Contents lists available at ScienceDirect

Acta Haematologica Polonica

journal homepage: www.elsevier.com/locate/achaem

Original research article/Praca oryginalna

Assessment of lineage-specific chimerism after allogeneic stem cell transplantation

Badanie chimeryzmu specyficznego liniowo po allogenicznym przeszczepieniu komórek macierzystych

Patrycja Zielińska *, *Mirosław Markiewicz*, *Monika Dzierżak-Mietła*, *Anna Koclęga*, *Krzysztof Białas*, *Małgorzata Kopera*, *Krystyna Jagoda*, *Agnieszka Karolczyk*, *Sławomira Kyrcz-Krzemień*

Department of Hematology and Bone Marrow Transplantation, Medical University of Silesia, Head: prof. dr hab. n. med. Sławomira Kyrcz-Krzemień, Katowice, Poland

ARTICLE INFO

Article history:
Received: 23.12.2013
Accepted: 31.10.2014
Available online: 13.11.2014

Keywords:
- Lineage-specific chimerism
- Allogeneic stem cell transplantation
- Erythrocyte chimerism

ABSTRACT

Introduction: Donor lineage-specific chimerism of hematopoietic cells enables very precise monitoring of engraftment in selected cell lines after allogeneic stem cell transplantation (allo-SCT). **Materials and methods:** The study group consisted of 12 acute leukemia patients who underwent allo-SCT in the Department of Hematology and Bone Marrow Transplantation in Katowice, Poland. Lineage-specific chimerism was assessed in B cells (CD19+ CD38−/+), plasma cells (CD19+ CD38++), T cells (CD3+ or CD7+ CD56−), monocytes (CD14+), and immature progenitor cells deriving from myeloid line (CD34+CD19). We also assessed erythrocyte chimerism by flow cytometry. **Results:** All patients engrafted. 8 out of 10 patients presented normal donor hematopoiesis. Lineage specific chimerism in these patients corresponded with chimerism analysis in unsorted material and with undetectable minimal residual disease (MRD). Relapse of the underlying disease was diagnosed in 2 patients. In both cases loss of donor chimerism occurred in leukemia specific cell line and corresponded with detectable MRD. One patient with secondary graft failure presented decreasing lineage specific chimerism in all subpopulations, with negative MRD status. In 10 patients normal hematopoiesis of donor-origin was assessed by flow cytometry. In one case no donor-derived erythrocytes were detected and the diagnosis of pure red cell aplasia was set. **Conclusions:** Lineage specific chimerism as a method of high sensitivity and specificity allows for precise assessment of donor chimerism especially in clinically ambiguous situations. Assessment of erythrocyte chimerism by flow cytometry is a reliable method of monitoring erythroblastic line engraftment. Presented results are preliminary and the study is being continued.

* *Corresponding author at*: Katedra i Klinika Hematologii i Transplantacji Szpiku Śląskiego Uniwersytetu Medycznego w Katowicach, ul. Dąbrowskiego 25, 40-031 Katowice, Poland. Tel.: +48 32 2591 281.
E-mail address: klinhem@sum.edu.pl (P. Zielińska).

http://dx.doi.org/10.1016/j.achaem.2014.10.005

Introduction

Stem cell transplantation is a complex procedure that leads to damaging the recipient's hematopoietic system and restoring a new hematopoiesis and immunological functions by transfusion of stem cells of HLA-matched donor. Molecular surveillance of hematopoietic chimerism has become part of the routine diagnostic program in patients after allogeneic stem cell transplantation. Chimerism testing permits early identification of successful engraftment, and facilitates early detection of impending graft rejection. In patients transplanted for treatment of malignant hematological disorders, monitoring of chimerism can provide an early data of incipient relapse of the disease [1–3].

The investigation of chimerism has therefore become an important tool for the management of patients during the post-transplant period. Peripheral blood or bone marrow is most often used for chimerism analysis, with or without further manipulation of different cell subpopulations. Increasing use of non-myeloablative conditioning regimens, which may be associated with prolonged duration of mixed chimerism (MC, coexistence of both donor and recipient hematopoiesis), has further increased the clinical importance of chimerism analysis. In patients with MC, the percentage of recipient hematopoietic cells may remain stable, increase, or spontaneously decrease over time. Therefore, there are three types of mixed chimerism: transient (coexistence of recipient and donor genotypes, with conversion to complete chimerism over time), persistent (coexistence of recipient and donor genotypes, and the state is present over time), progressive (gradual increase of recipient genotype in subsequent examinations) [1, 2]. The term 'split chimerism' indicates that donor-derived cells are found only in some cell lineages e.g. only in myeloid line.

Several methods may be applied for precise monitoring of post-transplant chimerism: biochemical, immunologic, cytogenetic and molecular ones [1–3]. At present, the most commonly used technical approach to the investigation of chimerism is microsatellite analysis by polymerase chain reaction (PCR) technique, which detects polymorphic changes in DNA sequences of the donor and the recipient using quantitative method. The most commonly used technique is short tandem repeats (STR) method which identifies informative microsatellite sequences of 2 up to 8 bp. The sensitivity of this method reaches 5×10^2 and the specificity is around 100% [1–3]. Nonetheless, the competition between alleles of the donor and of the recipient multiplied in one reaction, using the same pair of starters, may cause errors. The errors may also occur because of different reaction speed, which depends on the length of multiplied alleles or occurrence of unspecific reaction products. PCR method enables the assessment of specific tandem repeatable DNA sequences called VNTR (variable number of tandem repeats). The VNTR sequences consist of 9 up to 50 bp [1]. It is worth stressing that both PCR methods, STR and VNTR, are independent of the donor or recipient sex. In contrast to STR, VNTR is a more complex and less sensitive method. At present, it is possible to perform simultaneous assessment of several different STR/VNTR sequences. To increase the sensitivity, real time PCR or other methods may be applied [1–4].

In the last decades, a great progress has been made in terms of isolating cells subpopulations with specific phenotype. The investigation of chimerism within specific leukocyte subsets isolated from peripheral blood or bone marrow samples provides more precise information on processes underlying the dynamics of donor/recipient chimerism. Particular cell subpopulations may be isolated using immunomagnetic or cytometric methods. In the first method cells are conjugated with paramagnetic beads, and then separated into particular subpopulations using magnetic field. In the latter, particular cell population marked with monoclonal antibody may be differed by cell sorter using cytometric method. Lineage specific chimerism increases the sensitivity and specificity of chimerism assessment in comparison to unsorted material chimerism [1, 4].

