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Development and clinical application of a strategy for preimplantation genetic diagnosis of single gene disorders combined with HLA matching

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Preimplantation HLA matching has recently emerged as a tool for couples desiring to conceive a potential donor progeny for transplantation in a sibling with a life-threatening disorder. In this paper we describe a strategy optimized for preimplantation genetic diagnosis (PGD) of haemoglobinopathies combined with HLA matching. This procedure involves a minisequencing-based genotyping of HLA regions A, B, C and DRB combined with mutation analysis of the gene regions involved by mutation. Analysis of at least eight polymorphic short tandem repeat (STR) markers scattered through the HLA complex has also been included to detect potential contamination and crossing-over occurrences between HLA genes. The above assay can also be used for preimplantation HLA matching as a primary indication. The strategy was clinically applied for HLA matching in 17 cycles (14 for β-thalassaemia, one for Wiscott–Aldrich syndrome and two for leukaemia). A reliable HLA genotype was achieved in 255/266 (95.9%) of the blastomeres. In total, 22 (14.8%) embryos were obtained that were HLA-matched with the affected siblings, 14 (9.4%) of which were unaffected and transferred back to the patients. Four clinical pregnancies were obtained, three of which (one twin, two singletons) are ongoing and were confirmed as healthy and HLA-identical with the affected children. Minisequencing-based HLA typing combined with HLA STR haplotyping has been shown to be a reliable strategy for preimplantation HLA matching. The major advantage of this approach is that the validation of a single assay can be done once and then used for the majority of the patients, reducing notably time needed for preclinical set-up of each case.

Key words: haematopoietic stem cell transplantation/microsatellites/minisequencing/preimplantation HLA matching/preimplantation genetic diagnosis

Introduction

Preimplantation genetic diagnosis (PGD) of single gene disorders, combined with HLA matching, represents one of the most recent applications in reproductive medicine. This strategy has emerged as a tool for couples at risk of transmitting a genetic disease to select unaffected embryos of an HLA tissue type compatible with that of an existing affected child. At delivery, haematopoietic stem cells (HSC) from the newborn umbilical cord blood can be used to treat the affected sibling. This approach is particularly valuable for β-thalassaemia, Fanconi anaemia, and other similar life-threatening disorders that require an HLA-compatible HSC donor, where molecular HLA identity seems to provide the best chance of avoiding graft rejection and other serious complications of bone marrow transplantation (Petersdorf *et al*., 1998a; Sasazuki *et al*., 1998).

Termination of pregnancy would probably be requested if a resulting fetus were found not to be an HLA match with an affected sibling. However, HLA incompatibility would not be a clinically acceptable reason for termination when traditional prenatal genetic diagnosis was made. In these cases, PGD combined with preselection of embryos presenting a perfect HLA match for the affected siblings who need a transplant (instead of a 'blind' selection of embryos for transfer) seems to be acceptable on ethical grounds (Pennings *et al*., 2002; Robertson, 2003).

The first application of PGD for single gene disorders combined with the preselection of HLA-matched embryos for transfer was recently reported (Verlinsky et al., 2000, 2001). The great difficulty in finding an HLA-matched donor led to the application of preimplantation HLA matching for diseases such as leukaemia or lymphoma, in which PGD for HLA matching becomes the primary indication (Kuliev and Verlinsky, 2002).

The human MHC, or HLA, represents one of the most polymorphic regions of the human genome. This complex consists of three regions that contain genes encoding class I, class II and class III antigens. Comparative DNA sequence analysis of MHC has indicated the presence of a high number of alleles in this region (MHC Sequencing Consortium, 1999), determining a vast array of distinct haplotypes.

For genes presenting a heterogeneous spectrum of alleles identified, such as the HLA complex, the development of an allele-specific single cell DNA analysis strategy is labour-intensive, since standardization of PCR protocols is necessary for any specific allele combination. In fact, the design and the development of a diagnostic single cell strategy specific for each PGD family, presenting different HLA allele

Table I. Description of primers used for PCR amplification and minisequencing genotyping of the HLA regions investigated

HLA region	Exon	External primer sequence	Size (bp)	Internal primer sequence	Size (bp)	Minisequencing primers	
						Nucleotide position[a]	Sequence
A	2	F-5′-AGAAGCAASGGGCCCKCCKG-3′ R-5′-CTRTGASTGGGCCTTCAYWTTCC-3′	307	F-5′-CRGGTCTCAGCCACTSCTCG-3′ R-5′-GTCCCAATACTCMGGMCYCT-3′	208	199	5′-(T_2)TGGAGTGRGAGCCTGGGG-3′[b]
						208	5′-(T_5)CTGYTCGCCCCCAGGCTC-3′[c]
						228	5′-(T_3)GCTCYCACTCCATGAGGTATTTCT-3′[c]
						232	5′-(T_6)CCACTCCATGAGGTATTTCTHCACA-3′[c]
						274	5′-(T_{16})GTGTCGTCCACGTAGCCCAC-3′[b]
						301	5′-(T_{20})TACGTGGACGACACGCAGTT-3′[c]
						310	5′-(T_{25})ACACGCAGTTCGTGCGGTT-3′[c]
	3	F-5′-GACYCCGAGACCCTTGYCCC-3′ R-5′-GGAGAYCTAYAGGCGATCAGGG-3′	459	F-5′-CCGGTTTCATTTTCAGTTTAGG-3′ R-5′-GTCTCCTTCCCGTTCTCCAG-3′	356	656*	5′-TTTAGGCCAAAAATCCCCCC-3′[c]
						707*	5′-(T_{24})TCGGGGGACTGGGCTGACC-3′[c]
						726*	5′-(T_9)GTCGCAGCCATACATTATCTGGA-3′[b]
						733*	5′-(T_1)CCAGGTTCTCACACCVTCCAGA-3′[c]
						734*	5′-(T_{27})CCCACGTCGCAGCCATACAT-3′[b]
						756*	5′-ATGTATGGCTGCGACGTGGGG-3′[c]
						762*	5′-(T_{16})TACCCGCGGAGGAAGCGCC-3′[b]
						827†	5′-(T_5)CGGTCCAAGAGCGCAGGTC-3′[b]
						859†	5′-(T_6)TTGYGCTKGGTGRTCTGAGC-3′[b]
						863†	5′-(T_{15})CCACTTGYGCTKGGTGRTCT-3′[b]
						897†	5′-(T_7)TAGRCTCTCYRCTGCTCCGCC-3′[b]
						910†	5′-(T_{11})TGCCCTCCAGGTAGRCTCTC-3′[b]
						930†	5′-(T_{19})GGAGAGYCTACCTGGAGGGC-3′[c]
						942†	5′-(T_{25})TCCAGGTATCTGCGGAGCC-3′[b]
	4	F-5′-CTGGWGGAGTGTCCCATGAC-3′ R-5′-CTAARAGACATGACACCCCCRTCT-3′	369	F-5′-GCCTGAATKWTCTGACTCTTCC-3′ R-5′-TCTCCCTCCTTACCCCATCT-3′	317	1577	5′-GGTGGTGRGTCATATGYGTYTT-3′[b]
						1616	5′-(T_6)CACSCYRTCTCTGACCATGA-3′[c]
						1622	5′-CAGGGCCCAGCACYTCAG-3′[b]
						1641	5′-(T_9)TGATCTCCGCAGGGTAGAAGC-3′[b]
						1712	5′-(T_{15})AGACCCAGGACACGGAGCT-3′[c]
						1757	5′-(T_{18})GGRACCTTCCAGAAGTGGGC-3′[c]
						1758	5′-(T_{23})CCAGAAGGYACCACCACAG-3′[b]
B	2	F-5′-AGGAGMGAGGGGACCGCAG-3′ R-5′-CCTCRGGGRGGCGGATCTCG-3′	461	F-5′-CTCCCACTCCATGAGGTATT-3′ R-5′-CCTCGCTCTGGTTGTAGTAG-3′	266	225	5′-GCCGGGACAYGGMGGTGT-3′[b]
						270	5′-(T_9)TCGTCCACGTAGCCCACTGM-3′[b]
						293	5′-(T_{14})TGGGCTACGTGGACGACAC-3′[c]
						295	5′-(T_{17})CGCTGTCGAACCTCACGAAC-3′[b]
						321	5′-(T_{22})AGGTTCGACAGCGACGCC-3′[c]
						334	5′-(T_{25})ACGCCRCGAGTCCGAGRR-3′[c]
						400	5′-GGGACCGGRASACACAGATCT-3′[c]
						430	5′-(T_2)CAACACACAGACTKACCGAGAGA-3′[c]
						439	5′-(T_{28})ACCGAGAGRRCCTGCGGA-3′[c]
	3	F-5′-AGATCCGCCYCCCYGAGGCC-3′ R-5′-GCCATCCCCGGCGACCTATA-3′	493	F-5′-CAGTTGAGGCCAAAATCCCC-3′ R-5′-STCCTTCCCGTTCTCCAGGT-3′	341	727	5′-(T_2)GGGCCAGGGTCTCACAYY-3′[c]
						734	5′-(T_5)CASGTCGCAGCCRTACATS-3′[b]
						784	5′-(T_9)CCGTCGTAGGCGDMCTGGT-3′[b]
						791	5′-(T_{14})TCCGCGGGYATVACCAGK-3′[c]
						911	5′-(T_{17})GCCCTCCAGGTAGGYTCTC-3′[b]
						931	5′-(T_{22})AGARCCTACCTGGAGGGC-3′[c]
						932	5′-(T_{25})CGGAGCSACTCCACGCAC-3′[b]

