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Polymorphisms for the size of heterochromatic regions allow sex-independent quantification of post-BMT chimerism targeting metaphase and interphase cells

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ABSTRACT

Background and Objective. Fully quantitative cytological techniques for the analysis of hemopoietic chimerism are very limited and largely restricted to sex-chromosome detection after sex-mismatched bone marrow transplants (BMTs). The aim of the present investigation was to assess the usefulness of autosomal polymorphisms for the size of heterochromatic regions in the identification of donor and recipient cells and therefore in the quantification of the hemopoietic chimerism after sex-matched BMT.

Design and Methods. Hemopoietic chimerism was followed up in 3 transplanted patients targeting a polymorphism for the size of the pericentromeric heterochromatin (PCH) of chromosome 9, uncovered by restriction endonuclease (RE) *in situ* digestion (REISD) with the RE *Sau*3A, to differentiate donor and recipient cells on conventional bone marrow chromosome preparations.

Results. The polymorphism for the size of the PCH of chromosome 9 allowed differentiation of donor and recipient cells targeting both metaphase and interphase nuclei. The misidentification error for the polymorphism for the size of HPC of chromosome 9 was estimated as 1% for metaphases and 6-11% for interphases. The 3 cases studied showed complete chimerism in the first post-BMT sample analyzed, which was maintained in 2 of them. One patient relapsed and showed transient mixed chimerism. One month later, this patient achieved a second complete remission, showing complete chimerism again. In this patient, who received a sex-mismatched BMT, chimerism was also quantified by sex-chromosome identification using established methods, such as conventional cytogenetics and FISH, and the results obtained were similar to those rendered by *Sau*3A-REISD.

Interpretation and Conclusions. The polymorphism for the size of the PCH of chromosome 9 uncovered by *Sau*3A-REISD allows accurate quantification of the hemopoietic chimerism after sex-matched BMT.

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The biological and clinical significance of the hemopoietic chimerism achieved after an allogeneic bone marrow transplantation (BMT) is a matter of active research. Post-BMT hematopoietic chimerism appears to influence the outcome of the BMT; either failure or rejection of stable marrow engraftments, development of graft-versus-host disease (GvHD), or relapse of disease.[1,2] Although semi-quantitative DNA based methodology has been successfully used to assess post-BMT chimerism,[3] fully quantitative cytological techniques are very limited and largely restricted to sex-chromosome detection after sex-mismatched transplants.[4]

We have recently developed a quantitative sex-independent method, based on the detection of cryptic polymorphisms of highly repeated DNA sequences by restriction endonuclease (RE) *in situ* digestion (REISD), which is able to discriminate between donor and recipient cells.[5] One of the REs assayed, *Sau*3A, is particularly useful for this purpose. *Sau*3A extensively digests fixed human chromosomes producing a conspicuous banding pattern involving pericentromeric heterochromatin (PCH) of chromosomes 3 and 9.[6] While the PCH of chromosome 9 is never digested by this RE, that of chromosome 3 can be digested or not and, therefore, three different karyotypes can be observed: $9^{++}3^{--}$, $9^{++}3^{+-}$ and $9^{++}3^{++}$. These karyotypes can be identified not only in metaphase but also in interphase nuclei from peripheral blood (PB) and bone marrow (BM) samples.[5] If donor and recipient cells show a different digestion pattern after *Sau*3A-REISD, the origin of each cell post-BMT can be determined and the chimerism in the transplanted patient may thus be monitored. Our results show that around 25% of patients are amenable to post-BMT chimerism analysis by this approach. Therefore, hematopoietic chimerism still cannot be accurately quantified in

a high percentage of sex-matched BMT recipients. The development of alternative quantitative methods to identify the donor/recipient origin of hemopoietic cells when their sex-chromosomes do not differ, would represent an advance in the accurate analysis of chimerism.

We report here a technique based on *Sau*3A-REISD that reveals a polymorphism for the size of the PCH of chromosome 9 which allows sex-independent identification of donor/recipient metaphase and interphase cells.