The presence of recipient cells during the post-transplant period is an important prognostic indicator of transplant rejection or relapse of the disease. Early immunotherapeutic intervention (e.g. the reduction of immunosuppressive therapy or donor lymphocyte infusion) might be effective in these cases. Some other findings are predictive of graft-versus-host disease (GVHD). The most accurate timing if chimerism assessment is still under investigation.

In the present work, we assessed donor chimerism in nucleated cells (NCs) and lineage-specific cell subtypes chimerism, namely in B cells (CD19+ CD38−/+), plasma cells (CD19+ CD38++), T cells (CD3+ or CD7+ CD56−), monocytes (CD14+) and immature progenitor cells, mainly deriving from myeloid line (CD34+CD19−) after allo-SCT by multiplex STR-PCR in order to compare the sensitivity and specificity of both techniques and to compare them with minimal residual disease (MRD) evaluation. We also assessed the erythrocyte chimerism by flow cytometry after allo-SCT. Clinical data of the study group were also evaluated. The study design was approved by the local Bioethical Committee, Medical University of Silesia on June 21, 2011. Written informed consent was collected from each patient included into the study.

The aim of the study

The aim of the study was to assess lineage-specific donor chimerism in selected leukocyte populations and erythrocyte chimerism in acute leukemia patients who underwent allogeneic hematopoietic stem cell transplantation.

Material

Patient characteristics: 12 patients with acute leukemia were included in the study (median age 35 years, range: 19–61 years). The study group consisted of 8 males and 4 females. All of them were hospitalized in the Department of Hematology and Bone Marrow Transplantation, Medical University of Silesia, Katowice, Poland in years 2011–2012 and underwent hematopoietic stem cell transplantation from unrelated (11 patients) or sibling donor (1 patient). 8 patients

suffered from acute myelogenous leukemia and 4 from acute lymphoblastic leukemia. The major AB0 group mismatch was detected in 9 cases; in 2 donor-recipient pairs major and minor AB0 group incompatibility (bi-directional mismatch) was present. In one case the blood groups between the donor and the recipient showed no difference. The characteristics of the study group is presented in Table I.

Conditioning regimen prior to allo-SCT: Standard myeloablative conditioning regimen (BuCy: busulphan 16 mg/kg in days −10 to −6 and cyclophosphamide 120 mg/kg in days −5 to −3) was applied in 3 patients, in 3 patients total body irradiation (TBI) of total dose 12 Gy and cyclophosphamide (120 mg/kg) was applied, in 6 patients reduced-toxicity myeloablative conditioning with treosulfan 14 g/m^2/day in days −6 to −4 and fludarabine 50 mg/day in days −6 to −2. Cyclosporine at adjusted doses to concentration in serum and short course of methotrexate on days +1, +3 and +6, were used routinely as an immunosuppressive treatment in all patients. All patients transplanted from unrelated donor received anti-thymocyte globulin (ATG) at dose 15 mg/kg on three consecutive days preceding the transplantation.

Methods

Assessment of total and lineage-specific donor chimerism: Bone marrow samples (3–10 ml) were collected at predefined time points after transplantation, namely 1, 2, 3, 6, 9 and 12 months after allo-SCT. First of all, bone marrow leukocytosis was assessed. Then the material was diluted with phosphate-buffered saline (PBS) in 1:1 ratio, stratified on Ficoll-Paque 1.077 G/cm^3 gradient (4 ml of diluted bone marrow and 3 ml of gradient) and centrifuged according to the manufacturer instructions. The layer containing nucleated cells was pooled with pipette, rinsed with PBS, congested to the volume of 500 μl and leukocyte count was measured. Isolated nucleated cells (at concentration of WBC < 50 000/μl) were incubated in +4 °C with monoclonal antibodies marking B cells (CD19+ CD38−/+), plasma cells (CD19+ CD38++), T cells (CD3+ or CD7+ CD56−), monocytes (CD14+), and immature progenitor cells (CD34+CD19−). Selected cells were suspended in PBS and kept in darkness in +4 °C till sorting. The cells were sorted using cell sorter FACSAria III. The 4-way purity method was implemented, according to the manufacturer instructions, to get the best purity, recovery and viability of sorted cells. Granulocytes were isolated from bone marrow after discarding erythrocytes using BD PharmLyse, and then sorted using cell sorter FACSAria III, basing on their size and granularity. Lineage specific chimerism was assessed using STR (short tandem repeats) method by Genetic Analyzer 3130 (Applied Biosytems). AmpFISTR SGM Plus PCR Amplification Kits were used. We analyzed 11 loci. The material was recipient DNA (1.5–2 ng) isolated from sorted cells and donor DNA stored after harvest. The multiplex-PCR was then

Table I – The characteristics of the study group
Tabela I – Charakterystyka grupy badanej

UPN	Age/sex	Diagnosis	Sibling/ unrelated donor	Conditioning regimen	HLA match	AB0 incompatibility	CD34 × 10^6/kg	Relapse	Last FU	Status at 1 year
1	40/F	AML	URD	RIC TreoFlu + ATG	FM	Major	11.91	No	CC	Alive
2	27/F	AML	URD	RIC TreoFlu + ATG	Antigen B and C mismatch	Major	6.54	No	CC	Alive
3	19/M	ALL	URD	MAC TBI + + + Ctx + ATG	FM	Major	6.9	No	CC	Alive
4	61/M	AML	URD	RIC TreoFlu + ATG	Antigen A mismatch	Major	6.21	No	CC	Dead
5	34/M	ALL	URD	MAC TBI + Ctx + ATG	FM	Bi-directional	5.04	No	Graft loss	Dead
6	24/M	ALL	URD	MAC TBI + Ctx + ATG	FM	Major	3.3	Yes	MC	Dead
7	32/F	AML	Sibling	MAC BuCy	FM	Major	4.59	No	CC	Alive
8	35/F	AML	URD	MAC BuCy + ATG	FM	Bi-directional	6.47	No	CC	Alive
9	35/M	ALL	URD	MAC BuCy + ATG	FM	Major	4.03	No	CC	Alive
10	53/M	AML	URD	RIC TreoFlu + ATG	FM	Major	6.28	No	CC	Alive
11	39/M	AML	URD	RIC TreoFlu + ATG	FM	No	2.8	Yes	MC	Alive
12	44/M	AML	URD	RIC TreoFlu + ATG	FM	Major	4.3	No	CC	Alive

