Mutations in the let-7 binding site - a mechanism of RAS activation in juvenile myelomonocytic leukemia?

Doris Steinemann,[1] Marcel Tauscher,[1] Inka Praulich,[1] Charlotte M. Niemeyer,[2] Christian Flotho[2] and Brigitte Schlegelberger[1]

[1]Institute of Cell and Molecular Pathology, Hannover Medical School, Hannover, Germany; [2]Pediatric Hematology and Oncology, Center for Pediatric and Adolescent Medicine, University of Freiburg, Freiburg, Germany

Key words: RAS pathway, let-7, JMML.

Correspondence: Doris Steinemann, Institute of Cell and Molecular Pathology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany. E-mail: steinemann.doris@mh-hannover.de

Citation: Steinemann D, Tauscher M, Praulich I, Niemeyer CM, Flotho C and Schlegelberger B. Mutations in the let-7 binding site - a mechanism of RAS activation in juvenile myelomonocytic leukemia? Haematologica. 2010;95:1616-1616.

doi:10.3324/haematol.2010.024984

Juvenile myelomonocytic leukemia (JMML) is a rare hematologic malignancy in childhood and accounts for less than 3% of all childhood hematologic malignancies.[1] The role of hyperactive RAS in JMML is underlined by the fact that approximately 80% of JMML develop due to gain-of-function mutations in NRAS, KRAS, PTPN11 and SOS1 or homozygous loss-of-function mutations in NF1 or c-CBL.[2-4] These genes are all components of the RAS/ERK signaling network, implicating deregulation of this signaling pathway in JMML pathogenesis. Therefore, it may be speculated that JMML lacking known mutations of genes playing a role in RAS signaling may carry other mutations, which result in activation of this pathway. Recently, germline mi-RNA gene variations were proposed to affect the expression levels of tumor suppressor or oncogenes and, thereby, familial/hereditary cancer risk.[5] The let-7 mi-RNA family targets many important genes including cell cycle regulators such as CDC25A and CDK6, a number of early embryonic genes including HMGA2, Mlin-41 and IMP-1 and promoters of growth including RAS and C-MYC. In Caenorhabditis elegans let-7 mutant seam cells fail to exit the cell cycle and to terminally differentiate, thus demonstrating continuous proliferation, a hallmark of cancer.[6] Human RAS expression was also shown to be regulated by let-7.[7] Evidence of a role of let-7 in cancer came from the observation that lung tumor tissues display significantly reduced let-7 levels and significantly increased RAS protein levels relative to normal lung tissue.[8] Recently, an SNP (rs61764370) in a let-7 complementary site in the KRAS 3' UTR, which leads to KRAS overexpression, was shown to increase non-small cell lung cancer risk.[9] It is still unknown whether alterations of the let-7 binding site in target genes or if mutations of let-7 mi-RNAs play a role in the development and progression of JMML. To address this question, we sequenced the 3'UTR of N-RAS and K-RAS in bone marrow cells from 10 JMML patients who had no other known RAS pathway mutations. The let-7 complementary sites LCS1-LCS9 in the 3´ UTR of NRAS and the complementary sites LCS1-LCS8 of KRAS were amplified and PCR products were directly sequenced.[7] No sequence alterations were found in the 3´ UTR of NRAS or KRAS. However, in KRAS, the SNP rs61764370 T>G in the LCS6 was observed in one case. So far, only a few studies have investigated whether germline mutations in genomic mi-RNA sequences predispose to development of certain types of cancer or other diseases.[10,11] Recently, a germline mutation in mature miR-125a was found to be highly associated with breast cancer tumorigenesis, suggesting that miR-125a is likely to function as a tumor suppressor gene in human cancer.[12] Sequencing of let-7a-1 (MI0000060) as well as the neighboring let-7f-1 (MI0000067) genomic sequences in the 10 JMML cases did not identify any sequence alterations in these genomic regions.

In summary, we found no evidence that mutations in let-7 or in binding sites of let-7 mRNA targets lead to an upregulation of RAS genes in JMML. Yet, we cannot rule out that other mi-RNAs known to bind to NRAS- or KRAS-UTR or other let-7 family miRNAs may play a role in the development of JMML.