Design and Methods

Peripheral blood (PB) and bone marrow (BM) chromosome preparations were obtained by standard procedures from 3 patients (pre- and post-BMT) and their donors (Table 1). REISD was performed at 37°C for 16 h in a moist chamber with 30 units (6 µL) of the restriction endonuclease *Sau*3A ($^{\downarrow}$GATC) (Boehringer Mannheim) in 50 µL of incubation buffer per slide. After REISD, the slides were stained with 4,6-diamidino-2-phenyl indole (DAPI). FISH was performed following the conditions suggested by the supplier (Oncor Inc.). Two different digoxigenin conjugated DNA probes (Oncor Inc.) were used: an alphoid-classical satellite DNA cocktail probe specific for the Y chromosome (DYZ1-DYZ3) and a chromosome 9 specific classical satellite DNA probe (D9Z1). The bound probe was detected by incubation with anti-digoxigenin antibodies FITC conjugated (Oncor Inc.) and chromosomes were counterstained with propidium iodide.

The error rate for the identification of the polymorphism in metaphase and interphase nuclei was determined on pre-BMT samples from patients and donors by analyzing 50 metaphases and 300 interphases.[5] The error rate in metaphases was 1% (percentage of cells with a banding pattern not coincident with the majority) in all cases. In interphases, on the other hand, the estimates differed among individuals, ranging between 6-11% (percentage of cells with a banding pattern not coincident with that determined on metaphases), but were constant in PB and BM samples of each individual (Table 1).

Patients whose post-BMT percentage of residual recipient cells was statistically different (test for the equality of two percentages[7]) from the intrinsic error estimated for metaphase or interphase nuclei were considered to have a mixed hemopoietic chimerism. Complete chimerism was considered to have occurred in patients with a percentage of recipient metaphases or interphases not significantly different from the determined intrinsic error.

Table 1. Characterization of patients and donors with Sau3A-REISD and rate of misidentification (error rate) of the polymorphism for the size of the PCH of chromosome 9 on interphase cells from each individual studied.

Patient		Sex	Sau3A	PCH-9*	Error° (%)
#1	R	M	9++3--	S/S	8
	D	F	9++3--	S/L	6
#2	R	M	9++3--	S/L	11
	D	M	9++3--	S/S	8
#3	R	M	9++3--	S/L	7
	D	M	9++3--	S/S	6

*R; recipient; D; donor; F; female; M; male. + and –; respectively, positive and negative pericentromeric band revealed by Sau3A-REISD. *size of the pericentromeric heterochromatic band on chromosome 9. S; Small. L; Large. °rate of misidentification of the targeted polymorphism on interphase cells (see text).*

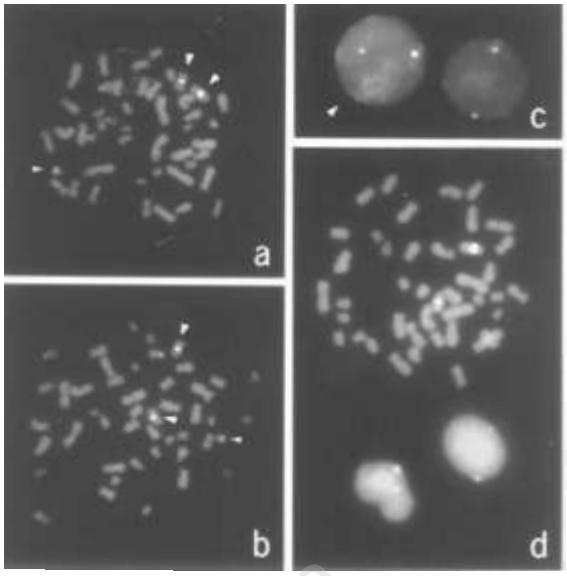

Figure 1. Sex-independent chimerism quantification using polymorphisms for the size of heterochromatic regions.
(a) DAPI-stained metaphase plate from patient #2 characterized as $9^{++}/3^{--}$ by Sau3A-REISD in which both chromosomes 9 show a conspicuous difference in the size of their PCH positive band (white arrows).
(b) Metaphase from the donor of patient #2, also characterized as $9^{++}/3^{--}$ by Sau3A-REISD, in which both chromosomes 9 show a similar small positive band (white arrows).
(c) The difference in the size of the PCH of chromosome 9 permits identification of recipient (arrow) and donor cells even targeting interphase nuclei.
(d) The difference in the size of the PCH observed in the chromosomes from patient #2 is also observed by FISH using a chromosome 9 specific classical satellite DNA probe (D9Z1). Note that both donor and recipient were men (the grey arrows in (a) and (b) point to the Y chromosomes).

