



Follow-up of chimerism status after allogeneic HLA-mismatched stem cell transplantation by detection of non-shared HLA alleles

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ABSTRACT

Background and Objectives. Chimerism studies after allogeneic transplantation are usually performed using cytogenetic analysis, PCR-VNTR or PCR-STR. Here, we report an alternative method for following the chimerism status after an HLA-mismatched stem cell transplantation (SCT), detecting the presence of non-shared HLA alleles by reference-strand mediated conformation analysis (RSCA).

Design and Methods. We tested this new approach on allogeneic related haploidentical SCT, unrelated cord blood transplantation, and HLA-mismatched unrelated donor SCT. The quantification of the chimerism was performed by laser detection of fluorescent-labeled primers on an automated DNA sequencer.

Results. In all cases this technique was able to detect mixed chimeras. The technique detected above 5% of residual cells when the analysis was based on HLA-class I and above 3% for HLA-class II. This sensitivity is similar to that of the PCR-VNTR analysis.

Interpretation and Conclusions. This method avoids the need to search for an informative locus (which is essential for PCR-VNTR or -STR). Moreover, we did not find the phenomenon of preferential amplification that is observed with most VNTR, thus avoiding the need for construction of standard curves to quantify mixed chimeras. We conclude that the detection of the non-shared HLA alleles by RSCA is a useful approach for chimerism follow-up after HLA-mismatched SCT.

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Key words: HLA-mismatch, chimerism analysis, RSCA, stem cell transplantation

Chimerism status follow-up after allogeneic stem cell transplantation (SCT) is essential to detect residual host hematopoiesis which could be responsible for relapse. The chimerism study is usually performed using cytogenetic analysis, fluorescent *in situ* hybridization (FISH) or polymerase chain reaction (PCR)-based analysis of polymorphic DNA regions such as variable number of tandem repeats (VNTR) and short tandem repeats (STR). A quantitative approach is desirable, because it now seems clear that progressive emergence of host hematopoiesis is predictive of relapse.¹⁻³

One problem in quantifying chimerism by PCR-VNTR is the phenomenon of preferential amplification: in a heterozygous sample for a polymorphism of variable length, it is possible that the shorter allele may amplify more efficiently than the longer allele.⁴ Because of this preferential amplification it is often not possible to correlate the obtained donor / host proportion to the real percentage of the donor in the analyzed sample. For this reason, it is essential to prepare amplification reconstruction standard curves in order to determine the percentage of chimerism when using PCR-VNTRs.

HLA is the most polymorphic human genetic system. Only 30% of patients who can benefit from allogeneic SCT have an HLA-matched sibling donor. For those patients lacking an HLA-identical related donor, there are alternative sources of allogeneic stem cells, such as unrelated volunteer donors, haploidentical-related donors or cord blood units. However, HLA mismatches, detected or not by the routine HLA-typing methods, are frequently involved in these transplants.⁵ These HLA mismatches are useful in following the chimerism status after SCT, avoiding the search for an informative locus, which is essential when performing PCR-VNTR or PCR-STR analysis. HLA typing methods used to follow chimerism status after transplantation include PCR-SSP and PCR-SSOP,^{6,7} but these methods are expensive, time-consuming and only allow a non-quantitative approach to mixed chimera analysis.

Reference strand mediated conformation analysis (RSCA) is a recently described technique, useful for mutation detection, as well as for HLA-typing and patient/donor HLA matching for stem cell transplantation.⁸⁻¹⁰

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Here, we report the usefulness of RSCA in the detection of non-shared HLA alleles not only for qualitative, but also for quantitative analysis of mixed chimerism after HLA-mismatched SCT.

Design and Methods

Patient and donor samples

RSCA-based chimerism studies were performed on six patients receiving an allogeneic HLA-mismatched graft. One patient was the recipient of a haploidentical related-donor SCT, three received cord blood transplants (all of them included in an experimental protocol of double cord blood transplantation), another one received an HLA-class II mismatched related donor SCT, and the last one received an allogeneic HLA-DQB1-mismatched unrelated SCT. The donors' samples were obtained prior to the stem cell collection. The cord blood DNA samples were sent by the supplier cord blood bank. Table 1 summarizes the type of transplantation for every patient, as well as the mismatched HLA locus.

