Mutations of the *GATA2* and *CEBPA* genes in paediatric acute myeloid leukaemia

Hereditary *GATA2* mutations show predisposition to acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS) (Hahn *et al*, 2011). These mutations have also been reported in chronic myeloid leukaemia (Zhang *et al*, 2008) and monocytopenia and mycobacterial infection (MonoMAC) syndrome (Hsu *et al*, 2011). More recently, *GATA2* mutations have been identified in *de novo* AML, especially in adult patients with biallelic *CEBPA* mutations (Greif *et al*, 2012; Green *et al*, 2013). GATA2 and CEBPA are transcription factors that are crucial for haematopoietic development. These findings prompted us to identify possible *GATA2* and *CEBPA* mutations in patients with various paediatric leukaemias.

Direct Sequencing of *GATA2* was performed in 157 *de novo* AML patients, including 13 patients with acute promyelocytic leukaemia (APL; French–American–British type-M3) and 10 with Down syndrome (DS; Table S1), 22 secondary AML patients, 40 juvenile myelomonocytic leukaemia (JMML) patients, 50 acute lymphoblastic leukaemia (ALL) patients, 70 cell lines (25 B-cell precursor-ALL, 15 T-cell-ALL, 22 AML, and 8 neuroblastomas), and 60 healthy subjects. *GATA2* mutation analysis was performed by direct sequencing for all coding exons (exons 2–6) using an ABI PRISM 3130 Genetic Analyser (Applied Biosystems, Branchburg, NJ, USA) (Table S2). For AML patients, *CEBPA* and *NPM1* mutations were also examined. Mutational analyses of *FLT3*, *KIT*, *WT1* and *RAS* genes in our AML patients was performed as described previously (Shimada *et al*, 2006). Informed consent was obtained from the patients or the patients' parents according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review boards of Gunma Children's Medical Centre approved this project.

GATA2 mutations were found in eight out of 157 AML patients (5·1%), including three APL patients (Fig 1A,B), but were absent in 18 patients with acute megakaryocytic leukaemia (FAB-M7; Table S3). Furthermore, there were no *GATA2* mutations in patients with other leukaemias, in the cell lines, or in the 60 healthy subjects, suggesting that *GATA2* mutations were indeed associated with leukaemogenesis in a subset of patients with *de novo* AML.

Germline *GATA2* mutations were also examined in five AML patients whose complete remission (CR) samples were available, and a germline mutation was identified in one patient. Furthermore, we performed *GATA2* mutation analyses of the patient's parents and two siblings, and identified the same *GATA2* mutations in her father (II-4) and brother (III-1) but not in her mother (II-5) or sister (III-2) (Fig 1C). Her father and brother lacked abnormalities in their full blood cell counts, lymphocyte subsets, or episodes of opportunistic infections. The proband experienced severe mycotic pneumonia during induction chemotherapy. Remarkably, she has been in CR for more than 11 years, despite discontinuation of chemotherapy. Three patients, for whom CR samples were not available, had no history of MonoMAC syndrome.

In addition, 16 *CEBPA* mutations (10·2%) and three *NPM1* mutations (1·9%) were found in 157 paediatric AML patients. Thirteen (81·3%) of 16 patients with *CEBPA* mutations had been in CR for more than 4 years, suggesting that *CEBPA* mutations may be associated with favourable outcomes. Although most *GATA2* mutations were found in patients with biallelic *CEBPA* mutations in adult AML (Greif *et al*, 2012; Green *et al*, 2013), only two of eight *GATA2* mutation-positive patients had monoallelic *CEBPA* mutations in this study (Table I).

We compared the clinical and molecular features between patients with and without *GATA2* mutations. However, there were no significant differences in terms of age, initial white blood cell count, gender, and cytogenetics (Table S3). Of the eight patients with *GATA2* mutations, one had a *WT1* mutation, one had a *KIT* mutation, and two patients had *RAS* mutations (Table I). *FLT3*-internal tandem duplication, *MLL*-partial tandem duplication, and *NPM1* mutations were not found in any patients with *GATA2* mutations (Table S3). All of the *GATA2* mutations were found in the intermediate risk subgroup or APL patients with t(15;17), whereas none were found in those with core-binding factor AML [i.e. t(8;21) and inv(16)]. *GATA2* mutations were found in two patients with 11q23 translocations, including t(11;19) and t(7;11), and three patients with complex chromosomal abnormalities, whereas most *GATA2* mutations were found in cytogenetically normal AML patients in previous reports (Table I) (Greif *et al*, 2012; Luesink *et al*, 2012).

GATA2 mutations were previously reported in patients with M1, M2, and M4 subtypes of AML (Greif *et al*, 2012; Luesink *et al*, 2012), which is in accordance with our results. *GATA2* mutations have not been previously reported in APL, but our study found these mutations in three APL patients. Of note, promyelocytic leukaemia protein has been shown to interact with GATA2 and potentiate its transactivation capacity (Tsuzuki *et al*, 2000).

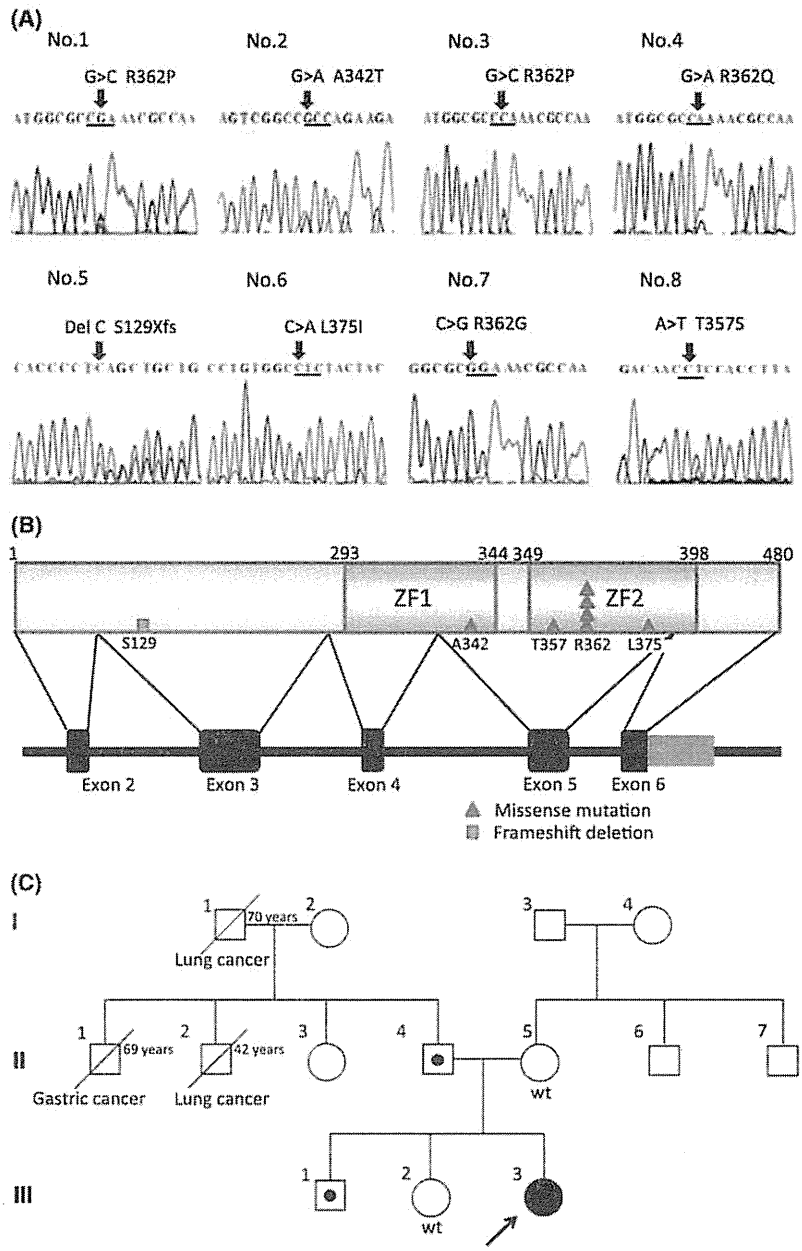

Fig 1. Identification of *GATA2* mutations by direct sequencing. (A) Eight *GATA2* mutations were identified in 157 Japanese paediatric *de novo* acute myeloid leukaemia (AML) patients (5·1%). Major missense mutations were R362 (R362P, R362Q, and R362G). Small vertical arrows indicate the mutated nucleotides. (B) Of the eight mutations, six mutations were identified in the ZF2 domain, one mutation was identified in the ZF1, and a mutation was identified on the outside of the ZF domain. (C) The family pedigree is shown. Squares indicate males and circles indicate females. The proband (III-3) is indicated by an arrow. The proband, her father (II-4), and her brother (III-1) harboured *GATA2* mutations (shown by squares containing dots). Her uncles and grandfather died of lung cancer (I-1 and II-2) and gastric cancer (II-1). wt, wild-type.

The outcomes of our patients with *GATA2* mutations was not poor (3-year overall survival and event free survival: 87·5%), which is in agreement with previous reports on *de novo* AML (Greif *et al*, 2012; Luesink *et al*, 2012): two of eight patients received autologous-stem cell transplantation (Auto-SCT), and one died of gastrointestinal haemorrhage after Auto-SCT. The remaining six patients who did not receive Auto-SCT were still alive (Table I).

In this study, one patient with a germline *GATA2* mutation developed AML. Her paternal grandfather (I-1) and second uncle (II-2) died of lung cancer at the age of 70 and 42 years, respectively, while her first uncle (II-1) died of gastric cancer at 69 years of age (Fig 1C). Increased GATA2 protein expression has been associated with biochemical recurrence and distant metastatic progression in prostate cancer (Böhm *et al*, 2009), as loss of GATA2 reduced the viability of Non-small cell lung cancer cells with RAS-pathway mutations, whereas wild-type cells were unaffected (Kumar *et al*, 2012). These facts indicate that GATA2 upregulation is strongly associated with maintenance of cancer cells. The association between *GATA2* mutations and solid tumours remains to be elucidated.