Results

The 6 individuals included in this study (3 patients and their donors) were karyotyped as $9^{++}3^{--}$ by *Sau*3A-REISD, i.e. only the PCH of chromosome 9 remained undigested. Patients #2 and #3, as well as the donor

of patient #1, showed a marked difference in the size of the PCH between both homologues of chromosome 9 (Figure 1a; Table 1). This difference was not observed in the donor/recipient partner in either of the cases (Figure 1b; Table 2). Chimerism quantification using this polymorphism to identify donor and recipient cells (Figure 1c; Table 2) showed complete chimerism in the first post-BMT samples obtained from the 3 patients. This status was maintained in two patients (#2 and #3) who were in complete remission. Patient #1 evolved to a mixed chimera 11 months after the transplant as the disease relapsed. In this patient, the relapse was successfully treated with donor lymphocyte infusion and complete chimerism was again achieved (Table 2).

Post-BMT chimerism quantification in patient #1 (who received a sex-mismatched BMT) using the sex chromosomes as markers of cell origin, both by conventional cytogenetics and FISH, rendered similar results to those obtained with *Sau*3A-REISD (Table 2).

FISH using chromosome 9 specific classical satellite sequences (Oncor Inc.) to probe a pre-BMT PB sample from patient #1 showed a polymorphism for the size of the hybridization signals similar to that uncovered by *Sau*3A-REISD. In this case, the rate of misidentification of the polymorphism on interphase nuclei was estimated as 2%.

Discussion

Different studies have pointed out the extreme variability that characterizes the size of heterochromatic regions in most species including humans.[8] Such polymorphisms can be revealed by C-banding and have been recently used to differentiate donor and recipient cells in an attempt to quantify post-BMT chimerism.[9] However, although C-banding allows the identification of metaphase cells from donor and recipient, the possible polymorphisms cannot be identified on interphase nuclei, due to the large number of positive bands produced by the technique. This considerably reduces the usefulness of C-like banding patterns for hematopoietic chimerism studies, considering that the number and quality of metaphases in post-BMT BM samples is usually low. In this sense, possible polymorphisms for the size of heterochromatic regions uncovered by REs such as *Sau*3A, which reveals a low number of chromosome regions, could be used to differentiate donor and recipient cells even when interphase nuclei are targeted.

In the 3 patients included in this study, post-BMT chimerism could not be determined on the basis of the polymorphism uncovered by *Sau*3A-REISD on chromosome 3 (Table 1). Moreover, two patients (#2 and #3) received a sex-matched BMT, which made it impossible to perform chimerism analysis using the sex chromosomes as markers of cell origin. Patients #2 and #3, as well as the donor of patient #1, showed a conspicuous difference in the size of the PCH between both homologs of chromosome 9 (Figure 1a; Table 1). This difference was not observed in the donor/recipient partner in either of the cases (Figure 1b; Table 2). In these patients, the polymorphism for the size of the PCH on chromosome 9 was successfully used to differentiate donor and recipient cells, and thus to document engraftment and quantify post-BMT hematopoietic chimerism (Figue 1c; Table 2).

