evolution. Our study demonstrates the ubiquitous nature of *Cbl* family mutations, in particular in the context of very circumscribed phenotypes of myelomonocytic neoplasms.

Several common clinical features were identified among 27 patients affected by the *Cbl* family of variants, including monoblast or monocyte proliferation (74%), splenomegaly (81%), and surface expression of c-kit on malignant cells (93%). Characteristic nuclear features included abnormal lobulation and hyperchromatic and raisinoid nuclei of megakaryocytes seen in patients with CMML (Appendix Fig A4, online only). By immunohistochemistry, *c-Cbl* mutant megakaryocyte nuclei displayed aberrant pSTAT5 staining (86%; Appendix Fig A4), while pSTAT5 was not expressed in patients without *c-Cbl* mutations. In three cases, pSTAT5 expression was not detected in specimens obtained before the mutation was present (Appendix Fig A4). The clinical phenotype of patients with *Cbl-b* mutations was not distinguishable from that of patients with *c-Cbl* mutations. The allelic pattern of *Cbl* family mutations plays an important role in disease phenotype in patients; in sAML eight (62%) of 13 *c-Cbl* family mutations were homozygous (Fig 3A).

To evaluate the impact of *Cbl* family mutations, we first compared mutant and WT cases on the basis of various clinical parameters (Table 1). In mutant cases, monocyte counts were significantly higher as compared with patients with WT *Cbl* family genes. A higher proportion of patients with *c-Cbl* mutations were treated with intense chemotherapy or stem cell transplantation, suggesting that these therapies were more frequently selected because of the aggressive biology of the *Cbl* mutation-associated disease. When we performed univariate analysis of survival impact of various clinical variables, significant differences in WBC, monocyte counts, disease risk, and the presence of *Cbl* family gene mutations (Table 2) were found. Chemotherapy was an adverse risk factor for survival, likely as it correlated with more advanced disease in multivariate analyses. The prognosis of patients with *Cbl* family mutations was poor, especially in those with homozygous mutations. The median overall survival was 5 months for all patients, with a significant difference of 1.7 months versus 20 months for patients with homozygous and heterozygous mutations, respectively (P = .04; Fig 3B). In multivariate analyses advanced disease (hazard ratio [HR], 5.64; 95% CI, 3.8 to 8.36) and *Cbl* family mutations (HR, 2.17; 95% CI, 1.18 to 4.02; Table 3) were shown to be independent adverse factors for overall survival.

DISCUSSION

In leukemia, cytogenetic abnormalities identify underlying pathophysiology and carry enormous prognostic and therapeutic significance. Various examples of gain of function and inactivating mutations show that homologous recombination may lead to duplication of affected alleles.[24] Consequently, areas of somatic UPD may point toward genes carrying putative pathogenic mutations. When high density SNP-A was applied as a karyotyping platform in a large number of patients with MDS and related disorders, we noted recurrent somatic UPD at chromosome 11q, particularly frequent in MDS/MPN. Commonly deleted region mapping and analysis of genes located within this region led us to hypothesize that *c-Cbl* may contain mutations. Sequencing of *c-Cbl* in patients affected by somatic UPD11q revealed RFD and linker sequence mutations present in patients with CMML, MDS/ MPN unclassifiable, and sAML derived from these conditions or MDS. We also found new mutations in *Cbl-b* with a clinical phenotype similar to that seen with *c-Cbl* RFD mutations. Consequently, our results imply that E3 ubiquitin ligases constitute a novel class of genes in whom mutations reflect a novel general mechanism of leukemogenesis. This notion is supported by the variety of pathomorphologic subentities of myeloid malignancies affected by mutations of the *Cbl* family. Moreover, a novel frame shift polymorphism was found in *Cbl-c* in patients with MDS/MPN. However, the relevance of this otherwise extremely rare polymorphism is not clear

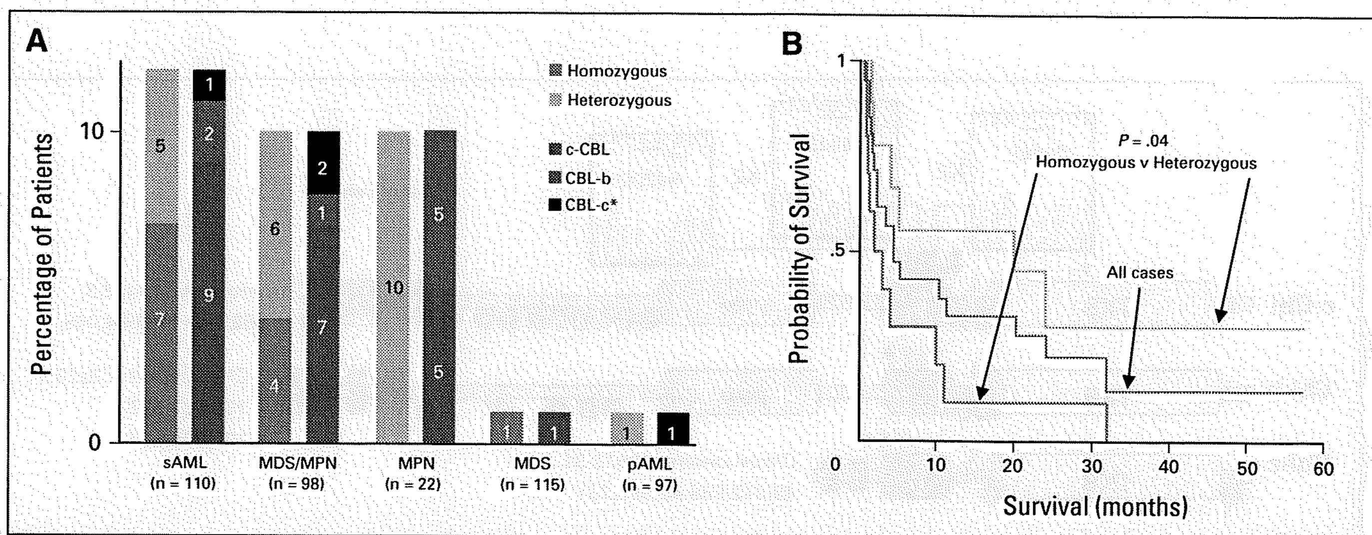

Fig 3. *Cbl* family mutations in myeloid malignancies and unique clinical characteristics of patients with mutations. (A) *Cbl* family mutations are frequently observed in secondary acute myelogenous leukemia (sAML; 12%), myelodysplastic/myeloproliferative neoplasms (MDS/MPN; 10%), and MPN (10%). Homozygous mutations of *c-Cbl* are more frequent in sAML (7%) than MDS/MPN (4%). *Cbl-b* mutations and/or *Cbl-c* frame shift polymorphism (*) are seen in all disease phenotypes. (B) Kaplan-Meier analysis shows overall survival in all cases (n = 20; gray line). Median survival is 5 months and the survival rate is 10%. By comparing patients homozygous for *Cbl* family mutation (n = 10; blue line) to those heterozygous (n = 10; gold line), there is a statistically significant difference in overall survival between these two cohorts regardless of treatment or remission/relapse.

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Table 1. Comparison of Clinical Characteristics Between Wild-Type and Mutant *Cbl* Family Genes

Variable*	*Cbl* Family Mutant (n = 17)	*Cbl* Family Wild Type (n = 307)	*P*
Age, years			
≥ 60	10	224	.21
< 60	6	62	
Sex			
Male	8	183	.2
Female	9	106	
WBC count, $\times 10^9$/L			
≥ 10	9	93	.11
< 10	8	197	
Monocyte count, $\times 10^9$/L			
≥ 1	13	53	< .0001
< 1	4	237	
Metaphase cytogetetics			
Abnormal	10	133	.62
Normal	7	139	
Disease risk†			
Advanced grade	12	141	.7
Low grade	4	142	
Therapy and response			
Chemotherapy‡	14	130	.001
Stem cell transplantation	4	21	.035
Complete remission	3	22	.73

*Some clinical data are not available.
†In advanced group, secondary acute myelogenous leukemia, refractory anemia with excess blasts, and chronic myelomonocytic leukemia 2. The others are in low grade group.
‡Chemotherapy includes mitoxantrone, idarubicin, daunorubicin, cytarabine, etoposide, hydroxyurea, fludarabine gemtuzumab, 5-azacitidine, decitabine, lenalidomide, arsenic trioxide, and valproic acid.

Table 2. Univariate Analysis of Overall Survival in Clinical Variables

Variable*	No. of Patients	Overall Survival: Mean (months)	Hazard Ratio	95% CI	*P*
Age, years					
≥ 60	220	32	1.23	0.83 to 1.82	.31
< 60	64	36			
Sex					
Male	176	31	1.37	0.98 to 1.91	.07
Female	108	36			
WBC count, $\times 10^9$/L					
≥ 10	79	25	1.83	1.30 to 2.57	.001
< 10	205	36			
Monocyte count					
≥ 1	48	24	1.79	1.22 to 2.64	.003
< 1	236	35			
Metaphase cytogetetics					
Abnormal	124	30	1.37	0.98 to 1.90	.063
Normal	140	35			
Disease risk†					
Advanced grade	141	18	6.25	4.27 to 9.15	< .0001
Low grade	142	46			
Cbl family gene					
Mutation	16	9	4.64	2.69 to 8.00	< .0001
Wild type	268	34			
Chemotherapy					
Treatment	131	26	2.13	1.54 to 2.93	< .0001
No treatment	153	40			
Stem cell transplantation					
Treatment	24	34	0.87	0.49 to 1.54	.63
No treatment	260	33			
Complete remission					
Yes	23	30	0.70	0.40 to 1.23	.72
No	95	25			

*Some clinical data are not available.
†In advanced group, secondary acute myelogenous leukemia, RAEB, and chronic myelomonocytic leukemia 2. The others are in low-grade group.

as the corresponding gene does not show significant expression in myeloid cells.

