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journal or publication title	Leukemia
volume	35
number	9
page range	2508-2516
year	2021-02-15
URL	http://hdl.handle.net/10422/00012919

doi: 10.1038/s41375-021-01157-w(https://doi.org/10.1038/s41375-021-01157-w)

Leukemia
https://doi.org/10.1038/s41375-021-01157-w

ARTICLE

Acute myeloid leukemia

Post-induction MRD by FCM and *GATA1*-PCR are significant prognostic factors for myeloid leukemia of Down syndrome

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Received: 2 September 2020 / Revised: 21 December 2020 / Accepted: 25 January 2021

Abstract

Myeloid leukemia of Down syndrome (ML-DS) is associated with good response to chemotherapy, resulting in favorable outcomes. However, no universal prognostic factors have been identified to date. To clarify a subgroup with high risk of relapse, the role of minimal residual disease (MRD) was explored in the AML-D11 trial by the Japanese Pediatric Leukemia/Lymphoma Study Group. MRD was prospectively evaluated at after induction therapy and at the end of all chemotherapy, using flow cytometry (FCM-MRD) and *GATA1*-targeted deep sequencing (*GATA1*-MRD). A total of 78 patients were eligible and 76 patients were stratified to the standard risk (SR) group by morphology. In SR patients, FCM-MRD and *GATA1*-MRD after induction were positive in 5/65 and 7/59 patients, respectively. Three-year event-free survival (EFS) and overall survival (OS) rates were 93.3% and 95.0% in the FCM-MRD-negative population, and 60.0% and 80.0% in the positive population. Three-year EFS and OS rates were both 96.2% in the *GATA1*-MRD-negative population, and 57.1% and 71.4% in the positive population. Adjusted hazard ratios for associations of FCM-MRD or *GATA1*-MRD with EFS were 10.98 ($p = 0.01$) and 27.68 ($p < 0.01$), respectively. Detection of MRD by either FCM or *GATA1* after initial induction therapy represents a significant prognostic factor for predicting ML-DS relapse.

Introduction

Myeloid leukemia of Down syndrome (ML-DS) shows unique characteristics that translate into good treatment response: predominance of acute megakaryoblastic leukemia, age predilection during the first 4 years of life, and higher sensitivity to chemotherapeutic agents. ML-DS also displays increased treatment-related toxicities compared to non-DS children with acute myeloid leukemia (AML). As a result, ML-DS children have been treated separately from non-DS AML children, with the application of less intensive treatment in recent clinical studies in developed countries resulting in long-term survival rates of 80–90% [1–3].

On the other hand, relapsed and refractory cases of ML-DS are rarely salvageable, even in patients receiving hematopoietic stem cell transplantation [4]. Although previous studies have tried to identify prognostic factors predicting high-risk patients who might benefit from more intensive chemotherapy in the frontline therapy, candidate factors such as chromosomal abnormalities, age, and morphological response have been poorly reproducible [3, 5, 6]. A previous study of children with de novo AML, however, suggested that risk stratification based on minimal residual disease (MRD) could improve outcomes [7]. The AML-D11 study by the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG), a nationwide clinical trial for ML-DS in Japan, was designed to evaluate the feasibility and utility of MRD in the risk stratification of patients with ML-DS, using flow cytometry (FCM) and deep sequencing targeting *GATA1* mutation.

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Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41375-021-01157-w.

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Published online: 15 February 2021

Patients and methods

Patients

Between March 2012 and February 2015, patients with ML-DS entered the AML-D11 study after informed consent was obtained from the guardians. Eligibility criteria for this study were as follows: (1) DS patients diagnosed with myeloid leukemia irrespective of blast percentage; (2) age >4 months and <18 years at diagnosis; (3) sufficient organ function (patients with cardiac disease were eligible unless serious complications were present); and (4) no history of previous chemotherapy [except cytarabine treatment for transient abnormal myelopoiesis (TAM)] or radiation therapy. Patients with current TAM or CNS leukemia were not eligible. The diagnosis was confirmed by a central morphology review, with peripheral blood (PB) and bone marrow (BM) smears reviewed by a pediatric hematologist, and BM trephine biopsies in cases whose BM aspiration was dry tap reviewed by a hematopathologist. Treatment response was also evaluated by central review and was defined as follows: M1 marrow, <5% blasts in the BM; M2 marrow, >5% and <25% blasts; M3 marrow, >25% blasts; and complete remission (CR), M1 marrow with regeneration of normal hematopoiesis and no leukemia-related symptoms or extramedullary leukemic infiltration. This study was approved by the JPLSG steering committee and the institutional review boards of all institutions participating in this study. This trial was registered with the UMIN Clinical Trials Registry (UMIN-CTR, URL: http://www.umin.ac.jp/ctr/index.htm), number UMIN000007237.

