Mixed chimerism following hematopoietic stem cell transplantation in pediatric thalassemia major patients: a single center experience

Pediatrik talasemi majör hastalarında hematopoetik kök hücre nakli sonrası karışık tip kimerizm-tek merkez sonuçları

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Abstract

Objective: Stable mixed chimerism (MC) may result in cure for thalassemia major patients following hematopoietic stem cell transplantation (HSCT), but rejection can occur. Twenty-eight HSCTs for thalassemia major were reviewed retrospectively to evaluate the clinical course of MC with possible risk factors and predictors of outcome, with a median follow-up of 1669 days (811-3576 days).
Materials and Methods: Chimerism was detected by fluorescence in situ hybridization (FISH) or multiplex polymerase chain reaction depending on the sex match between the donor and the recipient.
Results: Primary rejection, stable MC and full donor chimerism was detected in 3.6%, 17.8% and 78.6% of patients, respectively. Clinically, 4/5 patients with stable MC had thalassemia trait with donor chimerism as low as 14%. One patient was started on pRBC transfusions at 2.5 years postHSCT.
Conclusion: Stable MC can result in cure for thalassemia major patients. The clinical picture remains as the best guide for intervention until a more reliable predictor is available. *(Turk J Hematol 2010; 27: 8-14)*
Key words: Thalassemia major, mixed chimerism, pediatrics, chimerism, hematopoietic stem cell transplantation, non-malignant

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Özet

Amaç: Talasemi major (TM) hastalarında hematopoetik kök hücre transplantasyonu (HKHT) sonrasında stabil karışık-tip kimerizm ile kür mümkündür fakat rejeksiyonla sonuçlanabilir. TM tanısıyla yapılmış 28 HKHT karışık-tip kimerizm seyri, risk faktörleri ve sonuçlarını değerlendirmek üzere retrospektif olarak gözden geçirildi.
Yöntem ve Gereçler: İzlem süresi ortanca 1669 gündü (811-3576 gün). Kimerizm alıcı-verici cinsiyet uyumuna göre FISH veya multipleks PCR yöntemiyle bakıldı.
Bulgular: Primer rejeksiyon, stabil karışık tip kimerizm ve tam verici tip kimerizm sırasıyla % 3.6, % 17.8 ve % 78.6 idi. 4/5 hastada % 14 kadar düşük verici kimerizmine rağmen talasemi taşıyıcılığı kliniği vardı. Sadece 1 hastada HKHT sonrası 2.5 yıl sonra eritrosit ihtiyacı oldu.

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Sonuç: Stabil karışık-tip kimerizm, talasemi major hastalarında kür sağlayabilir ve bu hastalar için rejeksiyonu önceden gösteren bir belirteç bulunana kadar hastaların kliniği, herhangi bir müdahale için belirleyici olmalıdır.
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Anahtar kelimeler: Talasemi majör, karışık tip kimerizm, pediatri, kimerizm, hematopoetik kök hücre transplantasyonu, non-malign

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Introduction

Hematopoietic stem cell transplantation (HSCT) is the only curative treatment for thalassemia major [1-4]. However, graft rejection is the undesired possible outcome following mixed chimerism (MC) [4-7]. The evolution of MC after its first detection has been studied as a possible predictor of outcome, mainly to intervene in time in the necessary cases in order to prevent rejection. The risk factors in the occurrence as well as the outcome of MC remain elusive. For the occurrence of MC, the conditioning regimen has been shown to be a significant risk factor in some studies [8,9], but not in all [10]. For the outcome, the early detection and severity of MC were found to be significant in predicting the course of MC [5]. In this retrospective study, we evaluated the course of chimerism in patients with thalassemia major who received HSCT in our institution to determine the possible risk factors and the possible clinical predictors of outcome of MC.

Materials and Methods

Patient Population

Twenty-eight thalassemia major patients received 29 HSCT from an HLA-identical family donor from April 1999 to November 2007 at Ankara University Medical Faculty Pediatric Hematopoietic Stem Cell Transplantation Unit. One patient was removed from the study because of early treatment-related mortality. The remaining 27 patients and 28 transplants were evaluated retrospectively for the chimerism studies. One patient received a second transplant from the same donor following primary rejection. The median follow-up was 1669 days (range: 811-3576 days), median age was 8 years (range: 2-17 years) and the male/female ratio was 16/11. The patient characteristics are summarized in Table 1. Risk classification prior to HSCT was made as per Pesaro classification [2]. All patients above three years of age had a liver biopsy done to evaluate for fibrosis.