Abbreviations: UPN, unique patient number; FM, full match; URD, unrelated donor; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia, FU, follow up; CC, complete chimerism (defined as the presence of more than 95% donor cells); MC, mixed chimerism (defined as between 5 and 94% recipient cells); MAC, myeloablative conditioning; RIC, myeloablative with reduced intensity conditioning.

performed. Fluorescent marked PCR products were separated automatically with capillary electrophoresis using Genetic Analyzer 3130 (Applied Biosytems) and analyzed using GeneScan Analysis. Lineage-specific chimerism was calculated following the defined genetic profiles of the donor and the recipient. Total donor chimerism in unsorted bone marrow cells was assessed simultaneously using fluorescent-based STR-PCR and capillary electrophoresis as described above. Complete chimerism (CC) was defined as the presence of >95% donor-type hematopoietic cells after allo-SCT. Mixed chimerism (MC) was defined as the presence of 5–95% donor-type hematopoietic cells.

Assessment of donor erythrocyte chimerism by flow cytometry: Peripheral blood sample (100–250 μl) was taken to assess erythrocyte chimerism by flow cytometry at time points mentioned above to demonstrate the appearance of donor derived erythrocytes in cases with major AB0 blood group incompatibility. The percentage of donor-origin red blood cells was assessed in peripheral blood samples in cases with major blood group incompatibility. Monoclonal antibodies (anti-A, anti-B and anti-D) were used to assess the main blood groups. The erythrocytes were solidified by 0.1% glutaraldehyde in PBS before incubation with monoclonal antibody (MoAb) to avoid agglutination with them. Then, the specimen was incubated at first with MoAb I (anti-A, anti-B or anti-D), and later with MoAb II (fluorescein-conjugated) that detected the presence of MoAb I on erythrocytes. Monoclonal antibody bound erythrocytes were analyzed by flow cytometry using FACSCanto II (Becton Dickinson) and the percentage of A-, B- and/or D-positive red blood cells was measured (depending on the donor/recipient blood groups).

Minimal residual disease monitoring by flow cytometry: MRD assessment was performed in patients with known aberrant phenotype. Bone marrow cells were incubated with particular monoclonal antibody set marked by fluorochromes, and then erythrocytes were removed with the usage of BD PharmLyse. After rinse the cells were suspended in PBS and analyzed in FACSCanto II BD. The percentage of nuclear cells with aberrant phenotype among all bone marrow nuclear cells was calculated. MRD was regarded negative if less that 0.1 per 100 nucleated cells were found.

Results

Allo-SCT procedure: Peripheral blood was source of stem cells in all patients. A median number of transplanted nucleated cell (NC) was: 8.05 (3.98–16.68) $\times 10^8$/kg, including 5.73 (2.8–11.91) $\times 10^6$/kg of CD34+ cells and 17.11 (8.6–24.43) $\times 10^7$/kg of CD3+ cells. All patients engrafted. The median periods required for recovering the absolute counts of neutrophils (ANC $> 0.5 \times 10^3$/μl) and platelets (PLT $> 20 \times 10^3$/μl) were 16 days (range: 12–28 days) and 15 days (range: 13–58 days), respectively. None of the patients was given prophylactically hematopoietic growth factors to enhance engraftment. All patients were treated in reverse isolation using fungal prophylaxis and oral nonabsorbable antibiotics for gastrointestinal bacterial decontamination. All patients received prophylactic acyclovir and *Pneumocystis jiroveci* prophylaxis consisting of oral trimethoprim-sulfamethoxazole. Blood samples were obtained weekly for cytomegalovirus testing but no patient developed reactivation of CMV. Three patients developed severe complications: pneumonia, BK virus-induced (polyomavirus-induced) urinary tract infection, and *Clostridium difficile* infection.

Graft-versus-host disease: The diagnosis of GVHD was based on physical examination and laboratory tests. Viral, allergic, drug-related causes of symptoms were ruled out. Acute GVHD was graded according to the modified Seattle Glucksberg criteria. In 9 patients acute GVHD symptoms developed, most of the patients presented grade I. Skin involvement was noted most often. In 2 patients severe GVHD symptoms (grade III) were seen, with gastrointestinal tract and liver involvement. Topical steroids were applied in 9 patients and systemic steroid therapy was needed in 7 cases (the maximum dose of methylprednisolone was 2 mg/kg of body weight/day). None of the patients developed chronic GVHD symptoms.

Donor chimerism assessment and MRD monitoring after allo-SCT: Normal donor hematopoietic cells engraftment is presented in Table II (for AML patients) and Table III (for ALL patients).

Table II – Assessment of post-transplant donor chimerism and MRD status in AML patients – hematopoiesis of donor origin after allo-SCT monitored during one year follow up (n = 6, UPN 1, 2, 4, 7, 8, 10)
Tabela II – Ocena chimeryzmu potransplantacyjnego dawcy oraz statusu MRD u pacjentów z AML – hematopoeza dawcy po allo-SCT podczas roku obserwacji (n = 6, UPN 1, 2, 4, 7, 8, 10)

Time after allo-SCT (months)	MRD/100 nucleated cells	Donor chimerism in NC cells (%), median, range	Donor erythrocyte chimerism (%), median, range	Donor lineage-specific chimerism (%), median, range					
				Lymphocytes B	Lymphocytes T	CD34+	Plasma cells	Granulocytes	Monocytes
+1	<0.1	100 (99–100)	9.08 (6–17.6)	100	89.6 (87–100)	100	83.1 (39–100)	100	100
+2	<0.1	99 (99–100)	49.5 (45.5–80.4)	100	98.7 (96–100)	100	89.6 (38–100)	100	100
+3	<0.1	100	98.4 (84–97.4)	100	99.8 (99–100)	100	93.1 (47–100)	100	100
+6	<0.1	100	98.8 (98.2–99.5)	100	100	100	97.2 (64–100)	100	100
+9	<0.1	100	98.7 (99.8–99.8)	100	100	100	98.3 (96–100)	100	100
+12	<0.1	100	98.2 (96.3–97.8)	100	100	100	98.1 (92–100)	100	100

Abbreviations: MRD, minimal residual disease; NC, nucleated cells.

