REGULAR ARTICLE

blood advances

Transcriptome analysis offers a comprehensive illustration of the genetic background of pediatric acute myeloid leukemia

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Key Points

- Using RNA-seq in pediatric AML patients, 5 gene rearrangements were newly identified, including *NPM1* and *RUNX1* gene rearrangements.
- RNA-seq unmasked the complexity of gene alterations in pediatric AML by identifying disease-causing alterations in nearly all patients.

Recent advances in the genetic understanding of acute myeloid leukemia (AML) have improved clinical outcomes in pediatric patients. However, ~40% of patients with pediatric AML relapse, resulting in a relatively low overall survival rate of ~70%. The objective of this study was to reveal the comprehensive genetic background of pediatric AML. We performed transcriptome analysis (RNA sequencing [RNA-seq]) in 139 of the 369 patients with de novo pediatric AML who were enrolled in the Japanese Pediatric Leukemia/Lymphoma Study Group AML-05 trial and investigated correlations between genetic aberrations and clinical information. Using RNA-seq, we identified 54 in-frame gene fusions and 1 *RUNX1* out-of-frame fusion in 53 of 139 patients. Moreover, we found at least 258 gene fusions in 369 patients (70%) through reverse transcription polymerase chain reaction and RNA-seq. Five gene rearrangements were newly identified, namely, *NPM1-CCDC28A*, *TRIP12-NPM1*, *MLLT10-DNAJC1*, *TBL1XR1-RARB*, and *RUNX1-FNBP1*. In addition, we found rare gene rearrangements, namely, *MYB-GATA1*, *NPM1-MLF1*, *ETV6-NCOA2*, *ETV6-MECOM*, *ETV6-CTNNB1*, *RUNX1-PRDM16*, *RUNX1-CBFA2T2*, and *RUNX1-CBFA2T3*. Among the remaining 111 patients, *KMT2A*-PTD, biallelic *CEBPA*, and *NPM1* gene mutations were found in 11, 23, and 17 patients, respectively. These mutations were completely mutually exclusive with any gene fusions. RNA-seq unmasked the complexity of gene rearrangements and mutations in pediatric AML. We identified potentially disease-causing alterations in nearly all patients with AML, including novel gene fusions. Our results indicated that a subset of patients with pediatric AML represent a distinct entity that may be discriminated from their adult counterparts. Based on these results, risk stratification should be reconsidered.

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Introduction

Recent advances in the genetic understanding of cancer have drastically improved clinical outcomes in pediatric patients. However, ~40% of patients with childhood acute myeloid leukemia (AML) relapse, resulting in a relatively low overall survival (OS) rate of ~70%. AML is caused by various chromosomal aberrations, gene mutations/epigenetic modifications, and deregulated/overregulated gene expression, leading to increased proliferation and decreased differentiation of hematopoietic progenitor cells.[1-4] Several clinically important molecular markers have been discovered in patients with AML, which assisted in improving risk stratification. However, the evaluation of risk remains challenging even after incorporating these molecular markers in clinical practice.

Patients with biallelic *CEBPA* mutations, *NPM1* mutations, *RUNX1-RUNX1T1* fusions, or *CBFB-MYH11* fusions have relatively good outcomes in response to treatment with chemotherapy-based consolidation regimens.[5,6] However, the prognosis of patients with *FUS-ERG*, *NUP98-NSD1*, *CBFA2T3-GLIS2*, *NUP98-KDM5A*, or *KMT2A-MLLT3* with high *MDS1* and *EVI1* complex locus protein *EVI1* (*MECOM*, synonyms *EVI1* and *PRDM3*) expression was dismal, because many of these patients could not be during the first complete remission (CR), even with allogeneic transplantation.[7-10]

FMS-like tyrosine kinase 3-internal tandem duplication (*FLT3*-ITD) is a major alteration in pediatric and adult AML that is significantly associated with poor prognosis. However, the prognosis of patients with this alteration is not necessarily dismal, depending on concomitant genetic alterations.[11-13] In our previous studies, the overexpression of *PRD1-BF1-RIZ1* homologous domain containing 16 (*PRDM16*, also known as *MEL1*) was highly recurrent in patients with both pediatric AML and adult AML with intermediate- and high-risk cytogenetic profiles while being independently associated with an adverse outcome.[14,15]

Recently, Bolouri et al reported the seminal comprehensive molecular landscape and characteristics of pediatric AML in Children's Oncology Group AML trials.[16] However, the biologic basis differs among pediatric AML patients in the United States, Europe, and Japan. The objective of this study was to reveal the genetic alterations of all Japanese patients with pediatric AML and the differences in the genetic background between favorable and adverse groups using RNA sequencing (RNA-seq) by identifying new cytogenetic aberrations and comparing gene expression.

Methods

Study design and participants

In this study, we enrolled 485 patients with de novo AML who registered in the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) AML-05 trial between November 2006 and December 2010. The trial was registered with the University Hospital Medical Information Network Clinical Trials Registry (#UMIN000000511; http://www.umin.ac.jp/ctr/index.htm), and details of the schedules and treatment regimens have been described previously (supplemental Figure 2).[17] Patients diagnosed with acute promyelocytic leukemia or AML with Down syndrome were excluded from this study. Among the 485 patients with AML who were enrolled in the AML-05 trial, 116 were excluded because of sample insufficiency for diagnosis (n = 7), not meeting the criteria (n = 6), misdiagnosis (n = 25), lack of RNA samples (n = 74), or other reasons (n = 4). Therefore, a total of 369 patients were genetically analyzed. In these 369 analyzed patients, the age and initial white blood cell count were higher, whereas the proportion of M7 patients was lower compared with the 74 nonanalyzed patients. However, there were no significant differences observed between these groups in terms of mortality (20% vs 24%, log-rank $P = .84$) (supplemental Table 1). The present study was conducted in accordance with the Declaration of Helsinki and approved by the institutional review boards of Gunma Children's Medical Center and the participating institutes and the ethical review board of the JPLSG AML-05 trial. Written informed consent was provided by all patients or their parents/guardians.

Sample preparation

All leukemic samples were obtained from the bone marrow or peripheral blood at the time of diagnosis. DNA and total RNA samples were prepared using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Complementary DNA was prepared using 0.8 to 1.0 μg of total RNA and Ready-To-Go reverse transcription polymerase chain reaction (RT-PCR) beads (GE Healthcare, Buckinghamshire, United Kingdom).

RNA-seq

We performed RNA-seq for 139 out of the 369 patients with pediatric AML in order to obtain a complete registry of gene rearrangements, other genetic lesions, and gene expressions in pediatric AML. The RNA-seq data were available at the European Genome-Phenome Archive (EGAS00001003701). The cytogenetic characteristics of the analyzed patients are shown in supplemental Table 2. The study population mainly included patients with a normal karyotype (60/70 patients), *FLT3*-ITD (33/47 patients), *KMT2A*-PTD (12/13 patients), and high *PRDM16* expression (65/84 patients). All 137 patients with core binding factor AML (CBF-AML) and many patients with *KMT2A* rearrangements were excluded from RNA-seq, because these fusions are already known to affect leukemogenesis. The main reasons behind the inability to perform analyses were assay failure, sample availability, and/or sample quality. The quality of the extracted RNA was assessed using TapeStation system (Agilent Technologies, Santa Clara, CA). Sequencing libraries were prepared using an NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA), and prepared libraries were run on a HiSeq 2500 high-throughput sequencing system. Sequencing reads were aligned using bowtie and blat, and fusion genes were analyzed using Genomon-fusion.[18] Candidate gene fusions were validated by RT-PCR. Obtained reads were also analyzed using an in-house pipeline, GenomonExpression (https://github.com/Genomon-Project/GenomonExpression), to obtain fragments per kilobase million (FPKM) values. We used Cluster 3.0 to perform hierarchical clustering.[19] Briefly, genes that were not expressed (FPKM = 0) in >20% of samples and genes with a standard deviation <10 were excluded. For the remaining genes, their FPKM values were log transformed. Finally, an average linkage was calculated. Results were visualized using Java TreeView.[20]

