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CLINICAL CORNER: CASE REPORT

Complex preimplantation genetic diagnosis for beta-thalassaemia, sideroblastic anaemia, and human leukocyte antigen (HLA)-typing

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Abstract

Preimplantation genetic diagnosis (PGD) to select histocompatible siblings to facilitate curative haematopoeitic stem-cell transplantation (HSCT) is now an acceptable option in the absence of an available human leukocyte antigen (HLA) compatible donor. We describe a case where the couple who requested HLA-PGD, were both carriers of two serious haematological diseases, beta-thalassaemia and sideroblastic anaemia. Their daughter, affected with sideroblastic anaemia, was programmed to have HSCT. A multiplex-fluorescent-touchdown-PCR protocol was optimized for the simultaneous amplification of: the two *HBB*-gene mutated regions (c.118C > T, c.25-26delAA), four short tandem repeats (STRs) in chr11p15.5 linked to the *HBB* gene, the *SLC25A38* gene mutation (c.726C > T), two STRs in chr3p22.1 linked to the *SLC25A38* gene, plus eleven informative STRs for HLA-haplotyping (chr6p22.1-21.3). This was followed by real-time nested PCR and high-resolution melting analysis (HRMA) for the detection of *HBB* and *SLC25A38* gene mutations, as well as the analysis of all STRs on an automatic genetic analyzer (sequencer). The couple completed four clinical *in vitro* fertilization (IVF)/PGD cycles. At least one matched unaffected embryo was identified and transferred in each cycle. A twin pregnancy was established in the fourth PGD cycle and genotyping results at all loci were confirmed by prenatal diagnosis. Two healthy baby girls were delivered at week 38 of pregnancy. The need to exclude two familial disorders for HLA-PGD is rarely encountered. The methodological approach described here is fast, accurate, clinically-validated, and of relatively low cost.

Abbreviations: ART: Assisted Reproductive Technology; ESHRE: European Society of Human Reproduction and Embryology; HLA: human leukocyte antigen; HRMA: high-resolution melting analysis; HSCT: haematopoeitic stem-cell transplantation; IVF: in vitro fertilization; PGD: preimplantation genetic diagnosis; STR: short tandem repeat

Keywords

Beta-thalassaemia, high resolution melting analysis, preimplantation genetic diagnosis, sideroblastic anaemia, single cell HLA-typing

History

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Introduction

Preimplantation genetic diagnosis (PGD) with human leukocyte antigen (HLA)-matching to establish a pregnancy with histocompatible siblings to support a curative haematopoietic stem cell transplantation (HSCT) for an existing affected child in the family, was first reported in 2001 for a case of Fanconi anaemia. It is now a well-established procedure, with an increasing number of cycles performed every year [Moutou et al. 2014]. The present worldwide experience of preimplantation HLA typing probably includes more than a thousand cases, most of which have been performed within the last few years [Kuliev and Rechitsky 2014a].

Preimplantation HLA-typing can be performed as a sole indication, when the affected child requires transplantation to treat a non-inherited disorder related to the hematopoietic and/or immune system, for example certain leukemias, or simultaneously with PGD to exclude a familial single gene disease and establish a pregnancy with an unaffected and at the same time HLA compatible embryo. HSCT from an identical HLA sibling-stem cell donor provides the best treatment option, reducing the incidence of graft rejection and other complications [Soni et al. 2014; Tucunduva et al. 2014; Tur-Kaspa and Jeelani 2015].

Recent studies have provided data on a large number of HLA-PGD cycles, demonstrating the overall success of the procedure and the positive outcome following HSCT treatment. Kahraman et al. [2014] report 461 PGD cycles initiated

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for multiple HLA indications, performed within a period of 10 years, resulting in 80 live births and 44/48 successful HSCTs, using either cord blood as a sole source (in only three cases) or bone marrow, or a combination of bone marrow and cord blood. In the four unsuccessful cases graft failure occurred after HSCT for treatment of beta thalassaemia in older children with a high number of previous blood transfusions [Kahraman et al. 2014]. Kuliev and Rechitsky [2014a; 2014b; 2014c] have also presented their significant work of over 300 HLA-PGD cycles for haemoglobinopathies, immunodeficiencies or HLA-typing as a sole indicator, resulting in the birth and so far complete cure of affected siblings that underwent HSCT [Kuliev and Rechitsky 2014a; 2014b; 2014c].

According to the data from the European Society of Human Reproduction and Embryology (ESHRE) PGD consortium and published data from the aforementioned centers, beta-thalassaemia/sickle cell anaemia is the most common indication for PGD with concurrent HLA-typing. The need to exclude two familial hereditary disorders for HLA-PGD has not been previously reported. Here we describe the development and application of a PGD protocol for the diagnosis of two monogenic disorders plus HLA compatibility testing, involving a couple known to be both carriers for beta-thalassaemia mutations and also carriers for an identical mutation causing autosomal recessive nonsyndromic congenital sideroblastic anaemia.