Cas-Br-M, a retrovirus, contains *v-Cbl* which corresponds to about one third of the murine *c-Cbl* gene and contains only the murine phosphotyrosine binding domain.[25] This virus consistently induces a type of pre-B cell lymphoma in infected mice. The importance of *c-Cbl* in hematopoiesis has been previously demonstrated in knockout mice that show hyper-responsiveness to hematopoietic growth factors, expansion of the progenitor and stem cell pool, and mild myeloproliferative features.[17] However, recent results obtained with an RFD knock-in in a *c-Cbl−/−* mouse model parallels the phenotype observed in patients; the mutant mouse demonstrated a severe myeloproliferative phenotype (W.Y. Langdon, personal communication). Indeed, in patients mutations were predominantly located in the RFD and affected structurally essential cysteines, possibly led to inactivation of RFD function by frame shift, or created novel splicing sites resulting in larger transcripts. In addition, *c-Cbl* mutations were homozygous or hemizygous, implying that the presence of a WT allele is protective. It is likely that mutations do not lead to the simple knockout of *c-Cbl* function. Rather, by affecting the RFD, they render it a proto-oncogene, consistent with the oncogenic properties of *v-Cbl*. Previously, mutations of *c-Cbl* have been described in a limited number of patients with AML, but neither their function nor their clinical phenotype could be delineated without a comprehensive study of corresponding karyotypes and clinical outcomes.[26-28]

c-Cbl is a member of the *Cbl* family of E3 ubiquitin ligases, which poly- or monoubiquitinate a number of important tyrosine kinases serving as important transduction elements of proliferative signals and activated tyrosine kinase receptors, including Flt-3, c-kit, and M-CSF.[29,30] Consequently, inactivation of ubiquitination may lead to enhanced and prolonged signaling, a function which can explain the phenotype in patients (Fig 4). Based on this essential role of E3 ligases, we hypothesized that another *Cbl* family member, *Cbl-b*, may also be affected by mutations in myeloid malignancies. Sequencing of these genes in patients who did not harbor *c-Cbl* mutations revealed that

Table 3. Multivariate Analysis of Overall Survival in Clinical Variables

Variable*	Hazard Ratio	95% CI	*P*
Disease risk (advanced grade/ low grade)	5.64	3.8 to 8.36	< .0001
Cbl family gene (mutation/ wild type)	2.17	1.18 to 4.02	.013

*WBC, monocyte count, disease risk, chemotherapy, and *Cbl* family mutation were included in multivariate analysis.

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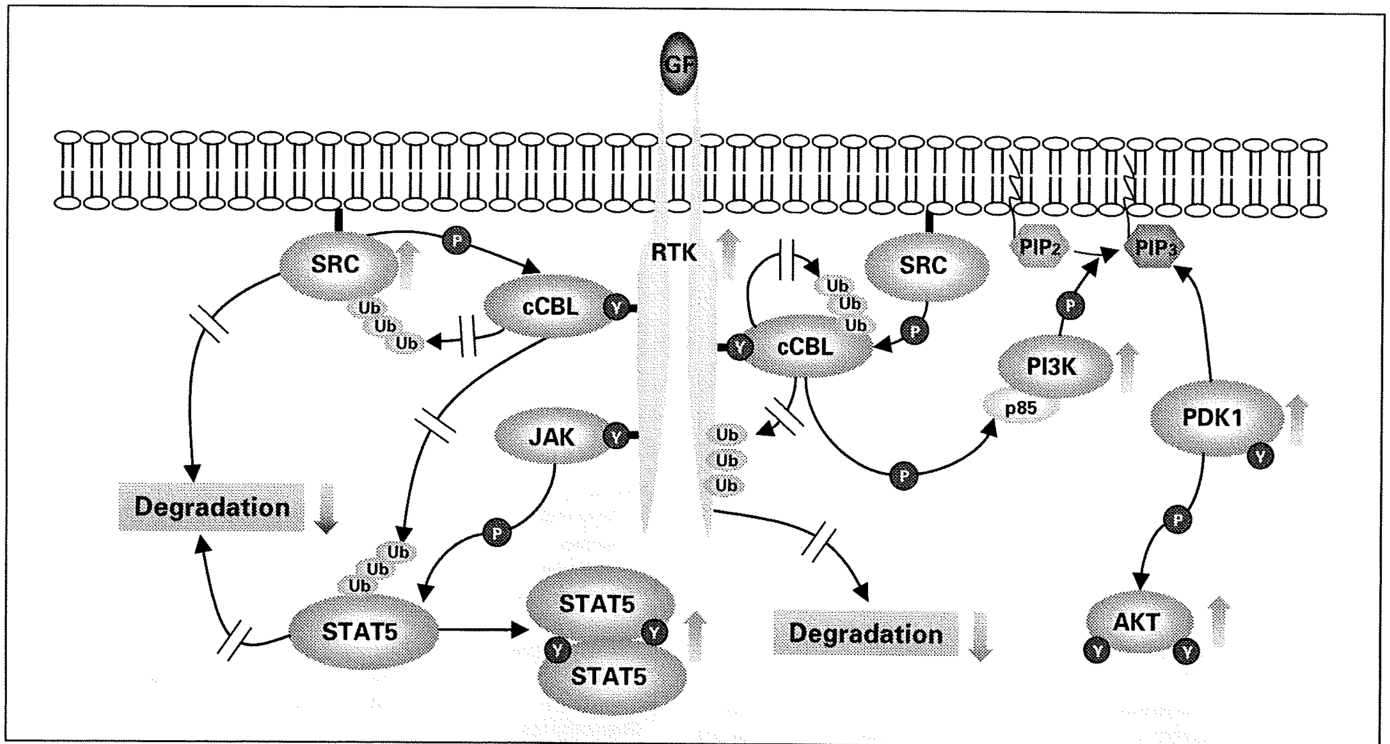

Fig 4. Potential intracellular consequences of *c-Cbl* mutations. *c-Cbl* is a member of the E3 ubiquitin ligase *Cbl* family, which poly- or monoubiquitinate a number of important receptor tyrosine kinases (RTK), including Flt-3, c-kit, and CSF-1, for degradation. Inactivation of ubiquitination activity through mutations occurring in RING finger domain may lead to enhanced and/or prolonged hematopoietic growth factor signaling, a function which can contribute to the clinical phenotype of patients with *c-Cbl* mutation. However, in addition to RTK, knockout of c-Cbl ubiquitination activity may result in enhanced phosphorylation of SRC kinase, STAT5, and also c-Cbl itself. Ultimately, the elevated level of important transduction factors such as STAT5 and PI3K as well as increased SRC kinase activity can lead to aberrant proliferative responses.

these genes can also be affected by mutations leading to the inactivation of the RFD. These patients displayed a clinical phenotype analogous to those with *c-Cbl* mutations. The clinical features corresponding to *c-Cbl* mutations included monocytic features, aberrant and increased phosphorylation of pSTAT5, and monocytoid blasts. An increased frequency of mutations in patients with frank AML may argue either that *c-Cbl* mutations lead to an invariant progression to an aggressive phenotype, or that they constitute a second hit event frequently occurring in the context of atypical myeloproliferative disorders. In fact, unlike other reports that looked at patients with inv16, we found no mutation of *Cbl* family genes in de novo AML, including French-American-British type M4 or M5.[28] Both theories are supported by the dismal prognosis of patients with *Cbl* family mutations. The close association of *c-Cbl* mutations with monocyte expansion, such as that seen in JMML, CMML, or sAML with monocytoid features, suggests a primary role of *c-Cbl* mutations in the pathogenesis of these diseases, while occurrence of *c-Cbl* mutations during evolution to AML in serially studied patients and the high proportion of cases with advanced leukemia affected by *c-Cbl* mutations argues for its auxiliary facilitator role.

Taken together, our data suggests that *Cbl* family mutations constitute a novel class of pathogenic molecular lesions associated with a spectrum of myeloid malignancies characterized by myeloproliferative features and poor prognosis. Inactivation of the RFD, and thereby ubiquitination involved in downmodulation of proliferative signaling, constitutes a general mechanism of leukemogenesis likely present in a variety of malignancies.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.
Employment or Leadership Position: None **Consultant or Advisory Role:** Mikkael A. Sekeres, Celgene (C), Pharmion (C); Jaroslaw P. Maciejewski, Eisai (C) **Stock Ownership:** None **Honoraria:** None **Research Funding:** Mikkael A. Sekeres, Celgene; Jaroslaw P. Maciejewski, Celgene, Genzyme **Expert Testimony:** None **Other Remuneration:** None

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Tacrolimus/Methotrexate versus Cyclosporine/ Methotrexate as Graft-versus-Host Disease Prophylaxis in Patients with Severe Aplastic Anemia Who Received Bone Marrow Transplantation from Unrelated Donors: Results of Matched Pair Analysis

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Tacrolimus (FK) and cyclosporine (CsA) have been shown to be effective in the prophylaxis of graft-versus-host disease (GVHD). However, no comparative studies have yet been conducted to examine the efficacy of FK/methotrexate (MTX) and CsA/MTX in patients with severe aplastic anemia (SAA) given unrelated donor bone marrow transplantation (U-BMT). We used matched-pair analysis to compare FK/MTX with CsA/MTX in patients with SAA who received U-BMT through the Japan Marrow Donor Program. Forty-seven pairs could be matched exactly for recipient age and conditioning regimens. Forty-five patients achieved engraftment in the FK group and 42 patients in the CsA group. The probability of grade II-IV acute GVHD (aGVHD) was 28.9% in the FK group and 32.6% in the CsA group ($P = .558$). The probability of chronic GVHD (cGVHD) was 13.3% in the FK group and 36.0% in the CsA group ($P = .104$). The 5-year survival rate was 82.8% in the FK group and 49.5% in the CsA group ($P = .012$). The study shows the superiority of FK/MTX over CsA/MTX in overall survival because of the lower incidence of transplantation-related deaths. A prospective randomized study comparing FK/MTX and CsA/MTX is warranted.