References

1. Hasle H, Wadsworth LD, Massing BG, McBride M, Schultz KR. A population-based study of childhood myelodysplastic syndrome in British Columbia, Canada. Br J Haematol. 1999; 106(4):1027-32.
2. Schubbert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer. 2007;7(4):295-308.
3. Flotho C, Kratz C, Niemeyer CM. Targeting RAS signaling pathways in juvenile myelomonocytic leukemia. Curr Drug Targets. 2007;8(6):715-25.
4. Loh ML, Sakai DS, Flotho C, Kang M, Fliegauf M, Archambeault S, et al. Mutations in CBL occur frequently in juvenile myelomonocytic leukemia. Blood. 2009;114(9):1859-63.
5. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer. 2006;6(11):857-66.
6. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature. 2000; 403(6772):901-6.
7. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, et al. RAS is regulated by the let-7 microRNA family. Cell. 2005;120(5):635-47.
8. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res. 2004;64(11):3753-6.
9. Chin LJ, Ratner E, Leng S, Zhai R, Nallur S, Babar I, et al. A SNP in a let-7 microRNA complementary site in the KRAS 3' untranslated region increases non-small cell lung cancer risk. Cancer Res. 2008;68(20):8535-40.
10. Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, et al. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell. 2006;9(6):435-43.
11. Shen J, DiCioccio R, Odunsi K, Lele S, Zhao H. Novel genetic variants in miR-191 gene and familial ovarian cancer. BMC Cancer. 2010;10(1):47.
12. Li W, Duan R, Kooy F, Sherman SL, Zhou W, Jin P. Germline mutation of microRNA-125a is associated with breast cancer. J Med Genet. 2009;46(5):358-60.

Short-term cryopreservation of allogeneic stem cells for optimization of transplant conditions in children

Cryopreservation is the technique of choice to store autologous peripheral blood stem cells (PBSC) and allogeneic umbilical cord blood stem cells. In contrast, donor stem cells for allogeneic hematopoietic stem cell transplantation (alloHSCT) are usually collected immediately prior to transplant and transfused "fresh" into the recipi-

ent. However, for medical reasons it may be necessary to delay conditioning and subsequent graft infusion when stem cell collection is already planned and cannot be rescheduled. In such situations allogeneic grafts may be cryopreserved and patients receive thawed products.

In 2009, 4 pediatric patients (male: female = 1:3; median age: 7 years) suffering from relapsed acute myeloid leukemia, Mucopolysaccharidosis I Hurler, relapsed severe aplastic anemia and metastatic osteosarcoma, respectively, were scheduled for allogeneic stem cell transplantation (patients' characteristics are summarized in Table 1). Conditioning had to be postponed for acute infections in 3 patients and for persistence of blasts in one patient. Stem cell donors (3 unrelated, one related) gave their consent to short-term stem cell cryopreservation and graft manipulation.

Cell doses of around 10×10^6/kg recipient body weight were requested. PBSC products were CD3/19-depleted, in 2 patients half of the product was CD34+selected; the median number of $CD34^+$cells/kg recipient body weight was 14.1×10^6/kg (median $CD3^+$cells/kg: 1.1×10^5). The manipulated products were split into aliquots/bags that could be infused several hours apart or on subsequent days with regard to potential dose-dependent DMSO-neurotoxicity especially in children with low body weight (≤ 10 kg).

The time interval from the end of the donation to freezing was 29, 28 and 27 h, respectively, for externally harvested apheresis products (n=3, patients 1-3), and 10 and 5 h, respectively, for in-house harvested products (n=2, patient 4).