Patient History: Case Report

The couple was referred to the Department of Medical Genetics of the University of Athens from the transfusion centre of the First Department of Paediatrics, University of Athens, in January 2012. At the time of referral, the maternal and paternal ages were 25 and 34 years, respectively. Both parents were known carriers of two different beta-thalassaemia mutations in the *HBB* gene (maternal mutation: c.118 C>T (Cd39 C>T) and paternal mutation: c.25-26 delAA (Cd8 delAA)). Their reproductive history indicated one previous pregnancy where prenatal diagnosis for β-thalassaemia was performed, which resulted in the birth of their daughter in 2010, unaffected for β-thalassaemia. However, at the age of 24 days she presented with febrile illness and pallor. She was found to have severe anaemia (haemoglobin 61 mg/L) which was hypochromic (MCHC 26.7 pg/dl) and microcytic (MCV 70fl) and was started on a transfusion regimen every three-four weeks. The clinical diagnosis of congenital sideroblastic anaemia was established at four months of age with the demonstration of ringed sideroblasts in bone marrow erythropoietic progenitors. The diagnosis was confirmed by analysis of the molecular defects for sideroblastic anaemia, performed at the Department of Biochemistry and Medical Biotechnologies, University Federico II of Naples, Italy (kindly provided by Professor Achille Iolascon). The results of this analysis showed that the propositus was homozygous for the mutation c.726C>T in the *SLC25A38* gene. This mutation, which was first reported in 2009 [Guernsey et al. 2009], was also identified in a heterozygous state in both parents. The patient continued regular transfusions, while iron chelation therapy was initiated after 15 transfusions, when iron overload became apparent. The patient responded well to the regular transfusion and chelation therapy, and her development milestones were in concordance with her age.

The only cure for congenital sideroblastic anaemia is HSCT [Wong et al. 2015]. In the absence of an HLA-compatible donor, the parents of the child were interested in undergoing PGD for HLA-typing to identify an embryo HLA compatible with their affected daughter and in addition unaffected for both familial single gene conditions, to support bone marrow transplantation. The decision to embark on the assisted reproductive technology (ART)-PGD-HSCT procedure was endorsed by the treating clinician and the pediatric bone marrow transplant unit of the ''Aghia Sophia'' Children's Hospital, Athens. The couple received reproductive assessment and a full consultation on the ART procedure with fertility specialists, as well as a separate consultation with the genetics team at the Department of Medical Genetics, University of Athens, on the procedure of genetic analysis (PGD). Ethical approval for performing an IVF/PGD cycle was acquired by the IVF center and the couple provided their informed consent prior to initiating cycle treatment.

Development of a PGD Protocol

A PGD protocol was developed for diagnosis of both monogenic disorders along with HLA-typing, following published guidelines from the ESHRE [Harton et al. 2011]. The strategy was based on a previously published generic PGD protocol for HLA-typing combined with concurrent diagnosis for beta-thalassaemia, modified to additionally include diagnosis for sideroblastic anaemia, in the first PCR, directly on the biopsied single embryo cells [Kakourou et al. 2014]. DNA was extracted from blood samples from the parents and their affected child for initial protocol optimization, while optimization at the single cell level was performed on isolated single lymphocytes from freshly drawn parental blood.

The couple was tested for a panel of 26 STRs covering the HLA-region, along with six STRs linked to the β-globin gene and newly identified STRs linked to the *SLC25A38* gene (for indirect disease genotyping). Of these, 17 informative STRs were selected for amplification in a first-round PCR, along with primers for amplification of the mutated regions in the *HBB* and *SLC25A38* genes, to enable direct mutation detection.

The first-round multiplex PCR involved the simultaneous amplification of 19 genetic regions in a single cell: (1) the *HBB* gene mutated region, (2) four STRs linked to the *HBB* gene: D11S1243, D11S2352, D11S1871 and D11S4891 (distance 4-138 kb), (3) the *SLC25A38* gene mutated region, (4) two STRs linked to the *SLC25A38* gene: *23TG*, *24TG* (distance 208-245kb), and (5) 11 STRs for HLA-haplotyping: 31C, 113, RF, D6S388, MIB, 9N, 16A, DQCAR-II, G5S1152, 148, D6S1560. All STRs were analyzed directly from the first round PCR product, on an automatic sequencer, apart from the MIB HLA STR and the 24TG STR, for which a nested PCR preceded fragment analysis on the automatic sequencer. Most primer sequences for direct and indirect mutation detection and HLA typing have been previously published and any re-designed or new primer sets are presented in Table 1

Table 1. Primer sequences for STRs linked to the *HBB* gene and primers used for direct and indirect diagnosis of sideroblastic anaemia.

		STR name	Primer sequence (5′-3′, F/R)	Fluorescent label
Beta-thalassaemia *HBB* gene	Linked STRs	D11S1243	F: CAGGCTGCTCTTTTGTTTGA R: AGTGAGCCTGGCCAAAGATA,	TAMRA
		D11S2352	F: GCCGTAAATTCTTATGCCACT R: TGTGCAGAGTTGCTGGACTT	HEX
		D11S1871	F: AGAAGTTGCCCTGATGTCTGA R: TGCCTCCCTTCTCATTTCTG	TAMRA
		D11S4891	F: AAATGGACCTCTGTCTCTCTCG R: GCAGGTGCAGGTCTATTCTACTT	HEX
Sideroblastic anaemia *SLC25A38* gene	Mutated region		SBL-mut F: TGTTCCTTCCACACCTTAAACAA SBL-mut R: TGATAGGTGACATACAGACCCCTG	
	Linked STRs	23TG	F: ACCCCTCCAGGATGGTATTC, R: AGCAGTGTGGGAAGGCTCTA	TAMRA
		24TG	F: GCTTAGCTGTCTGCATGGCTA R: AGAACTACAACACTGGTTCTCCTG	FAM
	LightScanner Probe		5′-TGGCCTCACAAGAAATXIACTGCTTCA—PH-3′	

Fluorescent label: where indicated, one primer of each pair was fluorescently labeled to facilitate fragment analysis of PCR products on the automatic sequencer.

