PGD for HLA Typing

4

Preimplantation HLA matching has never been indicated for prenatal diagnosis because of a possible clinical pregnancy termination after finding the fetus HLA-unmatched. However, PGD for such purposes should be acceptable because only a limited number of embryos are usually preselected for transfer anyway, which in this case will represent unaffected embryos with a perfect match for affected siblings in need of a transplant. The world's first case of preimplantation HLA typing was introduced in combination with mutation analysis for Fanconi anemia (FA), with the objective of establishing an unaffected pregnancy yielding a potential donor progeny for transplantation in an affected sibling [1, 2]. This historical case, which has opened a new chapter in reproductive medicine, is presented below.

4.1 Fanconi Anemia – World's First PGD for HLA Typing

One of the most severe congenital disorders requiring stem cell transplantation from a family member is FA, which actually was also the world's first disease for which cord blood transplantation was introduced [3]. FA 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 (FANCA, FANCB, FANCC, FANCD, and FANCE), one of the most severe being the FANCC mutation leading to aberrantly spliced transcripts (IVS4+4A-T), which result in inactivating the FANCC protein [4–6]. Bone marrow transplantation is the only treatment which restores definitively hematopoiesis in FA patients. However, because any modification of the conditioning is too toxic for these patients, leading to a high rate of transplant-related mortality, the HLA identical cord blood transplantation from a sibling is particularly valuable for FA, to avoid late complications due to severe GVH [7, 8].

A couple presented for PGD, with both parents being unaffected carriers of IVS 4+4A-T mutation in the FANCC gene. Their affected 6-year-old daughter had two copies of this mutation, requiring an HLA-compatible donor for bone marrow transplantation. The couple requested PGD for FANCC, together with HLA testing of embryos, in order to have an unaffected child who may also be a compatible cord blood donor for their affected daughter.

PGD was performed using a standard IVF protocol combined with micromanipulation procedure to biopsy single blastomeres from the day 3 cleaving embryos, as described in Chap. 2. Blastomeres were tested for IVS 4+4A-T mutation in the FANCC gene using polyacrylamide gel analysis of PCR product digested with *ScaI* restriction enzyme, according to the method of single-cell PCR analysis, also described in Chap. 2.

The outer primers, IVS4–1 (5′-GTCATAAAA GGCACTTGCAT-3′) and IVS4–2 (5′-GGCACA TTCAGCATTAAACA-3′), were designed for

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performing the first round of amplification, while using the previously described inner primers [9] for the second round of PCR. The second-round PCR produces a 131-bp product, undigested by the *ScaI* restriction enzyme, corresponding to the mutant allele, and two restriction fragments of 108 and 23-bp, corresponding to the normal allele, due to introduction of the restriction site by application of 4R inner primer with single base modification of T to G.

Nested PCR for specific amplification of HLA-A gene exons 2 and 3 was performed using gene-specific outer primers Asp5 (5′GCCCCG AACCCTC(CT)TCCTGCTA 3′) and Asp3 (5′CC GTGGCCCCTGGTACCCGT 3′) [10], followed by four separate second-round PCRs with allele-specific inner primers 085 (5′TCCTCGTCCCC AGGCTCT 3′) and 98 (5′GCAGGGTCCCCA GGTCCA 3′) for A2 allele, 140 (5′GGTTCTCAC ACCATCCAGATA 3′) and 142 (5′ CAGGTATCT GCGGAGCCCG 3′) for A1 allele[21], 140 (5′ GGT TCTCACACCATCCAGATA 3′) and 126 (5′CC ACTCCACGCACGTGCCA 3′) for A3 allele, and 118 (5′TCCATGAGGTATTTCTACACC 3′) and 145 (5′GCAGGGTCCCCAGGTTCG 3′) for allele A26 [11]. As haplotype analysis for the father, mother, and affected child showed different polymorphic short tandem repeat (STR) marker (GAAA)n (C2_4_4) located in between HLA-A and HLA-B (in HLA-E–HLA-C region), a heminested PCR system was designed to study the number of repeats in blastomeres from different embryos [12]. The first-round amplification cocktail for this system contained outer primers P1–1 (5′-GGCTTGACTTGAAACTCAGAG-3′) and P1–3 (5-TATCTACTTATAGTCTATCAC G-3′), while the second-round PCR used in addition to P1–1, the inner primer P1–2 (5′-CTTC AAACAATACGCAATGACA-3′). The nested PCR system for HLA-B allele discrimination included outer primers Bout 1(5′-GAGGGT CGGGCGGGTCTCAG-3′) and Bout 2 (5′-TG GGGGATGGGGAGTCGTGAC-3′) for the first round of amplification. The second round of amplification for HLA-B35 was performed using inner PCR primers CG4 (5′-GACGACAC CCAGTTCGTGA-3′) and 35in (5′-GAAGATC TGTGTGTTCCGG-3′). Accordingly, the second round of amplification of HLA-B41 was performed with primers CG3 (5′-CTCTGGTTGTAG TAGCCGC-3′) and 41 up (5′-CCACGAGTCCG AGGAAGG-3), and HLA-B44 with primers 41up and GC2 (5′-GCTCTGGTTGTAGTAGC GGA-3′) [13].

Blastomere genotyping for IVS 4+4A-T mutation in the FANCC gene was performed in four clinical cycles, involving 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. Of 14 embryos tested for mutation in the last cycle, only 1 was homozygous affected, 3 were homozygous normal, 2 did not amplify, with the remaining being 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 match for the affected sibling, requiring transplantation of stem cells. The results of HLA typing in 14 embryos in the last cycle revealed only 1 unaffected heterozygous embryo being HLA identical to the affected child, which therefore was transferred back to the patient. Similarly, 2 unaffected HLA-matched embryos were available for transfer in the first, 1 in the second, and one 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. Umbilical cord blood of the baby was collected at birth and transplanted to the affected sibling, resulting in a successful hematopoietic reconstitution. Nine embryos predicted normal but carrying HLA genes different from the sibling developed to the blastocyst stage and were frozen, while five affected embryos were exposed to PCR analysis, confirming the blastomere diagnosis.

As mentioned, the practical application of PGD has recently been extended for new indications, which appeared to be different from those used in prenatal diagnosis, such as the late-onset

disorders with genetic predisposition, which could have hardly been considered candidates for prenatal genetic diagnosis. HLA testing was the most recent and most unexpected addition to the indications for PGD. The results of this first case demonstrated feasibility of preimplantation HLA matching as part of PGD, with a prospect for the application of this approach to the other inherited conditions, such as thalassemias and other congenital disorders, also requiring an HLA-compatible donor for bone marrow transplantation. Although initially this was the first and only experience of PGD for HLA testing, it provided a realistic option for the couples desiring to avoid the birth of an affected child, together with the establishment of a healthy pregnancy, potentially providing an HLA match for an affected sibling. The data showed that the HLA testing in single blastomeres was accurate, and may also be applied as primary indication, that is, in cases not requiring mutation testing, such as for couples having affected children with leukemia or other cancers, awaiting 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 to benefit the affected member of the family.

Our experience of PGD with HLA typing is presented in Table 4.1, showing that among conditions requiring HLA-compatible stem cell transplantation, thalassemia is the most prevalent one, representing the commonest autosomal-recessive diseases in the Mediterranean region, Middle East, and South East Asia, with heterozygous frequency of thalassemia mutations reaching over 14% in Greece and Cyprus.

4.2 Thalassemia

As described in Chap. 3, beta-thalassemia is an autosomal-recessive disease affecting the production of beta-globin chains resulting in a severe anemia, which makes the patients transfusion-dependant starting from 6 months after birth, so bone marrow transplantation is the only option for radical treatment. At present, over 400 different mutations have been described in the beta-globin gene, located on chromosome 11 (11p15.5), causing congenital anemia of variable severity [14, 15]. Prenatal diagnosis has been applied widely for almost three decades resulting in considerable reduction of new cases of thalassemia up to 70% in many populations, including large countries in the Eastern Mediterranean region such as Turkey and Iran [16, 17]. A considerable progress has been achieved also in the treatment of the disease by bone marrow transplantation [18], the application of which is still limited to the availability of HLA-matched stem cells, making PGD an attractive option for couples with thalassemic children. PGD for thalassemia has already been provided for 15 years [19], so HLA typing is presently offered in the same framework, allowing couples not only to avoid the birth of another child with thalassemia, but also producing an unaffected child who may be an HLA match to the thalassemic sibling, and thus a potential stem cell donor.

Thalassemias are presently one of the major indications for PGD. PGD for thalassemia was first performed for couples who had previously undertaken prenatal diagnosis but had to terminate the pregnancy with an affected fetus on repeated attempts [19]. Then, it was offered as a primary option to the patients with infertility problems, and to those who could not accept the risk for prenatal diagnosis and termination of pregnancy [19–21]. This was followed by PGD for the couples with existing thalassemic children requiring HLA-compatible bone marrow transplantation [22–25]. The objective of PGD in these cases was not only to have a thalassemia-free child, but also to ensure that the resulting baby could serve as an HLA-compatible donor for bone marrow transplantation for the affected siblings.

In our experience, of a total of 293 PGD cycles for 161 couples at risk for producing offspring with thalassemia, 144 cycles were performed for HLA typing. PB or blastomere biopsy was performed to identify thalassemia mutations, and blastomere sampling was also used for

Table 4.1 Experience in PGD with HLA typing

Disease	Patients	Cycles	No. of embryo transfers	No. of embryos transferred	Pregnancy	Birth
Thalassemia/sickle cell disease	51	149	82	130	20	15
FANCA,FANCC, FANCD2,FANCF, FANCI, FANCJ	17	53	34	52	*7*	4
WAS	2	2	2	4	*1*	1
X-ALD	2	5	1	1	0	0
Hyper-IgM	5	8	6	9	*3*	2
HED + ID; IP	2	9	6	8	*2*	3
DBA	3	5	3	6	*2*	2
Krabbe	1	1	1	2	1	2
DM	1	2	1	2	1	2
Chronic granulomatous disease	1	3	3	5	1	1
Total	85	237	139	219	38	32

HLA testing, in order to identify the embryos containing the maternal and paternal chromosome 6 identical to the sibling with thalassemia, as described in detail elsewhere and summarized below [22, 25, 26].

HLA genes were tested simultaneously, using the short tandem repeats in the HLA region, by applying a multiplex heminested PCR system, involving only closely linked polymorphic short tandem repeat (STR) markers located throughout the HLA region [12], including D6S426, D6S291, Ring 3 CA, TAP1, G51152, D6S2447, LH1,DN, D6S273, 9N-2, TNF a,b,c,d; 62, MIC A, MIB, D6S276, D6S439, D6S1624, D6S265, D6S510; D6S248, RF, MOG a,b,c,d, D6S 258, D6S306, D6S464, D6S299, D6S461 (Fig. 4.1). The choice of alleles and markers was based on the information they provided about the presence of maternal and paternal matching or non-matching chromosomes. For each family, heterozygous alleles and markers were selected not to be shared by the parents. Such markers provided information about the origin of chromosome 6. A haplotype analysis for the father, mother, and affected child was performed for each family prior to preimplantation HLA typing. This allowed detecting and avoiding misdiagnosis due to preferential amplification and allele dropout (ADO), exceeding 10% in PCR of single blastomeres, potential recombination within the HLA region (see below), and a possible aneuploidy or uniparental disomy of chromosome 6, which may also affect the diagnostic accuracy of HLA typing of the embryo. The multiplex nature of the first round of PCR required a similar annealing temperature as the outside primers. Thirty cycles of PCR were performed with denaturation step at 95°C for 20 s, annealing at 62–50°C for 1 min and elongation at 72°C for 30 s [27]. Twenty minutes of incubation at 96°C was performed before starting cycling, and after cycling, 10 min of elongation at 72°C was performed. Annealing temperature for the second round was programmed at 55°C. The applied strategy provided a 100% HLA match, because the embryos with the same paternal and maternal chromosome 6 as in the affected siblings were preselected. Figure 4.2 presents PGD for HLA in the case of parents carriers of different mutations IVSI-5 and Cd8. Using PB1 and PB2 analysis, six mutation-free oocytes were identified prior to blastomere analysis, used mainly for selection of paternal mutation-free embryos (embryos #1, #3, #5, and #9) and HLA matching, simultaneously with linked marker analysis. In addition, HLA typing was also done in PB1 and PB2 to identify the oocytes with maternal HLA match, which is useful for interpretation of HLA-matching results in blastomeres. Only embryos #2 and #6 appeared to be HLA-matched to the affected sibling, which were transferred, resulting in the birth of a healthy child, who was confirmed to be thalassemia-free as well as HLA-matched to the affected sibling with thalassemia. The chances to identify

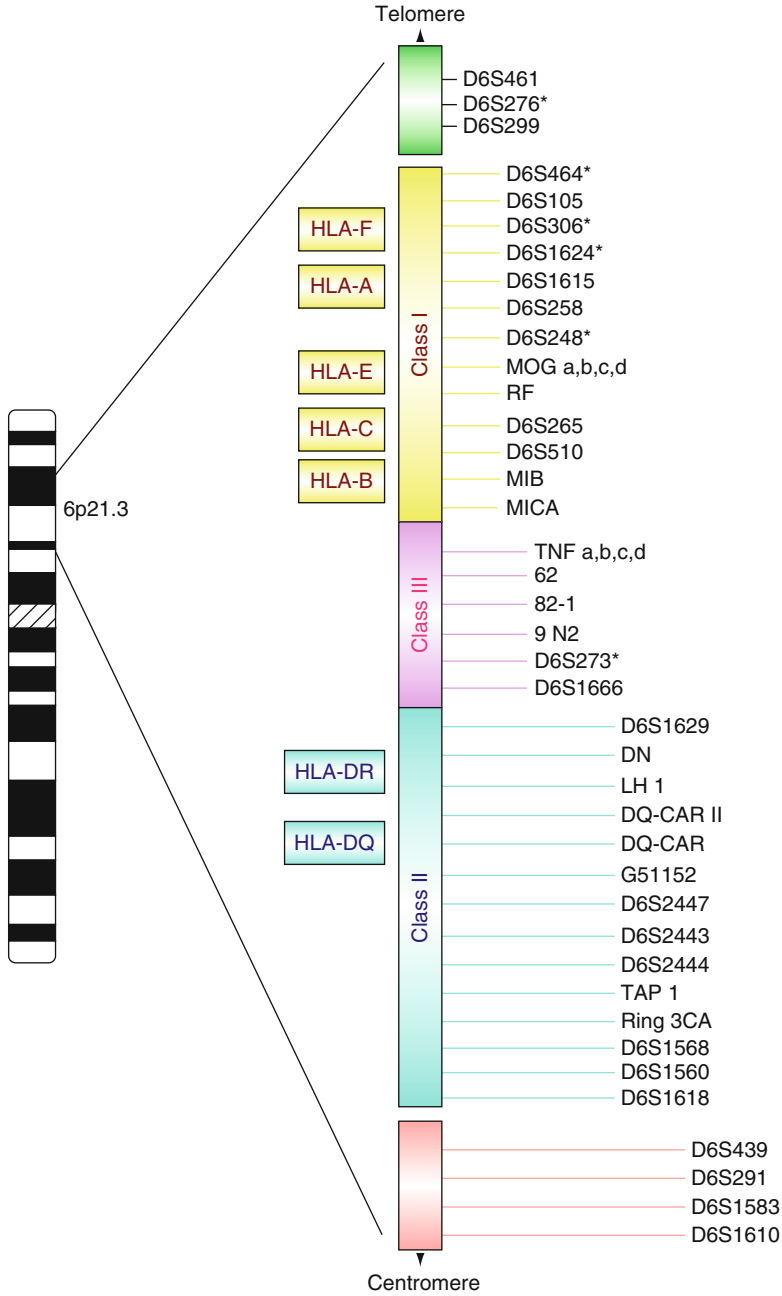


Fig. 4.1 Polymorphic markers in HLA region applied for preimplantation HLA typing

unaffected embryos fully matched to thalassemic siblings is 18.75% and, as for other autosomal-recessive conditions, there is a 25% chance of HLA match and 75% chance of having an unaffected embryo (see below).