Conditioning Regimen, GVHD Prophylaxis and Engraftment

Patients received conditioning regimen based on their risk classification as shown in Table 2. All Class III patients received conditioning regimen as per Pesaro Protocol 26 and antithymocyte globulin (ATG) except one patient who had received Pesaro Protocol 26 without ATG. Thiotepa was used in the conditioning regimen of two patients who received cord blood transplantation. Cyclosporine (CSA) and methotrexate (MTX) were used as graft-versus-host disease (GVHD) prophylaxis in 89.3% of the patients. CSA was started on day -1 at 3 mg/kg/day parenterally and was switched to peroral (PO) when the patient was clinically stable and could tolerate oral medications. CSA was tapered by 10% every other week beginning on day +60 and discontinued at +6 months if there was no evidence of GVHD. Dose adjustments were made to keep the

Table 1. Patient characteristics

	n	%
Number of Patients	27	
Number of Transplants	28	
Median Age in years (range)	8 (2-17)	
Sex		
Male	17	60.7
Female	11	39.3
Risk Classification		
Class I	5	17.8
Class II	8	28.5
Class III	14	50
2nd transplant	1	3.5
Donor Status		
Thalassemia Minor	18	64.3
Normal	10	35.7
Stem Cell Source		
Bone Marrow	15	53.5
Peripheral Blood	10	35.7
Cord Blood	2	7.2
Cord Blood + Bone Marrow (same donor)	1	3.6
Conditioning Regimen		
Busulphan+ Cyclophosphamide	11	39.3
Busulphan+ Cyclophosphamide+Thiotepa	2	7.2
Pesaro Protocol 26	1	3.5
Pesaro Protocol 26+ATG	14	50
GVHD Prophylaxis		
Cyclosporine+Methotrexate	25	89.3
Cyclosporine	3	10.7

GVHD: Graft versus host disease; ATG: Antithymocyte globulin

Table 2. Conditioning regimens

Risk Class	Conditioning Regimen	Dose	Days
Class I and Class II	Busulphan	480 mg/m^2	-9 to -6
	Cyclophosphamide	200 mg/kg	-5 to -2
	(Thiotepa)*	250 mg/m^2	-6
Class III	Hydroxyurea	25-30 mg/kg/day	-45 to -15
	Azathioprine	2.5-3 mg/kg/day	-45 to -15
	Fludarabine	120 mg/m^2 (total)	-14 to -10
	Busulphan	14 mg/kg (total)	-9 to -6
	Cyclophosphamide	40 mg/kg (total)	-5 to -2
	ATG	4-10** mg/kg/day	-4 to -1

*Thiotepa was used in 2 patients who received cord blood transplantation. For these patients, busulphan was given on days -10 to -7
ATG: Antithymocyte globulin
**Thymoglobulin: 4.0-4.5 mg/kg/day - ATG-Fresenius: 9-10 mg/kg/day

CSA trough levels between 150-200 ng/ml. Class I and Class II patients received MTX at 8 mg/m^2 on day +1 and 10 mg/m^2 on days +3 and +6, and Class III patients received MTX at 10 mg/m^2 on days +1, +3 and +6. GVHD prophylaxis was discontinued at six months postHSCT in the absence of GVHD. GVHD was graded according to the Glucksberg and Seattle consensus criteria [11,12].

Myeloid engraftment (neutrophil recovery) was defined as an absolute neutrophil count (ANC) ≥500/mm^3 for two consecutive days after nadir. Platelet engraftment was defined as a platelet count of ≥20,000/mm^3 independent of platelet transfusions for at least seven consecutive days.

Supportive Care: All patients were cared for in laminar air flow units, and received prophylactic fluconazole and acyclovir beginning on day -1 up to 75 and 180 days after transplantation, respectively. Co-trimoxazole was administered until day -1 and then resumed after neutrophil engraftment. Ciprofloxacin was used for antibacterial prophylaxis. Granulocyte colony-stimulating factor (G-CSF) was used at 5 μg/kg/day intravenously in 26 of the transplants. In three of these, G-CSF was used for delayed engraftment, and for 23 patients, it was started on day +5 until the ANC was ≥2.5x10^9 cells/L for two consecutive days and then was discontinued.