Table I *Continued*

HLA region	Exon	External primer sequence	Size (bp)	Internal primer sequence	Size (bp)	Minisequencing primers	
						Nucleotide position[a]	Sequence
C	2	F-5′-AGGARCGAGGKGCCCGCCC-3′ R-5′-CCTGGCGCTTGTACTTCTGTGTCTC-3′	302	F-5′-CCCAGGCTCCCACTCCATGA-3′ R-5′-ATACTCCGGCCCCTCCTGCT-3′	181	228	5′-(T_3)AGGCCGGGACACGGMKGTG-3′[b]
						235	5′-CCACTCCATGARGTATTTCKACACMKC-3′[c]
						256	5′-(T_{11})ACTGMGATGAAGYGGGGCTC-3′[b]
						272	5′-(T_{15})GTCGTCCACGTAGCCCACTG-3′[b]
						331	5′-(T_{20})ACAGCGACGCCGCGAGTCC-3′[c]
						343	5′-GAGTCCRAGAGGGGAGCC-3′[c]
	3	F-5′-CGGAGAGAGCCCYAGTCRCC-3′ R-5′-ATSCCGGGAGATCTAYRGGA-3′	447	F-5′-CRGTTTAGGCCAAAATCCCC-3′ R-5′-AGGTATCTGCGGAGCCACTC-3′	326	742	5′-(T_3)CACACCMTCCAGWGGATGT-3′[a]
						786	5′-(T_6)GCCGTCGTAGGCGRACTGGT-3′[b]
						793	5′-(T_{11})CTCCGCGGGTATRACCAGT-3′[c]
						833	5′-(T_{14})TACATCGCCCTGAAYGAGGA-3′[c]
						860	5′-CTGGGTGATCTGAGCCGC-3′[b]
						886	5′-(T_{18})TCAGATCACCCAGCGCAAGT-3′[c]
						913	5′-(T_{22})TGCCCTCCAGGTAGGCTCTS-3′[b]
	4	F-5′-GCTGYTGGAGTGTCSCAAGAG-3′ R-5′-GCTGAAGGGCTCCAGGACTT-3′	401	F-5′-TCAGAACMCCCAAAGACACA-3′ R-5′-ACTTCTGCTTTCYCTGAKAA-3′	329	1610	5′-(T_{10})TGGCCTCATGGTCAGAGACG-3′[b]
						1612	5′-(T_{15})ACACGTGACCCACCAYCCC-3′[c]
						1687	5′-GGAGATCACACTGACCTGGCAG-3′[c]
						1695	5′-(T_{17})GGTGTCCTGRGTYTGGTCCTC-3′[b]
						1815	5′-(T_3)GAGCAGAGATACACGTGCCATGT-3′[c]
						1833	5′-(T_{23})ATCTCAGGGTGAGGGGCTC-3′[b]
						1855	5′-CCCCTCACCCTGAGMTGG-3′[c]
DRB	2	F-5′-GTYATTTCTTCAAYGGGACGGA-3′ R-5′-CARCCCCGTAGTTGTRTCTGC-3′	212	F-5′-TTTCTTCAAYGGGACGGAGC-3′ R-5′-TTGTGTCTGCARTARKTGTCCA-3′	198	165	5′-GACGGAGCGRGTGYGGWW-3′[c]
						196	5′-(T_{10})CGCTGTYGWAGCGCRCGD-3′[b]
						197	5′-(T_{14})TCGCTGTYGWAGCGCRCG-3′[b]
						227	5′-(T_{18})ACAGYGACSTGGGGGAGT-3′[c]
						256	5′-(T_{22})ASGGAGCTGGGGCGGCCT-3′[c]
						266	5′-(T_{22})AGDTCCTTCTGGCTGTTCCAG-3′[b]
						286	5′-GAGYMCTGGAACAGCCAGAAGGAC-3′[c]
						308	5′-(T_{25})TGTCTGCARTARKTGTCCACC-3′[b]

[a]Nucleotide position number was obtained according to HLA Genomic Sequence Alignments, downloadable from IMGT/HLA Sequence Database (http://www.ebi.ac.uk./imgt/hla/align.html). Polymorphic positions are encoded by the NC-IUB codes as follows: R = G and A; K = G and T; S = G and C; W = A and T; M = A and C;Y = T and C; D = G and A and T; V = G and A and C; B = G and T and C; H = A and T and C.
[b]Antisense.
[c]Sense.
(T_n) indicates the number of thymidine nucleotides of the poly(T) track elongating the specific primer sequence. Minisequencing primers of each exon are used in multiplex. For exon 3, two different multiplexes (*, †) were performed.
F = forward; R = reverse.

combinations, may take several months. This becomes relevant for rapid progressing or well-developed diseases, in which time restrictions are very important. The development of a diagnostic PGD strategy that involves the use of a flexible protocol appropriate for the analysis of a wide spectrum of possible HLA genotypes, precluding the design of case-specific protocols each time, could be more feasible, shortening the preclinical development time necessary for future cases.

The application in PGD of a new method of genetic analysis, known as minisequencing, was recently reported (Bermudez *et al*., 2003; Cram *et al*., 2003; Fiorentino *et al*., 2003; Iacobelli *et al*., 2003). This technique, applied to preimplantation HLA matching, provides a common procedure for mutation analysis and HLA genotyping, irrespective of the specific mutations and genotypes involved. The development of different PGD protocols specific for each mutation and HLA genotype to be analysed is thus avoided.

The aim of this study was to optimize a flexible preimplatation HLA-matching protocol applicable to a wide spectrum of possible HLA genotypes, avoiding the design of different specific protocols for each single case. This strategy involves minisequencing-based genotyping of HLA regions A, B, C and DRB, combined with mutation analysis of the gene regions presenting mutations. Analysis of different polymorphic short tandem repeat (STR) markers, scattered throughout the HLA complex, is also included to increase accuracy of the analysis and to detect potential contaminations and crossing-over occurrences between HLA genes.

Materials and methods

HLA genotyping primer design

By searching the IMGT/HLA sequence database (Robinson *et al*., 2000), which is accessible through http://www.ebi.ac.uk/imgt/hla/, for all available nucleotide sequences of class I and II HLA alleles, locus-specific external and internal primers for amplification of all possible alleles of HLA-A, HLA-B, HLA-C and HLA-DRB regions were designed (Table I). The sequence of each external primer was chosen as the most suitable region for locus-specific PCR amplification of each HLA region to be investigated, also matching a region that is highly conserved in all class I and II genes. Selection criteria were restricted to the amplification of exons 2 and 3 of class I genes, since most polymorphisms are located in those exons. Furthermore, the analysis of exon 4 was included in the protocol, as its contribution to the discrimination of so many different alleles of the HLA-A and HLA-C regions seems to be relevant. For the HLA-DRB gene, typing was restricted to the amplification of exon 2 where the vast majority of allelic variability in this gene is located.

Minisequencing primers were designed for each exon by selecting the most recurrent and discriminating polymorphic positions in the different alleles of the HLA regions evaluated (Table I). The combination of these nucleotides defines a specific genotype (Table II).

STR markers selection criteria

Linked STR markers, selected to cover the extended HLA complex, are listed in Table III, and their position is illustrated in Figure 1. These microsatellites were selected to be relatively evenly spaced throughout the HLA region, with their position and linkage disequilibrium pattern with HLA genes also considered. Moreover, it was aimed at selecting markers with rather few alleles, but having a relatively high heterozygosity. STR markers were amplified using the primers listed in Table IV, following the protocol described below. One of the internal primers of each microsatellite was labelled with a fluorescent dye (e.g. 6-Fam, Hex, Ned) so it could be visualized on an automatic DNA Sequencer (ABI Prism® 3100; Applied Biosystems). STR markers with overlapping size ranges were labelled with different fluorochromes in order to analyse them in the same capillary electrophoresis run. Finally, the primer sequences of the individual STR markers were chosen to work in a multiplex PCR reaction format, in combination with the other STR markers selected, the primers specific for the HLA regions (listed in Table I) and the primers used to identify the disease under study (listed in Table V).

Preclinical work-up

In order to identify the most informative exons of each HLA region investigated and informative STR markers of HLA complex, HLA genotyping and STR haplotype analysis for family members (father, mother and affected child) were performed.

Our policy was to select, for the subsequent PGD, at least one exon for each HLA region containing six or more informative polymorphic sites and eight informative STR markers evenly spaced throughout the HLA complex. Analysis of family members permits identification of which alleles segregate from each parent to the affected child, and selection of the appropriate markers or polymorphic sites to be used in the following clinical PGD cases. A panel of 29 different STR was studied during the set-up phase to evaluate their informativity (Table III). An STR marker is considered to be informative for HLA matching if both partners of the couple are heterozygotes for that marker, so that segregation of each allele can be determined. It is also necessary that individuals who are heterozygotes for a specific STR marker do not present the same allele combination for that locus. For the selected exons of each HLA region, the six or more polymorphic sites chosen will determine a specific profile for each genotype of the specific HLA region evaluated, enabling distinction of the segregation of each haplotype (Figure 2).

Genomic DNA was extracted from 200 µl of peripheral blood in EDTA collected from both parents and affected child of each couple according to the phenol–chloroform procedure (Sambrook *et al*., 2000). Genomic DNA was also extracted from skin biopsy in the affected children, to resolve potential DNA profile discrepancy due to a recent blood transfusion.

PCR amplification of the HLA regions involved a nested PCR strategy, consisting of a region-specific external/nested PCR amplification using oligonucleotide primers listed in Table I. Microsatellites were grouped to assemble five multiplex reaction panels (Panels A–E), combining internal primer sets with similar optimal PCR conditions and adjusting primer concentrations of individual markers, to obtain balanced fluorescent signals. STR loci of each panel were amplified in a multiplex PCR format. The primer sequences, final working concentrations and size of the amplified fragments are reported in Table IV. A single round PCR amplification was performed, according to the conditions described below.

To identify disease-causing mutations, PCR amplification of the gene regions of interest was performed with the external primers listed in Table V. Mutation analysis was then carried out by direct sequencing of PCR products by means of Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA), according to the protocol provided by the manufacturer.

HLA genotyping and STR haplotyping were performed as described below.

Trial PGD testing on single lymphocytes

In order to evaluate the reliability of the protocol before proceeding to PGD, primers used for HLA genotyping, STR haplotyping and mutation detection were first tested on single lymphocytes collected from both parents and the affected child of each family.

Lymphocytes were isolated from 5 ml of unclotted blood in EDTA as previously described (Fiorentino *et al*., 2003). Single lymphocytes were loaded into 0.2 ml tubes containing 5 µl of alkaline lysis buffer [200 mmol/l KOH, 50 mmol/l dithiothreitol (DTT)] and subjected to PCR. A multiplex PCR protocol for simultaneous amplification of the selected informative exons of each HLA region, HLA STR markers, genetic regions containing the mutations and STR markers linked to these regions for ADO detection was performed, as described below. Twenty blank controls for each trial were also processed under the same conditions to check for the absence of contamination.