While the HLA-matched and thalassemia-free embryos were preselected for transfer back to the patient, based on the information about the mutation testing and polymorphic markers, those embryos predicted mutant or with insufficient

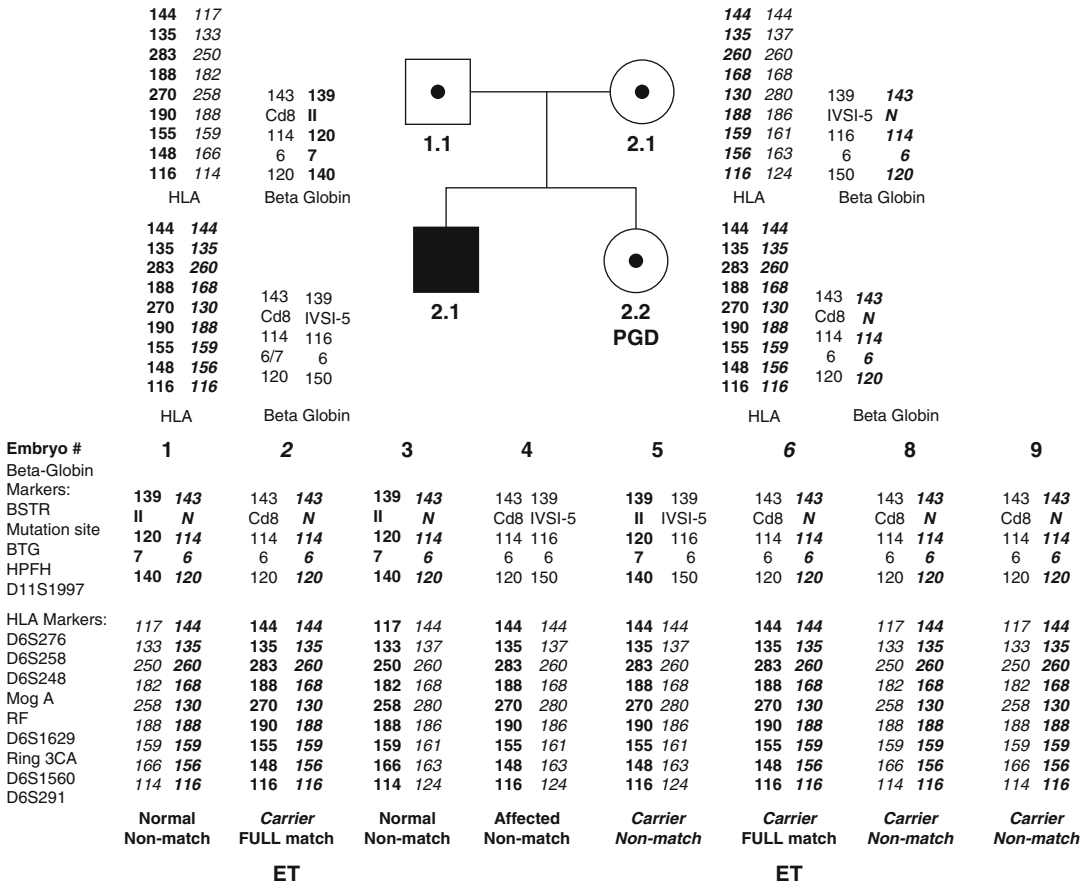


Fig. 4.2 Preimplantation HLA matching combined with PGD for thalassemia. (*Top panel*) Family pedigree with HLA haplotype analysis based on parental (1.1 and 1.2) and affected child's (2.1) genomic DNA testing. HLA marker order is presented on the *upper left* for the father and *upper right* for the mother. *Dark bars* represent the matching paternal and maternal HLA haplotypes, and the *non-bold bars* non-matching haplotypes. (*Middle panel*) Maternal mutation and linked polymorphic markers were first assessed by sequential multiplex polar body (*PB*) analysis. Two oocytes (#4 and #5) had affected alleles, while the remaining six (#1, #2, #3, #6, #8, and #9) were normal (data not shown). Based on blastomere results, one embryo was affected (#4), two were homozygous normal (#1 and #3), and five were carriers of paternal (#2, #6, #8, and #9) or maternal (#5) mutations. As seen from HLA typing blow (see *lower panel*), embryos #2 and #6 are also fully HLA-matched to the sick sibling (2.1; *upper panel*). (*Bottom panel*) HLA typing by short tandem repeats (*STRs*) along with mutation analysis was performed on blastomeres from eight embryos, two of which (#2 and #6) were predicted to be HLA-matched to that of the affected sibling (2.1), although carrying the paternal mutation (also see above). Prenatal testing confirmed these results, and a healthy baby girl with HLA type matching that of the sick sibling was born. Cord blood stem cells were collected during the delivery and frozen for the stem cell transplantation

marker information were exposed to confirmatory analysis. Non-matched unaffected embryos were frozen for future use by the couple.

Of more than two dozens of different beta-globin gene mutations tested, the most frequent ones were IVSI-110 mutation – 100 cases (33%), followed by IVS I-6 – 39 cases, IVSII-745 – 23 cases, codon 8 – 20 cases, IVSI-1 – 18 cases, and codon 39 and IVSI-5 –16 cases each (see Table 3.4). Among other mutations were IVSII-2, Codon 5, Codon 6, Codon 41/2, E121K, -29 (A-G) -87, R30T, Cap 1, deletion 69 kb, and deletion 13.4 kb. Mutation testing resulted in detection and transfer of 476 unaffected embryos (approximately two embryos per transfer) in 240 (81.9%) of 293 clinical cycles, yielding 67 (27.9%) unaffected

pregnancies and the birth of 70 thalassemia-free children. PGD for thalassemias currently represents 15% of our overall experience of 2,158 PGD cycles performed for single-gene disorders [28].

A total of 144 of these PGD cycles were performed for HLA typing, which allowed detecting and transferring unaffected HLA-matched embryos in 78 of them (Table 4.2). Of 824 embryos with conclusive results for testing of beta-globin gene mutations and HLA type, 602 (73.0%) were predicted to be unaffected carriers or normal, of which only 126 (15.3%) appeared to be HLA-identical to the affected siblings, which is not significantly different from the expectation [26]. As many as 123 of these embryos developed appropriately to be acceptable for transfer, resulting in 18 unaffected HLA-identical pregnancies and the birth of 13 healthy children. Umbilical cord blood was collected at birth of these children and transplanted or pending, resulting in a successful hematopoietic reconstitution in all of them.

Figure 4.3 demonstrates the case of PGD for HLA typing for a couple with two thalassemic children, resulting in preselection and transfer of unaffected embryos matched to each of the affected children. HLA typing showed that one of the embryos was matched to one of the affected siblings, another to the other affected sibling (embryos #4 and #9), and three were non-matched (embryos #6, #7, and #8), including one with a single

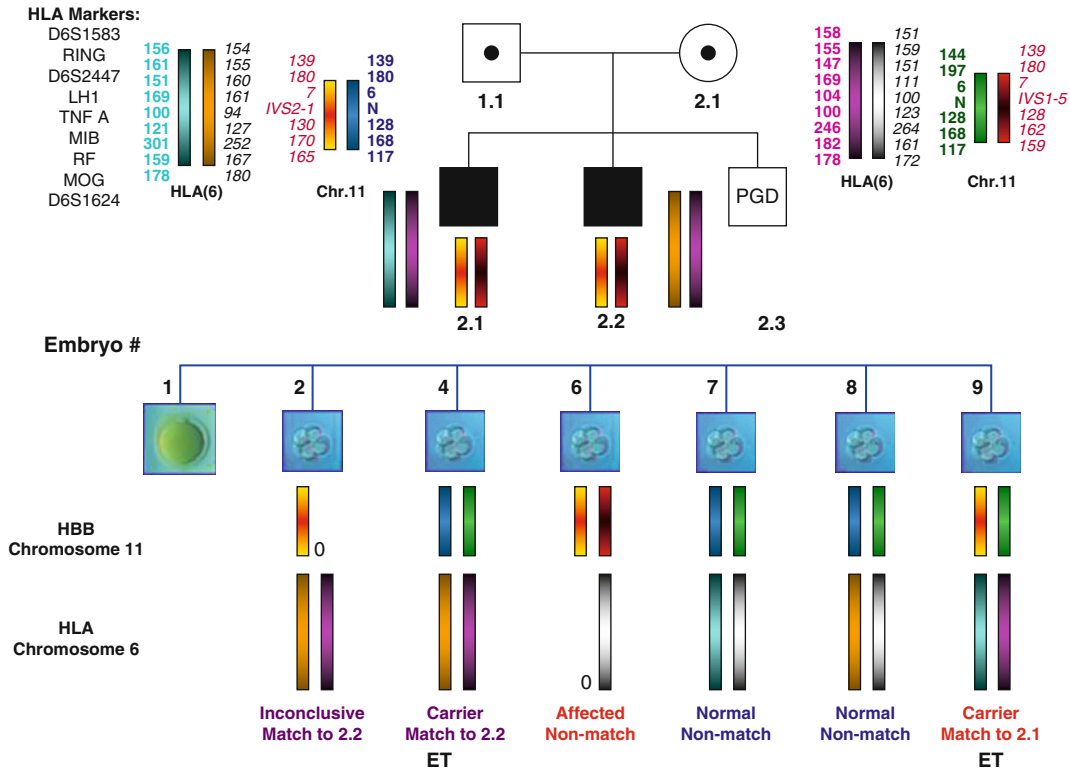


Fig. 4.3 PGD for HLA typing in a couple with two thalassemic children requiring HLA-matched bone marrow transplantation. (*Upper panel*) Family pedigree with HLA haplotype analysis based on parental (1.1 and 1.2) and affected children's (2.1; 2.2) genomic DNA testing. HLA marker order is presented on the *upper left* for the father and *right* for the mother. Paternal and maternal matching HLA haplotypes to the affected children (2.1; 2.2) are shown in *different colors*. Maternal and paternal mutations and the linked markers are also presented accordingly. (*Lower panel*) HLA typing by short tandem repeats (*STRs*) along with mutation analysis was performed on blastomeres from seven embryos, one of which (#4) was predicted to be a carrier and an HLA match to the affected sibling 2.2, and another (#9) also a carrier and match to the affected sibling 2.1. Three others (embryos #6–8) were non-matched, while embryo #2 was matched but had inconclusive results of mutation testing due to lack of maternal chromosome 11. Both carrier matched embryos were transferred, but singleton pregnancy was obtained with the birth of a thalassemia-free child matched to one of the affected siblings

Table 4.2 Results of PGD for thalassemia with HLA testing

Patient/cycle	No. of embryos total/ amplified	No. of normal embryos		No. of transfers/no. of embryos	Pregnancy/ birth
		Non-match	Match		
52/144	907/824	***476***	**126**	78/123	18/13
	91%			1.57	23%

chromosome 6 (embryo #6). A single aneuploid oocyte suggesting trisomy 22 in the resulting embryo was detected by FISH analysis of PB1 and PB2 (excluded from further analysis of the causative gene and HLA type), in addition to the embryos with monosomy 11 (embryo #2) and monosomy 6 (embryo #6) mentioned. Two unaffected embryos and HLA-matched to each of the affected children were also aneuploidy-free (embryos #4 and #9) and transferred, resulting in a singleton pregnancy and birth of a healthy baby, HLA-matched to one of the affected siblings with thalassemia.

With the current progress in the treatment of hemoglobin disorders, PGD may have an increasing impact on the decision of the well-treated patients to reproduce. In fact, the life expectancy of the patients with hemoglobin disorders has been dramatically improved with the increasing success rate of radical treatment by stem cell transplantation [18]. However, the further impact of this treatment will depend on the availability of HLA-identical donors.

As seen from the above experience, PGD for HLA typing is an efficient tool for couples at risk to ensure having thalassemia-free children HLA-identical to the affected siblings, to serve as potential donors for stem cell transplantation treatment. This currently is available for a wider application in those communities where thalassemia is highly prevalent, and will improve the access to HLA-matched bone marrow transplantation of thalassemia.

For example, in the second largest series of PGD for HLA typing in thalassemia performed in Turkey, 236 PGD cycles were performed resulting in the birth of 70 thalassemia-free children, stem cells of 19 of whom were used for cord blood or bone marrow transplantation, which resulted in successful bone marrow reconstitution in all of them [29, 30].

4.3 Immunodeficiencies

Severe congenital immunodeficiencies (SCID) are a large group of conditions requiring PGD for HLA typing, as without compatible bone marrow transplantation the patients with SCID cannot survive. HLA-matched stem cell transplantation improves or completely replenishes the immune system, so PGD is an obvious alternative for inherited forms of SCID, to ensure the birth of unaffected children, who may then also serve as potential stem cell donor progeny for the affected siblings. Our accumulated experience of PGD for SCID is presented below, including PGD for ataxia telangiectasia (AT), Omen syndrome (OMS) (OMIM, 2001), FANCA, hyperimmunoglobulin M syndrome (HIGM), X-linked adrenoleukodystrophy (X-ALD), Wiscott–Aldrich syndrome (WAS), and X-linked hypohidrotic ectodermal displasia with immune deficiency (HED-ID) (Table 4.3) [31].

A total of 23 PGD cycles for 11 couples for producing affected progeny with the above conditions were performed, including 8 cycles for HIGM, 3 for AT, 2 for WAS, 9 for HED-ID, and 1 for OMS (Table 4.3), confirming the usefulness of preimplantation HLA matching as part of PGD, which potentially provides an HLA-matched progeny for treatment of affected siblings.

