Successful pregnancies after combined human leukocyte antigen direct genotyping and preimplantation genetic diagnosis utilizing multiple displacement amplification

Ali M. Hellani, PhD, Siham M. Akoum, MD, Elias S. Fadel, MD, Hisham M. Yousef, MD, Khaled K. Abu-Amero, PhD, FRCPath.

ABSTRACT

الأهداف: ابتكار تقنية جديدة وبسيطة تساعد على اختيار الأجنة السليمة التي تساعد في الحصول على طفل سليم يكون متطابقاً في علاماته الوراثية مع إخوانه أو أخواته المصابين بأمراض وراثية مختلفة مثل فقر الدم المنجلي أو الثلاسيميا.

الطريقة: أجريت هذه الدراسة في مستشفى سعد التخصصي، الخبر، المملكة العربية السعودية وذلك خلال الفترة من مارس 2008م وحتى أبريل 2011م. لقد تم الحصول على الأجنة بعد 7 دورات من التلقيح الاصطناعي وتم تضخيم الحامض النووي المستخرج من خلية واحدة، وحُددت الطفرات الجينية الموجودة، وتم أيضاً تحديد العلامات الوراثية في هذه الخلايا.

النتائج: لقد خضعت 7 عائلات لسبع دورات من التشخيص الجيني قبل التلقيح الاصطناعي وذلك مع ظهور أنماط مستضد توافق الأنسجة وأنماط مستضد توافق الأنسجة المباشرة. وقد خضع مريضين للتشخيص الجيني قبل التلقيح الاصطناعي مع ظهور أنماط مستضد توافق الأنسجة من النوع I وII، فيما ظهر النوع II لدى 5 أشخاص. وقد نتج عن دورات التشخيص الجيني قبل التقيح الاصطناعي 3 حالات من الحمل من أصل 5 مرضى ظهر لديهم مستضد توافق الأنسجة متوافقاً مع الأجنة. وتم عمل زراعة للخلايا الجذعية لأحد العائلات في الدم وتكللت العملية بالنجاح.

خاتمة: إن هذا التقرير يعد من أول التطبيقات العملية لعملية التشخيص الجيني قبل التقيح الاصطناعي وفحص العلامات الوراثية من الحامض النووي المأخوذ من خلية واحدة.

Objectives: To devise a new and simple technique to help select normal embryos that are human leukocyte antigen (HLA) matched to their affected siblings for diseases, such as beta-thalassemia or sickle cell anemia, which are common in this part of the world.

Methods: This study was conducted between March 2008 and April 2011 at the preimplantation Genetic Diagnosis Laboratory, Saad Specialist Hospital, Al-Khobar, Kingdom of Saudi Arabia. Embryos were obtained after 7 *in vitro* fertilization preimplantation genetic diagnosis (IVF-PGD) cycles. Single cells were biopsied, and extracted DNA was amplified by the multiple displacement amplification (MDA) technique. Amplified DNA was then tested for mutations in the beta-globin gene, and directly HLA typed using a sequence specific primer technique.

Results: We report 7 families that underwent 7 PGD cycles with HLA typing and direct HLA loci-typing using an HLA conventional commercial kit. Two patients had PGD and HLA typing for class I and II, while the other 5 had class II. The PGD cycles resulted in 3 pregnancies out of 5 patients who had HLA matched and normal embryos. One family had a successful hematopoietic stem cell transplant.

Conclusion: This report demonstrates the first clinical application of PGD coupled with direct HLA loci-typing of DNA amplified by MDA from a single cell.

