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Impact of β-Globin Mutations on Outcome of Matched Related Donor Hematopoietic Stem Cell Transplantation for Patients with β-Thalassemia Major

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The clinical outcome of hematopoietic stem cell transplantation (HSCT) for patients with β-thalassemia major (β-TM) can be affected by several factors. We investigated the influence of *β-globin* gene mutation in patients with β-TM on the clinical outcome of HSCT and conducted a prospective study of consecutive β-TM patients who underwent allogeneic HSCT at our center. Among 87 included patients, 62 (71%) had homozygous and 25 (29%) had compound heterozygous *β-globin* gene mutations. Intervening sequence II-1 appeared to be the most common mutation, with an occurrence rate of 33% in *β-globin* alleles. With a median follow-up of 12 months, the thalassemia-free survival and overall survival probabilities were 83% (standard error, 4%) and 90% (standard error, 3%), respectively. Overall survival was not found to be associated with the *β-globin* gene mutation status, but thalassemia-free survival was significantly improved in patients with homozygous mutations compared with patients with compound heterozygous mutations in univariate (91.2% versus 64.0%, $P = .009$) and multivariable (hazard ratio, 3.83; $P = .014$) analyses. This is the first report on the impact of *β-globin* mutation status on the outcome of β-TM after allogeneic HSCT and helps to better illustrate the course and prognosis of β-TM after transplantation.

INTRODUCTION

β-thalassemia major (β-TM) is a hereditary anemia caused by mutation of the *β-globin* gene that is characterized by absent or defective *β-globin* chain synthesis, resulting in ineffective erythropoiesis [1]. Approximately 300 different mutations in the *β-globin* gene are responsible for β-TM; these mutations are mostly point mutations within the *β-globin* gene itself or in its immediate flanking sequence [2]. In most cases, β-TM patients become dependent of transfusions during the first 6 months of their lives because of a decrease in the expression of *γ-globin* genes. Although the life expectancy of β-TM patients has increased over the past few decades owing to chronic blood transfusions and iron chelation therapy, these treatments do not cure the disease. Hematopoietic stem cell transplantation (HSCT) has been used since the early 1980s for the treatment of β-TM and remains the only potentially curative treatment for these patients [3-8]. Although HSCT showed excellent results for these patients [9-11], different centers reported disparate outcomes for these patients [12-14].

Genetic profiling has proven to be 1 of the most valuable prognostic determinants in hematologic diseases. For example, in Fanconi anemia, the mutation type in *FANCA* gene has been shown to be associated with clinical outcome [15]. In β-TM, the genetic heterogeneity has been suggested to be associated with disease severity [16,17]. However, the molecular or genetic determinants of HSCT outcome in β-TM have not been investigated. We hypothesized that such determinants account for some of the disparities in transplantation outcomes. Therefore, we conducted a study to investigate the effects of type of *β-globin* mutation on HSCT outcomes in patients with β-TM. To our knowledge, this is the first report on the prognostic significance of *β-globin* gene mutation status on the outcome of subsequent transplantation procedures in β-TM patients. Our data suggest that *β-globin* gene mutation may be responsible for the significant heterogeneity in transplantation outcome in β-TM patients.

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PATIENTS AND METHODS

This study was approved by the institutional review board of Hematology Oncology and Stem Cell Transplantation Research Center. Because all of the study participants were children, written informed consent was obtained from their parents or legal guardians before study admission in accordance with the Declaration of Helsinki. All patients with β-TM who underwent HSCT between November 2009 and November 2011 were assessed for study eligibility. Patients who had cardiac or pulmonary diseases, human immunodeficiency virus, hepatitis B virus, hepatitis C virus, or progressive cirrhosis were excluded from the study. All patients underwent liver biopsy to determine liver fibrosis stage and were classified in risk class I, II, or III using the Pesaro classification.