DNA extraction

High-molecular weight genomic DNA was obtained from peripheral blood or bone marrow samples using the Camgen™ Mammalian Genomic DNA extraction kit (Cambridge Molecular Technologies Ltd., Cambridge, UK), according to the manufacturer's protocol. When the peripheral white blood cell count was lower than $1 \times 10^9/L$, DNA was extracted using silica membranes (QIAamp Blood and Tissue Test Kit; Qia-gen, Hilden, Germany).

HLA locus-specific PCR amplification

To perform HLA-class I locus-specific PCR, we used the primers and PCR cycling conditions described by Cereb *et al.*¹¹ The amplification was performed in a 100 μ L total volume, containing 1 μ g of genomic DNA, 10 x PCR reaction buffer (7.5 mM Tris-HCl pH 8.8, 0.2 mM $(NH_4)_2SO_4$, and 0.001% Tween), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 25 pmol of each primer and 1 U Taq polymerase.

To perform HLA-DQB1 locus-specific amplification, we used the primers and PCR conditions described at the International HLA Workshop.¹² The PCR products were resolved by 1.5 % agarose gel electrophoresis.

Table 1. Patients' characteristics.

UPN	Diagnosis	Type of SCT	HLA locus studied
1	ALL	Haploidentical related donor	HLA-B
2	CML	Double cord blood transplantation	HLA-DQB1
3	CML	Double cord blood transplantation	HLA-DQB1
4	ALL	Double cord blood transplantation	HLA-B and HLA-DQB1
5	RAEB-t	Unrelated HLA mismatched donor	HLA-DQB1
6	ALL	Related HLA-DQB1 mismatched donor	HLA-DQB1

Abbreviations: UPN: unique patient number, SCT: stem cell transplantation, ALL: acute lymphoblastic leukemia, CML: chronic myeloid leukemia, RAEB-t: refractory anemia with excess of blasts in transformation.

RSCA analysis

RSCA is a technique that allows the study of polymorphic genetic systems. The technique is based on three steps: 1) locus-specific PCR, 2) hybridization with a fluorescent-labeled reference (FLR) and, 3) polyacrylamide gel electrophoresis in a DNA automated sequencer.

The DNA sample analyzed is amplified for the desired locus, using specific primers. For HLA loci, we amplify the most polymorphic regions (exons 2 and 3 for HLA-class I loci, including intron 2, and exon 2 for HLA-class II loci). In this way, the amplified fragment has the same size for every sample, regardless of the HLA alleles present in the sample.

The concept of FLR is basic for this technique: the FLR is the PCR product obtained when amplifying a known homozygous sample for the studied locus. This PCR is performed with the same locus-specific primers and PCR conditions as for the studied samples. The only difference is that, in the case of the FLR, the forward primer is labeled with a fluorophore (Cy5), which confers fluorescent properties to this PCR product.

The alleles used as FLRs were B* 1801 and B* 4402 for the HLA-B locus, and DQB1* 0201 and DQB1* 0501 for the HLA-DQB1 locus. DNA was obtained from International Histocompatibility Workshop B-lymphoblastoid cell lines (SPO-010: HLA-B*4402 and COX: HLA-DQB1*0201) or from previously HLA-typed individuals.

Hybridization of the PCR from the samples with FLR was performed at a 3:1 ratio. The mixture was subjected to a three-step program (95°C, 55°C and 15°C). The second step facilitates the random hybridization between sense and antisense strands. This hybridization generates the formation of homoduplexes and heteroduplexes (see Figure 1). Only the combinations containing the sense strand of the FLR have fluorescent properties: the homoduplex and one for each allele present in the PCR product of the sample.

The size of the amplified PCR product and the degree of complementarity between the sense and the antisense strands determine the migration of double-stranded DNA in a non-denaturing polyacrylamide gel. When performing locus-specific amplification the size of the PCR product is constant, so the number of sequence mismatches between the FLR and the alleles present in the analyzed sample, as well as the position of such mismatches will confer a unique mobility pattern for each allele, allowing its identification.

