Chimerism analysis – critical diagnostic tool for the outcome assessment of allogeneic hematopoietic stem cell transplantation

Cristian Jinca[1*], Valentin Ordodi[2], Smaranda Arghirescu[1], Jijo John[3], Anca Isac[3], Luca Dehelean[3], Margit Şerban[1]

1. University of Medicine and Pharmacy „V. Babes”, IIIrd Clinic of Pediatrics, Center for Bone Marrow Transplantation
2. University of Medicine and Pharmacy „V. Babes”, Department of Biology, County Hospital no. 1, Center for Transplant Immunology,
3. Emergency Children's Hospital „L. Turcanu“, IIIrd Clinic of Pediatrics, Center for Bone Marrow Transplantation

Abstract

Purpose of the study. *The method used for the investigation of the presence of donor cells in the host after allogeneic hematopoietic stem cell transplantation (HSCT) is represented by the chimerism analysis. In this retrospective study, the objective was to evaluate the predictive value of chimerism analysis for the post-transplant outcome.* ***Material and methods.*** *The study was conducted on a lot of 16 patients who underwent allogeneic HSCT in the Centre for Bone Marrow Transplantation Timişoara. Chimerism was prospectively analyzed on days: + 30, +90, +180, + 270, +365 post-transplant. In six patients the investigation was done by fluorescent in situ hybridization whereas the rest were analyzed by means of real-time PCR. One patient died prior to the first chimerism assessment.* ***Results.*** *In 7 patients, the evolution towards a complete donor chimerism occured within the first six months post-transplant. In 3 patients, the last performed analysis revealed a progressive varied chimerism at least for one lineage, pattern which turned out to be highly predictive for the unfavorable outcome for two of them. Granulocytes, monocytes and CD 19 lymphocytes were 96 – 100 % donor cells already on day + 30 in the majority of patients.* ***Discussion and conclusions.*** *The two methods used for the assessment of chimerism offer predictive information for the outcome. Our patients with progressive mixed chimerism presented an unfavorable clinical outcome as compared to those with a full donor chimerism who were in complete remission at the time of evaluation.*

Keywords: *Hematopoietic stem cell transplantation, chimerism, polymerase chain reaction, fluorescent in situ hybridization.*

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[*]**Corresponding author:** Dr. Cristian Jinca, University of Medicine and Pharmacy „V. Babes”, IIIrd Clinic of Pediatrics, Center for Bone Marrow Transplantation, Str. I. Nemoianu, 2, 300011 Timişoara
Tel: 0256-295977, Fax: 0256-295978, E-mail: cristian_jinca@yahoo.com

Rezumat

Obiectivul studiului. *Investigarea după un transplant de celule stem hematopoietice (TCSH) alogenic a prezenței celulelor transplantate de la donator la receptor se efectuează prin analiza chimerismului. Prezentul studiu retrospectiv își propune evaluarea utilizării chimerismului ca analiză cu valoare predictivă a evoluției pacienților post-transplant.* ***Material şi metodă.*** *Studiul a fost efectuat pe un lot de 16 pacienți cu TCSH alogenic din Centrul de Transplant Medular Timişoara. Chimerismul a fost efectuat prospectiv în zilele: + 30, +90, +180, + 270, +365 post-transplant. La şase pacienți s-a aplicat hibridizarea fluorescentă in situ (FISH), în celelalte cazuri chimerismul a fost analizat prin real-time PCR.* ***Rezultate:*** *La 7 pacienți evoluția spre un chimerism complet de donator s-a produs în primele şase luni post-transplant. La alți 3 pacienți, ultima analiză efectuată a evidențiat un chimerism mixt progresiv cel puțin pentru o linie celulară, pattern evolutiv care s-a dovedit a avea o mare valoare predictivă pentru evoluția ulterioară nefavorabilă în cazul a doi dintre ei. Restul pacienților au prezentat un chimerism mixt pasager care a prefigurat apoi un chimerism complet. Granulocitele, monocitele şi limfocitele CD19 au fost de origine donatoare într-o proporție de 96 - 100% deja în ziua +30 la majoritatea pacienților.* ***Discuții şi concluzii.*** *Cele două metode aplicate pentru investigarea chimerismului la pacienții noştri, au avut o valoare predictivă înaltă. În acest context am observat că pacienții care au prezentat un chimerism mixt progresiv au avut o evoluție clinică nefavorabilă în timp ce pacienții care au prezentat un chimerism complet sunt în remisiune completă.*