Table III – Assessment of post-transplant donor chimerism and MRD status in ALL patients – hematopoiesis of donor origin after allo-SCT during one year follow up (*n* = 2, UPN 3, 9)
Tabela III – Ocena chimeryzmu potransplantacyjnego dawcy oraz statusu MRD u pacjentów z ALL – hematopoeza dawcy po allo-SCT podczas roku obserwacji (n = 2, UPN 3, 9)

Time after allo-SCT (months)	MRD/100 nucleated cells	Donor chimerism in NC cells (%), median	Donor erythrocyte chimerism (%), range	Donor lineage-specific chimerism (%), range				
				Lymphocytes B	Lymphocytes T	Plasma cells	Granulocytes	Monocytes
+1	<0.1	100	3.6–11	100	100	45–100	100	100
+2	<0.1	100	34.9–55.6	100	100	88–99	100	100
+3	<0.1	100	55.4–95.7	100	100	100	100	100
+6	<0.1	100	53.6–98.2	100	100	100	100	100
+9	<0.1	100	69.8–92.3	100	100	100	100	100
+12	<0.1	100	89.2–90.8	100	100	98	100	100

Abbreviations: MRD, minimal residual disease; NC, nucleated cells.

The data are presented separately since slightly different cell subsets were assessed. In all AML patients full donor chimerism was detected in myeloid line (granulocytes, monocytes and CD34 positive cells) during follow up period. It corresponded well with MRD status and donor chimerism in unsorted bone marrow. Plasma cell donor chimerism was within wide range, but over time we observed gradual increase in donor-derived plasma cells. Erythrocyte chimerism showed similar kinetics during our observation. In all ALL patients who presented normal donor hematopoiesis after allo-SCT complete donor chimerism was seen in most cell subtypes apart from plasma cells. It might be also partly explained by the fact that relatively low proportion of plasma cells is found in bone marrow.

Three out of 12 patients died in the early post-transplant period. One patient deceased at day +42 in the course of grade IV graft-versus-host disease showing complete donor chimerism in all subpopulations studied at 1 month after allo-SCT (UPN 4). Another patient developed relapse of Philadelphia chromosome-positive acute lymphoblastic leukemia 6 months after transplantation (UPN 6, see Table IV). In that case relapse was detected both by flow cytometry (detectable minimal residual disease) and by lineage-specific chimerism (complete loss of B cell donor chimerism). The patient started reinduction chemotherapy since material for DLI was unavailable. The patient died in course of severe pneumonia without achieving remission. Third patient with AB0 mismatch died 3 months after transplantation due to severe fungal pneumonia associated with secondary graft loss (UPN 5, see Table V). Graft failure diagnosis was based on morphology count and corresponded with lineage-specific chimerism data that showed rapid decrease in percentage of donor-derived hematopoietic cells in all subpopulations studied, also in erythrocyte chimerism. Relapse of the underlying disease was ruled out because of negative MRD status. Early relapse of AML was found in one case (UPN 11, see Table VI). Interestingly, we observed full donor chimerism in NC, whereas lineage-specific chimerism in CD34 positive cells decreased to 59% and MRD was detected in that patient 2 months after transplantation.

Erythrocyte chimerism assessment: Ten out of eleven patients presented with normal reconstitution of erythroblastic line despite major AB0-mismatched grafts. Our assessment of erythrocyte chimerism was based on the data that in the early post-transplant period the patients were transfused with group 0 red blood cells both in cases with major or bidirectional AB0 incompatibility. Cytometric assessment of erythrocyte chimerism is shown in Table VII. In cases with major AB0 incompatibility red blood cells derived from donor were easily distinguishable (Fig. 1). One patient in our study group developed pure red cell aplasia (PRCA) after allogeneic transplantation with major blood group incompatibility (see Table VIII and Fig. 2). This patient was transplanted in the presence of elevated isohemagglutinin titer. Parvovirus B19 infection was excluded by PCR. The patient required withdrawal of immunosuppression, intravenous immunoglobulin

Table IV – Assessment of post-transplant donor chimerism and MRD status – relapse of ALL (*n* = 1, UPN 6)
Tabela IV – Ocena chimeryzmu potransplantacyjnego dawcy oraz statusu MRD – wznowa ALL (n = 1, UPN 6)

Time after allo-SCT (months)	MRD/100 nucleated cells	Donor chimerism in NC cells (%)	Donor erythrocyte chimerism (%)	Donor lineage-specific chimerism (%)				
				Lymphocytes B	Lymphocytes T	Plasma cells	Granulocytes	Monocytes
+1	<0.1	100	10	100	100	100	100	100
+2	<0.1	98	36.7	100	100	99	100	100
+3	<0.1	100	73	100	100	100	100	100
+6	>0.1	77	97.2	0	100	78	89	98

Abbreviations: MRD, minimal residual disease; NC, nucleated cells.

Table V – Assessment of post-transplant donor chimerism and MRD – secondary graft failure (n = 1, UPN 5)
Tabela V – Ocena chimeryzmu potransplantacyjnego dawcy oraz statusu MRD – wtórna utrata wszczepu (n = 1, UPN 5)

Time after allo-SCT (months)	MRD/100 nucleated cells	Donor chimerism in NC (%)	Donor erythrocyte chimerism (%)	Donor lineage-specific chimerism (%)				
				Lymphocytes B	Lymphocytes T	Plasma cells	Granulocytes	Monocytes
+1	<0.1	100	8.1	100	99	93	100	100
+2	<0.1	100	46.5	100	100	99	98	100
+3	<0.1	78	12	0	87	56	78	82

Abbreviations: MRD, minimal residual disease; NC, nucleated cells.

infusion, subcutaneous erythropoietin supplementation and daily plasmaphereses. Cessation of transfusion requirements occurred after 6 weeks. The red cell parameters remained stable during the follow-up period up to one year after allo-SCT.