All patients received stem cell transplants from an HLA-identical related donor, with bone marrow or peripheral blood as a stem cell source. Sibling donors were matched at low resolution for HLA-A, -B, and -DR. Other related donors were matched at 10/10 loci by high-resolution typing. The donors had normal physical examination and blood test results and negative serology for human immunodeficiency virus and hepatitis viruses. The conditioning regimen administered before transplantation was based on conventional protocols described previously [11,18,19]. Briefly, for patients in Pesaro risk class I and II, the conditioning regimen was busulfan at 3.5 mg/kg/day from day -9 for 4 consecutive days, cyclophosphamide at 50 mg/kg/day as intravenous infusion from day -5 for 4 consecutive days, and rabbit antithymocyte globulin (Thymoglobulin, Genzyme, Cambridge, MA) at 1.25 mg/kg/day as intravenous infusion from day -3 for 2 consecutive days. The conditioning regimen for patients in risk class III consisted of busulfan at 3.5 mg/kg/day from day -9 for 4 consecutive days and cyclophosphamide at 40 mg/kg/day from day -5 for 4 consecutive days. Prophylaxis against graft-versus-host disease (GVHD) consisted of cyclosporine (1.5 mg/kg/day intravenously, from day -2 and then 3 mg/kg/day from day 7) with a short course of methotrexate (10 mg/m^2 on day 1 and 6 mg/m^2 on days 3, 6, and 11). Cyclosporine was continued orally for 6 to 7 months after HSCT and was discontinued in the absence of GVHD.

All patients were hospitalized in well-restricted and protective isolation and fed via hyperalimentation. The patients were checked for infections, cytomegalovirus DNA (using polymerase chain reaction analysis), or cytomegalovirus structural protein 65 antigen twice weekly. The patients also were given acyclovir and fluconazole to prevent viral and fungal infections, as well as sulfamethoxazole/trimethoprim to prevent *Pneumocystis jirovecii*. All patients had taken phenytoin for prophylaxis of busulfan-induced seizure.

Genotypic Analysis

Blood samples were collected in EDTA-containing tubes. DNA was extracted from blood samples using a standard salting out method [20]. Because β-globin mutations usually arise at multiple geographical locations and there are certain mutations accounting for the majority of thalassemia major in each location, the amplification refractory mutation system technique using primer sequences was used to detect common β-TM mutations [21,22]. DNA sequencing and multiplex ligation probe amplification were used when no mutation was detected with the amplification refractory mutation system.

Additionally, alpha-globin genes were analyzed by multiplex gap–PCR to detect common deletions. When no abnormalities were detected, multiplex ligation-dependent probe amplification assay was performed to detect other molecular alterations.

Endpoints

Outcome endpoints followed standard definitions. *Myeloid engraftment* was defined as the first of 3 consecutive days in which the absolute neutrophil cell count was $\geq .5 \times 10^9$/L, and platelet recovery was defined as platelets $\geq 20 \times 10^9$/L without transfusion support for 7 days. Acute grade II to IV GVHD and chronic GVHD were graded using standard criteria [23,24]. *Overall survival* (OS) was defined as the time from transplantation until death from any cause or the last follow-up for surviving patients. *Thalassemia-free survival* (TFS) was defined as the time from transplantation until first event (graft failure, a second transplantation, or death from any cause, whichever occurred first) or last follow-up for surviving patients.

Statistical Analysis

Continuous data were presented as median values and ranges, and categorical data were expressed as frequencies and percentages. OS and TFS were calculated using Kaplan-Meier curves and were compared between the levels of covariates using log-rank test statistic, and 95% confidence intervals (CI) were computed through log-transformed method. We applied the Cox proportional hazards model with a backward selection method to assess the multiple predictors of OS and TFS. Covariates with *P* values less than .10 in the univariate analyses were candidates to enter the multivariable model. Also, variables that were simultaneously associated with type of gene mutation and OS or TFS with *P* values less than .20 were considered potential confounders for gene mutation. The effects of covariates on OS and TFS were reported as hazard ratios (HR) with a 95% CI. The proportional hazards assumption was checked using the chi-square test of correlation coefficient between transformed survival time and Schoenfeld residuals. The "survival" package in R software, version 3.0.0 [25], was used to analyze the survival data.