Cuvinte cheie: *Transplant de celule stem hematopoietice, chimerism, reacția de polimerizare în lanț, hibridizare fluorescentă in situ..*

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT), using the peripheral blood, bone marrow or cord blood as its source, has a critical role in the therapeutic practice of many malignant or nonmalignant, congenital or hereditary diseases, serving as the salvage therapy for all these diseases. Chimerism is the term that defines the presence of donor cells in the bone marrow or peripheral blood of HSCT recipients. Chimerism is quantifiable from the donor and recipient cell proportion present in the recipient's organism at any moment following the transplant procedure. The importance of the chimersim exploration post HSCT lies not only in the capacity of the method to furnish information absolutely necessary for establishing the optimal prophylaxis regime of graft versus host diseases (GvHD) through immunosuppression but also especially in the early diagnosis of the failure or the graft rejection. Chimerism assessment is carried out from all cells (global chimerism) or following an initial sorting of leukocytes through fluorescence-activated cell sorting (FACS), the so-called lineage specific chimerism. The latter is the elective method in the analysis of mixed chimerism. The present study aims at a retrospective evaluation of chimerism in allogeneic transplant patients at the Bone Marrow Transplantation Center, Timisoara. This factor was correlated with the clinical evolution of patients.

Material and methods

This retrospective observational study included 16 patients with allogeneic HSCT, transplanted during the period 2003-2008 at the Bone Marrow Transplant Center, Timisoara. The pathology for which these patients underwent allogeneic transplant is categorized as follows: predominantly represented by acute myeloid leukemia (AML – 6), acute lymphoblastic leukemia (ALL - 5), chronic myeloid leukemia (CML - 1), very severe aplastic anemia (SAA - 3) and chronic granulomatous diseases (CGD - 1). One patient with ALL died prior to day +30 (*Table 2*).

The characteristics of patients enrolled in this study are illustrated in *Table 1*. During

Table 1. Characteristics of patients enrolled in the study

Parameter	Number (percentage)
Number of patients	16
Mean age	20.37
Gender of donors (male/female)	11/5 (68,75% / 31,25%)
Gender of recipients (male/female)	10/6 (62,5%/37,5%)
Diagnosis:	
ALL	6 (37,5%)
AML	5 (31,25%)
CML	1 (6,25%)
SAA	3 (18,75%)
CGD	1 (6,25 %)
Source of stem cells:	
Peripheral blood stem cells (apheresis)	13 (81,25 %)
Bone marrow	3 (18,75 %)
Conditioning regimen:	
Bu-Cy-ETO	5 (31,25%)
Bu-Cy	7 (43,75 %)
Cy-ATG	1 (6,25%)
Cy-Fludara-ATG	2 (12,5%)
Bu-Cy-Campath	1 (6,25%)
Immunosuppression:	
Pre-transplant (conditioning)-	
ATG	3 (23,1%)
Campath	1 (7,7%)
Post-transplant (cyclosporine A+methotrexate)	16 (100%)
Pre-transplant clinical status:	
CR1	4 (25%)
CR2	8 (50%)
Partial remission/progression	4 (25%)
Survival	
Alive	13 (81,25%)
Deceased	3 (18,75%)

Legend: *Bu = busulfan; Cy = cyclophosphamide; ETO = etoposide; ATG = anti-human activated T-lymphocyte globuline; CR1 = complete remission 1; CR2 = complete remission 2*

the time of transplant, 2 patients were below 5 years, 6 patients were in the 5 – 10 age group, 1 patient in the 10-15 age group and 7 patients were over the age of 15 years. All leukemia patients were in complete remission at the time of transplant and HLA histocompatibility in all the cases was 100%.

The method consisted of the analysis of peripheral blood samples, some of them after a prior sorting of the leucocyte subsets by FACS. Chimerism was prospectively analyzed by real-time PCR in donor-patient pairs of the same gender and by FISH in gender mismatched pairs. Timepoints for analysis were days + 30, +90, +180, + 270, +365 post-transplant.