Targeted gene mutation analysis

We performed targeted gene mutation analysis of *KIT* (exons 8 and 17), *CEBPA* (all coding regions), *NPM1* (exon 12), *WT1* (exons 7-10), *RUNX1* (exons 2-6), *NRAS* (exons 1 and 2), *KRAS* (exons 1 and 2), *ASXL1* (exon 11), *ASXL2* (exons 11 and 12), and *GATA2*

(exons 4-6). The allelic ratio (AR) of *FLT3*-ITD to wild-type alleles was further examined using GeneScan (Applied Biosystems, Foster City, CA), and a defined mutant/wild-type >0.40 was considered as high AR according to previous studies.[12,21-26] *KMT2A*-PTD was analyzed using previously described multiplex ligation-dependent probe amplification methods.[27] Gene fusions, namely, *RUNX1-RUNX1T1*, *CBFB-MYH11*, *KMT2A* rearrangements, *NUP98-NSD1*, *CBFA2T3-GLIS2*, *NUP98-KDM5A*, *FUS-ERG*, and *DEK-NUP214*, were also analyzed in all patients. In addition to these conventional gene fusions, we performed RT-PCR using samples from all patients to detect the *MYB-GATA1*, *NPM1-CCDC28A*, *MLF1-NPM1*, *NPM1-TRIP12*, *TBL1XR1-RARB*, *ETV6-CTNNB1*, *ETV6-NCOA2*, *ETV6-MECOM*, *RUNX1-FNBP1*, *RUNX1-CBFA2T2*, and *RUNX1-CBFA2T3* alterations identified through RNA-seq.

Quantification of *PRDM16* and *MECOM* gene expression

Quantitative RT-PCR analysis of the *PRDM16* and *MECOM* genes was performed using the 7900HT Fast Real Time PCR System, TaqMan Gene Expression Master Mix, and TaqMan Gene Expression Assay (Applied Biosystems), which have been previously described.[28]

Data collection

A standardized form was used to record clinical variables, including patient demographic information. Every 6 months, data forms were forwarded to the JPLSG AML-05 trial data coordination center at the National Center for Child Health and Development (Tokyo, Japan), reviewed for internal consistency and face validity, and transferred into an Excel database (Microsoft Corporation, Redmond, WA). The clinical data of patients in each risk group were followed up until December 2013 (censored for 3 years from the date of final registration). The JPLSG conducted a central review of morphological classification and karyotyping based on the World Health Organization's classification, French-American-British (FAB) classification and cytogenetic analysis using conventional G-banding. OS was defined as the time from AML diagnosis to death or censorship at the last follow-up. Event-free survival (EFS) was defined as the time from AML diagnosis to treatment failure, relapse, death, or last follow-up.

Statistical analysis

Univariate and multivariate Cox regression analyses were calculated using EZR (version 1.37; Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (version 3.4.1; The R Foundation, Vienna, Austria)[29] to extract the adverse and favorable risk factors related to OS. We defined the risk factors with a hazard ratio (HR) of <0.20 as favorable and those with a ratio of >2.0 as adverse. Continuous variables are presented as means ± standard deviations and/or medians with ranges. Categorical variables are represented by frequencies and percentages. For all analyses, the *P* values were 2 tailed, and $P < .05$ was considered statistically significant. A multivariate Cox regression analysis was used to identify independent genetic factors related to OS. Initially, we included all genetic variables in the first model and then sequentially removed the nonsignificant variables ($P \geq .05$). To test the probability of co-occurrence and mutual exclusivity of 2 parameters, we performed a Monte Carlo simulation using the total number of patients and the number of patients who were positive for each parameter.[30]

Results

Overview of RNA-seq

We performed RNA-seq in 139 patients with pediatric AML and identified a total of 54 in-frame fusions and 1 *RUNX1* out-of-frame gene fusion in 53 patients. Many of the recurrent gene fusions identified in this study have been previously reported as targets in AML (Figure 1). Among these 54 gene fusions, 5 gene rearrangements were newly identified, namely, *NPM1-CCDC28A*, *TRIP12-NPM1*, *MLLT10-DNAJC1*, *TBL1XR1-RARB*, and *RUNX1-FNBP1*. We also identified 17 rare gene rearrangements, including *MYB-GATA1*, *NPM1-MLF1*, *ETV6-NCOA2*, *ETV6-MECOM*, *ETV6-CTNNB1*, *RUNX1-PRDM16*, *RUNX1-CBFA2T2*, and *RUNX1-CBFA2T3* (Figures 1 and 2). The characteristics of the patients with these fusions are shown in Table 1. We identified 5 *RUNX1* rearrangements other than *RUNX1-RUNX1T1*, 4 *NPM1* rearrangements, 3 *ETV6* fusions, and 1 *TBL1XR1-RARB* fusion in FAB-M3 patients without the *PML-RARA* fusion.

Complete genetic landscape of the JPLSG AML-05 trial

We showed the demographic characteristics and genetic landscape of 369 patients with AML enrolled in the JPLSG AML-05 trial (Figure 1). *RUNX1-RUNX1T1* and *KIT* mutations were more frequently observed and *CBFB-MYH11*, trisomy 8, and *NPM1* were less frequently observed as a molecular characteristic in the Japanese cohort compared with the Children's Oncology Group (supplemental Figure 1). We also showed the result of univariate and multivariate Cox regression analysis in Table 2. In the univariate analysis, factors significantly associated with OS included *CBFB-MYH11* (HR, 0.11, 95% confidence interval [CI], 0.02-0.80), biallelic *CEBPA* (HR, 0.16; 95% CI, 0.02-1.13), *RUNX1-RUNX1T1* (HR, 0.19; 95% CI, 0.09-0.39), *NPM1* (HR, 0.19; 95% CI, 0.03-1.35), *FLT3*-ITD (HR, 2.92; 95% CI, 1.83-4.65), *CBFA2T3-GLIS2* (HR, 3.00; 95% CI, 2.54-6.48), *PRDM16* overexpression (HR, 3.09; 95% CI, 2.06-4.65), and *NUP98-NSD1* (HR, 5.07; 95% CI, 2.54-10.1). The multivariate analysis revealed that *CBFB-MYH11* (HR, 0.08; 95% CI, 0.01-0.58), biallelic *CEBPA* (HR, 0.10; 95% CI, 0.01-0.69), *RUNX1-RUNX1T1* (HR, 0.17; 95% CI, 0.08-0.35), *NPM1* (HR, 0.09; 95% CI, 0.01-0.64), *FLT3*-ITD (HR, 2.53; 95% CI, 1.57-4.08), *FUS-ERG* (HR, 4.54; 95% CI, 1.81-11.4), and *RPN1-MECOM* (HR, 5.44; 95% CI, 1.32-22.4) were independent prognostic factors associated with OS. Concerning CBF-AML, 9 of 137 patients died of primary disease or other causes (4 and 5 patients, respectively) (Table 3). Although CBF-AML patients with the *KIT* mutation exhibited a higher relapse rate than those without the *KIT* mutation, most of these patients were rescued by hematopoietic stem cell transplantation.[31] No fusion genes were identified in patients with the *NPM1* or biallelic *CEBPA* mutation, and their prognosis was absolutely favorable irrespective of the presence of *FLT3*-ITD. Among the 41 patients with the biallelic *CEBPA* or *NPM1* mutation, only 2 patients died, and the causes of death were acute respiratory disorder syndrome (ARDS) and primary disease (Table 3). On the other hand, the prognosis of patients with adverse gene fusions (eg, *FUS-ERG*, *NUP98-NSD1*, *RPN1-MECOM*, *KMT2A-AFDN*, *DEK-NUP214*, *CBFA2T3-GLIS2*, and *NUP98-KDM5A*) and adverse mutations (eg, *FLT3*-ITD, *RUNX1*, and