RESULTS

Patient Characteristics and β-globin Gene Mutations

A total of 89 patients were recruited for the study. Two patients were dropped from the study because of difficulties in access (they were living out of country). The source of stem cells was bone marrow in 30 patients and peripheral blood in 57 patients. The characteristics of the 87 patients analyzed are summarized in Table 1.

Because the patients had various combinations of *β-globin* gene mutations and to simplify genotype–phenotype analysis, the genotypes were classified into 2 groups: homozygous and compound heterozygous mutations. Sixty-two (71%) patients had homozygous mutations in the *β-globin* gene. In this group of 62 patients, intervening sequence II-I was the most prevalent mutation (40 alleles, 32%), followed by mutations at

Table 1
Clinical Characteristics of Patients and Transplantations

Characteristic	Value
No. of patients	87
Patient age, median (range), yr	8 (2-15)
Sex	
Male	49 (56)
Female	38 (44)
Pesaro classification*	
I	26 (30)
II	38 (44)
III	23 (26)
β-Globin gene mutation	
Homozygous	62 (71)
Compound heterozygous	25 (29)
Donor age, median (range), yr	13.5 (2.5-53)
Donor sex	
Male	46 (53)
Female	41 (47)
Donor-recipient sex	
Matched	44 (51)
Unmatched	43 (49)
Donor-recipient relationship	
Sibling	75 (86)
Other relative	12 (14)
Donor-recipient age difference	
Donor younger than recipient	27 (31)
Donor older than recipient <12 yr	31 (36)
Donor older than recipient ≥12 yr	29 (33)
Source of cells	
Peripheral blood	57 (66)
Bone marrow	30 (34)
Chimerism	
Full donor	77 (89)
Mixed	3 (3)
Graft rejection	7 (8)
Acute GVHD	39 (45)
I-II	22 (56)
III-IV	17 (44)
Chronic GVHD	14 (16)
Limited	12 (86)
Extensive	2 (14)

Data presented are n (%), unless otherwise indicated.
* From Lucarelli at al. N Engl J Med, 1990. [7]

Table 2
β-Globin Gene Mutations

Mutation	Homozygous (62 patients, 124 alleles)	Compound Heterozygous (25 patients, 50 alleles)
IVS II-1	40	17
Codons 36-37	24	6
IVS I 5	22	2
Frame shifts 8-9	6	6
Codon 44	6	2
Codon 5	4	3
Frame shifts 36-37	4	1
IVS I 6	2	3
IVS I 25bpdel	4	1
Codon 22	2	2
IVS II 745	2	1
Codon 15	2	0
IVS I 128	2	0
Codon 8	2	0
IVS I 110	0	2
Codons 37-38-39	2	0
Codons 25-26	0	1
Deletion	0	1
Codon 39	0	1
Connection domain 16	0	1

IVS indicates intervening sequence.

codons 36 to 37 (24 alleles, 19%). The distribution of *β-globin* gene mutations is shown in Table 2.

Survival and Factors Predicting Survival

With a median follow-up of 12 months, the TFS and OS probabilities were 82.5% (95% CI, 74.5% to 91.3%) and 89.9% (95% CI, 83.5% to 96.9%), respectively. Acute GVHD (grades I to IV) was observed in 39 patients (45%). During the follow-up period, a total of 9 patients died (7 from GVHD and 2 from infection).

In univariate analysis, younger recipient and donor age as well as Pesaro class I or II status were associated with significantly higher 1-year OS and TFS rates (Table 3). Donor-recipient age difference was significantly associated with TFS ($P = .032$). A donor-recipient age difference of less than 12 years (the median) was significantly associated with a higher TFS rate compared with a donor-recipient age difference of greater than 12 years (HR, .088; 95% CI, .011 to .686; $P = .02$).