Cryoprotectant solutions for unmanipulated PBSC consisted of 80% autologous plasma and 20% DMSO (CryoSure, WAK Chemie, Steinbach, Germany), for

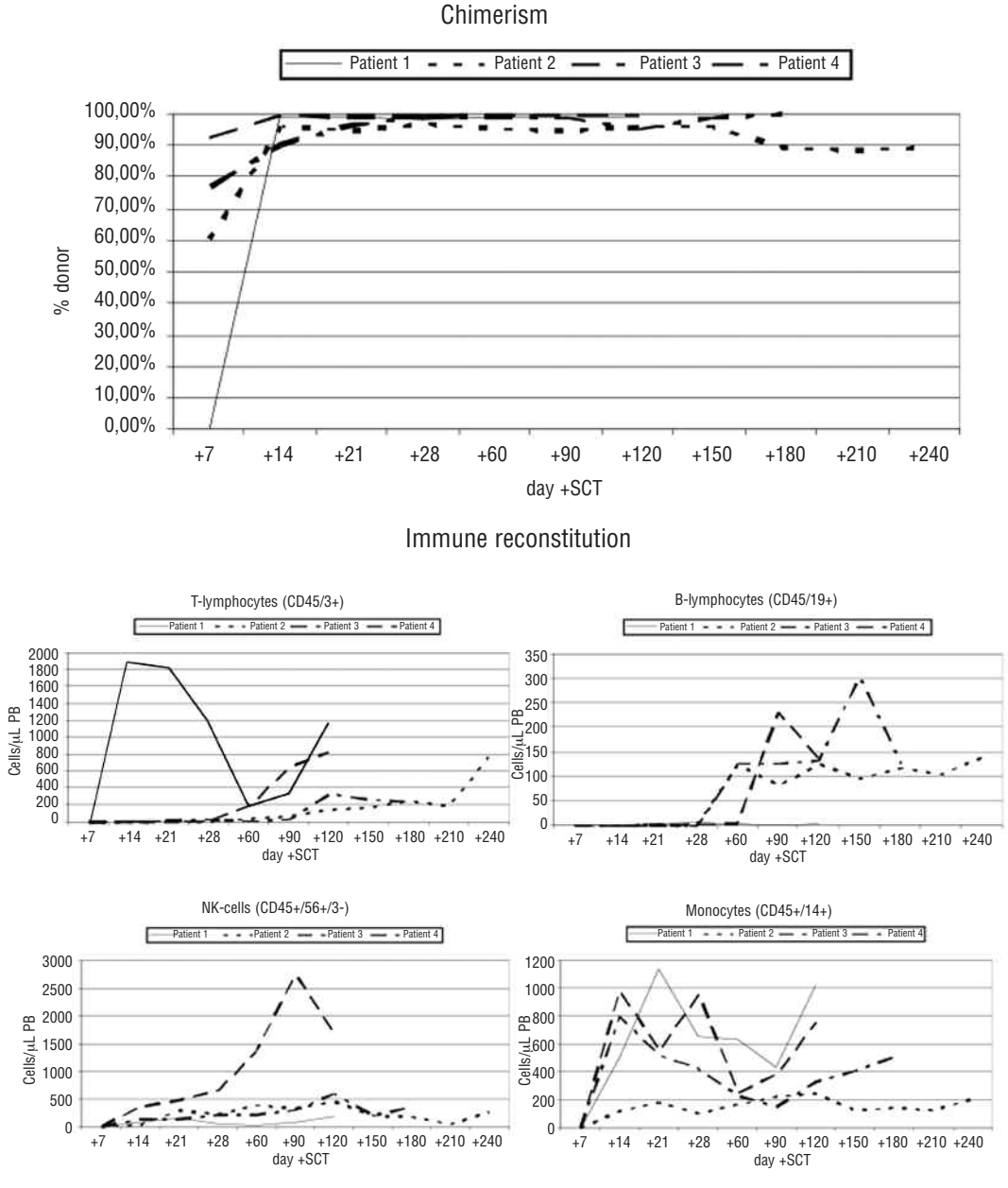

Figure 1. Donor chimerism of patients 1-4 as assessed by SNP-analysis on days +7,+14,+21,+28, + 60, +90, +120, +150, +180, +210 and +240 (upper panel). Absolute numbers of T-lymphocytes, B-lymphocytes, NK-cells, and monocytes at the same time points (lower panel).