Treatment

Treatment components in the AML-D11 study were identical to those in the prior AML-D05 study (Fig. 1 and Table S1) [3]. All patients received a single course of induction therapy comprising pirarubicin, intermediate-dose cytarabine (via 1-h intravenous infusion), and etoposide (CET), then were stratified into two risk groups according to the morphological BM response. Patients showing good response (M1 marrow after initial CET) were classified as standard risk (SR) and received less intensive chemotherapy, in which etoposide was omitted from the 2nd and 4th courses of CET. For patients displaying poor response (non-M1 marrow after initial CET) classified as high risk (HR), a salvage regimen with more intensive use of cytarabine was given by either 24-h continuous infusion or a high dose. No intrathecal chemotherapy was administered to either risk group. The study protocol included supportive care guidelines such as empirical antimicrobial therapy, anti-fungal prophylaxis, and monitoring and prophylactic supplementation of intravenous immunoglobulin.

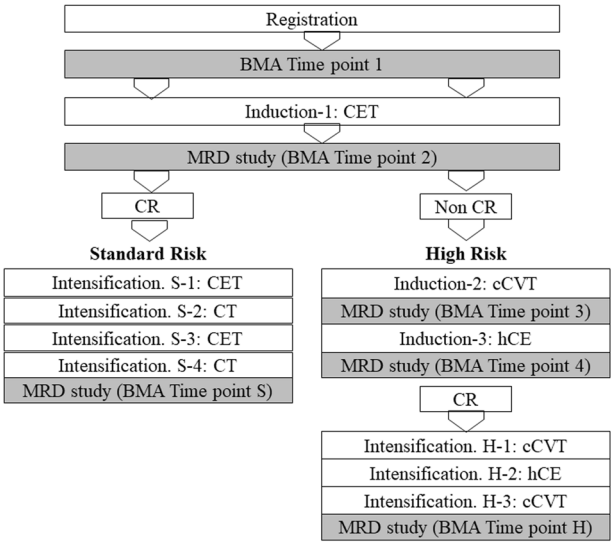

Fig. 1 Treatment outline of the AML-D11 study. Abbreviations: BMA: bone marrow aspiration, CET: Pirarubicin, etoposide, cytarabine (100 mg/m^2, 1 hr i.v.), CR: complete remission, CT: Pirarubicin, cytarabine (100 mg/m^2, 1 hr i.v.), cCVT: Pirarubicin, vincristine, cytarabine (100 mg/m^2, 24 hr continuous i.v.), hCE: etopiside, high-dose cytarabine (1 g/m^2, 2 hr i.v x2/day), MRD: minimal residual disease.

MRD study

MRD was measured by FCM (FCM-MRD) and deep sequencing for *GATA1* mutation (*GATA1*-MRD). Both FCM- and *GATA1*-MRD were evaluated at two time points for SR patients, after the induction therapy (time point 2; TP-2) and at the end of all chemotherapy (time point S; TP-S) (Fig. 1). For HR patients both FCM- and *GATA1*-MRD were evaluated at four time points, after induction therapy (TP-2), after re-induction therapies (Time points 3 and 4; TP-3 and TP-4, respectively), and at the end of all chemotherapy (Time point-H; TP-H).

FCM-MRD

FCM-MRD was performed at each time point in two reference centers. Samples were shipped from the referring institutions and processed within 24–48 h of collection using methods similar to those used in the previous report [7]. Briefly, mononuclear cells from diagnostic samples (PB samples were also allowed if BM was unavailable) were separated by centrifugation on a density step (Histopaque-1077, Sigma-Aldrich, St. Louis, MO). We incubated mononuclear cells with rabbit serum for 2 min to block Fc receptors and washed them in phosphate buffered saline containing 0.2% bovine serum albumin and 0.2% sodium azide (PBSA) and labeled with combinations of fluorochrome-conjugated monoclonal antibodies directed against surface antigens, or isotype-matched nonreactive

monoclonal antibodies. The marker antibody combinations used to study FCM-MRD were as follows: (1) anti-CD38 fluorescein isothiocyanate (FITC; Beckman Coulter), anti-CD11b phycoerythrin (PE; BD Biosciences), anti-CD34 peridinin chlorophyll protein (PerCP; BD Biosciences), anti-CD117 allophycocyanin (APC; Miltenyi Biotec), anti-CD33 phycoerythrin-Cy7 (PE-Cy7;BD Biosciences), anti-CD45 APC-H7 (BD Biosciences), anti-CD7 Brilliant Violet421 (BV421; BD Biosciences), and anti-HLA-DR BV510 (BD Biosciences); (2) anti-CD41 FITC (DAKO), anti-CD56 PE (BD Biosciences), anti-CD34 PerCP, anti-CD117 APC, anti-CD33 PE-Cy7p, anti-CD45 APC-H7, anti-CD7 BV421, and anti-CD4 Violet 500 (V500; Biolegend). After incubation for 10 min at 20 °C in the dark, we washed the cells in PBSA twice, fixed them in 0.5% paraformaldehyde, and analyzed using a three-lasers-FACSCantoII or FACSVerse flow cytometer with FACS-Diva™ software (Becton Dickinson, San Jose, CA). Side by side comparisons of the results obtained in diagnostic AML samples and in reference control samples were performed to define leukemia-associated immunophenotypes.