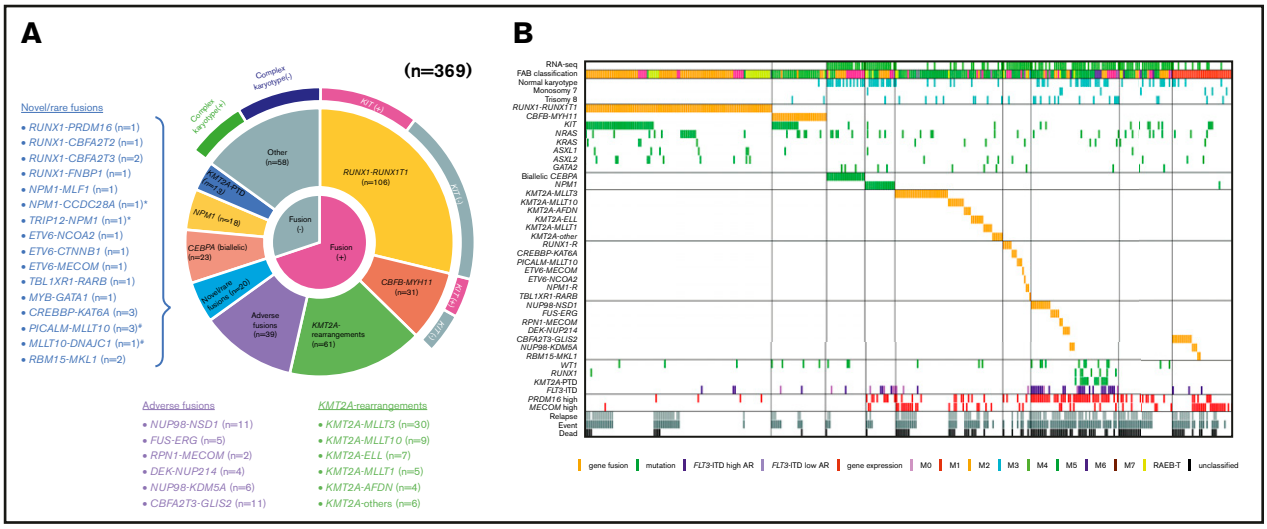

Figure 1. Clinical and genetic profiles of the 369 patients enrolled in the JPLSG AML-05 trial. (A) Pie chart represented the frequency of each gene alterations. (B) Landscape of 369 de novo pediatric AML patients. Each column indicates 1 patient. *,#Both gene fusions were observed in the same patient. *RUNX1*-R, *RUNX1* rearrangement; *NPM1*-R, *NPM1*-rearrangement.

KMT2A-PTD) was dismal (5-year OS, 31%; EFS, 9%). The characteristics of patients with unfavorable gene fusions are shown in supplemental Table 3. Although all of patients with *FUS-ERG*, *RPN1-MECOM*, *KMT2A-AFDN*, and *DEK-NUP214* were predicted by chromosomal G-banding, *NUP98-NSD1, NUP98-KDM5A*, and *CBFA2T3-GLIS2* were cryptic. No disease-causing genetic alterations were identified in 45 patients out of 369 patients with AML (29 patients with FAB non-M7 and 16 patients with FAB-M7) (supplemental Table 4). Ten patients were cytogenetically normal, whereas the remaining 35 patients had structural alterations in chromosomes, including complex karyotype (n = 23), monosomy 7 (n = 4), and trisomy 8 (n = 3). The prognosis of these patients tended to be dismal (3-year OS, 67%; 3-year EFS, 42%); thus, they should be treated as high-risk patients. Further research is warranted to confirm these observations.

Characteristics of patients with pediatric AML with normal karyotype

Sixty out of 70 patients with a normal karyotype were analyzed using RNA-seq. Although some cryptic gene fusions were identified (eg, *NUP98-NSD1* and *CBFA2T3-GLIS2*), fewer gene fusions were identified in this subset of patients. In particular, gene fusions were not identified in patients with normal karyotype with biallelic *CEBPA*, *NPM1*, and *KMT2A*-PTD aberrations, which are frequently observed in the normal karyotype (supplemental Figure 3).

Characteristics of patients with pediatric AML with *KMT2A* rearrangements

Sixty-one *KMT2A* rearrangements were identified in 369 patients with AML (16%). Among them, 7 *KMT2A* fusions not identified through conventional chromosomal G-banding were detected through RNA-seq. *KMT2A-MLLT3* was the most commonly observed alteration (30/61 patients). Intriguingly, the prognosis of these patients was highly dependent on the expression of the *MECOM* gene. Seven of 11 patients with high *MECOM* expression died compared with only 1 of 19 patients with low *MECOM* expression ($P < .001$). *KMT2A-ELL* and *KMT2A-MLLT1* were the second most commonly observed alterations in patients with pediatric AML (supplemental Table 5).

Characteristics of patients with pediatric AML with FAB-M7

In this study, we performed RNA-seq in only 9 of 34 patients with acute megakaryoblastic leukemia. Among them, we confirmed 3 types of recurrent gene fusions identified through RT-PCR, including 2 *RBM15-MKL1*, 3 *CBFA2T3-GLIS2*, and 1 *NUP98-KDM5A*. In the remaining 3 patients with acute megakaryoblastic leukemia, there were no gene fusions identified. Among 34 patients registered and treated in the JPLSG AML-05 trial, 19 of 34 patients showed high expression of the *MECOM* gene. Although high *MECOM* gene expression was associated with poor prognosis in other FAB patients, this association was not observed in patients with FAB-M7 (Figure 1). Interestingly, patients with the *CBFA2T3-GLIS2* and *NUP98-KDM5A* alterations, which were associated with poor prognosis, showed low *MECOM* gene expression, indicating the presence of a different pathogenesis mechanism.

Gene expression profiling

The RNA-seq expression profiles of 139 patients were subjected to all-gene–based unsupervised hierarchical clustering, and 3 clusters were generated (blue, orange, and green) (Figure 3A). Thirteen out of 14 patients with *KMT2A* rearrangement were classified into the blue cluster, and all patients with *FLT3*-ITD with high AR were classified into the orange or green cluster. On the contrary, 6 of 7 patients with *NUP98-NSD1* and 2 patients with *DEK-NUP214* were classified into the green cluster. The gene expression pattern of *NUP98-KDM5A* was different from the other *NUP* fusions in this study (Figure 3A). Furthermore, *HOXA7, HOXB3, HOXB6, HOXB7, RUNX1, FLT3, PRDM16*, *HIST1H1C,* and *HIST1H2BD* were overexpressed in patients with *FLT3*-ITD (Figure 3B-C), and *HOX* genes were underexpressed in patients with biallelic *CEBPA*. Intriguingly, *FLT3* and *HOXAs* were