CD34+ selected and CD3/19 depleted products of 70% MEM medium, 10% human albumin 20% and 20% DMSO-CryoSure. Apheresis products were added to equal volumes of cryoprotectant solution to a final DMSO-concentration of 10%. Cryopreservation was performed using a controlled phased freezing procedure within one hour to a temperature of -120°C (Ice-Cube 1810 Computer Freezer, Sylab). Cryo-bags were stored in liquid nitrogen at a temperature of -196°C. At the time of alloHSCT, frozen grafts were thawed rapidly in a warm water bath at 37°C and infused via a central venous catheter. Stem cell products were cryopreserved for a median 12 days (range 6-102 days).

Sterility testing by bacterial cultures of aliquots yielded negative results in all patients. CD34+ cells and CD3+ cells were measured by flow cytometry in aliquots of PBSC products before cryopreservation and after thawing; correlation coefficient for CD34+ cell numbers was 0.9557, for CD3+ cell numbers 0.9932. Viability after thawing was assessed by trypan blue staining and was 91±4.08%.

Three patients received fludarabine-based conditioning, one patient a busulfan-based regimen.

Stem cell infusion was tolerated without side effects.

All patients had three-lineage hematopoietic engraftment. The posttransplant course of patients 2 to 4 was uneventful and without signs of GVHD. Patient 1 with a refractory high-risk AML, who received a partly unmanipulated PBSC-graft, reactivated the hemophagocytic syndrome, which she had manifested just before the start of conditioning, in the early posttransplant period, and developed acute graft-versus-host-disease (GVHD) of skin and gut up to grade IV. She had a poor graft function due to the cumulative toxicity of the intense multimodal immunosuppressive and virostatic treatment, and succumbed to aspergillus pneumonia on day +140 in remission from her refractory AML.

Transplant characteristics as well as engraftment data are summarized in Table 1. Chimerism and immune reconstitution data are shown in Figure 1.

Informed consent was obtained from the patients' parents to stem cell transplantation including cryopreservation and to the study, which was approved by the Ethics Committee of the Medical University of Graz.

There are several advantages and disadvantages regarding cryopreservation of allogeneic stem cells as discussed in a recent review.[1] Data on the use of cryopreserved allo-

Table 1. Patients' characteristics, details of transplant procedure and posttransplant course.

	Patient 1	Patient 2	Patient 3	Patient 4
Diagnosis	Relapsed AML FAB M6	MPS I Hurler Syndrome	SAA – relapse	Metastatic osteosarcoma
Gender	f	m	f	f
Age at alloHSCT (years)	2.6	1.8	15.9	11.3
Recipient weight (kg)	15	12	45	45
Pre-treatment	Front-line AML-BFM 2004 polychemotherapy	ERT (Iduronidase)	IST for 3.5 yrs	EURAMOS polychemotherapy, surgery (primary site + lung metastases)
Reason for PBSC cryopreservation	Persistence of blasts after 2nd induction cycle: 3rd cycle	URTI: RSV+, Adeno +, HHV6 +	Suspected infection due to CRP-increase: antibiotic therapy	CRP-increase: ?endoprosthesis-inf. antibiotic therapy
Conditioning regimen	Bu 1.6 mg/kg x 8 VP-16 30 mg/kg x 1 Cyclo 60 mg/kg x 2 ATG 15 mg/kg x 3	TT 8mg/kg x 1 Fludara 40 mg/m²x 4 Treo 14 g/m² x 3 ATG 20 mg/kgx3	TT 8 mg/kg x 1 Fludara 40 mg/m²x 4 Cyclo 60 mg/kg x 2 ATG 20 mg/kg x 3	TT 10mg/kg x 1 Fludara 40 mg/m²x 4 Mel 70 mg/m² x 2 OKT3 0.1 mg/kg x20
Donor/ HLA-match	Unrelated/ 11/12	Unrelated/ 10/12	Unrelated/ 12/12	Mother/ haplo
Duration of cryopreservation (days)	6	13	11	102
Graft manipulation	1/3 unmanipulated 2/3:CD3/19 depleted	CD3/19 depleted	1/2: CD34+ selected 1/2: CD3/19 depleted	1/2: CD34+ selected 1/2: CD3/19 depleted
AlloPSCT:CD34+/kg	10.55×10^6	20.3×10^6	12.9×10^6	15.37×10^6
AlloPSCT: CD3+/kg	3.277×10^8	1.2×10^5	5.5×10^4	1.0×10^5
Volume (ml/kg)/ #bags	13.3 / 2	8.3 / 1	6.7 / 3	8 / 4
DMSO (mg/kg)	1463	913	737	880
Graft *vs.* host prophylaxis	MMF	MMF	MMF	MMF
ANC >0.5×10^9/L (day)	+17	+19	+10	+9
GVHD	aGVHD IV (skin+gut)	None	None	None
Follow-up time (days)	140	273	190	137
Outcome	Died of *aspergillus* pneumonia	A & W	A & W	A & W