Chimerism Studies

Patients: Patients were evaluated in two categories: [1] Twenty-eight transplants with at least one-year follow-up postHSCT were evaluated for the long-term follow-up of MC, and (2) Twenty transplants within this group with chimerism evaluation done both at the 1st month and the 3rd month postHSCT were evaluated for the evolution of early MC detected at the 1st month postHSCT.

Chimerism studies were done on a routine basis only after 2001. For patients who were transplanted after that date, chimerism studies were scheduled to be done at the 1st, 3rd, 6th and 12th month postHSCT. Patients who were transplanted before 2001 had their chimerism analysis done only after 2001 at whichever postHSCT date they were at. Written informed consent was obtained from all transplant patients.

Method: Chimerism was evaluated by fluorescence in situ hybridization (FISH) in 13 patients who had sex mismatch donors, and 14 who had sex match were evaluated by single nucleotide polymorphism (SNP) analysis done by multiplex polymerase chain reaction (PCR). Quantitative multiplex PCR-based method was performed by the detection of the short tandem repeats (STRs) on specific genes. DNA was extracted from whole peripheral blood/bone marrow (PB/BM) samples using the Invitek kit (Berlin, Germany). Multiplex STR-PCR was performed on 2 ng of genomic DNA using the AmpFlSTR SGM Plus (PEBiosytems, CA, USA) or AmpFlSTR Identifier Kits (Applied Biosystems, CA, USA), which contain 10-15 STR loci plus amelogenin gene, respectively. The repeat regions detected by AmpFlSTR SGM Plus included D3S1358, HUMvWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433, THO1, FGA, and amelogenin. The AmpFlSTR Identifier Kit additionally detects the D7S820, CSF1PO, D13S317, TPOX, and D5S818 repeats. Upon denaturation by deionized formamide, amplified PCR products were subjected to capillary electrophoresis in an ABI Prism 310 or ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using the conditions recommended by the manufacturer. All the fluorescence signals generated from the amplicons were recorded and analyzed by the Gene Scan 3.1 or Gene Mapper ver 4.0 softwares (Applied Biosystems). In order to increase specificity, only the markers in which recipient specific peaks showed no residual fluorescence might be generated from stutter peaks or spectral overlap, and the markers that were not homozygote for any defined allele were considered as informative and taken into account for the calculations. Chimerism was quantified as suggested by Fernández-Avilés et al. [13] using the peak areas calculated by the software. For FISH analysis, CEPX SO/CEPYSG direct-labeled fluorescent DNA probe kit was used (Abbott, Germany). Following the MNC isolation using Ficoll 1077, cells were fixated by Carnoy solution. Hybridization was performed as described by the manufacturer, and at least 500 cells were counted with Nikon E600 fluorescent microscope and the ratio of XX and XY signals was calculated.

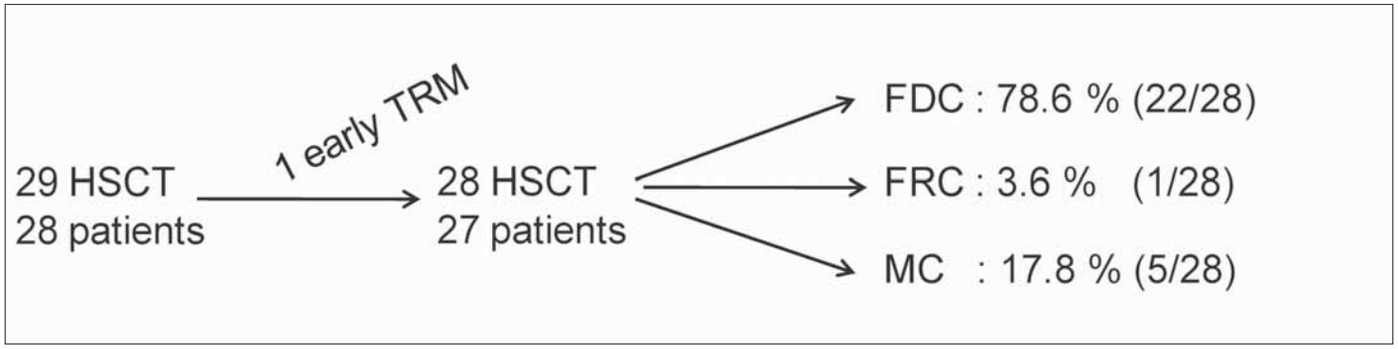

Figure 1. Summary of the chimerism outcome
MC: Mixed chimerism; FRC: Full recipient chimerism; FDC: Full donor chimerism