IVF and embryo biopsy procedure

Cleavage stage embryos were obtained using a standard IVF procedure, as described elsewhere (Kahraman *et al*., 2002). At 62–64 h post insemination, embryos with ⩾6 cells and <50% fragmentation underwent embryo biopsy. According to the internal biopsy policy, one blastomere was removed from embryos with <7 cells and two blastomeres from embryos with ⩾7 cells. After removal, each blastomere was washed twice in two drops of HEPES-buffered medium, transferred into a sterile 0.2 ml PCR tube containing 5 µl of alkaline lysis buffer (200 mmol/l KOH, 50 mmol/l DTT), and covered with mineral oil

Table II. The nucleotide sequences of the HLA-A alleles at positions where they differ

Nucleotide position number[a]	199	208	228	232	274	301	310	656	707	726	733	734	756	762	827	859	863	897	910	930	942	1577	1616	1622	1641	1712	1757	1758
A*010101	C	C	T	A	C	C	C	G	G	A	T	A	C	G	G	A	C	C	G	C	G	[b]	G	C	G	C	G	G
A*0103	T	–	–	–	–	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A*0104N	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–
A*0108	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–
A*0109	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A*020101	T	T	–	–	A	–	–	A	C	G	G	G	T	T	–	–	–	T	T	A	T	–	A	–	A	–	–	–
A*020102	–	–	–	–	A	–	–	A	C	G	G	G	T	T		–	–	T	T	A	T	–	–	–	–	–	–	–
A*020105	T	T	–	–	A	–	–	A	C	G	G	G	T	T	A	–	–	T	T	A	T	–	A	–	A	–	–	–
A*0202	T	T	–	–	A	–	–	A	C	C	G	G	T	T	–	–	–	T	–	A	T	–	A	–	A	–	–	–
A*0203	T	T	–	–	A	–	–	A	C	G	G	G	T	T	–	–	–	A	–	A	T	–	A	–	A	–	–	–
A*0204	T	T	–	–	A	–	–	A	C	G	–	G	T	T	–	–	–	T	T	A	T	–	A	–	A	–	–	–
A*0205	–	T	A	C	A	–	–	A	C	C	G	G	T	T	–	–	–	T	–	A	T	–	A	–	A	–	–	–
A*021702	T	T	–	–	A	–	–	A	C	C	–	G	T	T	–	–	–	T	T	A	T	–		–		–	–	–
A*0232N	T	T	–	–	A	–	–	A	C	G	G	G	T	T	–	–	T	T	T	A	T	–	A	–	A	–	–	–
A*0243N	T	T	–	–	A	–	–	A	C	G	G	G	T	T	–	–	–	T	T	A	T	–	A	–	A	–	–	–
A*0246	–	T	–	–	A	–	–	A	C	G	G	G	T	T	–	–	–	T	T	A	T	–	–	–	–	–	–	–
A*030101	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	G	–	A	T	A	T	–	–	–	–	–	–	–
A*110101	–	–	A	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	T	–	–	–	–	–	–	–
A*1105	T	–	A	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	T	–	–	–	–	–	–	–
A*2301	T	–	C	–	–	–	–	–	–	C	–	G	T	–	–	G	–	T	T	A	–	–	–	T	–	T	A	–
A*2306	T	–	C	–	–	–	–	–	–	C	–	G	T	–	–	G	–	T	T	A	–	–	–	T	–	T	A	–
A*2307N	T	–	C	–	–	–	–	–	–	C	–	G	T	–	–	G	–	T	T	A	–	C	–	T	–	T	A	–
A*24020101	T	–	C	–	–	–	–	–	–	C	–	G	T	–	–	G	–	T	A	A	–	–	–	T	–	T	A	–
A*24020102L		–	C	–	–	–	–	–	A	C	–	G	T	–	–	G	–	T	A	A	–	–	–	T	–	T	A	–
A*2405	T	–	C	–	–	–	–	–	–	C	–	G	T	–	–	G	–	T	A	A	–	–	–	–	–	–	–	–
A*2409N	T	–	C	–	–	–	–	–	–	C	–	G	T	–	–	G	–	T	A	A	–	–	–	T	–	T	A	–
A*2411N	–	–	C	–	–	–	–	–	–	C	–	G	T	–	–	G	–	T	A	A	–	–	–	T	–	T	A	–
A*2423	–	–	C	–	–	–	–	–	–	C	–	G	T	–	–	G	–	T	A	A	T	–	–	–	–	–	–	–
A*2427	–	–	C	–	–	–	–	–	–	C	–	G	T	–	–	G	–	T	A	A	–	–	–	–	–	–	–	–
A*2432	–	–	C	–	–	–	–	–	–	C	–	G	T	–	–	G	–	T	A	A	–	–	–	–	–	–	–	–
A*2611N	–	–	A	C	–	–	–	–	C	–	G	G	–	–	–	G	–	A	–	–	T	–	–	–	A	–	–	T
A*2615	–	–	A	C	–	–	G	–	C	–	G	G	–	–	–	G	–	A	–	–	T	–	–	–	A	–	–	T
A*29010101	–	–	C	–	–	–	T	–	–	–	–	G	T	–	–	G	–	T	T	A	T	–	–	–	A	T	–	T
A*29010102N	–	–	C	–	–	–	T	–	–	–	–	G	T	–	–	G	–	T	T	A	T	–	–	–	A	T	–	T
A*3002	–	–	C	–	A	–	–	–	–	–	–	–	T	–	–	G	–	G	T	A	T	–	–	–	–	–	–	–
A*3104	–	–	C	–	–	–	–	–	–	–	–	–	T	–	–	G	–	T	T	A	T	–	–	–	A	–	–	T
A*3306	–	–	C	–	–	–	–	–		–	–	G	T	–	–	G	–	T	T	A	T	–	–	–	A	–	–	T
A*680101	–	–	A	C	–	–	–	–	C	–	–	G	T	–	–	–	–	T	–	A	T	–	A	–	A	–	–	–
A*6808N	–	–	A	C	–	–	–	–	C	–	–	G	T	–	–	–	–	T	T	A	T	–	A	–	A	–	–	–
A*6811N	–	–	A	T	–	–	–	–	C	–	–	G	T	–	–	–	–	T	–	A	T	–	A	–		–	–	–
A*6817	–	–	A	T	–	–	–	–	C	–	–	G	T	–	–	–	–	T	–	A	T	–	A	–	A	–	–	–
A*6818N	–	–	A	C	–	–	–	–	C	–	G	G	–	–	–	–	–	T	–	A	T	–	A	–	A	–	–	–
A*6901	–	–	A	C	–	–	–	A	C	G	G	G	T	T	–	–	–	T	T	A	T	–	–	–	–	–	–	–
A*7404	–	–	–	–	–	–	T	–	–	–	–	G	–	–	–	–	–	T	T	A	T	–	–	–	–	–	–	–

The nucleotides identical to the allele A*010101 are indicated as '–'.

[a]The nucleotide position number was obtained according to HLA-A Genomic Sequence Alignments, downloadable from IMGT/HLA Sequence Database (http://www.ebi.ac.uk./imgt/hla/align.html).

[b]Insertion C at nucleotide 1577 (ACCCCCCC-C-AAGACACATAT).

Table III. Overview on selected STR markers in the HLA complex

STR locus	Approximate location	Heterozygosity	No. of alleles	References
D6S426	5 cM centromeric of DPB1	0.85	9	Gyapay *et al*. (1994)
D6S291	3.6–4 cM centromeric of DPB1	0.72	7	Gyapay *et al*. (1994)
D6S439	0.7 Mb centromeric of DPB1	0.6	8	Gyapay *et al*. (1994)
Ring3CA	36.4 kb centromeric of DMB	0.73	9	Cullen *et al*. (1997)
TAP-CA	4.8 kb centromeric of TAP1	0.58	9	Carrington *et al*. (1994)
G51152	37.2 kb centromeric of DQB1	0.81	9–11	Cullen *et al*. (1997)
D6S2447	12 kb centromeric of DQB1	0.86	9	GDB:5886537
DQCAR	1134 bp telomeric of DQB1	0.78	13	Macaubas *et al*. (1995)
DQCAR-II	13.8 kb telomeric of DQB1	0.88	15	Mignot *et al*. (1997)
DRA-CA	DRA gene	0.84	12	Cullen *et al*. (2003)
LH-1	Between DRA and NOTCH4	0.71	11	Lako *et al*. (1999)
D3A	Between PBX2 and TN-X	0.76	6	Lako *et al*. (1999)
D6S273	96 kb telomeric of HSP70	0.78	8	Gyapay *et al*. (1994)
9N-2	Between HOM and BAT5	0.72	7	Lako *et al*. (1999)
82-1	Between BAT5 and LTB	0.81	13	Lako *et al*. (1999)
TNFa	Between TNF-β and LkBL	0.84	13	Nedospasov *et al*. (1991)
TNFb	Between TNF-β and LkBL	0.75	5	Nedospasov *et al*. 1991)
62	Between LkBL and BAT1	0.82	14	Lako *et al*. (1999)
MICA	40 kb centromeric of HLA-B	0.70	8	Grimaldi *et al*. (1996)
MIB	24.9 kb centromeric of HLA-B	0.82	15	Grimaldi *et al*. (1996)
HLABC-CA	39.4 kb centromeric of HLA-C	0.78	12	Tamiya *et al*. (1998)
HLAC-CA	88.4 kb telomeric of HLA-C	0.72	10	Tamiya *et al*. (1998)
D6S1624	Between HLA-C and HLA-E	0.76	9	Dib *et al*. (1996)
D6S265	115 kb centromeric of HLA-A	0.76	14	Bouissou *et al*. (1995)
D6S510	37 kb centromeric of HLA-A	0.74	8	Gandon *et al*. (1994)
D6S248	82 kb telomeric of HLA-A	0.82	10	Orphanos *et al*. (1993)
MOGCA	262 kb telomeric of HLA-A	0.77	15	Roth *et al*. (1995)
D6S105	2–3 cM telomeric of HLA-A	0.81	10–12	Weber *et al*. (1991)
D6S276	3–4 cM telomeric of HLA-A	0.79	16	Gyapay *et al*. (1994)

GDB number refers to the accession number in the Genome Data Base (http://www.gdb.org).

(Sigma–Aldrich, Italy) before subjection to cell lysis. For each embryo biopsied, a blank control was prepared from the wash drops by adding 5 µl of this medium to a sterile 0.2 ml PCR tube.

Cell lysis and PCR

The cells were lysed by incubation at 65°C for 10 min. The alkaline lysis buffer was then neutralized by the addition of 5 µl of neutralization buffer (900 mmol/l Tris–HCl, 300 mmol/l KCl, 200 mmol/l HCl) before proceeding to PCR.