Biol Blood Marrow Transplant 15: 1603-1608 (2009) © 2009 American Society for Blood and Marrow Transplantation

KEY WORDS: Tacrolimus, Cyclosporine, Graft-versus-host disease, Prophylaxis, Aplastic anemia, Unrelated bone marrow transplantation

INTRODUCTION

Bone marrow transplantation (BMT) from a human-leukocyte antigen (HLA)-matched related donor is the treatment of choice for children and young adults with severe aplastic anemia (SAA) [1,2]. However, HLA-matched related donors are available for <30% of patients in developed countries. Immunosuppressive therapy (IST) has been used as an alternative treatment for patients without a HLA-matched related donor [3,4]. For nonresponders to IST, BMT from an unrelated donor (U-BMT) has been indicated [5]. Acute and chronic graft-versus-host disease (aGVHD, cGVHD) contribute to much of the morbidity and mortality associated with U-BMT. Effective prevention of these complications is therefore crucial for the success of U-BMT.

A combination of cyclosporine (CsA) and a short course of methotrexate (MTX) is the standard pharmacologic regimen for the prophylaxis of GVHD after BMT from both HLA-matched siblings and HLA-matched unrelated donors [6,7]. Tacrolimus (FK), a potent macrolide lactone immunosuppressant, inhibits T cell activation by forming a complex with FK binding protein-12, which blocks the serine-threonine phosphatase activity of calcineurin [8]. Although the mechanism of action is similar to that

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Financial disclosure: See Acknowledgments on page 1607.
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Received May 21, 2009; accepted August 15, 2009
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1083-8791/09/1512-0014$36.00/0
doi:10.1016/j.bbmt.2009.08.012

of CsA, the potency of FK in vitro is more than 100 times that of CsA [9]. This suggests that FK might also be more effective than CsA as GVHD prophylaxis in high-risk settings. In fact, in randomized studies of GVHD prophylaxis after matched related and unrelated BMT, the incidence of aGVHD was reduced in the treatment group receiving a combination of FK and a short course of MTX (FK/MTX) compared to the control group who received CsA/MTX [10-12]. However, although the number of deaths from GVHD was lower, an increased incidence of relapse was observed in the FK group, resulting in no difference in overall survival (OS) rate between the 2 groups. Nevertheless, because there is no risk of relapse in patients with nonmalignant disease, the results might be different in studies of malignant versus nonmalignant disease. Accordingly, FK/MTX could be associated with a lower incidence of aGVHD/cGVHD and better survival compared to CsA/MTX in patients with acquired SAA who received U-BMT.

METHODS

Patients and Controls

We collected U-BMT data from SAA patients who received FK/MTX for the prophylaxis of GVHD through the Japan Marrow Donor Program (JMDP) database. Forty-seven patients were recruited who underwent BMT between July 1997 and December 2002. For each patient receiving FK/MTX, we selected a control patient who received CsA/MTX for the prophylaxis of GVHD during the same period. Because our previous study identified that recipient age and conditioning regimens were the most important variables associated with treatment failure, we selected control patients matched for these 2 variables [13].

Transplantation data were collected using standardized forms provided by the JMDP. Baseline information and follow-up reports were submitted at 100 days, 6 months, 1 year, and then annually after transplantation. Analysis of patient outcome was performed using data from the last reported follow-up or the date of death.

Recipient-Donor HLA Matching

HLA matching between the recipient and donor was based on HLA serotyping according to the standard technique. In 69 (73%) of the 94 recipient-donor pairs, molecular analyses of HLA-A, -B, and -DRB1 loci were performed using DNA-based methods.

Transplantation Procedures

Various preconditioning regimens were used by individual transplantation centers and classified into 6 categories (Table 1): (1) cyclophosphamide (Cy; 120-200 mg/kg) + total body irradiation (TBI; 2-10 Gy) + limited field irradiation (LFI; 5-8 Gy) + antithymocyte globulin (ATG), (2) Cy + TBI + LFI, (3) Cy + TBI + ATG, (4) Cy + TBI, (5) Cy + LFI + ATG, and (6) Cy + LFI. For the prophylaxis of GVHD, FK was started at a dose of 0.03 mg/kg from day −1 and administered through continuous 24-hour i.v. infusion. Patients were converted from intravenous i.v. to oral intake when it could be tolerated at a ratio of 1:3 in 2 divided doses per day based on the last intravenous dose. Standard doses of CsA were 3 mg/kg by i.v. infusion and 6 mg/kg by oral intake. The MTX doses were 15 mg/m^2 on day 1 and 10 mg/m^2 on days 3, 6, and 11 after transplantation in both the FK and CsA groups.

Table 1. Patient/donor Characteristics and BMT Procedure

	Tacrolimus	Cyclosporine	P
Patient number	47	47	
Age (year)			.962
<10	11	11	
11-29	28	27	
>30	8	9	
Sex			.396
Male	31	27	
Female	16	20	
Recipient/donor sex			.71
Male/male	19	19	
Female/female	7	10	
Male/female	12	8	
Female/male	9	10	
HLA matching by DNA typing			.029
A, B, DRB1 match	20	34	
A mismatch	4	3	
B mismatch	7	0	
DRB1 mismatch	7	6	
2 alleles mismatch	3	2	
Unknown*	6	2	
Duration of disease before BMT			.359
1 year or less	6	11	
1-3 year	17	17	
3 year or more	24	19	
RBC transfusions before BMT			1
<20	7	7	
20 or more	38	38	
Unknown	2	2	
Platelet transfusions before BMT			.651
<20	9	7	
20 or more	36	36	
Unknown	2	4	
Conditioning regimens			1
Cy + TBI + LFI + ATG	3	3	
Cy + TBI + LFI	6	6	
Cy + TBI + ATG	18	18	
Cy + TBI	11	11	
Cy + LFI + ATG	3	3	
Cy + LFI	6	6	
Marrow cell dose			.764
$<3 \times 10^8$/kg	14	13	
3×10^8/kg or more	30	32	
Unknown	3	2	

BMT indicates bone marrow transplantation; Cy, cyclophosphamide; TBI, total body irradiation; LFI, local field irradiation; ATG, antithymocyte globulin; RBC, red blood cell.
*HLA was serologically matched or 1-antigen mismatched in these donor-recipient pairs.

Definitions and Statistical Analysis

Engraftment was defined as achievement of a peripheral blood (PB) absolute neutrophil count (ANC) of more than 0.5×10^9/L for 3 consecutive days. In evaluation of engraftment, patients who died before day + 22 without engraftment were not considered evaluable. aGVHD and cGVHD were evaluated according to the standard criteria [14,15]. Patients who died before engraftment were excluded from the analysis of aGVHD. For analysis of cGVHD, only those who survived 100 days after transplantation were included. The probabilities of overall survival and aGVHD and cGVHD were estimated from the time of transplantation according to the Kaplan-Meier product-limit method. The χ^2 test and log-rank statistics were used to assess significance of differences in variables and outcomes between the 2 groups. All probability values were 2 sided, and $P < .05$ was considered significant.

RESULTS

Patient, Donor, and Transplantation Characteristics

Patient, donor, and transplantation characteristics of the study population are summarized in Table 1. There was an imbalance in HLA-A, -B, and -DRB1 allele mismatches, with 21 of the mismatch pairs observed in the FK group and 11 in the CsA group ($P = .029$). Other variables were comparable between the 2 groups.

Engraftment

Engraftment took place in 45 patients (96%) in the FK group and 42 patients (89%) in the CsA group. Three patients, 1 in the FK group and 2 in the CsA group, died before day 21 and were considered not evaluable for engraftment. One of the 46 evaluable patients in the FK group and 3 of the 45 evaluable patients in the CsA group failed to engraft. Another patient in the CsA group experienced late graft failure. The median time to neutrophil recovery was 18 days in the FK group (range: 10-28 days) and 17 days in the CsA group (range: 12-26 days) ($P = .400$).

aGVHD

The probability of grade II-IV aGVHD was 28.9% (range: 15.3%-42.5%) in the FK group and 32.6% (range: 18.4%-46.8%) in the CsA group at 100 days (Figure 1; $P = .558$). aGVHD developed at a median of 30 days (range: 7-76 days) in the FK group and 13 days (range: 5-28 days) in the CsA group after transplantation. The distribution of GVHD grade and organ involvement is presented in Table 2. Despite the imbalance in HLA disparity, the incidence of grade II-IV aGVHD in the FK group was equal to that in the CsA group.

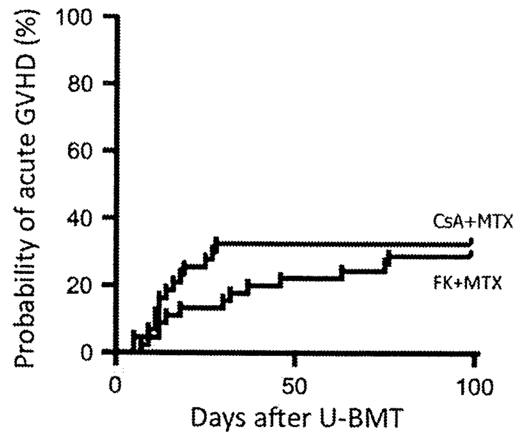

Figure 1. The probability of grade II-IV aGVHD in the FK/MTX group and the CsA/MTX group (28.9% versus 32.6%, $P = .558$).

cGVHD

Thirty-eight patients in the FK group and 35 in the CsA group were evaluable for cGVHD. Five patients in the FK group developed cGVHD at a median period of 4 months (range: 3-5 months) and 10 patients in the CsA group developed cGVHD at a median period of 4 months (range: 2-40 months). Overall, the probability of cGVHD was 13.3% (range: 2.1%-24.5%) in the FK group and 36.0% (range: 15.2%-56.8%) in the CsA group (Figure 2; $P = .104$). Three patients in the FK group and 4 in the CsA group developed an extensive type of cGVHD.