The real-time PCR method permits the analysis of chimerism even in the situation of a small number of cells and it may also provide information on engraftment kinetics in the early post-transplant phase when there is hardly morphological evidence of engraftment (3). The procedure based on the TaqMan technology is done by inserting a sequence specific hybridization probe between forward and reverse

primers. The fluorogenic probe is cleaved during the extension phase and emits a fluorescent signal analyzed by the thermocycler. A PE 9700 thermocycler was used. The DNA of 9 donor - patient pairs was extracted from peripheral blood, in 7 cases sorting of the different cell lines was performed prior to chimerism analysis. Purity of the extracted DNA sample was assessed by determination of optical density at 260 – 280 nm. PCRs were analyzed using an ABI PRISM 7700 (Applied Biosystems) apparatus. 6 – carboxifluoresceine (FAM) and 6 – carboxitetrametil-rodamine (TAMRA) served as fluorescent dyes. For each sample an amplification curve was plotted by the ABI PRISM 7700 software (Applied Biosystems). The reaction mix consisted of 250 ng of DNA mixed with 20 μL of Master Mix 2x buffer, 200 nM of TaqMan probe and 600 nM each primer in a volume of 40 μL. The PCR cycles were: 2 minutes at 50°C, 10 minutes at 95°C and 40 amplification cycles consisting of 45 seconds at 95°C and 60 seconds at 60°C. In order to evaluate the validity of the method, standard amplification curves were generated for donor and recipient specific allele PCRs out of chimeric DNA probes artificially obtained by serial halved dilutions of recipient DNA in donor DNA and donor DNA in recipient DNA respectively. Donor and recipient were genotyped before quantification using typing trays, stored at – 20°C, containing primers and probes for the genetic markers used also in the quantification assay, in the same concentrations. Moreover, a negative well was included in each tray and genotyping was performed by adding 100 ng of DNA in each well. An allele was considered informative when it was positive on recipient DNA and negative on donor DNA or vice versa.

FISH analysis was performed on interphasic nuclei, obtained from the peripheral blood samples of 6 patients with allogeneic HSCT in the pre- and posttransplant phase and from pre-transplant donor samples. The working protocol mentions running hemolysis in 1-2 ml of blood by adding erythrolysis solution. After applying a treatment with normal saline, the nuclei thus prepared were fixated with a fixating mixture at –20°C. The interphasic nuclei palette was resuspended in an appropriate density and exposed to heat and humidity. The drying of the slides was followed by the digestion with pepsin, after which the slides were washed with phosphate buffer saline and subsequently treated in cold formaldehyde (4°C) in dark for 10 min. Furthermore, different concentrations of ethanol are used for 2-3 min after which the slides were dried. The patient's DNA was denatured chemically at 73°C after which the ethanol (-20°C) is used in the different concentrations (70%, 85%, 95%) to prevent the renaturation process. CEP XY dual-color (Vysis Inc.) genetic tubes were used for X and Y chromosomes, followed by hybridization with the patient's DNA until day two in dark and humid conditions (37°C). The slides were treated with 0.4 X SSC at 65°C, washed with washing solution and rinsed with water, and later dried. Contrast substance (Dapi) was applied on the slide. Analysis was done with a microscope with florescence on a set of 500 nuclei.

Definitions for the results section:

According to the proportion of the donor cells, we define:

- *complete chimerism* (CC) – status in which all the lympho-hematopoietic lineage of the host is derived from the donor.
- *partial or mixed chimerism* (CM)- defined as the concomitant presence of own cells as well as that of the donors in different lympho-hematopoetic compartments of the host
- *dissociated chimerism* (CD) in which some of the hematopoietic cellular lineage is completely derived from the host while others from the donor (1, 3).

Mixed chimerism is classified as:

- *transient or decreasing mixed chimerism* (TMC) – mixed chimerism profile in which

Table 2. Evolution of chimerism

No.	Patient	Diagnosis	Gender recipient/ donator	Type of chimerism					Clinical outcome
				Day + 30	Day +90	Day +180	Day +270	Day +365	
1	R.A.	LAL	M / F	TMC	TMC	TMC	TMC	CC	CR
2	T.D.	AAS	M / M	TMC	TMC	TMC	PMC	PMC	relapse
3	G.G.	LAM	M / M	TMC	TMC	CC	TMC	CC	CR
4	V. S.	LAL	F / F	TMC	PMC	#	#	#	relapse
5	M. L.	AAS	F / M	TMC	CC	TMC	PMC	CC	CR
6	M. S.	LAM	M / F	TMC	TMC	TMC	CC	CC	CR
7	A.A.	BGC	M / M	TMC	TMC	PMC	#	#	CR
8	F.F.	LAM	F / M	TMC	TMC	TMC	#	#	CR
9	P.R.	LAM	F / M	TMC	TMC	CC	#	#	CR
10	S. A.	LAM	F / F	TMC	TMC	CC	#	#	CR
11	B. A.	AAS	M / F	TMC	#	#	#	#-	CR
12	T.M.	LAL	M / M	TMC	TMC	#	#	#	CR
13	P. O.	LAM	M / M	TMC	CC	#	#	#	CR
14	P.A.	LAL	M / M	TMC	TMC	CC	#	#	CR
15	B. A.	LMC	M / M	TMC	CC	CC	CC	CC	CR
16	P.I.	LAL	F / F	*	*	*	*	*	early death