Our results indicate that *GATA2* mutations are associated with a favourable outcome in paediatric AML. Therefore, less aggressive treatment strategies without SCT may be

Table I. Clinical and molecular characteristics of patients with *GATA2* mutations.

Pt	Sex	Age (years)	FAB	WBC ($\times 10^9$/l)	Chromosome	Risk	Tx	Relapse	Prognosis (months)	*GATA2* mutation	Germline	Additional mutations
1	M	3	M4	23·8	46, XY, t(11;19) (q23; p13·1)	IR	Auto	Yes	16	R362P	N/A	–
2	F	7	M0	3·7	45,XX,add(3)(p13),del(6)(q?), der(8) t(3;8)(p21;q24), −13	IR	Chemo	No	+141	A342T	Yes	*NRAS*
3	F	8	M1	1·8	46, XX	IR	Chemo	No	+56	R362P	No	*KRAS*
4	M	14	M1	440·0	46XY [2/8], 46, XY, del(6) (q15 q21), −7, −9, −10, +3mar[1/8], 46, XY, ?del(3) (p25)[1/8], 47, XY, −5, −8, −10, add(12)(q24·1), −16, −18, +6mar [1/8], 46, XY, −2, −6, −8, +3mar [1/8], 46, XY, −8, †mar [1/8], 46, Y, ?add(X)(p11·2) [1/8]	IR	Auto	No	+51	R362Q	No	*WT1*, *CEBPA*-SM
5	M	11	M3	16·1	46,XX,inv(9)(p11q13),t(15;17)(q22;q11·21)	M3	Chemo	No	+50	S129X	N/A	–
6	M	3	M3	11·6	46,XY,t(15;17)(q22;q11·21)	M3	Chemo	No	+45	L375I	No	*CEBPA*-SM
7	M	10	M3	13·6	47,XY, +8, t(15;17)(q22;q11·21)	M3	Chemo	No	+41	R362G	N/A	*KIT*
8	F	2	M4	12·7	48, XX, +6, +10, t(11; 7) (q23;q25)	IR	Chemo	No	+38	T357S	No	–

Pt, Patient; FAB, French–American–British classification; WBC, white blood cell count; Tx, Treatment; M, Male; F, Female; IR, Intermediate risk; Auto, Autologous stem cell transplantation; Chemo, Chemotherapy; N/A, not available; +, alive; SM, single mutation.

appropriate for paediatric AML patients with *GATA2* mutations, although most patients with *GATA2* mutations were classified into an intermediate risk group. Furthermore, the association between germline *GATA2* mutations and solid tumours remains to be elucidated.

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Author contributions

Y.H. designed the study; M.F., S.A., M.K., A.K., M.S., A.T., K.H. and I.T. collected patient samples and clinical data; N.S., K.O., M.-J.P., Y.M. and S.M. performed the laboratory research; N.S., M.-J.P. and Y.H. analysed and interpreted the data; N.S. performed the statistical analysis; N.S. and Y.H. wrote the manuscript; H.A. and Y.H. supervised the work; and all authors critically reviewed the manuscript and gave their final approval.

Conflicts of interest

The authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical and cytogenetically characteristics of 157 AML patients.

Table S2. PCR primers used for mutation screening.

Table S3. Clinical and molecular characteristics of *GATA2* mutation positive patients.

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BRIEF REPORT

Reduced Intensity Conditioning in Allogeneic Stem Cell Transplantation for AML With Down Syndrome

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Allogeneic hematopoietic stem cell transplantation (HSCT) has not been widely used in patients with acute myeloid leukemia (AML) and Down syndrome (DS) due to fear of transplantation-related toxicity. A retrospective analysis of the outcome of allogeneic HSCT was conducted in 15 patients with AML and DS. The five patients transplanted with the reduced intensity conditioning (4 in complete remission (CR) and 1 in non-CR) had a significantly better survival rate than 10 patients transplanted with a conventional conditioning (4 in CR and 6 in non-CR) (3-year EFS (95% confidence interval): 80.0% (20.4–96.9%) vs. 10.0% (0.6%–35.8%), $P = 0.039$). Pediatr Blood Cancer 2014;61:925–927. © 2013 Wiley Periodicals, Inc.

Key words: acute myeloid leukemia; allogeneic stem cell transplantation; Down syndrome; reduced intensity conditioning

INTRODUCTION

Patients with Down syndrome (DS) have a 10- to 20-fold increased risk of developing acute myeloid leukemia (AML), especially acute megakaryoblastic leukemia (AMKL) [1,2]. The introduction of reduced-dose chemotherapy regimens specifically designed for AML in patients with DS has improved the survival outcome [3–7]. However, approximately 15% of patients still experience induction failures or relapses of leukemia [7]. In the general population, allogeneic hematopoietic stem cell transplantation (HSCT) has been adopted as a promising treatment option for relapsed or high-risk leukemia. Nevertheless, HSCT has not been widely used in patients with DS because of the comorbidities associated with the condition and increased chemotherapy-related toxicity. Recently, reduced intensity conditioning (RIC) regimens have been extensively introduced for patients with comorbidities, such as elderly patients. Patients with DS may also be good candidates for HSCT with RIC regimens. Reports on transplant outcomes in patients with DS are very limited, with the majority of patients being transplanted with conventional conditioning regimens [8,9]. In the present study, the outcome of allogeneic HSCT was retrospectively analyzed in 15 AML patients with DS, including five patients transplanted with a RIC regimen.

PATIENTS AND METHODS

Patients

Using the Japan Society for Hematopoietic Cell Transplantation registry, 15 patients (10 males and 5 females) with DS who suffered from AML and had undergone allogeneic SCT between 1993 and 2008 were identified. The patients' characteristics, including sex, age, diagnosis, and disease status at SCT, are summarized in Table I. The patients' median age was 3 years (range, 0–14 years), and French-American-British (FAB) classifications were: M2 (n = 1), M4 (n = 1), and M7 (n = 13). Eight patients underwent transplantation in complete remission (CR) [first CR (n = 4), second CR (n = 3), and third CR (n = 1)], while seven patients underwent

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Conflict of interest: Nothing to declare.

Author Contributions: H.M. designed and performed the research, analyzed the data and wrote the manuscript. H.S., T.T., K.T., S.A., designed and performed the research. M.I., T.K., A.K., H.Y., E.A., Y.S., A.O., A.K., H.K., Y.O., K.K., performed research and treated the patients. K. Kawa, K. Kato, Y.A., K. Kudo collected patient information, analyzed data, and wrote the paper.

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TABLE I. Patient Characteristics

Patient no.	Age at SCT (years)	Diagnosis	Disease status at SCT	Days from diagnosis to SCT	Donor	Serological HLA mismatched loci	Graft	ATG	RIC/MA	Conditioning regimen	GvHD prophylaxis
1	1	M7	NCR	307	Unrelated	HLA-B, DR	CB	−	MA	Bu + TEPA	MTX
2	4	M7	NCR	188	Related	HLA-B	BM	−	MA	Bu + MEL	TAC + MTX
3	3	M7	3CR	872	Related	HLA-B	BM	−	RIC	FLU + MEL + TBI 2 Gy	TAC + MTX
4	1	M7	2CR	333	Related	HLA-A, B	BM	−	MA	Bu + CA + CY	TAC + MTX
5	1	M7	1CR	117	Related	Matched	BM	−	MA	Bu + MEL	CyA + MTX
6	0	M7	NCR	237	Related	HLA-B, DR	BM	−	MA	CA + CY + TBI 8 Gy	TAC + MTX
7	4	M7	2CR	543	Related	Matched	PBSC	−	MA	Bu + CA + CY	CyA + Steroid
8	1	M7	1CR	171	Unrelated	HLA-DR	CB	−	RIC	FLU + MEL + ETP	CyA + MTX
9	2	M7	2CR	368	Unrelated	Matched	CB	−	RIC	FLU + MEL + TBI 2 Gy	TAC + MTX
10	6	M2	NCR	378	Unrelated	Matched	CB	−	MA	CA + CY + FLU + ETP + TBI 6 Gy	TAC + MTX
11	3	M7	1CR	795	Related	HLA-B	BM	−	MA	CY + TBI 10 Gy	CyA + MTX
12	3	M7	NCR	503	Related	Matched	BM	−	MA	BU + CY	TAC
13	1	M7	1CR	263	Unrelated	Matched	CB	+	RIC	FLU + MEL	TAC + MTX
14	3	M7	NCR	236	Related	Matched	PBSC	−	MA	BU + CY + MEL + TEPA	CyA + MTX
15	14	M4	NCR	724	Unrelated	Matched	BM	+	RIC	FLU + MEL + TBI 3 Gy	TAC + MTX

SCT, stem cell transplantation; HLA, human leukocyte antigen; ATG, anti-thymocyte globulin; RIC, reduced intensity conditioning; MA, myeloablative conditioning; GvHD, graft versus host disease; AML, acute myeloid leukemia; NCR, no complete remission; 1CR, first complete remission; 2CR, second complete remission; 3CR, third complete remission; CB, cord blood stem cell; BM, bone marrow; PBSC, peripheral blood stem cell; Bu, busulfan; TEPA, tespamin; MEL, melphalan; TBI, total body irradiation; CA, cytosine arabinoside; CY, cyclophosphamide; ETP, etoposide; FLU, fludarabine; MTX, methotrexate; TAC, tacrolimus; CyA, cyclosporine A.

transplantation in non-CR (NCR). The median interval from diagnosis to transplantation was 333 days (range, 117–872 days). The ethics committee of Nagoya University Graduate School of Medicine approved this study.