[Kakourou et al. 2014; Vrettou et al. 2004; Zachaki et al. 2011].

The first round multiplex PCR was performed using Multiplex HotStart Taq (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. PCR was set-up in a final volume of 50 µl, including 35 µl of Multiplex HotStar Taq, 5 µl Q solution (Qiagen GmbH), 0.2 µM of the forward and the reverse primers for amplification of the *HBB* mutated region, 0.2 µM of the forward and reverse primers for amplification of the *SLC25A38* mutated region, 0.2 µM of each of the STRs linked to the *HBB* gene, 0.2 µM of each of the STRs linked to the *SLC25A38* gene, 0.4 µM of the MIB HLA STR, and 0.2 µM of each of the remaining HLA STRs.

Amplification was achieved by the following touchdown cycling conditions: an initial step at 95 °C for 15 minutes, followed by 12 amplification cycles of denaturation at 96 °C for 30 seconds (94 °C for the last 2 cycles), annealing at 60 °C for 2 minutes (decreasing 0.5 °C/cycle down to 54 °C), and extension at 72 °C for 1.5 minutes. This was followed by 32 amplification cycles of: denaturation at 94 °C for 30 seconds, annealing at 54 °C for 2 minutes, and extension at 72 °C for 1 minute, followed by a final extension at 72 °C for 15 minutes.

The nested PCR used to amplify the MIB HLA STR and the 24TG HLA STR was performed on diluted first round PCR products (1:1500) and PCR was set-up in a final volume of 15 µl, including 7.5 µl of HotStar Taq (Qiagen GmbH), 0.2 µM of the forward and the reverse primers for each STR. Amplification was achieved by a primary step at 95 °C for 15 minutes, followed by 35 amplification cycles of denaturation at 96 °C for 30 seconds, annealing at 58 °C for 1 minute and extension at 72 °C for 30 seconds, followed by a final extension at 72 °C for 15 minutes.

Mutation Detection for Beta-Thalassaemia and Sideroblastic Anaemia

For beta-thalassaemia, mutation detection was performed on diluted first round PCR products by real-time nested PCR using LightCycler hybridization probes as previously described [Vrettou et al. 2004]. For detection of the sideroblastic anaemia mutation, a second round PCR was performed on diluted (1:1000) first round PCR products. The second PCR reaction was an asymmetric PCR, performed in a final volume of 10 µl, including 4 µl of Idaho's LightScanner® master mix (Idaho Technology Inc., Salt Lake City, UT, USA), 1 µM of the SBL-mut Forward primer, 0,2 µM of the SBL-mut Reverse primer, 2 µM of the simple unlabelled oligo probe (Roche Molecular Diagnostics, GmbH, Germany), and 2 µl of diluted first round PCR product (Table 1). This was followed by high resolution melting analysis. Melting curves were imaged in a 96-well LightScanner system (Idaho Technology Inc.), using the commercial LightScanner software (Figure 1).

Optimization and Testing at the Single Cell Level

The PGD protocol was optimized and tested on single lymphocytes isolated from both parents. The final optimized protocol was tested on thirty single lymphocytes isolated from the mother as she was heterozygous at all amplified loci except HLA STR RF, and twenty lymphocytes from the father, to allow evaluation of amplification and allele drop-out rates (ADO) for each tested locus separately (beta-thalassaemia, sideroblastic anaemia, linked STRs, and HLA loci). Results on amplification efficiency and ADO adhered to PGD guidelines and the protocol was deemed ready for clinical application.

In the final optimized protocol, amplification efficiency was 95.5% for the beta-thalassaemia mutated region, 90% for the MIB HLA STR, and 100% for the remaining sixteen STRs included in the multiplex as well as the sideroblastic anaemia mutated region. The ADO rate was 0% for almost all of the amplified regions, with the following exceptions: 13% ADO for the HLA STR DQCAR-II, 10% ADO for the STRs D11S1243 and 24TG, and 4.7% for the sideroblastic anaemia mutated region. The high ADO for the HLA STR DQCAR-II was accepted as there were two flanking HLA STRs (16A and G5S1152, distance 403 kb and 56 kb, respectively) with 0% ADO.

Clinical PGD Cycles

The couple underwent a total of four clinical IVF-PGD cycles. ART treatment (stimulation, oocyte collection, intracytoplasmic sperm injection (ICSI), biopsy, and embryo transfer procedures) was performed by two assisted reproduction clinics following standard procedures [Kokkali et al.