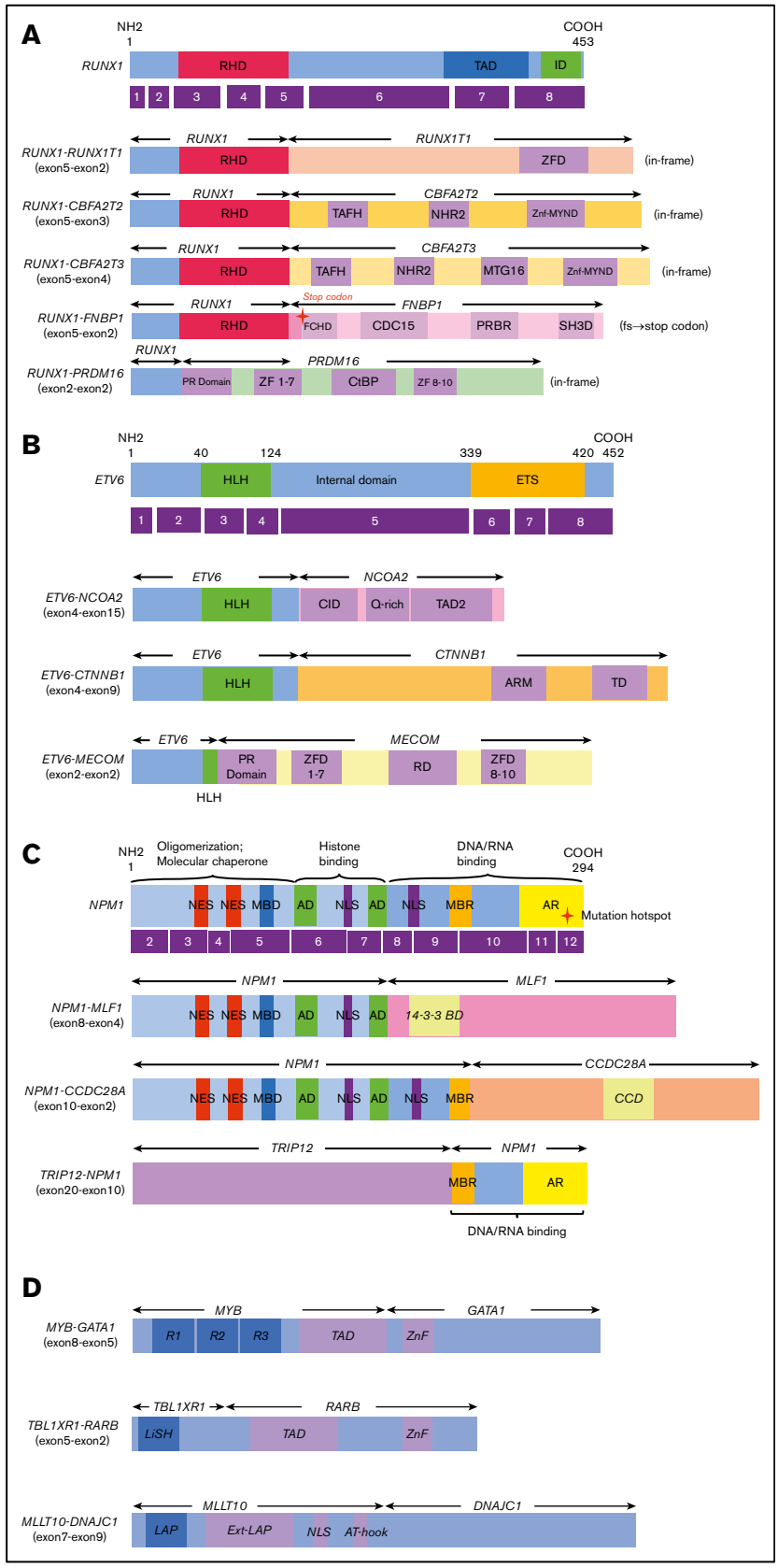

Figure 2. Novel/rare fusion genes identified in patients with de novo pediatric AML. (A-D) The structure of the detected fusion proteins. (A) Five *RUNX1* rearrangements. (B) Three *ETV6* rearrangements. (C) Three *NPM1* rearrangements. (D) Other novel gene fusions. R1, R2, and R3 are 51 ± 52 amino acid tandem repeats that comprise the DNA-binding domain Ext–leukemia-associated protein (LAP). 14-3-3 BD, 14-3-3 binding domain; AD, acidic domains; AT-hook, adenine-thymine hook; CCD, coiled-coil domain; CDC15, CDC15 homology region; CID, CBP interaction domain; CtBP, C-terminal binding protein; ETS, ETS domain; FCHD, FER-CIP1 homology domain; HLH, helix-loop-helix; ID, transcription inhibition domain; LiSH, Lis homology domain; MBD, metal-binding domain; MBR, moderately basic region; MTG16, myeloid translocation gene 16; NES, nuclear export signal; NHR2, nervy homology region 2; NLS, nuclear localization signal; PRBR, putative ρ-binding region; RD, runt domain; RHD, runt homolog domain; SH3D, Src homology 3 domain; TAD, transcription activation domain; TAFH, TAF homology; ZF, zinc finger; ZFD, zinc-finger domain; Znf-MYND, MYND zinc-finger domain.

Table 1. The clinical features of de novo pediatric AML patients with novel/rare fusions

RNA no.	Novel/rare	Fusion gene	Sex	Age, y	WBC, 10^9/L	FAB	Risk	Chromosome	*FLT3*-ITD	SCT	Relapse	Outcome
273	Rare	*RUNX1-CBFA2T2*	M	1.5	43.3	M1	Int	47,XY,+8,t(14;20)(q11.2;q11.2),del(21)(q21q22)[20]	–	–	–	Alive
227	Rare	*RUNX1-CBFA2T3*	F	4.4	5.0	M1	Int	46,XX,t(16;21)(q24;q22)[4]/46,sl,t(1;16)(q32;p13.3)[16]	–	–	–	Alive
308	Rare	*RUNX1-CBFA2T3*	F	1.6	35.4	M4	Int	46,XX,add(2)(q31),t(16;21)(q24;q22)[5]/45,sl,+2,-add(2),-11,add(18)(p11.2)[12]/91,sl,×2,-11[2]	–	–	–	Alive
177	Rare	*RUNX1-PRDM16*	M	2.9	25.2	RAEB-T	Non-CR	45,XY,−7[1]/45, sl, t(11;21)(q13;q22)[19]	–	+	–	Dead
440	Novel	*RUNX1-FNBP1*	F	0.2	10.0	M1	Int	46,XX,add(7)(q11.2),add(8)(q34),add(12)(p13)[20]	–	–	–	Alive
104	Rare	*KAT6A-CREBBP*	F	17	10.6	M4	Int	46,XX,t(8;16)(p11.2:p13.3)[19]/46,XX[1]	–	–	–	Alive
216	Rare	*KAT6A-CREBBP*	M	15.3	18.1	M5a	Int	46,XY,t(8;16)(p11.2;p13.3)[20]	–	–	+	Dead
271	Rare	*KAT6A-CREBBP*	M	1.8	9.8	M5b	Int	46,XY,add(7)(q22),t(8;16)(p11.2;p13.3)[17]/46,XY[3]	–	–	–	Alive
331	Rare	*ETV6-CTNNB1*	M	4.8	2.2	M5a	Int	46,XY,add(3)(p21),inv(9)(p12q13),add(12)(p11.2)[7]/46,idem,del(20)(q11.2q13.3),add(22)(q11.2)[11]/46,XY,inv(9)(p12q13)[2]	–	+	+	Dead
45	Rare	*ETV6-MECOM*	M	4.8	33.9	M5a	Non-CR	45,XY,-7[1]/45,sl,t(3;12)(q26;p13)[18]/46,XY[1]	–	+	+	Dead
333	Rare	*ETV6-NCOA2*	F	9.8	4.1	M0	Int	46,XX,t(8;12)(q11.2;p11.2)[20]	–	–	–	Alive
73	Rare	*MYB-GATA1*	M	0.3	20.2	M5a	IR	47,Y,del(X)(p11.2), -6,+8,+r[15]/46,Y,del(X)(p11.2),-6,add(19)(p13.1), +r[2]/46, XY[3]	–	–	–	Alive
171	Novel	*NPM1-CCDC28A TRIP12-NPM1*	F	1.4	16.6	M2	High	46,XX,add(2)(q31),add(5)(q35),add(6)(q15)[13]/46,XX[7]	+	+	–	Dead
58	Rare	*NPM1-MLF1*	M	12.9	14.4	RAEB-T	Int	46,XY,t(3;5)(q24;q33)[20]	–	+	–	Alive
274	Rare	*PICALM-MLLT10*	F	8.2	0.9	M1	Int	46,XX,t(10;11)(p12;q14)[19]/46,XX[1]	–	+	+	Dead
30	Rare/novel	*PICALM-MLLT10 MLLT10-DNAJC1*	F	4.1	26.5	M5a	Int	46,XX,add(9)(q27),add(8)(p11.2),t(10;11)(p12;q14),-16,add(19)(p13),+r1[13]/46,XX[7]	–	–	–	Alive
436	Rare	*PICALM-MLLT10*	M	10.2	29.9	M5a	Int	46,XY,add(1)(p36.1),add(5)(q31),add(7)(q22),del(9)(q),t(10;11)(p12;q14),add(17)(p11.2)[1]/46,sl,add(3)(q11.2),-9,+mar1[4]/46,sdl1,-4,-15,+der(?)t(?;4)(?;q12),+mar2[11]/46,XY[4]	–	+	+	Alive
161	Novel	*TBL1XR1-RARB*	F	0.9	30.5	M3	Int	47,XX,+6[2]/46,XX	–	+	+	Alive
5	Rare	*RBM15-MKL1*	M	0.8	12.0	M7	Int	61,XXX,der(1)t(1;22)(p13;q13),t(1;22)(p13;q13),-3,-4,-5,+7,-9,-11,-12,-13,-15,-18,+19,-22[10]/46,XX[10]	–	+	+	Dead
162	Rare	*RBM15-MKL1*	F	0.1	42.2	M7	Int	46,XY,der(1)t(1;22)(p13;q13)add(1)(q32),der(22)t(1;22)(add(1)(p22)[15]/46,XY[5]	–	–	–	Alive

