*Katarzyna Guz[1], Barbara Nasiłowska-Adamska[2], Kazimierz Hałaburda[2, 3], Agnieszka Orzińska[1], Bożena Mariańska[2], Ewa Brojer[1]

Early chimerism analysis using real-time PCR method as a predictor of graft-versus-host-disease following allogeneic haematopoietic stem cell transplantation

Analiza wczesnego chimeryzmu przy użyciu PCR w czasie rzeczywistym jako czynnik rokowniczy choroby przeszczep przeciwko gospodarzowi po allogenicznym przeszczepieniu hematopoetycznych komórek krwiotwórczych

[1]Department of Immunohaematology and Immunology of Transfusion Medicine, Institute of Haematology and Transfusion Medicine, Warsaw
Head of Department: prof. Ewa Brojer, MD, PhD
[2]Department of Haemopoietic Stem Cell Transplantation, Institute of Haematology and Transfusion Medicine, Warsaw
Head of Department: prof. Bożena Mariańska, MD, PhD
[3]Department of Haematology, Oncology and Internal Diseases, Medical University of Warsaw
Head of Department: prof. Wiesław Wiktor Jędrzejczak, MD, PhD

Summary

Assessment of donor chimerism has an established role in the follow-up of patients after allogeneic haematopoietic stem cell transplantation (alloHSCT).

Material and methods. In this retrospective study, chimerism was measured by real-time PCR using insertion/deletion polymorphisms every 5 days until day +30 following alloHSCT in 33 patients with haematological malignancies conditioned with standard (CST) or reduced intensity regimens (RIC). No T-depleted bone marrow (BM) or granulocyte-colony stimulating factor mobilized peripheral blood (PBSC) were the sources of stem cells.

Results. The statistically important differences in the assessment of early donor chimerism increase were observed between day +5 and +10 in patients transplanted with CST and PBSC (the faster donor chimerism increase) compared to those with CST and BM or after RIC. The analysis of early donor chimerism in patients transplanted with CST and PBCS, who developed acute and chronic graft-versus-host-disease (GvHD), showed significantly faster disappearance of autologous cells and the expansion of transplanted donor cells between day +5 and +10 as compared to patients with no GvHD.

Key words: RQ-PCR, stem cell transplantation, early chimerism, GvHD

Streszczenie

Ocena chimeryzmu dawcy odgrywa znaczącą rolę w rokowaniu pacjentów po allogenicznym przeszczepieniu hematopoetycznych komórek krwiotwórczych (alloHSCT).

Materiał i metody. W tej retrospektywnej pracy, chimeryzm oceniano metodą PCR w czasie rzeczywistym z użyciem polimorfizmów insercja/delecja co 5 dni, aż do +30 dnia po alloHSCT u 33. pacjentów z hematologicznym standardowym mieloablacyjnym kondycjonowaniem (CST) lub kondycjonowaniem o zredukowanej intensywności (RIC). Źródłem komórek macierzystych był szpik kostny bez usuwania limfocytów T (BM) lub komórki macierzyste krwi obwodowej (PBSC).

Wyniki. Statystycznie istotne różnice w tempie nabywania chimeryzmu dawcy obserwowano między +5 a +10 dniem u pacjentów z przeszczepieniem CST i PBSC, co wskazywało na szybsze nabywanie chimeryzmu dawcy w porównaniu z tymi po CST i BM lub po RIC. Analiza wczesnego chimeryzmu dawcy u pacjentów po przeszczepieniu z CST i PBSC, u których wystąpiła ostra lub przewlekła choroba przeszczep przeciwko gospodarzowi, wykazała znacząco szybszy zanik autologicznych komórek i ekspansję przeszczepionych komórek dawcy między +5, a +10 dniem w porównaniu z pacjentami bez tej choroby.