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Preimplantation genetic diagnosis (PGD) is an established procedure for the detection of genetic anomalies in embryos.[1-3] It allows couples carrying genetic diseases to have an unaffected child, without facing prenatal procedures and potential termination of pregnancy.[4] After the first case of PGD in 1991,[5] the last decade witnessed a substantial improvement in PGD, due to advancement of molecular genetics techniques and *in vitro* fertilization (IVF) enhancement protocols. A new technique of single cell DNA amplification technique in PGD was introduced in 2004,[5-7] where the multiple displacement amplification (MDA) technique was used to amplify the whole genome of a single cell. The MDA technique is gradually becoming widely used in PGD worldwide.[7-12] Initially, PGD applications utilizing the MDA technique were focused mainly on mutation screening and haplotyping. However, this has now been extended to include techniques, such as array comparative genomic hybridizations (array CGH) analysis.[8,13] Recently, PGD combined with human leukocyte antigen (HLA) matching has been used. This procedure allows selection and transfer of unaffected embryos (those with close HLA to their affected siblings).[14,15] In these cases, PGD proved itself as a technique not only to avoid the birth of affected children, but also to conceive healthy children. Those healthy children will be potential HLA-identical donors of hematopoietic stem cells (HSC) for transplantation in siblings with severe and life-threatening disorders. At delivery, HSC from the newborn umbilical cord blood (UCB) are collected, and used for the hematopoietic reconstruction of the affected sibling.[16] Most PGD trials and HLA matching have been applied to diseases that may be cured by hematopoietic stem cell transplant (HSCT).[17] Technically, PGD for HLA typing could be carried out indirectly by testing of single blastomeres by fluorescent multiplex polymerase chain reaction (PCR) analysis of polymorphic short tandem repeats (STR).[14] Direct genotyping of HLA loci was also successfully applied using a nested PCR based protocol for class I and II HLA loci.[14,15] Here, we report on 7 families (5 families suffering from beta-thalassemia, and 2 from sickle cell anemia) in whom we performed PGD coupled with HLA typing. Due to consanguinity in these families, there was a lack of informative linked markers in the HLA locus to apply the indirect typing method. We report the application of low resolution HLA typing by the sequence specific primer (SSP) technique on MDA products of an embryonic single cell (direct HLA typing). Such a protocol does not require any specific preparation, and could be applied to any couple seeking PGD and HSCT, especially consanguineous families, where haplotyping is not possible due to lack of heterozygous informative markers.

Methods. A total of 7 consanguineous Saudi families were included in the study. Inclusion criteria were as follows: i) a history of sickle and/or beta-thalassemia (2 families suffering from sickle cell anemia and 5 families with beta-thalassemia), ii) maternal age from 25-40, iii) an informative dopamine receptor beta (DRB)1 HLA matching, iv) a consent form stating that the couple's desire is to conceive normal babies, and if possible these babies would be HSC donors for the affected sibling (HLA matching and HSCT from UCB). The study adhered to the tenets of the Helsinki Declaration, and all the legal guardians of the patients signed an informed consent. The study was approved by Saad Specialist Hospital Institutional Review Board, and conducted between March 2008 and April 2011 at the Preimplantation Genetic Diagnosis Laboratory in Saad Specialist Hospital, Al-Khobar, Kingdom of Saudi Arabia.

Mutation screening and linked markers assessment in HLA locus. To assess the mutation causing sickle cell and beta-thalassemia in the families, genomic DNA was extracted and exons one and 2 (hot-spot area for mutations) of the beta-globin (HBB) gene were screened for mutation(s). The primer sequences and the PCR conditions are available upon request. Briefly, HBB gene sequences were amplified from 100-200 ng of DNA using specific primers (5 μM), dNTP (5 mM), PCR buffer 10X, and one unit of Fast start high fidelity Taq polymerase (Roche Applied Science, Mannheim, Germany). The PCR products were purified (Qiagen, Valencia, CA, USA) then assessed on a bio-analyzer capillary electrophoresis using the DNA 12000 chip (Agilent Technologies Inc., Santa Clara, CA, USA). The purified PCR product was sequenced on an ABI 3130xI (Applied Biosystems, Foster City, CA, USA)

Genetic analyzer using forward and reverse primers. A total of 21 STR14 in HLA locus were tested for each family to find the number of informative markers that could be used for the typing. The PCR conditions for beta-globin mutation screening and STR testing were as follows: one cycle of denaturation at 95°C for 10 minutes followed by 35 cycles of denaturation at 95°C

for 30 seconds; annealing at 60°C for 30 seconds; and extension at 72°C for 30 seconds. Final extension was performed at 72°C for 7 minutes. The PCR products were analyzed on a 3130xl Genetic analyzer for the labeled STR (Applied Biosystems, Foster City, CA, USA), or Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) for beta-globin before processing for sequencing. The purified PCR product was sequenced on an ABI 3130xI (Applied Biosystems, Foster City, CA, USA) Genetic Analyzer using the forward primers.

Controlled ovarian stimulation protocol. The Flare protocol described by Padilla et al[18] was followed for controlled ovarian stimulation using gonadotropin-releasing analogue ([GnRHa] Decapeptyl, 0.1 mg/day; Ipsen-Biotech, Paris, France), and human menopausal gonadotropin (hMG, Menogon, Ferring Pharmaceuticals Ltd, San Diego, CA, USA). Human chorionic gonadotropin (HCG-Pregnyl 10,000 IU [Organon BioSciences N.V, Oss, Netherlands) was administered when the 3 follicles reached a diameter of 18 mm. Oocytes were recovered transvaginally under conscious sedation, 35 hours after HCG administration.