To monitor FCM-MRD according to leukemia-associated immunophenotypes, marker combinations that allowed identification of MRD for each patient were selected. We acquired data from all mononuclear cells in each test tube ($>1 \times 10^5$).

To determine the proportion of mononuclear cells within each sample and to distinguish them from residual erythrocytes, platelet aggregates and debris, cells in one tube were stained with SYTO-13 (50 nmol/L; Molecular Probes, Eugene, OR). FCM-MRD-positivity pre-specified as ≥0.05% and leukemic cell clustering was reviewed by two operators.

In statistical analyses, patients for whom MRD positivity was not evaluable were excluded from the calculation of proportions. Results of MRD analysis were blinded to investigators and patients.

GATA1-MRD

GATA1 mutations were analyzed by Sanger sequencing using diagnostic samples, as previously described [3, 8–10]. Targeted deep sequencing for *GATA1* was retrospectively performed using pooled BM or PB samples at diagnosis and *GATA1*-MRD was monitored using BM samples at each time point, as previously described [3]. Briefly, genomic DNA was extracted from BM and/or PB using the QIAamp DNA Blood Mini Kit (QIAGEN, Venlo, the Netherlands). Polymerase chain reaction (PCR) was performed for exons 2 and 3 of *GATA1* using primers that flanked each of the exons. Primers used for PCR were 135 sense (5′-AGGTAGAAGCAGATGAGAGTGGA-3′) and AS3 (5′-GTGGGGTGGAGAGGAGAAGAGGGA-3′). Forty ng of genomic DNA was used as a template for PCR. PCR was performed in a total volume of 50 μL consisting of 5 μL of 10x PCR buffer for KOD-Plus-Neo, 0.2 mM of dNTPs, 1.5 mM of $MgSO_4$, 0.5 μM of each primer, and 1 μL of KOD-Plus-Neo (TOYOBO, Osaka, Japan). We performed PCR in a three-step cycle. Initial denaturation at 94 °C for 2 min, followed by 26 cycles of denaturation at 98 °C for 10 s, annealing at 67 °C for 30 s, and extension at 68 °C for 60 s. PCR products were purified using QIAquick PCR purification kit (QIAGEN). Each amplicon was subsequently used for sequencing library preparation using the Nextera XT library kit (Illumina, San Diego, CA). Sequencing was done on a MiSeq sequencer with 150 bp pair end reads. Each library was indexed with a barcode and reads were automatically partitioned post sequencing. After quality trimming, reads from each sample were aligned to the human genome reference sequence (GRCh37/hg19), and the CLC Genomic Workbench 7.5 (CLC Bio, Aarhus, Denmark) was used for mapping and variant calling with the low frequency variant detection methods. To eliminate sequencing errors, we excluded all variants found in a control sample (HEL cells). The cutoff for mutation-positive status was set as 0.3% for mutation detection at diagnosis. Patient-specific target mutations detected at allele frequencies ≥0.1% for males or ≥0.05% for females in follow-up samples were considered *GATA1*-MRD-positive, with this difference due to the fact that *GATA1* gene is located on the X chromosome. Cases with complex mutations were removed from the analysis because low frequency mutations may not be detected.

Definitions and statistics

The primary endpoint was MRD positivity after induction and intensification therapy (TP-2, -3, -4, -S, and -H). Secondary endpoints were evaluability of MRD, reasons for an unevaluable sample (e.g., dry tap), the proportion of patients with *GATA1* mutation, event-free survival (EFS) rate, overall survival (OS) rate, and adverse events during induction and intensification therapy. EFS was defined as the length of time from registration to failure to achieve remission, relapse, secondary malignancy, or death from any cause, whichever came first. OS was defined as the length of time from registration to death from any cause. Time-to-event for patients who did not experience the event in question was censored as of the date of last follow-up.