Słowa kluczowe: RQ-PCR, transplantacja komórek macierzystych, wczesny chimerizm, GvHD

INTRODUCTION

Although donor chimerism assessment is an established part of the follow-up procedure in patients after allogeneic haematopoietic stem cell transplantation (alloHSCT) (1), no sufficient data on kinetics and clinical importance of early chimerism (assessed within first 30 days following alloHSCT) are available. For this purpose routine methods of chimerism analysis cannot be used because of their insufficient sensitivity. New perspectives have been opened with the implementation of the very sensitive real-time PCR method (RQ-PCR). Several RQ-PCRs protocols using different primers and probes (SNP (2, 3) or insertion/deletion polymorphism (4, 5) as well as different methods of product detection [specific labeled probes (4, 5) or the nonspecific detection of PCR product by SYBR-Green (6)] have been proposed for assessment of early chimerism. The aim of our study was to develop a reliable RQ-PCR-based protocol for early chimerism evaluation as predictor of acute (a) and chronic (ch) graft-versus-host-disease (GvHD).

MATERIAL AND METHODS

We applied the RQ-PCR method based on TaqMan technology on ABI PRISM 7700 with specific labeled probes and primers (Applied Biosystems, USA).

PCRs were performed in 25 μl reagents: 12.5 μl TaqMan master mix (Applied Biosystems, USA), 200 nM appropriate probes and 300 nM appropriate primers. As stem cell donor and recipient markers we used 26 specific markers, described in other studies (4, 7, 8, 9, 10) and applied by us routinely for noninvasive prenatal diagnostics in RhD negative women (11): 6 null alleles (*SRY, S03, S06, GSTM1, GSTT1, RHD*), 10 insertion/deletion polymorphisms (*ACE, S01, S04, S05, S07, S08, S09, S10, S11, RHC/c*) and the housekeeping *CCR5* gene for both estimation of DNA concentration in each sample and the „normalizator" for chimerism analysis. The details of our RQ-PCR protocols for chimerism analysis can be read at previous publication (12).

In this retrospective study, we evaluated early chimerism in 33 consecutive patients transplanted in our center for haematological malignancies from fully HLA-matched related or unrelated donors. The baseline characteristics of patients enrolled to the study are presented in table 1. The study protocols were approved by the Ethical Committee.

Standard conditioning (CST) was administered in 28 patients. In this group, no T-depleted bone marrow (BM) in 8 patients or peripheral blood stem cells (PBSC) in 20 patients were the sources of stem cells. In the remaining 5 patients, reduced intensity conditioning (RIC) with PBSC infusion was applied. All patients undergoing CST were administered GvHD prophylaxis with CsA/MTX (15 mg/m^2 i.v. on day +1 and 10 mg/m^2 on days +3, +6, +11) or CsA/ /MTX/ATG from related or unrelated HLA-matched donors, respectively. In patients undergoing RIC transplantations, single agent prophylaxis with CsA was applied.

Blood samples were collected from donors and recipients prior to transplantation and from recipients on days +5, +10, +15, +20, +25 and +30 (± 1) following transplantation. Blood samples were also collected from the panel of 51 healthy volunteers. Mononuclear cells (MC) were isolated on Ficoll density gradient (Lymphoprep, Axis Shield PoC, Norway). DNA was extracted according to standard procedure (Qiagen, Germany) and stored at -80°C until RQ-PCR was performed. The concentration and purity of each DNA sample were measured spectrophotometrically at 260 and 280 nm and by RQ-PCR using primers and probes for *CCR5* gene.

Prior to transplantation, donor and recipient DNA were tested to identify the specific markers present in the recipient but absent in the donor and vice versa. The healthy volunteer-panel was also tested for the "calibrator" DNA for each transplanted patient. *SRY, RHD, RHC/c* alleles were chosen as markers in sex- or RhD/C mismatched recipient/ donor pairs. If more than one marker for one or for both individuals were informative, those with the most similar PCR efficiencies were selected.

To evaluate the sensitivity, accuracy and variability of each RQ-PCRs, standard amplification curves were plotted for all specific markers. PCRs with artificial chimeric DNAs were prepared by serial dilution of appropriate positive DNA (heterozygous for specific allele) in negative DNA (without the specific allele) with a constant final

Table 1. Baseline characteristics of study patiens.