Transplant Procedures

Features of SCT, including donor, graft, preparative regimen, and graft versus host disease (GvHD) prophylaxis, are shown in Table I. Three children received a graft from an HLA-matched sibling [bone marrow (BM) (n = 2) and peripheral blood stem cells (PBSC) (n = 2)], four received a graft from an HLA-matched unrelated donor [BM (n = 1) and cord blood stem cells (CB) (n = 3)], two received a graft from an HLA-mismatched unrelated donor [CB (n = 2)], and five received a graft from an HLA-mismatched family member [BM (n = 5)]. Anti-thymocyte globulin (ATG) was given to two patients. Five patients received a RIC regimen (fludarabine (FLU) + melphalan (MEL)-based regimen; patients 3, 8, 9, 13, and 15), seven received a busulfan (Bu)-based regimen (patients 1, 2, 4, 5, 7, 12, and 14), and three patients received a total body irradiation (TBI)-based regimen (6–10 Gy) (patients 6, 10, and 11). A GvHD prophylaxis regimen with methotrexate (MTX) alone was used in one patient, tacrolimus (TAC) ± MTX was used in nine patients, and cyclosporine A (CyA) ± MTX ± steroid was used in five patients.

RESULTS

Engraftment and GvHD

All 15 patients achieved neutrophil engraftment between days +10 and +34. One patient (patient 9) experienced secondary graft failure on day +33. The data for acute GvHD (aGvHD) were not available for patient 11. Grade II–IV aGvHD was observed in seven patients, with two (patients 2 and 6) classified as grade III–IV aGvHD. Chronic GvHD (cGvHD) was observed in seven patients (47%), with one being an extensive-type (patient 4).

Relapse, Transplant-Related Mortality, and Survival Outcome

Six patients relapsed, and all but one died (patients 1, 6, 7, 12, and 14). Four patients died of transplant-related complications [aGvHD (patient 2), cGvHD (patient 4), secondary graft failure (patient 9), and idiopathic pneumonia (patient 11)]. At the time of this report, six patients were still alive (patients 3, 5, 8, 10, 13, and 15). The 3-year event-free survival (EFS) and overall survival (OS) were 32.0% (95% CI, 10.9–55.7%) and 38.9% (95% CI, 15.3–62.2%), respectively. Eight patients transplanted in CR showed trend of better survival rates compared to seven patients transplanted in NCR [3-year EFS, 95% CI: 46.9% (12.0–76.3%) vs. 14.3% (0.7–46.5%), $P = 0.102$]. Although 4 of 5 patients transplanted in CR, patients transplanted with the RIC regimen (FLU + MEL-based regimen) had a significantly better survival rate than patients transplanted with the other conventional conditioning regimens [3-year EFS, 95% CI: 80.0% (20.4–96.9%) vs. 10.0% (0.6–35.8%), $P = 0.039$] (Table II).

DISCUSSION

This retrospective survey identified 15 DS children with AML who had undergone transplantation in Japan between 1993 and 2008. Six of these children survived. Previous reports of

TABLE II. Clinical Outcome of Stem Cell Transplantation in 15 Children With Acute Myeloid Leukemia and Down Syndrome

Patient no.	aGvHD	cGvHD	Relapse, graft failure	Cause of death	Outcome	Observation period, months
1	II	—	Relapse, day +35	Relapse	Dead	3
2	IV	—	—	aGvHD	Dead	4
3	I	Limited	—	—	Alive	+51
4	II	Extensive	—	cGvHD	Dead	26
5	—	Limited	—	—	Alive	+211
6	III	—	Relapse, day +55	Relapse	Dead	6
7	II	—	Relapse, day +64	Relapse	Dead	5
8	—	Limited	—	—	Alive	+22
9	—	—	Secondary graft failure, day +33	Graft failure	Dead	10
10	II	—	Relapse, day +233	—	Alive	+36
11	ND	Limited	—	IP	Dead	7
12	I	Limited	Relapse, day +56	Relapse	Dead	5
13	I	—	—	—	Alive	+28
14	II	Limited	Relapse, day +123	Relapse	Dead	7
15	—	—	—	—	Alive	+47

aGVHD, acute graft versus host disease; cGVHD, chronic graft versus host disease; ND, not determined; IP, idiopathic pneumonia.

transplantation in patients with DS have mainly involved patients with ALL [8,9]. Very recently, the retrospective study of 28 transplantations for DS-AML was reported from the Center for International Blood and Marrow Transplant Research (CIBMTR) [10]. Hence, the current study represents one of the largest DS cohorts with AML who received HSCT.

German and Austrian groups reported 11 transplanted children with DS (8 ALL, 3 AML) and showed that the main cause of death was relapsed leukemia (5/11) rather than transplant-related mortality (TRM) (2/11) [9]. In the present cohort, both relapse (6/15) and TRM (4/15) had an impact on survival. However, when five patients transplanted from HLA-mismatched family donors (patients 2, 3, 4, 6, and 11) were excluded, the majority of the remaining patients died of relapse (5/10) rather than TRM (1/10).

The present analysis showed that the RIC regimen (FLU + MEL-based) had a positive effect on survival in patients with AML and DS. Previous reports have focused mainly on full conditioning regimens including recent report from CIBMTR [8–10]. Thus, this is one of the first reports of transplantation with RIC in patients with DS. Of the five patients who received the RIC regimen, only one died of graft failure (patient 9). This patient, with graft rejection, received a relatively low dose of melphalan (40 mg/m^2) compared to the other four patients (120–180 mg/m^2). To ensure engraftment, we assume that the melphalan dose should not be reduced in the conditioning regimen for HSCT in patients with DS. Considering that even patients with advanced risk (patients 3 and 15) maintained EFS for a long time, the FLU + MEL-based regimen appears to have sufficient anti-leukemia activity for patients with AML and DS.

In conclusion, this retrospective analysis of 15 patients with AML and DS who underwent transplants in Japan demonstrated that a RIC regimen was well tolerated in patients with DS. A prospective clinical trial is required to further evaluate the present findings.

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ORIGINAL ARTICLE

Outcome of children with relapsed acute myeloid leukemia following initial therapy under the AML99 protocol

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Abstract The outcomes of children with relapsed acute myeloid leukemia (AML) are known to be poor, but remain obscure. We retrospectively analyzed 71 patients who had relapsed following first-line treatment under the AML99 protocol. We investigated the time and site of recurrence, response to re-induction therapy, and performance of hematopoietic stem cell transplantation (HSCT) in relapsed cases, and performed a multivariate analysis to identify prognostic factors. The 5-year overall-survival (OS) rate after relapse was 37 %. Of 71 patients, three died without any anti-leukemic therapy and two underwent allogeneic HSCT. The remaining 66 patients received re-induction chemotherapy, and 33 (50 %) achieved second CR (CR2). Twenty-two of 25 (88 %) late relapse patients and 11 of 41 (27 %) early relapse patients achieved CR2 ($P < 0.001$). Twenty-nine CR2 cases and 35 non-CR2 cases underwent allogeneic HSCT. The 5-year OS rate was significantly higher in patients who underwent HSCT in CR2 than those in non-CR2 (66 vs. 17 %, $P < 0.000001$). Multivariate analysis indicated that early relapse ($P < 0.05$) and the positivity of the FMS-like tyrosine kinase 3—internal tandem duplication ($P < 0.05$) were adverse prognostic factors for survival. In conclusion, the etiology of relapsed

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pediatric AML needs to be elucidated and effective chemotherapy should be administered to obtain CR2.

Keywords Acute myeloid leukemia (AML) · Relapse · Children · Hematopoietic stem cell transplantation (HSCT) · Second complete remission (CR2)

Introduction

The treatment of childhood acute myeloid leukemia (AML) in Japan has led to a complete remission (CR) rate of approximately 90 % and 5-year overall-survival rate (OS) of approximately 70 % [1, 2]. Western studies reported a 30–40 % relapse fraction among children with AML and the OS rate for these relapsing children was 24–36 % [3–9]. Relapsed AML in children appears to have some common characteristics in that the median relapse time is approximately 10 months and OS after relapse is less than 40 % in spite of various chemotherapy protocols in different countries [3–9]. Treatment strategies for recurrent AML children in Japan have been left to the discretion of each medical institution, and neither unified treatment guidelines nor nationwide clinical trials have been accomplished until now. To clarify the prognosis of relapsed pediatric AML, we retrospectively analyzed relapsed cases in the AML99 protocol of the Japanese Childhood AML Cooperative Study. In the AML99 protocol, children with newly diagnosed de novo AML were treated with continuous cytarabine-based induction therapy (Induction A, B, or C regimens, details have been described below) and stratified into three risk groups based on the initial treatment response, age, WBC, and cytogenetics [1]. Three or four courses of consolidation chemotherapy included high-dose cytarabine. Allogeneic hematopoietic stem cell transplantation (HSCT) was only indicated for intermediate-risk (IR) patients with a matched related donor and for high-risk (HR) subsets.

Although molecular data were not used for stratifying patients in AML99 protocol, we have analyzed the prognostic factors with several gene alterations such as FMS-like tyrosine kinase 3 (FLT3)—internal tandem duplication (ITD) in addition to the clinical characteristics.

Patients

A total of 240 children younger than 18 years with newly diagnosed de novo AML were registered in the AML99 protocol between January 2000 and December 2002. The Institutional Review Board approved the protocol and written informed consent was obtained from their parents or guardians. Of 209 patients who achieved CR, 73 had relapsed by December 2005. Two patients were excluded from our analysis because of insufficient data. Therefore, the total number of relapsed patients examined in the present study was 71.

Methods

Each medical institution diagnosed recurrence independently. We investigated the time and site of recurrence, response to re-induction therapy, the performance of HSCT after relapse, and cause of death in relapsed cases. Each institution chose treatment procedures for recurrent patients individually. When considering these procedures, we defined "re-induction therapy" as a single course of chemotherapy according to one of the AML99 induction regimens (Induction A, B or C; see below). Other treatment regimens and two or more courses of chemotherapy were classified as "Miscellaneous".