Survival

Of 47 patients in each group, 39 in the FK group survived at 4 to 61 months (median: 26 months), whereas 25 in the CsA group survived at 3 to 61 months (median: 38 months) after transplantation. The OS at 5 years was 82.8% (range: 71.9%-93.6%) in the FK group and 49.5% (range: 32.5%-66.4%) in the CsA group (Figure 3; $P = .012$). Eight patients in

Table 2. Distribution of Grade and Organ Involvement in Acute GVHD

	Tacrolimus (n = 47)	Cyclosporine (n = 47)
Grade		
0	22 (47%)	26 (55%)
I	7 (15%)	3 (6%)
II	8 (17%)	7 (15%)
III	4 (8%)	6 (13%)
IV	2 (5%)	1 (2%)
unevaluable	4 (8%)	4 (8%)
Organ involvement		
skin	5 (38%)	3 (21%)
skin + gut	6 (46%)	7 (50%)
gut + liver	1 (8%)	0 (0%)
skin + gut + liver	1 (8%)	4 (29%)

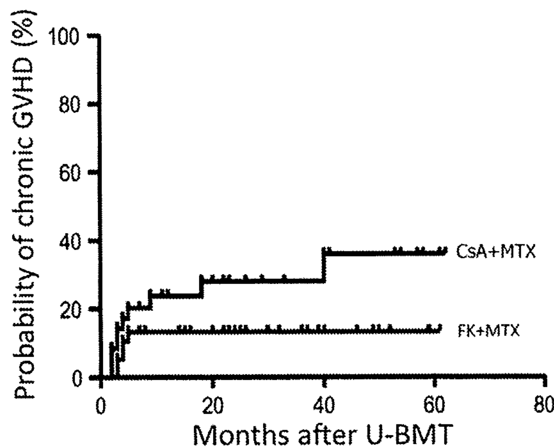

Figure 2. The probability of cGVHD in the FK/MTX group and the CsA/MTX group (13.3% versus 36.0%, $P = .104$).

the FK group and 22 in the CsA group died from transplantation-related toxicities ($P = .002$). Causes of death are summarized in Table 3. Graft failure and bacterial/fungal infection were the major causes of death.

DISCUSSION

Analyses of registration data suggest that the outcome of U-BMT in AA patients has substantially improved over the past 10 years. In analysis of 498 patients registered to the European Group for Blood and Marrow Transplantation (EBMT), 5-year survival increased from 32% ± 8% before to 57% ± 8% after 1998 [16]. Similarly, Maury et al. [17] analyzed the outcome of 89 patients in the French registry and found that 5-year survival increased from 29% ± 7% before and 50% ± 7% after 1998. An optimum conditioning regimen, GVHD prophylaxis and better donor selection may be responsible for these improvements.

In the late 1990s, HLA typing using molecular methods was introduced into clinical use. Matching for 10 alleles by high resolution technology replaced

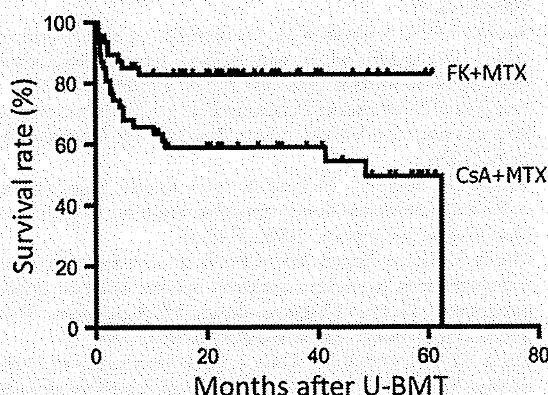

Figure 3. Kaplan-Meier estimates of OS in the FK/MTX group and the CsA/MTX group (82.8% versus 49.5%, $P = .012$).

Table 3. **Primary Causes of Death**

	Tacrolimus (n = 47)	Cyclosporine (n = 47)
Bacterial/fungal infection	4	4
Graft failure	1	4
Acute GVHD	1	2
Interstitial pneumonitis	0	3
Hemorrhage	1	1
EBLPD	1	1
Heart failure	0	2
Others	0	3
Total	8	22

EBLPD indicates Epstein Barr virus associated lymphoproliferative disorder; GVHD, graft-versus-host disease.

matching for 6 antigens by low resolution technology. A French study revealed that improved survival was associated with high-resolution HLA matching, suggesting that better donor selection might be a major factor in improving prognosis [17]. Recent attempts to improve the outcome in SAA patients include the use of low-dose TBI or a nonirradiation-fludarabine (Flu)-based regimen. In a prospective multicenter study sponsored by the National Marrow Donor Program (NMDP) using low-dose TBI, a low graft rejection rate of 5% and 5-year survival of 55% were achieved in 87 patients [18]. Moreover, a study by the EBMT using Flu, low-dose Cy and ATG showed a lower incidence of aGVHD and cGVHD and 5-year survival of 73% [19]. Although these novel pretransplant conditioning regimens are promising, all analyses failed to show the contribution of new regimens to the improved outcomes because of the small number of patients.

Different from patients with hematologic malignancies, there is no obvious benefit of GVHD for patients with AA. In fact, many studies have indicated adverse effects of aGVHD on the outcome of AA patients, suggesting that the most effective prophylactic regimen for GVHD should be employed for patients with AA. However, trials involved with lessening severe GVHD are limited in patients with AA. In a small number of studies, ex vivo T cell depletion by monoclonal antibodies (mAbs) or in vivo use of alemtuzumab instead of ATG has been attempted with encouraging results [20,21]. Although pharmacologic prevention with CsA/MTX is used as GVHD prophylaxis in the majority of AA patients, the role of alternative pharmacologic agents remains undetermined. Although previous randomized studies comparing CsA/MTX and FK/MTX did not show any survival benefits of FK despite a reduction in the incidence of aGVHD, most patients had malignant disease and only a few with AA were included [10-12].

The aim of the present study was to compare FK and CsA in the prophylaxis of GVHD using matched pair analysis. One drawback was the imbalance of HLA disparity between the 2 groups, with 21 mismatched pairs in the FK group and 11 in the CsA group. Our previous study showed that allelic mismatching of

HLA-A and -B antigens, but not HLA-DRB1 is the most crucial risk factor for survival of AA patients who received transplants from an unrelated donor [13]. More HLA class I mismatched pairs (HLA-A; 4, HLA-B; 7) were included in the FK group than in the CsA group (HLA-A; 3). Despite this disadvantage in terms of HLA disparity, the probability of grade II-IV aGVHD did not differ between the 2 groups. The probability of cGVHD tended to be marginally less in the FK group than in the CsA group ($P = .104$). The duration of CsA or FK after U-BMT may affect the incidences of cGVHD. However, we did not compare the difference of duration in this study because the actual duration of administration of these immunosuppressants was not available in our database.

The duration of follow-up in the FK506 group is less than in the CSP group. Although it may introduce a significant bias in the analysis, the current study showed that 5-year survival was significantly higher in the FK group than in the CsA group. Patients in the FK group showed a significant reduction in treatment-related mortality (TRM), resulting in better OS. To date, results of 3 previous randomized studies comparing FK and CsA have indicated a significantly lower incidence of aGVHD among patients receiving FK, but with no survival benefits having been demonstrated [10-12].

Yanada et al. [22] conducted a retrospective study comparing an FK-based regimen and CsA-based regimen for the prophylaxis of GVHD using registration data of the Japan Society for Hematopoietic Cell Transplantation (JSHCT). In their study, 777 patients who underwent BMT from an unrelated donor were analyzed (FK group: n = 191, CsA group; n = 586). Although the distribution of different diseases was not specified, the majority of the patients appeared to have hematologic malignancies. FK significantly reduced the risk of aGVHD and TRM without any increase in relapse, thus improving OS.

In conclusion, our matched pair analysis showed the superiority of FK/MTX over CsA/MTX in OS. However, our study was retrospective and a further study comparing FK/MTX and CsA/MTX as a prophylaxis of GVHD in AA patients who will receive U-BMT may be warranted.

ACKNOWLEDGMENTS

Financial disclosure: The authors have nothing to disclose.

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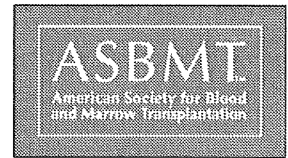

Outcome of 125 Children with Chronic Myelogenous Leukemia Who Received Transplants from Unrelated Donors: The Japan Marrow Donor Program

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Because of a small number of patients, only a few studies have addressed the outcome of bone marrow transplantation (BMT) in children with Philadelphia chromosome–positive (Ph+) chronic myelogenous leukemia (CML), who receive graft from a volunteer-unrelated donor (VUD), especially after practical application of imatinib mesylate. The outcomes of BMT from a VUD in 125 children with Ph+ CML were retrospectively reviewed. Patients were identified through the Japan Marrow Donor Program as having undergone BMT between 1993 and 2005 and were aged 1-19 years at the time of transplant (median age, 14 years). The probabilities of 5-year overall survival (OS) and leukemia-free survival (LFS) were 59.3% and 55.5%, respectively. Multivariate analysis identified the following unfavorable survival factors: infused total nucleated cell dose $< 314 \times 10^6$ /kg (relative risk [RR] = 2.43; 95% confidence interval [CI] = 1.33-4.44; P =.004), advanced phase (RR = 2.43; 95% CI = 1.37-4.31; P =.004), and no major cytogenetic response (MCyR) at the time of BMT (RR = 6.55; 95% CI = 1.98-21.6; P =.002). Of the 17 patients treated with imatinib, 15 (88%) achieved MCyR at the time of BMT, and this group had an excellent 5-year OS of 81.9%. Disease phase, infused total nucleated cell dose, and cytogenetic response were independent risk factors for survival of unrelated BMT. These findings provide important information for assessing the indications for and improving outcome in unrelated BMT for the treatment of pediatric CML.

Biol Blood Marrow Transplant 16: 231-238 (2010) © *2010 American Society for Blood and Marrow Transplantation*

KEY WORDS: Chronic myelogenous leukemia, Children, Unrelated donor, Stem cell transplantation, Bone marrow transplantation, Japan Marrow Donor Program

INTRODUCTION

Philadelphia-positive (Ph+) chronic myelogenous leukemia (CML) is a rare disease in children, accounting for only 3%-5% of all pediatric leukemia, with a incidence of <1 in 100,000 children [1]. Allogeneic hematopoietic stem cell transplantation (HSCT) is the only proved curative treatment for children with Ph+ CML. Reported event-free survival (EFS) in children with Ph+ CML who underwent transplantation in the

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Financial disclosure: See Acknowledgments on page 237.