A nested multiplex PCR assay was used to co-amplify all the selected loci. The first round PCR contained the external primers for the amplification of the informative HLA regions and HLA STR markers selected during the preclinical work-up of each PGD case, the gene regions involved by mutations and STR markers linked to these regions for ADO detection. The first round multiplex PCR was followed by separate second round PCR reactions for each locus. Primer sequences, final working concentrations and size of the amplified fragments are reported in Tables I, IV and V.

The first round PCR reaction was performed in a total volume of 50 µl containing 1.5 mmol/l $MgCl_2$, 200 µmol/l of each dNTP (Roche Diagnostic, Italy), 2.5 IU AmpliTaq Polymerase (Applied Biosystems), 10 pmol of each outer primer of the selected informative exons of HLA A, B, C and DRB regions, 5 pmol of the outer primers of the gene regions affected by mutations, 5 pmol of the outer primer of polymorphic marker linked to the mutation sites for ADO detection, and the outer primers of the selected informative HLA STR markers (the corresponding concentrations are reported in Table IV). The program used consisted of 35 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C. Each round of PCR was preceded by an initial 4 min denaturation step at 94°C and followed by a final extension step of 10 min at 72°C.

For the individual second round PCR reactions, 2 µl of the primary PCR products were added to another tube containing 5 µl of 10×PCR Buffer II (500 mmol/l KCl, 100 mmol/l Tris–HCl, pH 8.3; Applied Biosystems), 1.5 mmol/l $MgCl_2$, 200µmol/l of each dNTP (Roche Diagnostic, Italy), 2.5 IU AmpliTaq Gold Polymerase (Applied Biosystems), 10 pmol of each inner primer, and ultra-pure water to a total volume of 50 µl. A heminested approach was used to amplify D3A, LH1, DRA-CA, MOG-CA, D6S439, D6S105, DQCAR-II, HLAC-CA, HLABC-CA, TH01 and DXS6940 STR loci (Tables IV and V). All the reactions were cycled on a GeneAmp® PCR System 9700 (Applied Biosystems, USA). The cycling condition for second round PCR consisted of 35 cycle amplification involving an initial denaturation of 95°C for 10 min (to activate AmpliTaq Gold), 95°C for 30 s, 60°C for 30 s and 72°C for 30 s followed by a final extension of 72°C for 10 min. For STR markers, amplification annealing and final extension temperatures were reduced to 55 and 65°C respectively. Moreover final extension time was lengthened to 60 min in order to reduce the plus-A effect, which represents a potential source of error in STR genotyping.

During the PGD cycles, PCR tubes containing single lymphocytes isolated from each family member (two cells for each individual) were analysed simultaneously with the biopsied blastomeres as positive controls for DNA amplification and HLA genotype assignment.

Mutation analysis and HLA genotyping

Mutation analysis of gene regions affected by mutations and HLA genotyping were carried out on positively amplified blastomeres with the minisequencing method, following the protocol described elsewhere (Fiorentino *et al*., 2003). Polymorphic sites of HLA regions and mutation sites were interrogated simultaneously in a multiplex minisequencing reaction format. The size difference between minisequencing products was determined by the addition of non-homologous polynucleotide tails (poly-dTTP) of different lengths to the 5′ end of each primer.

For HLA STR and linked marker genotyping, 1 µl of each dye-labelled PCR product obtained after individual nested PCR was mixed in a single tube containing 0.5 µl of GS500 TAMRA (Applied Biosystems, USA) and 15 µl of Hi-Di Formamide (Applied Biosystems), and denatured for 4 min at 94°C, before the samples were resolved and detected by 20 min capillary electrophoresis on an automatic DNA sequencer ABI Prism® 3100 (Applied Biosystems). To reveal the electrophoresis data, the peak signal was analysed with GeneScan® Analysis Software (Applied Biosystems). The fragment sizes were automatically determined by the software by use of the second-order regression method to establish a best-fit curve generated from the internal size standard GS500 TAMRA.

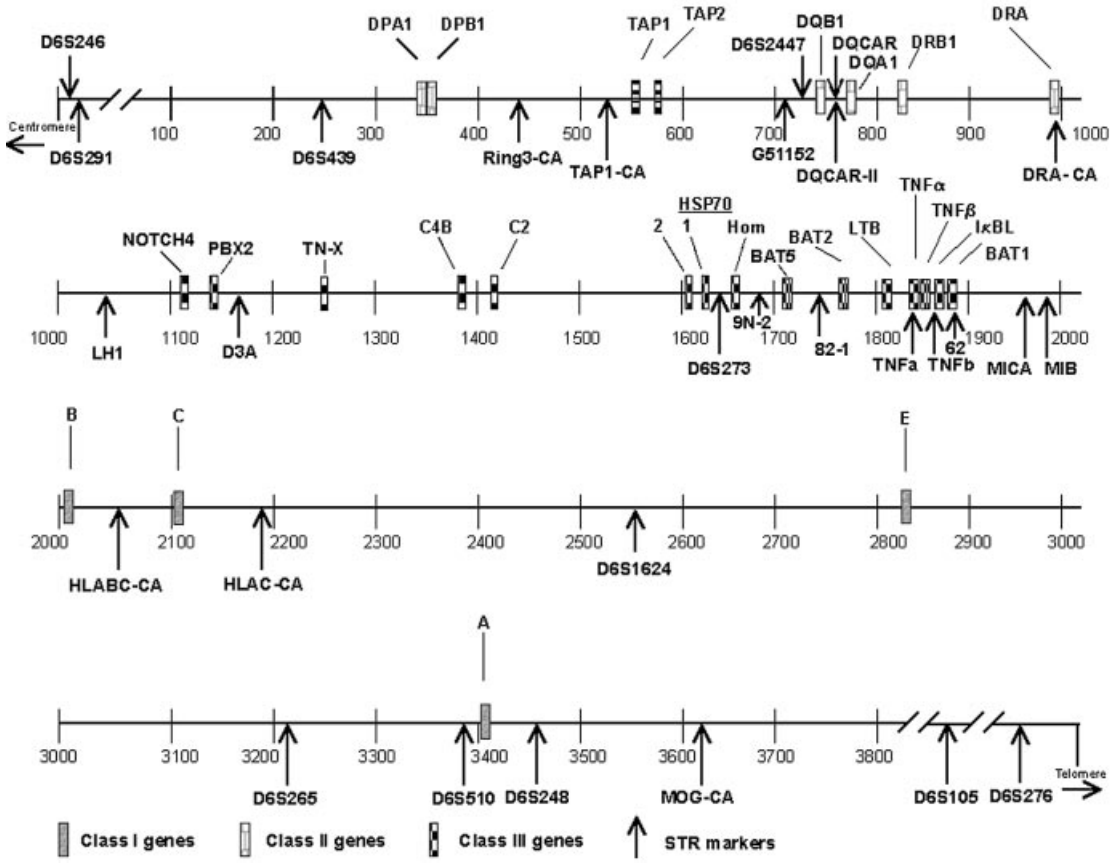


Figure 1. Selected microsatellite markers covering the extended HLA complex. Numbers correspond to kilobases.

Confirmation of PGD results

After the clinical cases, non-transferred and non-frozen embryos (affected or morphologically incompetent) had the zona pellucida removed; the disaggregated blastomeres were collected in individual tubes containing lysis buffer and reanalysed to verify the PGD results.

In cases in which pregnancies occurred, patients underwent conventional prenatal diagnosis to confirm the genetic status of the fetus.

Results

Preclinical work-up

During the preclinical stage of the study, before including the couples in the PGD program, HLA genotyping and STR haplotyping for family members (father, mother and affected child) were performed to select the most informative HLA exons and STR markers to be used in PGD.

A total of 15 couples was included in the preclinical work-up; all were found to be fully informative and therefore suitable for the PGD procedure. The chosen HLA exons and STR markers were thus included in the trial testing on single lymphocytes.

Mutation analysis of the genes of interest was also performed to identify the disease-causing mutations. The studied genes and the corresponding mutations are listed in Table V. Mutation screening of WAS gene, performed on the female partner of a couple with a child affected by Wiskott–Aldrich disease, revealed a novel mutation, T353C, changing a tyrosine into a histidine at codon 107 (Y107H). This mutation was also found in hemizygosity in the affected son of the couple, highly suggestive that this was the disease causing mutation.

Optimization of single cell multiplex PCR

In order to set up a multiplex PCR assay, the best conditions to obtain reproducible results for each locus were first determined. Multiplex PCR was designed to amplify simultaneously at least one exon from each HLA region, HLA STR markers, gene regions involved by mutation and linked STR markers which were found to be informative for the couple during the preclinical work for their PGD case. The PCR conditions were previously set up separately for each locus on DNA samples during the work-up phase, and were then adjusted for multiplex reaction by manipulating primer concentrations until satisfactory results for all the regions were obtained. Finally, the optimized multiplex PCR was evaluated on the couples' own single lymphocytes for single cell amplification efficiency, and reliability of the protocol with respect to allele drop-out (ADO) and contamination.

A summary of the amplification results is shown in Table VI. A total of 118 single lymphocytes was analysed from the selected couples. The number of markers/loci included in the first round multiplex PCR varied from 12 to 15. Amplification rates were generally high for all loci tested, ranging from 90.5 to 100%, with an overall amplification rate of 97.5%. No amplification signal for any of the markers was obtained in three out of 118 single lymphocytes, probably because individual cells were not successfully transferred into the reaction tubes. Successful amplification of all the markers was achieved in 92.2% (106/115) of single lymphocytes with positive amplification. Eleven cells (9.6%) were affected by ADO, involving 18 different markers for a total of 31 ADO occurrences; the ADO rates varied among the different loci investigated, ranging from 0 to 12.5%.

None of the 20 blank controls included in the single cell test series displayed amplification signals, indicating absence of contamination.

