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Clinical significance of RAS pathway alterations in pediatric acute myeloid leukemia

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Contributions

T.K., G.Y., N.S., and Y. Hara designed and performed the research, analyzed the data, and wrote the paper. Y. Hayashi designed the research, led the project, and wrote the paper. K.Y., Y.S., S.M., and S.O. performed the research. K.T. performed the research and bioinformatics analysis. K.O., M.S., H.A., H.M., A.S.,

T. Taki., N.K., D.T., K.H., T. Taga and S.A provided patient samples and data. All authors critically reviewed and revised the manuscript.

Disclosures

None.

Abstract

RAS pathway alterations have been implicated in the pathogenesis of various hematological malignancies. However, their clinical relevance in pediatric acute myeloid leukemia (AML) is not well characterized. We analyzed the frequency, clinical significance, and prognostic relevance of RAS pathway alterations in 328 pediatric patients with de novo AML. RAS pathway alterations were detected in 80 (24.4%) out of 328 patients: *NF1* (n = 7, 2.1%), *PTPN11* (n = 15, 4.6%), *CBL* (n = 6, 1.8%), *NRAS* (n = 44, 13.4%), *KRAS* (n = 12, 3.7%). Most of these alterations were mutually exclusive and were also mutually exclusive with other aberrations of signal transduction pathways such as *FLT3*-ITD ($p = 0.001$) and *KIT* mutation ($p = 0.004$). *NF1* alterations were frequently detected in patients with complex karyotype ($p = 0.031$) and were found to be independent predictors of poor overall survival (OS) in multivariate analysis ($p = 0.007$). At least four of seven patients with *NF1* alterations had bi-allelic inactivation. *NRAS* mutations were frequently observed in patients with *CBFB-MYH11* and were independent predictors of favorable outcomes in multivariate analysis [OS, $p = 0.023$; event-free survival (EFS), $p = 0.037$]. Patients with *PTPN11* mutations more frequently received stem cell transplantation ($p = 0.035$) and showed poor EFS

than patients without *PTPN11* mutations ($p = 0.013$). Detailed analysis of RAS pathway alterations may enable a more accurate prognostic stratification of pediatric AML and may provide novel therapeutic molecular targets related to this signal transduction pathway.

Introduction

Acute myeloid leukemia (AML) is characterized by considerable genetic heterogeneity. Several chromosomal aberrations and gene alterations have been identified in these patients; some of these have been found useful for risk stratification.¹ Aberrations of signal transduction pathways (such as RAS family members, *KIT*, and *FLT3*) are considered as one of the most important pathogenetic factors in AML.²

Recently, aberrations of *NF1* and *PTPN11* were reported to be associated with a poor prognosis in adult patients with AML.^{3,4} *NF1* and *PTPN11* are the family of RAS pathway genes and constitute the granulocyte-macrophage colony stimulating factor signaling pathway. Among the broad family of RAS pathway genes, mutations of *CBL*, *NRAS* and *KRAS* were also commonly detected in AML.² These RAS pathway alterations have also been implicated in the causation of juvenile myelomonocytic leukemia (JMML).⁵

Mutations of *PTPN11*, *NRAS*, and *KRAS* have been reported in 3%–4%,^{6,7} 7%–13%, 6%–11%^{8,9} of pediatric patients with AML, respectively. However, there is no clear consensus on the clinical significance of RAS pathway gene mutations especially *NF1* and *CBL* mutations.^{10,11} The reported frequency of

detection of *CBL* mutations and *NF1* mutations or deletions in adult patients with AML is 0.6%–0.7%^{12,13} and 3.5%–10.5%¹⁴⁻¹⁶, respectively. However, the prognostic relevance of these mutations is not well characterized, particularly in pediatric AML patients.

In this study, we analyzed *NF1*, *PTPN11*, *CBL*, *NRAS*, and *KRAS* alterations in 328 pediatric patients with AML to determine the clinical significance of these alterations. We also examined the correlation of RAS pathway alterations with other genetic aberrations, cytogenetic alterations, and clinical characteristics.

Methods

Patients

Between November 2006 and December 2010, 443 pediatric patients with de novo AML (age <18 years) participated in the Japanese AML-05 trial conducted by the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG). Treatment, data collection, and other details of the AML-05 study are presented in Supplementary Methods and Supplementary Figure S1. This study was conducted in accordance with the Declaration of Helsinki and approved by the

institutional review board of the Gunma Children's Medical Center and the ethical review board of the JPLSG.