Two microliters of the hybridization product and 6x Ficoll loading buffer were loaded in a 6 % non-denaturing Long Ranger gel (FMC Bioproducts, Rockland, Maine, USA). Electrophoresis was performed with 1 x TBE running buffer, on an ALFlexpress II automated sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden), at 30 W constant power. The running time for an 8-cm long gel was 120 minutes for HLA-class I. This electrophoresis was faster for HLA-DQB1 (80 minutes). Fluorescent peaks were detected by the laser system and analyzed using the ALFwin Fragment Analyser v. 1.00 software (Amersham Pharmacia Biotech, Uppsala, Sweden).

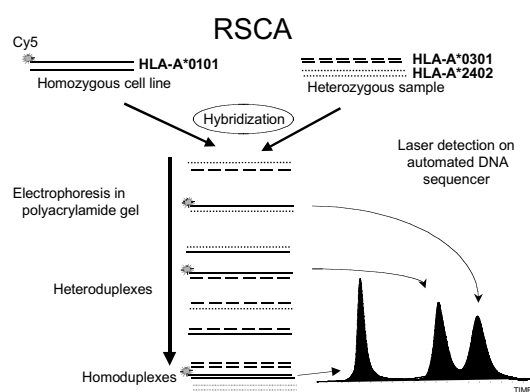


Figure 1. Schematic representation of the RSCA technique. A homozygous cell line (or individual) for HLA-A*0101 is used as a fluorescently-labeled reference (FLR). After locus-specific PCR, this FLR is hybridized with the HLA-A PCR product of the unknown sample. Only the homoduplex and the heteroduplexes corresponding to the union of the sense FLR strand and the antisense strand of each allele present in the sample are labeled with Cy5, generating a fluorescent peak after electrophoresis on polyacrylamide gel and laser detection. As each heteroduplex migrates according to the number of mismatches between the FLR and the corresponding allele, as well as to the position of such mismatches, each allele has a unique mobility pattern, allowing its identification.

PCR-VNTR amplification

The VNTR loci used for routine chimerism analysis in our laboratory are D1S80, D17S30, D4S95 and Apo-B. The amplification primers for each locus have been previously described.¹³⁻¹⁶ The forward primer is Cy5-labeled, in order to obtain a fluorescent PCR product that can be analyzed on the ALExpress II automated DNA sequencer. This approach allows the quantification of mixed chimeras, based on the fluorescent intensity of the peaks.

Quantification of mixed chimerism

The calculation of the degree of mixed chimerism based on the area of the fluorescent peaks has been previously published for PCR-STR and PCR-VNTR.^{17,18} We determined the ratio of donor and recipient by calculation of the proportion of the peak areas corresponding to donor signals as compared to the sum of peak areas of the donor and recipient signals for each locus. When donor and recipient were heterozygous, but shared one allele, only the area of the non-shared alleles was considered for the analysis. The same calculation system was used for the cell dilution assays.

Dilution assays

To explore the sensitivity of the RSCA technique in the detection and the quantification of low percentages of chimerism we performed cell dilution assays between unrelated individuals. We did not perform DNA dilutions to avoid mistakes due to the measurement of DNA concentration. We mixed selected numbers of cells from each individual to obtain dilutions from 90% to 0.5%. All of them had at least one

Table 2. Evaluation of chimerism in patients receiving allogeneic HLA-mismatched SCT.

UPN	Marker	Days after SCT	Source	% Donor	% Host	Comments
1	HLA-B	+15	PB	100	0	Engraftment
		+20	PB	95	5	Neutropenia
		+26	BM	60	40	Neutropenia
2	HLA-DQB1	+6	PB	23	70	+ 7 expanded CB
		+6	BM	20	60	+ 20 expanded CB
		+14	PB	100	0	
		+14	BM	92	8	
		+21	PB	100	0	Engraftment
		+21	BM	100	0	Engraftment
		+35	PB	100	0	
		+35	BM	100	0	
		+46	PB	100	0	
		+78	PB	100	0	
3	HLA-DQB1	+7	PB	13	4	+ 83 expanded CB
		+7	BM	4	78	+ 18 expanded CB
		+14	PB	100	0	
		+14	BM	64	36	
		+19	PB	100	0	
		+19	BM	100	0	
4	HLA-B & HLA-DQB1	+7	PB	62	38	
		+7	BM	44	56	
		+13	PB	100	0	
		+13	BM	92	8	
		+19	PB	100	0	Engraftment
		+19	BM	100	0	Engraftment
5	HLA-DQB1	+19	PB	100	0	Engraftment
		+19	BM	100	0	Engraftment
6	HLA-DQB1	+16	PB	100	0	Engraftment

