo develpment. The removed blastomere nuclei were washed twice in HEPES medium and transferred to sterile microtubes containing 3 mL of lysing solution. For each biopsied embryo a negative control was prepared with lysing solution.

Mutation and HLA analysis

Multiplex PCR was carried out in biopsied blastomeres, in negative controls and maternal and paternal isolated lymphocytes, as positive controls. The amplicons were electrophoresed (ABI 3130 - Applied Biosystems, Foster City, CA, USA), according to the protocol: 9.0 mL of Hi-Di ™ Formamide (Applied Biosystems, Foster City, CA, USA), 0.25 μL GeneScan ™ 500 ROX ™ Standard size (Applied Biosystems, Foster City, CA, USA) and 0.75 μL of the PCR product. Fragments analyses were done by GeneMapper ID® Software Version 3.2 (Applied Biosystems, Foster City, CA, USA).

Results

Blastomere genotyping for mutation was performed in 10 embryos. Of 10 embryos with results, 5 were heterozygous carriers, 3 were homozygous affected, and 2 were homozygous normal. Testing for HLA in the 10 embryos revealed 3 embryos with HLA antigen match for the affected sibling: one homozygous affected, one heterozygous carrier and one homozygous normal. Two compact embryos were transferred on day 5 of development. Both embryos were found to be HLA matching. One embryo was homozygote normal for β-thalassemia and the other was heterozygous carrier.

The embryo transfer resulted in a clinical pregnancy and birth of a healthy baby, homozygote normal for β-thalassemia, on February 2012. Stem cells from the baby's umbilical cord were frozen and will be used for bone marrow transplantation for her sister at the end of 2012.

Discussion

The advent of single-cell PCR has presented the opportunity for combined PGD and HLA antigen testing. This is a novel and useful way to preselect a potential donor for an affected sibling requiring stem cell transplantation. The possibilities for couples with a history of genetic illnesses are considerably increasing. However, PGD with tissue typing is only currently available for a limited number of genetic conditions, usually those treatable through stem cell transplant.

Fourteen years after the introduction of PGD for thalassemias [6], haemoglobin disorders are presently one of the major indications for PGD [7]. Bone marrow transplantation remains the only definitive cure currently available for patients with thalassemia. In patients with favorable pretransplantation clinical conditions, stem cell transplantation from an HLA identical sibling has a disease free survival rate over 90 % [3].

This strategy is particularly interesting in the treatment of β-thalassaemia because: (i) β-thalassaemia is caused by point mutations in the b-globin gene that can be detected by PCR/PGD; (ii) β-thalassaemia is a very common single-gene disorder and therefore, the number of candidate

couples requesting such therapeutic option may be significant; and (iii) β-thalassaemia is an autosomal recessive genetic disorder with a high frequency of heterozygotes (silent carriers or β-thalassaemia minor), which is a relatively mild medical condition. As a consequence, only embryos that are affected have to be excluded for transfer, thereby increasing the likelihood of finding suitable candidate embryos for transfer. The limitations are: (i) the financial cost is high (approximately $7000 for the ICSI cycle and $2700 for the PGD and HLA analysis); (ii) the lower the number of embryos, the lower the probability of having a suitable one. Theoretically, one in four embryos are HLA identical and three out of four will not carry the mutation or be heterozygous so that only three out of 16 will be transferable; and (iii) the implantation capacity of the embryos is highly variable and the chance of a successful pregnancy may be small.

The selection of a savior sibling involves the selection of embryos knowing that most of them will be destroyed. However, these embryos are conceived with the intention of bringing a human being into the world. Actually, this is not the only intention, as the procedure is started with the intention of saving the life of an already existing child. This further intention has led critics of this procedure to argue that it involves 'instrumentalizing' the child that is created by bringing it into existence as a means to the end of saving another child's life [9, 15].

The selection of savior siblings has been the topic of ethical and social dilemmas, and several authorities concluded that it is ethical and also legally permissible in a number of jurisdictions [13]. According to the ESHRE PGD Consortium guidelines, consideration should be given to the time required for the PGD test to be developed and applied and for an HLA-matched sibling to be born: cases in which the affected child has an acute medical condition prohibiting safe stem cell transplantation or an extremely low life expectancy should be excluded [5].

In conclusion, PGD for single gene disorders combined with HLA typing has recently emerged as a therapeutic tool. For couples who are at risk of passing on a genetic disease to their offspring, preimplantation embryos can be selected according to their genetic status as well as a possible HLA matching with the affected sibling. Stem cells from the resulting baby's umbilical cord blood have, therefore, a great therapeutic value for haematopoietic and other life threatening diseases, as stem cells in the cord blood from a HLA-compatible newborn can be used for transplantation without graft rejection, thus saving an affected child's life.

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