AML99 Induction A (ECM) regimen: etoposide (VP-16) 150 mg/m^2 × 5 days, cytosine arabinoside (Ara-C) 200 mg/m^2 × 7 days and mitoxantron (MIT) 5 mg/m^2 × 5 days.

AML99 Induction B (sqECI) regimen: VP-16 100 mg/m^2 × 3 days, [Ara-C 500 mg/m^2 + idarubicin (IDA) 8 mg/m^2] × 3 days and [VP-16 200 mg/m^2 + Ara-C 500 mg/m^2] × 3 days.

AML99 Induction C (CIEC) regimen: [Ara-C 500 mg/m^2 + IDA 8 mg/m^2] × 3 days and [VP-16 200 mg/m^2 + Ara-C 500 mg/m^2] × 3 days.

The length of first CR (CR1) was calculated as the time from CR1 to the first relapse. Early relapse was defined as relapse within 1 year of entering CR1, and late relapse was

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defined as relapse after more than 1 year in CR1. OS after relapse or HSCT was defined as the period from relapse or HSCT to death by any cause. Disease-free survival (DFS) was the time from second CR (CR2) to a second relapse or death by any cause. Surviving patients were censored on the last date when they were known to be alive at each medical institution. Statistical analysis using χ^2 tests was performed in order to assess the relationship between various clinical characteristics and CR2 by chemotherapy. We used the Kaplan–Meier method to estimate survival rates and the Cox proportion hazards model for the multivariate analysis of prognostic factors. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Japan) [10], a graphical user interface for R (The R Foundation for Statistical Computing), or more precisely, a modified version of R commander designed to add statistical functions frequently used in biostatistics.

Genetic alterations and Wilms tumor 1 mutation (WT1) mRNA expression were examined in 40 relapsing individuals from the diagnostic BM samples. Mutation analysis for ITD within the JM domain and D835 mutation (D835Mt) within the TK2 domain of FLT3 were performed as previously described [11, 12]. Mutation analysis of the KIT gene was performed with real-time polymerase chain reaction (RT-PCR) followed by direct sequencing as previously reported [13]. Mixed-lineage leukemia (MLL)—partial tandem duplication (PTD) was examined by simple first round RT-PCR with 35 cycles using the primer pair as previously described [14–16]. Exon 2 and 3 of the N-RAS and K-RAS genes were amplified by RT-PCR and directly sequenced using primer pairs as previously reported [17]. Total RNA extracted from the BM samples was reverse transcribed to cDNA Synthesis Kit (Amersham Bioscience, Tokyo, Japan). WT1 mRNA expression was measured using RT-PCR system (ABI 7700, Applied Biosystems) with primers and controls as previously reported [18].

Results

Relapse occurred in 71 cases 34 to 1,156 days (median 312 days) after achieving CR1. The median follow-up after relapse in all survivors was 9.5 (range 7.5–12.2) years. Table 1 shows the clinical characteristics of the 71 patients examined in the present study. A total of 6 patients underwent allogeneic HSCT in CR1, including 3 IR patients and 3 HR patients. In 45 out of 71 patients (63.4 %), relapse occurred within 1 year after entering CR1 (early relapse), whereas late relapse occurred in 26 patients (36.6 %).

Figure 1 shows the clinical outcomes of all cases. Of 71 patients analyzed in this study, three patients died without any anti-leukemic therapy and two patients proceeded to allogeneic HSCT skipping chemotherapy. The other 66 patients received re-induction chemotherapy and 33 children (50 %) achieved CR2. Twenty-nine cases in CR2 and a total of 35 cases in non-CR2 underwent subsequent allogeneic HSCT. One patient remained in long-term CR after missing HSCT due to a severe infection. Of the 47 cases that died, 42 were in relapse or the refractory phase. Three cases died in CR2 due to multiple organ failure caused by sepsis on day 13 of HSCT, a hemothorax 6 months after HSCT, and sudden death of an unknown cause 9 years after HSCT. Two patients died in third CR (CR3) due to graft-versus-host disease after the second HSCT.

Table 1 Clinical characteristics ($n = 71$)

Characteristics	Number	%
Age at AML diagnosis (year)		
<2	19	27
2–9	28	39
≥10	24	34
Male sex	36	51
WBC ≥100 × 10^3/μL	13	18
FAB; M7	6	9
Extramedullary infiltration	19	27
Initial induction response; M1 marrow	63	89
Risk group in the AML99 protocol		
Low	29	41
Intermediate	34	48
High	8	11
Relapse site; BM only	62	87
Relapse after allogeneic HSCT in CR1	6	9
Early relapse (CR1 <1 year)	45	63

AML acute myeloid leukemia, *WBC* white blood cell count, *FAB* French American British classification, *M1 marrow* bone marrow blast <5 % of the total nuclear bone marrow cell count, *BM* bone marrow, *HSCT* hematopoietic stem cell transplantation, *CR* complete remission, *CR1* first CR

The overall CR2 rate by heterogeneous re-induction regimens was 50 %. As described in detail in Table 2, CR2 rate of late relapse patients was significantly higher than that of early relapse patients (88 vs. 27 %, $P < 0.001$). Most of late relapse patients received ECM or sqECI regimens as re-induction chemotherapy, which were the same as the initial induction chemotherapy in the AML99 protocol. On the other hand, CR rate of various chemotherapy regimens (miscellaneous) for early relapse patients was 21 %. No patient achieved CR2 by chemotherapy for relapse after allogeneic HSCT in CR1.

As summarized in Table 3, CR2 rate and 5-year OS rate were 69 and 46 % in favorable cytogenetic risk group ($n = 13$), 39 and 31 % in intermediate-risk group,

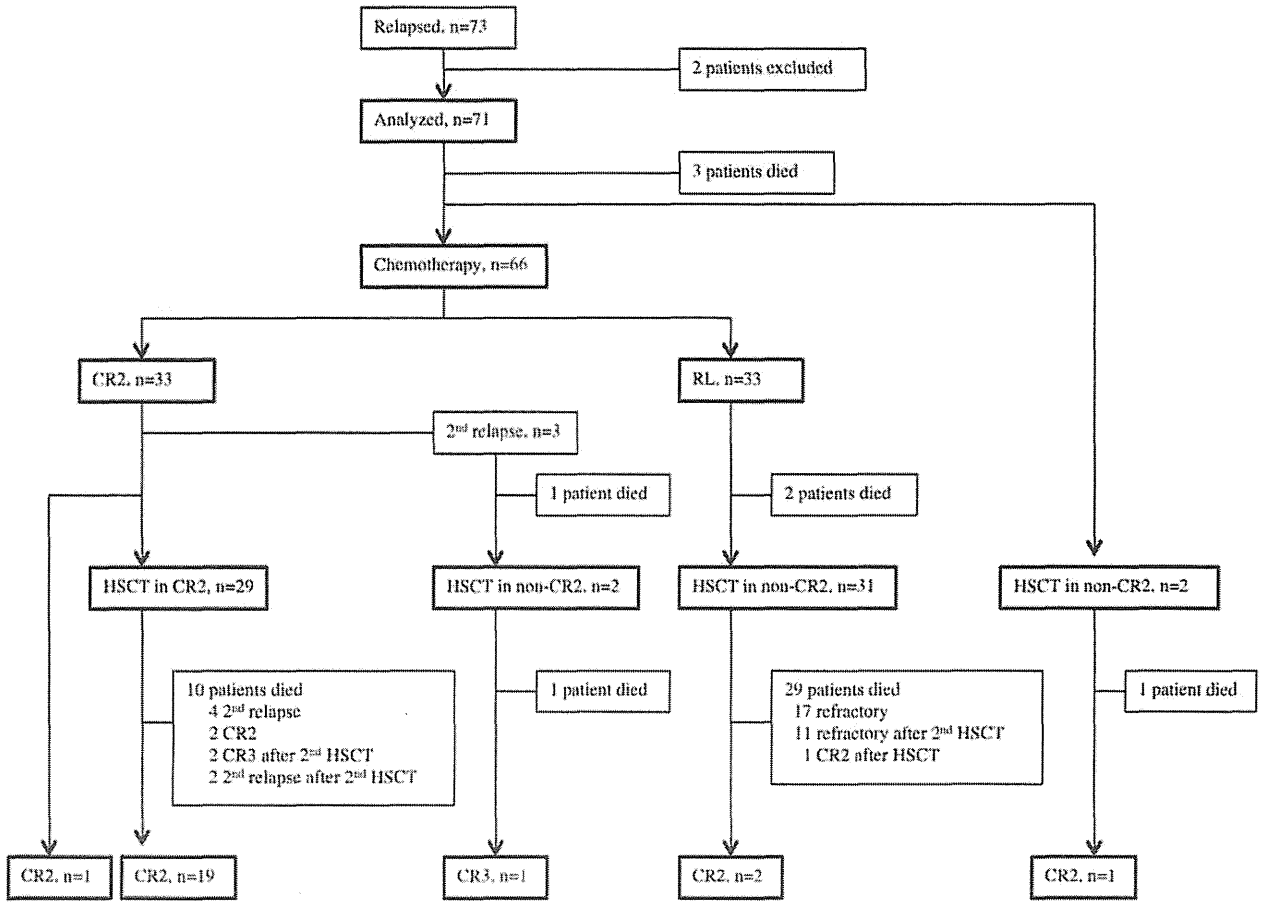

Fig. 1 Clinical outcomes of 71 relapsed cases following initial therapy with the AML99 protocol. *Thick border* indicates surviving patients and *thin border* shows deceased patients. *CR2* second complete remission, *CR3* third complete remission, *HSCT* hematopoietic stem cell transplantation

Table 2 Re-induction chemotherapy and CR2 rates ($n = 66$)