Table IV. Oligonucleotide primer sequences for the first- and second-round amplification of selected STR markers scattered through HLA complex

Panel[a]	STR locus	External primer sequence	Internal primer sequence	Size range (bp)[b]	Conc. (μmol/l)	Fluorescent label
Panel A	D6S276	F-5′-GTGTACACATCAATCAAATCATC-3′ R-5′-CCTCTTCAGTAGTTTTGCTACAG-3′	f-5′-TCAATCAAATCATCCCCAGAAG-3′ R-5′-GGGTGCAACTTGTTCCTCCT-3′	190–220	0.3	HEX
	D6S291	F-5′-CTCAGAGGATGCCATGTCTAAAATA-3′ R-5′-GGGGATGACGAATTATTCACTAACT-3′	f-5′-GTCTAAAATATCCATCCGGCAT-3′ R-5′-TTAATTGTGGTGATGGTTTCAC-3′	156–166	0.2	6-FAM
	D6S426	F-5′-CATGTGCTCTGCACCATAAGTG-3′ R-5′-GGAACCAGAAACTGTGGCATAG-3′	f-5′-ACTCCCCCAAAAATGTAGTCAT-3′ R-5′-AAAATGCACGTACCTAGTCCTC-3′	112–130	0.1	NED
	D6S273	F-5′-ATGTAAGATGTTAGCATTAGGAGA-3′ R-5′-AGTTAAAACCAAACTTCAAATTT-3′	f-5′-TGAGTATTTCTGCAACTTTTCTGTC-3′ R-5′-AAACCAAACTTCAAATTTTCGG-3′	135–146	0.25	HEX
	D6S265	F-5′-CCTTCTATCTGACTGTACGTTCGT-3′ R-5′-AGCTTTAAAAGGTTGATCTAATCG-3′	f-5′-TCGTACCCATTAACCTACCTCTCT-3′ R-5′-TCGAGGTAAACAGCAGAAAGATAG-3′	110–125	0.15	6-FAM
	MICA	F-5′-CTCTGCCCAGTGTATAACAAGTCC-3′ R-5′-GGGCCTTACCATCTCCAGAAAC-3′	f-5′-GAAAGTGCTGGTGCTTCAGAGTC-3′ R-5′-CTTACCATCTCCAGAAACTGCC-3′	170–180	0.2	NED
Panel B	TNFa	F-5′-ATGCAAGAAGGGTAAAGCCTCTAG-3′ R-5′-TTTTGAGGTTGCAGTGGGCTAT-3′	f-5′-GCCTCTAGATTTCATCCAGCCAC-3′ R-5′-CCTCTCTCCCCTGCAACACA-3′	97–121	0.1	6-FAM
	TAP1CA	F-5′-CTGTTCATATCCTCATACATCTGCT-3′ R-5′-GTTTTTCTTAAGGTAAGGAGGACAA-3′	f-5′-TCATACATCTGCTTTGATCTCCC-3′ R-5′-GGACAATATTTTGCTCCTGAGG-3′	195–215	0.2	NED
	TNFb	F-5′-ATGCAAGAAGGGTAAAGCCTCTAG-3′ R-5′-TTTTGAGGTTGCAGTGGGCTAT-3′	f-5′-TGTGTTGCAGGGGAGAGAGG-3′ R-5′-GGCCACAGAGCAAGACACCA-3′	100–118	0.2	HEX
	D6S2447	F-5′-CCATGGTCACTGTTTATCTGCA-3′ R-5′-GGACAATGTGTTTGAGAGGTGTG-3′	f-5′-CTGCATTTCTCTTCCTTATCACTTC-3′ R-5′-TTTGAGAGGTGTGCATGTTACC-3′	180–202	0.25	6-FAM
	D6S510	F-5′-AACACACTGATTTCCATAGCAGGT-3′ R-5′-CTGCAATGGGCTACTACTTCACA-3′	f-5′-TTTGTCTTTCCCAATGTACTACAC-3′ R-5′-GCTACTACTTCACACCAATTAGGA-3′	140–155	0.2	HEX
Panel C	D3A	F-5′-CATCCATGACAGAAAGCAGAGC-3′ R-5′-GATGTGTGACCTTTTGGCACAG-3′	[c] R-5′-CCTGCCTTCTGTAAGCCTCAG-3′	181–215	0.3	NED
	62	F-5′-GCAAGAAGGGTAAAGCCTCTAGA-3′ R-5′-GCAAGACACCATGTCAAAAGAA-3′	f-5′-GATTTCATCCAGCCACAGGA-3′ R-5′-TCCAATCACCTCTGCTCACC-3′	140–170	0.2	HEX
	82–1	F-5′-ATTACAAGGGCTTTAGGAGGTCTG-3′ R-5′-AGGTCAAAGCTGCAGTAGCCAT-3′	f-5′-GAGCCAGGATGGAGACCAAA-3′ R-5′-CCTGGATAACAGAACGAGACCC-3′	100–122	0.2	6-FAM
	G51152	F-5′-AGACAGCTCTTCTTAACCTGCC-3′ R-5′-GGTAAAATTCCTGACTGGCCT-3′	f-5′-GGAAAAGAGCTCACGCACAT-3′ R-5′-CCTGCCATCATGACTTCAAG-3′	145–158	0.15	NED
	LH-1	F-5′-GAACAGAGACAATGCCTAGAACC-3′ R-5′-ACCTTACTGGGCACAAATTCAC-3′	f-5′-GCTAGTCTGTGCCAAGGAACTC-3′ [c]	126–160	0.1	6-FAM
	Ring3CA	F-5′-TTGTCCCACAGTTTAATTGGG-3′ R-5′-TTGCATACACTCTGAAGCAGC-3′	f-5′-GCCGCAGTTTAACTGTTCCTT-3′ R-5′-GAAATGTTAGGTCAGAACCACAGA-3′	124–130	0.2	HEX
Panel D	MOG-CA	F-5′-ACCAGGCTAGGATCTGTCACA-3′ R-5′-TTGACCATGGGTAACTGAAGC-3′	f-5′-AGATCACCTCGAGTGAGTCTCTT-3′ [c]	205–235	0.25	6-FAM
	DRA-CA	F-5′-ACTTTCCTAATTCTCCTCCTTC-3′ R-5′-CATCCATGTAAAACAGAGACC-3′	R-5′-GCATGAGTAAACTATGGAATCTC-3′	122–140	0.2	HEX
	D6S439	F-5′-GGCTTCACAACTTTGGCACTAC-3′ R-5′-CAGCCTCAGGGAAGACACATT-3′	f-5′-CCCCTATTCTCCACCCACTAGA-3′ [c]	116–130	0.2	NED
	DQCAR	F-5′-CTGCATTTTTTCTATCTCCGTG-3′ R-5′-TTTGAGAGGTGTGCATGTTACC-3′	f-5′-CTGCATTTCTCTTCCTTATCACTTC-3′ R-5′-TGGCCAATCAGAATCTTTCCTA-3′	150–175	0.15	6-FAM
	9N-2	F-5′-GTCAGGAGAATTGCTTGAACCC-3′ R-5′-ATTCCTCTGCTCTCTGGGATTG-3′	f-5′-TGGGTAACAGAGCAAGACTCTGT-3′ R-5′-TGGGATTGCAGATGTGTTACAC-3′	100–110	0.1	HEX

Table IV *Continued*

Panel[a]	STR locus	External primer sequence	Internal primer sequence	Size range (bp)[b]	Conc. (μmol/l)	Fluorescent label
Panel E	MIB	F-5′-ATCCTGCCTCAGAATTAGAACATC-3′	f-5′-CGTTTTCAGCCTGCTAGCTTAT-3′	155–186	0.2	HEX
		R-5′-TCTAGAACCACTCTTCGTACCACA-3′	R-5′-CCACAGTCTCTATCAGTCCAGATTC-3′			
	D6S105	F-5′-AACAAGAGCAAAACTCCGTCTC-3′	[c]	141–155	0.2	NED
		R-5′-CTGATCACCCTCGATATTTTACC-3′	R-5′-TCACCTTGATATCTTATTACCCTGG-3′			
	DQCARII	F-5′-TTGAGGACTGAGTCTGAGTTGG-3′	f-5′-TTGGGCAGCATTTGTAGATTTC-3′[c]	118–138	0.15	6-FAM
		R-5′-GCAAGAATCCAGCATTTTGG-3′	[c]			
	HLABC-CA	F-5′-GTCAAGCATATCTGCCATTTGG-3′	[c]	113–144	0.15	HEX
		R-5′-GCCCAGTAGTAAGCCAGAAGCTA-3′	R-5′-ACTTGGGCAATGAGTCCTATGA-3′			
	HLAC-CA	F-5′- ACCCGTCAAACCTTGACTGTTC	F-5′-CGGCAAGAGACTCTGATGAGAA-3′[c]	156–173	0.1	NED
		R-5′- GTAGCTGGGATTACAGGTGCCT	[c]			
	D6S248	F-5′-GGCAGGAGAATGGCATGAAC-3′	f-5′-CGAGATCAAGCCACTGCACT-3′	151–165	0.25	HEX
		R-5′-TTGATCAGGAATGGTGAGAAGGG-3′	R-5′-CAGGAATGGTGAGAAGGGAAA-3′			
	D6S1624	F-5′-CCCCAGAGGCCTATATATAACCC	f-5′-TATAACCCCAGGTGTTTGTGG-3′	200–220	0.2	6-FAM
		R-5′-CGTCCTATCAGTTAACACTTCATGG	R-5′-GGAAGTCTTCAGTGGAGAGAGTG-3′			

[a]STR loci are grouped in panels to be amplified in multiplex PCR format during the set-up phase.
[b]Size ranges refer to the internal primers.
[c]A heminested approach was used to amplify D3A, LH1, DRA-CA, MOG-CA, D6S439, D6S105, DQCAR-II, HLAC-CA and HLABC-CA loci.
F = forward; R = reverse.

Moreover, no contamination was observed resulting in the presence of polymorphisms or mutations which were absent in the individual from whom the cells were taken. In view of these results, the assay was considered to be robust enough to proceed to clinical application.

The electropherograms shown in Figure 2 illustrate an example of minisequencing-based HLA-A genotyping results obtained from single lymphocytes of a β-thalassaemia case, combined with analysis of IVS-I-110 G-A mutation of β-globin gene. Figure 2A, B and C shows the profiles of father (genotype A*2615–A*0103), mother (genotype A*020101–A*030101) and affected child (genotype A*0103- A*020101) respectively. Seven informative polymorphic sites, located in exon 3 of the HLA-A gene, and IVS-I-110 G-A mutation were detected by single nucleotide extension of primers annealing directly adjacent to the nucleotide of interest. The size of specific primer sequences ranged from 20 to 47 nucleotides (Table I). These primers were synthetically elongated at their 5′ end with a poly(T) tail, which varied in size to facilitate electrophoresis separation of minisequencing products. During the minisequencing reaction, carried out with multiple primer/template combinations, the different polymorphic sites are interrogated simultaneously in the same reaction, producing one (homozygote) or two (heterozygote) coloured peaks for each site, depending on the single nucleotide polymorphism or genotype present at this site. The selected polymorphic sites will determine a specific profile for each genotype of the specific HLA region evaluated, making it possible to distinguish the segregation of each haplotype.