Mutation analysis of RAS pathway alterations

We analyzed *PTPN11* (exons 2–4, and 13), *CBL* (exons 8–9), *NRAS* (exons 1–2), and *KRAS* (exons 1–2) mutations using Sanger sequencing according to the previous studies.^{9,17,18} For *NF1* gene, all coding exons were captured using the SureSelect custom kit (Agilent Technologies, Santa Clara, CA, USA), and sequenced using HiSeq 2500. Somatic mutations in *NF1* were identified as described elsewhere.¹⁹

Molecular characterization

We analyzed *KIT* (exons 8, 10, and 17)²⁰, *NPM1* (exon 12)²¹, *CEBPA* (exons 1–4)²², *CSF3R* (exons 14 and 17)²³, *WT1* (exons 7–10)²⁴, *ASXL1* (exon 12), *ASXL2* (exons 11 and 12)²⁵, all exons of *BCOR*, *BCORL1*²⁶, *RAD21*, *SMC3*, *STAG2*²⁷, *RUNX1*²⁸, *FLT3-ITD*²⁹, and gene rearrangement of *NUP98-NSD1*³⁰ and *FUS-ERG*³¹ using Sanger sequencing. *KMT2A*-partial tandem duplication (PTD) was analyzed using the multiplex ligation-dependent probe amplification

(MLPA) method.³² 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, Foster City, CA, USA), as described elsewhere.³³

Copy number analysis

Copy number (CN) analysis was performed as previously reported³⁴ using an in-house pipeline CNACS (https://github.com/papaemmelab/toil_cnacs); the total number of reads covering each bait region and the allele frequency of heterozygous single-nucleotide polymorphisms (SNP) (n = 1,216) detected by targeted sequencing were used as input data. Based on the previous reports¹⁵, we set the total CN < 1.5 as the definition of *NF1* deletion.

Statistical methods

All statistical analyses were performed using the EZR software (version 1.35; Saitama Medical Center, Jichi Medical University, Saitama, Japan).³⁵

Between-group differences with respect to clinical characteristics were assessed using the Fisher's exact and Mann-Whitney U tests. Survival rates were estimated using the Kaplan–Meier method and compared using the log-rank test.

Overall survival (OS) was defined as the time from diagnosis to death or last follow-up. Event-free survival (EFS) was defined as the time from diagnosis to the date of failure (induction failure, relapse, second malignancy, or death) for patients who experienced treatment failure or to the date of last contact for all other patients. Cox proportional hazards model was used to estimate hazard ratios and 95% confidence intervals. For all analyses, two tailed p -values < 0.05 were considered indicative of statistical significance.

Results

Frequencies of RAS pathway alterations in 328 pediatric AML patients

Out of the 443 patients, 115 patients were excluded from this study because of unavailability of genomic DNA samples. Therefore, 328 samples were analyzed in this study. We did not analyze germline alterations because of the lack of nonhematological or remission samples. The clinical characteristics of patients with available samples ($n = 328$) and those with no available samples ($n = 115$) are summarized in Supplementary Table S1. White blood cell (WBC) count at diagnosis was significantly higher in the sample available group than in the sample unavailable group ($p < 0.001$). There were more patients who were at a

low risk and there were less patients who were at an intermediate risk in the sample available group as compared with the sample unavailable group (low risk, $p = 0.046$; intermediate risk, $p = 0.003$). Cytogenetic features and prognosis were not significantly different between the available and unavailable samples (Supplementary Table S1).

RAS pathway alterations were detected in 80 (24.4%) of the 328 patients; most of these alterations were mutually exclusive (Figure 1). The mutation sites and clinical characteristics of patients with RAS pathway alterations are summarized in Figure 2 and Tables 1-2, Supplementary Tables S2-3, respectively.