Table 3. Characteristics of patients with mixed chimerism

Pt#	Age (yrs)	Sex	Risk Classification	Stem Cell Source	Donor Trait Status	Conditioning Regimen	Time of Engraftment (Days)		#of Cells Infused		GVHD Prophylaxis	GVHD	Last Chimerism (%)	Follow-up (Months)
							Myeloid	Platelet	Nucleated Cell Count (10^8/kg)	CD34 + Cell Count (10^6/kg)				
1	2	Male	Class I	Bone Marrow	Trait	BU+CTX	26	26	4.00	2.90	CSA+MTX	_	40	90
2	3	Male	Class II	Bone Marrow	Trait	BU+CTX	15	25	2.83	1.83	CSA+MTX	_	53	82
3	7	Female	Class II	Bone Marrow	Trait	BU+CTX	29	26	3.88	7.86	CSA+MTX	_	47	90
4	11	Female	Class III	Peripheral Blood	Trait	Pesaro Protocol 26+ATG	15	17	6.40	8.00	CSA+MTX	_	18	37
5	9	Male	Class III	Peripheral Blood	Normal	Pesaro Protocol 26+ATG	12	20	15.00	8.37	CSA+MTX	_	88	31

BU: Busulphan; CTX: Cyclophosphamide; ATG: Antithymocyte globulin; GVHD: Graft versus host disease; CSA: Cyclosporine; MTX: Methotrexate

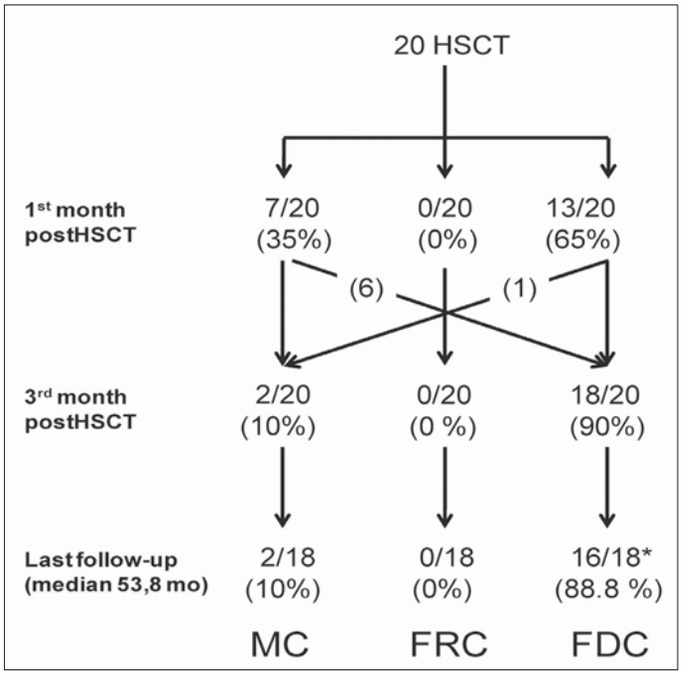

Figure 2. Evolution of the early MC detected at 1st month postHSCT
MC: Mixed chimerism; FRC: Full recipient chimerism; FDC: Full donor chimerism.
*Two patients who died after 1 year postHSCT had FDC

Definitions of Chimerism Status: MC was defined as the presence of >5% residual host hematopoietic cells. Rejection was defined as >90% residual host hematopoietic cells with relapse of thalassemia and packed red blood cell (pRBC) transfusion dependence [14]. Stable MC was defined as fluctuations in the percentage of recipient cells over time without complete loss of donor cells [15]. Patients were also evaluated as per Nesci et al.'s [5] classification. Patients were included in level I if the residual host cells were less than 10%, level II if the residual host cells were 10-30% and level III if the residual host cells were >30%.

Statistical Analysis*:* Results are presented as medians with specified ranges of data sets. The relationship between clinical parameters and chimerism was determined using Mann-Whitney test for quantitative variables. Comparison of the percentages between two groups was performed using χ^2 test or Fisher's exact test where appropriate. Differences with a P value less than 0.05 were considered significant. SPSS version 15.0 (Statistical Package for Social Sciences) statistical software was used for analyses.