Abbreviations: UPN: unique patient number, PB: peripheral blood, BM: bone marrow, CB: cord blood.

HLA disparity. We also selected an informative PCR-VNTR locus for these mixtures, in order to compare the sensitivity and accuracy of both techniques.

Results

Chimerism follow-up after SCT

Six patients undergoing HLA-mismatched allogeneic SCT were studied. Table 2 shows the chimerism data and the clinical follow-up of these patients. Chimerism status was determined by both RSCA and PCR-VNTR in all cases. There was a concordance between the RSCA results and the PCR-VNTR data.

With this technique, the reappearance (or persistence) of the HLA alleles belonging to the receptor indicated mixed chimerism. The RSCA chimerism evolution after haploidentical related donor transplant for patient #1 is shown in Figure 2.

The existence of a clinical trial of double cord blood transplantation (simultaneous transplant of a non-manipulated cord blood unit and a CD34 positive-selected and *in vitro* expanded unit) allowed us to test the ability of RSCA to detect mixed chimerism when more than two individuals are involved. We tested 3 patients included in this protocol, and in all three cases we were able to detect mixed chimeras in the first

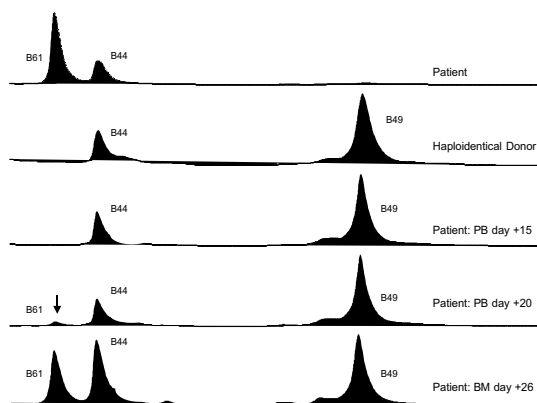


Figure 2. Analysis of HLA-B mobility pattern after allogeneic haploidentical related donor transplantation. Patient and donor share HLA-B44, but are different for the other allele (B61 for the patient and B49 for the donor). After engraftment (day +15), full chimerism is observed. On day +20, the analysis of peripheral blood shows the reappearance of the B61 allele (arrow), developing mixed chimerism. The last lane shows an increasing peak of the recipient allele in the bone marrow on day +26. FLR was HLA-B*1801. The homoduplex peak is not shown in the figure. *Abbreviations: PB: peripheral blood. BM: bone marrow.*

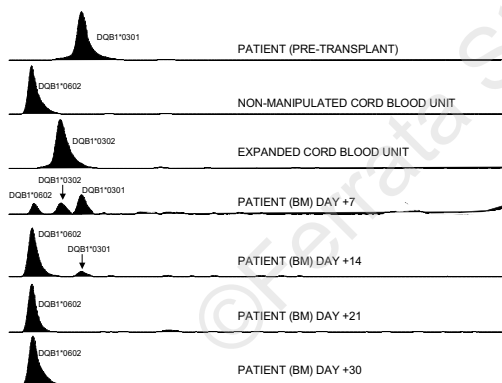


Figure 3. Analysis of HLA-DQB1 after double cord blood transplantation. HLA typing of the patient: HLA-DQB1*0301, 0501; non-manipulated cord blood: HLA-DQB1*0501, 0602; expanded cord blood: HLA-DQB1*0302, 0501. On day +7 after infusion, we were able to detect all three non-shared HLA-DQB1 alleles in bone marrow aspirate, corresponding to mixed chimerism between the patient and both cord blood units. On day +14, the allele corresponding to the expanded cord blood unit has disappeared, and the allele corresponding to the patient (DOB1*0301) had lower fluorescent intensity. The analysis of bone marrow on day +21 and +30 shows only the allele corresponding to the non-manipulated cord blood unit (full donor chimerism). FLR used: HLA-DQB1*0501. The homoduplex peak is not shown in the figure. *Abbreviations: BM: bone marrow.*