Discussion

Chimerism analysis is routinely performed after allo-SCT to monitor engraftment. Achieving complete hematopoietic chimerism after allogeneic transplantation is one of the main factors that contribute to the successful outcome. The issues of chimerism analysis in selected cell subsets (lineage-specific chimerism) is still under investigation. Nowadays, monoclonal antibodies are widely used to detect specific surface antigens (CD – cluster of differentiation). Thus, chimerism analysis can be performed in B (CD19+, CD20+) and T (CD3+) lymphocytes, NK cells (CD56+), granulocytes (CD15+, CD16+), monocytes (CD14+), dendritic cells, macrophages, platelets and megakaryocytes (CD61+), erythrocytes (CD71+) and progenitor cells (CD34+). This precise evaluation enables to confirm the donor-origin hematopoiesis. When mixed chimerism is found it may provide valuable information about further immunotherapy, such as donor lymphocyte infusion or immunosuppression therapy adjustment [5]. Moreover, chimerism analysis provides data about imminent relapse or risk of GVHD development. The most important factor is to investigate chimerism changes over time. There are no strict recommendations about the timing of chimerism assessment. Generally, it is recommended to investigate chimerism in days +30, +90, and then after 6 months and 12 months after allo-SCT [2]. Most authors agree to monitor chimerism most often, even once a week, in patients after DLI procedure. In presented study we performed chimerism evaluation more frequently in order to collect more precise data. In cases where no molecular marker or minimal residual disease phenotype is present, chimerism analysis is one of the most reliable parameters to be analyzed in order to monitor disease status after allo-SCT. In our study group MRD status and chimerism assessments were done at the same time points and in two cases of relapse MRD assessment corresponded well with lineage-specific chimerism evaluation, but not always with chimerism in unsorted material (NC chimerism) (see Table V).

The most important factor in lineage-specific chimerism evaluation is to determine which cells would provide the most valuable data. In general, it is advised to assess chimerism in at least a few different cell lines. In this study we decided to focus on sorting the following cell lines: B cells (CD19+ CD38−/+), T cells (CD3+ or CD7+ CD56−), plasma cells (CD19+ CD38++), monocytes (CD14+), and immature progenitor cells from myeloid line (CD34+CD19−). It is worth mentioning, that CD34 positive blastic cell phenotypes are found only in around 70% of AML patients, so anti-CD34 antibody is not a perfect marker of relapse. It is advisable then to adjust the assessment of specific cell lines to particular patient looking closely at the leukemic cell phenotype detected at the time of diagnosis. It is also worth noting that expression of CD34 may change in the course of the disease.

Some authors proved that measurements performed in cell line that was the origin of the clonal (malignant) disorder (leukemia lineage specific chimerism) are the most important clinically [6–8]. In our material we observed relapse of acute myelogenous leukemia with detectable MRD that was associated with decrease of donor chimerism in CD34 positive cells down to 59% (UPN 11). An interesting

Table VI – Assessment of post-transplant donor chimerism and MRD status – relapse of AML (n = 1, UPN 11)
Tabela VI – Ocena chimeryzmu potransplantacyjnego dawcy oraz statusu MRD – wznowa AML (n = 1, UPN 11)

Time after allo-SCT (months)	MRD/100 nucleated cells	Donor chimerism in NC (%)	Donor erythrocyte chimerism (%)	Donor lineage-specific chimerism (%)					
				Lymphocytes B	Lymphocytes T	Plasma cells	Granulocytes	Monocytes	CD34+ cells
+1	<0.1	97	11	97	100	–	100	100	100
+2	>0.1	100	80.4	100	100	94	100	100	59

Abbreviations: MRD, minimal residual disease; NC, nucleated cells.

Table VII – Assessment of erythrocyte chimerism by flow cytometry in major (*n* = 8) AB0 incompatibility and bi-directional (*n* = 2) after allo-SCT. Donor-origin erythroblastic line regeneration
Tabela VII – Ocena chimeryzmu erytrocytów z zastosowaniem cytometrii przepływowej u chorych z dużą niezgodnością grup krwi pomiędzy dawcą i biorcą (n = 8) oraz w przypadku małej i dużej niezgodności grup krwi (n = 2) po allo-SCT. Regeneracja układu erytroblastycznego z komórek dawcy

Time after allo-SCT (months)	Donor erythrocyte chimerism (%), median, range (*n* = 10)
+1	9.4 (3.6–17.6)
+2	44.2 (34.9–80.4)
+3	66.2 (55.4–97.4)
+6	78.1 (53.6–99.5)
+9	80.8 (69.8–99.8)
+12	93.4 (89.2–97.8)

issue has been raised by Lange et al. [11], who concluded that post-transplant monitoring of WT1 expression in peripheral blood and CD34(+) donor chimerism in bone marrow seems to be good predictors of early relapse not only in AML patients, but also in patients suffering from myelodysplastic syndromes who underwent hematopoietic cell transplantation with reduced-intensity conditioning.

Serrano et al. [9] studied chronic myelogenous leukemia patients and found that all MC patients who expressed recipient type CD15+ cells after allo-SCT relapsed. That was also consistent with data provided by van Leeuwen et al. [10], who suggested that the identification of persistent host cells within the leukemia lineage can be associated with leukemia relapse. The regrowth of a clonal cells being a consequence of an inefficient immune surveillance is easily detected and found first in the myeloid compartment and then occurs progressively in other cell lines [9].

In 2007 Mohty et al. [12] presented retrospective single center analysis of a group of 102 oncological patients after allo-SCT. They found that early full donor T-cell chimerism is associated with grade 2–4 acute GVHD. The authors concluded that monitoring of the kinetics of donor T-cell chimerism is mandatory after RIC allo-SCT, and can improve patients' outcome. Similar data were provided by El-Cheikh et al. [13] who found that acute GVHD was predictive of full donor T-cell chimerism after RIC allo-SCT. Early assessment of T- and NK-cell chimerism was proved to be instrumental in the risk assessment and therapeutic management of imminent graft rejection [13–16]. Detection of predominant host-origin T and NK cells were strongly predictive of graft loss in the majority of patients despite therapeutic interventions [13]. In our experience, one ALL patient (UPN 5) experienced secondary graft failure with undetectable MRD and decreasing lineage specific chimerism in all lines studied. NK cell chimerism was not assessed in our study.