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Received July 18, 2009; accepted September 24, 2009

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1083-8791/10/162-0010$36.00/0
doi:10.1016/j.bbmt.2009.09.022

chronic phase with a matched related donor is 60%-75% [2-4]; however, this approach is limited by the availability of HLA-matched family donors. The majority of children who lack an HLA-matched donor receive a transplant from an alternative donor, such as a volunteer-unrelated donor (VUD). EFS is less favorable in this setting, ranging from 30% to 55% [3-5].

Since the introduction of the novel tyrosine kinase inhibitor imatinib mesylate, the treatment for Ph+ CML has been completely revised [6]. Imatinib can induce complete hematologic and cytogenetic remission in the majority of patients, and follow-up data on patients treated only with imatinib indicate that complete cytogenetic and major molecular responses are durable, while drug toxicity is low [7]. The number of transplantations for Ph+ CML has declined rapidly [8]. But, despite significant cytogenetic and molecular responses, there is no evidence that imatinib is curative, and imatinib's long-term side effects remain to be determined. Some patients have successfully stopped imatinib without recurrence, but some who were polymerase chain reaction (PCR)-negative for a period stopped and then experienced recurrence [9,10]. Stopping imatinib may be possible, but effective strategies have yet to be developed.

This is particularly important for pediatric patients, in whom the goal is cure of the disease rather than palliation, and for whom long-term survival is particularly anticipated. The presence of molecular disease and the emergence of resistant clones in patients treated with imatinib suggest the need for caution with regard to abandoning curative therapy by SCT. The need for information on the current status of SCT for Ph+ CML and up-to-date results when considering the treatment of children with Ph+ CML, even in the imatinib era, is evident; however, few studies have specifically analyzed outcomes of SCT in children with Ph+ CML [2-5]. The aim of the present study was to analyze data from 125 children with Ph+ CML who underwent bone marrow transplantation (BMT) from a VUD and identify factors influencing outcome.

PATIENTS AND METHODS

Patients

A retrospective analysis was conducted on behalf of the Japan Marrow Donor Program (JMDP) and the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) CML Committee. Data were collected from 125 children (age at transplantation < 20 years) whose donors were identified through the JMDP and who underwent allogeneic BMT from a VUD for Ph+ CML between 1993 and 2005. Table 1 summarizes the patient, donor, and transplant characteristics. Patient characteristics in the first chronic phase (CP1) and in the advanced phase are described separately. All patients or their guardians gave written informed consent for transplantation and submission of data to the JMDP for further research. This study

Table 1. Patient, Donor, and Transplant Characteristics

	CP1 (n = 88)	Advanced Phase (n = 37)	Total (n = 125)
Year of transplantation			
1993-1998	45	22	67
1999-2005	43	15	58
Stage of CML at BMT			
CP1	88	0	88
CP2	0	12	12
CP3	0	1	1
Advance phase	0	11	11
Blast crisis	0	13	13
Cytogenetic response at BMT			
With MCyR	29	4	33
Without MCyR	39	25	64
Unknown	20	8	28
Pretransplantation therapy with IFN-α			
No	22	8	30
Yes	66	29	95
Pretransplantation therapy with imatinib			
No	72	36	108
Yes	16	1	17
Recipient sex, M/F	56/32	25/12	81/44
Donor–recipient sex			
Female donor to male recipient	20	10	30
Other	68	27	95
Median age at BMT, years (range)	13 (1-19)	17 (2-19)	14 (1-19)
Median time from diagnosis to transplantation, months (range)	14 (2-111)	19 (5-103)	14 (2-111)
Patient CMV antibody			
Negative	25	14	39
Positive	54	21	75
Unknown	9	2	11
ABO mismatch			
Match	41	15	56
Major mismatch	29	11	40
Minor mismatch	17	9	26
Unknown	1	2	3
Recipient–donor HLA DNA typing			
Match (10/10)	33	8	41
1 alleles mismatch	9	5	14
2 alleles mismatch	19	9	28
3 alleles mismatch	8	3	11
4 alleles mismatch	2	2	4
6 alleles mismatch	0	1	1
Unknown	17	9	26
Conditioning regimen			
TBI regimen	66	30	96
Non-TBI regimen	22	7	29
GVHD prophylaxis			
CsA + MTX	59	22	81
Tacrolimus + MTX	28	15	43
MTX alone	1	0	1
Administration of ATG			
No	76	34	110
Yes	12	3	15
Median infused total nucleated cell dose, $\times 10^6$ /kg (range)	315 (27-880)	298.5 (29-750)	314 (27-880)

ATG indicates antithymocyte globulin; BMT, bone marrow transplantation; CML, chronic myelogenous leukemia; CP, chronic phase; CMV, cytomegalovirus; CsA, cyclosporine; IFN, interferon; GVHD, graft-versus-host disease; MCyR, major cytogenetic response; MTX, methotrexate; TBI, total body irradiation.

was approved by the Data Management Committee of the JMDP and by the Ethical Committee of Nagoya University Graduate School of Medicine.

The 125 children in the study included 81 boys (65%) and 44 girls (35%). The median age at the time of BMT was 14 years (range, 1-19 years). Disease phase at the time of transplantation was defined according to International Bone Marrow Transplant Registry (IBMTR) criteria [11]. Eighty-eight patients (70%) underwent transplantation in CP1. Of the 37 children who underwent transplantation in an advanced phase of CML, 12 were in CP2, 1 was in CP3, 11 were in the accelerated phase (AP), and 13 were in blast crisis (BC). Cytogenetic response data at the time of BMT were available for 97 patients (78%), of whom 68 were in CP1 and 29 were in an advanced phase. Major cytogenetic response (MCyR; ≤ 35% Ph+ cells) was achieved in 33 patients (29 patients in CP1 and 4 patients in CP2). Ninety-five recipients (76%) were given interferon (IFN)-α, and 17 (14%) were given imatinib before transplantation. The patients treated with imatinib proceeded to BMT regardless of their response, according to each institutes' therapeutic strategy. The median interval from diagnosis to transplantation was 14 months (range, 2-111 months). Fifty-seven patients (46%) underwent transplantation within 12 months, and 68 (54%) did so after 12 months. Imatinib began to be used in Japan in 1999, and its use was approved by the Japanese Health and Welfare Ministry in 2002. In our cohort, 17 patients (16 in CP1, 1 in AP) received imatinib before transplantation.

Transplantation Procedures and Recipient–Donor HLA Matching

All 125 recipients received a BM graft from a VUD identified through the JMDP. Various preconditioning regimens were used by individual centers. Of the 125 recipients, 96 (77%) received a preparative regimen with total body irradiation (TBI). Fifteen recipients (12%) received antithymocyte globulin (ATG). Cyclosporine A (CsA)–based GVHD prophylaxis was used in 81 patients (65%); tacrolimus-based prophylaxis, in 43 (34%). One patient received only methotrexate (MTX) as GVHD prophylaxis. HLA-matching data based on high-resolution DNA typing for HLA-A, -B, -C, -DRB1, and -DQB1 antigens were available in 99 patients (79%). Of these 99 patients, 41 (41%) were fully matched at 10/10 alleles, 14 (14%) were mismatched at 1 HLA allele, 28 (28%) were mismatched at 2 HLA alleles, and 16 (16%) were mismatched at more than 3 HLA alleles.

Definitions, Data Collection, and Statistical Analysis

The outcomes were analyzed on the basis of engraftment, grade II-IV acute and chronic GVHD (aGVHD, cGVHD), treatment-related mortality (TRM), relapse, overall survival (OS), and leukemia-free survival (LFS). The date of engraftment was defined as the first of 3 consecutive days with a neutrophil count exceeding 0.5×10^9 /L. aGVHD and cGVHD were classified according to published criteria [12]. Only patients surviving for >100 days after transplantation were considered eligible for evaluation of cGVHD. Relapse of CML was defined by hematologic or cytogenetic evidence of disease. (Data on molecular evidence of relapse were not available.) Transplantation data were collected using standardized forms provided by the JMDP. After transplantation, patient baseline information and follow-up reports were submitted at 100 days, 6 months, 1 year, and annually thereafter.

Comparisons between groups were performed using Fisher's exact test for categorical variables and the Mann-Whitney *U* test for continuous variables. Survival and time to events were calculated from the date of transplantation. OS and LFS were estimated by the Kaplan-Meier method and compared using the log-rank test. Cumulative incidence curves were created for TRM. The Cox proportional hazard model was used to obtain the estimates and the 95% confidence interval (CI) of the relative risk (RR) for predictive factors and to evaluate predictive factors for TRM, LFS, and OS in a multivariate analysis. The following variables were evaluated: patient age at the time of BMT (≥ 15/< 15 years), patient sex, sex mismatch, year of transplantation (1993-1998/1999-2005), period from diagnosis to transplantation (≥ 12 months/ < 12 months), infused total nucleated cell dose (≥ 314×10^6/kg/< 314×10^6/kg), TBI-containing regimen (yes/no), use of ATG (yes/no), GVHD prophylaxis (CsA + MTX ± steroids/FK ± MTX), full HLA matching (yes/no), disease phase at the time of BMT (CP1/advanced phase), MCyR at the time of BMT (yes/no), ABO mismatch (match/mismatch), recipient cytomegalovirus (CMV) antibody (negative/ positive), history of interferon therapy (yes/no), and history of imatinib therapy (yes/no). Variables with more than 2 categories were dichotomized for the final multivariate model. The cutoff points of the variables were chosen to make optimal use of the information, with the proviso that smaller groups contained at least 20% of the patients. The cutoff points of continuous variables were chosen from the 25th, 50th, and 75th percentiles; consequently, the median of continuous variables was dichotomized as follows: age (≥15/ < 15 years), year of transplantation (1993-1998/ 1999-2005), and infused total nucleated cell dose (≥ 314×10^6/kg/< 314×10^6/kg). SPSS version 15.0 (SPSS Inc, Chicago, IL) was used for all statistical calculations except estimation of the cumulative incidence, which was performed using Stata version 10.0 (StataCorp, College Station, TX).