alloHSCT: allogeneic hematopoietic stem cell transplantation; PBSC: peripheral blood stem cells; HLA: human leukocyte antigen; alloPSCT: allogeneic peripheral blood stem cell transplantation; Volume ml/kg: volume of graft in ml/kg; #bags: number of bags; DMSO: dimethylsulfoxide; ANC >0.5×10^9/L: absolute neutrophil count greater than 0.5×10^9/L; GVHD: graft versus host disease; AML: acute myeloid leukemia; MPS I : Mucopolysaccharidosis I; SAA : severe aplastic anemia; ERT: enzyme replacement therapy; IST: immunosuppressive therapy; URTI: upper respiratory tract infection; RSV: respiratory syncitial virus; Adeno: adenovirus; HHV6: human herpesvirus 6; CRP: C-reactive protein; Bu: busulfex; VP16: etoposide; Cyclo: cyclophosphamide; ATG: antithymocyte globulin fresenius; TT: thiotepa; Fludara: fludarabine; Treo: treosulfan; Mel: melphalan; haplo: haploidentical; MMF: mycophenolate mofetil; A&W: alive and well.

geneic grafts are limited and almost exclusively restricted to adults. For cryopreserved compared to fresh bone marrow transplants from related and unrelated donors no differences were found with regard to time to myeloid or platelet engraftment, incidence or severity of acute and chronic GVHD, day 100 survival and long-term survival.[2-4] A trend toward less acute GVHD in patients who received cryopreserved bone marrow reported by one group[5] was not confirmed by others.[2] Although the use of cryopreserved allogeneic PBSC grafts has increased over recent years,[1] few reports have been published so far demonstrating conflicting results.[6-8]

In 2006, Frey et al. stated in their review that the "available literature does not sufficiently justify the dogmatic use of fresh over frozen allografts".[1] Since then, contradictory data on cryopreserved alloPBSC grafts were added. In modern and increasingly sophisticated transplant settings including alloHSCT from haploidentical family donors and posttransplant adoptive immunotherapy, graft manipulation is a prerequisite and temporary cryopreservation of certain grafts as well as donor lymphocytes is being performed.[9-11]

There is a concern that grafts might be cryopreserved in advance but not utilized and that donors might be subjected unnecessarily to the potentially harmful procedure of stem cell collection.[12] This could be met by keeping the time interval between harvest and the definitive start of the transplant procedure as short as possible (e.g. around 30 days) as suggested by Frey based on reported median storage times ranging from 10.5 to 38 days.[1]

We conclude that short-term cryopreservation of unrelated PBSC allografts in pediatric patients is feasible for compelling medical reasons. The advantages of the use of cryopreserved grafts must be individually outweighed against the concerns raised but not definitely answered by the available data. Looking to the future, and in view of the increasing use of manipulated grafts, we suggest that short-term cryopreservation might be an option to ensure graft quality and to enhance procedure safety for the patient without increasing the risk for the donor. Therefore, further studies regarding cryopreservation of allogeneic PBSC, including manipulated grafts on a larger and more homogenous patient cohort, are required.

Petra Sovinz,[1] Wolfgang Schwinger,[1] Herwig Lackner,[1] Andrea Nebl,[1] Sabine Sipurzynski,[2] and Christian Urban[1]

[1]Division of Pediatric Hematology/ Oncology, Department of Pediatrics and Adolescent Medicine, and [2]Department of Blood Group Serology and Transfusion Medicine; Medical University of Graz, Austria

Correspondence: Wolfgang Schwinger, Division of Pediatric Hematology/Oncology, Department of Pediatrics and Adolescent Medicine, Medical University of Graz; Auenbruggerplatz 30, 8036 Graz, Austria. Phone: international +43.316.38583329. Fax: international +43.316.38513450. E-mail: wolfgang.schwinger@medunigraz.at

Acknowledgments: the authors would like to thank Andrea Raicht, B.Sc., and Barbara Egner, B.Sc., for performing SNP-and FACS analyses and for their technical assistance.