Characteristics	Patiens (N=33)
Sex-N	
Female	15
Male	18
Age-yr	
Median	38.4
Range	18-60
Diagnosis-N	
Acute lymphoblastic leukemia	4
Acute myelogenous leukemia	13
Myelodysplastic syndrome	1
Chronic myeloid leukemia	11
Chronic lymphocytic leukemia	2
Non – Hodgink's lymphoma	2
Conditioning regimens-N	
CST:	28
BuCy	21
BEAMMAbCampath	1
TreoFlu	1
TBICy	5
RIC:	5
2CdABuMabCampath	5
HSCT-N	
allogeneic (HLA matched):	30
related	3
unrelated	
WBC > 1,0 x 10^9/l (days after HSCT)	
Mean (range)	19.3 (10-35)
ANC > 0,5 x 10^9/l (days after HSCT)	
Mean (range)	20.3 (12-50)
PLT > 20 x 10^9/l (days after HSCT)	
Mean (range)	16.7 (7-57)
PLT > 50 x 10^9/l (days after HSCT)	
Mean (range)	24-2 (10-76)

DNA amount of 50 ng. Positive and negative DNA were obtained from healthy volunteers pre-genotyped for all markers. The experimentally established detection limit for RQ-PCR-based chimerism analysis was 4/7575 copies of negative DNA (0.05%); Ct value for 1 copy (0,013% chimerism) was between 37.328 and 41.081. The slope of regression curve varied from -3.191 to -4.198 and the R^2 correlation coefficient, from 0.972 to 0.999. The inter- and intra-assay variabilities were examined according to Jiménez-Velasco et al. (5) and the CV range was between 0.24-1.42 and 1.00-3.26, respectively. The standardization results of our RQ-PCR protocol for chimerism analysis and comparison with STR-PCR method was previously published on the larger DNA samples from recipient alloHSCT including material collected after +30 day following transplantation (13).

The marker informativity of our system was 100% in unrelated pairs and 97.1% in related individuals presented in this article. In 85.3% of the last group markers were found for both donor and recipient and in 11.8%, only for recipient. In one case (2.9%), the marker was found only for donor and was not further analyzed.

For chimerism analysis, the simultaneous quantitative analysis of the stem cell donor and recipient polymorphisms and the referent *CCR5* gene were performed in duplicates using 50 ng of DNA per reaction. As calibrators we used DNA of 6/51 tested volunteer blood donors. The "calibrator" DNA from healthy volunteers were of the same genotype and zygosity as the stem cell donor and recipient prior to transplantation.

Chimerism percentage was calculated separately for donor and recipient using Pfaffl's formula: (E target) ΔCt target (C-U)/(Eref)ΔCt ref (C-U), where Etarget/ /ref = 10(-1/slope target) – efficiency of PCR for target polymorphism or reference *CCR5* gene; ΔCt – difference between results of calibrator DNA (C) and sample DNA (U) either for the target recipient/donor polymorphism or for the *CCR5* gene.

The < 0.1 result was directly recalculated for the chimerism percentage. For a > 0.1 result of Pfaffl's formula, the donor and recipient results were added and treated as 100%. The chimerism percentage was calculated by proportion.

The t-test was used for statistical analysis. The percentage of donor chimerism in patients with and with no GvHD was compared; the $p < 0.05$ differences were treated as statistically significant.

RESULTS

In 32 patients the observed donor profile was 91.1 to 100% by day +30 but the tempo of disappearance of autologous cells and the expansion of transplanted donor cells was different (one patient died before +30 day).

The statistically important differences in the engraftment tempo were observed at +5 and between day +5 and +10 in patients transplanted with CST and PBSC (the fastest donor chimerism increase) compared to those with CST and BM ($p = 0.0015$ at day +5 and 0.0012 between day +5 and +10) or after RIC (0.0099 at day +5 and 0.037 between day +5 and +10) (fig. 1A).

As for GvHD: we found symptoms of aGvHD in 9 patients (27,6%) and of chGvHD in 15 (45,4%). In table 2, we present the frequency of GvHD in our patients groups with different conditioning and source of stem cells.

Table 2. Frequency of GvHD in patiens groups with differend conditioning and source of stem cells.

GvHD	CST/PBSC (N = 20)	CST/BM (N = 8)	RIC (N = 5)
No a/ch GvHD-N*(%)	2 (10)	2 (25)	0
aGvHD-N(%)	6 (30)	3 (37.5)	0
chGvHD-N(%)	12 (60)	3 (37.5)	0

N – number of patiens.