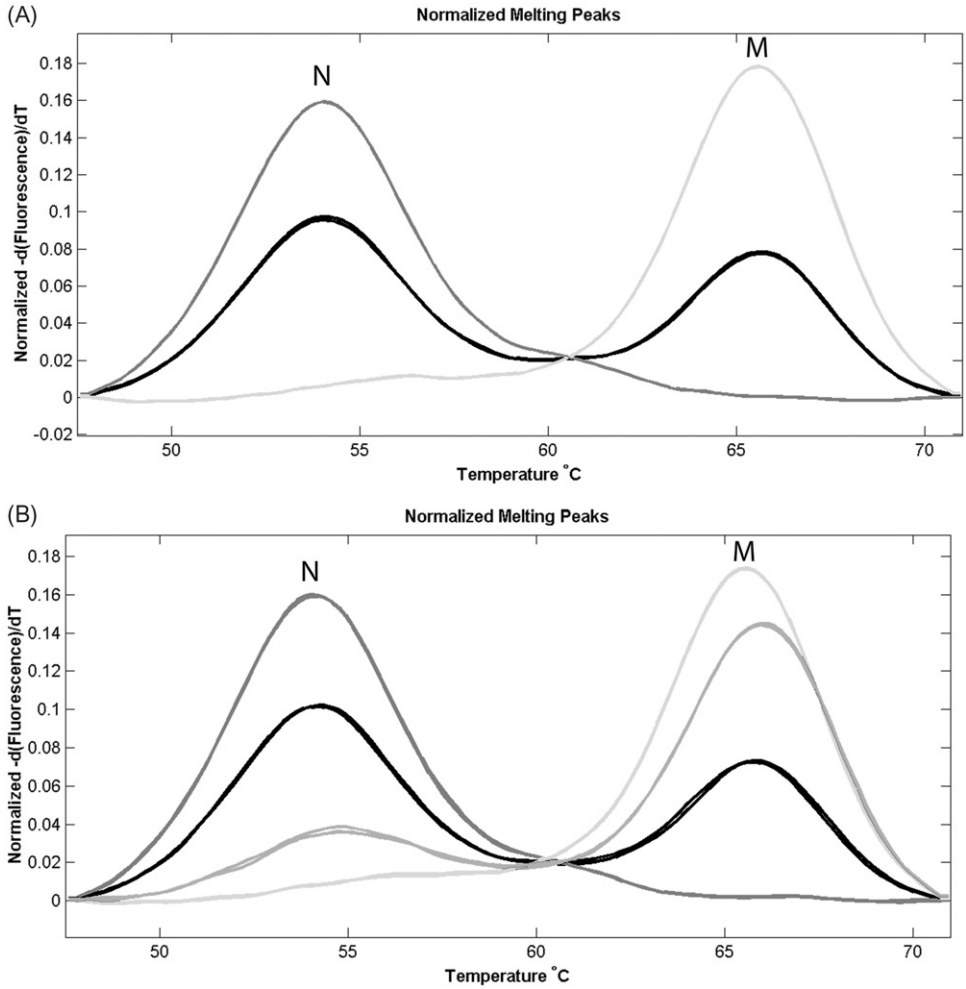

Figure 1. Detection of the sideroblastic anaemia mutation by high resolution melting analysis. A Luna probe was used to obtain the melting profiles for genotyping. Melting curves were analyzed with the commercial LightScanner software. (A) DNA samples of the carrier parents (black peaks), the affected child (light grey peak), and a wild-type sample (dark grey peak) were run parallel to the blastomere samples, as controls. (B) Melting profile result examples from four single blastomere samples, run in duplicate. Black: carrier, light grey: affected, dark grey: unaffected. The medium grey sample indicates a single blastomere of carrier status, showing preferential amplification of the mutant allele. N: unaffected allele melting profile, M: affected allele melting profile.

2007; Traeger-Synodinos et al. 2003]. Overall, 77 oocytes were collected, 67 were fertilized (87.01%), and 56 embryos were successfully biopsied on day 3. Embryo biopsy was performed on cleavage stage (day 3) embryos, each biopsied blastomere was washed in PBS, transferred in 5 µl of alkaline lysis buffer (50 mM DTT/200 mM KOH), and incubated at 65 °C for 10 minutes, prior to PCR set-up. The molecular analysis was performed at the Department of Medical Genetics, University of Athens. One blastomere failed to amplify at all loci. Amplification efficiency for all 19 loci ranged from 87.8–100% and ADO from 0–10% (average 2.79%).

A complete diagnosis (diagnosis for beta-thalassaemia, sideroblastic anaemia, and HLA typing) was obtained for 49 out of 55 amplified blastomeres. Reasons for incomplete diagnosis were failure of amplification for all loci tested on chromosome 6 (1 blastomere), failure of amplification or ADO at some of the tested loci, resulting in insufficient evidence for the presence of unaffected or matched alleles (3 blastomeres), or detection of maternal-only or paternal-only alleles in the tested sample (2 blastomeres in cycle 1, one showing maternal alleles for β-thalassaemia, another showing paternal HLA STRs). There was another blastomere in cycle 4 showing only maternal alleles for the HLA region; however, these were not matched to those of the affected child, so this embryo was scored as not-matched. Results from the four PGD cycles are summarized in Table 2. Diagnosis was completed within 24 hours, enabling fresh embryo transfer. Unaffected HLA-matched embryos were identified in each cycle and seven unaffected HLA-matched embryos were identified in total.

