Preimplantation Diagnosis for Fanconi Anemia Combined With HLA Matching

Yury Verlinsky, PhD
Svetlana Rechitsky, PhD
William Schoolcraft, MD
Charles Strom, MD, PhD
Anver Kuliev, MD, PhD

Preimplantation genetic diagnosis (PGD) was introduced for couples at high risk for producing progeny with genetic disorders to avoid prenatal diagnosis followed by pregnancy termination. Preimplantation genetic diagnosis has become an alternative to traditional prenatal genetic diagnosis and also an integral part of assisted reproduction, providing an important tool for improving the efficiency of in vitro fertilization (IVF) through avoiding the transfer of aneuploid embryos. Preimplantation genetic diagnosis has been performed in more than 2500 clinical cycles for at-risk couples, resulting in approximately 600 clinical pregnancies and births of at least 500 healthy children.

While PGD has been performed for more than 2 dozen different genetic disorders, with indications being in general similar to those in prenatal diagnosis, a few new indications have recently emerged, including preimplantation HLA antigen matching for preselection of potential donor progeny for bone marrow transplantation. Single-cell polymerase chain reaction (PCR) presents the opportunity for combined PGD and HLA antigen testing, which is a novel and useful way to preselect a potential donor for an affected sibling requiring stem cell transplantation. HLA antigen testing as part of PGD should be a reasonable option, because only 2 to 3 of the 10 to 12 embryos resulting on average from a hormonal hyperstimulation in IVF are actually selected for transfer. Therefore, instead of a "blind" selection of embryos for transfer, only those representing a match for an affected sibling needing a transplant are preselected.

One of several congenital disorders requiring stem cell transplantation from a family member is Fanconi anemia (FA), which was the first disease for which cord blood transplantation was introduced. Fanconi anemia is an autosomal recessive disorder, characterized by inherited bone marrow failure, congenital malformations, and an increased predisposition to the development of leukemia. It is genetically heterogeneous, involving different complementation groups (FA-A, FA-B, FA-C, FA-D, and FA-E), one of the most severe being FA-C mutation leading to aberrantly spliced transcripts (IVS 4 +4 A→T), which result in inactivating FA complement C (FANCC) protein.

Bone marrow transplantation is the only treatment that definitively restores hematopoiesis in FA patients. However, because modification of the conditioning by high-dose chemotherapy and ablative radiation is too toxic for these patients, the HLA antigen–identical cord blood transplantations are preselected.

Context The advent of single-cell polymerase chain reaction (PCR) has presented the opportunity for combined preimplantation genetic diagnosis (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.

Objective To perform in vitro fertilization (IVF) and preimplantation HLA matching combined with PGD for Fanconi anemia (FA).

Design DNA analysis for the IVS 4 +4 A→T (adenine to thymine) mutation in the FA complement C (FANCC) gene in single blastomeres, obtained by biopsy of embryos, to identify genetic status and HLA markers of each embryo before intrauterine transfer.

Setting In vitro fertilization programs at large medical centers in Chicago, Ill, and Denver, Colo.

Participants A couple, both carriers of the IVS 4 +4 A→T mutation in the FANCC gene with an affected child requiring an HLA-compatible donor for cord blood transplantation.

Main Outcome Measures DNA analysis of single blastomeres to preselect unaffected embryos representing an HLA match for the affected sibling.

Results Of 30 embryos tested in 4 IVF attempts, 6 were homozygous affected and 24 were unaffected. Five of these embryos were also found to be HLA-compatible, of which 2 were transferred in the first and 1 in each of the other 3 cycles, resulting in a pregnancy and birth of an unaffected child in the last cycle.

Conclusion To our knowledge, this is the first PGD with HLA matching, demonstrating feasibility of preselecting unaffected embryos that can also be an HLA-compatible source for stem cell transplantation for a sibling.

JAMA. 2001;285:3130-3133

For editorial comment see p 3143.
tion from a sibling is particularly valuable for FA, especially in avoiding late complications due to severe graft-vs-host reaction.\textsuperscript{15,16}

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

**METHODS**

A couple presented for PGD with both parents being unaffected carriers of IVS 4+4 A→T mutation in the FANCC gene (Figure 1). Their affected 6-year-old daughter had 2 copies of this mutation, requiring an HLA-compatible donor for bone marrow transplantation. The couple requested PGD for FA, together with HLA antigen testing of embryos, in order to have an unaffected child who could be a compatible cord blood donor for their affected daughter. The study was approved by the Illinois Masonic Medical Center institutional review board of Chicago.