Sample size of the study was derived by prespecifying the width of the 95% confidence interval (CI) of estimated proportions of MRD-positive patients. We specified that the width of the 95% CI was narrower than 0.2. The required sample size was 62 patients for a true proportion of 20%. Assuming a 5–10% rate of ineligible

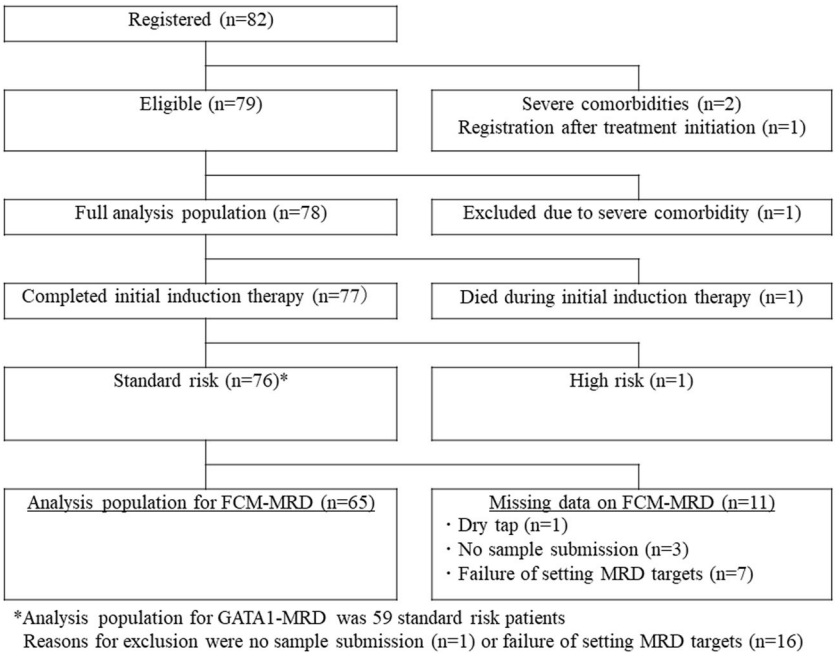

Fig. 2 Flow diagram of patients in the AML-D11 study. FCM flow cytometry, MRD minimal residual disease.

patients, the target number of registered patients was set at 75.

MRD after induction and intensification therapy and adverse events were described using proportions and 95% CI. EFS and OS rates were described using the Kaplan–Meier method. Prognostic factors for EFS and OS of AML-DS were explored using Cox regression analysis. Potential prognostic factors identified in the prior AML-D05 (age, sex, mosaic 21 trisomy, history of TAM, cardiac complications, FAB classification, white blood cell count, hemoglobin level, platelet count, abnormal cytogenetics, and *GATA1* mutation) were initially screened using univariate log-rank tests. Only factors identified as significant from univariate analyses were included in multivariate Cox regression analysis. Proportional hazards assumptions were confirmed from log-negative log graphs.

All reported *p*-values were two-sided, and we considered values of $p < 0.05$ as statistically significant. An academic statistician conducted all analyses using SAS version 9.4 software (SAS Institute, Cary, NC). The date of data cutoff was as of July 13, 2018.

Results

Patient characteristics

A total of 82 patients were registered to this study. Four patients were judged ineligible due to severe comorbidities ($n = 3$), and initiation of chemotherapy prior to registration ($n = 1$). As a result, 78 patients were eligible for analysis (Fig. 2). The relevant initial clinical and hematological data of the 78 patients in this study are shown in Table 1. Median age at diagnosis was 16 months (range, 4–48 months). Ten patients had mosaic trisomy 21 and 43 patients (58%) had a history of TAM. Seventy-three percent of patients ($n = 47$) showed cardiac disease at presentation. Forty-five patients (58%) had BM blast exceeding 20%, whereas 33 (42%) had <20% of BM blasts. Karyotype analysis showed monosomy 7 in 15 patients, complex karyotype (defined as presence of a clone with at least three unrelated cytogenetic abnormalities) in 8 patients, and 20 patients with normal karyotype and sole constitutional trisomy 21. *GATA1* mutations were identified in 76 of 78 patients (97%).

Treatment outcome

After induction therapy, 76 of 78 patients (97.4%) achieved CR and were stratified to the SR group, whereas one patient did not achieve CR and received intensive chemotherapy according to the HR regimen. Another patient died of cardiac failure during initial CET. No therapy-related deaths were observed during intensification therapy.

During the follow-up period (median, 4.0 years; range, 0.7–5.7 years), seven patients showed relapse in BM within 5–20 months after registration; all seven patients were in the SR group. One patient in the HR group could not achieve CR after intensive salvage therapy and died of disease progression. No extramedullary relapse including CNS was observed. The 3-year EFS and OS rates in the entire population ($n = 78$) were 87.2% (95% CI, 77.5–92.9%) and 89.7% (95% CI, 80.5–94.7%). The 3-year EFS and OS rates in the SR patients

Table 1 Baseline characteristics of patients in total and standard risk populations for which FCM-MRD was assessable at time point 2.