Omen syndrome (OMS) is an extremely rare autosomal-recessive disease with a prevalence of 1 in over 50,000, for which there is still no available cure other than stem cell transplantation. OMS is an early-onset fatal immunodeficiency with the absence of B cells and excess production of highly restricted T lymphocytes, which is caused by mutation in recombinase-activating genes RAG1 and RAG2 located on chromosome 11p, coding the lymphoid-specific proteins responsible for the process of variable, diversity, and joining (V (D) J) segment recombination

Table 4.3 Results and outcomes of PGD for immunodeficiencies

Disease/gene/mutations	Patient/cycle	Cell type tested	No. of embryos total/ amplified	No. of normal embryos	No. of abnormal embryos	No. of transfers/ no. of embryos	Pregnancy/ birth
HIGM/TNFSF (CD40L) gene/C218X, exon 4 c.437–438 ins A, exon 4 c.397 ins T	5/8	Pbs, BL; BL	46/43	31	12	6/9	3/2
OMS/RAG1 gene/ R 396 C; L86 AA del	1/1	Pbs, BL	5/5	3	2	1/2	1/2
HED-IP/ IKBKG(NEMO)gene/ D113N; Q348X; L153R	2/9	Pbs, BL	36/35	24	11	6/8	2/3
AT/ATM gene/ K1807E; A2262P; Unknown	1/3	Pbs, BL	20/20	10	10	2/4	1/1
WAS/WASP gene/ L39P; Nt. 361 (–2) A→G	2/2	Pbs, BL: BL	10/10	6	4	1/2	1/1
Total	11/23	N/A	117/113	74	39	16/25	8/9

required for generation of the T- and B-cell repertoire (MIM 603554). This severe primary immunodeficiency disease is characterized by generalized erithrodermia, protracted diarrhea, repeated infections, hepatomegaly, and leukocytosis with eosinophilia and elevated immunoglobulin E. The large phenotypic variability of patients may be determined by different mutations in RAG1 and RAG2 genes, involving missense and splice mutations or deletions. Despite specific therapy for dermatitis and lymphoadenitis, using immunosuppression or replacement therapy with intravenous immunoglobulins, persistent viral, bacterial infections and chronic diarrhea resulting in inanition may be responsible for death, with the only cure being HLA-identical bone marrow transplantation.

A couple at risk for producing a progeny with OMS had two previous children, including the younger daughter with a severe OMS resulting in death (Fig. 4.4). The child was double heterozygous, with the inherited paternal

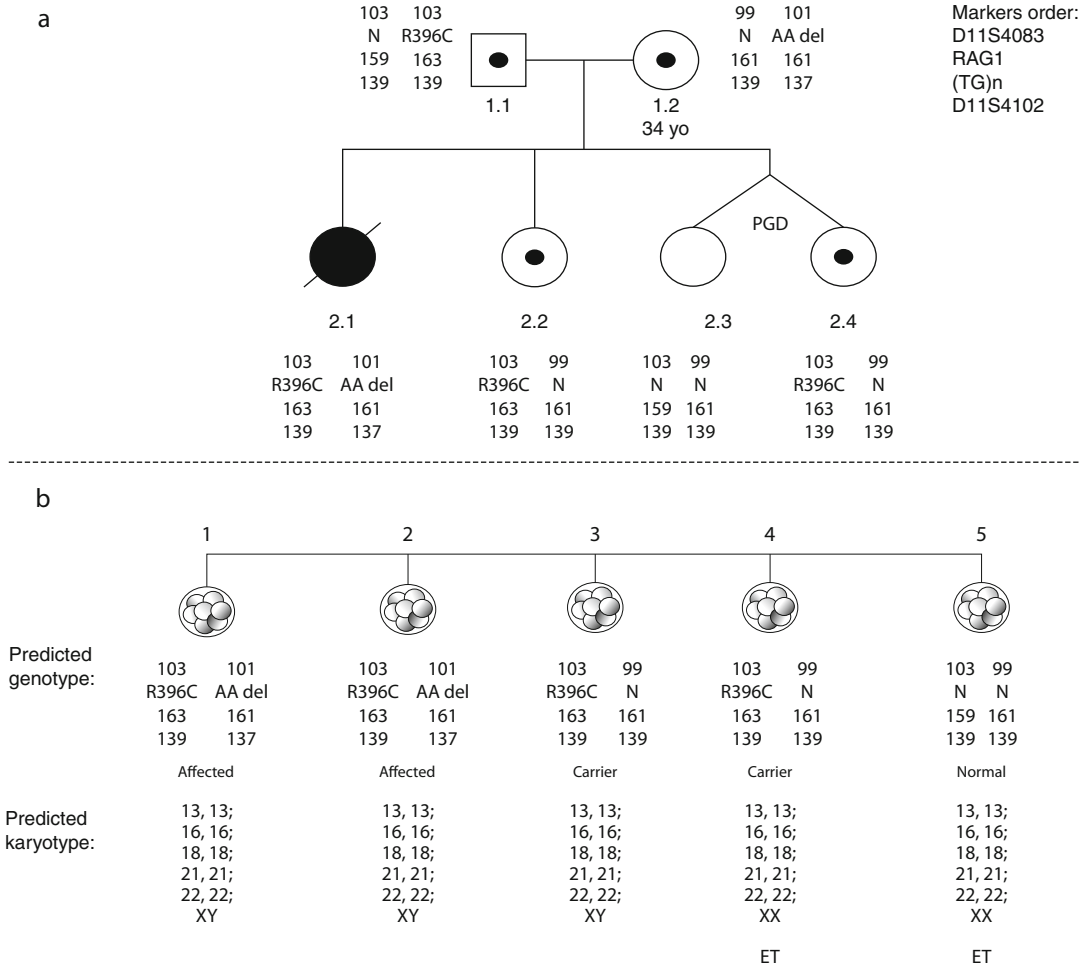


Fig. 4.4 PGD for OMS with aneuploidy testing. (**a**) Family pedigree with the mutation and haplotype analysis of parents (1.1 and 1.2) and children (2.1 affected, and 2.2 healthy heterozygous carrier of paternal mutation). (**b**) (*Top*) Blastomere analysis involving mutation analysis of five embryos, including two affected (embryos #1 and #2), two carriers of paternal mutation (embryos #3 and #4), and one free of both paternal and maternal mutations (embryo #5). (**b**) (*Bottom*) Blastomere analysis for aneuploidy, showing normal chromosomal sets for all five embryos, two of which were transferred (embryos #4 and #5) resulting in the birth of healthy twins (2.3 normal, and 2.4 heterozygous carrier of the paternal mutation)

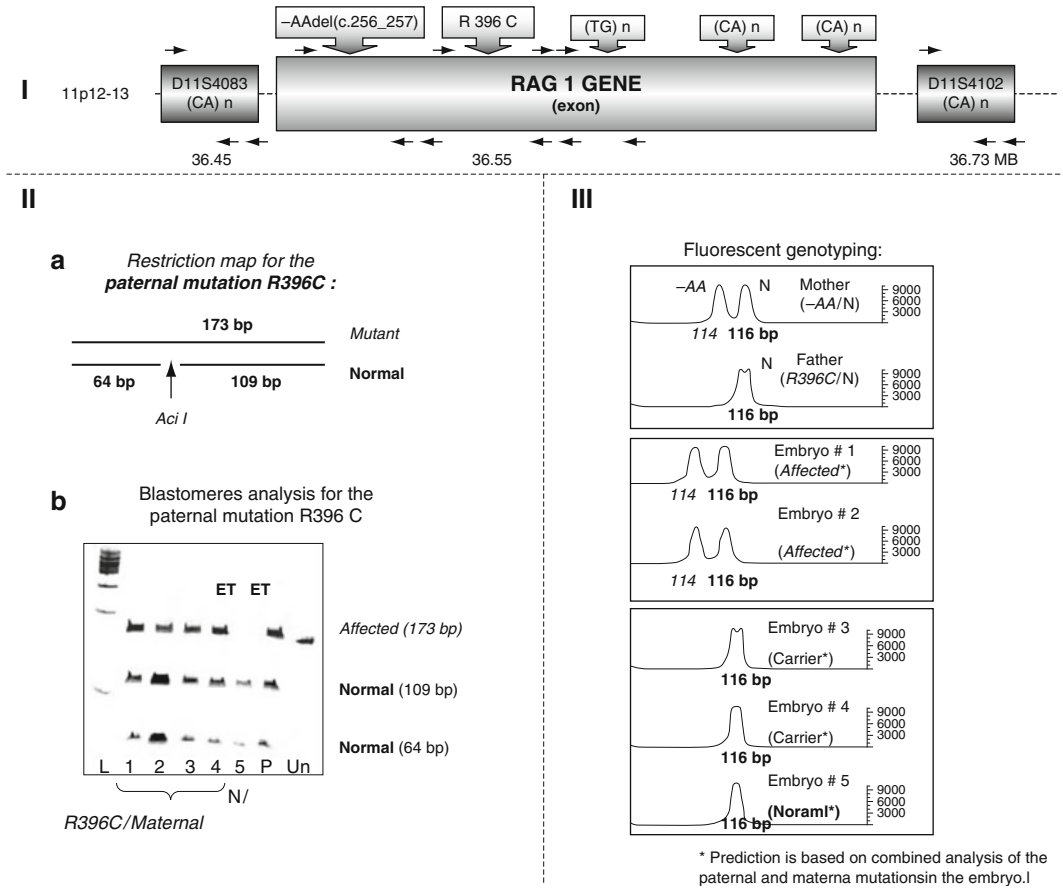


Fig. 4.5 PGD for OMS by blastomere analysis of paternal and maternal mutations. (**I**) Position of parental mutations and informative linked polymorphic markers used in PGD. (**IIa**) Restriction map of the paternal mutation R396C, following Aci I digestion, creating two fragments of 64 and 109 bp in PCR product of the normal gene. (**IIb**) The polyacrilamide gel electroforegram of the Aci I-digested PCR products of biopsied blastomeres from five embryos for the paternal mutation R396C, showing that only embryo #5 is free of paternal mutation, while the remaining embryos contain the paternal mutation. (**III**) Capillary electroforegram of fluorescently labeled PCR products of RAG1 gene obtained from mother, father, and five embryos, showing normal fragment of 116 bp for the father, two fragments of 114 pp (AA del) and 116 bp for the mother, the same two fragments including AA del for embryos #1 and #2, and one normal 116 bp fragment for embryos #3, #4, and #5, the latter two of which were transferred, resulting in the birth of healthy twins

R396C mutation, representing a sequence change from arginine to cysteine at amino acid position 396, caused by a single C to T sequence change (CGG to TGG) in codon 396 of the RAG1 gene, and maternal mutation c.256_57 del AA within the lysine 86 codon, causing a frameshift mutation that results in a premature termination signal 32 codons downstream. The older heterozygous unaffected daughter inherited only the paternal A396C mutation in the RAG1gene.

The paternal mutation was tested by Aci I digestion, which creates two fragments of 64 and 109 bp in the PCR product of normal RAG1 gene, leaving the mutant allele uncut. The two "A" nucleotide deletions in codon 86 of the maternal allele of RAG1 gene were detected by capillary electroforesis of the fluorescent labeled PCR product, visualizing the 114 bp fragment vs. the 116 bp fragment in the normal allele (Fig. 4.5).

This case represented the world's first PGD for OMS, which was performed using PB1 and

PB2 and blastomere analysis, and resulted in the transfer of two unaffected and aneuploidy-free embryos, yielding the birth of healthy twins. Because in this case the affected sibling died early in childhood, there was no need for HLA typing, but the couples with previous OMS children will definitely be potential candidates for performing PGD with HLA typing to provide also an identical HLA donor progeny for stem cell transplantation.

Ataxia Telangiectasia (*AT*) is a progressive, neurodegenerative childhood disease that affects the brain and other body systems (MIM 208900). A weakened immune system makes the patients susceptible to recurrent respiratory infections. The disease presents between 1 and 4 years of age, as a delayed development of motor skills, poor balance, and slurred speech. Telangiectasias appear in the corners of the eyes or on the surface of the ears and cheeks. Patients with AT may develop cancer, such as acute lymphocytic leukemia or lymphoma. Other features may include mild diabetes mellitus, premature graying of the hair, difficulty swallowing, and delayed physical and sexual development. Although the currently used symptomatic and supportive treatment, including high-dose vitamin regimens, physical and occupational therapy, and gammaglobulin injections to supplement a weakened immune system may be helpful, the prognosis is very poor, and patients still die in their teens. More than 500 unique mutations are known in the ataxia telangiectasia-mutated (ATM) gene associated with AT, resulting in the absence of serine–protein kinase coded by the ATM gene located on chromosome 11q22.3 (MIM 607585). Sequence analysis detects as many as 90% mutations, and others may be identified by the linkage analysis, which is extremely accurate, based on testing for intragenic markers presently available.

PGD was performed for the couple at risk for producing a progeny with AT, who had one affected child who died in early infancy and one spontaneous abortion (Fig. 4.6). The mother was a carrier of two ATM sequence changes, involving exon 38 (5419A>G, K1807E) and exon 48 (6784G>C, A2262P). As it is not known which of these two mutations is responsible for AT, both were tested in PB1 and PB2 and blastomeres. As shown in Fig. 4.7, maternal mutation K1807E was identified by Bsm AI digestion, creating two fragments of 96 and 92 bp in the PCR product of the mutant gene. In contrast, maternal mutation A2262P was not cut by Hae III restriction digestion, but created two fragments of 35 and 99 bp in the normal gene.

As the paternal mutation in the ATM gene was not identified, it was traced using four closely linked markers, listed in Fig. 4.6a. So we performed sequential PB1 and PB2 removal following maturation and fertilization of oocytes, to identify the mutation-free oocytes, as shown in Fig. 4.7IIb, and then tested the resulting embryos for paternal mutation by linkage analysis, simultaneously with testing for aneuploidy (Fig. 4.6c). As shown in Fig. 4.6, the transfer of two unaffected carrier embryos resulted in a singleton pregnancy and the birth of a healthy baby boy, confirmed to be an unaffected carrier of maternal mutations.

The case of PGD for AT was reported previously for a Saudi family with three affected children [32]. The disease was caused by a large deletion of more than two-thirds of the AT gene, which was detected by amplification of one of the deleted exons (exon 19). Of three embryos available for biopsy and testing, one was deletion-free and transferred, resulting in an unaffected pregnancy.

Fanconi anemia complementation group A (*FANCA*), similar to FANCC, described above in Sect. 4.1, is an autosomal-recessive disorder causing an inherited bone marrow failure with increased predisposition to leukemia. As mentioned, bone marrow transplantation is the only treatment for FA, as it restores hematopoiesis in FANCA patients. However, because any modification of the conditioning is too toxic for these patients, as in FANCC, leading to a high rate of transplant-related mortality, the HLA-identical cord blood transplantation from a sibling is particularly valuable, to avoid late complications due to severe GVH, as mentioned above.