One blastocyst stage embryo was transferred in the first cycle on day 5 but a pregnancy was not established; two embryos were transferred in the second cycle on day 5 (an eight cell embryo and a morula), the patient indicated positive human chorionic gondadotropin (hCG) but the pregnancy was lost; one blastocyst-stage embryo was transferred on day 5 in the third PGD cycle but a pregnancy was not established. Finally in the fourth cycle, three unaffected good quality hatching blastocysts HLA-matched embryos were identified and transferred on day 6 post insemination and a triplet pregnancy was established. One of the embryos was lost at the eighth week of gestation. Prenatal diagnosis testing was performed at 17 weeks of pregnancy following an amniocentesis of the twin pregnancy and the genotypes characterized

Table 2. Summary of the four clinical PGD-HLA cycles performed in the Department of Medical Genetics, University of Athens for the couple carriers of β-thalassaemia and sideroblastic anaemia mutations, requesting PGD with HLA typing.

		Cycle 1	Cycle 2	Cycle 3	Cycle 4	Total (%)
Age at treatment (years)		25	26	26	27	
Number of oocytes collected		24	18	15	20	77
Number of oocytes normally fertilized		22	13	13	19	67 (87.01%)
Number of embryos biopsied		19	11	12	14	56 (83.6%)
Number of blastomeres showing amplification		18	11	12	14	55 (98.2%)
Number of embryos diagnosed for b-thalassaemia		17	11	11	12	51 (92.7%)
Number of embryos diagnosed for sideroblastic anaemia		17	11	12	14	54 (98.2%)
Number of embryos with a result for HLA typing		18	11	11	14	54 (98.2%)
Number of HLA-matched embryos	Unaffected for both SGDs	1	2	1	3	7 (12.7%)
	Affected for both SGDs	0	0	2	0	2
	Affected for β-thalassaemia only	0	0	1	0	1
	Affected for sideroblastic anaemia only	1	1	0	2	4
Number of non- HLA matched embryos	Unaffected for both SGDs	7	4	5	4	20
	Affected for both SGDs	1	0	1	1	3
	Affected for β-thalassaemia only	0	4	1	2	7
	Affected for sideroblastic anaemia only	5	0	0	0	5
Incomplete/inconclusive diagnosis		3	0	1	2	6
Embryos indicating recombination in the HLA region		2 (one maternal, one paternal)	1 (paternal)	0	0	3
Embryos with other findings		2 (one embryo showing maternal alleles only for β-thalassaemia loci, another embryo showing paternal alleles only for the HLA STRs)	0	0	1 (one embryo indicating maternal alleles only for HLA STRs)	3
Number of transferred embryos		1	2	1	3	7
Clinical pregnancy		NO	+ve hCG but pregnancy lost	NO	YES	

SGDs: single gene disorders; hCG: human chorionic gonadotropin.

during the HLA-PGD analysis were successfully confirmed in both fetuses. Two healthy baby girls were delivered at week 38 of pregnancy. Cord blood was collected at birth and HSCT with a combination of cord blood and bone marrow is planned for when the donor reaches one year of age.

Discussion

Preimplantation HLA haplotyping by linkage analysis was first described by Verlisnky et al. [2001] and since then, several approaches have been reported with the aim of developing flexible and reliable methodologies for HLA-PGD analysis [Fiorentino et al. 2004; Van de Velde et al. 2004; Verlinsky et al. 2001]. To our knowledge this is the first report whereby a 19-plex PCR is applied directly on a single cell followed by direct analysis of almost all STR loci from the first round PCR product, without either prior whole genome amplification (WGA) of the biopsied sample or the need of subsequent multiple separate nested or hemi-nested PCR steps, which significantly increase the laboratory expense, workload, and time to diagnosis [Altarescu et al. 2007; Brezina et al. 2011; Fernandez et al. 2014; Fiorentino et al. 2006; Fiorentino et al. 2005; Kahraman et al. 2014; Natesan et al. 2014; Shen et al. 2013; Van de Velde et al. 2009].

The protocol described here employed three different technologies for single-cell genotyping, following the initial multiplex PCR: fragment analysis, real-time PCR, and high-resolution melting analysis (HRMA). Use of the HRMA methodology was recently reported by our group for prenatal diagnosis of cystic fibrosis, while a robust, single-cell HRMA protocol has also been employed on other preimplantation genetic diagnosis cycles [Poulou et al. 2014].

In this study four PGD cycles were completed for the couple, who were both carriers of beta-thalassaemia and sideroblastic anaemia mutations. In each cycle one or two embryos were genetically suitable and selected for transfer. To our knowledge, this is the first report of a 'triple factor' preimplantation genetic diagnosis for HLA matching involving parallel testing for two single gene disorders. Most previously published protocols combining diagnosis of more than one indication in a PGD cycle, either employed an initial step of WGA of the biopsied embryo sample or involved double embryo biopsy (either double blastomere biopsy or biopsy at more than one stages of development). It should be noted, however, that WGA has been associated with high ADO, while double embryo biopsy may be detrimental to subsequent embryo development [Obradors et al. 2008; Obradors et al. 2009; Rechitsky et al. 2013]. Another reported case involved trophectoderm biopsy followed by analysis of each half of the biopsied sample by different protocols. This strategy also required cryopreservation to allow completion of the genetic analysis [Treff et al. 2009].