	Total		Early relapse		Late relapse	
	Number	CR2 (%)	Number	CR2 (%)	Number	CR2 (%)
ECM = Induction A of AML99	17	13 (77)	3	2 (67)	14	11 (79)
sqECI = Induction B of AML99	15	10 (67)	7	2 (29)	8	8 (100)
CICE = Induction C of AML99	7	2 (29)	7	2 (29)	–	–
Miscellaneous	27	8 (30)	24	5 (21)	3	3 (100)
All	66	33 (50)	41	11 (27)	25	22 (88)

Early relapse was defined as relapse within 1 year of entering CR1, and late relapse was defined as relapse after more than 1 year in CR1

CR2 second complete remission, *ECM* AML99 Induction A, etoposide 150 mg/m^2 × 5 days + cytosine arabinoside 200 mg/m^2 × 7 days + mitoxantron 5 mg/m^2 × 5 days, *sqECI* AML99 Induction B, etoposide 100 mg/m^2 × 3 days + [cytosine arabinoside 500 mg/m^2 - + idarubicin 8 mg/m^2] × 3 days + [etoposide 200 mg/m^2 + cytosine arabinoside 500 mg/m^2] × 3 days, *CIEC* AML99 Induction C, [cytosine arabinoside 500 mg/m^2 + idarubicin 8 mg/m^2] × 3 days, [etoposide 200 mg/m^2 + Ara-C 500 mg/m^2] × 3 days

respectively, and there were no statistically significant differences among three risk groups [19–21]. However, it should be noted that nearly half of the patients having t(8;21) or inv(16), who had been treated without allogeneic HSCT in CR1 following AML99 protocol, were rescued by allogeneic HSCT even after relapse.

The 5- and 10-year OS rates were 36.6 % (95 % CI 25.6–47.7) and 32.9 % (95 % CI 22.0–44.2) for 71 cases

(Fig. 2a). The 5-year OS rate correlated with the initial risk group: 56.8 % in the LR, 34.8 % in the IR, and 0 % in the HR groups ($P < 0.0001$, Fig. 2b), respectively. Regarding the 5-year OS rates, significant differences were observed between an age older and younger than 10 years (25.0 vs. 42.6 %, $P = 0.023$), FAB-M7 and others (16.7 vs. 38.5 %, $P = 0.025$), initial induction response: M1 and others (41.3 vs. 0 %, $P < 0.0001$), relapse after allogeneic HSCT in CR1 and others (0 vs. 40.0 %, $P < 0.00001$), and early and late relapse (22.2 vs. 61.5 %, $P < 0.0001$).

Based on genomic data in diagnostic BM samples from the AML99 study, we calculated CR2 rate and 5-year OS of 40 relapsed cases with analysis for C-KIT, N-RAS, K-RAS, FLT3-ITD, D838Mt, MLL-PTD and WT1 mutation >10,000 copies (Table 4). CR2 rate of patients with

Table 3 Cytogenetic risk group and CR2 and 5-year OS rates after relapse ($n = 71$)

Risk group	Karyotype	*n*	CR2 (%)	5-year OS (%)
Favorable		13	9 (69)*	46**
	t(8;21)(q22;q22)	12	8 (67)	42
	Inv(16)	1	1 (100)	100
Intermediate		51	20 (39)*	31**
	Normal	22	6 (27)	23
	11q23-non-adverse risk	14	8 (57)	29
	Trisomy 8	2	0 (0)	0
	Other abnormalities	13	6 (46)	54
Adverse		6	3 (50)*	50**
	t(6;11)(q27;q23)	2	1 (50)	50
	Complex	1	1 (100)	100
	t(6;9)(q23;q34)	1	0 (0)	0
	t(7;12)(q36;p13)	1	1 (100)	100
	7q-	1	0 (0)	0
No data		1	1 (100)	100
p value, among three risk groups			0.135*	0.251**

n number, *CR2* second complete remission, *OS* overall survival

There were no statistically significant differences among three risk groups in CR2 rate (*) and 5-year OS (**)

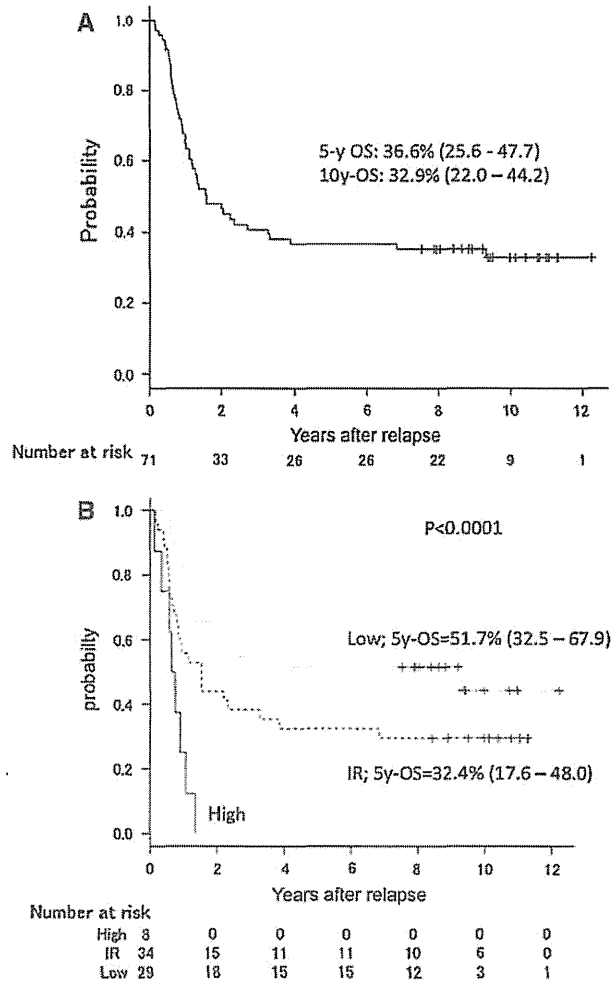

Fig. 2 a The 5- and 10-year overall-survival (OS) rates for 71 cases. b The 5-year OS rate correlated with the initial risk group in AML99 ($n = 71$): 56.8 % in the LR, 34.8 % in the IR, and 0 % in the HR groups ($P < 0.0001$)

Table 4 Molecular abnormalities related to CR2 and 5-year OS rates ($n = 40$)

Molecular abnormalities	Number	CR2 (%)	*P* value	5-year OS, % (95 % CI)	*P* value
C-KIT	7	5 (71)	0.226	42.9 (9.8–73.4)	0.466
N-RAS	2	0 (0)	0.488	0	0.303
K-RAS	5	4 (80)	0.172	40.0 (5.2–75.3)	0.67
FLT3-ITD	7	0 (0)	<0.01	0	3.15e−07
FLT3-D835Mt	1	0 (0)	0.475	0	0.978
MLL-PTD	10	4 (40)	0.721	30.0 (7.1–57.8)	0.846
WT1 >10,000	18	5 (28)	<0.05	27.8 (10.1–48.9)	0.107

CR2 second complete remission, *OS* overall survival, *CI* confidence interval, *C-KIT* proto-oncogene tyrosine-protein kinase kit (CD117), *N-RAS* neuroblastoma-rat sarcoma gene, *K-RAS* Kirsten-rat sarcoma gene, *FLT3-ITD* FMS-like tyrosine kinase 3 gene internal tandem duplication, *D835Mt* D835 mutation within the tyrosine kinase domain of the FLT3 gene, *MLL-PTD* mixed-lineage leukemia (MLL)-partial tandem duplication, *WT1* Wilms tumor 1 mutation

Table 5 Prognostic factors for 5-year OS rates by multivariate analyses ($n = 40$)

	Comparison	Hazard ratio	95 % CI	*P* value
Age ≥10 years	<10 years	2.35	0.74–7.47	0.149
FAB; M7	Non-M7	1.38	0.40–4.76	0.612
Cytogenetics; non-CBF	CBF	1.28	0.38–4.28	0.686
Induction response; M2 or M3	M1	1.01	0.21–4.98	0.987
Relapse after allo-HSCT in CR1	Others	1.26	0.29–5.42	0.756
Early relapse (CR1 <1 year)	Late relapse	3.06	1.09–8.60	<0.05
FLT3-ITD positive	Negative	5.88	1.27–27.15	<0.05

OS overall survival, *n* number, *CI* confidence interval, *HR* hazard ratio, *FAB* French American British classification, *CBF* core-binding factor, *M2* bone marrow blasts ≥5 and <25 % of the nuclear bone marrow cell count, *M3* bone marrow blasts ≥25 % of nuclear bone marrow cell count, *HCST* hematopoietic stem cell transplantation, *Early relapse* relapse within 1 year of first complete remission, *CR1* first complete remission, *FLT3-ITD* FMS-like tyrosine kinase 3 internal tandem duplication