Of 17 couples at risk for producing a progeny with FA (Table 4.1), in addition to two carriers of IVS 4+4A-T mutation in the FANCC gene, three

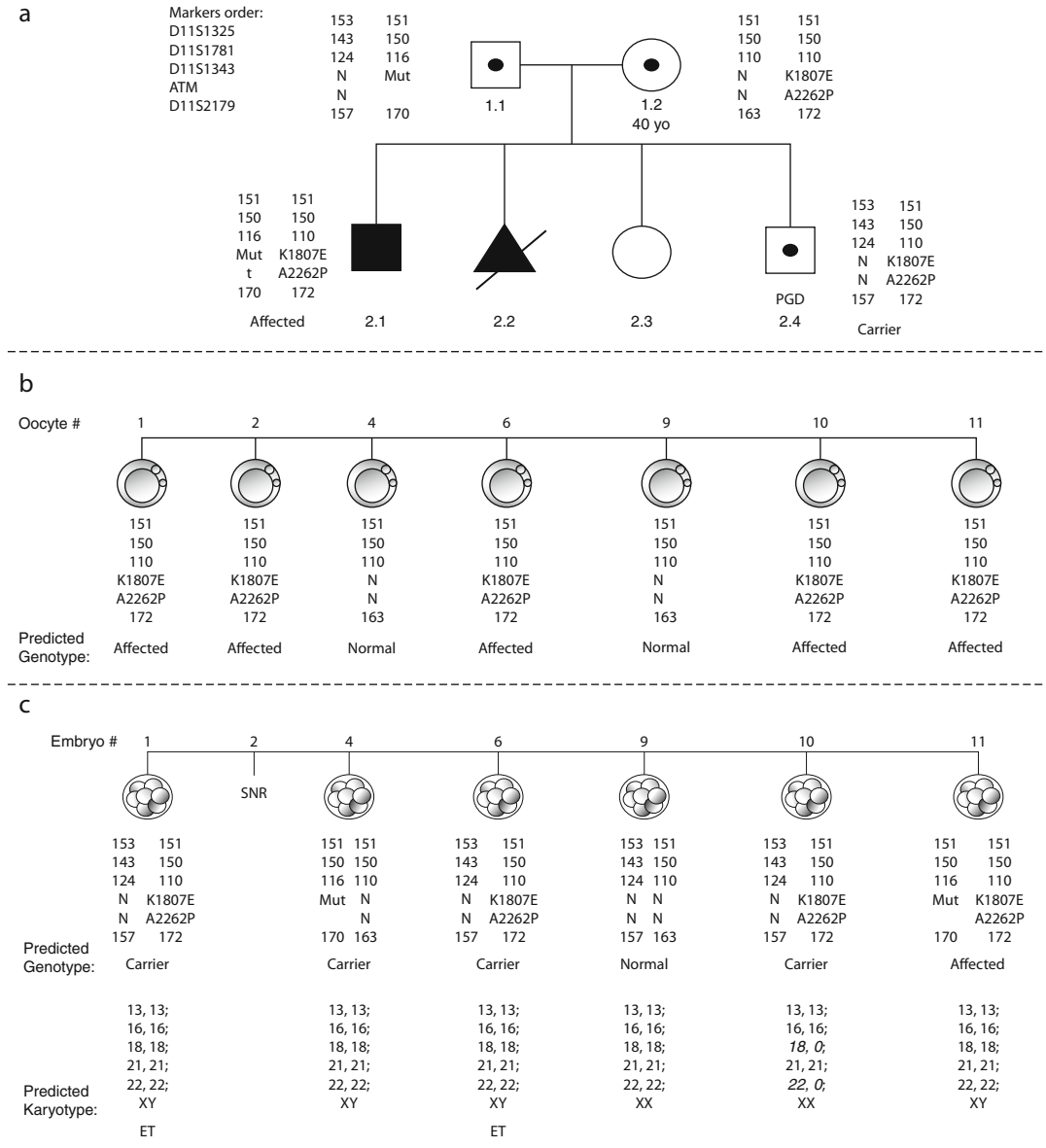


Fig. 4.6 PGD for AT with aneuploidy testing. (**a**) Family pedigree showing the results of mutation and haplotype analysis in the parents (1.1 and 1.2) and the affected child (2.1). (**b**) Results of PB analysis of seven oocytes, only two of which (oocytes #4 and #9) were free of mutation, based on mutation and marker analysis. The remaining five oocytes were affected, containing both maternal mutations tested. (**c**) (*Upper panel*) Results of mutation and linked marker analysis of six embryos originating from the above oocytes (no sample was available from the embryo originating from oocyte #2). Five of these six embryos were either normal (embryo #9) or carriers (embryos #1, #4, #6, and #10), while the remaining one embryo (embryo #11) was affected, inheriting both maternal and paternal mutations. (**c**) (*Bottom panel*) Results of aneuploidy testing for chromosomes 13,16,18,21,22,X, and Y, showing one double monosomy 18 and 22 in heterozygous unaffected embryo #10

were carriers of FANCD2, FANCF, FANCI, FAMCCJ, and FANCA gene mutations, including one with different maternal and paternal mutations, the maternal one involving A*T*G to A*A*G substitution in exon 1, resulting in methionine to lysine amino acid substitution, and the paternal-14 bp deletion in exon 2, representing a frameshift mutation. The paternal mutation was detected as the

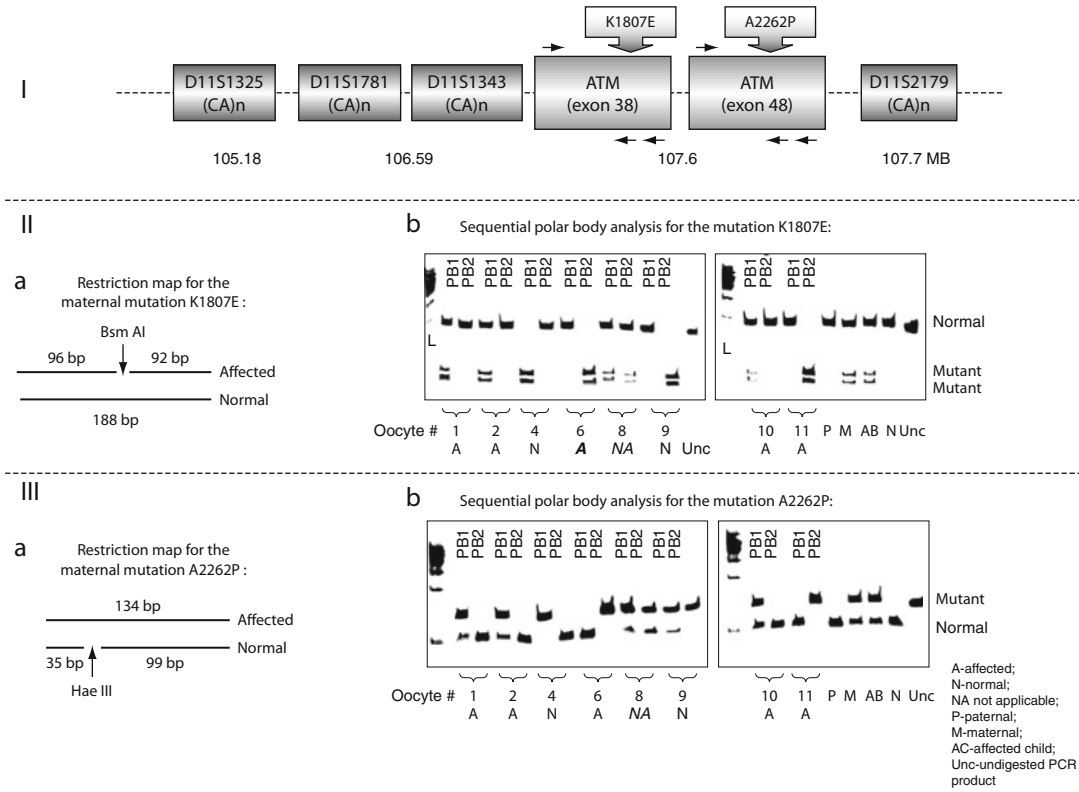


Fig. 4.7 PGD for AT by sequential PB1 and PB2 analysis of maternal mutations. (**I**) Position of two different mutations and informative linked polymorphic markers used in PGD. (**IIa**) Restriction map of maternal mutation K1807E, following Bsm AI digestion, which creates two fragments in the PCR product of the mutant gene. (**IIb**) The polyacrilamide gel electroforegram of the Bsm AI-digested PCR products of PB1 and PB2 from eight oocytes, of which only two (oocytes #4 and #9) were free of maternal mutation. Five oocytes were mutant, and one, with the heterozygous status of both PB1 and PB2, excluded from further study, due to possible DNA contamination. (**IIIa**) Restriction maps for the maternal mutation A2262P following Hae III restriction digestion, which creates two fragments of 35 and 99 bp in a normal allele, leaving the mutant allele uncut. (**IIIb**) The polyacrilamide gel electrophoregram of the Hae III-digested PCR products of PB1 and PB2 from eight oocytes, of which two (oocytes #4 and #9) were free of maternal mutation, five oocytes (oocytes #1, #2, #6, #10, and #11) were mutant, and one, with the heterozygous status of both PB1 and PB2, excluded from the further study, due to possible DNA contamination, as in the analysis for K1807E mutation

size difference in capillary electroforesis of the PCR product, while the maternal mutation was detected by Nla*II* restriction digestion, which cuts the normal sequence, leaving the mutant sequence uncut. In the other couple, only paternal mutation was known, representing T1131A mutation, due to *A*CT to *G*CT substitution in exon 34, which creates a restriction site for Fsp4H*I*. In addition, another restriction enzyme, TspR*I*, was used, which cuts the normal sequence on the opposite end. When a mutation was not identified, unaffected embryos were chosen by linkage analysis, using five closely linked polymorphic markers. Overall, 52 unaffected HLA-matched embryos were transferred in 34 cycles, resulting in seven unaffected pregnancies and four FA-free and HLA-matched children, as potential donors for their siblings (Table 4.1).

X-linked adrenoleukodystrophy (*X-ALD*) affects the nervous system and the adrenal cortex, with three main phenotypes. One of them manifests between ages 4 and 8 as attention deficit disorder, followed by progressive impairment of cognition and behavior, vision, hearing, and motor function, leading to total disability within 2 years. The other phenotype, called adrenomyeloneuropathy, manifests in the late twenties as progressive paraparesis, sphincter disturbances, and hearing loss, while the third presents with primary adrenocortical insufficiency by 7–8 years of age. Regardless of the presence of symptoms,

99% of patients have an elevated concentration of very long chain fatty acids (VLCFA). The disease is caused by mutations of ABCD1 gene, with more than 200 different mutations reported by the present time, which may be detected by PCR and direct sequencing, except for large deletions identified by Southern blot analysis. Carrier screening and prenatal diagnosis is available and the same method may be applied for PGD with simultaneous HLA typing.

PGD was performed for two couples, involving the testing for G343D mutation, representing a sequence change from aspartic acid to glycine at amino acid position 343, caused by a single (G to A) sequence change in the nucleotide 1414 (G1414A) of the ABCD gene. PGD was based on Fok *I* restriction digestion, which creates two fragments in the PCR product of a normal gene, leaving the mutant one uncut. A total of five PGD cycles were performed, which resulted in only one unaffected HLA matched embryo identified for transfer, which however failed to produce a clinical pregnancy (Table 4.1).

Hyperimmunoglobulin M Syndrome (HIGM) (Table 4.1) is a rare immunodeficiency characterized by normal or elevated serum IgM levels, with absence of IgG, IgA, and IgE, which results in an increased susceptibility to infections, manifested in the first few years of life, and a high frequency of autoimmune hematologic disorders, accompanied by gingivitis, ulcerative stomatitis, fever, and weight loss. HIGM is caused by mutation in the CD40 ligand gene (CD40LG), located on chromosome Xq26, which leads to a defective CD40 ligand expression resulting in the failure of T cells to induce IgE synthesis in interleukin-4-treated B cells. Although a regular administration of intravenous immunoglobulins may be used for treatment, the best results were obtained by HLA-matched bone marrow transplantation, which makes PGD the method of choice for those who cannot find an HLA match among their relatives.

PGD was performed for five couples with HIGM, (Table 4.1) one with C218X mutation in exon 5 of CD40 ligand gene (CD40LG), three with maternal mutations C218X exon 4 c.437_38 ins A, and one with exon 4 c.397 ins T, using the primers listed in (Table 4.4). The maternal mutations were analyzed by PB1 and PB2, followed by HLA and aneuploidy testing in biopsied blastomeres. CYS218STOP mutation in exon 5 was detected by restriction digestion, which eliminates the restriction site for *Cac 81*, creating two fragments in PCR product from the normal gene, leaving the mutant gene product uncut. For higher accuracy, another restriction enzyme (*Mnl I*) was applied, which creates three fragments in the mutant PCR product, compared to two fragments in the normal gene (Fig. 4.8).

Figure 4.9 presents the case of PGD and HLA typing for a couple at risk for producing offspring with HIGM. Of 15 oocytes tested by PB1 and PB2, 5 of 11 oocytes with conclusive results

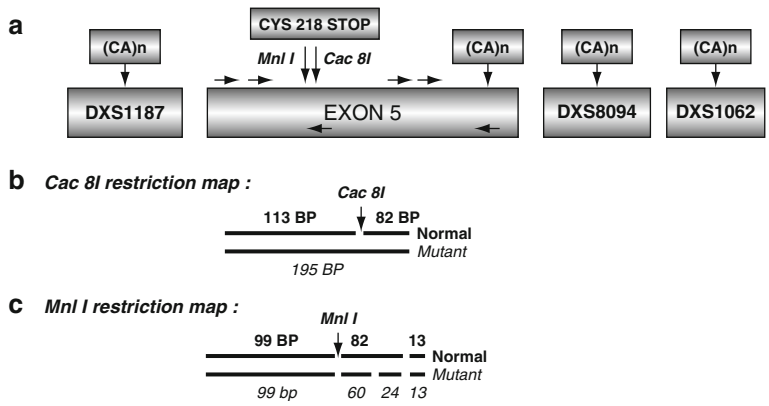


Fig. 4.8 PGD design for HIGM in combination with HLA typing (see also Fig. 4.5). (**a**) Position of the C218X mutation in exon 5 of CD40 ligand gene (Xq26.3) and tightly linked dinucleotide polymorphic markers inside the gene (exon 5) and outside the gene (DXS1187, DXS8094, DXS1062). Horizontal arrows represent primer positions. Vertical arrows indicate the location of *Mnl*I and *Cac*8I restriction sites, and the positions of the dinucleotide polymorphic markers. (**b**) Restriction map of the *Cac*I restriction digestion. (**c**) Restriction map for the Mnl I digestion