The case described here has demonstrated that direct amplification of multiple loci from a single cell biopsy sample remains a satisfactory strategy. In addition, the case highlights the need to develop an appropriate diagnostic protocol to maximize the chance of identifying transferable embryos in order to achieve a pregnancy. For diagnosis of two autosomal recessive monogenic conditions along with HLA matching, the chance for detection of an unaffected HLA matched embryo was: $0.75 \times 0.75 \times 0.25 = 0.14$ (14%). The real number of embryos, however, found to be unaffected or affected, matched or non-matched, during diagnosis cannot be predicted and therefore, the successful outcome of the diagnosis in each PGD cycle cannot be assured. The high diagnostic efficiency of the protocol was ensured prior to clinical application based on results from single cell testing. In the 4 HLA-PGD cycles performed, seven out of the fifty-six (12.5%) biopsied embryos were found to be genetically suitable. The number of transferable embryos identified was also limited by some additional findings, such as evidence of recombination in the HLA region in three out of the 55 embryos (5.45%) (in agreement with reports from other studies), and the indication of only maternal or paternal alleles for loci on one of the analyzed chromosomes, which was observed in three embryos [Kuliev and Rechitsky 2014c]. With regards to potential recombination issues and monogenic disease diagnosis, it is worth underlining that the protocol developed enables both direct and indirect mutation detection by use of polymorphic markers on either side of the mutated region. For this reason, a recombination event near the mutated region would most likely not prevent a confident diagnosis of the mutational status of an embryo, and therefore, not limit the number of diagnosed embryos, unlike approaches that rely on linkage analysis alone. In any case, all potential limitations noted above (recombination, ADO, aneuploidy, failure of amplification) should be communicated to the patients before starting treatment [Tur-Kaspa and Jeelani 2015].

It was recently reported that due to the advanced maternal age of many couples requesting PGD with HLA typing, there may be benefit from additional preselection of euploid preimplantation embryos. The combined approach of PGD with HLA typing and 24-chromosome aneuploidy testing was applied in 26 HLA-PGD and 5 HLA-only cycles. The methodology involved WGA from a single biopsied blastomere, followed by a multiplex nested PCR approach using a specific custom-made PGD design for each couple. This resulted in a decreased number of transferable embryos identified (the authors estimate the chance of identifying a transferable embryo between 6.25-9.4% depending on the monogenic condition diagnosed), but a higher pregnancy rate per embryo transfer in these cycles compared to HLA-PGD cycles performed without concurrent aneuploidy testing [Rechitsky et al. 2015]. The usefulness of this approach could be further validated by collecting data from a greater number of cycles. In addition to this, results from ongoing randomized controlled trials are expected to clarify the value of aneuploidy screening for different patient groups.

Finally, it is worth noting that new technologies such as SNP arrays and next-generation sequencing are expected to further facilitate simultaneous testing of different conditions in a single cell and may offer more generic approaches for genetic diagnosis, simplifying protocol work-up and allowing wider application. However, it may be some time until they become available to a great number of PGD centers, mostly due to the high cost of equipment and reagents involved [Natesan et al. 2014; Thornhill et al. 2015; Treff et al. 2013; Vendrell and Bautista-Llacer 2012].

The described single multiplex touchdown PCR protocol allowed completion on the day of PGD, enabling fresh

embryo transfer. The PCR protocol has shown high flexibility for multiple primer combinations and has previously been applied in several other PGD protocols, maintaining successful amplification of all loci from a single cell and excluding the need for multiple nested PCR reactions in most cases.

For the time being, as demonstrated here, the optimization of simple multiplex PCR protocols, employing different analytical methodologies on a single cell sample, remains an adequate strategy even for the most complex PGD indications. In this particular case the family is expected to benefit by the birth of two healthy siblings for their affected daughter, along with the potential of curing their child through the option of having a HSCT treatment with fully compatible donors.

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Declaration of interest

The authors report no declarations of interest.

Author contribution

Protocol design and optimization of protocol for preimplantation genetic diagnosis, preimplantation genetic diagnosis procedure, embryo results analysis, preparation of draft manuscript, and preparation of final manuscript for submission: GK; Preimplantation genetic diagnosis procedure, embryo results analysis, critical revision of draft manuscript, approval of final manuscript: CV, AD, MP, MM; Treating clinician of the affected child, clinical evaluation and follow-up of the patient, provisional diagnosis and arrangements for laboratory confirmation of the sideroblastic anaemia mutation, critical revision and approval of final manuscript: AK; Completion of two cycles of assisted reproductive technology, embryo biopsy, embryo transfer, critical revision of draft manuscript, approval of final manuscript:GK, KP; Completion of two cycles of assisted reproductive technology, embryo biopsy, embryo transfer, critical revision of draft manuscript, approval of final manuscript: SD; Patient counseling for preimplantation genetic diagnosis, approval of diagnostic results, critical revision and approval of final manuscript: SK-T, EK; Patient counseling for preimplantation genetic diagnosis, overall supervision of protocol optimization and project design, participation in preimplantation genetic diagnosis procedure, embryo results analysis, approval of diagnostic results, critical revision of draft manuscript, critical discussion and approval of final manuscript: JT-S.