Legend: *CC = complete chimerism; CR = complete remission; TMC = transient mixed chimerism;*
** = death before day +30; # = not yet analyzed.*

a small percent of the cells(1-5%) with host origin is detected in the organism in the first six months post transplant

- *stable mixed chimerism* (SMC) –in which a percent of the cells (1 – 20%) are of the host origin and remain detectable at a constant level for a long time.
- *progressive or increasing mixed chimerism* (PMC) – in which a proportion of the cells of host origin increases with time by more than 5 - 10% (3, 7) .

Results

The majority of transplanted patients in our center presented TMC during the first analysis done at 30 days post transplant; chimerism that progressed during the following six months towards a complete donor chimerism in 7 patients. In 3 patients, the last analysis showed a PMC for at least one cell lineage; among two of these patients such a progressive pattern had a very significant early predictive value for a later stage unfavorable outcome. One patient could not be evaluated due to death before +30 day post transplant. The rest of the patients presented TMC which prefigured a CC (*Table 2*). The evolutive pattern of chimerism, from a TMC towards a complete donor chimerism was different from one patient to the other. Taking into consideration the evolution of chimerism for granulocytes (GRA), monocytes (MONO) and lymphocytes CD19+, we observed in 12 out of the 15 patients who could be evaluated, that these were of the donor origin with a proportion of 96-100% already at 30 days post transplant whereas a different pattern was observed in T lymphocytes CD4+ and CD8+, as well as with natural killer cells CD16+CD56+. *Figures 1- 6* depict each type of pathology.

Discussion

The quantitative follow-up of post-transplant chimerism can provide early information about engraftment or non-engraftment, re-

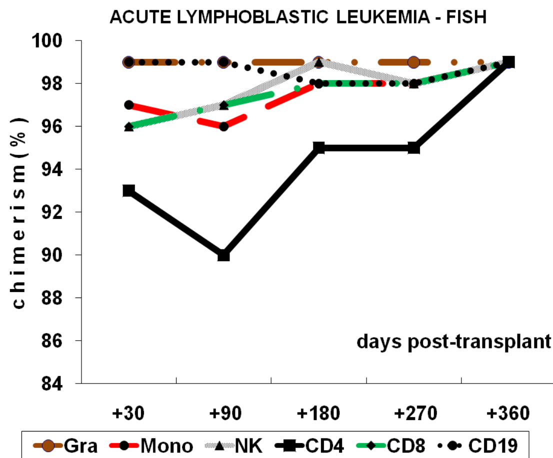

Figure 1. Patient R.A. (lineage specific chimerism, FISH)

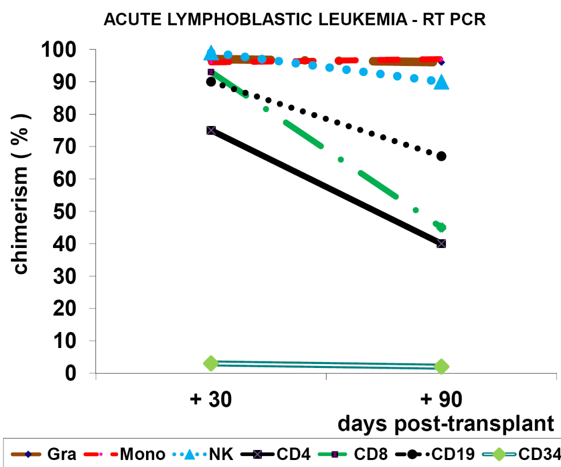

Figure 2. Patient V. S. (lineage specific chimerism, RT-PCR)