F, female; Int, intermediate; M, male; SCT, stem cell transplantation; WBC, white blood cell count.

Table 2. Univariate and multivariate Cox regression analysis of OS in AML-05 trial

	No. of deaths/patients		Univariate analysis				Multivariate analysis			
					95% CI				95% CI	
OS	Negative	Positive	HR	*P*	Lower	Upper	HR	*P*	Lower	Upper
CBFB-MYH11	93/338	1/31	0.11	.03	0.02	0.80	0.08	.01	0.01	0.58
Biallelic *CEBPA*	93/346	1/23	0.16	.07	0.02	1.13	0.10	.02	0.01	0.69
RUNX1-RUNX1T1	86/263	8/106	0.19	<.001	0.09	0.39	0.17	<.001	0.08	0.35
NPM1	93/351	1/18	0.19	.10	0.03	1.35	0.09	.02	0.01	0.64
KIT	80/283	14/86	0.43	.02	0.21	0.85				
KMT2A rearrangement	75/308	19/61	1.10	.73	0.64	1.88				
PICALM-MLLT10	93/366	1/3	1.22	.84	0.17	8.78				
WT1	86/345	8/24	1.33	.44	0.64	2.74				
KAT6A-CREBBP	93/366	1/3	1.40	.74	0.19	10.1				
RBM15-MKL1	93/367	1/2	1.76	0.57	0.24	12.7				
MECOM overexpression	71/312	23/57	1.91	.007	1.20	3.07				
KMT2A-PTD	89/356	5/13	2.28	.04	1.06	4.94				
FLT3-ITD	70/322	24/47	2.92	<.001	1.83	4.65	2.53	<.001	1.57	4.08
CBFA2T3-GLIS2	87/358	7/11	3.00	.005	2.54	6.48				
PRDM16 overexpression	52/285	42/84	3.09	<.001	2.06	4.65				
KMT2A-AFDN	91/365	3/4	3.15	.051	1.00	9.97				
NUP98-KDM5A	91/363	3/6	3.20	.049	1.01	10.2				
RUNX1	89/361	5/8	3.80	.004	1.54	9.38				
DEK-NUP214	91/365	3/4	4.91	.007	1.39	15.6				
NUP98-NSD1	85/358	9/11	5.07	<.01	2.54	10.1				
FUS-ERG	89/364	5/5	6.80	<.001	2.75	16.8	4.54	<.001	1.81	11.4
RPN1-MECOM	92/367	2/2	7.84	.04	1.92	32.0	5.44	.02	1.32	22.4

GVHD, graft-versus-host disease; TMA, thrombotic microangiopathy.

overexpressed in patients with *KMT2A*-rearrangements. Herein, we showed the detail of gene expression pattern focused on *HOXAs*, *HOXBs*, *PRDM16*, *FLT3*, *RUNX1*, and *HIST1s* in Figure 3B.

Discussion

Using RNA-seq, we identified 54 in-frame gene fusions and 1 out-of-frame fusion in 53 of 139 patients with pediatric AML. Moreover, we found ≥258 gene fusions in 369 patients (70%) through RT-PCR and RNA-seq. Only a few fusion genes were detected in patients with AML with a normal karyotype. Only 2 patients had 2 in-frame gene fusions, which resulted from 3-way translocations (*PICALM-MLLT10* and *MLLT10-DNAJC1*; *NPM1-CCDC28A* and *TRIP12-NPM1*). This finding suggested that most of the in-frame gene fusions are able to facilitate AML. In the remaining 111 patients, *KMT2A*-PTD, biallelic *CEBPA*, and *NPM1* mutations were found in 11, 23, and 17 patients, respectively. These mutations were completely mutually exclusive with any gene fusions. The *FLT3*-ITD alteration was more frequently observed in patients with adverse risk factors than in those with favorable risk factors, such as co-occurring mutations, and was considered to amplify the malignant potential.

In this study, we identified 5 novel gene rearrangements, namely, *NPM1-CCDC28A*, *TRIP12-NPM1*, *NPM1-DNAJC1*, *TBL1XR1-RARB*, and *RUNX1-FNBP1*, and 17 rare fusions. Concerning patients with *NPM1* rearrangements, *TRIP12* encodes E3 ligase, which ubiquitinates *CDKN2A*. *NPM1* is a major component of the nucleolus, with important functions in regulating cell growth, proliferation, and transformation. Intriguingly, as an additional important effect, *NPM1* stabilizes the protein levels of CDKN2A.[32] *CDKN2A* is known to suppress aberrant cell growth by activating the *p53* response.[33] The function of *CDKN2A* may be inhibited by fusing *NPM1* and *TRIP12*. *NPM1-MLF1* has been associated with therapy-related AML and myelodysplastic syndrome with good prognosis.[34,35] Our patient was diagnosed as RAEB-T and maintained CR for >3 years (Table 1). Regarding the roles of *NPM1-MLF1* and *NPM1-CCDC28A*, the fusion resulted in the loss of DNA/RNA-binding domains. This may deregulate the function of *NPM1* and facilitate leukemogenesis because the mutational hotspot is located within the DNA/RNA binding domain at exon 12. Interestingly, *CCDC28A* has been reported as the partner gene of *NUP98* in T-ALL.[36] Regarding the *RUNX1-CBFA2T2* gene fusion, the expression of the *CBFA2T2* gene was high in AML cells following the activation of the *RUNX1* promoter compared with that observed in normal bone marrow. Thus, the *RUNX1-CBFA2T2* gene fusion may be associated with leukemogenesis.[37] In addition, *RUNX1-CBFA2T3* showed a gene expression pattern similar to that of *RUNX1-RUNX1T1*.[38] Moreover, we identified novel out-of-fusion of *RUNX1-FNBP1* (Figure 2). The affected *RUNX1* is truncated at the end of the runt homolog domain, forming a stop codon within the *FNBP1* gene, 88 to 90 bp downstream of the