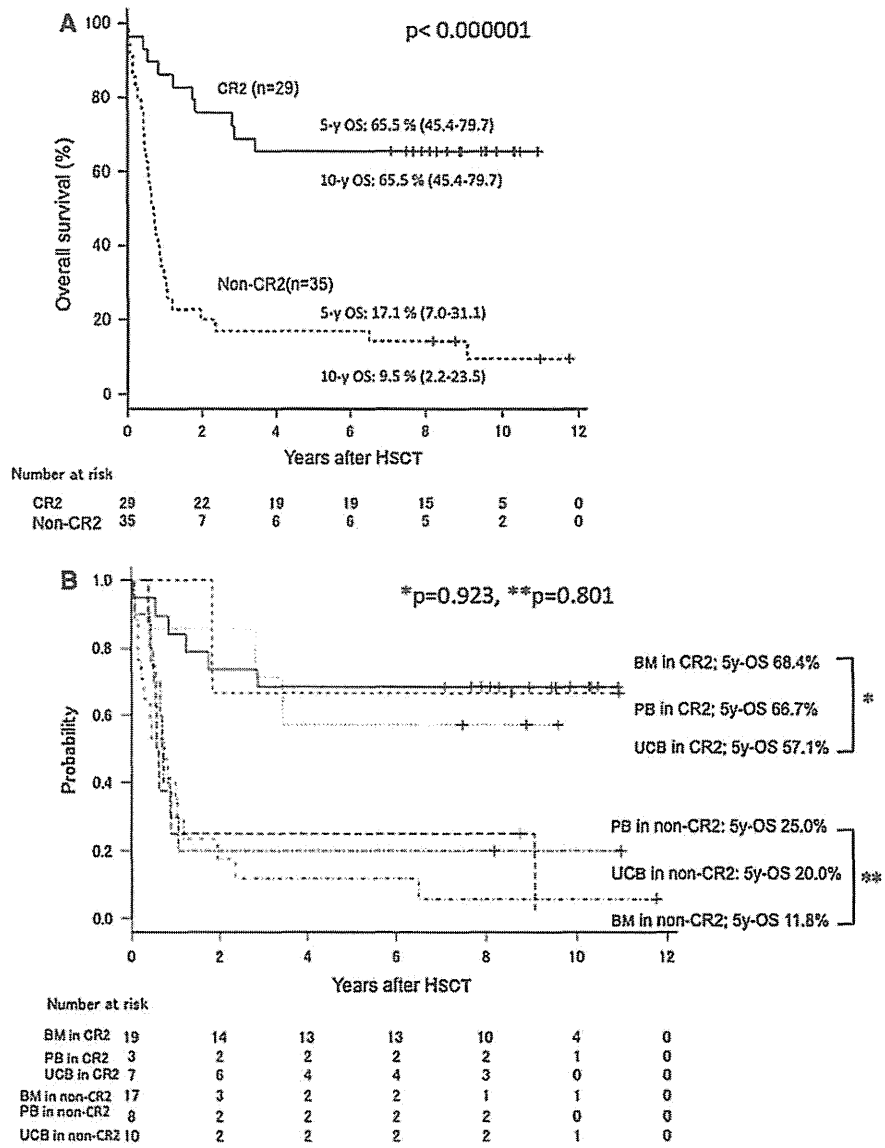

Fig. 3 **a** The 5- and 10-year OS after HSCT in patients with CR2 by re-induction chemotherapy ($n = 29$) and in those with non-CR2 ($n = 35$) (65.5 vs. 17.1 %, 65.1 vs. 9.5 %, $P < 0.000001$). **b** The 5-year OS by the stem cell source after HSCT following ($n = 64$). No significant difference by stem cell sources in CR2 patients ($P = 0.923$) and non-CR2 patients ($P = 0.801$). *HSCT* hematopoietic stem cell transplantation, *OS* overall survival, *CR2* second complete remission, *non-CR2* non-second complete remission, *CI* confidence interval, *BM* bone marrow, *PB* peripheral blood, *UCB* umbilical cord blood

Table 6 Literature review of relapsed AML in children

References First-line treatment	[3] MRC-AML10	[4] LAME89/91	[6] NOPHO88/93	[7] BFM87/98	[8] TACL	This study AML99
Number of cases	125	106	146	379	99	71
Time to relapse (months)	10	10	11	12	10	11
Early relapse (%)	65	62	55	51	61	63
Relapse site; BM only (%)	82	79	87	79	78	87
Risk group; good/low (%)	34	N.D.	N.D.	N.D.	18	29
Second therapeutic attempt (%)	70	91	84	83	91	96
CR2 (%)	70	71	77	63	62	50
HSCT in CR2 (%)	51	60	62	40	N.D.	88
5-year OS rate (%)	24 (3 years)	33	34	23	29	37
Prognostic factors by multivariate analysis for OS	N.D.	aHSCT-CR1	N.D.	aHSCT-CR1	aHSCT-CR1	Early relapse
		Early relapse		Early relapse	Early relapse	FLT3-ITD
				Age ≥0 years		
				Cytogenetics		

MRC Medical Research Council, *LAME* Leucémie Aiqë Myéloïde Enfant, *NOPHO* the Nordic Society for Pediatric Hematology and Oncology, *BFM* Berlin Frankfurt Muenster study group, *TACL* a Therapeutic Advances in Childhood Leukemia, *BM* bone marrow, *CR2* second complete remission, *OS* overall survival, *aHSCT-CR1* allogeneic hematopoietic stem cell transplantation in first CR, *ND* no data, *FLT3-ITD* FMS-like tyrosine kinase 3 internal tandem duplication

FLT3-ITD and WT1 mutation >10,000 copies were significantly low ($P < 0.05$). However, only positivity for FLT3-ITD correlated with a significantly poor OS after relapse ($P = 3.15e{-}7$).

We selected age at diagnosis (older than 10 years), FAB classification (M7), cytogenetics (core-binding factor; CBF), initial induction response, relapse phase (after allogeneic HSCT), early/late relapse and FLT3-ITD for multivariate analyses for OS in order to identify prognostic factors after the relapse of AML (Table 5). Only an early relapse and positivity of FLT3-ITD were identified as adverse prognostic factors for survival (Cox regression).

The 5- and 10-year OS rates were significantly higher in patients who underwent HSCT in CR2 than those in non-CR2 (65.5 vs. 17.1 %, $P < 0.0000001$; 65.5 vs. 9.5 %, $P < 0.0000001$, Fig. 3a). The 5-year DFS rate in CR2 was 65 %. No significant difference was observed in the 5-year OS rate in CR2 and non-CR2 with regard to the stem cell source (Fig. 3b).

Discussion

This study showed that the CR2 rate by heterogeneous re-induction regimens was 50 % and 5-year OS was 37 %. Western studies reported that the CR2 rate by chemotherapy was 62–77 % and 5-year OS rate was 23–36 % in childhood AML (Table 6), respectively [3–9]. The percentage of patients that relapsed early (63 %) was consistent with previous findings, whereas the performance rate of HSCT following the first relapse was higher (CR2 cases, 88 %; all cases, 90 %), which may have contributed to the salvage of many cases. Although low-risk (LR) patients having t(8;21) or inv(16) were treated without allogeneic HSCT in CR1, half of the recurrent patients in LR could be rescued by allogeneic HSCT even after relapse (Table 6).

The CR2 rate of early relapse patients was lower than that of late relapse patients, which was in agreement with the findings of non-Japanese studies [3–9]. The combination of fludarabine, Ara-C and granulocyte-colony stimulating factor (FLAG) with or without an anthracycline has induced CR2 rates of 58–78 % [4, 6, 7, 9, 22, 23]; however, we identified only one patient that was treated with FLAG + idarubicin and achieved CR2. Furthermore, no patient in this study received gemtuzumab ozogamicin [24, 25] or clofarabin [26, 27] as re-induction therapy. As many frontline AML chemotherapy protocols include a total anthracycline dose exceeding 350 mg/m^2, effective chemotherapy without anthracyclines [28, 29] should be introduced to obtain CR2.

No relationship was observed between the cytogenetic risk classification at initial diagnosis and CR2 or 5-year OS. FLT3-ITD mutations are known to be a strong prognostic factor, independent of other factors including genetic

chromosomal abnormalities [30–34]. However, FLT3-ITD was not available as a stratification factor in the AML99 protocol. We had not only molecular abnormality analysis at diagnosis of all cases, but also cytogenetic data at relapse. In recent protocols using FLT3-ITD as a high-risk factor, allogeneic HSCT in CR1 for AML patients with FLT3-ITD is recommended. On the other hand, high WT1 expression after induction chemotherapy was reported to be a poor prognostic factor [34]. Cytogenetic and molecular studies at relapse as well as diagnosis may be able to more precisely predict the prognosis of patients.

OS after HSCT in CR2 was 68 % for allogeneic BMT and 57 % for UCB transplant; therefore, the stem cell source did not appear to be relevant [35, 36]. UCB transplants for AML patients younger than 16 years have been performed in approximately 289 cases in Japan, and their 5-year OS rates in CR1 and CR2 were 67.3 and 61.4 % [37]. This source for HSCT might facilitate a timely transplant.

There were some limitations in this study. We only analyzed relapses that occurred during the first 3 years from the end of the AML99 study. Another limitation may be that each participating institution diagnosed recurrence independently without a central confirmation. In conclusion, further clinical trials with cytogenetic and molecular data are necessary in order to verify the genetic or molecular background of relapsed pediatric AML, and the swift introduction of new promising drugs [28, 29] is imperative for relapsed AML children to obtain CR [38].

Most of the results of the present study were presented at the 48th Annual Meeting of the Japan Pediatric Society of Hematology (November 2006, Osaka) and the 9th Childhood Leukemia Symposium (April 2014, Prague).

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Normal karyotype is a poor prognostic factor in myeloid leukemia of Down syndrome: a retrospective, international study

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ABSTRACT

Myeloid leukemia of Down syndrome has a better prognosis than sporadic pediatric acute myeloid leukemia. Most cases of myeloid leukemia of Down syndrome are characterized by additional cytogenetic changes besides the constitutional trisomy 21, but their potential prognostic impact is not known. We, therefore, conducted an international retrospective study of clinical characteristics, cytogenetics, treatment, and outcome of 451 children with myeloid leukemia of Down syndrome. All karyotypes were centrally reviewed before assigning patients to subgroups. The overall 7-year event-free survival for the entire cohort was 78% (±2%), with the overall survival rate being 79% (±2%), the cumulative incidence of relapse 12% (±2%), and the cumulative incidence of toxic death 7% (±1%). Outcome estimates showed large differences across the different cytogenetic subgroups. Based on the cumulative incidence of relapse, we could risk-stratify patients into two groups: cases with a normal karyotype (n=103) with a higher cumulative incidence of relapse (21%±4%) than cases with an aberrant karyotype (n=255) with a cumulative incidence of relapse of 9% (±2%) (P=0.004). Multivariate analyses revealed that white blood cell count ≥20x10^9/L and age >3 years were independent predictors for poor event-free survival, while normal karyotype independently predicted inferior overall survival, event-free survival, and relapse-free survival. In conclusion, this study showed large differences in outcome within patients with myeloid leukemia of Down syndrome and identified novel prognostic groups that predicted clinical outcome and hence may be used for stratification in future treatment protocols.