Table 2. Patient Clinical Outcomes

	CP1 (n = 88)	Advanced Phase (n = 37)	Total (n = 125)	P Value
Engraftment				.336
Yes/No	85 / 3	34 / 3	119 / 6	
Acute GVHD				.186
None	21	11	32	
Grade I	34	9	43	
Grade II	18	5	23	
Grade III	11	7	18	
Grade IV	4	5	9	
Chronic GVHD				.393
None	49	25	74	
Limited	15	6	21	
Extensive	24	6	30	
5-year TRM (95% CI)	28.3% (23.4-33.2)	56.5% (48.0-65.0)	36.5% (32.5-40.5)	.002
5-year relapse rate (95% CI)	11.8% (8.1-15.5)	29.0% (18.7-39.3)	15.4% (11.7-19.1)	.098
5-year LFS (95% CI)	65.2% (60.0-70.4)	32.4% (24.7-40.1)	55.5% (51.0-60.0)	.001
5-year OS (95% CI)	70.7% (65.7-75.7)	32.4% (24.7-40.1)	59.3% (54.8-63.8)	<.001

GVHD indicates graft-versus-host disease; LFS, leukemia-free survival; OS, overall survival; TRM, treatment-related mortality.

RESULTS

Engraftment

A total of 119 recipients (95%) were successfully engrafted. Neutrophil engraftment occurred at a median of 18 days after BMT (range, 11-37 days). Six patients (5%) experienced primary graft failure (Table 2), all of whom died.

aGVHD and cGVHD

Grade II-IV aGVHD occurred in 50 patients (40.7%; 95% CI = 36.3%-45.1%), and grade III-IV aGVHD occurred in 27 patients (22.6%; 95% CI = 16.1%-31.2%). Fifty-one patients (50.1%; 95% CI = 45.0%-55.2%) developed cGVHD (extensive type, n = 30; limited type, n = 21).

Relapse

Seventeen patients (11 recipients in CP1 and 6 in an advanced phase) experienced a relapse. The 5-year cumulative incidence of relapse was 19.7% (95% CI = 15.1%-24.3%). The median time for occurrence of relapse for the entire study cohort was 7 months (range, 1-97 months).

Survival

LFS

The 5-year LFS rate was 55.5% (95% CI = 51.0%-60.0%) for the entire cohort (Figurel). The LFS rate was significantly higher in children undergoing BMT in CP1 (65.2%; 95% CI = 60.0%-70.4%) than those undergoing BMT in an advanced phase (32.4%; 95% CI = 28.7%-36.1%; $P = .001$) (Table 2).

On univariate analysis, the following factors were significantly associated with LFS: age at the time of BMT ($P = .047$), infused total nucleated cell dose ($P = .002$), disease phase ($P = .002$), and cytogenetic response at the time of BMT ($P = .001$). Multivariate analysis also identified infused total nucleated cell dose (RR = 2.320; 95% CI = 1.326-4.061; $P = .003$), disease phase (RR = 2.051; 95% CI = 1.187-3.545; $P = .010$), and cytogenetic response at the time of BMT (RR = 2.890; 95% CI = 1.264-610); $P = .012$) as independent risk factors for LFS.

OS

The 5-year OS rate was 59.3% (95% CI = 54.8%-63.8%) for the entire cohort (Figure 1). The OS rate was significantly higher in the children undergoing BMT in CP1 (70.7%; 95% CI = 65.7%-75.7%) than in those undergoing BMT in an advanced phase (32.4%; 95% CI = 24.7%-40.1%; $P < .001$) (Table 2).

On univariate analysis, the following risk factors were significantly associated with OS: age at the time of BMT ($P = .037$), interval between diagnosis and

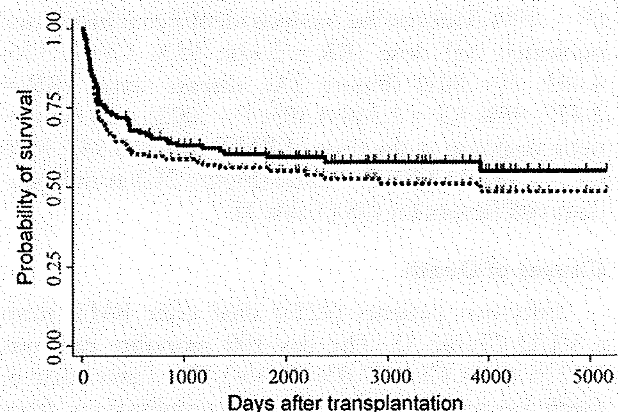

Figure 1. OS and LFS in children with Ph+ CML. In Kaplan-Meier curves graph, solid line shows the probabilities of OS (5-year OS = 59.3%; 95% CI = 54.8%-63.8%) and the dotted line shows that of LFS (5-year LFS = 55.5%; 95% CI = 51.0%-60.0%).

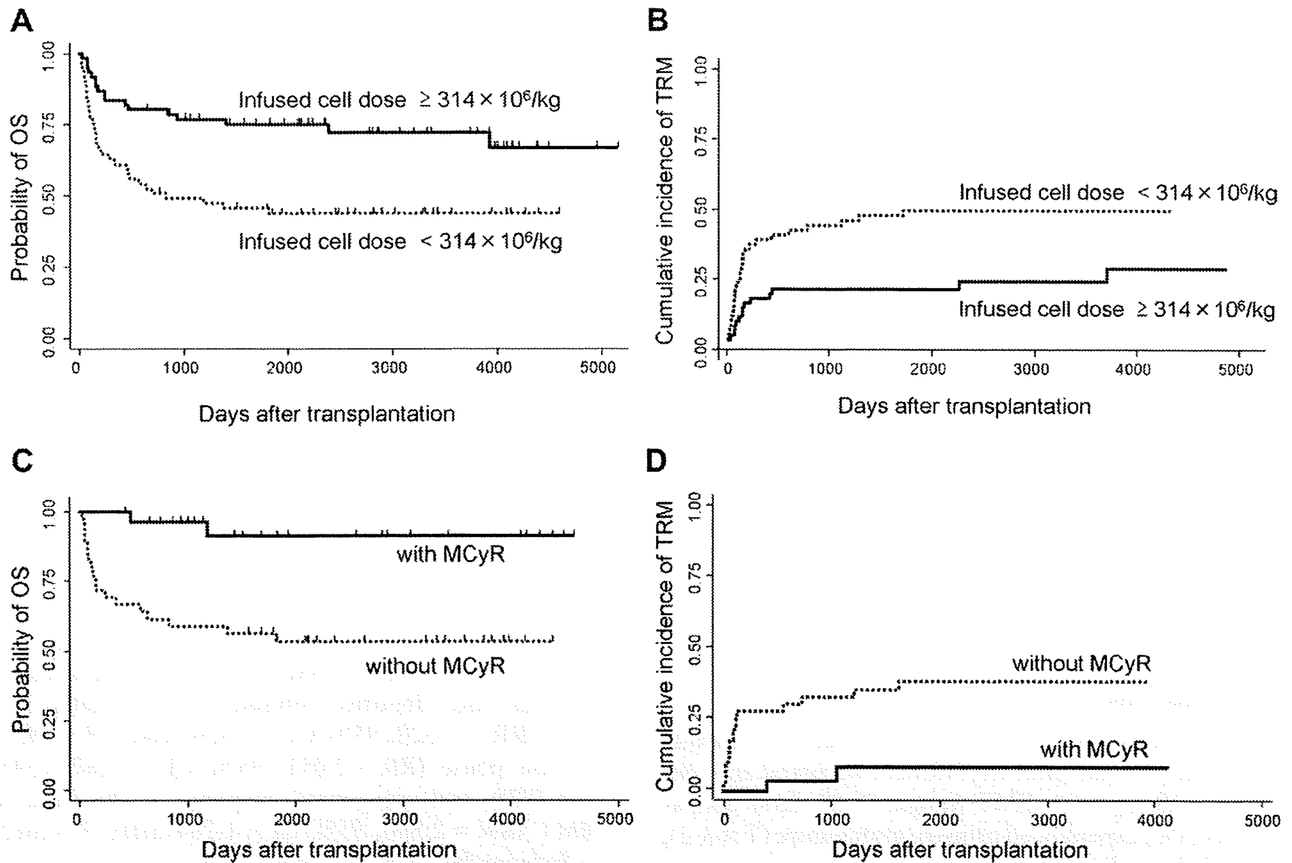

Figure 2. A and B, Relationship among infused total nucleated cell dose, OS (A), and TRM (B) in children with Ph+ CML. In the entire cohort, OS was significantly higher for children who received a higher infused total nucleated cell dose than those who received a lower dose ($\geq 314 \times 10^6$/kg vs $< 314 \times 10^6$/kg; $P = .001$). TRM was significantly higher for children who received a lower cell dose than for those who received a higher cell dose ($\geq 314 \times 10^6$/kg vs $< 314 \times 10^6$/kg; $P = .003$). Solid lines show the probabilities of OS and TRM for children who received a higher infused total nucleated cell dose and the dotted lines show the probabilities for those who received a lower infused total nucleated cell dose. C and D, OS (C) and TRM (D) of Ph+ CML children in CP1 with or without an MCyR. OS was significantly higher for children who achieved MCyR at the time of BMT (n = 29) than for those who did not (n = 39) (OS; $P < .001$) (C). TRM was also significantly higher for children who did not achieve MCyR ($P = .005$) (D). The solid lines show the probabilities of OS and TRM for children with MCyR at the time of BMT, and the dotted lines show the probabilities for those without.

BMT ($P = .042$), infused total nucleated cell dose ($P = .002$), disease status ($P < .001$), and cytogenetic response at the time of BMT ($P = .002$). A history of imatinib therapy before BMT marginally affected OS ($P = .099$). Multivariate analysis identified infused total nucleated cell dose (RR = 2.426; 95% CI = 1.326-4.441; $P = .001$) (Figure 2A), disease status (RR = 2.427; 95% CI = 1.368-4.305; $P = .002$), and cytogenetic response at the time of BMT (RR = 6.547; 95% CI = 1.982-21.629; $P = .002$) (Figure 2C) as independent risk factors for OS (Table 3).