Decreasing chimerism in B cells was widely studied by Zetterquist et al. [17] and was proved to be associated with incipient relapse of B cell leukemia. Similar data were observed in our study (UPN 6), where decrease in total donor chimerism was noted. Lineage specific chimerism evaluation showed complete lack of donor B-cells after 6 months

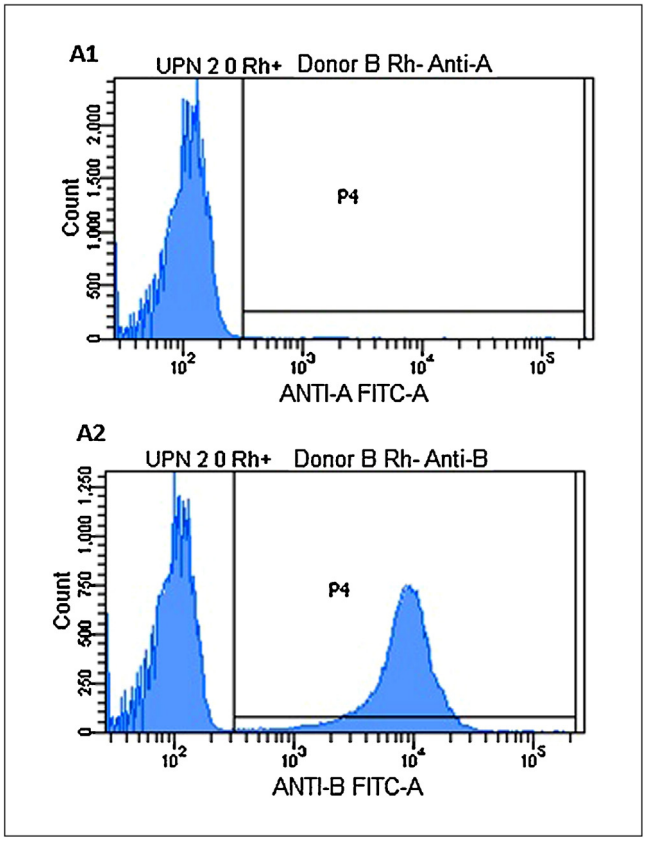

Fig. 1 – Donor-origin erythropoiesis 2 months after allo-SCT (UPN 1). Assessment of group 0 red blood cells (recipient-origin and transfused) and group B red blood cells (donor-origin) by flow cytometry using anti-A (A1) and anti-B (A2) monoclonal antibodies
Ryc. 1 – Erytropoeza dawcy 2 miesiące po allo-SCT (UPN 1). Ocena krwinek czerwonych grupy 0 (biorcy i pochodzących z transfuzji) oraz grupy B (dawcy) za pomocą cytometrii przepływowej z zastosowaniem przeciwciał monoklonalnych anty-A (A1) oraz anty-B (A2)

after allo-SCT, whereas other cell lines were still mostly donor-derived. This data corresponded with detectable minimal residual disease by flow cytometry.

As thrombocytopenia often occurs in post-transplant period, some authors put an effort to assess platelet and

Table VIII – Assessment of erythrocyte chimerism by flow cytometry – pure red cell aplasia (*n* = 1, UPN 12)
Tabela VIII – Ocena chimeryzmu erytrocytów z zastosowaniem cytometrii przepływowej. Aplazja czystoczerwonokrwinkowa (n = 1, UPN 12)

Time after allo-SCT (months)	Donor erythrocyte chimerism (%)	Isohemagglutinins titer
+1	0.1	Anti-B: IgG 256, IgM 256
+2	0.2	–
+3	0	Anti-B: IgG 1024, IgM 64

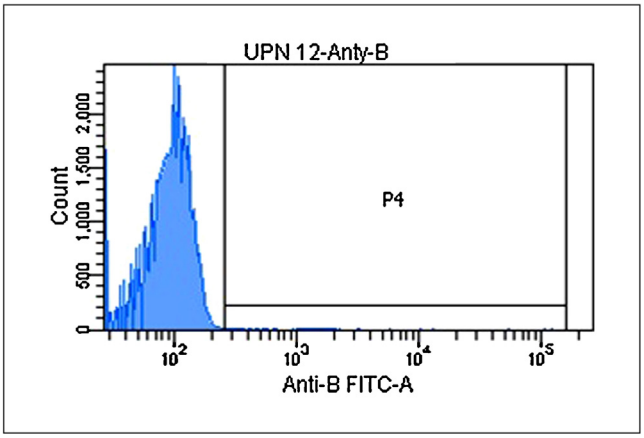

Fig. 2 – Pure red cell aplasia (UPN 12). Assessment of group 0 red blood cells (recipient's-origin and transfused) by flow cytometry using anti-B monoclonal antibodies and absence of group B red blood cells (donor's origin)
Ryc. 2 – Aplazja czystoczerwonokrwinkowa (UPN 12). Ocena krwinek czerwonych grupy 0 (pochodzących od biorcy oraz z transfuzji) za pomocą cytometrii przepływowej z zastosowaniem przeciwciał monoklonalnych anty-B. Brak krwinek czerwonych grupy B (pochodzących od dawcy)

megakaryocyte chimerism [18, 19], but due to frequent platelet transfusions those data have some obvious limitations. It was proved that prolonged platelet regeneration might be associated with mixed chimerism. In our material we did not focus on these assessments.

Similar constraints may be encountered while assessing erythroblastic line [18, 20–22]. The additional obstacle is longer red blood cell half-life. In our study group most of the patients presented major blood group incompatibility so erythrocyte chimerism was assessed by flow cytometry using secondary (indirect) immunofluorescence. The median of erythrocyte donor chimerism in 10 patients in our study group at +1 month after allo-SCT was 9.4% (range: 3.6–17.6%), and was increasing in the course of observation, achieving at 12 months post allo-SCT 93.4% (range: 89.2–97.8%). The erythrocyte phenotypes of the patient, of the donor and the transfused red blood cells in these cases were easily distinguishable. This method, which is reasonably inexpensive and quick, enables strict monitoring of the group of patients who are at increased risk of developing pure red cell aplasia. Detecting erythrocyte chimerism by flow cytometry comprised a valuable tool to set the diagnosis, apart from histopathological bone marrow specimen examination, reticulocyte count and isohemagglutinin titer. In PRCA case we observed very low (0.1%) erythrocyte chimerism since the first assessment 1 month after transplantation. Monitoring of this patient enabled quick diagnosis and treatment that resulted in resolving of this complication.