Introduction

Children with Down syndrome (DS) have an increased risk of developing leukemia, including acute myeloid leukemia (AML) and acute lymphoblastic leukemia.[1,2] These children develop a unique type of AML referred to as myeloid leukemia of Down Syndrome (ML-DS), which is recognized as a separate entity in the new World Health Organization classification of leukemias.[3] ML-DS is characterized by a low diagnostic white blood cell (WBC) count, myelofibrosis with a low number of leukemic blasts in the marrow,[3] mostly French-American-British (FAB) M7 morphology, young age at diagnosis (it occurs almost exclusively in children <5 years old), and superior clinical outcome when treated with reduced intensity chemotherapy protocols without stem cell transplantation.[4-10] ML-DS patients have an increased risk of side effects, hence there is a delicate balance between antileukemic efficacy and treatment-related toxicity. Drug resistance profiles showed that ML-DS blasts are particularly sensitive to various chemotherapeutic drugs *in vivo* and *in vitro*,[11,12] which enables dose reduction.

Somatic mutations in the gene encoding for the transcription factor *GATA1*, localized on the X chromosome (Xp11.2), are pathognomonic for ML-DS.[13,14] This transcription factor regulates the differentiation of megakaryocytes and erythrocytes. Mutations mainly occur in exon 2 and lead to the truncated protein GATA1s, and are unique to each patient.[15,16]

Age has been recognized as a prognostic factor in ML-DS, with an inferior outcome in the limited number of children aged over 4 years.[17] In fact, it has been proposed that DS chil-

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dren who present over 4 years of age are in fact suffering from sporadic AML occurring in a child with DS, rather than from 'true' ML-DS.[18] In addition, ML-DS patients with a history of transient myeloproliferative disease have a significantly better outcome than children with ML-DS without documented transient myeloproliferative disease.[19] Until now, no other prognostic factors have been identified in ML-DS.

The leukemic blasts from the majority of patients with ML-DS (72%) show additional cytogenetic changes apart from the constitutional trisomy 21.[20] A previous international-BFM study, performed by Forestier *et al.*, showed that the most frequent gains involved chromosomes 8 (27%), 21 (23%), 11 (8.1%), and 19 (7.4%), whereas chromosomes X (3.2%; only females), 5 (1.5%), and 7 (2.2%) were commonly monosomic. The most frequent partial imbalances were duplication 1q (16%), deletion 7p (10%), and deletion 16q (7.4%).[20] However, the potential clinical impact of these cytogenetic abnormalities is not known and has not been well studied, mainly due to the small numbers of patients in individual series.[9,10,20-22]

In current treatment protocols of non-DS pediatric AML patients, stratification is based on cytogenetics and response to therapy.[23] In ML-DS, no prognostic cytogenetic groups have yet been identified, nor any other prognostic factors allowing a risk-stratified approach.

We, therefore, conducted a large international study of clinical and outcome data including cytogenetic records from children with ML-DS collected from 13 collaborative study groups. Our aim was to identify differences in outcome related to cytogenetics and clinical characteristics in ML-DS. This was approached by analyzing differences in the cumulative incidence of relapse (CIR), reflecting leukemia resistance, and hence avoiding the influence of toxic (non-leukemic) events on survival estimates. This may result in risk-group stratification and risk-group directed therapy for these patients in the future. In addition, we compared the outcome of ML-DS patients in the different cytogenetic groups with that of non-DS AML patients from the same era treated on AML-BFM regimens as a reference cohort.

Methods

Patients

Data on 451 patients with ML-DS were collected from 13 collaborative study groups participating in the International AML-BFM Study Group. For comparison, a reference cohort of non-DS AML patients (n=548) from the same treatment era, kindly provided by the AML-BFM Study Group, was used. This study was approved by the Institutional Review Boards in accordance with local legislation and guidelines.

Patients were eligible if diagnosed between January 1, 1995 and January 1, 2005. Patients who were not treated with curative intent from diagnosis were excluded. The data collected at diagnosis comprised karyotype, sex, age, white blood cell (WBC) count, hemoglobin level, platelet count, immunophenotypic data and FAB morphology. In addition, we collected data on treatment, such as therapy protocol, including stem-cell transplantation, and all events during follow-up. Only patients between 6 months and up to 5 years of age were included in the analyses. Patients with transient myeloproliferative disease were excluded. Patients were treated in national or collaborative group AML trials.

Cytogenetic results

All karyotypes were provided after review by a national collaborative group, and centrally reviewed by two cytogeneticists (EF, BJ). Fluorescence *in situ* hybridization analyses were not performed routinely. Of the 451 cases, karyotypes were available for 358 (79%). As there was no *a priori* knowledge on the prognostic impact of the various cytogenetic groups in ML-DS, the classification of the cases was based on the premise that all groups should be mutually exclusive, i.e. each patient was included only once. Only groups that were sufficiently large (≥5 cases) were analyzed in more detail to allow meaningful statistical analyses.

The numerically largest group was formed of 103 patients (29%) with a normal karyotype (NK). Another entity that was readily delineated consisted of 49 cases with trisomy 8 (14% of all cases), either as a single abnormality (n=16), or with additional cytogenetic aberrations (n=33). Next, a group of 82 cases (23%) with losses of chromosome 5/7 material (excluding those with +21) was distinguished. Other smaller groups consisted of 28 cases (6%) with a gain of chromosome 21 (in addition to +21c); 14 cases (4%) with a duplication of chromosome 1q; and 9 cases (3%) with a deletion of chromosome 16q. Finally, a group of 73 cases (20%) remained, harboring other aberrations that could not be sub-categorized further (Figure 1 and *Online Supplementary Figure S1*).

Statistical analyses

Continuous variables were categorized according to cut-off points; age < or ≥3 years, WBC count < or ≥20x10^9 and Ara-C < or ≥20,000 mg/m^2. The χ^2 or Fisher exact test was used to compare discrete variables among groups; the Mann-Whitney U test was used for continuous variables. All *P* values are descriptive and explorative, and were considered statistically significant if ≤0.05. Statistical analyses were performed using SAS software (SAS-PC, Version 9.1).

More details on the methods are provided in the *Online Supplementary Material*.

Results

Clinical characteristics

The median age of all ML-DS patients (n= 451) was 1.8 years (range, 6 months - 5.0 years) and the median WBC count was 7.0x10^9/L (range 0.8 – 290x10^9/L). The male - female distribution was almost equal (49.9% *versus* 50.1%). Only two (0.5%) patients had central nervous system involvement. The characteristics of the entire cohort of patients are presented in detail in Table 1.

The median follow-up of survivors was 4.9 years. Forty-three percent (192 patients) received therapy reduction, or were treated with adjusted DS treatment protocols. Outcome parameters did not differ significantly between these groups. Six patients were also treated with irradiation: three patients received central nervous system irradiation, whereas the radiation target was not specified for the three other patients.

Ninety-two percent of all patients reached complete remission. The 7-year event-free and overall survival rates of all included 451 patients were 78% (± 2%) and 79% (± 2%), respectively. The 7-year CIR was 12% (± 2%), and cumulative incidence of toxic death was 7% (± 1%) (Figure 2). Of all patients with evaluable karyotypes (n=358), the complete remission rate was 92% and the 7-year event-free and overall survival rates were 77% (± 2%) and 79% (± 2%), respectively. The 7-year CIR was 13% (± 2%), and the cumulative incidence of toxic death

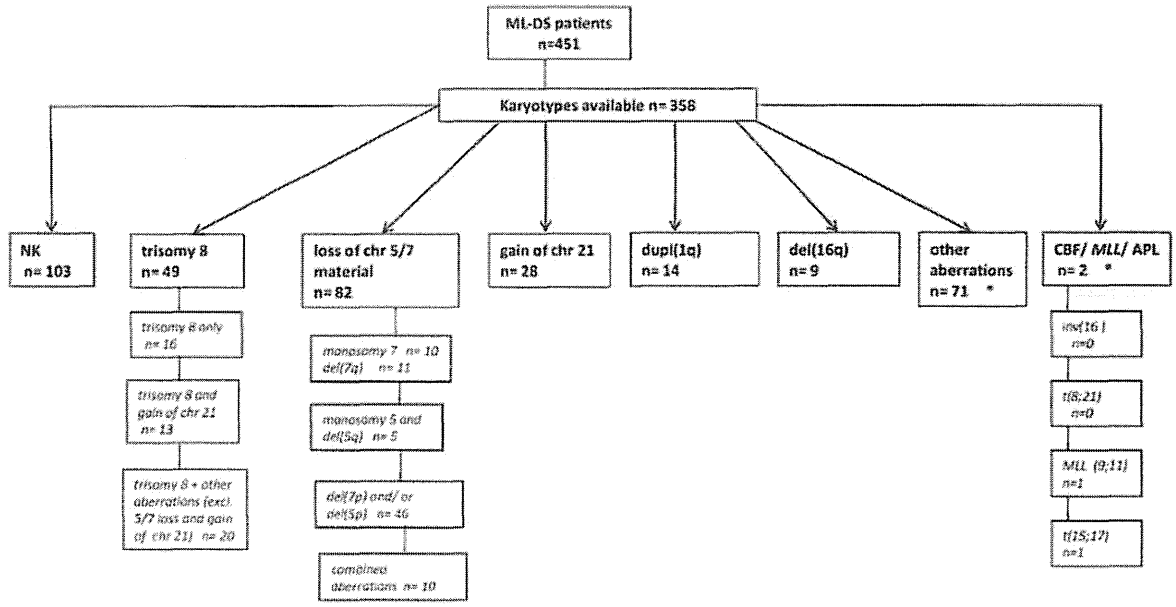

Figure 1. **Hierarchy of cytogenetic groups within ML-DS delineated in the present study.**

was 7% (±1%) (Figure 3). There were no statistically significant differences between these two groups when comparing various outcome estimates. We, therefore, conclude that there was no selection bias between the entire study population and the subgroup with informative karyotypes.