Causes of Death

Fifty-two patients (42%) died after BMT from a VUD (Table 4). The day-100 mortality rate was 15.2 % (95% CI = 12.0%-18.4%). The main cause of death was transplantation-related complications, from which 46 patients (37%) died between day 8 and 10 years (median, 4 months) after transplantation. These included 18 transplantation-related deaths occurring before day 100 after transplantation. Death was associated with treatment-resistant GVHD in 14 patients (9 with aGVHD and 5 with cGVHD). Infection was the cause of death in 12 patients. Six patients died from recurrent CML between 3 and 28 months (median, 13 months) after transplantation.

Univariate analysis revealed that infused cell dose ($P = .013$), disease phase ($P = .006$), and cytogenetic response at the time of BMT ($P = .001$) were significant risk factors for TRM. The interval between diagnosis to BMT ($P = .083$) and HLA mismatch ($P = .087$) were marginally associated with TRM. In the multivariate model, infused cell dose (RR = 2.347; 95% CI = 1.195-4.610; $P = .013$) (Figure 2B) and cytogenetic response at the time of BMT (RR = 9.055; 95% CI = 2.151-38.127; $P = .003$) (Figure 2D) were independent risk factors for TRM (Table 3).

Effects of HLA Compatibility

The influence of HLA compatibility between recipient and donor on aGVHD, TRM, and OS was assessed by univariate analysis. aGVHD (grade II-IV)

Table 3. Risk Factors for TRM and OS on Multivariate Analysis

Covariates	RR (95% CI)	*P* value
TRM		
Infused cell dose		
≥ 314 × 10⁶/kg	(1)	
< 314 × 10⁶/kg	2.347 (1.195-4.610)	.013
Cytogenetic response at BMT		
With MCyR	(1)	
Without MCyR	9.055 (2.151-38.127)	.003
OS		
Infused total nucleated cell dose		
≥ 314 × 10⁶/kg	(1)	
< 314 × 10⁶/kg	2.426 (1.326-4.441)	.004
Disease phase at BMT		
CP1	(1)	
Advanced phase	2.427 (1.368-4.305)	.002
Cytogenetic response at BMT		
With MCyR	(1)	
Without MCyR	6.547 (1.982-21.629)	.002

BMT indicates bone marrow transplantation; MCyR, major cytogenetic response; OS, overall survival; TRM, treatment-related mortality.

was less frequent in patients with fully matched donors than in those with mismatched donors (RR = 2.044; 95% CI = 1.055-3.961; *P* = .034). TRM (RR = 1.902; 95% CI = 0.894-4.045; *P* = .095) and OS (RR = 1.572; 95% CI = 0.817-3.027; *P* = .176) tended to be worse in mismatched transplantation, but the difference was not statistically significant. In the analysis of each single allele mismatch, only the HLA-A allele mismatch significantly affected OS (RR = 2.837; 95% CI = 1.347-5.977; *P* = .006). HLA-C mismatch marginally affected OS (RR = 1.639; 95% CI = 0.945-2.843; *P* = .078), whereas HLA-B, -DRB1, and -DQB1 mismatch were not significant. On multivariate analysis, HLA compatibility was not identified as an independent risk factor for acute GVHD, TRM, or OS.

Table 4. Causes of Death

	CP1 (n = 88)	Advanced Phase (n = 37)	Total (n = 125)
TRM	26	20	46
Infections			
Bacterial	4	1	5
Fungal	1	0	1
Viral	3	1	4
Pneumocystis jirovecii	1	0	1
Unknown	0	1	1
Rejection	0	1	1
Acute GVHD	5	4	9
Chronic GVHD	4	1	5
Idiopathic interstitial pneumonitis	6	4	10
Cardiac failure	0	1	1
Respiratory failure	0	1	1
Renal failure	1	1	2
Hemorrhage	0	2	2
Secondary malignancy	1	0	1
Unknown	0	2	2
Relapse	1	5	6

CP indicates chronic phase; GVHD, graft-versus-host disease; TRM, treatment-related mortality.

Effect of Cytogenetic Response at Transplantation

Cytogenetic response data were available in 68 of 88 patients (77%) who underwent transplantation in CP1. Sixteen patients received imatinib, 35 received IFN-α, and 3 received neither imatinib nor IFN-α. MCyR at the time of BMT was achieved in 15 of the 16 patients (94%) treated with imatinib and in 14 of the 35 patients (40%) treated with IFN-α.

Patients with MCyR at the time of BMT (n = 29) had significantly better OS and LFS than those without MCyR (n = 39): 5-year OS = 91.4%, 95% CI = 85.4%-97.4% versus 53.4% and 45.3%-61.5% (*P* = .001); 5-year LFS = 81.0 %, 95% CI = 73.2%-88.8% versus 50.9% and 42.8%-59.0% (*P* = .02) (Figure 2C). Although no significant difference in relapse rate was seen between the 2 patient groups (*P* = .91), TRM was significantly lower in those who achieved MCyR at the time of BMT (n = 29) than in those who did not (n = 39): 5-year TRM = 9.6 %, 95% CI = 3.0%-16.2% vs 41.0% and 32.7%-49.3% (*P* = .005) (Figure 2D).

Effect of Pre-BMT Imatinib Therapy

In this cohort, 17 patients received imatinib before transplantation, and 15 of them (88.2%) achieved MCyR in CP1 before transplantation. This percentage was significantly higher than that in the patients who did not receive imatinib (88.2% vs 22.2%; *P* < .01). A history of imatinib therapy had a positive effect on survival (5-year OS = 81.9 %, 95% CI = 72.4%-91.4% vs 56.4% and 51.6%-61.2%; *P* = .086), but this effect was not statistically significant.

DISCUSSION

Because of the small number of patients, to date only a few studies have addressed the outcome of children with Ph+ CML undergoing BMT with a VUD [3-5]. The number of patients in the present study is comparable to that of the largest previous study, which included 132 children with CML undergoing BMT from a VUD [4]. Furthermore, unlike that previous study, our data set contains detailed information on infused total nucleated cell dose, high-resolution HLA compatibility, and cytogenetic response at the time of BMT. Until now, these variables have not been evaluated in a pediatric CML population.

In clinical settings [13-15], as well as in animal models [16,17], larger cell dose is recognized as an important predictor of a favorable outcome for allogeneic BMT. When an adult patient with CML receives a transplant from a VUD, a lower infused total nucleated cell dose is associated with an increased incidence of TRM [18]. Our findings also demonstrate an association between lower infused total nucleated cell dose

and lower OS and LFS and a higher incidence of TRM. These correlations are independent of recipients' age. Moreover, all 6 patients who experienced graft failure were in the lower infused total nucleated cell dose group. Based on our findings, we recommend BM harvest teams attempt to collect a higher number of nucleated cells for infusion in CML patients undergoing BMT from a VUD.

Cytogenetic response to previous treatment with IFN-α [19] and imatinib [20] has been reported to be predictive for survival after allogeneic SCT in Ph+ CML. In the multivariate model of our entire cohort, MCyR at the time of BMT was an independent predictive factor for transplantation outcome. Furthermore, subgroup analysis of the patients in CP1 confirmed that the lower TRM rate in patients with MCyR at the time of BMT contributed to a better survival rate (Figure 2C), suggesting that MCyR is important for better transplantation outcome in CP1 CML as well. Recently, the Center for International Blood and Bone Marrow Transplant Research reported a significantly lower TRM and a better OS in imatinib-treated patients undergoing allogeneic SCT [21]. In our cohort, the imatinib-treated patients tended to have a higher OS ($P = .086$), but the difference was not statistically significant; however, our imatinib-treated group was small (17 of 125 patients), which may have reduced the statistical power.

We have now multiple treatment modalities for pediatric CML, including allogeneic SCT, imatinib, and, more recently, second-generation tyrosine kinase inhibitors. Although only few small studies have analyzed the data on pediatric imatinib monotherapy [22,23], those studies have reported comparable results to adult large clinical trials [24-26]. Growth disturbance as a side effect of imatinib in a pediatric CML patient was reported recently [27]; this effect could be a serious drawback to long-term imatinib therapy in the future. Of course, allogeneic SCT also has potential long-term sequelae, including growth retardation. We are currently planning a study comparing the long-term outcomes and complications of therapy with tyrosine kinase inhibitors and allogeneic SCT in the imatinib era.

In summary, disease phase, infused total nucleated cell dose, and cytogenetic response at the time of BMT were found to be independent risk factors for OS, LFS, and TRM in BMT from a VUD for the treatment of pediatric CML. These results provide important information for evaluating indications and improving outcome in children with CML undergoing unrelated BMT.

ACKNOWLEDGMENTS

Financial disclosure: The authors have nothing to disclose.