The authors reported no potential conflicts of interest.

Citation: Sovinz P, Schwinger W, Lackner H, Nebl A, Sipurzynski S, and Urban C. Short-term cryopreservation of allogeneic stem cells for optimization of transplant conditions in children. Haematologica 2010; 95(9):1616-1619. doi:10.3324/haematol.2009.021592

References

1. Frey NV, Lazarus HM, Goldstein SC. Has allogeneic stem cell cryopreservation been given the "cold shoulder"? An analysis of the pros and cons of using frozen versus fresh stem cell products in allogeneic stem cell transplantation. Bone Marrow Transplant. 2006;38(6):399-405.
2. Stockschläder M, Krüger W, Kroschke G, Zeller W, Hoffknecht M, Löliger C, et al. Use of cryopreserved bone marrow in allogeneic bone marrow transplantation. Bone Marrow Transplant. 1995;15(4):569-72.
3. Stockschläder M, Krüger W, tom Dieck A, Horstmann M, Altnöder M, Löliger C, et al. Use of cryopreserved bone marrow in unrelated allogeneic transplantation. Bone Marrow Transplant. 1996;17(2):197-9.
4. Stockschläder M, Hassan HT, Krog C, Krüger W, Löliger C, Horstmann M, et al. Long-term follow-up of leukaemia patients after related cryopreserved allogeneic bone marrow transplantation. Br J Haematol. 1997;96(2):382-6.
5. Eckardt JR, Roodman GD, Boldt DH, Clark GM, Alvarez R, Page C, et al. Comparison of engraftment and acute GVHD in patients undergoing cryopreserved or fresh allogeneic BMT. Bone Marrow Transplant. 1993;11(2):125-31.
6. Stockschläder M, Löliger C, Krüger W, Zeller W, Heyll A, Schönrock-Nabulsi P, et al. Transplantation of allogeneic rhG-CSF mobilized periperal CD34+cells from an HLA-identical unrelated donor. Bone Marrow Transplant. 1995;16(5):719-22.
7. Kim DH, Jamal N, Saragosa R, Loach D, Wright J, Gupta V, et al. Similar outcomes of cryopreserved allogeneic peripheral stem cell transplants (PBSCT) compared to fresh allografts. Biol Blood Marrow Transplant. 2007;13(10):1233-43.
8. Lioznov M, Dellbrügger C, Sputtek A, Fehse B, Kröger N, Zander AR. Transportation and cryopreservation may impair haematopoietic stem cell function and engraftment of allogeneic PBSCs, but not BM. Bone Marrow Transplant. 2008;42(2):121-8.
9. Sohn SK, Jung JT, Kim DH, Lee NY, Seo KW, Chae YS, et al. Prophylactic growth factor-primed donor lymphocyte infusion using cells reserved at the time of transplantation after allogeneic peripheral blood stem cell transplantation in patients with high-risk hematologic malignancies. Cancer. 2002;94(1):18-24.
10. Amrolia PJ, Muccioli-Casadei G, Huls H, Adams S, Durett A, Gee A, et al. Adoptive immunotherapy with allodepleted donor T-cells improves immune reconstitution after haploidentical stem cell transplantation. Blood. 2006;108(6):1797-808.
11. Schlegel PG, Wölfl M, Schick J, Winkler B, Eyrich M. Transient loss of consciousness in pediatric recipients of dimethylsulfoxide (DMSO)-cryopreserved peripheral blood stem cells independent of morphine co-medication. Haematologica. 2009;94(10):1473-5.
12. Halter J, Kodera Y, Urbano Ispizua A, Greinix HT, Schmitz N, Favre G, et al; for the European Group for Blood and Marrow Transplantation (EBMT) activity survey office. Severe events in donors after allogeneic hematopoietic stem cell donation. Haematologica. 2009;94(1):94-101.