Table 4.4 Primers and reaction conditions for PGD of immunodeficiencies

Gene/polymorphism	Accession no.	Heterozy gosity index	No. of alleles	Upper primer	Lower primer	Annealing T_m (°C)
ATM K 1807 E (Heminested) *Bsm AI* cuts mutant sequence	AH004875	NA	NA	Outside: 5′ AGTTTTTAGAAGTACCCA GATTTGA 3′	5 TAGATAAACAGGTCATAA ACAAGGA 3′	62–45
				Inside: 5′ AGTTTTTAGAAGTACCCA GATTTGA 3′	5′ TCTTCTTACTTCACACAT TGGCT 3′	55
ATM A 2262 P (Heminested) *Hae III* cuts normal sequence	AH004875	NA	NA	Outside: 5′ AAGGAAATGGACAACTC ACAAAG 3′	5′ CCCTCAGGCTTTCTGT TTTTTA 3′	62–45
				Inside: 5′ TCTCACCAAACACCTTGTA GAACTC 3′	5′ CCCTCAGGCTTTCTGT TTTTTA 3′	55
D11S1325 (Heminested)	Z23828	0.52	3	Outside: 5′ AACATCAAATGGTTCTTG CTTC 3′	5′ TTTTATCTCTTTTTCAATA CAATGC 3′	62–45
				Inside: 5′ AACATCAAATGGTTCTTG CTTC 3′	5′ *Fam* GGGATTCTGCTTTTT TCCTTTA 3′	55
D11S1781 (Heminested)	Z52108	0.34	4	Outside: 5′ GGGGATGAGTAATGATATAAGA CAA 3′	5′ ACTTCTACTGTGTATATTT ACGGCA 3′	62–45
				Inside: 5′ GGGGATGAGTAATGATATAAGA CAA 3′	5′ *Fam* CGGCATATAACATAGTG TTATTTTG 3′	55
D11S1343 (Heminested)	Z24175	0.56	5	Outside: 5′ CTCCTTCCCAAACAATCCACT 3′	5′ CCTGGTTCATGTAGCAG TTCCT 3′	62–45
				Inside: 5′ CTCCTTCCCAAACAAT CCACT 3′	5′ *Fam* CCCCTACTGTTTTATG ACCCA 3′	55
D11S2179 (Heminested)	AF119249	NA	8	Outside: 5′ CTCCTCATTCTAAACAA CAACTG 3′	5′ GCTTGCAACATCTACTATA TATTTT 3′	62–45
				Inside: 5′ *Fam* TTCTCTTCTATGAATATAAC AGGAG 3′	5′ GCTTGCAACATCTACTATA TATTTT 3′	55
RAG1 R 396 C (Heminested) *AciI* cuts normal sequence	NT_009237	NA	NA	Outside: 5′ CCACATCTCAAGTCACAA GGAA 3′	5′GCCAGCAGGAACAAG GTCAT 3′	62–45
				Inside: 5′ CCACATCTCAAGTCACA AGGAA 3′	5′ ACTTCACATCTCCAC CTTCTTCT 3′	55

RAG1 c.256_57 del AA (Nested)	NT_009237	NA	NA	Outside: 5′ GAAACCCTCTCTGGAGCAATCT 3′	5′ GCTCTAAAAGAATTCC CACAGA 3′	62–45
				Inside: 5′ ACAAGGCTGATGGTCAGAAG 3′	5′ *Fam* TTGGCTTGATGG ATCGCTT 3′	55
D11S4083 (Heminested)	Z52164	0.87	15	Outside: 5′ GGACTCTTGGAACTCTGGACT 3′	5′ TTGGGGATCATGTG TACCC 3′	62–45
				Inside: 5′ GGACTCTTGGAACTCTGGACT 3′	5′ *Fam* AGGGCAGAGTATTTAC AAAGAAG 3′	55
RAG1 (TG)n (Heminested)	NT_009237	NA	4	Outside: 5′ AGAAGTTTGTTGGTTTCATTTA	5′ GTATCCAGCAGAGT GCCTAGT 3′	62–45
				Inside: 5′ *Fam* CCTTGCTTCTTTAGTTGCTTT	5′ GTATCCAGCAGAG TGCCTAGT 3′	55
D11S4102 (Heminested)	Z52543	0.77	10	Outside: 5′ ATCCTCACCTTATTCACCCTG 3′	5′ AATCCTGGAAAG CCCTGG 3′	62–45
				Inside: 5′ ATCCTCACCTTATT CACCCTG 3′	5′ *Hex* TAGGGATTTTAGGAGG GATGACT 3′	55
WAS Nt.361 A--G (Heminested) *HpyCH 4 V* cuts normal sequence	AF115548, AF115549	NA	NA	Outside: 5′ AGGAGATGGGAAAGTTGCGG 3′	5 CCAACTTCCTTTCCT CCCCTG 3′	62–45
				Inside: 5′ AGGAGATGGGAAAGTTGCGG 3′	5′ GATTCCTTTTTTGTATC TTCTCCTG 3′	55
WAS L 39 P (Heminested) *ScrF I* cuts mutant sequence	AF115548, AF115549	NA	NA	Outside: 5′ TCAGCAGAACATA CCCTCC 3′	5′ AGAGAGAGAAGGAG GAGAGG 3′	62–45
				Inside: 5′ TCAGCAGAACATAC CCTCC 3′	5′ GAAGAAACGGTGGG GAC 3′	55
DXS1003 (Heminested)	Z17201	0.8	11	Outside: 5′ AGAAGCCGTTATTGGTGGA CTC 3′	5′ ACACTGCTACTCCTTGG GAAATC 3′	62–45
				Inside: 5′ AGAAGCCGTTATTGGTGG ACTC 3′	5′ *Hex* CATTCCTCACTGGC AAGTTTTA 3′	55
GATA160B08 (Heminested)	G10694	0.71	5	Outside: 5′ CCAATTGCCTACTGGA TATTACCAA 3′	5′ TGGGAACAAAACAGGC AAAGTC 3′	62–45
				Inside: 5′ CCAATTGCCTACTGGATA TTACCAA 3′	5′ *Fam* TTTGCCCTCATGG AGTGCC 3′	55

(continued)

Table 4.4 (continued)

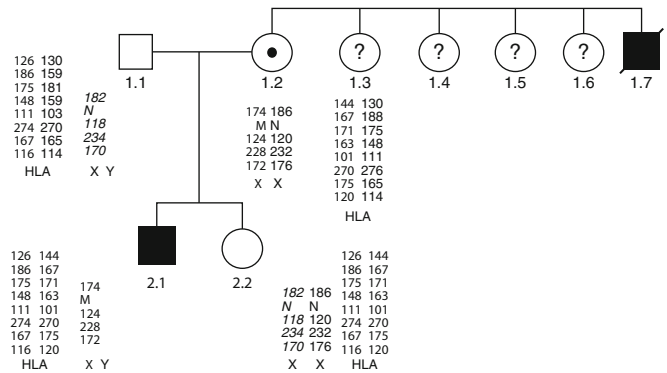
DXS1208 (Heminested)	Z23944	0.54	8	Outside: 5′ TCTAAAGCCCAGGACCCCG 3′	5′ TGGTTAAAGGATTT GGGAGGC 3′	62–45
				Inside: 5′ *Hex* TCAGGGCTCCAACTCCAGG 3′	5′ TGGTTAAAGGATTTG GGAGGC 3′	55
DXS1039 (Heminested)	Z23372	0.56	9	Outside: 5′ CCCTCTTCACTTTTCCAGTCAAT 3′	5′ GGAAAGGGAAGAA GAATGCC 3′	62–45
				Inside: 5′ *Fam* TGTTCCTGGTATGTGACAATGC 3′	5′ GGAAAGGGAAGA AGAATGCC 3′	55
DXS8023 (Heminested)	Z52342	0.57	9	Outside: 5′ GTGCAAACTGTTCCACCTGG 3′	5′ CTCAAAGAATGAAG TAGAATAAGGATA 3′	62–45
				Inside: 5′ GTGCAAACTGTTCCACCTGG 3′	5′ *Fam* TTGATAAAGTAGTCAGG AAAGGCT 3′	55
HED-ID (IKBKG gene) Q 348 X *Hpy188 III* cuts normal sequence	NT_025965	NA	NA	Outside: 5′ GGGAGTACAGCAAACTGAAGGC 3′	5′ CCCTAACCCAGAACA CCAGG 3′	62–45
				Inside: 5′ GGGAGTACAGCAAACTGAAGGC 3′	5′ CCATCCGTCTCCTG TGGTC 3′	55
HED-ID (IKBKG gene) D 113 N *Mbo I* cuts normal sequence	NT_025965	NA	NA	Outside: 5′ AGGAGTTCCTCATGTGCAAGTT 3′	5′ CCTTGTGGAACAC TGGCG 3′	62–45
				Inside: 5′ AGGAGTTCCTCATGTGCAAGTT 3′	5′ GTTTTCAGAACCT GGCCCTG 3′	55
TNFSF (CD40 gene) Exon4 C.397 ins T Exon4 C.437_38 ins A (Heminested)	D31796	NA	NA	Outside: 5′ TTTGGTTCCATTTCAGGTGATC 3′	5′ AACATGACTTCGGC ATCCCA 3′	62–45
				Inside: 5′ TTTGGTTCCATTTCAGGTGATC 3′	5′ *Fam* CGCTCAGATGCTGTGT GACTTAC 3′	55

appeared to be free of maternal mutation, but only one was a maternal HLA match (embryo #2 in Fig. 4.9). In addition, 3 of 5 oocytes with maternal mutation were HLA-matched (embryos #11, #13, and #15 in Fig. 4.9). However, embryos #13 and #15 were affected and a non-paternal match, while only a maternal mutant chromosome was detected in embryo #11. Only one embryo (embryos #2), predicted to be mutation-free and a maternal match by PB analysis, appeared to be a normal female with also a paternal match. The transfer of this single embryo resulted in a singleton pregnancy, confirmed to be unaffected and HLA-matched by amniocentesis, yielding the birth of a healthy HLA-matched baby girl.

The first transplantation treatment was done using cord blood stem cell obtained from this child, but no engraftment was achieved. So the second transplantation was performed 1 year later, using bone marrow mixed with the remaining portion of the cord blood sample, which resulted in a successful engraftment and reconstitution of the sibling's bone marrow, resulting in a total cure of the patient.

Wiscott–Aldrich syndrome (WAS) is a lethal X-linked immune deficiency, in which lymphocyte dysfunction and thrombocytopenia result in severe infections, bleeding episodes, and increased risk of lymphoproliferative malignancies. While supportive therapy may increase survival rate, the only hope for avoiding early mortality is bone

a



b

Embryo #	1	2	3	4	5	6	7	9	10	11	13	15
X-chromosome markers:												
DXS1187	186	*182* 186	*182* 186	*182* 174	*182* 174	186	*182* 186	186	*182* 186	174	174	174
CD40	N	*N* N	*N* N	*N* M	*N* M	N	*N* N	N	*N* N	M	M	M
(CA)n	120	*118* 120	*118* 120	*118* 124	*118* 124	120	*118* 120	120	*118* 120	124	124	124
DXS8094	232	*234* 232	*234* 232	*234* 228	*234* 228	232	*234* 232	232	*234* 232	228	228	228
DXS1062	176 Y	*170* 176	*170* 176	*170* 172	*170* 172	Y 176	*170* 176	Y 176	*170* 176	172	Y 172	Y 172
HLA markers:	130 130	126 144	126 130	130 130	126 130	126 130	130 144	126 130	126 130	126 144	130 144	130 144
D6S276	159 188	186 167	186 188	159 188	186 188	186 188	159 167	186 188	186 188	186 167	159 167	159 167
Moga	181 175	175 171	175 175	181 175	175 175	175 175	181 171	175 175	175 175	175 171	181 171	181 171
D6s265	159 148	148 163	148 148	159 148	148 148	148 148	159 163	148 148	148 148	148 163	159 163	159 163
D6S510	103 111	111 101	111 111	103 111	111 111	111 111	103 101	111 111	111 111	111 101	103 101	103 101
TNF a	270 276	274 270	274 276	270 276	274 276	274 276	270 270	274 276	274 276	274 270	270 270	270 270
D6S273	165 165	167 175	167 165	165 165	167 165	167 165	165 175	167 165	167 165	167 175	165 175	165 175
LHI D6S291	114 114	116 120	116 114	114 114	116 114	116 114	114 120	116 114	116 114	116 120	114 120	114 120
Predicted genotype	Normal male Non-match	Normal female Match ET	Normal female Non-match	Carrier Non-match	Carrier Non-match	Normal male Non-match	Normal female Non-match	Normal male Non-match	Normal female Non-match	*X 0 Match*	Affected Male Non-matched	Affected Male Non-matched

Fig. 4.9 Preimplantation HLA typing combined with PGD for X-linked hyperimmunoglobulin M syndrome (**a**) Family pedigree in three generations. Marker order is located next to maternal haplotypes. Paternal (2.1), maternal (2.2), and the affected sibling (3.1). CD 40 gene haplotype assignment is based on genomic DNA testing. Paternal and maternal matching HLA haplotypes are shown in *bold face*. (**b**) (*Upper panel*) PCR analysis of blastomeres removed from 12 embryos showed that all but three embryos (#11, #13, and #15) were predicted to be unaffected. (**b**) (*Lower panel*) HLA typing was performed simultaneously with mutation analysis of all blastomeres. Embryo #2 was predicted to be a normal female and to have the same HLA profile as the affected sibling (3.1). The transfer of this embryo resulted in pregnancy and the birth of a healthy unaffected HLA-matched baby girl (3.2). Cord blood stem cells were collected at birth for stem cell transplantation

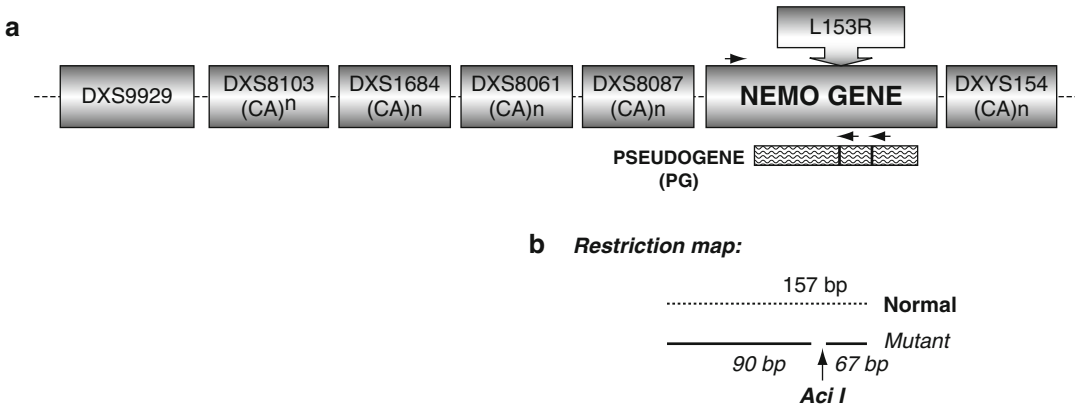


Fig. 4.10 PGD design for the mutation in NEMO gene in combination with HLA typing (see also Fig. 4.11). (**a**) Position of the L153R mutation in exon 5 of CD40 ligand gene (Xq26.3) and tightly linked dinucleotide polymorphic markers inside the gene (exon 4). (**b**) Restriction maps for *Aci*I restriction enzyme

marrow transplantation. WAS is caused by a mutation in the WAS gene mapped to the Xp11.22–11.23 region, which results in actin polymerization, with T lymphocytes of males exhibiting a severe disturbance of the actin cytoskeleton. The gene has 12 exons that encode a 502 amino acid cytosolic protein, expressed exclusively in hematopoietic cells.