Fertilization, embryo culture, and biopsy. Oocytes collected were injected with the fathers' sperm using the intra-cytoplasmic sperm injection technique (ICSI).[19] The ICSI was performed to eliminate contamination by sperm when performing subsequent embryo biopsy. The zona pellucida was pierced using the Saturn Laser System (Research Instrument, Cornwall, UK). Two blastomeres were carefully aspirated through the piercing, and transferred to separate 0.5 ml PCR tubes containing lysing buffer (albumin, 0.2 M potassium hydroxide, and 50 mM dithiothreitol) (Sigma-Aldrich Co, Taufkirchen, Germany). The last wash drop served as a blank. After 15 minutes incubation at 4°C, 3 µl of neutralization buffer (900 mmol/l Tris-hydrogen chloride [HCl], 300 mmol/l potassium chloride, 200 mmol/l HCL) were added to the solution. The cell lysates were used directly for whole genome amplification (WGA) using MDA (GE Healthcare, Waukesha, WI, USA) by adding 20 µl of the master mix in total volume of 30 µl. The mix was then incubated at 31ºC for 2 hours, followed by heat inactivation at 65ºC for 10 minutes. The MDA yield was quantified on a fluorometer using PicoGreen quantification kit (Molecular Probes Inc, Eugene, OR, USA). Blanks did not show any amplification. Diluted MDA product (10 ng/µl) was used for the mutation screening, while the concentrated product (≈100 ng/µl) was used for HLA typing (only normal or carrier embryos).

Human leukocyte antigen typing. Amplification was performed by PCR-SSP[20] using a Micro SSP™ kit (One Lambda Inc, Canoga Park, CA, USA). This assay is optimized for DNA typing of class II and class I. Pre-optimized primers for the amplification of genes were coated in wells of the PCR plate for later addition of DNA samples, Taq polymerase (Promega, Madison, WI, USA) and dNTP-buffer mix. The PCR was performed following manufacturer instructions using the Mastercycler EP gradiant S Eppendorf PCR system (Eppendorf, AG, Hamburg, Germany). After amplification, electrophoresis in 2.5% Tris-borate-EDTA agarose gel was undertaken at 150 volts for approximately 5 minutes, and then examined under UV illumination and recorded.[21] A pair of specific primers amplifying a limited region of HBB gene present in all DNA samples was used as an internal control.

Results. Table 1 summarizes the different mutations found in the beta-thalassemia and sickle cell anemia affected families. A sickle cell mutation was found in 2 families, while beta-thalassemia mutations

Table 1 - Summary of the 7 preimplantation genetic diagnosis cycles found in the beta-thalassemia and sickle cell anemia affected families included in a study conducted at the Genetic Diagnosis Laboratory of Saad Specialist Hospital, Al-Khobar, Kingdom of Saudi Arabia.

Identification	Mutation	Number of embryos generated	Number of normal/ abnormal embryos	Number of carrier embryos	Number of embryos matching	Pregnancy test	Engraftment
Family 1	c.118C>T c.93+21_96del25	15	5/5	5	2	Positive	Yes
Family 2	c.17A>T	12	5/4	3	1	Negative	N/A
Family 3	c.93+5G>C	8	3/3	2	None	Positive	N/A
Family 4	c.93+1G>C	16	5/6	5	2	Negative	NA
Family 5	c.17A>T	8	4/3	1	None	Negative	N/A
Family 6	c.118C>T	10	5/3	2	1	Positive	Not yet
Family 7	c.93+21_96del25	12	4/3	5	2	Positive	Not yet

Family 1 is compound heterozygous for the mutations indicated. The rest of the families are homozygous. N/A - not applicable.

Table 2 - Illustrative results of HLA typing for patients who had pregnancy test, positive for normal and matching embryos in subjects included in a study conducted at the Genetic Diagnosis Laboratory of Saad Specialist Hospital, Al-Khobar, Kingdom of Saudi Arabia.