Preimplantation genetic diagnosis was performed using a standard IVF protocol combined with micromanipulation procedure to biopsy single blastomeres from the day 3 cleaving embryos.\textsuperscript{17} Blastomeres were tested for IVS 4+4 A→T mutation in the FANCC gene using polyacrylamide gel analysis of PCR product digested with Scal restriction enzyme, according to the earlier described method of single-cell PCR analysis\textsuperscript{19} (Figure 1). The biopsied single cells were placed directly into a lysis solution, consisting of 0.5 µL of 10 × PCR buffer, 0.5 µL of 1% Tween 20, 0.5 µL of 1% Triton X-100, 3.5 µL of water, and 0.05 µL of proteinase K (20 mg/mL in 0.5-mL PCR tube). After spinning down, the samples were covered with 1 drop of mineral oil and incubated at 45°C for 15 minutes in a thermal cycler. Proteinase K was then inactivated at 96°C for 20 minutes. The cycling conditions were as follows: 45°C for 15 minutes, 96°C for 20 minutes, 72°C for 7 minutes (hot start); 96°C for 20 seconds, 55°C for 1 minute, 72°C for 20 seconds, for 10 cycles; and 72°C for 7 minutes, for 18 cycles. When the thermal cycler reached the 72°C step, each sample in a tube was brought up to a final volume of 50 µL, using the round 1 PCR master mix, which consisted of PCR solution with the final concentration of each dNTP (dATP, dCTP, dGTP, and dUTP; Pharmacia, Piscataway, NJ), 400 µM, 1 × Promega PCR buffer (Promega, Madison, Wis), Taq polymerase (1.25 U), 1.5-mM magnesium chloride, 5% dimethylsulfoxide, and 0.5 µM of each set of outside upstream and downstream primers for the FANCC gene, HLA, and polymorphic markers under the mineral oil.

We designed the outer primers IVS4-1 (5′-GTG TAT TTT AAT TA TTA A-3′) and IVS4-2 (5′-GGCA CATT CAC CATT AACA-3′) for performing the first round of amplification, and used previously described inner primers\textsuperscript{19} for the second round of PCR. As seen from Figure 1, second-round PCR produces a 131-base pair (bp) product, undigested by Scal restriction enzyme, corresponding to the mutant allele, and 2 restriction fragments of 108 bp and 23 bp, corresponding to the normal allele, generated by the introduction of the restriction site using 4R inner primer with single base modification of A→G.\textsuperscript{10}

Nested PCR for specific amplification of HLA antigen gene exons 2 and 3 was performed using gene-specific outer primers Asp5 (5′-GCC CGAC CCT ACCT (CT) TC CTT GCTA A-3′) and Asp3 (5′-CC GT GGG CTC TGT TA GA CTT A-3′) and inner primers (5′-GTG TAT TTT AAT TA TTA A-3′) and IVS4-1 (5′-GTG TAT TTT AAT TA TTA A-3′). The inner primers were designed to amplify a 131 bp insert specifically for the mutant allele.

**Figure 1.** Preimplantation Diagnosis for IVS 4+4 A→T Mutation in Fanconi Anemia

**A** Gene

<table>
<thead>
<tr>
<th>Gene</th>
<th>5′ Exon 4</th>
<th>3′ Intron 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant Allele</td>
<td>GTG TAT TTT AAT TA TTA A</td>
<td>3′</td>
</tr>
<tr>
<td>Normal Allele</td>
<td>GTG AG TAT TA TTA A</td>
<td>3′</td>
</tr>
</tbody>
</table>

**4R Primer**

<table>
<thead>
<tr>
<th>Primer</th>
<th>5′ Exon 4</th>
<th>3′ Intron 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>4R Prime</td>
<td>CAT GA TAT TA A</td>
<td>5′</td>
</tr>
</tbody>
</table>