	Total ($n = 78$)		Standard risk population with FCM-MRD ($n = 65$)	
	N	%	*N*	%
Age at diagnosis (months)				
Median (Range)	16.2 (4.0–48.1)		15.6 (4.0–48.1)	
4–23	60	76.9	50	76.9
24 or older	18	23.1	15	23.1
Girl	37	47.4	32	49.2
21 trisomy mosaic				
Yes	10	13.2	6	9.5
No	66	86.8	57	90.5
Not available	2		2	
History of TAM				
Yes	43	58.1	36	58.1
No	31	41.9	26	41.9
Not available	4		3	
Cardiac complication				
Yes	47	73.4	39	75.0
No	17	26.6	13	25.0
BM blast percentage				
30% or more	27	34.6	18	27.7
20–29%	18	23.1	16	24.6
5–19%	31	39.7	29	44.6
>5%	2	2.6	2	3.1
White blood cell (10^9/L): median (range)	5.6 (1.5–54.2)		5.7 (1.5–54.2)	
Hemoglobin (g/dL): median (range)	9.8 (3.6–15.8)		9.8 (3.6–15.8)	
Platelet (10^9/L): median (range)	49 (5–250)		43 (5–191)	
Cytogenetics				
Normal karyotype*	20	26.0	17	26.2%
Monosomy 7	7	9.1	6	9.2%
Monosomy 7 (complex)	8	10.4	7	10.8%
Sole trisomy 8	2	2.6	1	1.5%
Complex	8	10.4	7	10.8%
1q loss	7	9.1	5	7.7%
7p loss	10	13.0	10	15.4%
Acquired +21	10	13.0	7	10.8%
Other	32	41.6	27	41.5%
Not available	1		0	
GATA1 mutation				
Negative	2	2.6	1	1.5%
Positive	76	97.4	64	98.5%

MRD minimal residual disease, *TAM* transient abnormal myelopoiesis
*Normal karyotype means 47, XX, + 21c or 47, XY, + 21c.

($n = 76$) were 89.5% (95% CI, 80.1–94.6%) and 92.1% (95% CI, 83.3–96.4%), respectively (Fig. 3).

Toxic events

As grade 4 adverse events, sepsis was observed in two patients (2 events) during initial induction therapy and elevation of liver enzyme was observed in one patient (1 event) during the intensification phases. One toxic death due to cardiac failure was observed during remission, but no other severe cardiac sequelae were observed during follow-up. Secondary cancer was also not reported. The therapy-related mortality rate in this study was thus 1.3%.

FCM-MRD

Longitudinal profiles of FCM-MRD at TP-2, -3, -4, and at TP-S, -H are shown in Table S2. Of the 76 patients in the SR group, 65 patients (85.5%) were evaluable for FCM-MRD at TP-2. Eleven patients (14.5%) could not be evaluated because of dry tap ($n = 1$), no sample submission ($n = 3$), and failure of setting MRD targets ($n = 7$). The 3-year EFS and OS rates in the analysis population for FCM-MRD ($n = 65$) were 90.8% (95% CI, 80.6–95.7%) and 93.8% (95% CI, 84.4–97.6%), respectively. Five patients (7.5%) were positive (range, 0.088–1.441%), and all five samples had the cluster of blasts. Two of these five patients showed relapse in BM, at 7 and 20 months after completion of chemotherapy. Of 60 patients whose end-of-induction FCM-MRD was negative, three patients relapsed. At TP-S, one of the 55 patients with evaluable samples was positive. FCM-MRD in this patient was also positive after initial induction therapy and the patient was alive without relapse as of final follow-up. As expected, one patient in the HR group was MRD-positive (30.2%) at TP-2.

GATA1-MRD

In the SR group, 59 patients (77.6%) were evaluable for *GATA1*-MRD at TP-2, whereas 17 patients could not be evaluated because of no sample submission ($n = 1$) or failure of setting MRD targets ($n = 16$) (Table S3). Of the seven positive patients (11.9%), three patients relapsed and two subsequently died. In contrast, 2 of 52 patients with negative *GATA1*-MRD suffered relapse. At TP-S, 53 samples (70.1%) were evaluable and one patient was positive; this patient survived without relapse. The mean coverage of sequencing in each sample ranged 59,922x to 281,121x with a median of 170,693x (Figure S1).