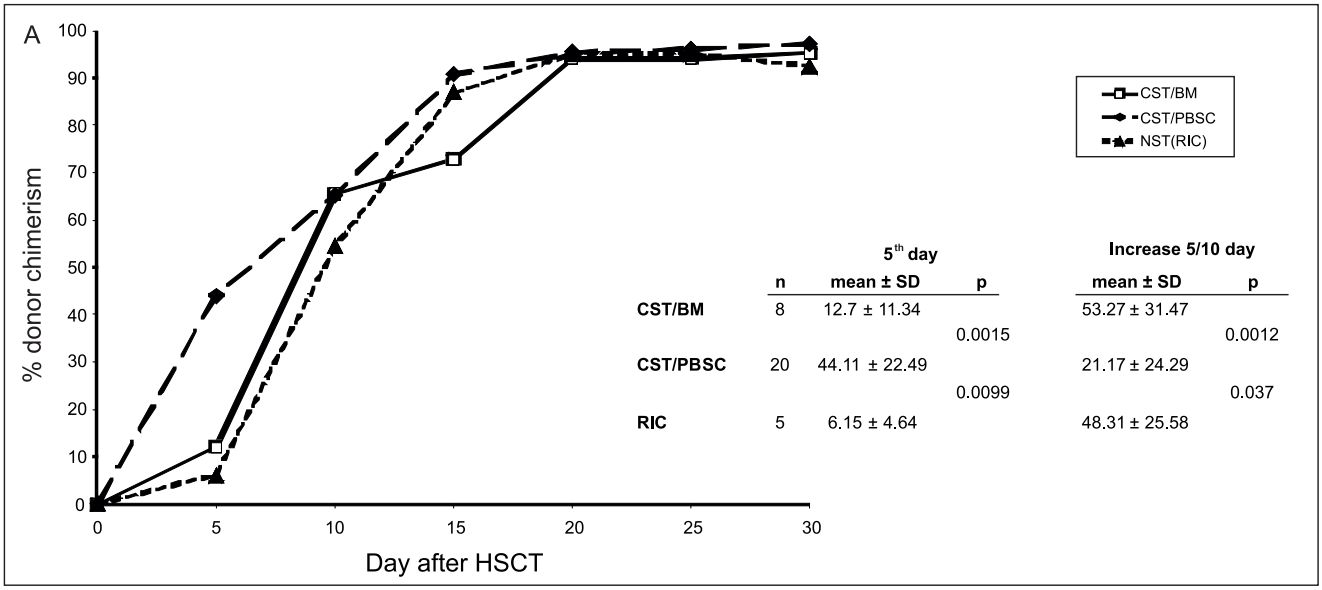

Fig. 1. The comparision of early chimerism incrising in patients:
A – transplanted with CST/BM, CST/PBSC and RIC.