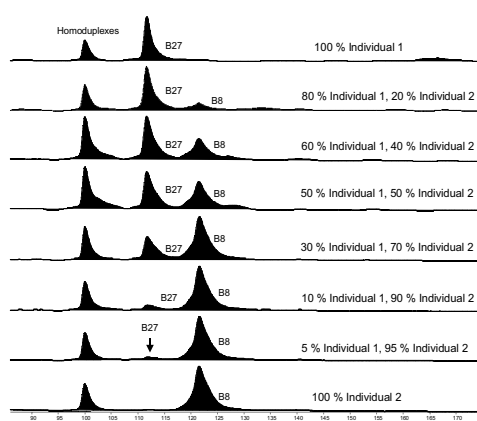


Figure 4. Electropherogram showing the RSCA pattern for HLA-B in one cell dilution assay: the area of each peak is correlated with the amount of cells corresponding to each individual. The lanes show the actual cell mixtures. The FLR used was B*4402.

days after transplantation, demonstrating the progressive evolution to a full-donor chimerism (belonging to the non-manipulated cord blood unit). Figure 3 shows the evolution of the chimerism pattern obtained by HLA-DQB1 RSCA analysis of patient #2.

The goal of this study was not to collect a high number of cases of HLA-mismatched SCT, but to demonstrate the feasibility of this technique and to determine the sensitivity of RSCA, as well as the accuracy of chimerism quantification. For this reason, we simulated different clinical situations of mixed chimerism with cell dilution assays.

Cell dilution experiments

We examined dilution steps covering the range between 0 and 100% of mixed cells from unrelated individuals.

A minor cell population corresponding to 5% was reproducibly detected after independent experiments for HLA-class I (Figure 4). If the mismatch involved HLA-class II, the technique was able to detect up to 3% of residual cells. We believe that this better sensitivity of HLA-class II is caused by the shorter size of the amplified fragment.

The reproducibility of the results was confirmed by three consecutive experiments for each informative locus. The median standard deviation from mean was 2.09% (range: 0.35-4.95) for HLA-class I, and 1.97% (range: 0.40-3.55) for HLA-DQB1. The median deviation of the calculated values from the theoretical values was 1.71% for HLA-class I (range: 0.35-3.89) and 3.59% (range: 0.16 - 6.98) for HLA-DQB1.

To obtain information about the accuracy of the RSCA method, an informative PCR-VNTR locus was selected for each dilution experiment. We compared the quantification data obtained by RSCA with those obtained by PCR-VNTR. A linear correlation was found between the proportion of mixed cells and the data obtained for HLA-A (r^2 : 0.991), HLA-B (r^2 : 0.993) and HLA-DQB1 (r^2 : 0.987). The lower coefficient of correlation obtained for HLA-DQB1 may be explained by the coamplification of the HLA-

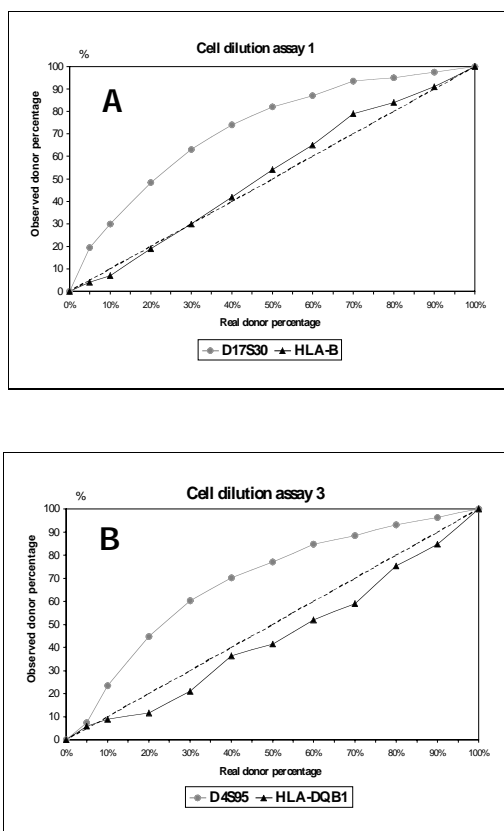


Figure 5. Cell dilution assay for quantification of mixed chimerism.