Clinical PGD

Following the set-up on lymphocytes, the technique was applied to 17 PGD cycles (14 for β-thalassaemia, one for Wiscott–Aldrich syndrome and two for leukaemia) performed for 15 couples involving the testing of 145 embryos for HLA typing in combination with a genetic disease, and four embryos for HLA typing only. The results are summarized in Tables VI and VII. A total of 278 blastomeres was analysed, 266 (95.7%) of which produced amplification products for at least one locus. A total of 250 blastomeres (94.0%) gave positive amplification for all the loci investigated (Table VII). Amplification failure for all the markers resulted in 12 blastomeres, probably because the blastomeres were anucleate or individual cells were lost during transfer into the reaction tubes. The amplification efficiency on the blastomeres was similar to that obtained from single lymphocytes, ranging from 94.5 to 100% (Table VI), with an overall amplification rate of 97.6%.

The PCR products obtained for HLA Class I and II loci from these 266 blastomeres were analysed by minisequencing to determine their HLA genotype. Diagnosis was assigned only when both cells yielded the same results or, as in the cases of only one cell result available (one cell biopsy or total amplification failure in one cell), when investigated STR loci and HLA genotyping provided concordant results.

A reliable HLA genotype was obtained in 95.9% (255/266) of the blastomeres with positive PCR results. In total, 22 (14.8%) embryos revealed an HLA match with the affected siblings. In the 15 cycles of HLA matching combined with single gene disorder, 15 HLA-identical embryos resulted in an unaffected genotype; 14 of them were transferred back to the patients. In five embryos, a recombination event occurred; in one of these embryos the genotype was still unaffected and HLA compatible, but was not considered for transfer because of recombination (Table VII). Seven embryos remained without diagnosis because they were found to be haploid. Four clinical pregnancies resulted from the above PGD cycles, three of which (one twin, two singletons) are ongoing and have been confirmed both as healthy and HLA compatible with the affected sibling by prenatal

Table V. Description of genetic regions amplified, mutations investigated and primers used

Disease	Gene	Exon	External primers (5′ to 3′)	Internal primers (5′ to 3′)	Minisequencinq Primers for mutation detection		Primers (5′ to 3′) for ADO detection	
					Mutation Investigated	Sequence (5′ to 3′)	Name	Sequence (5′ to 3′)
β-Thalassaemia	HBB	1	F-5′-ctgtcatcacttagacctca-3′	f-5′-CATCACTTAGACC TCACCCTGT-3′	-30 T→A	5′-(T_{15})GATGGCTCTGC CCTGACTTTT-3′	Cod.2 C→T	5′-(T_{25})GCAGACTTCTCCT CAGGAGTCAG-3′[a]
			R-5′-TGGTCTCCTTAAA CCTGCTTG-3′	R-5′-TCTCCTTAAACCT GTCTTGTAACC-3′	Cod.5 – CT	5′-(T_{18})ACACCATGGTG CACCTGACTC-3′[b]	IVS-II-16 C→G	5′-(T_{27})ACTTCAGGGTGAG TCTATGGGAC-3′[a]
					Cod.6 – A	5′-CATGGTGCACCGAC TCCTG-3′	TH01 Ext F	5′-ATTCAAAGGGTATCTG GGCTCTGG-3′
					Cod.8 – AA	5′-(T_{25})TGCACCTGACT CCTGAGGAG-3′	TH01 Ext R	5′-GTGGGCTGAAAAGCTC CCGATTAT-3′
					IVS-I-1 G→A	5′-(T_9)CCTGTCTTGTAA CCTTGATACCAA-3′[c]	TH01 int F	5′-GTGATTCCCATTGGCCTGTTCCTC-3′[d]
		2	F-5′-ACTGGGCATGTGG AGACAGAGAAGA-3′	f-5′-TGGGTTTCTCATA GGCACTGA-3′	IVS-I-110 G→A	5′-(T_7)GGCACTGACTCT CTCTGCCTATT-3′	E-C Ext F[e]	5′-GTATACCCAGACTAGG GCCA-3′
			R-5′-TGTACCCTGTTAC TTCTCCCCTTCC-3′	R-5′-AAAGAAAACATC AAGGGTCCC-3′	IVS-I-130 G→C	5′-CTATTGGTCTATTTT CCCACCCTTA-3′	E-C Ext F[e]	5′-GCTTCTACTCTGTGAAT GGA-3′
					Cod.39 C→T	5′-(T_{22})TGGTGGTCTAC CCTTGGACC-3′	E-C int F[e]	5′-AGACTGGAGTAAAGGA ATGG-3′[d]
					IVS-II-1 G→A	5′-CGTGGATCCTGAGA ACTTCAGG-3′	E-C int R[e]	5′-GATGCCACAGCAGGTG-3′
		3	F-5′-TATCATGCCTCTTT GCACCATT-3′	f-5′-ATTTCTGGGTTAA GGCAATAGC-3′	IVS-II-745 C→G	5′-CATATTGCTAATAG CAGCTACAATCCAG-3′		
			R-5′-GGCCTAGCTTGGA CTCAGAATA-3′	R-5′-AATAATCCAGCC TTATCCCAAC-3′				
Wiskott–Aldrich syndrome	WAS	3	F-5′-ACTGTGCCTCCCAC CCTACAC-3′	f-5′-TACACCTCTCCAG GCTGGTC-3′	Y107H	5′-(T_3)AAGAAGGGGGT GGGGGTGGAGT-3′[c]	DXS6940 Ext F	5′-ACATCCTGGACATACA CCAACA-3′
			R-5′-GTCCTCGTCTGCA AAGTTCAGC-3′	R-5′-GCAACTTTCCCAT CTCCTCTC-3′			DXS6940 Ext R	5′-CAGCTTGTGTGGTTTTT GATCT-3′
							DXS6940 Int R	5′-GTGTGTTTCCTAGAAT TTGGA-3′[d]

[a]Minisequencing primers.
[b]Sense.
[c]Antisense.
[d]Labelled in 5′ with 6-FAM.
[e]Intergenic region between delta and beta globin genes.
(T_n) indicates the number of thymidine nucleotides of the poly(T) track elongating the specific primer sequence.
F = forward; R = reverse.

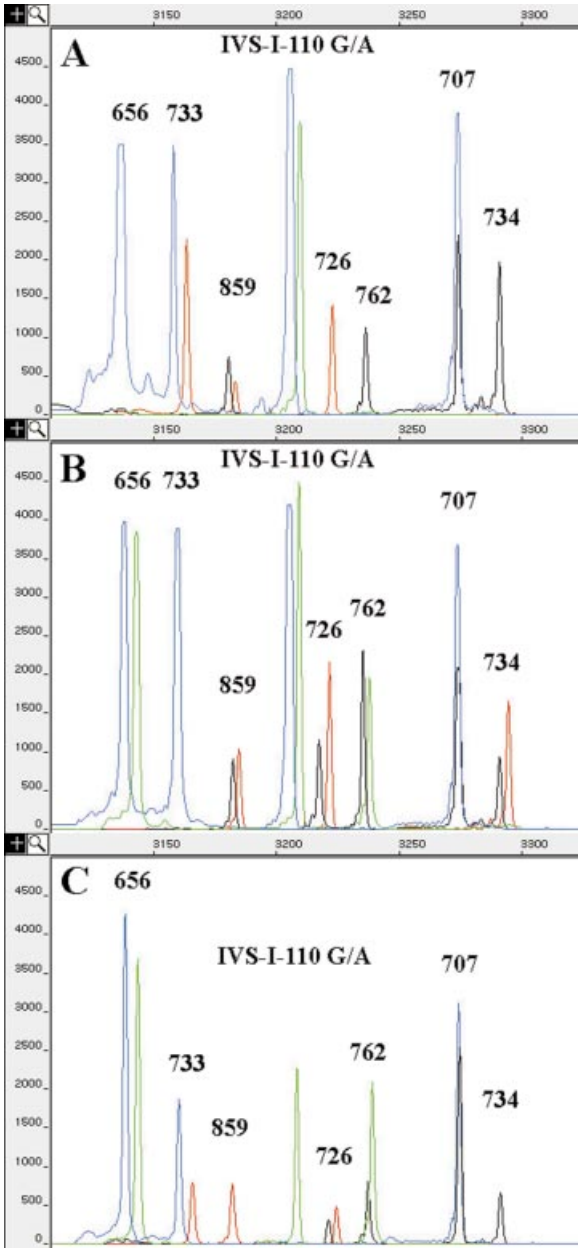
diagnosis. One pregnancy of a β-thalassaemia cycle resulted in early abortion.

In the couples undergoing preimplantation HLA typing for their children with leukaemia, none of the embryos analysed appeared to be HLA compatible with the affected children and no transfer was performed.

ADO was detected on 47 blastomeres, involving 20 different markers for a total of 99 ADO occurrences. The ADO rates varied between the different loci investigated, ranging from 0.0 to 14.3% (Table VI).

In all cases, single cell PCR positive controls were diagnosed reliably. No contamination was detected in blank controls collected during the biopsy procedure nor in the blanks from the PCR reagents.

The electropherograms in Figure 3 show an example of preimplantation HLA matching results combined with analysis of IVS-I-110 G-A mutation. Figure 3A shows the HLA-A genotype profile obtained from the affected child, compared with two HLA-identical blastomeres, one carrier (Figure 3B) and one affected (Figure 3C), and one homozygous unaffected HLA-non-identical blastomere (Figure 3D).



Reanalysis of non-transferred embryos

After the clinical cases, 33 non-transferred embryos (affected or morphologically incompetent) were disaggregated into single blastomeres and reanalysed under the same PCR conditions and procedure as that used for clinical PGD samples to confirm the diagnosis. PCR amplification was performed on 115 blastomeres. The amplification rates were similar to those obtained during clinical diagnosis, ranging from 91.4 to 100%, with an overall amplification rate of 96.8%. ADO was detected in 9/115 (7.8%) blastomeres; the ADO rates varied between the different loci investigated, ranging from 0 to 7.1%, with an average ADO rate of 4.2% (data not shown).