Approximately 15–25% of HLA identical sibling donor/recipient pairs differ in AB0 blood groups. The figure is higher for alternative donor transplants. The impact of donor–recipient AB0 incompatibility on long-term HCT outcomes remains controversial [23, 24]. However, pure red cell aplasia and delayed red blood cell engraftment were proved by some authors to be more frequent in HCT recipients with major AB0 mismatched donors [25]. Delayed red blood cells engraftment was noted to be associated with post-transplantation hyperferritinemia and increased mortality risk [23]. AB0-mismatched SCT might have an unfavorable impact on transplant outcomes, but it does not

Table IX – Preliminary data (month +1 and +2 after allo-SCT) illustrating mixed donor chimerism in AML patients after RIC allo-SCT
Tabela IX – Wstępne dane (miesiąc i 2 miesiące po alloprzeszczepieniu) ilustrujące mieszany chimeryzm dawcy u chorych z ostrą białaczką szpikową po allo-SCT z kondycjonowaniem o zredukowanej toksyczności

Time after allo-SCT (months)	MRD/100 nucleated cells	Donor chimerism in NC (%)	Donor erythrocyte chimerism (%)	Donor lineage-specific chimerism (%)					
				Lymphocytes B	Lymphocytes T	Plasma cells	Granulocytes	Monocytes	CD34+
Patient B.S., MC → relapse 2 months after SCT									
+1	–	84	23.6	100	73	32	87	90	16
+2	–	100	35.1	100	92	–	91	52	3
Patient L.G., MC → CC									
+1	<0.1	100	48.1	100	78	84	100	99	100
+2	<0.1	100	97.5	100	100	100	100	100	100
Patient K.K., MC → CC									
+1	–	100	68.2	99	88	66	100	99	99
+2	–	100	88.5	100	98	83	100	100	100
Patient M.Z., MC									
+1	>0.1	100	23	100	–	–	99	97	51

Abbreviations: MRD, minimal residual disease; NC, nucleated cells; MC, mixed chimerism; CC, complete chimerism.

affect either graft rejection or GVHD since AB0 antigens are not expressed on primitive stem cells. Nonetheless, selection of AB0-compatible donors whenever possible, strategies to prevent PRCA, modifications in peritransplant transfusion practice, also iron chelation are recommended to improve transplant outcomes.

To summarize, the most important advantage of lineage specific chimerism is the ability to predict the risk of relapse and to monitor precisely the engraftment itself [26]. The present work presents preliminary results of lineage-specific donor chimerism assessment in a selected group of patients. The most important question is when to assess the hematopoietic chimerism. The expected obstacle is the cost effectiveness of this method. The question arises whether there is a specific subgroup of patients, that would benefit the most. An important aspect is the monitoring of hematopoietic chimerism after nonmyeloablative conditioning [27].

In our material all patients underwent myeloablative conditioning prior to allo-SCT, in 6 cases – reduced intensity conditioning based on treosulfan (total dose 42 mg/m^2) was applied. Interesting observation has been done by our team only recently. These are partial data not included in patient characteristics since the observation is not completed yet. Nonetheless, the data is presented in Table IX. Four AML patients who underwent allo-SCT preceded by nonmyeloablative conditioning are currently under investigation. First of all we noticed that reduced intensity conditioning with BuFlu (busulphan 6.4 mg/kg body weight, fludarabine 125 mg/m^2) resulted in mixed donor chimerism in all analyzed cases. In one patient we observed that low donor chimerism in CD34 positive cells preceded early relapse of acute leukemia. The second patient with low CD34 positive chimerism and detectable MRD is under close observation with decreasing immunosuppression therapy. These patients had high risk of relapse, since they did not achieve complete remission before allo-SCT. In our opinion, these are very promising observations that need further investigation in a larger group of patients. Based on these cases, donor chimerism in CD34 positive cells seems to be crucial for retaining complete remission in AML patients. These data are preliminary but indicate a very interesting direction for further investigation.

Conclusions

Following hematopoietic stem cell transplantation, monitoring the proportion of donor and recipient hematopoiesis in the patient is an influential tool in directing further treatment decisions. However, there is no consensus on when and how often to monitor post-transplant chimerism.

Currently, fluorescence-based PCR amplification of short tandem repeats is the gold standard for analyzing post-transplant chimerism and has been used in most studies. Lineage specific chimerism allows for precise monitoring of the patients after allogeneic stem cell transplantation. The unquestionable advantages of this method are high sensitivity and specificity. The method itself is cost and time consuming, requires qualified staff and appropriate equipment. Lineage specific chimerism may be worth applying in selected patients with high risk of relapse or graft failure. The optimal timing of these diagnostic interventions is a critical issue and has to be further optimized. Whether this will ultimately improve the survival of patients with leukemia after transplantation has to be shown in prospective studies. Only very frequent monitoring of chimerism status by highly sensitive methods might identify impending relapse and allow early immunological intervention.

Assessing erythrocytes chimerism by flow cytometry is a reliable method of monitoring erythroblastic line engraftment in high risk cases. The sensitivity of erythrocyte antigens assessment is estimated around 0.04 up to 3% in all patients, irrespective of their blood groups. We conclude that cytometric evaluation of erythrocyte phenotype is an easy and valuable method in patients with major incompatibility across AB0-barrier since donor and recipient antigens, as well as transfused red blood cells are easily detectable and their evaluation provides data with very high sensitivity and specificity.

Presented data obtained from a limited group of patients are preliminary. We conclude that the methodology used in the study, especially concerning cell sorting may be applied in further studies. The study is being continued with aim to select a group of patients that may benefit the most from careful monitoring and to select the best timeframes for attentive assessment of donor chimerism after allogeneic transplantation. We also plan to investigate donor chimerism after reduced intensity conditioning and compare the data to myeloablative conditioning regimens.

Authors' contributions/Wkład autorów

PZ – study design, data collection, statistical analysis, data interpretation, manuscript preparation, literature search, funds collection. MM – study design, data collection and interpretation, manuscript preparation, literature search, funds collection. MD-M – study design, data collection and interpretation, literature search. SK-K – study design. AgK, – data collection and interpretation, manuscript preparation. MK – data collection. AK – data collection, literature search. KJ – data interpretation, manuscript preparation. KB – literature search.

Conflict of interest/Konflikt interesu

None declared.

Financial support/Finansowanie

This research was supported by Medical University of Silesia.

Ethics/Etyka

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical

Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform Requirements for manuscripts submitted to Biomedical journals.

REFERENCES/PIŚMIENNICTWO

[1] Dawidowska M, Guz K, Brojer E, Wachowiak J, Witt M. Molecular hematology – pathogenesis, pathomechanisms and methods of investigation. In: Witt M, Szczepański T, Dawidowska M, editors. Chimerism after allogeneic transplantation of hematopoietic stem cells. Poznań: Ośrodek Wydawnictw Naukowych; 2009 [Chapter III-3].

