HOW CAN WE GET THE BEST OUTCOMES FROM CORD BLOOD STEM CELL TRANSPLANTATION IN SEVERE THALASSEMIC PATIENTS: WHEN DOES THE CELL NUMBER REALLY MATTER?

Kleebsabai Sanpakit,[1] Bunchoo Pongtanakul,[1] Nattee Narkbunnam,[1] Vip Viprakasit,[1,2] Gavivann Veerakul,[1] Voravarn STanphaichitr,[1] Surapol Issaragrisil[3] and Vinai Suvatte[1]

[1]Division of Hematology and Oncology, Department of Pediatrics, [2]Thalassemia Center; [3]Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Abstract: Hematopoietic stem cell transplantation (HSCT) is the only curative treatment for severe thalassemia. The umbilical cord blood (CB) is one of the alternative sources of stem cells (SC) for transplantation. We performed a retrospective study to analyze the outcomes of children with severe thalassemia who had undergone CBSC transplantation (CBSCT) at Siriraj Hospital between June 1993 and August 2016. Seventeen CBSCTs were performed on 16 patients [12 Hemoglobin (Hb)] E/beta-thalassemia; 4 Beta-thalassemia major]. The median age at CBSCT was 4.0 years (range: 1.9–7.6 years). All except one were 8/8 HLA-matched CBSCTs. The conditioning regimen included oral busulfan and intravenous cyclophosphamide. Graft versus host disease (GVHD) prophylaxis was cyclosporine with or without methotrexate. The median, total mononuclear (TMN) and CD34+ cells were 3.1 x 10^7 and 1.7 x 10^5 cells/kg, respectively. The median times for neutrophil, erythroid and platelet engraftment were 21.5, 31.5 and 32.0 days, respectively. Nine patients were cured after the first CBSCT; however, 3 had mixed chimerism, but could maintain Hb levels > 10 g/dl without transfusion. Four patients recurred, 3 of whom successfully underwent a second bone marrow HSCT from the same donor. The fourth, who received 3/8 mismatched CBSCT, underwent a second CBSCT from another 8/8 matched-sibling donor, but she died from infection. Three patients died from infections after the first HLA-matched CBSCT. The overall survival and the thalassemia-free survival [or event-free survival (EFS)] rates of the matched-sibling CBSCTs were 75.0% and 56.3%, respectively, with a median follow-up time of 17.1 years. In 13 matched-sibling CBSCT patients, the EFS was better for post-thawing TMN cell counts of ≥ 3.0 x 10^7 cells/kg than < 3.0 x 10^7 cells/kg (70.00%; 95% CI: 32.87-89.19; versus 33.33%; 95% CI: 4.61-67.56; $p = 0.18$). HLA-matched-sibling CBSCT could be an alternative treatment for severe thalassemia; however, a relatively low number of TMN cells in CBSC results in a less favorable outcome. Moreover, delayed hematopoietic engraftment leading to a high rate of severe infections is the main contributing factor for mortality in CBSCT. Therefore, stringent selection of CBSCs with adequate TMN cells is important for successful treatment.

Keywords: severe thalassemia, children, hematopoietic stem cell transplantation, umbilical cord blood

Correspondence: Kleebsabai Sanpakit, MD, Division of Hematology and Oncology, Department of Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Wanglang Road, Bangkok Noi, Bangkok 10700, Thailand. Tel: 66 (0) 2419 5972; Fax: 66 (0) 2866 3021 Email: kleebsabai.sap@mahidol.ac.th