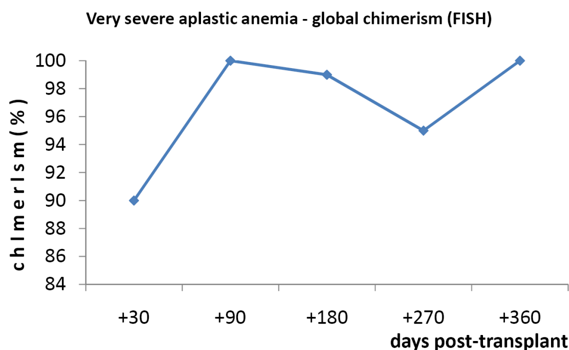

Figure 3. Pacient M. L. (global chimerism, FISH)

ject or relapse (1, 3, 8). The results point out the fact that CD4+ T lymphocytes had the slowest evolution of chimerism and TMC maintained for a period up to 6 months post-transplant. Two of the patients had a particular evolution of the CD4+ chimerism. One of the patients with allogeneic bone marrow transplant for CGD (*Figure 5*) presented at 90 days post-transplant 93% donor CD4+ lymphocytes followed at day +180 by a PMC with a proportion of 89% donor cells. Figure 6 presents the evolution of the chimerism of a patient with AML, who presented a particular pattern with complete donor chimerism for granulocytes, monocytes and NK cells at 30 days post-transplant whereas the complete chimerism for CD8+ lymphocytes and CD 19+ lymphocytes was reached at day +90 and for CD4+ only at day +180. After the complete chimerism was achieved, the clinical evolution of the patient was very good. The patient currently is in complete remission at 5 years post-transplant.

Figures 2 and 4 will be discussed in detail because in these situations the evolution of chimerism was correlated with the clinical manifestation of the disease and constituted a predictive parameter for the unfavorable clinical evolution. *Figure 2* refers to a case of ALL that presented a PMC concomitant with a post-transplant bone marrow and CNS relapse and illustrates the decrease of all leukocyte series beginning with day 30 +, a pattern of evolution that suggested an early failure of the graft or an imminent graft rejection. Further clinical evolution confirmed the prediction with a relapse of leukemia at 3 months post-transplant. The suggestive chimera constellation allowed the analysis of CD34+ progenitor cells chimerism which revealed only 3% donor population of CD34+ at 30 days post-transplant .

Figure 4 presents the state of the chimerism of one patient with SAA. It is known that the majority of aplastic anemia cases with HSCT from related HLA matched donor develop only a mixed chimerism (2, 4, 5). The dy-

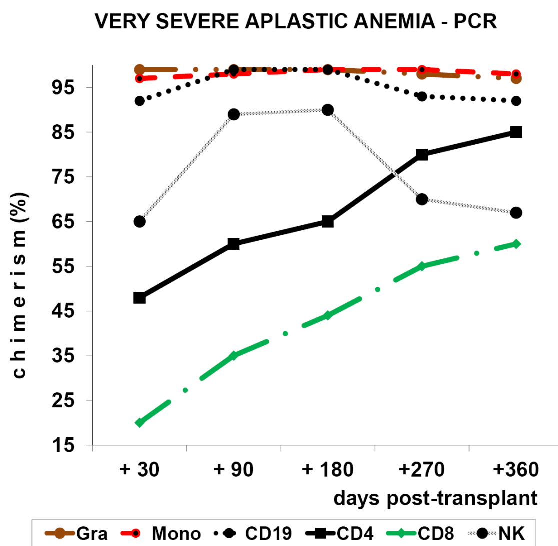

Figure 4. Pacient T. D.
(lineage specific chimerism, RT-PCR)

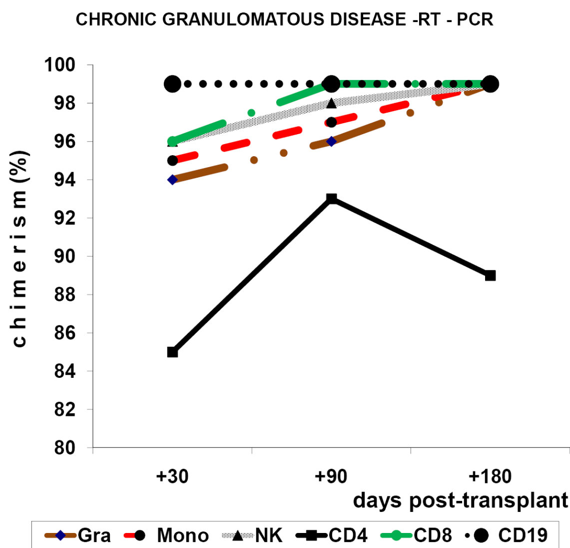

Figure 5. Patient A. A.
(lineage specific chimerism, RT-PCR)

namic investigation of the chimerism is motivated by the clinical practice. There are some patterns with different predictable value. As such, a decreased donor CD8+ lymphocyte population suggests the risk of rejection associated with a decreased risk of GVHD and vice versa, while a decreased donor CD3+ CD4+ and CD34+CD38- population is significant for the