Results

Long-term Follow-Up and Evaluation of Stable MC

All 28 transplants were evaluated for the long-term follow-up. The median time for follow-up was 1669 days (range: 811-3576 days). The overall survival was 92.9%. Twenty-two

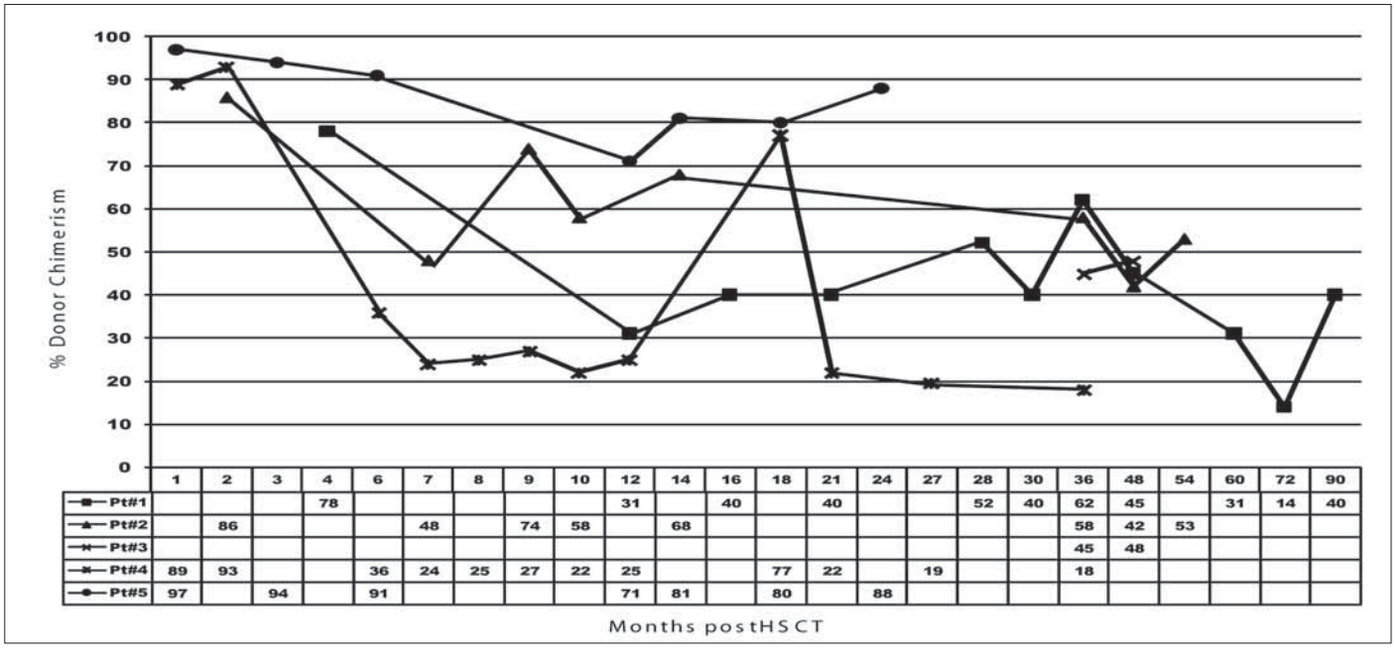

Figure 3. Course of chimerism in patients with MC

Table 4. Clinical course of patients with MC

Pt#	ABO Status	Median Hemoglobin (g/dL) (Range)	PostHSCT Day of Last pRBC Transfusion	Last Hemoglobin without Transfusion (g/dl)	Last chimerism (%)	Current Follow-up postHSCT (months)	Clinical Status
1	I	9.3 (5.4-11.1)	119	9.8	40	90	Thalassemia Trait
2	I	10.3 (4.8-12.2)	334	10.8	53	82	Thalassemia Trait
3	C	9.8 (7.8-11.4)	43	9.4	47	90	Thalassemia Trait
4	C	7.1 (5.2-9.0)	16	5.2	18	37	Thalassemia Intermedia
5	C	9.8 (7.4-10.7)	40	9.3	88	31	Thalassemia Trait

I: ABO incompatible; C: ABO compatible

patients had full donor chimerism (FDC) (78.6%) and 5 had MC (17.8%), with one of these patients starting pRBC transfusions at 2.5 years postHSCT. Only one patient had primary rejection (3.6%) (Figure 1). Two patients died with infection 394 and 507 days following HSCT. One patient who had primary rejection had autologous recovery and received a second transplant 1.5 years after the first. He had FDC following the second transplant.