We identified that among patients with intervening sequence II-I mutation in the *β-globin* gene, homozygous mutation holders had a higher TFS rate than those with compound heterozygous mutations in the *β-globin* gene (homozygous mutation 94.4% [95% CI, 84.4% to 100%] versus compound heterozygous mutations 70.6% [95% CI, 51.9% to 95.9%], $P = .048$). Because of various combinations of *β-globin* gene mutations, we couldn't analyze other mutations separately; therefore, we compared homozygous with compound heterozygous mutations in all *β-globin* gene mutations.

Table 4 compares variables between gene mutation levels. Age was the only variable that differed between the levels of gene mutation. However, there was no difference in the clinical status of patients (evaluated by Pesaro classification) among the 2 groups. The survival analysis of all patients revealed a highly significant association between homozygous mutation and a higher TFS rate (HR, .25; 95% CI, .09 to .71; unadjusted $P = .009$). After adjustment of this association for age, it remained significant (HR, .328; 95% CI, .113 to .952; $P = .04$). Further analysis showed that better TFS in homozygous *β-globin* mutation holders may contribute to less graft rejection (HR, .166; 95% CI, .034 to .826; $P = .028$). Treatment-related mortality was not significantly different in homozygous versus compound heterozygous *β-globin* mutations holders (HR, .403; 95% CI, .108 to 1.503; $P = .176$). The final multivariable model (Table 5) demonstrated that the main determinants of a higher TFS rate were homozygous gene mutation, younger donor than recipient, and Pesaro class I or II status.

Table 3
Univariate Effects of Covariates on OS and TFS

Variables	Group	OS		TFS	
		HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Patient age		1.24 (1.03-1.51)	.025	1.29 (1.11-1.50)	.001
Sex	Male	1	.589	1	.897
	Female	.69 (.17-2.75)		.93 (.33-2.62)	
Pesaro classification	I, II	1	.013	1	.001
	III	4.70 (1.22-18.03)		6.14 (2.15-17.55)	
Mutation type	Compound heterozygous	1	.27	1	.009
	Homozygous	.48 (.13-1.83)		.25 (.09-.71)	
Donor age		1.06 (1.01-1.11)	.014	1.08 (1.04-1.13)	<.001
Donor sex	Male	1	.682	1	.675
	Female	1.31 (.35-4.89)		1.24 (.45-3.43)	
Donor-recipient sex matching	Unmatched	1	.786	1	.473
	Matched	1.20 (.32-4.46)		1.46 (.52-4.10)	
Donor-recipient relationship	Other relative	1	.069	1	.022
	Sibling	.3 (.07-1.21)		.29 (.10-.84)	
Blood group matching	Unmatched	1	.619	1	.361
	Matched	.72 (.19-2.69)		.62 (.23-1.72)	
Stem cell source	Peripheral blood	1	.651	1	.763
	Bone marrow	1.35 (.36-5.07)		1.17 (.42-3.31)	
Donor-recipient age difference	Donor younger than recipient	1	.136	1	.032
	Donor older than recipient <12 yr	.48 (.04-5.31)	.55	.23 (.03-2.03)	.184
	Donor older than recipient ≥12 yr	3.03 (.61-15.17)	.177	2.58 (.81-8.28)	.11
Acute GVHD	No	1	.206	1	.535
	Yes	2.36 (.59-9.46)		.72 (.26-2.03)	
Chronic GVHD	No	1	.635	1	.683
	Yes	1.46 (.30-7.01)		.73 (.17-3.25)	