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a well-known curative treatment for children with severe thalassemia syndrome. Since Thomas and colleagues performed the first successful HSCT for a thalassemic patient in 1981 (Thomas *et al*, 1982), more than 3,000 HSCTs have been reported worldwide in the treatment of this genetic disease (Angelucci *et al*, 2014). The most preferred approach for HSCT in cases of severe thalassemia is the use of human leukocyte antigen (HLA)-matched-sibling donor with a myeloablative conditioning regimen (Angelucci *et al*, 2014; Alfraih *et al*, 2016). One important factor that affects HSCT outcomes is the source of the hematopoietic stem cells (HSC) (Issaragrisil and Kunacheewa, 2016). Bone marrow (BM) is the preferred source of stem cells (SC), having a lower incidence of graft versus host disease (GVHD) than peripheral blood stem cells (PBSC). However, PBSCs are associated with faster engraftment and a lower incidence of graft rejection. Large prospective studies are still required to prove the benefits of PBSC compared with standard bone marrow stem cells (BMSC) (Ghavamzadeh *et al*, 2008; Iravani *et al*, 2010; Mathews *et al*, 2014; Alfraih *et al*, 2016). Cord blood (CB) is another source of SC, having been used for HSCT for many types of diseases, including thalassemia (Issaragrisil *et al*, 1995). The advantages of cord blood stem cells (CBSC) are a low probability and severity of GVHD, a very low risk of viral transmission (such as cytomegalovirus and Epstein-Barr virus), the ease of HSC collection, and the absence of clinical risk to the donor during the HSC harvest (Boncimino *et al*, 2010; Locatelli *et al*, 2013). As for matched-sibling donors, sufficient numbers of SC from the CB can be obtained at birth, and there is no need to wait until the sibling donor is older to become a BM donor. Consequently, HSCT can be performed earlier, with less chance of alloimmunization developing in the patient from multiple exposure to blood transfusions and less tissue injury resulting from iron overload, which can, in turn, result in better transplant outcomes (Issaragrisil, 2002). Our center performed the first successful CBSC transplantation (CBSCT) on a patient with hemoglobin (Hb) E/beta-thalassemia in June 1993 (Issaragrisil *et al*, 1995). Since then, we have performed CBSCTs in several cases of severe thalassemia. An analysis of our experiences will enable us to identify important factors that can be used to predict CBSCT outcomes and improve our management of future cases.

MATERIALS AND METHODS

A retrospective chart review was performed on cases of children with severe thalassemia syndrome who had undergone a CBSCT at the Department of Pediatrics, Faculty of Medicine Siriraj Hospital between June 1993 and August 2016. This study was approved by the Ethics Committee of the Faculty of Medicine, Siriraj Hospital, Mahidol University.

The data obtained from our hospital charts and hematology records included demographic and hematological data, serum ferritin levels, pre-CBSCT-transfusion duration, ABO blood group, complications, and the CBSCT outcomes. Histocompatibility was determined by serologic or DNA typing with intermediate- or high-resolution in class I (A, B) and II (DR, DQ) for 8 loci, using standard techniques. CBSC from an unaffected sibling was collected and cryopreserved by the method described elsewhere (U-prataya *et al*, 2003). The conditioning regimens consisted of oral busulfan (14-18 mg/kg/day for 4 days), and intravenous cyclophosphamide (200 mg/kg/day for 4 days). Rabbit antithymocyte globulin (ATG; 5 mg/kg/day for 3 days) was added for high-risk patients, who received long duration of transfusion pre-CBSCT or 3/8 antigens HLA-mismatched-sibling CBSC or a second CBSCT. The GVHD prophylaxis consisted of cyclosporine A with or without methotrexate (MTX). All patients underwent gastrointestinal tract decon-

tamination with oral gentamicin, and received prophylactic antifungus with oral nystatin or clotrimazole for the first 100 days of CBSCT. It was continued beyond day 100 if patients had GVHD and required additional immunosuppression. Trimethoprim-sulfamethoxazole was initiated after stable engraftment and continued until the immunosuppression was stopped. GVHD was defined as per established criteria (Glucksberg *et al*, 1974). Livers and spleens were sized pre-transplantation or before splenectomy.

The time to engraftment for neutrophils was defined as the first of 3 consecutive days on which the ANC was > 500 cells/mm^3. The times to engraftment for erythrocytes and platelets were defined as the first of 3 consecutive days on which the Hb was more than 10 g/dl and the platelet count was more than 20,000/mm^3 without transfusion, respectively. The engraftment was documented by the determination of donor-type DNA alleles using analyses of restriction-fragment-length polymorphisms (RFLP), as described by Schreiner *et al* (1994). Thalassemia-free or event-free survival (EFS) was defined from the time of the CBSCT to an event, which was graft failure or rejection, or death. Overall survival (OS) was defined as the time from the CBSCT to death due to any cause, or the time from the CBSCT to the second transplantation.