Table 3. The cause of death of pediatric patients with favorable alterations

RNA no.	Alterations	Mutations	Sex	Age, y	WBC, 10^9/L	FAB	Chromosome	Risks	Relapse	Cause of death
13	*RUNX1-RUNX1T1*	*KIT*	M	15.7	29.2	M2	46,XY,t(8;21)(q22;q22) [20]	Low	+	Acute GVHD grade
31	*RUNX1-RUNX1T1*	—	M	17.9	6.9	M2	45,X,-Y,t(8;21)(q22;q22)[18]/46,XY[2]	Low	+	Dyspnea due to GVHD and TMA
134	*RUNX1-RUNX1T1*	*KIT*	F	3.6	11.7	M2	46,XX,t(8;21)(q22;q22)[12]/46,XX[8]	Low	+	Infection, cardiac failure
146	*RUNX1-RUNX1T1*	—	M	5.4	9.8	RAEB-T	45,X,-Y,t(8;21)(q22;q22)[20]	Low	+	Primary disease
172	*RUNX1-RUNX1T1*	—	M	7.8	9.3	RAEB-T	45,X,-Y,t(8;21)(q22;q22)[19]/46,XY[1]	Low	+	Primary disease
200	*RUNX1-RUNX1T1*	*KIT*	F	6.1	18.7	M2	46,XX,t(8;14;21)(q22;q24;q22)[20]	Low	+	Primary disease
355	*RUNX1-RUNX1T1*	-	M	14.5	19.2	RAEB-T	46,XY,t(8;18;21)(q22;q21;q22)[12]/46,XY[2]	Low	+	Sepsis
383	*RUNX1-RUNX1T1*	*KIT, RUNX1*	M	9.9	38.7	M2	46,XY,t(8;19)(q22;p13) or t(8;19;21)(q22;p13;q22)[20]	Non-CR	–	Primary disease
209	*CBFB-MYH11*	*FLT3*-ITD	M	16.2	172.2	M4Eo	48,XY,+6,+8,inv(16)(p13q22)[8]/49, sl,+21[5]/ 50, sl1,+10[5]/ 49, sl2, +8[2]/49, sl3,+11[2]	Induction death	–	Intracranial hemorrhage (day 16)
26	biallelic *CEBPA*	—	M	6.8	10.7	M1	46,XY[20]	Intermediate	–	ARDS
97	*NPM1*	—	F	11.6	38.2	RAEB-T	47,XX,+18[1]/46,XX[19]	Intermediate	+	Primary disease

break point (Figure 2A). In this study, patients with *RUNX1-CBFA2T3*, *RUNX1-CBFA2T2*, and *RUNX1-FNBP1* maintained CR without relapse for >3 years. On the other hand, the patient with *RUNX1-PRDM16* did not achieve CR and eventually expired owing to primary disease following hematopoietic stem cell transplantation in non-CR. This patient showed the highest *PRDM16* gene expression among the 369 patients with AML, whereas other patients with *RUNX1* fusions showed low *PRDM16* expressions. This finding indicates that the *RUNX1-PRDM16* fusion gene has a function distinct from those of other *RUNX1* fusions (eg, *RUNX1-RUNX1T1*) in terms of pathogenesis. *RUNX1-PRDM16* has already been reported in myelodysplastic syndrome, de novo AML, therapy-related AML, and chronic myelogenous leukemia and is associated with high *PRDM16* gene expression and dismal outcomes.[39-43] We showed the genetic landscape of patients with AML with high levels of *PRDM16* expression (supplemental Figure 4). Many adverse risk factors were observed in patients with high *PRDM16* gene expression, and numerous patients died, indicating that high *PRDM16* expression is associated with high-risk factors and dismal outcomes.[4]

Previous studies suggested *MYB-GATA1* as the cause of acute basophilic leukemia.[44] This fusion gene commits myeloid cells to the granulocyte lineage, blocking their differentiation.[44] This fusion gene has been reported to be a cause of infant AML FAB-M5 as a result of reducing the expression of the *GATA1* gene.[45] Recently, this fusion was also reported in a pediatric patient with acute erythroid leukemia.[46] The break point was quite the same as in our patient, and the characteristics were very similar, including age, FAB-M5, and favorable outcomes. Thus, the phenotype may depend on the co-occurrence of genomic alterations.

It has been reported that *ETV6-NCOA2* in acute leukemia is associated with the coexpression of T-lymphoid and myeloid markers. Our patient was diagnosed with peroxidase-negative acute leukemia including minimally differentiated FAB-M0. As blast cells were positive for CD7, CD13, CD33, CD34, CD56, CD117, and HLA-DR by surface marker analysis, myeloid-natural killer cell acute leukemia should be considered. Consistent with the results of previous studies, this finding suggests that this fusion gene may be specific for a common myeloid-lymphoid progenitor cell.[47,48] Intriguingly, similar to *ETV6-NCOA2*, *ETV6-CTNNB1* has been previously reported in patients with T-cell acute lymphoblastic leukemia.[49] *ETV6-MECOM* was previously reported in patients with chronic myelogenous leukemia or therapy-related leukemia.[50] Our patient with this fusion showed the eighth highest *MECOM* expression among the 369 patients. *ETV6* may promote the expression of the *MECOM* gene and turn on the critical role in progression to the blast crisis of AML. Interestingly, all 3 patients with *MECOM* rearrangements and 1 patient with *PRDM16* rearrangement harbored monosomy 7, suggesting that PRDM alterations may be associated with monosomy 7, and showed dismal outcomes (supplemental Table 3).

Of the 61 patients (16%) with *KMT2A* rearrangements, 16 patients did not show 11q23 breakage through conventional chromosomal G-banding. This suggested that fluorescence in situ hybridization analysis of *KMT2A* may be useful for the detection of rare *KMT2A* rearrangements, because >100 partner genes are already known (supplemental Table 5). *KMT2A-MLLT3* was the most common *KMT2A* rearrangement. Moreover, *MECOM* gene expression was