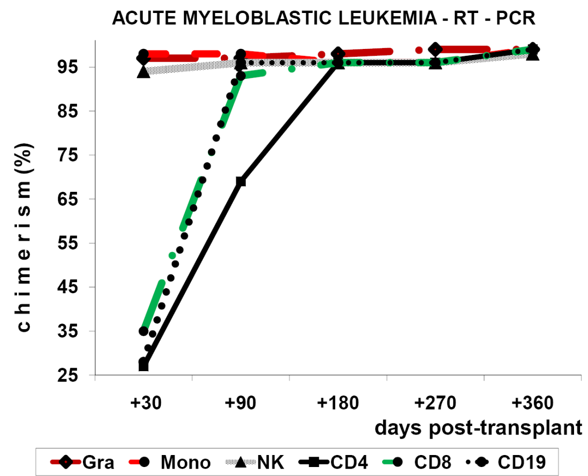

Figure 6. Patient G.G.
(lineage specific chimerism, RT-PCR)

risk of graft failure (1, 2, 3, 8). We noticed in our case a very slow ascending evolution of a mixed chimerism towards a TMC for CD4+ and CD8+ lymphocytes. There was a concomitant evolution to a PMC for NK and CD19+. This pattern correlated with a graft failure and the decrease of hematologic parameters with pancytopenia at 14 months post-transplant.

Our results confirm findings from specialty literature, the long term success of a transplant could be ascertained by reaching the status of complete donor chimerism for the CD4 and CD8 T lymphocyte and NK cells population(3, 4, 5). Both methods employed in the investigation of chimerism were equally accurate and informative as found in data published in literature (6).

In the classic concept of HSCT, the transplant is preceded by a myeloablative conditioning regimen which is expected to achieve the complete destruction of the recipient's hematopoiesis. In such cases, the CC status is reached more rapidly and if the indication for transplantation was a hematological malignancy, the appearance of recipient cells in a progressive mixed chimerism can be an early prediction of relapse. Once reduced- intensity conditioning regimes were introduced, the TMC is prevalent. With the progression to-

wards complete donor chimerism, the donor cells exert the graft versus leukemia or graft versus malignancy effect known as adaptive immunotherapy (9).

The exploration of the chimerism is a mandatory procedure in the follow-up of the disease according to international consensus of this specialty. It is desirable to analyze the specific chimerism of the different cells lineages. This procedure offers pertinent information about the necessity of intensification or depression of post-transplant immunosuppression, respectively regarding the opportunity of intensification of donor graft by donor lymphocytes infusion for the stabilization of the decline of the graft and the induction of a graft versus host hematopoiesis reaction.

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Disclosure statement on conflict of interest

No actual conflict of interest exists among the authors of this study nor is any future conflict of interest foreseen. No special funds have been raised for the preparation of this retrospective study.

Abbreviations

ALL – acute lymphoblastic leukemia;
AML – acute myeloid leukemia;
CC –complete chimerism;
CGD – chronic granulomatous disease;
DC –dissociated chimerism;
ELB – erythrolyse buffer;
FACS – fluorescence activated cell sorting;
FAM - 6 – carboxyfluorescein;
FISH – fluorescence *in situ* hybridization;
GRA - granulocytes;
GvHD – graft versus host disease;
HLA – human leukocyte antigene;
HSCT – hematopoietic stem cell transplantation;
LMC – chronic myeloid leukemia;
MC –mixed chimerism;
MONO - monocytes;
NK –natural killer cells.
PBS - phosphate buffered saline;
PMC –progressive mixed chimerism;
RT-PCR – real time PCR;
SAA – very severe aplastic anemia;
SMC –stable mixed chimerism;
TAMRA - 6 – carboxytetramethil-rhodamine;
TMC – transient mixed chimerism;

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