**Copy of Normal Allele With Scal Restriction Site**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Copy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant Allele</td>
<td>GTG AG TAT TA TTA A</td>
</tr>
<tr>
<td>Normal Allele</td>
<td>CAT GA TAT TA A</td>
</tr>
</tbody>
</table>

**B** Scal Restriction Digestion

<table>
<thead>
<tr>
<th>Allele</th>
<th>Scal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant Allele</td>
<td>131 bp</td>
</tr>
<tr>
<td>Normal Allele</td>
<td>108 bp</td>
</tr>
</tbody>
</table>

**C** Polyacrylamide Gel Analysis

<table>
<thead>
<tr>
<th>Allele</th>
<th>Ladder (standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant Allele</td>
<td>ET</td>
</tr>
<tr>
<td>Normal Allele</td>
<td>108 bp</td>
</tr>
</tbody>
</table>

\(\text{FANCC}\) gene, showing sites and location of IVS 4+4 A mutation, and fragment-length polymorphism. Colored arrows indicate outer primers 4-1 and 4-2 and inner primers 4F and 4R. B, Primer design and restriction map for normal and abnormal alleles. C, Polyacrylamide gel analysis of Scal restriction digestion, showing 1 homozygous affected (embryo 6), 3 homozygous normal (embryos 7, 9, and 10), and 8 heterozygous unaffected embryos (embryos 8 and 11 are not amplified). IVS indicates intervening sequence (intron); bp, base pair; ET, embryo transfer; and L, ladder (standard).
3'), followed by 4 separate second-round PCR with allele-specific inner primers 085 (5'-CTCTTGACCCAGGCTCT-3') and 98 (5'-GCAGGGTCCCAGGAGGCGCCG-3') for A2 allele, and 126 (5'-GGTTCTCACACCATCCAGATA-3') and 142 (5'-CAGGTATCTGGCGAGCCCG-3') for A1 allele, and 118 (5'-TCCATGAGGTATTTCTACACC-3') and 145 (5'-GCAGGGTCCCCAGGTTCG-3') for allele A26. As haplotype analysis for father, mother, and the affected child showed different polymorphic short-tandem repeat (STR) marker (GAAA)n (C2_4_4) located between HLA-A and HLA-B and in the HLA-E to HLA-C region (Figure 2), a hemi-nested PCR system was designed to study the number of repeats in blastomeres from different embryos. The first-round amplification cocktail for this system contained outer primers P1-1 (5'-GGCTTGACTTGAAAAGTGAT-3') and P1-3 (5'-TATCTACTTATAGTCTATCACG-3'), while the second round PCR used in addition to P1-1, the inner primer P1-2 (5'-CTTCAAACAATACGCAATGACA-3'). The nested PCR system for HLA-B allele discrimination included outer primers Bout 1 (5'-GAGGGTCGGGGCGGGTCTCAG-3') and Bout 2 (5'-TGGGGGATGGGGAGTCGTTGAC-3') for the first round of amplification. The second round of amplification for HLA B35 was performed using inner PCR primers CG4 (5'-GACGACACCCAGTTCGTGA-3') and 35in (5'-GAAGATCTGTGTGTTCGAGG-3'). Accordingly, the second round of amplification of HLA B44 was performed with primers CG3 (5'-CTCTGGTTAGTAGCGGAG-3') and 41up (5'-GCCAGTGGTTAGTAGCGGAGG-3'), and HLA B44 with primers 41up and GC2 (5'-GCTCTGGTTAGTAGCGGAGG-3').

Unaffected embryos, which were also HLA antigen compatible with the affected sibling, were transferred back to the patient, while the other unaffected embryos were frozen to be available for future transfer. With consent of the parents, all affected embryos were exposed to PCR analysis to confirm the blastomere diagnosis. The follow-up analysis was also performed in the established pregnancy in the last cycle, using chorionic villus sampling (CVS) at the first trimester.

**Figure 2.** Design for Preimplantation Haplotype Analysis of HLA Genes

Haplotypes of family members with A, B, and C representing polymorphic short-tandem repeat (STR) alleles corresponding to specific markers. HLA-A1, HLA-A2, HLA-A3, and HLA-A26 are alleles of the HLA-A gene; HLA-B35, HLA-B41, and HLA-B44 are alleles of the HLA-B gene; IVS4+4 represents the mutant allele in the FANCC gene.