References

Altarescu, G., Brooks, B., Margalioth, E., Eldar Geva, T., Levy-Lahad, E. and Renbaum, P. (2007) Simultaneous preimplantation genetic diagnosis for Tay-Sachs and Gaucher disease. Reprod Biomed Online **15**:83–88.

Brezina, P.R., Benner, A., Rechitsky, S., Kuliev, A., Pomerantseva, E., Pauling, D. and Kearns, W.G. (2011) Single-gene testing combined with single nucleotide polymorphism microarray preimplantation genetic diagnosis for aneuploidy: a novel approach in optimizing pregnancy outcome. Fertil Steril **95**:1786 e1785–1788.

Fernandez, R.M., Pecina, A., Lozano-Arana, M.D., Sanchez, B., Guardiola, J., Garcia-Lozano, J.C., et al. (2014) Experience of preimplantation genetic diagnosis with HLA matching at the University Hospital Virgen del Rocio in Spain: technical and clinical overview. Biomed Res Int 2014. **2014**:560160.

Fiorentino, F., Biricik, A., Karadayi, H., Berkil, H., Karlikaya, G., Sertyel, S., et al. (2004) Development and clinical application of a strategy for preimplantation genetic diagnosis of single gene disorders combined with HLA matching. Mol Hum Reprod **10**:445–460.

Fiorentino, F., Biricik, A., Nuccitelli, A., De Palma, R., Kahraman, S., Iacobelli, M., et al. (2006) Strategies and clinical outcome of 250 cycles of Preimplantation Genetic Diagnosis for single gene disorders. Hum Reprod **21**:670–684.

Fiorentino, F., Kahraman, S., Karadayi, H., Biricik, A., Sertyel, S., Karlikaya, G., et al. (2005) Short tandem repeats haplotyping of the HLA region in preimplantation HLA matching. Eur J Hum Genet **13**: 953–958.

Guernsey, D.L., Jiang, H., Campagna, D.R., Evans, S.C., Ferguson, M., Kellogg, M.D., et al. (2009) Mutations in mitochondrial carrier family gene SLC25A38 cause nonsyndromic autosomal recessive congenital sideroblastic anemia. Nat Genet **41**:651–653.

Harton, G.L., De Rycke, M., Fiorentino, F., Moutou, C., SenGupta, S., Traeger-Synodinos, J., et al. (2011) ESHRE PGD consortium best practice guidelines for amplification-based PGD. Hum Reprod **26**: 33–40.

Kahraman, S., Beyazyurek, C., Yesilipek, M.A., Ozturk, G., Ertem, M., Anak, S., et al. (2014) Successful haematopoietic stem cell transplantation in 44 children from healthy siblings conceived after preimplantation HLA matching. Reprod Biomed Online **29**:340–351.

Kakourou, G., Destouni, A., Vrettou, C., Traeger-Synodinos, J. and Kanavakis, E. (2014) A generic, flexible protocol for preimplantation human leukocyte antigen typing alone or in combination with a monogenic disease, for rapid case work-up and application. Hemoglobin **38**:49–55.

Kokkali, G., Traeger-Synodinos, J., Vrettou, C., Stavrou, D., Jones, G.M., Cram, D.S., et al. (2007) Blastocyst biopsy versus cleavage stage biopsy and blastocyst transfer for preimplantation genetic diagnosis of beta-thalassaemia: a pilot study. Hum Reprod **22**: 1443–1449.

Kuliev, A. and Rechitsky, S. (2014a) Preimplantation Genetic Diagnosis for Congenital Immunodeficiencies. J Blood Disorders Transf **5**(**8**):234–236.

Kuliev, A. and Rechitsky, S. (2014b). Preimplantation HLA typing for stem cell transplantation treatment of hemoglobinopathies. Thalassemia Reports **4**(**51**):99–101.

Kuliev, A. and Rechitsky, S. (2014c) Preimplantation HLA typing: Practical tool for stem cell transplantation treatment of congenital disorders. World J Med Genet **4**(**4**):105–109.

Moutou, C., Goossens, V., Coonen, E., De Rycke, M., Kokkali, G., Renwick, P., et al. (2014) ESHRE PGD Consortium data collection XII: cycles from January to December 2009 with pregnancy follow-up to October 2010. Hum Reprod **29**:880–903.

Natesan, S.A., Handyside, A.H., Thornhill, A.R., Ottolini, C.S., Sage, K., Summers, M.C., et al. (2014) Live birth after PGD with confirmation by a comprehensive approach (karyomapping) for simultaneous detection of monogenic and chromosomal disorders. Reprod Biomed Online **29**:600–605.

Obradors, A., Fernandez, E., Oliver-Bonet, M., Rius, M., de la Fuente, A., Wells, D., et al. (2008) Birth of a healthy boy after a double factor PGD in a couple carrying a genetic disease and at risk for aneuploidy: case report. Hum Reprod **23**:1949–1956.

Obradors, A., Fernandez, E., Rius, M., Oliver-Bonet, M., Martinez-Fresno, M., Benet, J., et al. (2009) Outcome of twin babies free of Von Hippel-Lindau disease after a double-factor preimplantation genetic diagnosis: monogenetic mutation analysis and comprehensive aneuploidy screening. Fertil Steril **91**:933 e931–937.