PGD was performed for two couples at risk for producing a progeny with WAS. One of them had two affected sons carrying the missence Leu39Pro mutation in exon 1 of WAS gene, due to a single nucleotide (CTT to CCT) substitution at position 150, which leads to substitution of leucine by proline at position 39. The mutation testing was done using Scr FI restriction digestion, cutting the mutant and leaving the normal gene product intact. A total of four unaffected HLA-matched embryos were detected, resulting in one singleton pregnancy and the birth of an unaffected HLA-matched child, as a potential donor for bone marrow transplantation of her affected sibling (Table 4.1).

X-linked hypohidrotic ectodermal displasia with immune deficiency (*HED-ID*) is a congenital disorder of the teeth, hair, and eccrine sweat glands, inherited as an X-linked recessive condition, caused by approximately two dozens of different mutations in the IKK-gamma gene (IKBKG, or NEMO) located in Xq28. The gene consists of ten exons and codes for a scaffold protein that binds IKK-alpha and IKK-beta, being essential for forming a functional IKK complex. The disease is characterized by susceptibility to microbial and streptococcal infections, dys-gamma-globulinemia, poor polysaccharide-specific antibody responses, and depressed antigen-specific lymphocyte proliferation. Intravenous immunoglobulins and prophylactic antibiotics may be useful in improving clinical status, but bone marrow transplantation is required to prevent early mortality.

One of the PGD cycles for HED-ID with HLA typing is presented in Figs. 4.10 and 4.11. The mother was a carrier of a L153R mutation, resulting from T to G change (CTG->CGG) in exon 4 of the NEMO (IKBKG) gene, replacing leucine with arginine at position 153 of the resultant protein. Because of the presence of a closely linked pseudogene with a normal sequence at the position of the mutation, which is co-amplified with the transcribed gene, a special design was developed to avoid misdiagnosis (Fig. 4.10).

A total of 16 embryos were analyzed, of which 6 were derived from oocytes free of maternal mutation, based on PB1 and PB2 testing, but none of these was a maternal HLA match. As seen from Fig. 4.11, of 16 resulting embryos, for which blastomere biopsy results were available both for mutation analysis and HLA typing, 3 were affected males (embryos #17, #20, and #21; only the latter being HLA-matched), 4 female carriers, 2 of which were non-matched (embryos #3 and #4), one HLA-recombinant (embryo #13), and one HLA-matched (embryo #12). The remaining 7 embryos were unaffected, including 2 male non-matched embryos (embryos #16 and #24), the former containing extra maternal X-chromosome, and 5

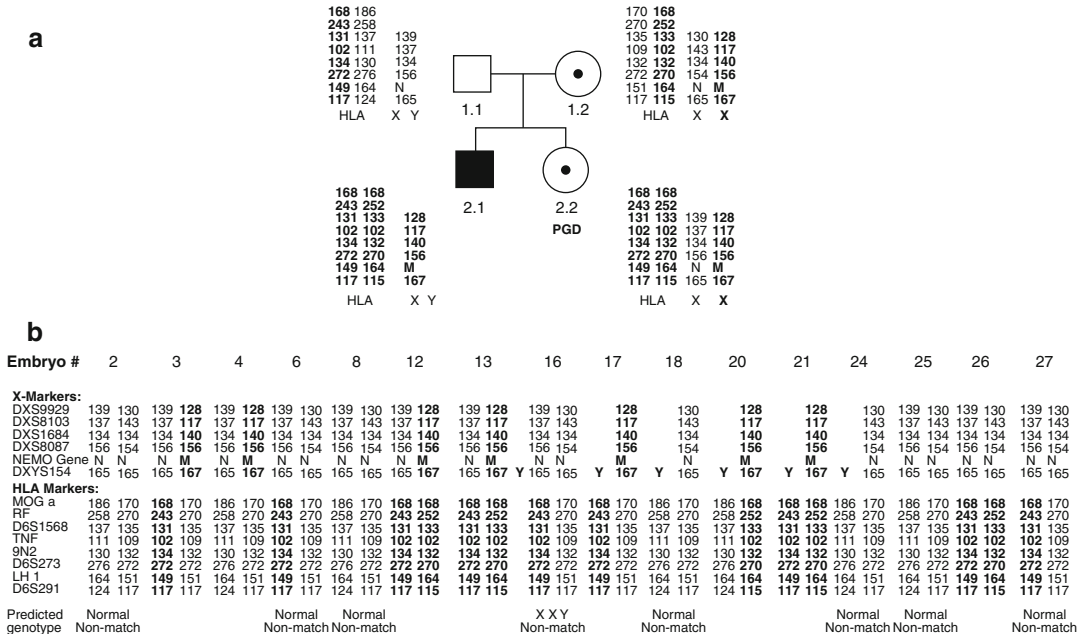


Fig. 4.11 PGD for the mutation in NEMO gene with preimplantation HLA typing. (**a**) Family pedigree showing maternal and paternal matching HLA haplotypes in *bold face*. Marker order for testing NEMO gene is located next to the maternal haplotypes. Paternal (1.1), maternal (1.2), and affected sibling (2.1) NEMO gene haplotype assignment is based on genomic DNA testing. (**b**) (*Upper panel*) Results of blastomere DNA analysis from 16 embryos showing that 3 embryos were affected (embryos #17, #20, and #21), one with an extra X chromosome, suggesting the XXY genotype, with the remaining being either carriers or unaffected. (**b**) (*Lower panel*) HLA typing was performed simultaneously with mutation analysis of all blastomeres, showing that 2 of the unaffected embryos (#12 and #26) were also HLA-matched to the affected sibling. The transfer of this embryo resulted in pregnancy and the birth of a healthy unaffected HLA-matched baby girl (2.2). Cord blood stem cells were collected at birth for stem cell transplantation

normal female embryos, of which only 1 (embryo #26) was HLA-matched. While the normal embryos, which were not HLA-matched to the affected sibling, were frozen for future use by the couple, embryo #26, together with embryo #12, which was a normal female carrier, was transferred, resulting in a singleton pregnancy and the birth of an unaffected child that was confirmed to be HLA-matched to the affected sibling. Cord blood from this child was collected and transplanted to the affected sibling, resulting in a complete cure.

The presented data show the usefulness of PGD for SCID, as there is no effective treatment for these conditions other than stem cell transplantation. PGD provides the couples at risk with the option to avoid the affected pregnancy and have a progeny free of SCID. If there is already an affected child in the family, PGD with HLA typing makes it also possible to have access to the HLA-identical stem cell transplantation through selection and transfer of those unaffected embryos which are also HLA-matched to the sibling. Because the finding of the HLA-identical stem cell donor is the key for achieving the success in stem cell transplantation [33], a complete cure was observed in both cases of stem cell transplantation in siblings with HIGM and HED-ID.

4.4 Preimplantation HLA Matching Without PGD

The first report of experience of preimplantation HLA matching without testing for a causative gene included 13 IVF cycles initiated for 9 couples wishing to have another child who may also be a potential cord blood donor for the affected siblings with leukemia or Diamond–Blackfan anemia (DBA), the conditions requiring bone

Table 4.5 Preimplantation HLA typing with and without PGD

Preimplantation testing	Patients	Cycles	No. of embryo transfers	No. of embryos transferred	Pregnancy/ birth
HLA testing only	46	98	65	99	24/19
HLA + mutation	85	237	139	219	38/32
Total	131	335	204	318	62/51

marrow transplantation or cord blood transplantation treatment [34]. Although the latter condition was sporadic and did not require mutation testing, with a sole indication being of HLA typing, mutation analysis may be also required for patients at risk of producing offspring with DBA, caused by mutations in the gene encoding ribosomal protein S19 on chromosome 19 (19q13.2), and other genes mapped to chromosome 8 (8p23.3-p22). However, the majority of DBA are sporadic with no mutation detected, such as in both cases performed in our experience [34].

There was no difference in performing preimplantation HLA testing without PGD, except in limiting the analysis of day 3 or day 5 embryo to only HLA typing with the sibling requiring stem cell transplantation, using a multiplex heminested PCR system (see Chap. 2). A haplotype analysis for the father, mother, and affected child was performed for each family prior to preimplantation HLA typing, using a set of polymorphic STR markers located throughout the HLA region, as shown in Fig. 4.1. This allowed detecting and avoiding misdiagnosis due to preferential amplification and ADO, potential recombination within the HLA region, and a possible aneuploidy or uniparental disomy of chromosome 6, which may also affect the diagnostic accuracy of HLA typing of the embryo.

At the present time, a total of 98 clinical cycles from 46 couples were performed, in which 99 HLA-matched embryos were preselected for transfer (Table 4.5). The proportion of embryos predicted to be HLA-matched to the affected siblings was 21.5%, not significantly different from the expected 25% (Table 4.6). The transfer of 99 HLA-matched embryos in 65 clinical cycles resulted in 24 singleton clinical pregnancies and 19 HLA-matched children born. These results suggest that testing of an available number of embryos per cycle allows preselecting a sufficient number of the HLA-matched embryos for transfer to achieve a clinical pregnancy and birth of an HLA-matched progeny.

Table 4.6 Chances for detection of disease-free and HLA-matched embryo in preimplantation HLA typing

HLA MATCH only – ¼ (25%)
Autosomal-recessive or X-linked free + HLA MATCH – ¾ × ¼ = 3/16 (18.75%)
Autosomal-dominant free + HLA MATCH – ½ × ¼ = 1/8 (12.5%)
Autosomal-recessive or X-linked free + HLA MATCH + ANEUPLOIDY-free – ¾ × ¼ × ½ = 3/32 (9.4%)
Autosomal-dominant free + HLA MATCH + ANEUPLOIDY-free – ½ × ¼ × ½ = 1/16 (6.25%)

The usefulness of detection of recombination within the HLA region is demonstrated in Table 4.7, describing the results of HLA typing of one of the cycles resulting in the birth of an HLA-matched child to a sibling with acute lymphoid leukemia (ALL). Of 10 embryos tested simultaneously for 11 alleles within the HLA region in this family, crossing over between D6S2426 and Ring alleles was observed in embryos #4, #7, and #9. Of the remaining 7 embryos, 3 were fully matched (embryos #2, #6, and #8), while the other 4 were HLA-incompatible to the affected sibling, as seen from the haplotypes of the mother, father, and affected child, presented in Table 4.7.

Recombinations were detected also in both cases of preimplantation HLA typing for DBA presented in Table 4.8 and Fig. 4.12. In one of them, maternal recombination is seen in the embryo #8 results (Table 4.8), while HLA testing of the other six embryos revealed three HLA-matches to the affected sibling and were transferred, resulting in a singleton pregnancy and the

Table 4.7 Preimplantation HLA typing resulting in the birth of an HLA-matched baby for the affected sibling with acute lymphoid leukemia

HLA genes and STRs	Embryo no.												
	1	2	3	4	5	6	7	8	9	10	Father	Mother	Affected baby
D6S276*	*130/118*	**144/139**	*130/118*	*130/118*	*130*/**139**	**144/139**	*130/118*	**144/139**	*130*/**139**	*130*/**139**	**144**/*130*	*118*/**139**	**144/139**
HLA A	*3/2*	**1/32**	*3/2*	*3/2*	*3*/**32**	**1/32**	*FA*	**1/32**	*3*/**32**	*3*/**32**	**1**/*3*	*2*/**32**	**1/32**
D6S510	*16 167*	**148/163**	*163/167*	*163/167*	*163*/**163**	**148/163**	**148**/*167*	**148/163**	*163*/**163**	*163*/**163**	**148**/*163*	*167*/**163**	**148/163**
HLA B	*AD 27*	**27/18**	*ADO/27*	*FA*	**FA**	**27/18**	*ADO/27*	**27/18**	*ADO*/**18**	*ADO*/**18**	**27**/*57*	*27*/**18**	**27/18**
MIB	*118/114*	**114/124**	*118/114*	*118/114*	*118*/**124**	**114/124**	*118/114*	**114/124**	*118*/**124**	*118*/**124**	**114**/*118*	*114*/**124**	**114/124**
TNF a	*94/102*	**110/102**	*94/102*	*94/102*	*94*/**102**	**110/102**	*94/102*	**110/102**	*94*/**102**	*94*/**102**	**110**/*94*	*102*/**102**	**110/102**
D6S273*	*275/273*	**273/271**	*275/273*	*275/273*	*275*/**271**	**273/271**	*275/273*	**273/271**	*275*/**271**	*275*/**271**	**273**/*275*	*273*/**271**	**273/271**
HLA-DRB1	*ADO/1*	**11/10**	*ADO/1*	*ADO/1*	*ADO*/**10**	**11/10**	*ADO/1*	**11/10**	*ADO*/**10**	*FA*	**11**/*7*	*1*/**10**	**11/10**
G51152	*181/208*	**181/191**	*181/208*	*181/208*	*181*/**191**	**181/191**	*181/208*	**181/191**	*181*/**191**	*181*/**191**	**181**/*181*	*208*/**191**	**181/191**
Ring 3CA	*162/162*	**160/155**	*162/162*	*162/162*	*162*/**155**	**160/155**	*162/162*	**160/155**	*162*/**155**	*162*/**155**	**160**/*162*	*162*/**155**	**160/155**
D6S426	*144/130*	**140/144**	*144/130*	**140**/*130*	*144*/**144**	**140/144**	*144/130*	**140/144**	*144/130*	*144*/**144**	**140**/*144*	*130*/**144**	**140/144**
Predicted genotype	*Non-match*	**Match**	*Non-match*	*Non-match recombinant*	*Non-match*	**Match**	*Non-match recombinant*	**Match**	*Non-match recombinant*	*Non-match*	**NA**	**NA**	**NA**

ADO allele dropout, *FA* failed amplification. *HLA* matched alleles are shown in **bold**

birth of an HLA-matched baby. In the other case, one embryo with a maternal (embryo #8) and another (embryo #16) with both maternal and paternal crossing over (both in Ring allele) were detected in testing of 16 embryos (only 8 embryos shown). There was another embryo with trisomy 6 (embryo #5) with an extra maternal chromosome 6, making this and the other two above also unacceptable for transfer. However, five embryos appeared to be HLA-matched, of which two were transferred back to the patient, resulting in the birth of an HLA-matched baby (Fig. 4.12). The cord blood collected from this baby was transplanted to the affected sibling, resulting in a complete cure.