Data were analyzed with descriptive statistics (SPSS Version 16; SPSS, Chicago, IL). The probabilities of EFS and OS following a CBSCT were estimated by the Kaplan-Meier method, and the significance of the number of TMN cells to survival rate was assessed by a log-rank test. Pearson correlation was applied to analyze the correlation between viable TMN cells and cord blood volume, viable CD34s, and time to engraftment. A p-value < 0.05 was considered statistically significant.

RESULTS

This study analyzed 17 CBSCTs in 16 severe-thalassemic patients (10 boys; 62.5%). The majority were transfusion-dependent Hb E/beta-thalassemia. The median age at the time of the CBSCT was 4.0 years (range: 1.9-7.6 years). Three patients (17.6 % of the 17 transplants), who had a spleen size of 4-7 cm below the left costal margin, were splenectomized pre-CBSCT. Eleven patients (64.7% of the 17 transplants) received regular transfusion and iron chelation before the CBSCT. The median duration of regular transfusions pre-CBSCT was 13 months (range: 2-84 months). The median, pretransplant, serum ferritin level was 1,582 ng/ml (range: 182-4,490 ng/ml). Eleven patients (64.7% of the 17 transplants) had no hepatosplenomegaly at the time of the CBSCT. Three (17.6%) and 2 (11.8%) patient/donor pairs were major and mixed major/minor ABO blood group incompatibility, respectively. All patients were transplanted first from HLA-matched-sibling CBSC except one, who received 2/6 or 3/8 HLA-mismatched (HLA-B, DRB1, DQB1) sibling CBSC. The median CBSC volume was 90 ml (range: 60-120 ml). The median viability of CBSC post-thawing was 75% (range: 55%-95%). The median numbers of viable, TMN and CD34 cells after thawing cryopreserved CBSC were 3.1 x 10^7 cells/kg (range: 1.5-7.0 x 10^7 cells/kg) and 1.7 x 10^5 cells/kg (range: 1.1-3.3 x 10^5 cells/kg), respectively. The percentage of CD34 cells in the CB ranged from 0.2% to 1.3 % of TMN cells. The last CBSCT in this cohort was in December 2003. The patients' characteristics and the number of viable TMN cells in CBSC are shown in Table 1.

An analysis of the engraftment showed the median time of the myeloid engraftment was 21.5 days (range: 9-42 days). This was faster than the erythroid engraftment (31.5 days; range: 19-67 days) and the platelet engraftment (32.0 days; range: 18-59 days). Only 1 patient (5.8%) had acute GVHD, and none had venoocclusive disease. Four patients (23.5%) had a recurrence of thalassemia. Two patients had a primary graft failure at 2 months post-CBSCT, while 2 had a late graft rejection at 1

Table 1
Characteristic of thalassemic patients undergoing 17 cord blood stem cell transplantations (CBSCT), and number of viable total mononuclear (TMN) cells in stored cord blood cells.

Variables	Number of CBSCT (%)
Age	
≤ 7 years old	16 (94.1)
>7 years old	1 (5.9)
Diagnosis	
Hb E/ beta-thalassemia	12 (70.6)
Beta-thalassemia	5 (29.4)
Regular transfusion	
Yes	15 (88.2)
No	2 (11.8)
Iron chelation	
Yes	11 (64.7)
No	6 (35.3)
Liver size pretransplantation	
≤ 2 cm	14 (82.4)
>2 cm	3 (17.6)
Splenectomy	
Yes	5 (29.4)
No	12 (70.6)
ABO blood group compatibility	
No mismatch	11 (64.7)
Major mismatch	3 (17.6)
Minor mismatch	1 (5.9)
Mixed major and minor mismatch	2 (11.8)
Number of viable total mononuclear cells *	
< 3 x 10^7 cells/kg	6 (42.9)
≥ 3 x 10^7 cells/kg	8 (57.1)

*Data were missing for 3 transplants.