Identification	Parents		Affected siblings	
	Mother	Father		
Family #1				
	DRB1*12, DRB1*16	DRB1*14, DRB1*16	DRB1*12, DRB1*14	
	E #1and 6*	**E #2, 7 and 13**	**E #4, 8 and 15**	**E #9 and 10**
	DRB1*12, DRB1*14	DRB1*16, DRB1*16	DRB1*12, DRB1*16	DRB1*16, DRB1*14
Family#6				
	B*18, B*35	B*35, B*35	B*18, B*35	
	DRB1*16, DRB1*16	DRB1*16, DRB1*11	DRB1*16, DRB1*11	
	E#1*	**E#2 and 5**	**E #3 and 9**	**E#4**
	B*18, B*35	B*18, B*35	B*18, B*35	B*35, B*35
	DRB1*16, DRB1*11	DRB1*16, DRB1*16	DRB1*16, DRB1*16	DRB1*16, DRB1*11
Family #7				
	DRB1*13, DRB1*11	DRB1*16, DRB1*11	DRB1*13, DRB1*16	
	E#4 and 10*	**E#2, 3 and 7**	**E #8, 12 and 6**	**E #5**
	DRB1*13, DRB1*16	DRB1*16, DRB1*16	DRB1*16, DRB1*16	DRB1*16, DRB1*11

E - embryo, DRB - dopamine receptor beta; *indicates embryos with matching human leukocyte antigen (HLA) with the affected sibling; Families #1 and #7 underwent class II (DRB), and family #6 class I and II (DRB). Normal or carrier embryos are shown in this table.

were identified in 5 families (family one compound heterozygous, families 3, 4, 6, and 7 homozygous). For STR-analysis in the HLA locus, we tested 21 STR in the vicinity, and within the HLA loci on the 7 families. The results presented, showed one or 2 fully informative markers in 6 families, and none in the seventh family.

Table 2 summarizes the results of blastomeres analysis for HLA typing. This table contains families who had pregnancy with matching embryos. The DRB1 typing was not fully informative in the families. Families with no informative DRB1 underwent class I (B) typing. Indeed, family 6 had uninformative DRB1 typing. They underwent class I (B) and II (DRB) typing. A combination of the 2 tests enabled us to choose the normal and HLA matching embryos (**Table 2**). Families with no informative class I and II were excluded. Normal and HLA matching embryos were found in 5 families. A pregnancy test showed positive results in 3 out of the 5 couples. The other 2 families accepted to transfer normal, but not matching embryos. Upon delivery, HSCT[17] from the UCB was performed successfully on family one who had a delivery on a normal and matching boy. Cord blood stem cells, collected during deliveries are stored for the 2 other families (delivery of a baby girl each) until HSCT is carried out.

Discussion. This report demonstrated the successful clinical application of HLA typing on blastomere DNA amplified by MDA using direct genotyping of class I and II by routine PCR-SSP. Previously reported data on PGD-HLA were achieved using a complex set of primers.[14] Such a labor-intensive procedure makes the

application of PGD-HLA on a single cell very difficult. High consanguinity rates in some societies, which can reach to up to 65% in some areas of Saudi Arabia,[19] result in lower chances of finding informative STR markers (fully or semi-informative), and consequently HLA typing using STR could not be applied (some semi-informative markers could be useful for embryo screening [depends on the markers inherited by the affected babies who need HLA typing], fully informative markers are ideal for HLA typing regardless of the inherited alleles). We used 21 reported highly polymorphic markers, while this list could reach over 50 linked markers, we opted to use direct genotyping of HLA. Such a method was successfully applied,[14,20] using a mini sequencing approach. However, the complexity of the technique makes it difficult to apply in routine settings. Further, the nested PCR technique was applied with a very complex set of primers. In the procedure we applied here, there is no need for any optimization and the MDA product is treated like any normal DNA routinely used for PCR-SSP HLA genotyping. The current report shows for the first time the clinical application of HLA SSP on embryonic cell DNA amplified by MDA.

The quality of MDA product obtained was suitable for HLA matching proved by an interpretation of the entire blastomeres tested (blastomeres found normal, or carriers for mutation in beta-globin gene). We did not observe any case of allele dropout or failure of amplification (during the HLA typing) due probably to the high amount of DNA, the multiplex PCR protocol used in the HLA typing test, and finally the length of

the probe used in the typing. However, the percentage of embryos found carriers by PCR (23 out of 81) for beta-globin mutation is less than the theoretical percentage (40 out of 81) probably due to allelic drop out. The importance of HLA typing using PCR-SSP is evidenced in 2 of the 7 patients. These 2 couples had homozygous alleles at DRB1 in HLA class II, and consequently HLA typing could not be performed. We applied HLA typing class I (A and B) in conjunction with DRB1 to differentiate between the types of HLA in the embryos.