Table 4
Comparison of Variables between Gene Mutation Levels

Variables	Group	Compound Heterozygous (n = 25)		Homozygous (n = 62)		*P* Value
		Freq.	%	Freq.	%	
Sex	Male	16	64.0%	33	53.2%	.359
	Female	9	36.0%	29	46.8%	
Pesaro classification	I/II	17	68.0%	47	75.8%	.455
	III	8	32.0%	15	24.2%	
Donor sex	Male	12	48.0%	34	54.8%	.563
	Female	13	52.0%	28	45.2%	
Donor-recipient sex matching	Unmatched	12	48.0%	31	50.0%	.866
	Matched	13	52.0%	31	50.0%	
Donor-recipient relationship	Other relative	3	12.0%	9	14.5%	1.000
	Sibling	22	88.0%	53	85.5%	
Blood group matching	Unmatched	9	36.0%	21	33.9%	.850
	Matched	16	64.0%	41	66.1%	
Stem cell source	Peripheral blood	16	64.0%	41	66.1%	.850
	Bone marrow	9	36.0%	21	33.9%	
Donor-recipient age difference	Donor younger than recipient	10	40.0%	17	27.4%	.472
	Donor older than recipient <12 yr	7	28.0%	24	38.7%	
	Donor older than recipient ≥12 yr	8	32.0%	21	33.9%	
Acute GVHD	No	15	60.0%	33	53.2%	.565
	Yes	10	40.0%	29	46.8%	
Age at treatment	Median (range)	10 (4-15)		7 (2-15)		.003
Donor age	Median (range)	13 (2.5-43)		14 (2.5-53)		.642
Follow-up, mo	Median (range)	11 (2-75)		12 (2-106)		

Freq. indicates frequency.

DISCUSSION

Our results show that the type of *β-globin* mutation (homozygous versus compound heterozygous) as well as the Pesaro classification and donor-recipient age difference are able to predict survival outcomes of HSCT in β-TM patients. To our knowledge, we are the first to report that homozygous mutation in the *β-globin* gene is a prognostic biomarker of a favorable TFS after HSCT in β-TM patients.

Improvements in transplantation technologies and patient care increased the OS and TFS of thalassemia patients to greater than 90% and more than 80%, respectively [9]. With pretransplantation risk assessment by Pesaro group, β-TM patients were divided into 3 risk classes based on their clinical risk factors, including liver size, presence of liver fibrosis, and chelation history [7,8,26]. Pesaro risk stratification has been established and used by many bone marrow transplantation programs worldwide; however, results of some studies were different from Pesaro results [12-14], which might be explained by differences in the patients' genetic profiles.

The molecular pathophysiology of thalassemia was identified during the 1970s [27-29] and was characterized later with the advancement of molecular analysis techniques. Previous studies have shown that the molecular defects in β-TM are heterogeneous [30-32] and this heterogeneity might be associated with disease severity in β-TM [16,17]. Also, a possible association between genotype and response to hydroxyurea therapy in β-TM has been suggested in some studies [33,34]. In addition, different distributions of β-globin mutations are expected in different patient populations [35,36]. This different geographical prevalence of β-globin mutations [37,38] might explain the different clinical outcome in these patients. Therefore, we hypothesized that genotype may affect the HSCT outcome in β-TM. In our study, patients with compound heterozygous β-globin mutation showed a nearly 4-times worse TFS compared with those with homozygous mutations. Our understanding of the mechanisms and details of this effect is limited and needs further investigation. Also, the heterogeneity of *β-globin* mutations limited our ability to analyze each mutation individually. Analysis of the outcome for each mutation can reveal more details of the role of β-globin mutation on treatment outcome. In addition, future studies might uncover other molecular determinants of outcome in β-TM.

Although limited by the small sample size and short median follow-up time, our study is the first to underscore the role of *β-globin* gene mutation in TFS after transplantation for patients with β-TM. Further large multicenter studies are needed to clarify this role and to confirm our results.

Table 5
Multiple Predictors of TFS and OS

Variables	Group	TFS		OS	
		Hazard Ratio (95% CI)	*P* Value	Hazard Ratio (95% CI)	*P* Value
Mutation	Homozygous	1	.014		
	Compound heterozygous	3.83 (1.31-11.20)			
Donor-recipient age difference	Donor younger than recipient	1	.045		
	Donor older than recipient <12 yr	.401 (.043-3.72)	.421		
	Donor older than recipient ≥12 yr	3.12 (.954-10.20)	.06		
Pesaro classification	I, II	1	.008	1	.007
	III	4.31 (1.46-12.74)		6.87 (1.67-28.19)	
Donor-recipient relationship	Other relative			1	.021
	Sibling			.18 (.04-.77)	

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