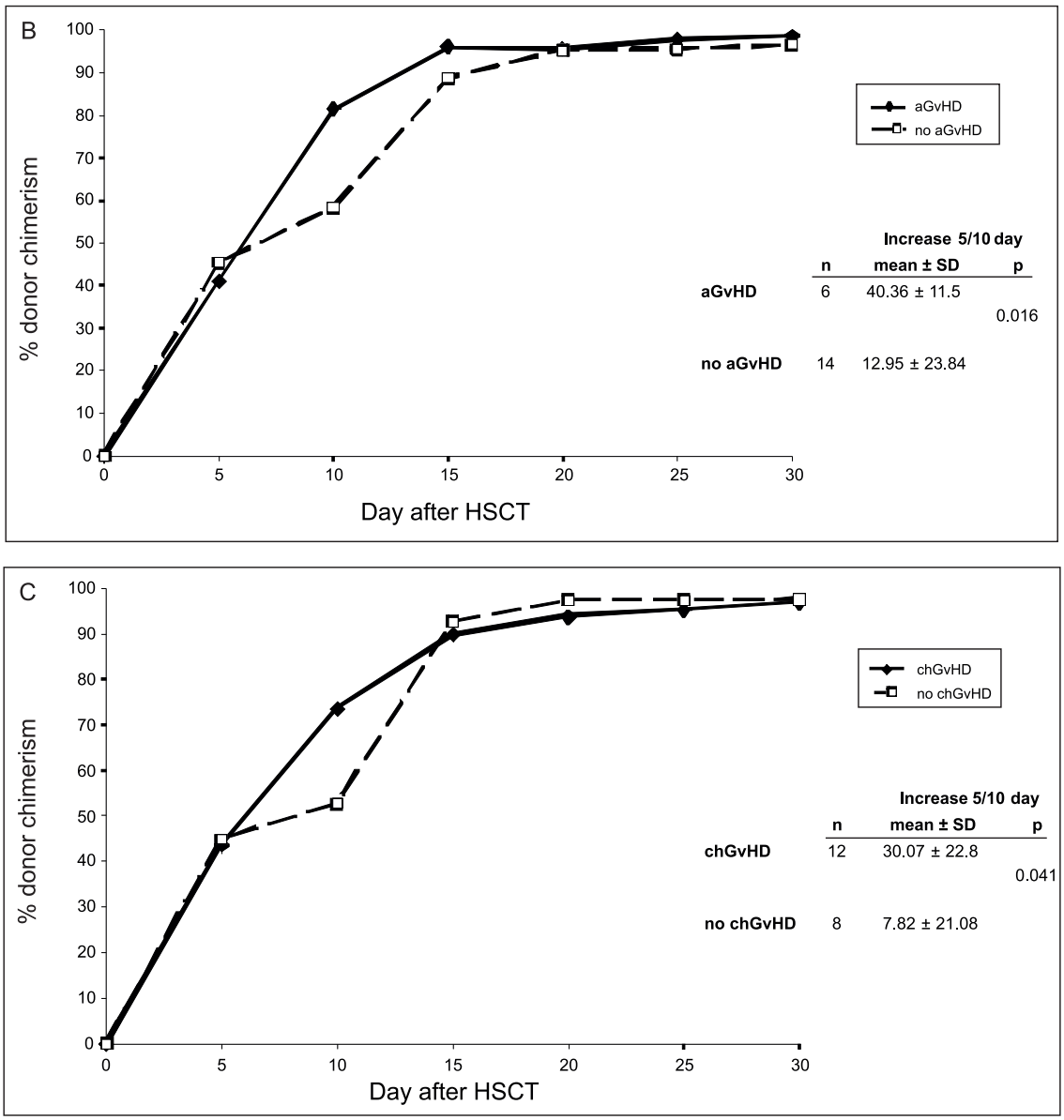

Fig. 1. The comparision of early chimerism incrising in patients:
B – after CST/PBSC with and with no acute GvHD,
C – after CST/PBSC with and with no chronic GvHD.

The analysis of early chimerism in patients transplanted with CST and PBCS who developed aGvHD (fig. 1B) and chGvHD (fig. 1C) showed faster disappearance of autologous cells and the expansion of transplanted donor cells between day +5 and +10 as compared to patients with no disease. For aGvHD the results were statistically significant ($p = 0.016$) and for chronic disease they were on the border of significance ($p = 0.041$). No significance was observed between early chimerism and GvHD in patients transplanted with CST and BM.

Four patients (12,1%) died on days 25, 34, 52 and 57 following SCT; two of them of veno-occlusive liver disease (CST/PBSC), one of fourth stage liver aGvHD resistant to several immunosuppressive agents (CST/BM) and one of disease progression (RIC).

DISCUSSION

In general, our methodology for early chimerism evaluation is based on that presented by Alizadeh et al. (4) but with some modifications to improve clinical application.

The informativity of our method was high (97.1%), close to that of Masmas et al. (14) who used RQ-PCR method based on single nucleotide polymorphisms and higher than that of others [80.3% (5); 90% (4)]. This was achieved by extending the null alleles and insertion/deletion polymorphism panel to 16 as compared to the 10-11 panel used by Alizadeh et al. (4), Jimenez--Velasco et al. (5) and Masmas et al. (14). We were however, unable to assess chimerism in one patient with only donor but no recipient signals since no appropriate marker could be detected. In such cases

we postpone the chimerism analysis because the accuracy of the RQ-PCR method is too low for higher autologous or donor signals. This issue is discussed by Alizadeh et al. (4), Willasch et al. (15) and Masmas et al. (14) with the conclusion that it is difficult to achieve high accuracy with the target concentration of more than 40-50%.