Concordance between FCM-MRD and GATA1-MRD

Correlations between MRD results are depicted in Figure S2. Among the 54 SR patients for whom both FCM-MRD

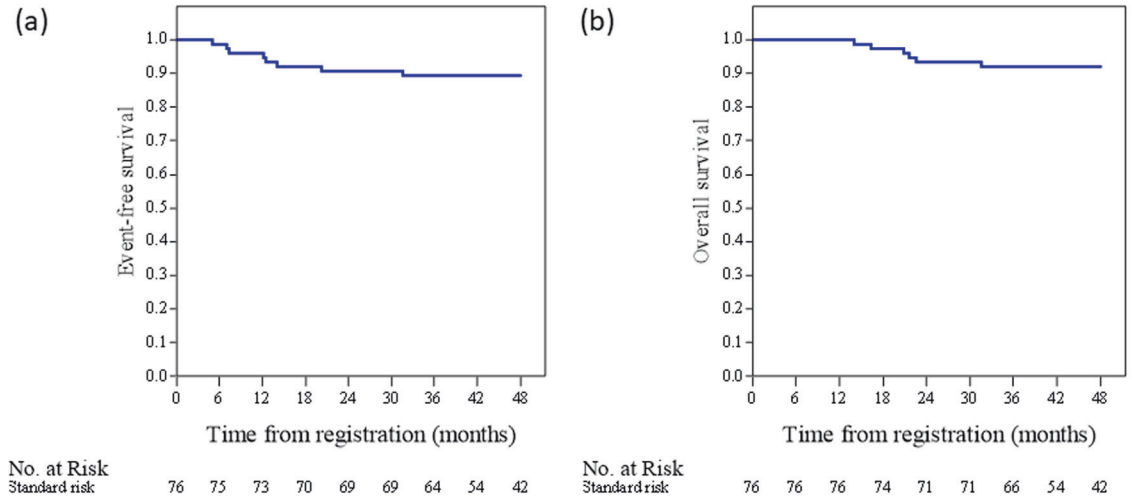

Fig. 3 Survival curve of the patients with standard risk ($n = 76$). (**a**) Event-free survival and (**b**) overall survival for patients showing good response after initial induction therapy.

Table 2 Multivariate Cox regression of FCM-MRD or *GATA1*-MRD for event-free and overall survivals in the standard risk population.

	EFS			OS		
	HR	95%CI	*p*	HR	95%CI	*p*
Analysis of FCM-MRD ($n = 65$, including 5 MRD-positive patients)						
MRD at Time point 2						
Negative	Ref			Ref		
Positive	10.98	1.70–70.89	0.01	5.74	0.52–63.50	0.15
Cytogenetics						
Other abnormalities	Ref			Ref		
1q loss	4.61	0.45–47.54	0.20	6.31	0.54–73.74	0.14
Platelet (per 10^{10}/L increase)	1.17	0.98–1.39	0.09	1.13	0.90–1.41	0.31
Analysis of *GATA1*-MRD ($n = 59$, including 7 MRD-positive patients)						
MRD at Time point 2						
Negative	Ref			Ref		
Positive	27.68	2.84–269.89	<0.01	15.08	1.37–166.56	0.03
Cytogenetics						
Other abnormalities	Ref			Ref		
1q loss	11.55	0.68–197.24	0.09	10.19	0.62–168.08	0.10
Platelet (per 10^{10}/L increase)	1.14	0.93–1.41	0.20	1.09	0.85–1.38	0.50

MRD minimal residual disease, *EFS* event-free survival, *OS* overall survival *HR* hazard ratio *CI* confidence interval

and *GATA1*-MRD were analyzed, 50 patients (92.6%) showed concordant classification based on FCM-MRD and *GATA1*-MRD (either both positive or both negative). All four patients showing discordant results survived without relapse. Among the seven relapses in SR patients, five patients each were evaluable by FCM-MRD and *GATA1*-MRD, respectively. Two patients were positive in both FCM-MRD and *GATA1*-MRD, one was positive only in *GATA1*-MRD, and four were negative or missing in both FCM-MRD and *GATA1*-MRD.

Prognostic factors

Table 2 and S4 show results of uni- and multivariate Cox regression analysis for prognostic factors among SR patients. FCM-MRD and *GATA1*-MRD after initial induction therapy (TP-2) represented significant prognostic factors for predicting relapse in SR patients. Three-year EFS and OS rates were 93.3% (95% CI, 83.2–97.4%) and 95.0% (95% CI, 85.3–98.4%) in the FCM-MRD-negative population, compared to 60.0% (95% CI, 12.6–88.2%) and 80.0% (95% CI, 20.4–96.9%) in the FCM-MRD-positive