We detected six *NF1* mutations in four patients; all of these were frameshift or nonsense mutations (Figures 1-2). Two patients concomitantly had two types of mutations, respectively (Table 1). In addition, we also detected four patients with microdeletion within chromosome 17q containing *NF1* (Table 1, Supplementary Figure S2). Since one patient had both *NF1* mutation and CN alteration, *NF1* alterations were detected in 7 (2.1%) patients (Figure 1, Table 1). UPN 57 and 415 were considered to have heterozygous deletion. Besides, UPN 57 with variant allele frequency (VAF) 0.83 was considered to have nonsense

mutations in the remaining allele. UPN 50 with VAF 0.94 had 17q uniparental disomy (UPD) (Supplementary Figure S2). UPN 105 and 333 had two or three different CN regions in *NF1*, respectively. These were considered to have partially homozygous deletion. Other two patients (UPN 262 and 367) had two types of mutations each. However, it was not clear whether these alterations were mono-allelic or bi-allelic (UPN 262, VAF 0.28 and 0.26; UPN 367, VAF 0.28 and 0.08). Thus, we concluded that at least four patients (UPN 50, 57, 105, and 333) had bi-allelic *NF1* inactivation. Next, on the basis of the VAF of each mutation, we estimated whether *NF1* mutations were somatic or germline. If a mutation is a heterozygous germline mutation, then the VAF would be around 0.5.³⁶ We considered mutations with UPN 262 (VAF 0.28 and 0.26) and 367 (VAF 0.28 and 0.08) as somatic mutations. Regarding UPN 50 (VAF 0.94) and 57 (VAF 0.83), it was not possible to predict whether these mutations were somatic or germline because their VAF were high owing to their coexistence with heterozygous deletion or UPD. On the contrary, these two mutations were determined as somatic in the COSMIC v90 (URL: <https://cancer.sanger.ac.uk/cosmic>). R1241X detected in UPN 57 was previously observed in adult AML and E1561X detected in UPN 50 was previously detected

in nonhematological malignancies.^{37,38}

PTPN11 mutations were detected in 15 (4.6%) patients (Figure 1). Of these, 14 were located in exon 3 or exon 13, which are known mutation hotspots in AML and JMML (Figure 2).³⁹ As previously observed³⁹, codon 76 represented a mutational hot spot (4/15, 27%) with three different amino acid substitutions (Figure 2), and 13 of the 15 mutations have been reported as somatic mutations.³⁹⁻⁴¹ Although the remaining two mutations (V45L and T493I) have not been confirmed as somatic mutations, V45L was earlier detected in lung carcinoma and showed an association with activation of protein-tyrosine phosphatase.⁴¹ However, T493I has not been reported in any hematological or other disease. These two variants have not been reported as SNPs on any database such as COSMIC v90, ClinVar, mutations taster, Ensembl GRCh37, or db SNPs (URL: <https://www.ncbi.nlm.nih.gov/snp/>, <https://www.ncbi.nlm.nih.gov/snp/>, <http://grch37.ensembl.org/index.html>, <https://www.ncbi.nlm.nih.gov/clinvar/>, and <http://mutationtaster.org/>); therefore, we recognized these as novel disease-causing mutations.

CBL mutations were found in six (1.8%) patients (Figure 1). Among these, four were deletions or insertions and deletions in exon 8 and two were missense

mutations in exon 9. Five of these mutations were in the linker region or the RING finger domain which were previously reported as the affected regions in myeloid malignancies with *CBL* mutations (Figure 2).^{12,13,18} None of the six mutations have been reported as SNPs or germline mutations in any online databases or previous reports.⁴² *CBL* mutations especially missense mutations were shown to exhibit a strong association with 11q-acquired UPD.¹⁸ 11q UPD was detected in only one patient with missense mutation (UPN 97) by CN analysis (Supplementary Figure S2).

NRAS and *KRAS* mutations were detected in 44 (13.4%) and 12 (3.7%) patients, respectively (Figure 1). All *NRAS* and *KRAS* mutations were missense mutations in codon 12, 13, or 61, which are well known hotspots (Figure 2).⁴³ Six patients concomitantly had two missense mutations in *NRAS*.

Clinical and cytogenetic characteristics of patients with RAS pathway alterations

The clinical characteristics of patients with RAS pathway alterations are summarized in Supplementary Table S4. Patients with RAS pathway alterations showed significantly higher frequency of detection of monosomy 7 as compared to those without RAS pathway alterations ($p < 0.001$). *FLT3*-ITD and *KIT*

mutations were significantly less frequent in patients with RAS pathway alterations (*FLT3*-ITD; $p = 0.001$, *KIT* mutations; $p = 0.004$). Age, gender, or relapse rate were not significantly different between patients with or without each specific RAS pathway alteration.