No discrepancies were observed with the diagnosis obtained for single blastomeres from clinical cases in all non-transferred embryos reanalysed.

Discussion

Allogeneic HSC transplantation represents the only curative option for severe cases of haematopoietic disorders, including β-thalassaemia which has been classified as a public health problem (Weatherall and Clegg, 1996). The best possibilities of cure are provided by transplantation with HLA-identical donors, because transplantation using donors other than HLA-identical siblings is associated with high morbidity and poor survival. Unfortunately, this cannot be applied in the majority of cases because of the difficulty of finding HLA-matched donors, even among family members.

Umbilical cord blood from HLA-identical siblings has been reported as an excellent source of stem cells (Petersdorf *et al*., 1998b; Sasazuki *et al*., 1998; Frassoni *et al*., 2003). Therefore, an increasing number of couples with a child affected by such a disease are requesting preimplantation HLA matching to conceive a healthy child who could become a future donor of HSC, to provide radical

Figure 2. Example of minisequencing-based HLA-A genotyping results obtained from single lymphocytes of a beta thalassemia case, combined with mutation analysis of IVS-I-110 G-A β-Globin gene mutation. **A**) Father, genotype A*2615 – A*0103. **B**) Mother, genotype A*020101 – A*030101. **C**) Affected child, genotype A*0103- A*020101. Numbers indicate polymorphic nucleotide positions, according to HLA-A Genomic Sequence Alignments, downloadable from IMGT/HLA Sequence Database (http://www.ebi.ac.uk./imgt/hla/align.html). The combination of these nucleotides defines a specific genotype.
The genotyped region corresponds to exon 3 of HLA-A gene. Primers used for PCR amplification are the follow: HLA-A ex.3 external F: 5′-GACYCCGAGACCCTTGYCCC-3′; HLA-A ex.3 external R: 5′-GGAGAYCTAYAGGCGATCAGGG-3′; HLA-A ex.3 internal F: 5′-CCGGTTTCATTTTCAGTTTAGG-3′; HLA-A ex.3 internal R: 5′-GTCTCCTTCCCGTTCTCCAG-3′. Only minisequencing primers for the informative polymorphic sites were selected on the basis of their size and included in the multiplex reaction. Minisequencing primer sequences are listed in Table I. The expected bases for each nucleotide position are the following: **656**: G (Blue) / A (Green); **707**: G (Blue) / C (Black) / A (Green); **726*:** C (Black) / T (Red) / G (Blue); **733**: G (Blue) / T (Red); **734*:** C (Black) / T (Red); **762*:** C (Black) / A (Green); **859*:** C (Black) / T (Red). * Minisequencing primers in these positions were designed in antisense. For the IVSI-110 G-A β-Globin gene mutation, the blue peak (G) refers to the normal base, and the green peak (A) to the mutant base. In our design, a dTTPs tail of different length was added to the 5′ end of each minisequencing primer. Multiplex single base extension produces minisequencing products that differ significantly in size, so that it is possible to distinguish easily the different sites in a single capillary electrophoresis run.

Table VI. Single cell multiplex PCR of the HLA microsatellite markers and HLA A, B, C and DRB regions from single lymphocytes and single blastomeres: amplification efficiencies and allele drop-out (ADO) rates

HLA marker/ gene region	Lymphocytes					Blastomeres[a]				
	No. of cells analysed	No. of cells with positive amplification	PCR efficiency (%)	No. of ADO occurrence	ADO rate (%)	No. of cells analysed[b]	No. of cells with positive amplification	PCR efficiency (%)	No. of ADO occurrence	ADO rate (%)
D6S426	42	41	97.6	1	2.4	129	126	97.7	4	3.2
D6S291	20	20	100.0	0	0.0	59	59	100.0	0	0.0
D6S439	25	24	96.0	3	12.5	83	82	98.8	5	6.1
Ring3CA	12	12	100.0	0	0.0	2	2	100.0	0	0.0
TAP1-CA	15	15	100.0	1	6.7	15	15	100.0	1	6.7
G51152	12	12	100.0	0	0.0	14	14	100.0	0	0.0
D6S2447	12	12	100.0	1	8.3	7	7	100.0	1	14.3
DQCAR	12	12	100.0	0	0.0	6	6	100.0	1	16.7
DQCAR-II	42	42	100.0	0	0.0	152	152	100.0	4	2.6
DRA-CA	42	41	97.6	0	0.0	139	139	100.0	0	0.0
LH-1	15	15	100.0	0	0.0	99	99	100.0	1	1.0
D3A	25	25	100.0	2	8.0	54	53	98.1	2	3.8
D6S273	25	25	100.0	0	0.0	87	87	100.0	0	0.0
9N-2	15	14	93.3	0	0.0	9	9	100.0	0	0.0
82-1	12	12	100.0	0	0.0	27	27	100.0	0	0.0
TNFa	42	42	100.0	4	9.5	212	211	99.5	10	4.7
TNFb	15	15	100.0	0	0.0	6	6	100.0	0	0.0
62	25	25	100.0	1	4.0	125	125	100.0	4	3.2
MICA	15	15	100.0	0	0.0	27	26	96.3	0	0.0
MIB	42	42	100.0	2	4.8	151	150	99.3	7	4.7
HLABC-CA	28	28	100.0	1	3.6	58	57	98.3	0	0.0
HLAC-CA	30	29	96.7	1	3.4	57	56	98.2	0	0.0
D6S1624	20	19	95.0	0	0.0	7	7	100.0	0	0.0
D6S265	34	34	100.0	1	2.9	87	86	98.9	1	1.2
D6S510	42	40	95.2	3	7.5	122	119	97.5	7	5.9
D6S248	11	11	100.0	0	0.0	11	11	100.0	0	0.0
MOG-CA	42	38	90.5	0	0.0	127	120	94.5	4	3.3
D6S105	25	24	96.0	1	4.2	78	77	98.7	6	7.8
D6S276	42	40	95.2	2	5.0	186	180	96.8	6	3.3
HLA A	42	40	95.2	2	5.0	266	254	95.5	16	6.3
HLA B	42	39	92.9	2	5.1	266	255	95.9	14	5.5
HLA C	42	41	97.6	2	4.9	266	256	96.2	12	4.7
HLA DRB	42	40	95.2	1	2.5	266	257	96.6	15	5.8

[a]Blastomeres from clinical PGD cases.
[b]Blastomeres in which total PCR failure occurred were not considered.
The number of markers/loci included in the first round multiplex PCR, for single lymphocytes as well as for blastomeres, varied from 12 to 15 (13 on average).

Table VII. Preimplantation HLA-matching results from 17 PGD cycles

Disease	No. of cells analysed	No. of cells with total PCR failure	No. of cells with positive amplification of all loci analysed	No. of cells with positive HLA typing	HLA-compatible embryos resulted			No. of recombination events occurred	HLA-compatible embryos obtained	HLA-compatible embryos transferred	Clinical pregnancies
					Affected	Carrier	Normal				
β-Thalassaemia	252	10	228 (94.2)	231 (95.5)	6	9	5	5 (3.8)	20 (15.2)	13 (9.8)	3[a]
Wiskott–Aldrich syndrome	20	2	16 (88.9)	18 (100.0)	1	1	0	0 (0.0)	2 (15.4)	1 (7.7)	1
Leukaemia	6	0	6 (100.0)	6 (100.0)	0	0	0	0 (0.0)	0 (0.0)	0 (0.0)	0
Total	278	12	250 (94.0)	255 (95.9)	7	10	5	5 (3.4)	22 (14.8)	14 (9.4)	4

Values in parentheses are percentages.
[a]One pregnancy of a beta thalassaemia cycle resulted in early abortion.

treatment for the existing affected sibling. The first successful HSC transplantation from an unaffected HLA-identical sibling donor, selected using PGD, was recently reported (Grewal *et al*., 2004).

This study describes the development and clinical application of a preimplantation HLA-matching strategy involving minisequencing-based genotyping of HLA Class I and II loci, combined with the analysis of different polymorphic STR markers located along the HLA complex. This approach permits HLA genotyping in preimplantation embryos combined with analysis of single gene disorders, by use of a flexible protocol that can be applied to a wide spectrum of different HLA allele combinations, as demonstrated by its validation in 17 couples with different haplotypes. As a result, the time needed for the set-up of a clinical PGD case is now significantly reduced (~1 week), enabling a substantial shortening of the waiting time for new couples. This is extremely important, as one of the main practical obstacles to preimplantation HLA matching is the time necessary to optimize the experimental conditions for all patients, due to the high polymorphism of the HLA complex. The size of the region, the presence of different loci with a high number of alleles, and the relatively high recombination rate detected within the region, render the design and the development of a diagnostic single cell strategy, specific for each family, labour intensive and time consuming. For couples needing a matched donor for their affected child with a rapidly progressing disease, time restrictions are crucial. Following the strategy presented here, the selection of unaffected, HLA-matched embryos can be performed without the need to develop a specific diagnostic experimental design for each couple, and thus with a substantial shortening of the preliminary phase.

A multiplex PCR assay has been optimized, based on simultaneous amplification of at least one exon from each HLA region, the selected HLA STR markers, the gene regions involved by mutation and linked STR markers for ADO detection. The most critical factor in achieving an efficient multiplex amplification was the choice of reliable and compatible primer sequences. The optimized single cell multiplex PCR requires exact reaction conditions that allow each probe to amplify equally, especially as the number of the primers within the PCR increases. Careful design of primers and PCR conditions is absolutely necessary to avoid artefacts and non-specific amplification.

Locus-specific primers (Table I) for amplification of all the possible alleles of HLA-A, HLA-B, HLA-C and HLA-DRB regions were designed and involved separate amplification of exons 2, 3 and 4 of class I genes, and exon 2 of the DRB gene. These primers have been shown not only to be highly specific for amplification of the corresponding HLA region, but also to be able to amplify most of the alleles with the highest efficiency. Primers specific for the amplification of polymorphic STR markers scattered throughout the HLA complex were also designed to increase the accuracy of the assay. The selected STR markers displayed high heterozygosity, broad distribution of alleles and identifiable allelic size ranges, and showed strong amplification efficiency (Table VI), with the absence of non-specific background interference to confound data interpretation.