year 26 days and 1 year 52 days post-CBSCT. None were splenectomized. Three underwent a second HSCT (from the same HLA-matched-sibling donor, and using BMSC) 4-6 years after the first CBSCT, with successful engraftment and free from thalassemia. One patient, who had received 3/8 loci HLA-mismatched-sibling CBSC, had a primary graft failure at 2 months post-CBSCT even though the number of viable TMN cells post-thawing was as high as 6.98 x 10^7 cells/kg. This patient received a second CB-SCT from another sibling with 8/8 HLA-matched CBSC (TMN cells post-thawing: 5.7 x 10^7; and CD34: 1.2 x 10^5 cells/kg). Unfortunately, she died from pulmonary aspergillosis with dis-seminated intravascular coagulation (DIC), and renal and liver failure, at day 33 after the second CBSCT, without evidence of engraftment. Three

patients died after the first CBSCT. One of these was splenectomized pre-CBSCT and died from primary graft failure with methicillin-resistant *Staphylococcus aureus*, *Candida* septicemia and encephalitis at day 26 post-CBSCT. The second case was also splenectomized pre-CBSCT and had myeloid engraftment on day 17. This patient was discharged from the hospital on day 101, but had *Streptococcus* group D septicemia and died at 10 months post-CBSCT. The third case had *Klebsiella pneumoniae* septicemia and DIC with gastrointestinal bleeding. He died on day 5 post-CBSCT before engraftment. Two out of 4 patients who died and 2 out of 4 patients who recurred with thalassemia received additional MTX for GVHD prophylaxis. Eight out of 17 CBSCTs had MTX in the GVHD prophylaxis regimens. Eleven patients from all the CBSCTs (64.7%) had septicemia. The incidences of causative agents were approximately the same for gram-negative and gram-positive bacteria. The overall mortality and rejection rates of all the CBSCTs were 23.5% each. The median number of transfusions during admission for packed red cells (PRC) and platelets was 11 (range: 3-20 transfusions) and 14 (range: 2-34 transfusions), respectively.

Survival analysis of HLA-matched-sibling CBSCT patients (n = 16), with a median follow-up time of 17.1 years (range: 5 days-22.6 years), showed an EFS of 56.25% (95% CI: 29.54-76.22) and an OS of 75.0% (95% CI: 46.34-89.80) (Fig 1), and a graft rejection rate of 18.75%. Three out of 9 patients (33.33%), who were cured of thalassemia, had stable mixed chimerism from the first month post-CBSCT, and the level of the recipient's DNA ranged from 15% to 33% while maintaining a baseline Hb > 10.0 g/dl without PRC transfusion. The median Hb level at 1 year in the cured thalassemic patients post-CBSCT was 11.0 g/dl (range: 10.0-15.5 g/dl). The EFS rates for the matched-sibling CBSCT patients (n = 13), who had received viable TMN cells post-thawing of $< 3.0 \times 10^7$ versus $\geq 3.0 \times 10^7$ cells/kg, were 33.33% (95% CI: 4.61-67.56) versus 70.00% (95% CI: 32.87-89.19), respectively, but without statistical significance (Fig 2). We did not analyze the OS in these two groups of TMN cells due to the limited data. A Pearson correlation failed to show a statistically significant correlation between the number of viable TMN cells post-thawing and the cord blood volume, the number of viable CD34 cells, and the time to engraftment (data was not shown).

DISCUSSION

Both HLA-matched-sibling and unrelated donors for CBSC have been widely used for hematological disorders, including thalassemia (Boncimino *et al*, 2010; Locatelli *et al*, 2013). CBSC has many favorable characteristics, especially a low incidence and severity of GVHD, which allows for less stringent HLA-matching and brings about the possibility of extending the number of HLA-mismatches of CBSC to 1-2 out of 6 HLA loci. However, the problem of delayed HSC engraftment that can contribute to severe infections and a high risk of graft failure, especially in severe thalassemia, is of particular concern. This is because thalassemic patients have a prolonged exposure to minor histocompatibility antigens from chronic pre-HSCT blood transfusions, which can lead to an increased risk of alloimmunization and strengthen the host's immunological barriers to engraftment (Boncimino *et al*, 2010; Kanathezhath and Walters, 2010; Locatelli *et al*, 2013).