Patients with *CBL* mutations had significantly higher WBC count at diagnosis ($p = 0.026$, Supplementary Table S4, Supplementary Figure S3). The frequency of stem cell transplantation (SCT) was significantly higher in patients with *PTPN11* mutations ($p = 0.035$), and significantly lower in patients with *NRAS* mutations ($p = 0.022$, Figure 1, Supplementary Table S4). *PTPN11* mutations were significantly fewer ($p = 0.024$) in patients with low risk, i.e., core binding factor (CBF)-AML, and *NRAS* mutations were significantly higher ($p = 0.017$) in these patients (Figure 1, Supplementary Table S4). The frequency of detection of *NF1* alterations was significantly higher in patients with complex karyotype ($p = 0.031$) and *MECOM* high expression ($p = 0.013$, Figure 1, Supplementary Table S4). *PTPN11* mutations were significantly more frequently detected in patients with monosomy 7 ($p = 0.047$), *RUNX1* mutations ($p = 0.004$), *PRDM16* high expression ($p = 0.002$), and *MECOM* high expression ($p = 0.004$) (Figure 1, Supplementary Table S4). *NRAS* mutations were frequently detected

in *inv(16)(p13q22)/CBFB-MYH11* ($p = 0.001$) and monosomy 7 ($p = 0.013$). *NRAS* mutations were also mutually exclusive with *FLT3-ITD* ($p = 0.005$) and *KIT* mutations ($p = 0.040$) (Figure 1, Supplementary Table S4). Although there was no significant difference, three of 6 patients with *CBL* mutations were identified in CBF-AML ($p = 0.411$) (Figure 1, Supplementary Table S4).

Prognosis of patients with RAS pathway alterations

We analyzed the prognosis of patients with or without RAS pathway alterations using the Kaplan–Meier method (Figure 3, Supplementary Figure S4). Despite the small sample size, alterations of *NF1* and *PTPN11* showed a significant association with poor prognosis. Although there was no significant difference in EFS between patients with or without *NF1* alterations, the OS of patients with *NF1* alterations was significantly worse than that of patients without *NF1* alterations (2-year OS, 42.9% vs. 82.3%, $p = 0.003$) (Figure 3 A-B). Although no significant differences were observed in OS, *PTPN11* mutations were significantly associated with poor EFS (2-year EFS, 30.0% vs. 59.8%, $p = 0.013$) (Figure 3 C-D). The OS and EFS of patients with *NRAS* mutations were significantly better than those of patients without *NRAS* mutations (2-year OS,

97.7% vs. 79.0%, $p = 0.014$; 2-year EFS, 74.9% vs. 55.9%, $p = 0.021$) (Figure 3 E-F). Presence of *CBL* or *KRAS* mutations showed no significant impact on prognosis (Supplementary Figure S4). With respect to prognosis, patients with *CBL* mutations were divided into two distinct groups based on the presence of CBF. All CBF-AML patients with *CBL* mutations achieved complete remission and were alive. However, all non-CBF-AML patients relapsed and died (Table 2).

Next, we performed multivariate analysis using the Cox regression analysis to determine the prognostic impacts of RAS pathway alterations (Table 3). Besides RAS pathway mutations, we used $t(8;21)(q22;q22)/RUNX1-RUNX1T1$, *CBFB-MYH11*, monosomy 7, complex karyotype, *FLT3-ITD*, 5q-, *FUS-ERG*, *NUP98-NSD1*, and *PRDM16* high expression as explanatory variables in the multivariate analysis; these cytogenetic aberrations were used for risk classification in the AML-05 trials (Supplementary Figure S1) or were recently shown to affect the prognosis.^{33,44} Remarkably, *NF1* alterations were associated with inferior OS in multivariate analysis [hazard ratio (HR), 4.109; 95% confidence interval (CI), 1.471–11.48; $p = 0.007$] (Table 3). In univariate analysis, *PTPN11* mutation was associated with inferior EFS (HR, 2.142; 95% CI, 1.157–3.965; $p = 0.015$) (Table 3). However,