The relevance of aneuploidy testing for chromosome 6 for accuracy of diagnosis is seen from the results of HLA typing in the other cycle, resulting in the birth of a baby who was HLA-matched to the sibling with ALL (Fig. 4.13). Two of ten embryos tested in one case (of which only eight embryos are shown in Fig. 4.13) appeared to have only maternally derived chromosomes 6, one with only one maternal chromosome (embryo #1), and the other with two maternal chromosomes, representing uniparental maternal disomy of chromosome 6 (embryo #2). In addition, crossing over between D6S291 and class II HLA alleles was evident, making one embryo unacceptable for transfer. Of the remaining embryos, only two were HLA-matched to the affected sibling, which were transferred resulting in the birth of an HLA-matched baby.

Presented data show feasibility of preimplantation HLA matching for families with affected children with bone marrow disorders who may wish to have another child as a potential HLA-matched donor of stem cells for transplantation treatment of the affected sibling. As seen from our data, HLA-matched embryos were preselected and transferred in all cycles, resulting in clinical pregnancies and the birth of HLA-matched children in almost every second transferred cycle.

The results also demonstrate the prospects for the application of this approach to other conditions, requiring an HLA-compatible donor for stem cell transplantation. This provides a realistic option for those couples who would like to have another child anyway, as they may potentially provide an HLA-matched progeny for an affected sibling. In addition to leukemias and sporadic forms of DBA, the method may be applied for any other condition, such as for couples having affected children with different cancers awaiting an HLA-compatible donor with no success for years. These new indications make preimplantation testing 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 to benefit the affected member of the family.

4.5 Limitations and Future Prospects of PGD for HLA Typing

Presented data demonstrate that PGD for HLA typing may become a practical option, available for a wider application in order to further improve the radical treatment for congenital and acquired bone marrow failures by stem cell transplantation. Despite the high rate of preferential amplification and ADO in PCR analysis of single blastomeres, a potential recombination within the HLA region described in our material, and a high rate of mosaicism for aneuploidies at the cleavage stage (see Sect. 5), the approaches described above appear to be highly accurate in preselecting HLA-matched embryos for transfer. The approaches involve a multiplex PCR analysis involving simultaneous testing for HLA alleles together with STR markers within HLA and flanking regions, allowing avoidance of misdiagnosis due to ADO, aneuploidy, or recombination of HLA alleles, which cannot be detected by other currently used DNA methods of HLA typing.

One of the major limitations of PGD for HLA typing is a relatively high frequency of recombination in the HLA region, with a few possible hot spots, which may affect not only the accuracy of preimplantation HLA typing, but also the outcome of the procedure. In our experience, of

Table 4.8 Preimplantation HLA typing resulting in the birth of an HLA-matched baby for the affected sibling with Diamond-Blackfan anemia

HLA genes and STRs	Embryo no.									
	2	3	7	8	9	10	11	Father	Mother	Affected child
D6S461*	**225/227**	**225/227**	*229*/**227**	*229*/**227**	**225/227**	*229/229*	*229/229*	**225**/*229*	*229*/**227**	**225/227**
D6S276*	**143/143**	**143/143**	*141*/**143**	*141*/**ADO**	**143/143**	*141/117*	*141/117*	**143**/*141*	*117*/**143**	**143/143**
D6S258	**144/132**	**144/132**	*135*/**132**	*135*/**132**	**144/132**	*135/135*	*135/135*	**144**/*135*	135/**132**	**144/132**
D6S248*	**253/278**	**253/278**	*276*/**278**	*276*/**278**	**253/278**	*276/278*	*276/278*	**253**/*276*	*278*/**278**	**253/278**
MOG a	**159/169**	**159/169**	*167*/**169**	*167*/**169**	**159/169**	*167/169*	*167/169*	**159**/*167*	*169*/**169**	**159/169**
RF	**275/269**	**275/269**	*257*/**269**	*257*/**269**	**275/269**	*257/263*	*257/263*	**275**/*257*	*263*/**269**	**275/269**
9N-2	**129/133**	**129/133**	*131*/**133**	*131/127*/**133**	**129/133**	*131/127*	*131/127*	**129**/131	*127*/**133**	**129/133**
D6S273	**276/270**	**276/270**	*274*/**270**	*274*/**270**	**276/270**	*274/270*	*274/270*	**276**/*274*	*270*/**270**	**276/270**
LH 1	**163/168**	**163/168**	*163*/**168**	*163*/**168**	**163/168**	*163/179*	*163/179*	**163**/*163*	*179*/**168**	**163/168**
D6S2447	**147/159**	**147/159**	*152*/**159**	*152*/**159**	**147/159**	*151/151*	*151/151*	**147**/*151*	*151*/**159**	**147/159**
TAP 1	**205/220**	**205/220**	*205*/**220**	*205*/**220**	**205/220**	*205/207*	*205/207*	**205**/*205*	*207*/**220**	**205/220**
Ring 3CA	**159/155**	**159/155**	*155*/**155**	*155*/**155**	**159/155**	*155/159*	*155/159*	**159**/*155*	*159*/**155**	**159/155**
D6S439	**125/125**	**125/125**	*123*/**125**	*123*/**125**	**125/125**	*123/125*	*123/125*	**125**/*123*	*125*/**125**	**125/125**
D6S291	**114/123**	**114/123**	*116*/**123**	*116/114*	**114/123**	*116/114*	*116/114*	**114**/*116*	*114*/**123**	**114/123**
D6S426	**129/129**	**129/129**	*144*/**129**	*FA*	**129/129**	*144/142*	*144/142*	**129**/*144*	*142*/**129**	**129/129**
Predicted genotype	**Match**	**Match**	*Non-match*	*Non-match recombinant*[c]	**Match**	*Non-match*	*Non-match*	**NA**	**NA**	**NA**

HLA matched alleles are shown in bold

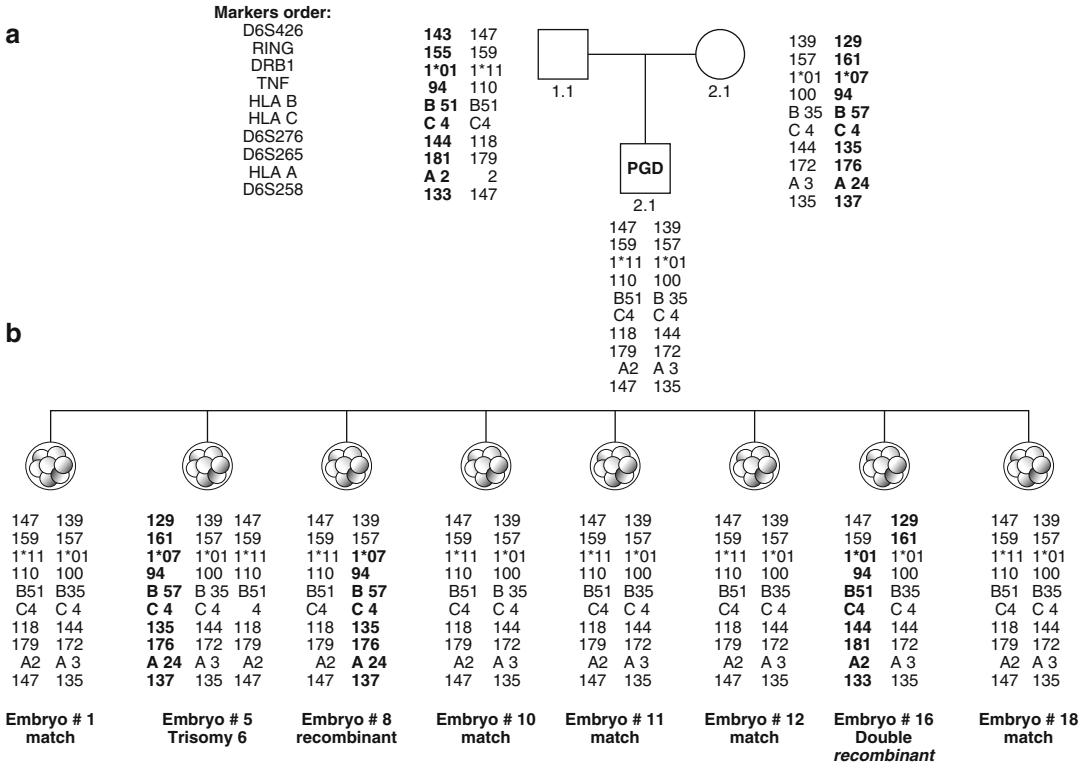


Fig. 4.12 Preimplantation HLA typing for Diamond–Blackfan anemia, resulting in the birth of an HLA-matched child. (**a**) Family pedigree with marker order and haplotypes of the mother, father, and affected child. HLA matching haplotypes are shown in non-bold face. (**b**) Results of HLA typing of biopsied blastomeres from eight embryos (other eight embryos which were also tested are not shown). Embryos #1, #10, #11, #12, and #18 are HLA-matched to the affected sibling (see **a** *panel*). Embryo #8 is a maternal non-match due to maternal recombination in the Ring allele, as well as embryo #16, which is both a paternal and maternal non-match, due to double recombination in the paternal and maternal Ring alleles. Embryo #5 is also a non-match due to an extra maternal chromosome, suggesting trisomy 6

1,713 embryos tested for HLA, 1,634 (95.5%) were non-recombinant, 52 (3%) with maternal, and 27 (1.5%) with paternal recombination. The prevalence was even higher based on family evaluation, performed prior to PGD in a series of 114 families: in 104 of these families tested, recombination in siblings requiring HLA-compatible bone marrow transplantation was identified in 7 (6.1%), suggesting that preimplantation HLA typing may never be able to identify the HLA match for these siblings. Therefore, haplotype analysis prior to initiation of the actual cycle is strongly required, so that the couples may be informed about their possible options. For example, in one of our cases performed for thalassemia, the fact that the child was recombinant became obvious only during PB1 analysis, without which maternal haplotypes cannot be established. While paternal haplotypes may be identified through sperm typing, the testing for maternal haplotypes requires maternal somatic cell haploidization, which may be performed by somatic cell nuclei transfer and fusion with matured oocytes, as described in Chap. 2 [35]. As shown in Fig. 4.14, the preparatory testing identified the sibling with maternal recombination, so it could have been unrealistic to identify the exact match, and therefore the couples should be informed that only relatively close matches may be possible, which may be discussed with the pediatric hematologist in the preselection process of the embryos for transfer.

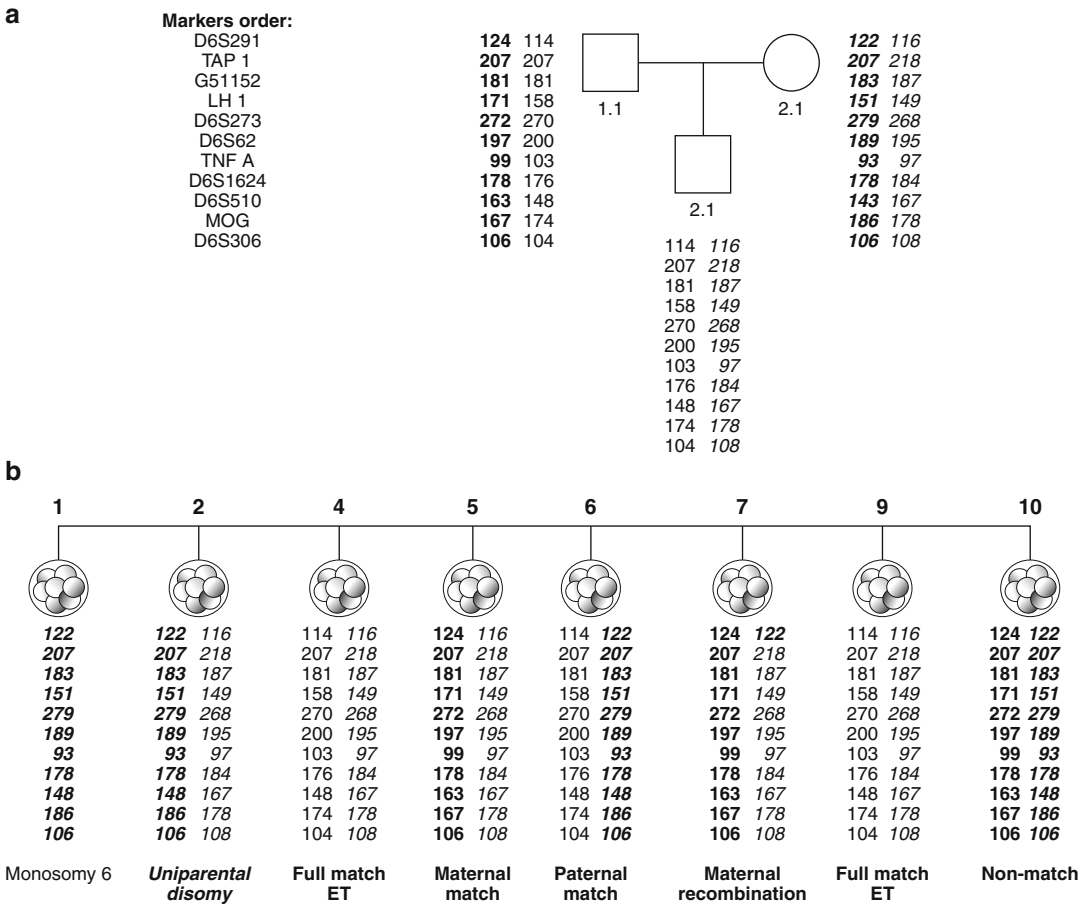


Fig. 4.13 Preimplantation HLA typing for acute lymphoid leukemia (*ALL*), resulting in the birth of an HLA baby. (**a**) Family pedigree with marker order and haplotypes of the mother, father, and affected child. Matching maternal and paternal haplotypes are shown in *non-bold face*. (**b**) Results of HLA typing of biopsied blastomeres from eight embryos. Embryos #4 and #9 are HLA-matched to the affected sibling (see **a** *panel*). Embryo #1 is a maternal non-match with no paternal chromosome present (monosomy 6). Embryo #2 is also a non-match due to only maternal chromosomes present (uniparental disomy). Embryo #7 is both a paternal and maternal non-match, the latter being due to maternal recombination in the Ring allele. The other two embryos are a non-match, embryo #6 being a paternal non-match and embryo #10 both a maternal and paternal non-match

Despite the need for further improvement of the technique as mentioned, the presented results show that the couples with affected children requiring HLA-compatible stem cell transplantation have a realistic option to undergo IVF and PGD with a combined preimplantation HLA typing, so as to have an unaffected HLA-matched child as a potential donor of compatible stem cells for the sibling.

The other important limitation is that most patients requesting preimplantation HLA typing are of advanced reproductive age, so the outcome of the procedure has not yet been sufficiently high, and many patients still undergo two or more attempts before they become pregnant and deliver an HLA-identical offspring. So testing for age-related aneuploidy may appear useful for improving the reproductive outcome of preimplantation HLA typing, which will also minimize the risk of delivering a child with chromosomal disorders, providing reassurance for patients who are usually concerned about their pregnancy outcomes.