Our retrospective study showed that all patients except one were 7 years old or younger. This young age might result from the limitation of HSC numbers in CB, so only small patients could receive enough HSCs per body weight from CB for transplantation. The major advantages of performing CBSCTs in the early life of thalassemic patients are less exposure to transfusion and possibly fewer complications related to iron overload. Eighty-two percent of our patients had a liver size $\leq$ 2 cm, and the median duration

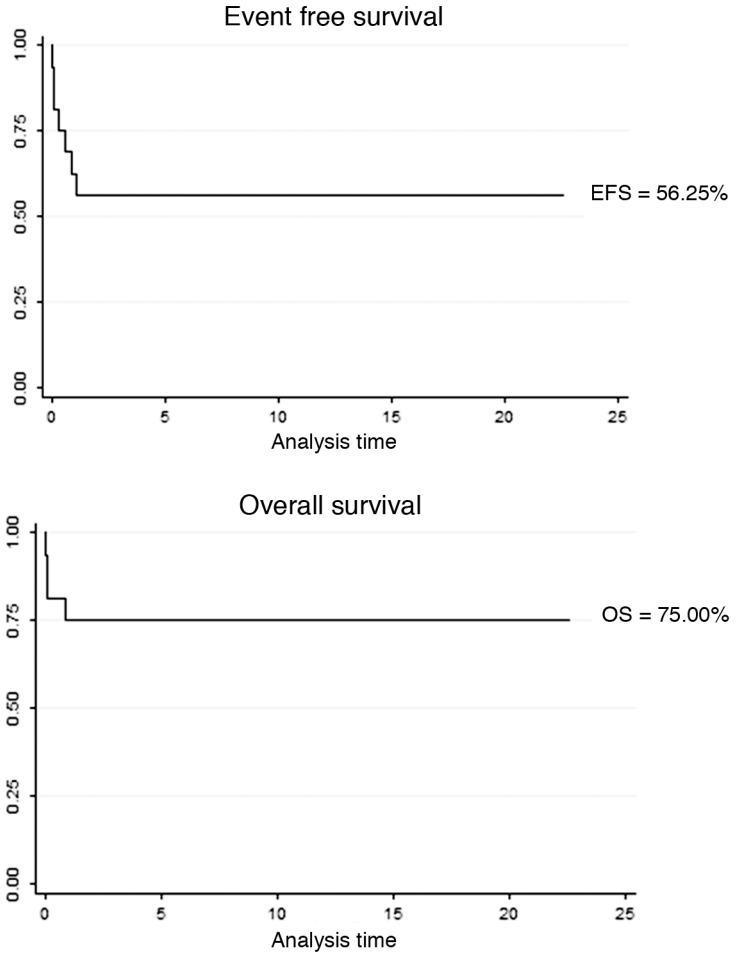

Fig 1– Probability of event-free (or thalassemia-free) survival (EFS) and overall survival (OS) of matched-sibling cord blood stem cell transplantation in severe thalassemia syndromes (number of transplants, *n* = 16; time in years).

of transfusion was only 13 months, which placed those patients in the low-risk group, based on Lucarelli's classification (Lucarelli *et al*, 1990; Alfraih *et al*, 2016).

We applied the most widely used myeloablative conditioning regimen, which consisted of busulfan and cyclophosphamide, and used cyclosporine with or without MTX for GVHD prophylaxis. One study reported an unfavorable EFS when using MTX-based GVHD prophylaxis following a CBSCT (Locatelli *et al*, 2013). Our data cannot confirm this point since the number of patients in whom thalassemia recurred or who died was the same for those receiving and not receiving MTX. ATG for T-cell depletion was added to the conditioning regimens to reduce the rejection rate in those patients who received long duration of transfusion pre-CBSCT or underwent a mismatched or a second CBSCT. In 2013, Eurocord and the European Society for Blood and Marrow Transplantation (EBMT) reported a slower neutrophil and platelet recovery after CBSCT than with bone marrow stem cell