Stable MC was detected in 17.8% (5/28) of patients and the patient characteristics are summarized in Table 3. The course of chimerism during the follow-up of these patients is shown in Figure 2. The median follow-up for patients with MC was 2548 days (974-2814 days). Four of the five patients with MC (Pts 1, 2, 3 and 5) had a clinical picture of thalassemia trait as confirmed by hemoglobin electrophoresis and with near normal hemoglobin values. Patients 3 and 5 had not required pRBC transfusions after day 43 and 40 postHSCT. Pt 1 and Pt 2 had major ABO incompatibility and required pRBC transfusions till day 119 and day 334 postHSCT, respectively. Pt 2 received erythropoietin mainly to reduce the pRBC requirements at 125 u/kg/day three times a week between days 250-411 postHSCT. Following the erythropoietin treatment, hemoglobin values improved and transfusion requirements decreased. Remarkably, Pt 1 has not required any pRBC transfusions despite very low donor chimerism of 14% detected at 72 months postHSCT.

Pt 4, who has been followed for 3.1 years postHSCT with a clinical picture of thalassemia intermedia, was started on pRBC transfusions with a hemoglobin of 5.2 g/dl at 2.5 years postHSCT. Her median hemoglobin had been 7.1 g/dl (5.2-9.0) until then and did not require any transfusions. Erythropoietin was used between days 209-307 postHSCT with her first sign of thalassemia intermedia to improve the clinical picture with no change in hemoglobin values. No additional immunosuppressive agent was prescribed. Currently she is 3.1 years postHSCT and considered for a second HSCT. Table 4 summarizes the clinical course of patients with MC.

Evolution of Early Chimerism Status

Twenty patients who had chimerism studies done both at the 1st month and the 3rd month postHSCT were evaluated for the evolution of the chimerism during the first few months and results are summarized in Figure 3. At the 1st month, MC was detected in 7/20 patients (35%). At the 3rd month evaluation, only 1 of these patients (14.3%) continued to have MC and the

remaining 6 (85.7%) achieved FDC. One of the 20 patients who had FDC at the 1st month regressed to MC at the 3rd month evaluation. All patients who had FDC at the 3rd month continued to have FDC with a median follow-up of 1159 days (394-1974 days). When the 7 patients who had MC at the 1st month were classified according to the criteria suggested by Nesci et al. [5], 4/7 were at MC level I and 3/7 were at MC level II. The only patient within this group who continued to have MC in the long-term was at MC level II with 89% donor chimerism at the time of the 1st month evaluation.

In the long-term, when all 28 transplants were evaluated regardless of having chimerism studies done at the 1st month, 4 patients had MC at the 3rd month evaluation and all had stable MC in the long-term. Additionally, all 16 patients who had FDC at the 3rd month evaluation had FDC in the long-term.

Evaluation of Risk Factors and Potential Predictors for MC

The recipient's age, sex, disease class, conditioning regimen, nucleated cell and CD34+ cells infused, time of myeloid engraftment, duration and regimen for GVHD prophylaxis, presence of acute (≥ grade II) or chronic GVHD, and donor sex and thalassemia trait status were evaluated as the potential risk factors and predictors. None was found to be statistically significant.

Discussion

Mixed chimerism following HSCT occurs both in malignant and non-malignant disorders, and cure or clinically stable disease can be achieved with stable MC in patients with thalassemia major [5,6,16,17]. Possible rejection is the main concern with the initial detection of MC. The evolution of MC after its first detection has been studied mainly to intervene in time to prevent possible rejection. In our study, chimerism evaluations at the first month following HSCT demonstrated 65% FDC. Of the 35% who had MC at the first evaluation, 85.7% recovered to FDC at the second evaluation done at the 3rd month postHSCT. At the first-year follow-up, 17.8% of the patients had stable MC. These findings correlate with the literature in terms of the early and later incidence of MC following HSCT for thalassemia major. Early MC was reported as 31% and 36.5% by Amrolia et al. and Nesci et al., respectively [5,14]. In the same study, at the longer follow-up (8-46 months postHSCT), stable MC was detected in 20% of the patients. Additionally, it has been shown that the early detection of MC does not always correlate with the chimerism outcome in the long-term [5,6]. In our study, at the second evaluation, which was done at 3 months, most patients who had MC initially recovered to FDC, and the only patient who did not continued to have MC at 3.1 years follow-up. Thirty-five percent of patients (7/20) had changed their chimerism status from the first evaluation to the second (6 patients to FDC and 1 patient to MC). Although the study group was very small, our study suggests that the later evaluation of chimerism (in our study the 3rd month postHSCT) might be more representative of the outcome than the first month evaluation for this group of patients.