A. The graph shows the correlation between actual cell mixture and the calculated ratio of peak areas for informative loci HLA-B and D17S30 in one assay. The discontinuous line represents the expected value for the cell mixture. The percentage of donor cells obtained by calculation of the relationship between the area of the peaks corresponding to the donor and the area of the peaks corresponding to the donor and the recipient is closer to the expected value when using RSCA. This is due to the preferential amplification of short alleles of D17S30. This preferential amplification requires the construction of standard curves to quantify mixed chimeras by PCR-VNTR, whereas the observed percentage may be assumed to be the real percentage when using detection of HLA by RSCA. The coefficient of linear correlation was 0.993 for HLA-B and 0.866 for D17S30.

B. Standard curve obtained with informative loci D4S95 and HLA-DQB1 in a second cell dilution assay. The HLA-DQB1 curve is not influenced by the preferential amplification observed with the D4S95 locus. The coefficient of linear correlation was 0.987 for HLA-DQB1 and 0.891 for D4S95.

DQB2 gene with the available primers. These coefficients were higher than D17S30 (r^2 : 0.866) or D4S95 (r^2 : 0.891). Figure 5 shows the comparison of the standard curves obtained with HLA or VNTRs. The absence of preferential amplification when using the RSCA method makes it unnecessary to construct a standard curve for quantifying the chimerism for each patient/donor pair. Figure 6 shows the electropherogram obtained when comparing HLA-B and

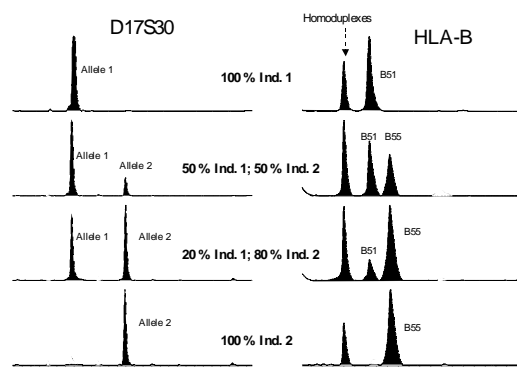


Figure 6. Comparison between PCR-VNTR and HLA-RSCA for quantitation of mixed chimerism. Electropherogram of one cell dilution assay analyzed by PCR-VNTR (D17S30) or HLA detection by RSCA (HLA-B). The D17S30 analysis shows a phenomenon of preferential amplification. The difference becomes obvious in the 50% dilution (lane 2), because the area of allele 1 is much larger than the area of allele 2. In contrast, the HLA-B analysis does not show this preferential amplification: the proportion of the peak areas corresponds to that expected (about 50%). This makes it easier to quantify mixed chimeras with the HLA-RSCA method.

D17S30 in a cell dilution assay, and how the preferential amplification only takes place with PCR-VNTRs.

Discussion

The follow-up of chimerism after allogeneic SCT is essential for the management of the patient and for identifying an increasing recipient chimerism pattern which can be predictive of relapse. At present DNA-based technologies are the methods of choice for chimerism analysis, mainly due to their sensitivity in detecting a minor clone of recipient cells after transplantation.¹⁹

The detection of HLA mismatches to monitor chimerism status has been previously described. However, conventional HLA techniques such as PCR-SSP or PCR-SSOP do not offer the possibility of a quantitative analysis. Our approach, a PCR-based method, allows the quantification of the ratio of patient/donor hematopoiesis in mixed chimerism, by calculation of the area of the fluorescent peaks. Analysis of peripheral blood samples from patients before engraftment, with fewer than $1 \times 10^9/L$ white blood cells, can be performed easily.