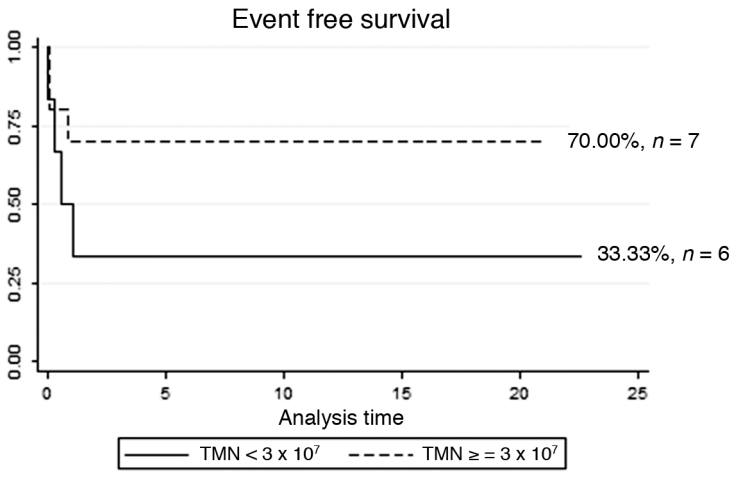

Fig 2– Probability of event-free (or thalassemia-free) survival in severe thalassemia patients undergoing matched-sibling CBSCT, comparing number of viable TMN post-thawing < 3.0 x 10^7 and ≥ 3.0 x 10^7 cells/kg (n = 13; time in years; p = 0.18).

transplantation (BMSCT) (Locatelli *et al*, 2013). However, the delay in engraftment was not associated with an increased risk of bleeding or fatal infectious complications. In contrast, all patients in our study died from severe infections, with 75% dying early during the CBSCT without evidence of engraftment. This result emphasizes the possible adverse effects of a CBSCT involving a delayed neutrophil engraftment, causing a higher risk of transplant-related infection and, eventually, mortality. The median number of TMN cells was 3.9 x 10^7 cells/kg (1.5-14 x 10^7 cells/kg), and the time to neutrophil and platelet recovery after the CBSCT in the Eurocord study was 23 days (range: 9-60 days) and 38 days (range: 13-125 days) respectively (Locatelli *et al*, 2013), which were comparable to our findings. The graft failure rate of matched-sibling CBSCTs in our study was as high as 18.75 %; this might reflect the importance of the TMN-cell dose because 46.15 % of patients received CBSC with viable TMN cells after thawing of < 3 x 10^7 cells/kg. For cases of severe thalassemia, Eurocord has recommended a minimum TMN cell number of at least 3.5 x 10^7 cells/kg in cryopreserved CBSC before thawing for effective sibling CBSCT (Locatelli *et al*, 2013). The incidence of acute GVHD in our study was much lower than in the Eurocord study of matched-sibling CBSCTs (5.8% *vs* 11.0%) (Locatelli *et al*, 2013). This difference may be due to the different ethnic groups. Sustained donor/recipient mixed chimerism of circulating leukocytes was reported in a significant proportion of thalassemia major patients who had undergone an HLA-identical sibling CBSCT (Lisini *et al*, 2008; Locatelli *et al*, 2013). The relatively high proportion of patients with mixed chimerism in our study (33%) might reflect that CBSC promotes reciprocal tolerance between the donor and recipient cells (Locatelli *et al*, 2013). However, the percentage of donor cells in a chimerism study required to maintain the Hb level in a patient needs to be determined. Since the risk of graft rejection is directly correlated with the percentage of mixed chimerism of the patients, especially early in the course, the chimerism study should be monitored closely post-CBSCT (Andreani *et al*, 2014; Issaragrisil and Kunacheewa, 2016). The EFS, OS and graft rejection rates of the HLA-matched-sibling CBSCTs in our study were 56.3%, 75.0% and 18.8%, respectively. The comparison of CBSCT

Table 2
Comparison of HLA-matched-related cord blood stem cell transplantation (CBSCT) data and clinical outcomes of this study with selected studies (Lisini *et al*, 2008; Locatelli *et al*, 2013).

Variables	Lisini *et al*	Locatelli *et al*	This study
Years of CBSCT	1998-2006	1994-2005	1993-2003
Number of patients	27	96	17*
Diseases	Beta-thalassemia major 100%	Beta-thalassemia major 69% Sickle cell disease 31%	Hb E/beta-thalassemia 71% Beta-thalassemia 29%
Median age; range (years)	6; 0.8-18	5.9; 2-20	4; 1.9-7.6
Conditioning regimens	Bu/Flu/TT 89% Bu/Cy/TT 11% (Plus ATG 100%)	Bu/Cy 56% Bu/Flu/TT 22% Bu/Cy/TT 11% Bu/Cy/Flu 5% Bu/Flu 4% (Plus ATG 54%)	Bu/Cy 100% (Plus ATG 24%)
Median number of TMN cells ; range (x10^7 cell/kg)	3.3; 1.5-6.0	3.9; 1.5-14.0	3.1; 1.5-7.0
MTX in GVHD prophylaxis	0%	30%	47.1%
Acute GVHD	0%	11%	5.8%
Chronic GVHD	0%	5%	0%
Treatment-related mortality rate	0%	3%	23.5%
Graft failure/rejection	0%	9%	18.8% **
Overall survival	100%	97%	75.0% **
Event-free survival	100%	Thalassemia major 80% Sickle cell diseases 90%	56.3% **