Other than the timing, severity of MC was studied as a predictor of long-term outcome of MC in the literature. Nesci et al. [5] graded the MC in three groups according to the degree of MC and evaluated the outcome for each group. In their study, MC level III, defined as having >30% recipient chimerism, correlated with rapid graft rejection within the first year following HSCT. In our study group, no patient was at MC level III at the first evaluation. Of the patients with stable MC, only two had an evaluation done at the first month. One was at MC level I and the other at MC level II. Our study group was too small to evaluate for the influence of the MC level on long-term stable chimerism; larger study groups may be helpful to draw final conclusions in this regard.

The risk factors for MC have been studied by different groups. So far, only the conditioning regimen was found to have a significant role in the occurrence of MC for thalassemia major. In 1992, Nesci et al. [5] found significant influence of the conditioning regimen at the early MC evaluated at two months postHSCT. At later follow-ups, this significance disappeared but MC remained higher in the group of patients who received reduced doses of cyclophosphamide (120 mg/kg versus 200 mg/kg) (Pesaro Protocol 12 versus Protocol 6), and the reduced immunosuppression was discussed as a possible reason for the incomplete suppression of the host residual cells [5]. In another study, the busulphan levels were not found to affect the chimerism status statistically, but patients with MC tended to have lower levels of busulphan [10]. In our study, there was no significant difference between the two groups of patients receiving different conditioning regimens. Compared to the previous report by Nesci et al. [5] in 1992, the conditioning regimen used in our patients is more intense for Class III disease as well as for Class I and II. Our group used busulphan as per m^2 for Class I and II patients as opposed to per kg dosing used in the Pesaro protocol. The per kg equivalent dose of busulphan used for Class I and II patients in this study was a median 19.8 mg/kg (range: 18.1 mg/kg-20.6 mg/kg). Considering the intensity of the regimens increasing over time to prevent rejection in thalassemia patients, the rate of MC seems to stay approximately the same. The role of the conditioning regimen remains unclear for the occurrence of MC. None of the other risk factors evaluated in this study including age, sex, disease class, conditioning regimen, use of G-CSF after stem cell infusion, nucleated cell and CD34+ cells infused, time of myeloid engraftment, GVHD prophylaxis regimen and duration, presence of GVHD, and sex and thalassemia trait status of the donor was found to be statistically significant. Further studies are necessary to clarify the role of the conditioning regimen and to identify the risk factors for the occurrence of MC. The issue of intervention for MC has been controversial. In this study, one patient had a donor chimerism of 14% with near normal hemoglobin values whereas another patient with 19% donor chimerism started requiring pRBC transfusions. When and how to intervene in MC remain the critical questions to be answered. Erythropoietin, donor lymphocyte infusion (DLI) and/or changing the dose of immunosuppression have been tried to augment the donor chimerism [8,14,18]. No change in the immunosuppression schedule was made in any of our patients with MC because of the stable

hemoglobin values. Erythropoietin was tried in two patients with no increase or change in either donor chimerism or hemoglobin levels. DLI had been reported to be useful by some authors although the majority of the experience with DLI was following T-cell depleted HSCT [18]. The complications following DLI include GVHD and aplasia. The risks and benefits of this intervention should be considered carefully in view of the lack of strong evidence and clinical studies. The clinical status of the patient may be the only guidance so far for any intervention. But the question remains whether we can prevent rejection or thalassemia intermedia with the guidance of the chimerism studies. Although the answer to how and when to intervene is clearer for malignant disorders, further studies are necessary for thalassemia major.

Conflict of interest

No author of this paper has a conflict of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included in this manuscript.

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