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Mutations of an E3 ubiquitin ligase *c-Cbl* but not *TET2* mutations are pathogenic in juvenile myelomonocytic leukemia

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Juvenile myelomonocytic leukemia (JMML) is a rare pediatric myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. When we investigated the presence of recurrent molecular lesions in a cohort of 49 children with JMML, neurofibromatosis phenotype (and thereby *NF1* mutation) was present in 2 patients (4%), whereas previously described *PTPN11*, *NRAS*, and *KRAS* mutations were found in 53%, 4%, and 2% of cases, respectively. Consequently, a significant proportion of JMML patients without identifiable pathogenesis prompted our search for other molecular defects. When we applied single nucleotide polymorphism arrays to JMML patients, somatic uniparental disomy 11q was detected in 4 of 49 patients; all of these cases harbored RING finger domain *c-Cbl* mutations. In total, *c-Cbl* mutations were detected in 5 (10%) of 49 patients. No mutations were identified in *Cbl-b* and *TET2*. *c-Cbl* and *RAS* pathway mutations were mutually exclusive. Comparison of clinical phenotypes showed earlier presentation and lower hemoglobin F levels in patients with *c-Cbl* mutations. Our results indicate that mutations in *c-Cbl* may represent key molecular lesions in JMML patients without *RAS/PTPN11* lesions, suggesting analogous pathogenesis to those observed in chronic myelomonocytic leukemia (CMML) patients. (Blood. 2010;115:1969-1975)

Introduction

Juvenile myelomonocytic leukemia (JMML) is a special subtype of myelodysplastic syndrome/myeloproliferative disorder (MDS/MPD) that, analogous to chronic myelomonocytic leukemia (CMML), is characterized by excessive proliferation of myelomonocytic cells, but unlike CMML it occurs in young children and shows characteristic hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF).[1-3] Mutations of genes involved in GM-CSF signal transduction, including *RAS* and *PTPN11*, can be identified in a majority of children with JMML.[3-5] Constitutional mutations of *NF1* can be found in another 10% of patients with JMML.[1,6,7] Recent studies show that a common mechanism of *NF1* inactivation is uniparental disomy (UPD) resulting in duplication of the mutant *NF1* allele.[7,8] *NF1* is a GTPase activating protein for RAS and thereby acts as a tumor suppressor.[9] Oncogenic *RAS* mutations at codons 12, 13, and 61 have been identified in approximately 20% to 25% of patients with JMML.[4,10] These mutations lead to elevated levels of RAS-GTP, the active form of RAS.[11] Somatic mutations in *PTPN11*, coding for tyrosine phosphatase Src homology 2 domain-containing protein, have been reported in 35% of patients with JMML,[5,12,13] and induce hematopoietic progenitor hypersensitivity to GM-CSF due to hyperactivation of the RAS signaling axis.[14,15]

Based on the proposed paradigm that recurrent areas of somatic copy-neutral loss of heterozygosity can point toward the presence of homozygous mutations contained within the corresponding region,[16] we have identified various recurrent areas of acquired segmental UPD, in particular in patients with MDS/MPD, including CMML. Such analyses have shown that, in addition to the recently identified *Jak2V617F* mutation associated with UPD9p, other known mutations can be duplicated by homologous recombination, including, for example, *c-Mpl* (UPD1p), *FLT-3* ITD (UPD13q), *TET2* (UPD4q), and others.[17-22] Based on the observation of recurrent somatic UPD11q23.3, we have discovered homozygous *c-Cbl* mutations in the RING finger domain (RFD) occurring frequently in MDS/MPD and especially CMML or secondary acute myeloid leukemia (AML) evolved from CMML.[23] When we analyzed other members of *Cbl* gene family, mutations were also found in *Cbl-b* and *Cbl-c* and were associated with an indistinguishable clinical phenotype.[24] The *Cbl* gene family codes for E3 ubiquitin ligases (ULs) with the ubiquitination activity mediated via the RFD. They are involved in degradation of activated phosphotyrosine receptors and other phosphotyrosine kinases such as ζ-chain–associated protein kinase 70 involved in signal transduction.[25] Thus, mutations in the RFD can lead to decreased receptor degradation and, analogous to *PTPN11* mutations, result in augmentation of proliferative signals mediated by various growth factor receptors. In a $c\text{-}Cbl^{-/-}$ mouse model a mild myeloproliferative phenotype with expansion of stem cells and hyperresponsiveness to growth factors is found,[26] whereas a RFD mutant knock-in model shows a severe myeloproliferative phenotype (W. Langdon, University of Western Australia, oral communication, January 2009). These observations, together with the transforming effects of the

Submitted June 8, 2009; accepted November 14, 2009. Prepublished online as *Blood* First Edition paper, December 11, 2009; DOI 10.1182/blood-2009-06-226340.

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The online version of this article contains a data supplement.

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v-Cbl oncogene lacking the RFD, suggest that E3 UL activity is essential for the tumor suppressor function of *c-Cbl,* whereas the N-terminal portion of the protein may be oncogenic.

Based on the morphologic similarities of JMML and typical CMML, presence of growth factor hypersensitivity, and observation of UPD11q in children affected by JMML, we hypothesized that *Cbl* family mutations may also be present in a subset of patients with JMML. Here, we investigated 49 JMML patients with the goals of (1) identifying pathogenic molecular lesions, including mutations in *Cbl* gene family members, and (2) correlating clinical outcomes to presence and location of other pathogenic molecular lesions, including *PTPN11, NRAS, KRAS,* and *TET2*. Of note is that during review of our paper, *c-Cbl* mutations were reported in JMML.[27]

Methods

Patients

We studied 49 children (32 boys and 16 girls; 1 patient's sex was unknown) with JMML diagnosed between 1988 and 2008 in 28 institutions throughout Japan. Written informed consent for sample collection was obtained at appropriate institutions from patients' parents according to the institutional protocols and the Declaration of Helsinki. The sample repository was located at Nagoya University Graduate School of Medicine. Molecular analysis of the mutational status was approved by the Ethics Committee of Nagoya University Graduate School of Medicine. The diagnosis of JMML was based on the internationally accepted criteria previously published.[26] We excluded patients with Noonan syndrome. The clinical and hematologic characteristics of the patients are summarized in Table 1. The median age at diagnosis was 28 months (range, 1-75 months). Karyotypic abnormalities were detected in 11 patients, including 7 patients with monosomy 7. Two children had clinical evidence of *NF1*. Of 49 patients, 32 underwent hematopoietic stem cell transplantation.

Table 1. Characteristics of JMML patient cohort

Variable	Total cohort, N = 49
Median age at diagnosis, mo (range)	32 (1-75)
Sex, male/female/unknown	32/16/1
NF1 by clinical diagnosis, yes/no	2/47
Median Hb, g/L (range)	0.96 (0.49-1.20)
Median HbF, % (range)	23.6 (1.0-62.0)
Median WBC, $\times 10^9$/L (range)	28.0 (10.9-126.2)
Median monocyte in PB count, $\times 10^9$/L (range)	4.5 (1.0-31.6)
Median plt, $\times 10^9$/L (range)	49 (1.4-320)
Metaphase cytogenetics, no. of patients (%)	
Normal karyotype	35 (71.4)
Monosomy 7	8 (16.3)
Trisomy 8	1 (2.0)
Other abnormalities	3 (6.1)
Unknown	2 (4.1)
Hematopoietic stem cell transplantation	
Yes	32
No	13
Unknown	4
Status at last follow-up	
Alive	24
Dead	21
Unknown	4
Median observation period, mo (range)	14 (1-216)

NF1 indicates neurofibromatosis type1; Hb, hemoglobin; WBC, white blood cell; PB, peripheral blood; and plt, platelet.

SNP-A karyotyping analysis

Mononuclear cells were isolated using Ficoll-Hypaque density gradient centrifugation and cryopreserved until use. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN). High-density Affymetrix single nucleotide polymorphism array (SNP-A; 250 K) was applied as a karyotyping platform to identify loss of heterozygosity (LOH), microamplification, and microdeletion as previously described.[28]

Bioinformatic analysis

Signal intensity was analyzed and SNP calls were determined using Gene Chip Genotyping Analysis Software Version 4.0 (GTYPE). Copy number and areas of UPD were investigated using a Hidden Markov Model and CN Analyzer for Affymetrix GeneChip Mapping 250-K arrays (CNAG Version 3.0) as previously described.[28]

We excluded germline-encoded copy number variation and nonclonal areas of gene copy number–neutral LOH from further analysis using a bioanalytic algorithm based on lesions identified by SNP-A in an internal control series (N = 713) and reported in the Database of Genomic Variants (http://projects.tcag.ca/variation).[29] Through calculation of their average sizes, we defined a maximal size of germline LOH in controls and consequently excluded all defects of this type in patients' samples; according to 95% confidence interval, stretches of UPD larger than 25.8 Mb were considered unlikely of germline origin. In addition, all nonclonal areas of UPD seen in controls were interstitial.

PTPN11, NRAS, KRAS, TET2, and *E3* ubiquitin ligase mutational screening

To screen for *PTPN11* mutations, we polymerase chain reaction amplified genomic DNA corresponding to exons 2, 3, 4, 7, 8, 12, and 13 as previously reported.[12,30,31] *NRAS* and *KRAS* mutations in codons 12, 13, and 61 were identified as previously described[32,33] and were confirmed by sequencing. To screen patients for mutations in E3 ubiquitin ligase genes and *TET2,* direct genomic sequencing of exons constituting the RFD of *Cbl* family members (exons 8 and 9 of *c-Cbl,* exons 9 and 10 of *Cbl-b,* exons 7 and 8 of *Cbl-c,* and exons 3-11 of *TET2*) was performed. For sequencing, 250 ng of polymerase chain reaction product, 3μM original forward or reverse primer, 2 μL of Big Dye Version 3.1 (Applied Biosystems), and 14.5 μL of deionized H_2O were amplified under the following conditions: 95°C (2 minutes) followed by 25 cycles of 95°C (10 seconds), 50°C (5 seconds), and 60°C (4 minutes). Sequencing was performed as previously described.[22]

GM-CSF hypersensitivity assay

GM-CSF hypersensitivity assays were established as described previously.[2] Briefly, we used cytokine-free methocult H4230 (StemCell Technologies), and added 1×10^3 $CD34^+$ bone marrow cells that were prepared by positive selection with magnetic-activated cell sorting beads (Miltenyi Biotec). Recombinant human GM-CSF (R&D Systems) was added at the time the cultures were initiated. Cultures were performed in duplicate, and colonies of 40 or more cells were scored after 14 days of incubation. The data are expressed as percentage of maximal numbers of granulocyte-macrophage colony-forming units (CFU-GMs). This approach more accurately reflects changes in sensitivity and does not bias the results compared with graphing actual counts because most JMML samples had considerably higher total numbers of CFU-GMs than controls, although there was considerable patient-to-patient variability.

Statistical analysis

When appropriate, Kaplan-Meier statistics were applied to assess survival. For comparison of the frequency of mutation or other clinical features between disease groups, categoric variables were analyzed using the Fisher exact test and continuous variables were tested using the Mann-Whitney *U* test.