ATG, antithymocyte globulin; Bu, busulfan; Cy, cyclophosphamide; Flu, fludarabine; GVHD, graft versus host disease; MTX, methotrexate; TMN, total mononuclear cells; TT, thiotepa.
* All transplantations were HLA-matched except one was 3/8 loci HLA-mismatched-sibling CBSCT.
** Calculated only HLA-matched-sibling CBSCT.

data and clinical outcomes of our study with selected studies are shown at Table 2 (Lisini *et al*, 2008; Locatelli *et al*, 2013). Historical data reported by Siriraj Hospital in 1998 indicates that the EFS and OS rates for BMSCTs for severe thalassemia were 77.2% and 85.7%, respectively, with a graft rejection rate of 14.3% (Suvatte *et al*, 1998). Lucarelli and others reported in 2001

that thalassemic patients aged below 16 years (who had undergone BMSCTs at approximately the same time as our patients received CBSCTs) showed EFS and OS rates of 71% and 78%, respectively (Angelucci and Lucarelli, 2001). Our disappointing CBSCT results, with lower EFS and OS rates but a higher graft-rejection rate than shown by earlier BMSCT data, undermined the benefits of CBSCT as a preferred choice of stem cells. The major obstacle was a generally rather low number of TMN cells among the cord blood cells. However, the number of TMN cells did not statistically correlate with the time of engraftment in our study. The better EFS-rate (70%) in our study was associated with a viable TMN cell post-thawing of $\geq 3 \times 10^7$ cells/kg, which was close to previously reported EFS rate for BMSCT, suggesting the importance of the TMN cell count to promote engraftment (Suvatte *et al*, 1998; Angelucci and Lucarelli, 2001). All 3 patients who had failed at the first attempt were subsequently successfully treated with BMSCTs from the same donor. This suggests that BMSCT could rescue patients who had failed a CBSCT. A combination of low TMN-CBSC with BMSC harvested from the same sibling donor might improve the CBSCT outcomes to ensure engraftment (Locatelli *et al*, 2013). Considering high rate of graft rejection in our study, the conditioning regimen adding more potent myeloablative agent such as thiotepa and T-cell depleted agent such as ATG may help to compete the patient's toward the donor's HSCs and sustain donor engraftment (Lisini *et al*, 2008; Locatelli *et al*, 2013). However, the potential more toxicities from conditioning regimen which may lead to treatment-related mortality should be weighted carefully against the benefit from declining of graft rejection.

Our study had some limitations, including that there was a small cohort of patients, which affects the ability to achieve statistical significance. Moreover, it was a retrospective study. Our last case underwent a CBSCT 13 years ago; supportive care at that time might have been inferior to contemporary standards, and there was a lack of data on the prestoraged TMN number in CBSC. Due to our unfavorable experiences with CBSCTs, we changed our standard of practice in 2004 so that we now wait for the donors of HLA-matched CBSC to grow to at least one year of age and weigh at least 50% of the patient's weight before collecting BMSC. The marrow is used in combination with cryopreserved CBSC if the number of TMN cells in the cryopreserved CB is insufficient. A future, prospective, multicenter study for both siblings and unrelated CBSC donors, with or without a combination of bone marrow stem cells, would be helpful to clarify the role and risks/benefits of CBSCTs for severe-thalassemic patients. Addressing the importance of adequate number of TMN cells with successful engraftment in CBSCT, further development in CBSC manipulation especially in the field of CBSC expansion may overcome the limitation from inadequate cell doses and increase chance of patients to be performed successful CBSCT in the future.

In conclusion, HLA-matched-sibling CBSCTs could be an alternative treatment for severe-thalassemic patients with modest, transplant-related outcomes. A stringent selection of CBSCs that have an adequate number of TMN cells is important for successful treatment. Delayed hematopoietic engraftment, which causes high rates of severe infections, is the main complication leading to mortality for CBSCTs.

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