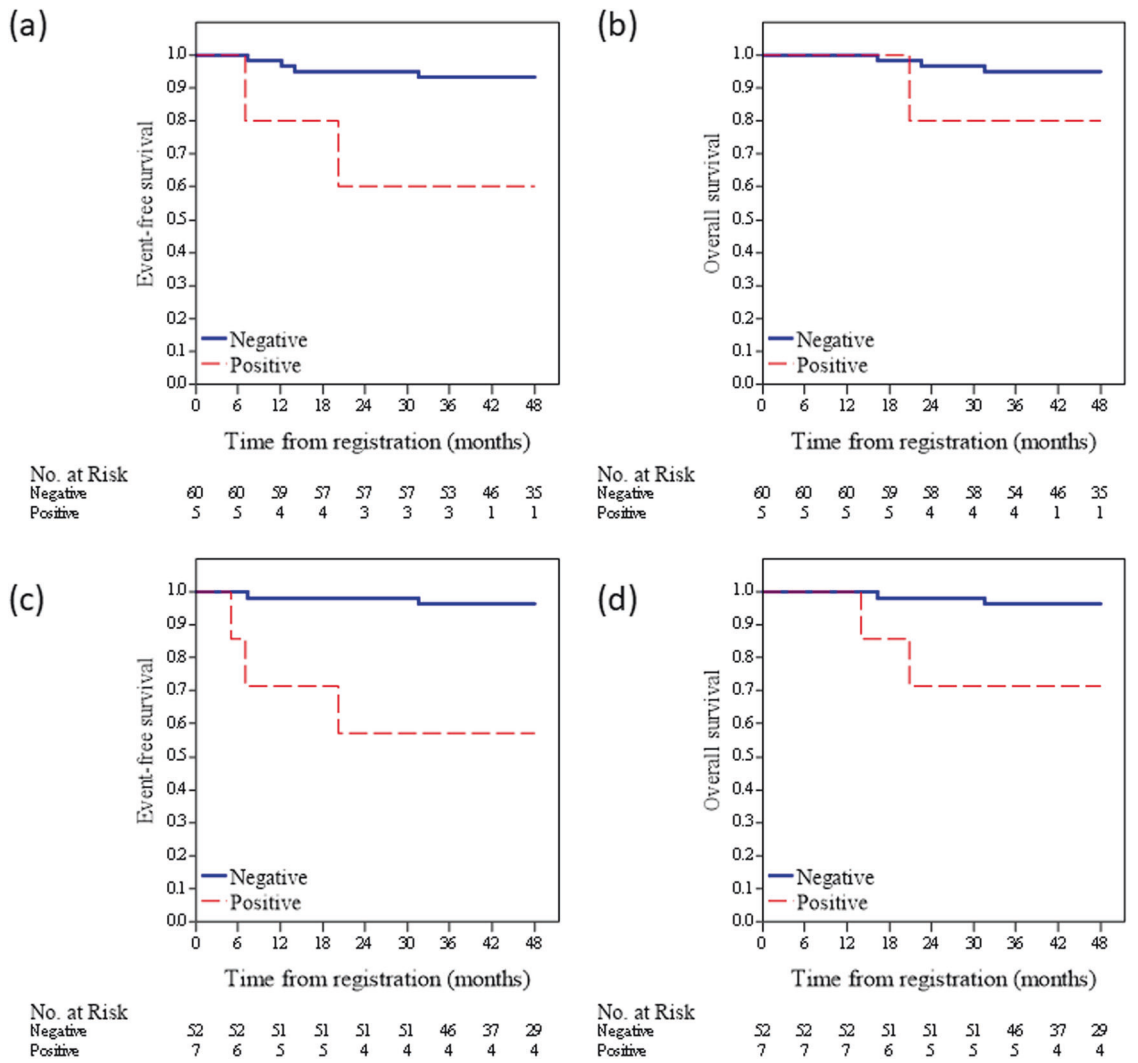

Fig. 4 Event-free survival and overall survival of patients with standard risk according to FCM-MRD ($n = 65$) or *GATA1*-MRD ($n = 59$). (**a**) event-free survival according to FCM-MRD, (**b**) overall survival according to FCM-MRD, (**c**) event-free survival according to *GATA1*-MRD, (**d**) overall survival according to *GATA1*-MRD.

population, respectively (Fig. 4a, b). Three-year EFS and OS rates were both 96.2% (95% CI, 85.5–99.0%) in the *GATA1*-MRD-negative population, while 57.1% (95% CI, 17.2–83.7%) and 71.4% (95% CI, 25.8–92.0%) in the *GATA1*-MRD-positive population, respectively (Figs. 4c, d). Hazard ratios for the associations of FCM-MRD and *GATA1*-MRD with EFS were 10.98 (95% CI, 1.70–70.89; $p = 0.01$) and 27.68 (95% CI, 2.84–269.89; $p < 0.01$), respectively, after adjusting for other prognostic factors (Table 2).

Irrespective of methodologies, MRD at the end of chemotherapy (TP-S) did not have any prognostic impact. Age at diagnosis <2 years that was significantly associated with favorable prognosis in the previous study [3], did not show any impact in the present study. Furthermore, significant prognostic factors in univariate analyses such as chromosomal abnormalities (1q loss) and platelet count were not associated with unfavorable EFS or OS rates in multivariate analysis.