**Figure 3.** Preimplantation HLA Analysis

Top: HLA detection system by allele-specific primers. Bottom: A, results of HLA typing of single blastomeres from embryos in the last cycle showing that only 1 of the unaffected embryos (embryo 3) corresponds to the HLA type of the affected sibling (S). B, polyacrylamide gel analysis results of confirmation of blastomere diagnosis in chorionic villus sampling (CVS) obtained from the resulting pregnancy, compared with the results of HLA typing of paternal (P), maternal (M), and affected S DNA samples. STR indicates short-tandem repeat; ET, embryo transfer; and L, ladder (standard). Colored arrows indicate primers.
RESULTS
Blastomere genotyping for IVS 4+4 A→T mutation in the FANCC gene was performed in 4 clinical cycles, involving the mutation analysis in 33 embryos, including 7 in the first, 4 in the second, 8 in the third and 14 in the fourth cycle. Of 30 embryos with results, 19 were heterozygous carriers, 6 were homozygous affected, and 5 were homozygous normal. Figure 1 shows the results of mutation analysis of 14 embryos in the last cycle, of which only 1 was homozygous affected (embryo 6), 3 were homozygous normal (embryos 7, 9, and 10), 2 did not amplify (embryos 8 and 11), and the rest were heterozygous unaffected.

Testing for HLA-A (A2, A26) and HLA-B (B35, B44) in these 24 unaffected embryos, including 19 heterozygous and 5 homozygous normal embryos, revealed 5 heterozygous unaffected embryos for transfer with HLA antigen match for the affected sibling. Figure 3 shows the results of mutation analysis in 14 embryos in the last cycle, of which only 1 unaffected heterozygous embryo (embryo 3) was HLA identical to the affected child and therefore transferred back to the patient. Similarly, 2 unaffected HLA antigen–matched embryos were available for transfer in the first, in the second, and in the third cycle. However, only the transfer in the last cycle resulted in a clinical pregnancy and birth of a healthy carrier of the FANCC gene, following confirmation of the results of both mutation analysis and HLA matching by CVS (Figure 3).

Umbilical cord blood was collected at birth and transplanted to the affected sibling, resulting in a successful hematopoietic reconstitution (J. Wagner, MD, University of Minnesota, unpublished data, 2001). A total of 9 embryos that were predicted to be normal but found carrying HLA genes different from the sibling developed to the blastocyst stage and were frozen, while 5 affected embryos were exposed to PCR analysis, confirming the blastomere diagnosis.

COMMENT
The practical application of PGD has recently been extended for new indications that appear to be different from those used in prenatal genetic diagnosis. For example, there is a growing interest for PGD for the late-onset disorders with genetic predisposition. 5,24 HLA antigen testing is one of the latest and most unexpected additions to the indications for PGD. Presented results demonstrate feasibility of preimplantation HLA matching as part of PGD, with a prospect for the application of this approach to other inherited conditions, such as thalassemias, which also require an HLA-compatible donor for bone marrow transplantation. Although this is the first and only experience (to our knowledge) of PGD for HLA antigen testing, it provides a realistic option for couples desiring to avoid the birth of an affected child, together with the establishment of a healthy pregnancy, potentially providing an HLA antigen match for an affected sibling. Our data show that the HLA antigen testing in single blastomeres is accurate and may also be applied as a primary indication (ie, in cases not requiring mutation testing), such as for couples having affected children with leukemia or other cancers, waiting for an HLA-compatible donor with no success for years. These new indications make PGD a genuine alternative to conventional prenatal diagnosis, providing patients with important prospects not only to avoid an inherited risk without facing termination of pregnancy, but also to establish a pregnancy with particular genetic parameters that benefit an affected member of the family.

Author Contributions: Study concept and design: Verlinsky, Rechitsky, Kuliev. Acquisition of data: Verlinsky, Rechitsky, Schoolcraft. Analysis and interpretation of data: Verlinsky, Rechitsky, Strom, Kuliev. Drafting of the manuscript: Verlinsky, Rechitsky, Kuliev. Critical revision of the manuscript for important intellectual content: Schoolcraft, Strom, Kuliev. Obtained funding: Verlinsky. Study supervision: Verlinsky, Rechitsky, Schoolcraft, Strom, Kuliev.

REFERENCES