Each multiplex PCR designed for the amplification of the above loci was successfully adapted to single lymphocytes during the preclinical work-up, and to blastomeres from clinical cases, showing high overall amplification rates in the preclinical setting (97.5%) as well as in clinical PGD (97.6%). Successful amplification of all the markers was achieved in 92.2% of single lymphocytes and in 94.0% of the blastomeres analysed. These results confirm that multiplex PCR with up to 14 primers set could be efficient, reliable and accurate.

The application of minisequencing to determine HLA genotypes in blastomeres obtained after embryo biopsy has been shown to be extremely useful. The best option was to choose a minisequencing-based typing of single cell PCR products to determine the HLA

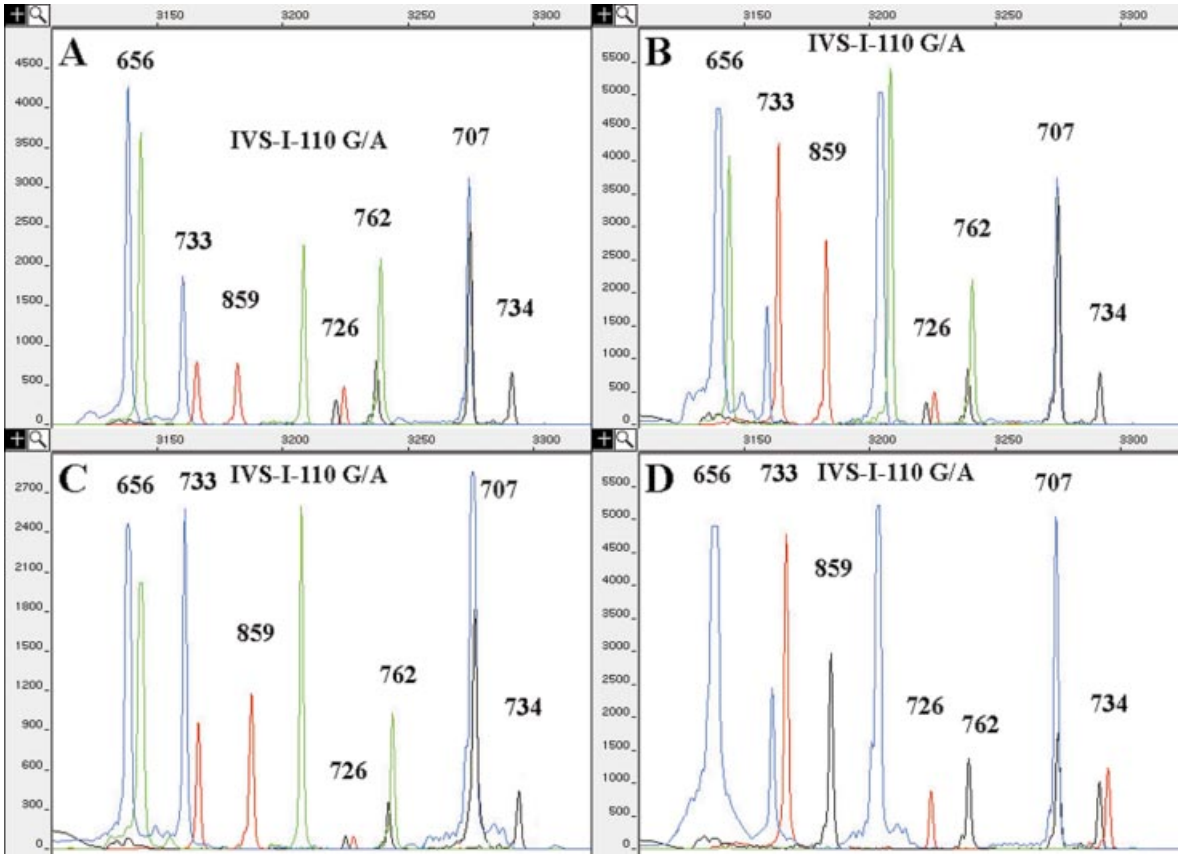


Figure 3. Example of preimplantation HLA matching results combined with mutation analysis from a beta thalassemia case, in which the mutation involved was IVS-I-110 G-A. **A**) HLA-A minisequencing profile obtained from the affected child (genotype A*0103- A*020101) compared with the profiles of two HLA identical blastomere, one carrier (**B**) and one affected (**C**), and (**D**) one normal HLA non-identical blastomere (genotype A*2615, A*030101). Numbers indicate polymorphic nucleotide positions. Expected bases for each nucleotide position and primers used are described in the Figure 2 legend.

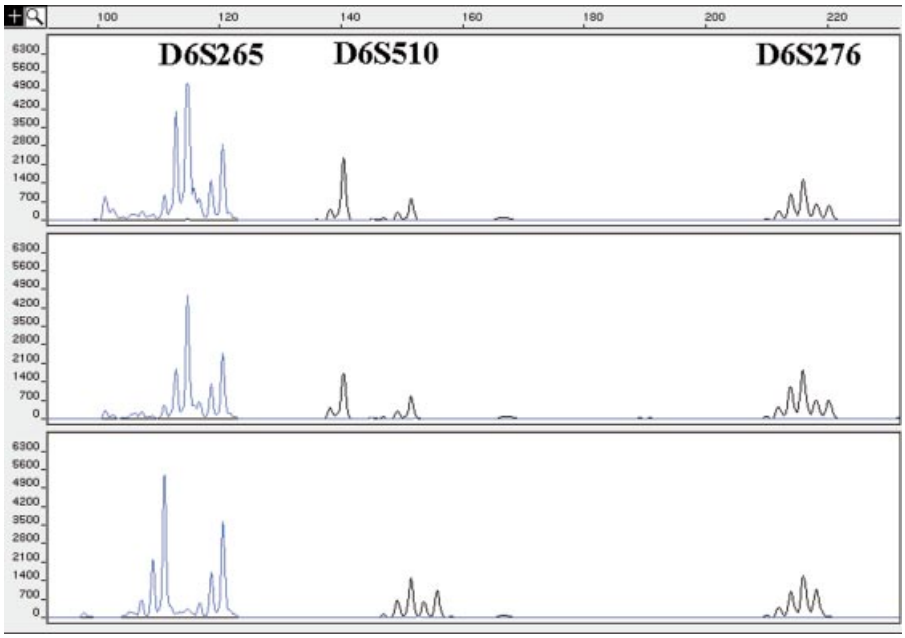


Figure 4. Capillary electrophoresis of fluorescent PCR products after analysis of 3 HLA STR markers on single lymphocytes (first line) and single blastomeres (second and third lines). **A**) Affected child HLA STR profile; **B**) profile of a blastomere derived from a HLA compatible embryo. **C**) STR profile from a HLA incompatible blastomere. The appearance of the fluorescent peaks is typical of dinucleotide microsatellites with an intrinsic stuttering pattern generating shorter extra bands separated from the main product in steps of two nucleotides.

genotype, the mutations carried by the couple and also eventual ADO occurrences, simultaneously. The main advantage of minisequencing-based HLA genotyping is that the same procedure can be applied to any genotype combination. Moreover, the opportunity to interrogate, simultaneously, different mutation sites with optimal discrimination in the same reaction provides a useful feature that significantly reduces analysis time. Furthermore, minisequencing uses standard conditions for all templates and does not appear to be very sensitive to changes in day-to-day use.

The minisequencing-based preimplantation HLA-matching strategy described in the present study has been shown to be accurate, as confirmed by the genotyping results obtained from the reanalysis of non-transferred embryos and by prenatal diagnosis of ensuing pregnancies. The minisequencing-based HLA genotyping has also proven to be highly efficient, as it provided a high rate of positive results in the investigated blastomeres (95.9%, 255/266).

Another important feature of the above strategy is its ability to minimize errors due to ADO as more than one point of the sequence is interrogated (provided that couples are informative).

The presence of two nucleotides at one position demonstrates the presence of two PCR products, confirming the amplification of two alleles, whereas the presence of a single nucleotide, when two were expected, is the consequence of an ADO occurrence. The combined use of a multiplex STR marker system provided an additional control for contamination with exogenous DNA, as other alleles differing in size from those of the parents would be detected.

Moreover, by using STR markers, the whole HLA complex can be covered and this allows the detection of recombination events between HLA genes. Genes in the MHC are tightly linked and usually inherited in block as a haplotype, with the exception of recombination events, that occur in ~1–3% of the haplotypes. Recombination occurrences, if not detected, could represent a source of misdiagnosis in preimplantation HLA matching. Single recombination events do not result in erroneous transfer of HLA-non-identical embryos, but rather in the exclusion of these embryos for transfer. On the contrary, double recombination between flanking markers of the paternal or maternal haplotype may lead to HLA-genotyping misdiagnosis, and an embryo can be erroneously diagnosed as HLA identical. However, the risk of transferring an HLA-non-identical embryo with a double recombination event is very low (<0.01%).

Multiplex PCR of HLA STR markers could also be used as a diagnostic tool for indirect HLA matching evaluation, providing further confirmation of the HLA genotyping results obtained with the minisequencing-based HLA-genotyping procedure (Figure 4). Microsatellite markers are particularly useful for this purpose, since they may provide information on identity over a greater distance within the MHC compared to matching strictly for the classic HLA genes, making haplotypes composed of both microsatellite and HLA alleles more accurate in predicting compatibility.

In conclusion, the relative ease, sensitivity and robustness of minisequencing-based HLA genotyping, combined with the sensitivity obtained with fluorescent PCR of STR sequences, makes this technique an attractive strategy for preimplantation HLA matching. This approach is efficient, as the validations of a single assay can be used for the majority of the patients, making testing of extensive series of lymphocytes prior to each specific case unnecessary. The current data confirm the feasibility of preimplantation HLA matching as a part of PGD, providing a realistic option for couples desiring to have an HLA-compatible child for the treatment of affected siblings, although some limitations must be considered. The most important is represented by the number of embryos available for screening. A good response to hormonal hyperstimulation and a high fertilization rate are critical, as the expected probability of having HLA-identical, unaffected embryos corresponds to 3/16 (one embryo out of four is expected to be HLA identical, and three embryos out of four will be transferable as they are wild type or carry the mutation in the heterozygous state).

The possibility of applying this strategy to a variety of severe pathological conditions enlarges the number of potential candidates who may benefit from programming a pregnancy with a known HLA type. Its clinical application may represent an alternative approach to the treatment of children with haemoglobinopathies who can benefit from allogenic haematopoietic stem cell transplantation, including β-thalassaemia and leukaemia.

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