In all our patients the chimerism analysis were performed every 5 days following alloHSCT until day 30 and in all cases only peripheral blood was used as analysis material (not bone marrow). In none of our patients was a lack of engraftment observed in the early days following alloHSCT. We hope that, due to the accuracy of the quantitative analysis of chimerism kinetics, our method will permit early differentiation between the absence of engraftment and engraftment delay as well as the identification of patients liable to relapse (4).

The sensitivity of our modified method was 0.05%, slightly lower than that observed by Jimez-Valesco et al. (5) (0.01%) but higher than the 0.1% observed by others in RQ-PCR (4, 6, 14, 15) or modified STR-PCR (16) and much higher than the 1-5% obtained with most classical PCR based methods (5, 17).

We have modified the method of chimerism calculation. In most studies the calculation was based on the $\Delta\Delta$Ct formula that disregarded the referent gene PCR efficiency (4, 14). The problem was solved by introducing the Pfaffl's formula which was used by us according to Jimenez-Velasco et al. (5). In our study additional modifications were introduced; for the result of > 0.1 in Pfaffl's formula, the results for donor and recipient were added together, treated as 100% and the chimerism percentages were calculated by proportion.

As additional modification, we introduced calibrators different than those used by others. To examine polymorphism we used the heterozygous DNA of volunteer blood donors instead of the separate donor and recipient DNA isolated prior to transplantation. From the panel of 51 healthy volunteers, DNA of 6 included all combinations of tested polymorphisms and were sufficient calibration for all patients. The approach of using marker specific standard dilution DNA series from healthy volunteers to avoid the use of DNA of patients and their donors, has been also published by Willasch et al (15). The use of DNA of healthy volunteers instead of frozen pre-transplant patient and donor DNA eliminates the problems and errors related to the shortage and low quality of pre-transplant patient DNA, inaccurate dilutions and measurement of DNA quantity. The use of the same calibrator is also very important for prospective patient monitoring.

We tested chimerism as early as on day +5 and confirmed the observation of other authors that kinetics of early chimerism depends on the conditioning therapy and stem cell source (18). In our group of patients we observed that the evaluation of early chimerism in MNC could predict aGvHD (faster donor chimerism increase predicts disease). Similar observations were made by Gyger et al. (19) and Jaksch et al. (20) who tested chimerism in T cells. In our patients, early chimerism occurred to predict also chGvHD.

CONCLUSIONS

Summing up, our method is highly informative, sensitive and precise and can be used as a routine method for early chimerism analysis and for evaluation of chimerism in lymphocyte subpopulations, especially in RIC transplantations.