Aneuploidy testing is currently offered as an integral component of preimplantation HLA typing to the patients of advanced reproductive age,

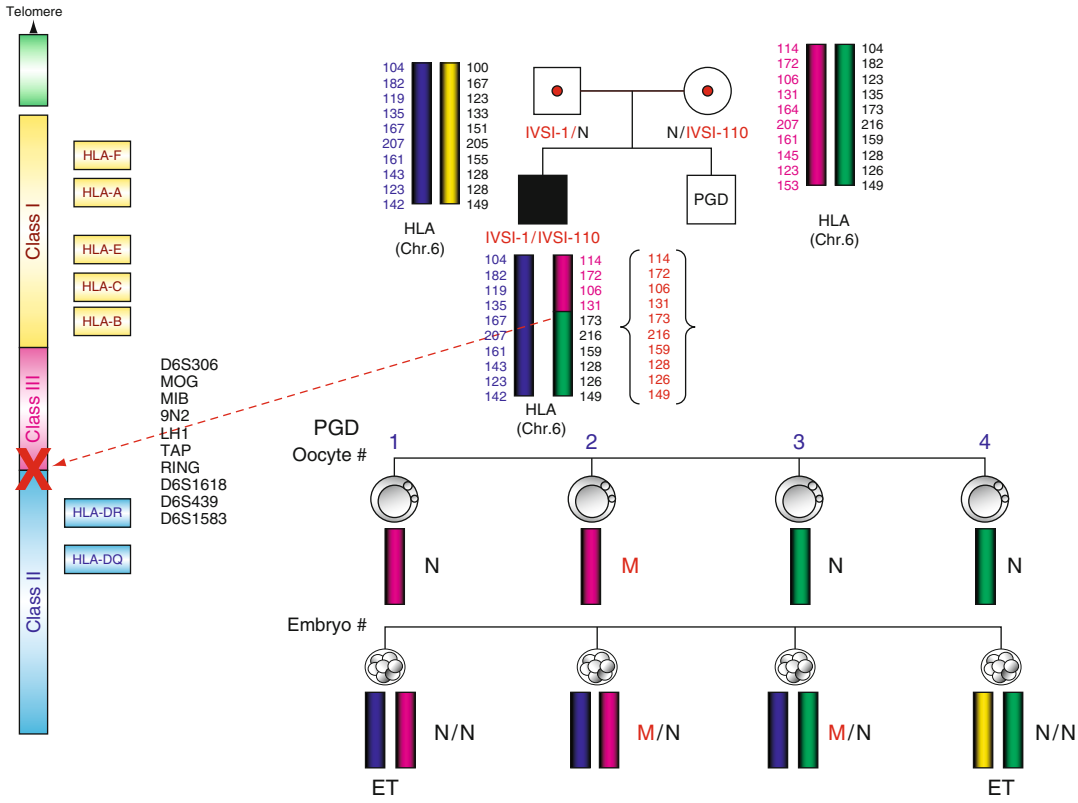


Fig. 4.14 Maternal recombination detected in thalassemia major sibling in preimplantation HLA typing combined with PGD. (*Top panel*) Family pedigree with HLA haplotype analysis based on parental and affected child's genomic DNA testing. HLA marker order is presented on the *upper left* for the father, who was a heterozygous carrier of thalassemia gene IVS 1–1, and on the *upper right* for the mother, a heterozygous carrier of thalassemia mutation IVS1–110. Paternal and maternal HLA haplotypes are shown in different colors: paternal in *blue/yellow*, and maternal in red/green. As seen from the HLA haplotypes of the affected child in need for transplantation, for whom HLA matching is performed, the maternal HLA contribution is recombinant (*red and green* instead of the expected *red or green*) between HLA-DR and HLA-B genes, shown schematically on chromosome 6 (on the *far left*). (*Middle panel*) Sequential multiplex polar body analysis for maternal mutation, linked polymorphic markers, and HLA haplotypes, showing that the oocyte #2 is affected (IVS1–110), while oocytes #1, #3, and #4 are normal, with no recombination in the HLA cluster. (*Bottom panel*) Blastomere results revealed two heterozygous carrier embryos (embryos #2 carries maternal mutation IVS1–110 and embryo #3 carries paternal mutation IVS1–1) and two homozygous normal ones (#1 and #4). HLA typing (presented by respective colors) shows that neither of these embryos is fully HLA-matched to the sick sibling. Two embryos, #1 and #4, predicted to be homozygous normal, and partially HLA-matched, were transferred back to the mother, yielding a singleton pregnancy and the birth of a thalassemia-free baby, who may still be considered for possible bone marrow transplantation, as there is no probability of producing a completely HLA-matched offspring for the affected sibling with recombinant HLA haplotypes

performed in an increasing number of preimplantation HLA typing cycles combined with or without PGD. Although the chances of preselecting unaffected HLA-matched embryos that could be also euploid are very low (see Table 4.6), our preliminary results of the reproductive outcome comparison between the groups of combined PGD/HLA with and without aneuploidy testing showed a significant difference (Table 4.9). Despite transferring a lower number of embryos, the pregnancy rate was higher in the aneuploidy testing group, suggesting the potential utility of aneuploidy testing in preimplantation HLA typing, allowing the avoidance of transfer of those HLA-identical embryos that are chromosomally abnormal which are destined to be lost anyway

Table 4.9 Outcome of preimplantation HLA typing with and without aneuploidy testing

	HLA	HLA plus Aneuploidy testing	Total
Patient/cycle	11/25	14/27	25/52
Total embryos	224	204	428
Matched embryos	48	21 (36)	69 (84)
Non-matchedembryos	176	168	344
Transfers	21 (84%)	13 (48%)	34
No. of embryos transferred	33 (1.6)	19 (1.4)	52
Pregnancy	6 (28.5%)	7 (53.8%)	13 (38%)
Birth	6	6	12

either before or after implantation. Alternatively, incidental transfer of aneuploid embryos in the absence of chromosomal testing should lead to implantation and pregnancy failures in preimplantation HLA typing cycles, or may compromise the pregnancy outcome through spontaneous abortions.

Although more data are still needed to further prove the impact of aneuploidy testing on the outcomes of preimplantation HLA typing, the presented data suggest that approximately half of the aneuploidy-free embryo transfers, following preimplantation HLA matching, resulted in pregnancy and the birth of HLA-matched children, compared to the 28.5% pregnancy rate following the transfer of HLA-matched embryos not tested for aneuploidy. In a comparable number of cycles performed with or without aneuploidy testing, despite unavailability of aneuploidy-free embryos for transfer in over half of the cycles, compared to only 26% of cycles without aneuploidy testing, comparable numbers of pregnancies and births of HLA-matched children were observed, indicating a possible clinical relevance of avoiding chromosomally abnormal embryos from transfer in preimplantation HLA typing.

As mentioned, the addition of aneuploidy testing expected to identify at least 50% of chromosomally abnormal embryos in patients of advanced reproductive age, will be also lowering the probability of detecting the embryos for transfer by half. In fact, the mean number of embryos for transfer was approximately 1.0 on the average, which also reflects the lower probability of identification of HLA-matched unaffected embryos free of aneuploidy, taking into consideration the average number of available embryos with results, which is usually much lower in women of advanced reproductive age (under ten embryos on the average in our experience). With one in two embryos expected to be aneuploid, one in four HLA-matched, and three in four unaffected in autosomal-recessive conditions, the overall probability of finding a suitable embryo for transfer could not be expected to be higher than one in ten embryos (see also Table 4.6). So with the availability of only under ten embryos on the average with conclusive results in our material, only one HLA-matched unaffected euploid embryo may have been expected to be available for transfer, assuming also that not all embryos develop to the status acceptable for transfer, which is of course below the optimal number of embryos to be replaced to ensure a clinical pregnancy and birth outcome. However, with present tendency of limiting the transfer to only one blastocyst, to avoid multiple pregnancies, the availability of a single euploid embryo for transfer is quite sufficient to obtain a clinical pregnancy and birth of an HLA-identical progeny for stem cell transplantation for the affected siblings.

The usefulness of aneuploidy testing is also obvious for the diagnostic accuracy improvement, as the error in the copy number of chromosomes may lead to misdiagnosis in testing for the causative gene and HLA typing. For example, our data further confirm an approximately 6% aneuploidy rate for chromosome 6 (see Chap. 5), which could affect the HLA typing results. Comparable aneuploidy rates for other chromosomes on which causative genes tested are located, such as beta-globin gene on chromosome 11, may also affect PGD results [25]. Thus, in addition to avoiding chromosomally abnormal embryos from transfer, testing for the copy number of chromosomes may become an important

requirement for achieving the accuracy of PGD and preimplantation HLA typing. In fact, the follow-up analysis of the mutant oocytes and embryos and the pregnancy outcomes in our experience did not find any misdiagnosis, suggesting an extremely high specificity and sensitivity in the presently used molecular genetic analysis.

Therefore, patients should be fully aware of the limits of the expected successful outcome of the above testing, which was shown to result in preselection and transfer of the HLA-matched unaffected embryos in only 13.7% of the embryos tested, which is even a bit lower than may have been predicted. Despite such a relatively moderate success rate, the number of PGD requests in combination with HLA typing has been increasing overall, with the recent emergence of a considerable proportion of cases involving preimplantation HLA typing without PGD.

4.6 Practical Implications of PGD for HLA Typing

Preimplantation HLA typing opens an important possibility of PGD application for stem cell therapy. Because of limited availability of the HLA-matched donors even among family members, this approach appeared to be attractive for couples with children requiring HLA-matched bone marrow transplantation.

It is well known that to achieve an acceptable engraftment and survival in stem cell therapy requires the finding of an HLA-identical stem cell transplant. However, there remain a large number of patients for whom no HLA-matched family member exists, so the search is extended to haplotype-matched unrelated donors. This has allowed successful application of stem cell transplantation to some individuals without a matched related donor, despite resulting in severe complications in more than half of the patients [36].

The experience of bone marrow transplantation for hemoglobinopathies presently comprises thousands of patients, showing 68% probability of cure in the world's largest center [18, 33]. The success rate is reported as 87%, 85%, and 80% for Class 1 (patients with regular iron chelation therapy who have neither hepatomegaly nor liver fibrosis), Class 2 (with regular/irregular chelation, borderline hepatomegaly, and fibrosis), and Class 3 (with irregular chelation, hepatomegaly and fibrosis) patients under age 17, respectively, suggesting that this may have wider implication for congenital bone marrow failures, depending primarily on the availability of HLA-matched donors.

Due to the small number of children per family, only one-third of patients are able to find an HLA-identical sibling, which may further be improved by 3% using an extended family search for a matched related donor with one or two identical ancestral haplotypes [37]. In the remaining patients, the only resort is the identification of a matched unrelated donor, which might be maximized by establishing national registries. These registries allow overcoming to some extent the genetic heterogeneity in the populations, which may affect the frequency of unique haplotypes, thus improving the donor selection process.

Stem cell transplantation obtained from umbilical cord blood provides the potential for further expanding the donor pool to patients without a suitable family match [38], which have presently been performed for thousands of children and adults, allowing for a greater degree of HLA disparity in choosing donor cord blood units, thus increasing the likelihood that a suitable unit can be identified for any particular patient. However, the advantages of this alternative source of stem cells can be more fully realized in the setting of the availability of matched related donor cord blood units, such as from siblings, providing the advantages of earlier transplantation, lower risk of complications, and lower treatment-related mortality.

HLA-identical sibling donors of cord blood stem cells are the only solution for patients affected with FA, allowing successful treatment in up to 85% of cases. So, over two decades, these families were offered the option of prenatal diagnosis combined with HLA typing on cells derived from chorionic villus sampling or amniocentesis

[39]. Of more than 80 pregnancies conceived during this time, one resulted in the first successful use of cord blood transplant in 1988 [3], opening the era of an alternative to bone marrow transplantation. However, because the probability of having an unaffected child who may be also an HLA match for an affected sibling is only one in five, these families often went through multiple cycles of pregnancy before conceiving an unaffected HLA match. So PGD provides a much more attractive approach, because a sufficient number of embryos may be tested at a time, increasing the chances to identify an appropriate match. In addition, PGD allows identification of the match before pregnancy, obviating the risk for termination of pregnancy for the HLA type alone, which cannot be acceptable on ethical grounds.

Presented data show that couples undergoing preimplantation HLA typing may be expected to require a repeated cycle to be able to preselect and transfer HLA-matched embryos. Even with the probability of selecting only one HLA-matched embryo from five tested, an acceptable pregnancy rate was observed, despite transferring only one or two embryos on the average, suggesting the usefulness of preimplantation HLA matching as part of PGD. The data provide a realistic option for the couples desiring to establish a pregnancy potentially providing an HLA match progeny for the treatment of the affected family member(s). However, preimplantation HLA typing raises important ethical, legal, and social issues, which are discussed in detail in Chap. 8.

Despite ethical issues involved in preimplantation HLA typing, there is an increase in the attractiveness of this option for couples with affected children requiring HLA-compatible stem cell transplantation, providing a practical option for those couples who would like to have another child anyway. This and other new indications above make preimplantation testing a genuine alternative to conventional prenatal diagnosis, providing patients with important prospect not only to avoid an inherited risk without facing termination of pregnancy, but also to establish a pregnancy with particular genetic parameters, which may also benefit the affected member of the family.

The present experience of PGD for HLA typing includes over 1,000 cases, resulting in the birth of more than 200 HLA-matched children, whose HLA-identical stem cells have already been used for a successful transplantation therapy in up to 100 affected siblings [27, 29, 30, 40]. The world's largest two experiences include preimplantation HLA typing in over 700 cases, with the accuracy rate per transfer of 99.4% [27, 29, 30]. The majority of cases were performed in combination with PGD for various genetic disorders, including thalassemia, sickle cell disease, FA, WAS, X-ALD, HIGM1, HED-ID, Krabbe disease, inherited form of DBA, and X-Linked chronic granulomatous disease (CGD), involving the preselection of unaffected children who were also HLA-identical to the affected sibling. With the introduction of aneuploidy testing, this may also expand the practical application of preimplantation HLA typing to patients of advanced reproductive age, allowing an improvement of their chances to become pregnant and deliver an HLA-matched progeny for stem cell transplantation in the affected siblings. This also makes it possible to apply this approach to HLA-compatible stem cell transplantation for older affected siblings, which has already been performed in our experience for the 14-year-old sibling with thalassemia, resulting in 10% of donor cell engraftment with neither acute nor chronic GVHD (unpublished data).

In conclusion, despite ethical issues involved in preimplantation HLA typing [41–43], the presented results show the increasing attractiveness of this option for couples with affected children requiring HLA-compatible stem cell transplantation. It is also important that no embryo is discarded based on the results of preimplantation HLA typing, as all unaffected embryos are frozen for future use by the couple. So the couples at risk of having children with congenital bone marrow disorders have to be informed about presently available options not only of avoiding the birth of an affected child, but also of selecting a suitable stem cell donor for their affected siblings, which may presently be the only hope for treating siblings with congenital bone marrow failures.

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