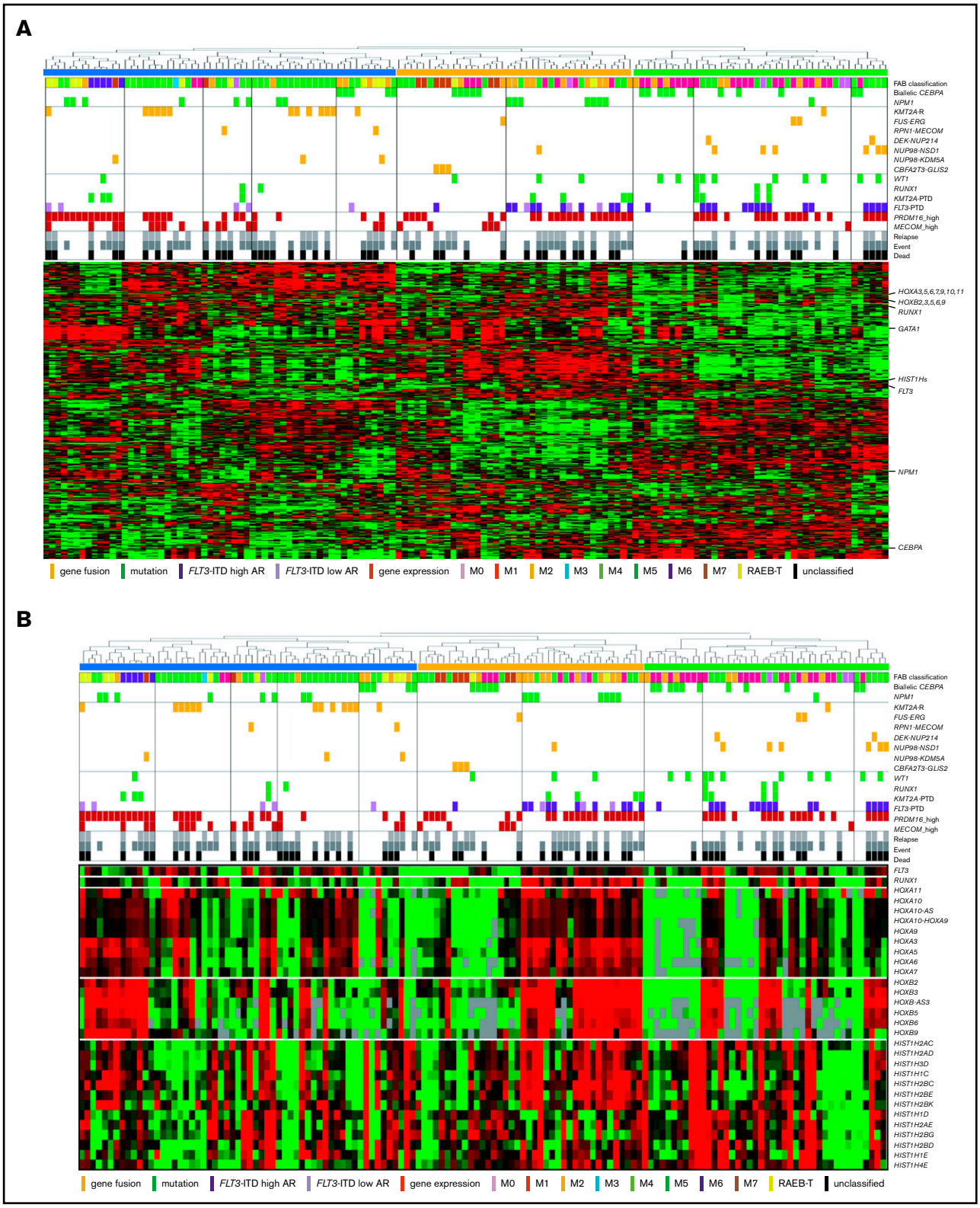

Figure 3. Unsupervised hierarchical clustering analysis of the 139 patients with pediatric AML. (A) Two-dimensional hierarchical clustering analysis of the 139 patients was performed using 3106 probe sets that were differentially expressed in 139 patients. (B) We showed the detail of gene expression pattern focused on the *FLT3*, *RUNX1*, *HOXA*s, *HOXB*s, and *HIST1*s in 139 patients. Each column represents a patient and each row represents a probe set. FAB classification, biallelic *CEBPA*, *NPM1*, *WT1*, *RUNX1*, *KMT2A*-PTD, *KMT2A* rearrangements, adverse gene fusions, *PRDM16* and *MECOM* gene expression status, and outcome of each patient are indicated. Relative expression levels normalized to the average for each probe set are indicated by color, where red and green represent high and low expression, respectively. (C) Messenger RNA expression levels of *FLT3*, *RUNX1*, *PRDM16*, *HOXA7*, *HOXB3*, *HOXB6*, *HIST1H2BD*, and *HIST1H1C* in 139 AML patients. *KMT2A*-R, *KMT2A* rearrangement.

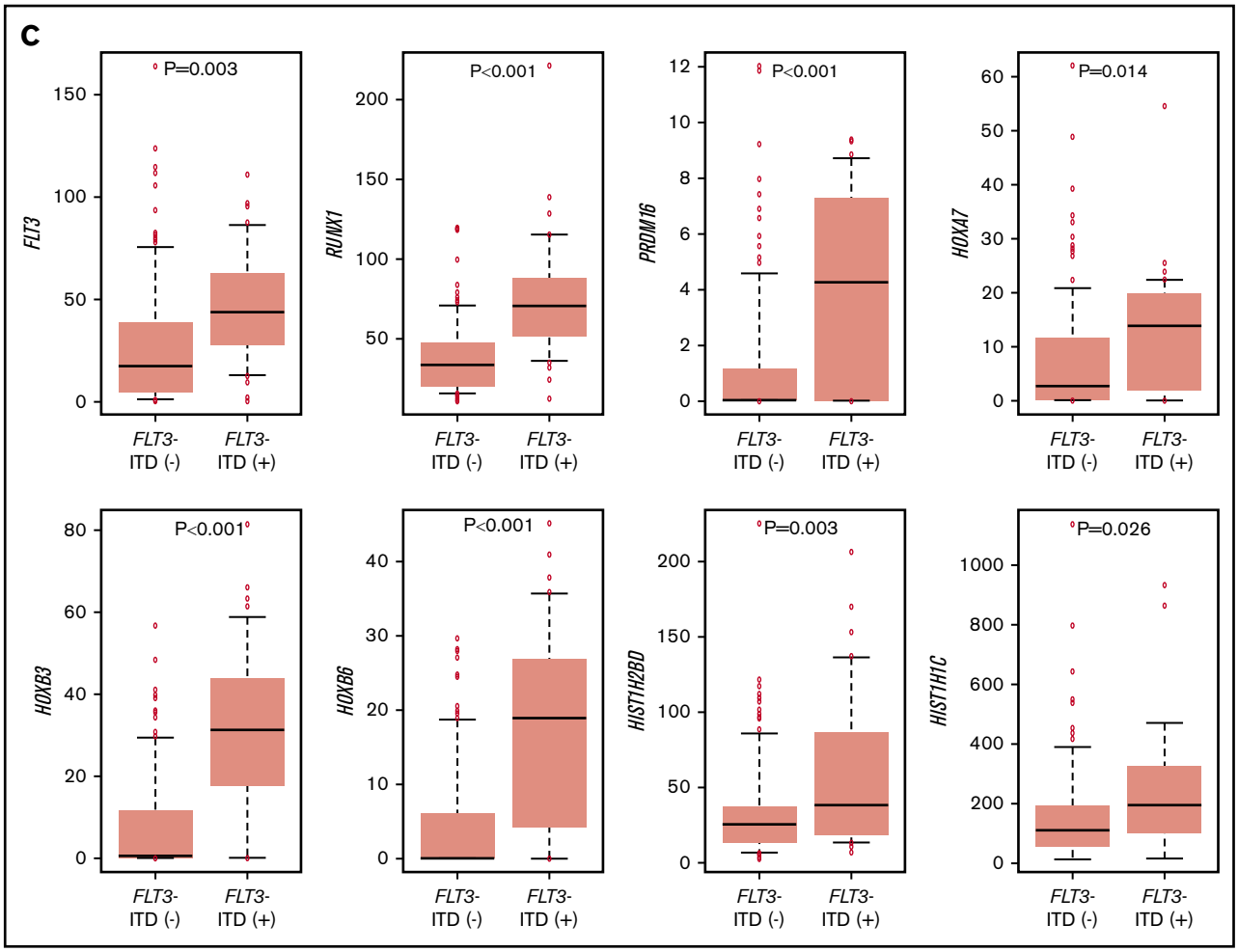

Figure 3. (Continued).