In the largest reported single center experience of PGD for thalassemia from the Reproductive Genetics Institute in Chicago, 197 PGD cycles were performed for hemoglobin disorders, of which 54 were combined with HLA typing.[21] The large denominator was, in part, due to samples from Cyprus being sent for analysis, but the numerator was only one successful HSCT known to have been achieved at the time of reporting. A smaller single center experience in California including 8 families affected with thalassemia: 6 cases of beta-thalassemia major, and 2 cases of transfusion-dependent hemoglobin E beta-thalassemia.[22] A total of 14 cycles of PGD were performed and 2 pregnancies occurred, one of which resulted in a cord blood transplant (CBT) and engraftment.

The mainstay of therapy for severely affected beta-thalassemia patients has included supportive blood transfusion and iron chelation therapy. The HSCT using HLA-identical donors, however, has demonstrated a 90% cure rate among affected children early in the course of disease.[16] On the other hand, transplantation utilizing an unrelated HLA-matched donor has had less impressive results. Moreover, related umbilical cord transplants, as an alternative source of stem cells, demonstrate a nearly similar outcome to related HLA-matched bone marrow stem cells in addition to a lower risk for graft-versus-host disease.[23]

There is increasing support for a more permissive view that allows pre-implantation testing for embryonic characteristics that may be highly relevant for the health of third parties, particularly PGD-HLA testing. The ethical debate has so far disregarded the possible dynamics of PGD-HLA testing. It is important to distinguish between HLA typing with a delivery of normal baby (type 1) in families suffering from a HSCT curable genetic disease, and the possible alternative type, where embryos generated in IVF will not be transferred to the mother (type 2), and will be used only to cure siblings for couples suffering from genetic conditions curable by HSCT. Although the former can be morally justified, the risks, pitfalls, and practical limitations of

this procedure make it imperative to consider the second option as an alternative. From an ethical point of view, the sensitive point with type 2 is that it involves the creation of embryos purely for therapeutic use. Considering the dominant view that the pre-implantation embryo has only limited moral value, this alternative may be morally justified. Both the prohibition of the creation of embryos for therapeutic use (either in research, or in future therapy), and the restrictive interpretation of the principle of subsidiary are questionable. If safe and effective, type 2 is prima facie, even the better option from a medical, psychosocial, and ethical point of view. Adequate preclinical studies with embryos donated for research with respect to the efficiency and safety of PGD-HLA testing type 2 (including methods to expand the cells ex vivo prior to transplantation) are of utmost importance. Since developmental biology is evolving fast, these laboratory experiments and the ethical discussion should be conducted simultaneously.

In general, HLA genes are inherited as a unit from each parent (haplotype) because of their close linkage on the chromosome. Thus, the best possible donor is an HLA matched family member of the patient. However, recombination in families has been observed between each of the HLA loci. Indeed, recombination events, documented by family study, occur at a rate of approximately 0.01-0.013 between HLA-B and -DRB1 (separated by approximately 1200 Kb), and at a rate of approximately 0.008 between HLA-DQB1 and DPB1 (separated by about 400 Kb). However, recombination between DRB1 and DQB1 (less than 100 Kb separated) is rarely documented within studied families. These recombination events could lead to the initial false assumption of HLA identity for the patients and donor in BMT, which could result in intractable acute graft versus host disease that could lead to the patient's demise. In PGD protocol, undetected recombination occurrences are a source of misdiagnosis, which ends with an erroneous result. Such recombination could be detected by 2 procedures: use the high resolution HLA typing method for class I and class II, or testing informative linked markers along the HLA loci. With regard to the first procedure, we demonstrated the possibility of typing class I and class II. Due to the low amount of DNA available, we can run low resolution class I and II, and consequently decreasing the possibility of recombination. The second procedure of testing linked markers was not possible due to consanguinity. One more approach is the use of 3-generation pedigrees[24] to determine DNA segments that are shared identical by descent between grandparent and grandchild, allowing

identification of parental recombination events in the HLA loci. Again, such an approach needs several informative linked markers to be tested, which is not an easy task in many consanguineous families.

In conclusion, this report has importance in introducing PGD-HLA matching as a routine standard technique easily performed in any facility. Populations in parts of the world, where hemoglobinopathies and consanguinity are prevalent will greatly benefit from such a technique. However, the incapability of detecting parental recombination at the embryo level is considered the main limitation of such a study.

Improving the PGD-HLA typing using the next generation sequencing could be an interesting future field of research where a small amount of DNA could yield high resolution HLA typing at MDA product of single cells.

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