Discussion

In this study of 78 patients with ML-DS, FCM identified 5 MRD-positive patients among 65 patients who achieved CR, while *GATA1*-targeted deep sequencing detected 7 MRD-positive patients among 59 patients with CR. These patients with MRD positivity after achieving morphological CR were at significantly high risk of relapse, revealing EFS rates around 60%. Although a small subset of patients whose end-of-induction MRD was negative eventually relapsed (four patients with FCM-MRD and two patients with *GATA1*-MRD), hazard ratios for the associations of FCM-MRD and

GATA1-MRD with EFS were striking (10.98 and 27.68, respectively).

To establish a therapeutic approach for ML-DS with reduced dose intensity than that applied for non-DS AML patients, identification of prognostic factors is essential for appropriate risk stratification. Several factors have been identified as prognostic in past ML-DS studies worldwide, but no universal prognostic factors have been established to date. The prognostic impact of FCM-MRD after initial induction therapy has been shown in several studies for pediatric de novo AML [7, 11] and also in the COG AAML0431 study for ML-DS [2]. In this AML-D11 study, the prognostic impact of MRD was shown not only by FCM, but also by deep sequencing for *GATA1*. Although *GATA1*-MRD was analyzed retrospectively, the results of outcome and FCM-MRD were masked at the time of *GATA1*-MRD analysis. *GATA1* mutation is well known as the hallmark of ML-DS blasts, but no previous reports have examined MRD using *GATA1* mutation in ML-DS patients. A high rate (98%) of detecting *GATA1* mutation from screening both BM and PB samples at initial diagnosis could contribute to showing the impact and effectiveness of *GATA1*-MRD in this study [10]. Taken together, MRD after initial induction therapy could offer a universal prognostic factor for ML-DS. Because obstacles to the introduction of *GATA1*-MRD into clinical use remain, due to costs and a labor-intensive procedure, applying FCM-MRD for the risk stratification of treatment for ML-DS appears reasonable. Therefore, it is necessary to refine the marker combination panels to improve the sensitivity and specificity of FCM-MRD.

Less intensive chemotherapy compared with Western countries has been used in Japanese studies for ML-DS without compromising treatment outcomes [12]. Moreover, further dose reduction was successfully shown in the JPLSG AML-D05 study [3], the backbone of which we applied in the AML-D11 study. Combined with the results from the Toronto group study with an ultra-low-dose cytarabine-based regimen that contained no anthracyclines and no etoposide [13], further dose reductions might be possible for specific subgroups. As many patients with ML-DS show comorbidities, pursuit of possibilities for appropriate dose reduction among patients expected to show good prognosis has been one of the strategies for the Japanese pediatric AML committee. On the other hand, our retrospective study of patients ineligible for enrollment in clinical trials showed that an excessively reduced intensity of chemotherapy might not be curative [14]. To address this issue, dose modification according to the end-of-induction FCM-MRD is attempted in the ongoing Japan Children's Cancer Group AML-D16 study (jrct.niph.go.jp, jRCTs041190047). Although the number of patients who will be influenced by refined risk stratification based on the MRD analyses might be small, treatment intensification in end-of-induction MRD-positive patients and reduction in treatment burden in patients whose MRD clearance is excellent may contribute to further improvement in not only treatment outcome but also quality of life of DS patients with AML. As an HR patient who did not achieve CR in this study eventually succumbed to the disease, establishing treatment strategies for refractory/relapsed patients is also imperative.

Acknowledgements The authors deeply appreciate the invaluable cooperation of the large number of physicians working at the institutions, central diagnostic laboratories, and the office and data center of the JPLSG. We also wish to thank Dario Campana and Elaine Coustan-Smith (National University of Singapore) for helpful discussions regarding FCM-MRD analysis. This work was supported by a Grant for Clinical Cancer Research and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour, and Welfare of Japan. This work was supported in part by the Project Promoting Clinical Trials for Development of New Drugs (JP19lk0201061t0004) and Practical Research for Innovative Cancer Control (JP19ck0106329) from the Japan Agency for Medical Research and Development (AMED) and by Grants-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (KAKENHI: 26253061, 18H04039).

Author contributions T.Tag (principal investigator), Daisuke H, TM, YH, HM, HN, HT, AS, and DT participated actively in the study conception and design; Ta.Ta., ST, Daisuke H, and DT reviewed the data analysis and interpretation and were the main authors of the paper; AMS and ST conducted the statistical analyses; SI and HH were responsible for immunodiagnostics and FCM-MRD analysis; KT, EI, and T.Tok were responsible for *GATA1* mutation and *GATA1*-MRD analysis; T.Tak was responsible for coordinating the molecular biological analyses; AH was responsible for diagnosis and treatment response by morphology; MI was responsible for diagnosis by pathology; KK, Daiichiro H, and AH recruited patients; and Ta.Ta., ST, and SA contributed financial and administrative support for the study. All authors contributed to the conduct of the trial and were involved in the review of the results and the final approval of the paper.

Compliance with ethical standards

Conflict of interest The authors declare no competing interest.

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