In total 25 (5.5%) patients were transplanted in first complete remission. One patient underwent autologous stem cell transplantation, three patients were transplanted with a graft from an allogeneic HLA sibling, and three patients received a matched family donor transplant; these specifications were not known for any of the other patients. Forty percent of all transplanted patients died (10/25), half of them due to the leukemia.

Outcome of cytogenetic subgroups

There were no significant differences in the frequency distribution of the various cytogenetic subgroups between the collaborative groups apart from the French cohort, which consisted of a relatively large proportion of NK ML-DS cases. This, however, did not influence the outcome estimates significantly, so there was no study group effect in the overall results.

Interestingly, outcome estimates differed largely across the different cytogenetic subgroups (Figure 4). An overview of all outcome estimates per subgroup is given in Table 2.

Based on the CIR estimates, patients could be divided into groups with a high CIR (> 20%), comprising those with NK and del(16q) (n=112), and a low CIR (< 20%), comprising all other patients (n=246). Since the former group consisted of NK cases (92%), with only nine cases with del(16q) with two events, we decided to perform further analyses comparing the NK cases (29%) with all cases

Table 1. **Clinical characteristics of the ML-DS patients.**

Clinical characteristics of the ML-DS patients	All patients	Patients with evaluable karyotypes	P
N.	451	358	
Male sex, n.(%)	225 (49.9)	183 (51.0)	0.78
Median age (years)	1.8 (0.5-5.0)	1.8 (0.5-5.0)	0.76
< 3 years (%)	399 (91.1)	317 (90.8)	
≥ 3 years (%)	39 (8.9)	32 (9.2)	
Median WBC (x10⁹/L)	7.0 (0.8-290)	7.0 (0.8-290)	1.0
< 20 x10⁹/L (%)	363 (81.8)	289 (81.9)	
≥ 20 x10⁹/L (%)	81 (18.2)	64 (18.1)	
CNS involvement, n.(%)	3 (0.7)	2 (0.6)	0.54
Hepatomegaly, n.(%)	247 (54.8)	193 (53.8)	0.9
Splenomegaly, n.(%)	180 (39.9)	147 (40.9)	0.9

WBC white blood cell count; CNS central nervous system.

with aberrant karyotypes (71%). Clinical characteristics did not differ between these two groups (Table 3). The rate of complete remission was significantly lower in NK ML-DS than in cases with an aberrant karyotype (87% *versus* 96%; $P<0.01$). The NK patients had significantly worse survival outcomes: 7-year CIR of 21% (± 4%) *versus* 9% (± 2%) ($P=0.004$), 7-year overall survival of 68% (± 5%) *versus* 84% (± 2%) ($P=0.0008$), and 7-year event-free survival of 65% (± 2%) *versus* 82% (± 5%) ($P=0.0005$). The cumulative incidence of toxic death was not significantly different between NK ML-DS and patients with aberrant karyotypes: 6% (± 2%) *versus* 7% (± 2%) ($P=0.58$) (Figure 5). Regarding the rate of complete remission, a significantly small proportion of NK-patients than patients with

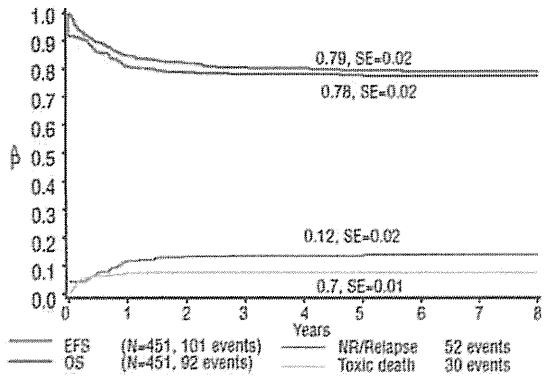

Figure 2. Survival curves of all 451 ML-DS patients included in this study. The 7-year overall survival (OS) was 79% (±2%); the 7-year event-free survival (EFS) 78% (±2%); the 7-year cumulative incidence of relapse was 12% (±2%); and the cumulative incidence of toxic death at 1.5 years from diagnosis was 7% (±1%). NR: non-remitters.

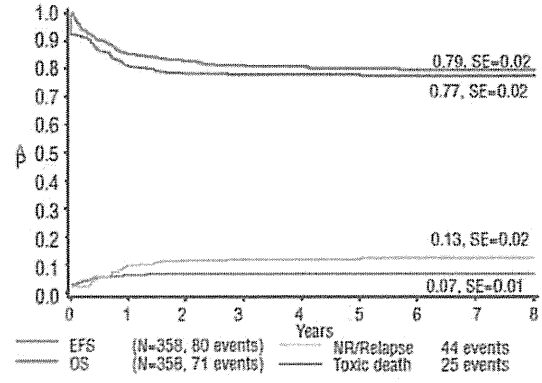

Figure 3. Survival curves of the 358 ML-DS patients with informative karyotypes. The 7-year overall survival (OS) was 79% (±2%); the 7-year event-free survival (EFS) 77% (±2%); the 7-year cumulative incidence of relapse was 13% (±2%); and the cumulative incidence of toxic death at 1.5 years from diagnosis was 7% (±1%). NR: non-remitters.

aberrant karyotypes reached complete remission (87% *versus* 96%; *P*< 0.01).

Chromosome 7 aberrations

Given the presence of a large number of cases with chromosome 7 abnormalities in our ML-DS cohort and the specific prognostic relevance of chromosome 7 abnormalities in non-DS AML, we focused on this group separately.[24] ML-DS patients with chromosome 7 aberrations did not have significantly different survival parameters compared to all other patients (*P*=0.63). This group was further subdivided into cases with a monosomy 7 (n=10) and those with a del(7q) (n=11). Patients with monosomy 7 tended to have worse survival estimates than patients with a del(7q), but this was not statistically significant: 7-year event-free survival 67% (± 14%) *versus* 81% (± 10%) (*P*=0.40), 7-year overall survival 59% (± 17%) *versus* 88% (± 8%) (*P*=0.2), and 7-year CIR 9% (± 9%) *versus* 20% (± 14%) (*P*=0.36) (*Online Supplementary Figure S2*).

Regarding the five patients with chromosome 5 aberrations, four of them were alive after at least 4 years of follow up, although one of them suffered from severe infections during treatment. One of them died 2 months after diagnosis due to sepsis in induction; this patient also had a congenital heart defect.

Other prognostic factors

Patients with high WBC counts (≥20x10^9/L tended to have a worse 7-year event-free survival rate than patients with a lower WBC count (< 20x10^9/L): 79% (± 2%) *versus* 70% (± 5%); (*P*=0.047). However, this did not translate into a significant difference in 7-year overall survival [80% (± 2%) *versus* 73% (± 5%); *P*=0.07]. This was due to the occurrence of events in the induction phase; the complete remission rate was significantly lower in patients with high WBC counts (93% *versus* 81%; *P*=0.007). The 7-year cumulative incidence of toxic death and CIR did not differ significantly: cumulative incidence of toxic death 6% (± 3%) *versus* 7% (± 1%) (*P*=0.83) and CIR 16% (± 4%) *versus* 10% (± 2%) (*P*=0.1) (*Online Supplementary Figure S3*).

In addition, after evaluating various cut-off points for age, patients aged <3 years had significantly better 7-year event-free survival and CIR than had patients aged ≥3 years [event-free survival 78% (± 2%) *versus* 65% (± 7%) (*P*=0.04) and CIR 11% (± 2%) *versus* 21% (± 6%) (*P*=0.05)] (*Online Supplementary Figure S4*). This was also due to events in induction, with a higher borderline statistically significant complete remission rate for patients aged <3 years (93% *versus* 84%; *P*=0.08). The cumulative incidence of toxic death was not significantly different between these two age groups [7% (± 1%) *versus* 5% (± 3%)(*P*=0.58)] nor was the overall survival rate [80% (± 2%) *versus* 69% (± 7%)(*P*=0.10)].

Immunophenotyping

ML-DS cases positive for the lymphoid co-expression marker CD7 (n=187/221) had a borderline better event-free survival rate [79% (± 3%) *versus* 64% (± 8%); *P*=0.054] (*Online Supplementary Figure S5*). However, no significant differences were seen for overall survival, CIR or cumulative incidence of toxic death. Expression of CD56 (neural cell adhesion molecule) (n=92/169) was not significantly associated with any of the outcome estimates (*Online Supplementary Figure S6*), whereas CD34 (expressed on early hematopoietic cells) positive cases (n=94/221) had a worse event-free survival [70% (± 5%) *versus* 82% (± 3%); *P*=0.049] and a higher CIR (16 ± 4% *versus* 7 ± 2%; *P*=0.04) than CD34-negative cases (*Online Supplementary Figure S7*).

Treatment

No significant differences in outcome estimates, CIR or cumulative incidence of toxic death were seen between groups treated with different cumulative dosages of anthracyclines and etoposide. Patients treated with higher cumulative dosages of cytarabine (≥20 g/m^2) had a significantly better 7-year event-free survival [84% (± 3%) *versus* 75% (± 3%); *P*=0.043] and a trend towards a better 7-year overall survival [85% (± 3%) *versus* 77% (± 3%); *P*=0.056] than patients treated with lower doses (<20 g/m^2) (*Online Supplementary Figure S8*). There was also a trend for lower 7-year CIR in patients treated with higher doses [7% (±