The feasibility of the RSCA technique in the quantification of chimeras by detection of the non-shared HLA alleles has been evaluated in clinical situations and cell dilution experiments. The sensitivity of the approach for quantitative analysis is similar to that described for PCR-VNTR or -STR (which detect up to 5% of residual cells).^{17,18} RSCA can detect and quantify up to 5% of minority cells using HLA class-I and up to 3% when using HLA class-II. This difference in sensitivity may be attributable to the different size of the PCR products (shorter for HLA class-II). Although we were not able to detect the non-shared alleles

below this level, it seems that a residual host-derived cell population after SCT would be not predictive of relapse, unless an increasing amount of these cells were observed. Our approach can detect this early progressive increase of mixed chimerism, allowing the identification of patients with high risk of relapse.

An advantage of the RSCA-based method is that, knowing the HLA alleles involved prior to SCT, there is no need to search for an informative locus, which is essential for PCR-VNTR or PCR-STR. This may be important when only a small amount of recipient DNA is available before transplantation.

Another advantage is that individual standard curves are not required for the quantification of chimerism. These standard curves are essential for quantification by the PCR-VNTR approach. This advantage is due to the lack of preferential HLA allele amplification when RSCA is used, because the amplified PCR product has the same size irrespective of the alleles present in the sample.

Moreover, as RSCA is based on a locus-specific PCR, only one set of primers (one forward and one reverse primer) is required for each HLA locus, independently of the alleles expressed by the patient and the donor. This is important, because the methods that have been used to monitor chimerism by detection of non-shared HLA alleles (PCR-SSP or PCR-SSOP) are expensive and time-consuming.

The choice of the FLR may seem to be a problem because the mobility pattern of each HLA allele depends on which FLR is used. In this way, two different alleles may have a similar pattern when using a single FLR, but their mobility can be dramatically different with a second FLR.⁸ As the pattern with two different FLRs has been previously published for each HLA-class I locus,⁹ these data can be used in order to select the FLR that offers the best separation for the alleles involved in each individual case.

A limitation of RSCA is the difficulty of performing HLA-DRB1 chimerism monitoring because the available primers amplify all the DRB chains that are present in the sample (DRB1 as well as DRB3, DRB4 or DRB5), making the electropherogram difficult to analyze. When HLA-DRB1 was present, we explored the HLA-DQB1 locus, because of the high linkage disequilibrium between these two loci, to detect extra mismatches not detected by the routine typing methods. The growth of panels in national unrelated donor registries and cord blood banks, as well as the use of haploidentical related donors could increase the number of patients receiving an alternative donor stem cell transplant. As most of these transplants are HLA-mismatched at the molecular level, for these patients, the follow-up of chimerism status using RSCA for the detection of non-shared HLA alleles is an alternative option to the classic methods.

Funding

The alloreactivity unit cooperates with the Deutsche José Carreras Leukämie-Stiftung e.V. This work was partially financed by grant JCLFAG/99, from the Fundación José Carreras para la lucha contra la leucemia. Dr. M. Rodríguez-Luaces is a recipient of grant BEFI 99/9136, from the Spanish Ministry of Health. Dr. Juan I. Aróstegui is a recipient of a grant from the Fundació Catalana de Transplantament.

Contributions and Acknowledgments

DG was the investigator involved in the design of the study and the analysis of the data. MR and JA were responsible for the laboratory management of the samples. DG, MR and JA wrote the paper. CF and JB were responsible for the clinical management of the patients included in the study. SQ was responsible for obtaining the cord blood samples. JG and AG critically reviewed the manuscript. All the authors gave their critical contribution and approved the final version of the manuscript. The authors are listed in an order reflecting their individual contribution to the article.

We thank Dr. J.A. Madrigal for encouraging us to perform the RSCA analysis. We also thank Helen Krueyer for her critical review of the manuscript.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received April 5, 2000; accepted June 20, 2000.

Potential implications for clinical practice

- ◆ This method can be used after haploidentical stem cell transplantation, cord blood transplantation or HLA-mismatched (related or unrelated) donor stem cell transplantation. It will offer another tool to follow the chimerism status after these transplants.

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