Poulou, M., Destouni, A., Kakourou, G., Kanavakis, E. and Tzetis, M. (2014) Prenatal diagnosis for CF using High Resolution Melting

Analysis and simultaneous haplotype analysis through QF-PCR. J Cyst Fibros **13**:617–622.

Rechitsky, S., Pakhalchuk, T., San Ramos, G., Goodman, A., Zlatopolsky, Z. and Kuliev, A. (2015) First systematic experience of preimplantation genetic diagnosis for single-gene disorders, and/or preimplantation human leukocyte antigen typing, combined with 24-chromosome aneuploidy testing. Fertil Steril **103**:503–512.

Rechitsky, S., Verlinsky, O. and Kuliev, A. (2013) PGD for cystic fibrosis patients and couples at risk of an additional genetic disorder combined with 24-chromosome aneuploidy testing. Reprod Biomed Online **26**:420–430.

Shen, J., Cram, D.S., Wu, W., Cai, L., Yang, X., Sun, X., et al. (2013) Successful PGD for late infantile neuronal ceroid lipofuscinosis achieved by combined chromosome and TPP1 gene analysis. Reprod Biomed Online **27**:176–183.

Soni, S., Boulad, F., Cowan, M.J., Scaradavou, A., Dahake, J., Edwards, S., et al. (2014) Combined umbilical cord blood and bone marrow from HLA-identical sibling donors for hematopoietic stem cell transplantation in children with hemoglobinopathies. Pediatr Blood Cancer **61**:1690–1694.

Thornhill, A.R., Handyside, A.H., Ottolini, C., Natesan, S.A., Taylor, J., Sage, K., et al. (2015) Karyomapping-a comprehensive means of simultaneous monogenic and cytogenetic PGD: comparison with standard approaches in real time for Marfan syndrome. J Assist Reprod Genet **32**(**3**):347–56.

Traeger-Synodinos, J., Vrettou, C., Palmer, G., Tzetis, M., Mastrominas, M., Davies, S., et al. (2003) An evaluation of PGD in clinical genetic services through 3 years application for prevention of beta-thalassaemia major and sickle cell thalassaemia. Mol Hum Reprod **9**:301–307.

Treff, N., Tao, X., Su, J., Northrop, L., Kamani, M., Bergh, P., et al (2009) SNP microarray based concurrent screening of 24 chromosome aneuploidy, unbalanced translocations, and single gene disorders in human embryos: first application of comprehensive triple factor PGD. Biol Reprod **81**:188.

Treff, N.R., Fedick, A., Tao, X., Devkota, B., Taylor, D. and Scott Jr., R.T., (2013) Evaluation of targeted next-generation sequencing-based preimplantation genetic diagnosis of monogenic disease. Fertil Steril **99**:1377–1384 e1376.

Tucunduva, L., Volt, F., Cunha, R., Locatelli, F., Zecca, M., Yesilipek, A., et al. (2014) Combined cord blood and bone marrow transplantation from the same human leucocyte antigen-identical sibling donor for children with malignant and non-malignant diseases. Br J Haematol **169**(**1**):103–110.

Tur-Kaspa, I. and Jeelani, R. (2015) Clinical guidelines for IVF with PGD for HLA matching. Reprod Biomed Online **30**: 115–119.

Van de Velde, H., De Rycke, M., De Man, C., De Hauwere, K., Fiorentino, F., Kahraman, S., et al. (2009) The experience of two European preimplantation genetic diagnosis centres on human leukocyte antigen typing. Hum Reprod **24**:732–740.

Van de Velde, H., Georgiou, I., De Rycke, M., Schots, R., Sermon, K., Lissens, W., et al. (2004) Novel universal approach for preimplantation genetic diagnosis of beta-thalassaemia in combination with HLA matching of embryos. Hum Reprod **19**:700–708.

Vendrell, X. and Bautista-Llacer, R. (2012) A methodological overview on molecular preimplantation genetic diagnosis and screening: a genomic future? Syst Biol Reprod Med **58**:289–300.

Verlinsky, Y., Rechitsky, S., Schoolcraft, W., Strom, C. and Kuliev, A. (2001) Preimplantation diagnosis for Fanconi anemia combined with HLA matching. Jama **285**:3130–3133.

Vrettou, C., Traeger-Synodinos, J., Tzetis, M., Palmer, G., Sofocleous, C. and Kanavakis, E. (2004) Real-time PCR for single-cell genotyping in sickle cell and thalassemia syndromes as a rapid, accurate, reliable, and widely applicable protocol for preimplantation genetic diagnosis. Hum Mutat **23**:513–521.

Wong, W.S., Wong, H.F., Cheng, C.K., Chang, K.O., Chan, N.P., Ng, M.H., et al. (2015) Congenital sideroblastic anaemia with a novel frameshift mutation in SLC25A38. J Clin Pathol **68**:249–251.

Zachaki, S., Vrettou, C., Destouni, A., Kokkali, G., Traeger-Synodinos, J. and Kanavakis, E. (2011) Novel and known microsatellite markers within the beta-globin cluster to support robust preimplantation genetic diagnosis of beta-thalassemia and sickle cell syndromes. Hemoglobin **35**:56–66.