BIBLIOGRAPHY

1. Dawidowska M, Guz K, Brojer E et al.: Chimeryzm po allogenicznej transplantacji macierzystych komórek krwiotwórczych. [In:] Witt M, Szczepański T, Dawidowska M (ed.): Hematologia molekularna patogeneza, patomechanizmy i metody badawcze. Ośrodek Wyd Nauk 2009; 177-194.
2. Maas F, Schaap N, Kolen S et al.: Quantification of donor and recipient hematopoietic cells by real-time PCR of single nucleotide polymorphisms. Leukemia 2003; 17: 621-629.
3. Koldehoff M, Steckel NK, Hlinka M et al.: Quantitative analysis of chimerism after allogeneic stem cell transplantation by real-time polymerase chain reaction with single nucleotide polymorphisms, standard tandem repeats and Y-chromosome-specific sequences. Am J Hematol 2006; 81: 735-746.
4. Alizadeh M, Bernard M, Danic B et al.: Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. Blood 2002; 99: 4618-4625.
5. Jiménez-Velasco A, Barrios M, Román-Gómez J et al.: Reliable quantification of hematopoietic chimerism after allogeneic transplantation for acute leukemia using amplification by real-time PCR of null alleles and insertion/deletion polymorphisms. Leukemia 2005; 19: 336-343.
6. Bai L, Deng YM, Dodds AJ et al.: A SYBR green-based real-time PCR method for detection of haemopoietic chimerism in allogeneic haemopoietic stem cell transplant recipients. Eur J Haematol 2006; 77: 425-431.
7. Legler T, Lynen R, Maas J et al.: Prediction of fetal Rh D and Rh CcEe phenotype from maternal plasma with real-time polymerase chain reaction. Transfus Apheresis Sci 2002; 27: 217-223.
8. Lo Y, Lau T, Chan L et al.: Quantitative analysis of bidirectional fetomaternal transfer of nucleated cells and plasma DNA. Clin Chem 2000; 46: 1301-1309.
9. Lo YMD, Tein M, Lau TK et al.: Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998; 62: 768-775.
10. Zhang J, Fidler C, Murphy F et al.: Determination of fetal RhD status by maternal plasma DNA analysis. Ann NY Acad Sci 2000; 906: 153-155.
11. Brojer E, Żupanska B, Guz K et al.: Non-invasive determination of fetal RHD status by examination of cell-free DNA in maternal plasma. Transfusion 2005; 45: 1473-1480
12. Dawidowska M, Jółkowska-Baraniak J, Guz K et al.: Analiza chimeryzmu poprzeszczepowego metodą STR-PCR i RQ-PCR.

[In:] Witt M, Szczepański T, Dawidowska M (ed.): Hematologia molekularna patogeneza, patomechanizmy i metody badawcze. Ośrodek Wyd Nauk 2009; 239-252.
13. Guz K, Smolarczyk-Wodzyńska J, Dawidowska M et al.: Evaluation of chimerism after allogeneic hematopoietic stem cell transplantation using the RQ-PCR method – its standardization and comparison with the STR-PCR method. Acta Haematol Pol 2010; 41: 535-544.
14. Masmas TN, Madsen HO, Petersen SL et al.: Evaluation and automation of hematopoietic chimerism analysis based on real-time quantitative polymerase chain reaction. Biol Blood Marrow Transplant 2005; 11: 558-566.
15. Willasch A, Schneider G, Reincke BS et al.: Sequence polymorphism systems for quantitative real-time polymerase chain reaction to characterize hematopoietic chimerism-high informativity and sensivity as well as excellent reproducibility and precision of measurement. Lab Hematol 2007; 13: 73-84.
16. Buno I, Nava P, Simon A et al.: A comparison of fluorescent in situ hybridization and multiplex short tandem repeat polymerase chain reaction for quantifying chimerism after stem cell transplantation. Haematologica 2005; 90: 1373-1379.
17. Gallardo D, Rodríguez-Luaces M, Aróstegui JI et al.: Follow-up of chimerism status after allogeneic HLA-mismatched stem cell transplantation by detection of non-shared HLA alleles. Haematologica 2000; 85: 848-854.
18. Frankel W, Chan A, Corringham RE et al.: Detection of chimerism and early engraftment after allogeneic peripheral blood stem cell or bone marrow transplantation by short tandem repeats. Am J Hematol 1996; 52: 281-287.
19. Gyger M, Baron C, Forest L et al.: Quantitative assessment of hematopoietic chimerism after allogeneic bone marrow transplantation has predictive value for the occurrence of irreversible graft failure and graft-vs.-host disease. Exp Hematol 1998; 26: 426-434.
20. Jaksch M, Uzunel M, Remberger M et al.: Molecular monitoring of T-cell chimerism early after allogeneic stem cell transplantation may predict the occurrence of acute GVHD grades II-IV. Clin Transplant 2005; 19: 346-349.

received/otrzymano: 07.05.2012
accepted/zaakceptowano: 04.06.2012

Address/adres:
*Katarzyna Guz
Department of Immunohaematology
and Immunology of Transfusion Medicine,
Institute of Haematology and Transfusion Medicine
ul. Chocimska 5, 00-957 Warszawa
tel.: +48 (22) 349-66-00 w. 154, fax.: +48 (22) 349-66-14
e-mail: kguz@ihit.waw.pl