[2] Rupa J, Lewandowski K. Practical aspects of hematopoietic chimerism monitoring. Acta Haematol Pol 2006;37:443–474.

[3] Jółkowska J, Witt M. Cellular chimerism after bone marrow transplantation. Acta Haematol Pol 2004;35:321–328.

[4] Antin JH, Childs R, Filipovich AH, et al. Establishment of complete and mixed donor chimerism after allogeneic lymphohematopoietic transplantation: recommendations from a workshop at the 2001 Tandem Meetings. Biol Blood Marrow Transplant 2001;7:473–485.

[5] Khan F, Agarwal A, Agrawal S. Significance of chimerism in hematopoietic stem cell transplantation: new variations on an old theme. Bone Marrow Transplant 2004;34:1–12.

[6] Mattsson J, Uzunel M, Tammik L, Aschan J, Ringden O. Leukemia lineage-specific chimerism analysis is a sensitive predictor of relapse in patients with acute myeloid leukemia and myelodysplastic syndrome after allogeneic stem cell transplantation. Leukemia 2001;15:1976–1985.

[7] Rosenow F, Berkemeier A, Krug U, et al. CD34(+) lineage specific donor cell chimerism for the diagnosis and treatment of impending relapse of AML or myelodysplastic syndrome after allo-SCT. Bone Marrow Transplant 2013;48:1070–1076.

[8] Lion T, Breuer S, Daxberger H, et al. Recipient chimerism in the T-cell lineage is indicative of impending graft rejection in pediatric patients undergoing allogeneic hematopoietic stem cell transplantation. Haematologica 2008;93(330) [Abs.0830].

[9] Serrano J, Roman J, Sanchez J, et al. Molecular analysis of lineage-specific chimerism and minimal residual disease by RT-PCR of p210BCR-ABL and p190BCR-ABL after allogeneic bone marrow transplantation for chronic myeloid leukemia: increasing mixed myeloid chimerism and p190BCR-ABL detection precede cytogenetic relapse. Blood 2000;95:2659–2665.

[10] Leeuwen JEM, Tol MJD, Joosten AM, Wijnen TJh, Hhan PM, Vossen JM. Mixed T-lymphoid chimerism after allogeneic bone marrow transplantation for hematologic malignancies of children is not correlated with relapse. Blood 1993;82:1921.

[11] Lange T, Hubmann M, Burkhardt R, et al. Monitoring of WT1 expression in PB and CD34(+) donor chimerism of BM predicts early relapse in AML and MDS patients after hematopoietic cell transplantation with reduced-intensity conditioning. Leukemia 2011;25:498–505.

[12] Mohty M, Avinens O, Faucher C, Viens P, Blaise D, Eliaou JF. Predictive factors and impact of full donor T-cell chimerism after reduced intensity conditioning allogeneic stem cell transplantation. Haematologica 2007;92:1004–1006.

[13] El-Cheikh J, Vazquez A, Crocchiolo R, et al. Acute GVHD is a strong predictor of full donor CD3+ T cell chimerism after reduced intensity conditioning allogeneic stem cell transplantation. Am J Hematol 2012;87:1074–1078.

[14] Goh RY, Kim SH, Han JY. Lineage-specific chimerism analysis in nucleated cells, T cells and natural killer cells after myeloablative allogeneic hematopoietic stem cell transplantation. Korean J Hematol 2011;46:18–23.

[15] Breuer S, Preuner S, Fritsch G, et al. Early recipient chimerism testing in the T- and NK-cell lineages for risk assessment of graft rejection in pediatric patients undergoing allogeneic stem cell transplantation. Leukemia 2012;26:509–519.

[16] Childs R, Clave E, Contentin N, et al. Engraftment kinetics after nonmyeloablative allogeneic peripheral blood stem cell transplantation: full donor T-cell chimerism precedes alloimmune responses. Blood 1999;94:3234–3241.

[17] Zetterquist H, Mattsson J, Uzunel M, et al. Mixed chimerism in the B cell lineage is a rapid and sensitive indicator of minimal residual disease in bone marrow transplant recipients with pre-B cell acute lymphoblastic leukemia. Bone Marrow Transplant 2000;25:843–851.

[18] Thiele J, Wickenhauser C, Kvasnicka HM, et al. Mixed chimerism of erythro- and megakaryopoiesis following allogeneic bone marrow transplantation. Acta Haematol 2003;10:117–118.

[19] Miura Y, Tanaka J, Toubai T, et al. Analysis of donor-type chimerism in lineage-specific cell populations after allogeneic myeloablative and non-myeloablative stem cell transplantation. Bone Marrow Transplant 2006;37:837–843.

[20] Lion T. Detection of impending graft rejection and relapse by lineage-specific chimerism analysis. Methods Mol Med 2007;134:197–216.

[21] Zeiser R, Spyridonidis A, Wäsch R, et al. Evaluation of immunomodulatory treatment based on conventional and lineage-specific chimerism analysis in patients with myeloid malignancies after myeloablative allogeneic hematopoietic cell transplantation. Leukemia 2005;19: 814–821.

[22] Rowley SD. Hematopoietic stem cell transplantation between red cell incompatible donor-recipient pairs. Bone Marrow Transplant 2001;28:315–321.

[23] Kimura F, Sato K, Kobayashi S, et al. Impact of AB0-blood group incompatibility on the outcome of recipients of bone marrow transplants from unrelated donors in the Japan Marrow Donor Program. Haematologica 2008;93:1686–1693.

[24] Ozkurt ZN, Yegin ZA, Yenicesu I, et al. Impact of AB0-incompatible donor on early and late outcome of hematopoietic stem cell transplantation. Transplant Proc 2009;41:3851–3858.

[25] Willasch A, Eing S, Weber G, et al. Enrichment of cell subpopulations applying automated MACS technique: purity, recovery and applicability for PCR-based chimerism analysis. Bone Marrow Transplant 2010;45:181–189.

[26] Sugita J, Tanaka J, Hashimoto A, et al. Influence of conditioning regimens and stem cell sources on donor-type chimerism early after stem cell transplantation. Ann Hematol 2008;87:1003–1008.

[27] Saito B, Fukuda T, Yokoyama H, et al. Impact of T cell chimerism on clinical outcome in 117 patients who underwent allogeneic stem cell transplantation with a busulfan-containing reduced-intensity conditioning regimen. Biol Blood Marrow Transplant 2008;14:1148–1155.