In order to have an external control of the quality of the approach proposed, the sex chromosomes were used for chimerism quantification in the sex-mismatched BMT case (patient #1). In the 3 post-BMT samples analyzed, similar results were obtained when hematopoietic chimerism was quantified with *Sau*3A-REISD and with established methods such as conventional cytogenetics and FISH (Table 2). It can be concluded, therefore, that the proposed method is useful for accurately quantifying post-BMT chimerism, even

Table 2. Post-BMT chimerism quantification in the 3 patients studied using the size of the PCH on chromosome 9 to determine the donor/recipient origin of hematopoietic cells. Identification of sex chromosomes by conventional cytogenetics and FISH in the sex-mismatched BMT case (patient #1) was used as control of the proposed method.

			Post-BMT chimerism°					
					Sau3A			
Patient	Sex R/D	Months post-BMT	Cyto. XY Meta.	FISH XY Inter.	Meta.	Inter.	Chimerism status	Disease status
#1	M/F	+4	0/20 (0%)	6/272 (2.2%)	2/20 (10%)	18/211 (8.5%)	CC	CR
		+11	2/20 (10%)	144/400 (36%)*	4/25 (16%)*	65/235 (27.7%)*	MC	Rel.
		+12	0/25 (0%)	12/300 (4%)	1/25 (4%)	12/246 (4.9%)	CC	CR
#2	M/M	+1			1/30 (3.3%)	8/217 (3.7%)	CC	CR
		+3			0/25 (0%)	11/250 (4.4%)	CC	CR
#3	M/M	+3			0/20 (0%)	6/232 (2.6%)	CC	CR
		+11			1/30 (3.3%)	15/228 (6.6%)	CC	CR

*R: recipient; D: donor; F: female; M: male; mo.: months; Cyto.: conventional cytogenetics (GTG banding); Meta.: metaphases; Inter.: interphases; CC: complete chimerism; MC: mixed chimerism; CR: complete remission; Rel.: relapse. °Recipient cells/Total cells studied (percentage). *Result significantly different from the intrinsic error determined (see text).*

targeting interphase cells. This method has the advantage over other cytological approaches of being applicable after sex-matched BMTs, although it has a higher error rate than FISH for the Y chromosome (1-2% in our laboratory).

Although differences in the size of the PCH between homologous chromosomes are common, they are usually not conspicuous enough to be accurately identified on interphase cells by the naked eye. In fact, in the samples we have analyzed (25 BMT cases), seven cases (28%) showed differences between patient and donor in the size of the PCH of chromosome 9. However, such differences could be consistently identified on interphase cells, even by unexperienced observers, only in 3 of them (12%). In any case, we are developing an image processing and analysis (IPA)-assisted procedure which has already proven able to discriminate between donor and recipient cells when small differences in the size of PCH regions are involved. This approach would allow chimerism quantification in those cases which cannot be studied by the naked eye. Even in those cases in which the naked eye does allow identification of donor/recipient cells, the IPA-assisted procedure proves more accurate and considerably reduces the error rate for the identification of the targeted polymorphisms.[10]

Finally, a second FISH experiment was performed on a pre-BMT PB sample from patient #1 using chromosome 9 specific classical satellite sequences (Oncor Inc.) as the probe. The results obtained (Figure 1d) demonstrated that the polymorphism for the size of the PCH of chromosome 9 is caused by a variation in the amount of such sequences. Furthermore, the identification of the targeted polymorphism is more accurate using this approach (error rate 2%) than using *Sau*3A-REISD. These results open up new possibilities for the study of post-BMT hematopoietic chimerism since polymorphisms for the size of the PCH of different chromosomes can be targeted using the appropriate probes to quantify accurately the post-BMT chimerism, even on interphase nuclei.

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IB, JLD-M, JG: design of the study and assessment of the patients; JLD-M, CL-F, JG: partial funding of the study; JLD-M, CL-F, JG: direct supervision, recruitment of patients, contact with participants; IB, JLF: laboratory work and data collection; JLD-M: clinical data collection and data handling; IB, JLD-M, JG: interpretation of the results; IB: writing of the paper; IB, JLD-M, JG: reviewing of the manuscript; all authors: final approval of the definitive version. The order of the authors has been decided on the basis of their dedication to the project, except for the last author, who is the head of the laboratory. The authors wish to thank N Polo for her excellent technical assistance.

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Disclosures

Conflict of interest: none.

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