useful in stratifying the prognosis of patients with *KMT2A-MLLT3* (low expression [3-year OS, 92%; 3-year EFS, 86%] vs high expression [3-year OS, 39%; 3-year EFS, 9%]). However, the expression of the *MECOM* gene was not correlated with poor prognosis in patients with FAB-M7. One of the reasons for this may be that *MECOM* expression is also associated with the differentiation of megakaryocytes, despite the association of high *MECOM* expression with stem cell maintenance through the expression of the *GATA2* gene in other FAB subtypes.[51] *KMT2A-MLLT10*, *ELL*, and *MLLT1* were common partner genes found in 9, 7, and 5 patients with de novo pediatric AML, respectively (supplemental Table 5). Of the 7 patients with *KMT2A-ELL*, 3 patients expired. Intriguingly, these 3 patients were <1 year old, and 2 of them died due to ARDS. On the other hand, the prognosis of patients with *KMT2A-MLLT10* tended to be worse than that of patients with other *KMT2A* gene fusions (ie, 3 of 9 patients died due to primary disease) (supplemental Table 5). Consistent with the findings of previous studies,[52] the prognosis of patients with *KMT2A-AFDN* was extremely dismal, indicating a different pathogenesis from other *KMT2A* rearrangements.

The co-occurrence and mutual exclusivity test identified that various clinical biomarkers may reflect different aspects of the same poor prognostic subgroup of patients with AML (Figure 4). *RUNX1-RUNX1T1* and *CBFB-MYH11* formed a unique entity that was mutually exclusive of any fusions and mutations other than *KIT* mutation. High *MECOM* expression was significantly associated with *KMT2A*-R and monosomy 7, whereas high *PRDM16* gene expression was significantly associated with *FLT3*-ITD, *KMT2A*-PTD, and trisomy 8. These could potentially be used as surrogate biomarkers for this distinct subgroup, which shares several molecular signatures and predict the prognosis.

In view of the gene expression results, *HOX* genes were upregulated in patients with *FLT3*-ITD or *KMT2A*-rearrangements. Moreover, overexpression of some *HOX* is known to enhance the self-renewal of hematopoietic stem and progenitor cells and perturb differentiation.[53] Additionally, *FLT3*, *RUNX1*, and some *HIST1*s were upregulated in these patients, suggesting that the aberrant expression of these genes plays a crucial role in leukemogenesis and indicates poor prognosis. Tiberi et al reported that the expression of *HIST1* was significantly higher in patients with AML with low levels of H3K27me3 than in those with high levels of H3K27me3.[54] Repressing H3K27me3 levels affects the transcriptional repression in patients with AML harboring aberrant expression of *HOX*, *FLT3*, *RUNX1*, and *HIST1*.

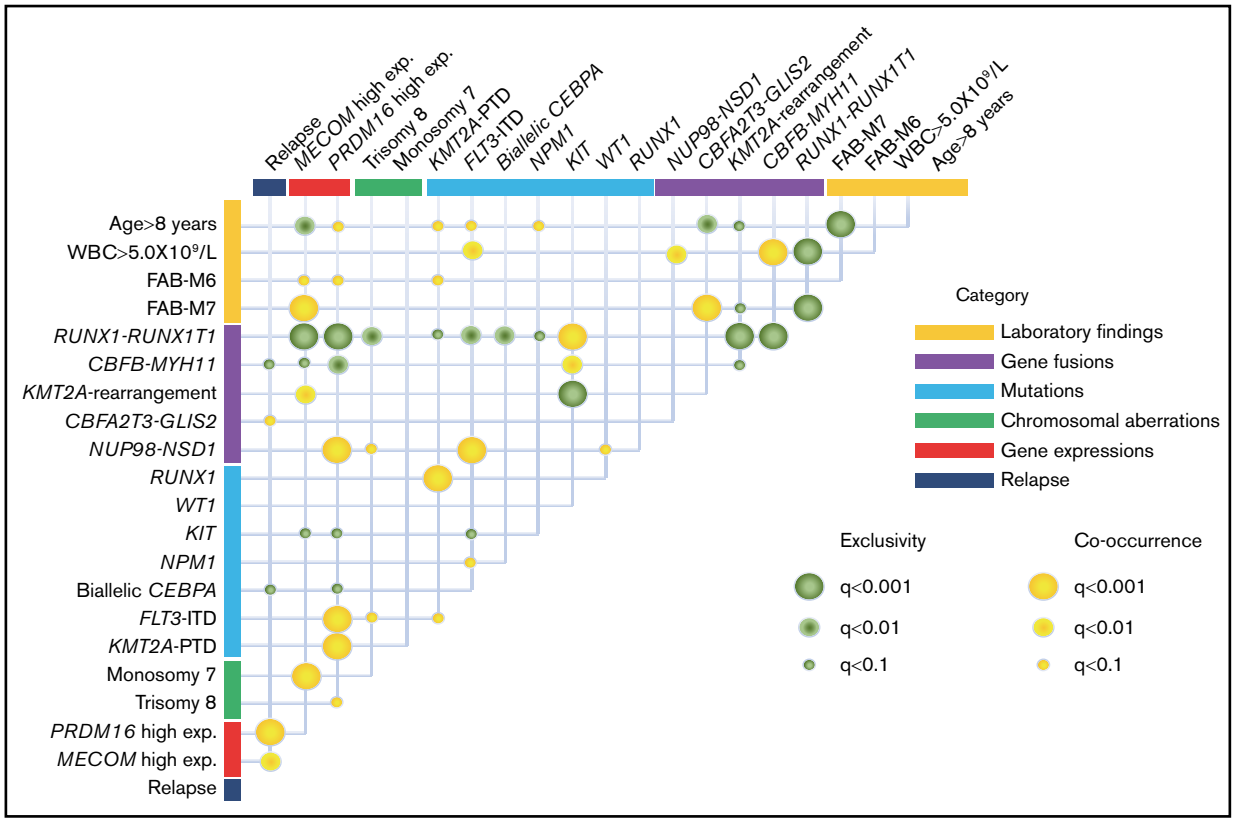

Figure 4. Correlations between established risk factors for pediatric AML. The diagram indicates co-occurrence or mutual exclusivity between pairs of clinical features, genetic alterations, gene expressions, and outcomes. The circles indicate that crossed factors are statistically co-occurrent (yellow) or exclusive (green).

In the present study, it was shown that RNA-seq unmasked the complexity of gene alterations in pediatric AML. We identified potentially disease-causing alterations in nearly all patients with AML, including novel gene fusions. Our results indicated that a subset of patients with pediatric AML represent a distinct entity that may be discriminated from their adult counterparts through an investigation of the spectrum of gene rearrangements and mutations. Furthermore, we found that a complex interplay of genetic events contributes to the pathogenesis of AML in individual patients. These findings suggest that gene rearrangements in conjunction with mutations play essential roles in pediatric AML. Based on the results of the RNA-seq, the prognostic factors currently used for risk stratification in pediatric AML may be reconsidered, particularly for intermediate/high-risk patients.

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Authorship

Contribution: N.S. and Y. Hayashi designed the study; N.S., K.Y., Y. Hara, G.Y., T.K., H.M., K.O., and A.S. performed the experiments; Y. Hayashi and S.O supervised the work; N.S., K.Y., Y. Hara, G.Y., Y.S., T.K., M.T., Y.O., and K.O. analyzed the results; N.S. constructed the figures; N.S., K.Y., and Y. Hayashi wrote the paper; and all authors critically reviewed the draft and approved the final version for publication.

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