PTPN11 mutation was not associated with inferior EFS (HR, 1.239; 95% CI, 0.616–2.494; $p = 0.548$) in multivariate analysis; this indicated that co-occurring aberrations contributed to worse outcomes (Table 3). In multivariate analysis, *NRAS* mutation was a favorable prognostic factor for both OS and EFS (OS: HR, 0.309; 95% CI, 0.112–0.849; $p = 0.023$; EFS: HR, 0.530; 95% CI, 0.293–0.961; $p = 0.037$) (Table 3). These results suggested that alterations of *NF1* and *NRAS* were independent predictors of prognosis in pediatric patients with AML. *CBFB-MYH11* could not be evaluated accurately for OS in the Cox regression analysis because 27 patients with *CBFB-MYH11* enrolled in this study were all alive. The OS of patients with *CBFB-MYH11* was significantly better than that of patients without *CBFB-MYH11* in the Kaplan–Meier method ($p = 0.005$). (Supplementary Figure S5)

Discussion

In this study, we detected RAS pathway alterations in 80 (24.4%) out of the 328 patients with AML [*NF1* (n = 7, 2.1%), *PTPN11* (n = 15, 4.6%), *CBL* (n = 6, 1.8%), *NRAS* (n = 44, 13.4%), *KRAS* (n = 12, 3.7%)]. Most of these were mutually exclusive and were also mutually exclusive with aberrations involving other

signal transduction pathways such as *FLT3*-ITD and *KIT* mutation (Figure 1).

Loss of the wild-type allele of *NF1*, either through deletions or mutations, has been implicated in the pathogenesis of hematological malignancies.¹¹ We have summarized previous reports on *NF1* alterations in adult and pediatric AML in Supplementary Table S5. *NF1* deletions have been reported in 3.5%–10.5% of adult patients with AML; in addition, 20%–50% of patients with *NF1* deletions had concomitant *NF1* mutations in the remaining allele.¹⁴⁻¹⁶ In this study, the frequency of *NF1* alterations was less than that in previous reports pertaining to adult patients. In addition, at least four of the seven (57%) patients with *NF1* alterations had bi-allelic *NF1* inactivation (Table 1). *NF1* alterations have been frequently reported in complex karyotype AML; in addition, *NF1* alterations were shown to be associated with poor prognosis in adult AML.³ In the contemporary literature, there are few reports about *NF1* alteration in pediatric AML. Balgobind et al. detected *NF1* deletion in 2 out of the 71 AML patients with *KMT2A* rearrangement, 1 of whom experienced relapse.¹¹ Consistent with previous reports, *NF1* alterations were frequently detected in complex karyotype, and were associated with poor OS in this study (Figure 3, Table 3). None of the four patients with relapse or induction failure were rescued by SCT (Figure 1, Table

1). Our findings suggest that more intensive primary chemotherapy may be an option to rescue AML patients with *NF1* alterations including use of novel molecular targeted therapy such as mTOR inhibitors. In a study by Parkin et al., *NF1* null blasts showed sensitivity to rapamycin-induced apoptosis.^{3,14}

We also detected 15 *PTPN11* mutations including two novel mutations (Table 2). In several previous studies, *PTPN11* mutations have been reported to be associated with acute monoblastic leukemia (FAB-M5),^{4,7} however, no such tendency was observed in this study (data was not shown). *PTPN11* mutations in our cohort were frequently detected in AML, minimally differentiated (FAB-M0) ($p = 0.026$) and erythroleukemia (FAB-M6) ($p = 0.047$). Goemans et al. also reported that the prevalence of *PTPN11* was not increased in acute monoblastic leukemia (FAB-M5) suggesting that differences could exist in the ethnic background of the patients studied.⁴⁵ In a study by Alfayez et al., *PTPN11* mutation in adult AML patients was associated with adverse prognosis.⁴ However, the prognostic relevance of *PTPN11* has not been reported in pediatric AML.^{6,7} In this study, patients with *PTPN11* mutations had high frequency of *RUNX1* mutations, *MECOM* high expression, and *PRDM16* high expression which are strongly associated with poor prognosis (Figure 1, Supplementary

Table S4).^{30,33,46-48} In our study, *PTPN11* mutations were associated with poor EFS in univariate analysis; however, multivariate analysis revealed no significant impact of *PTPN11* mutations on EFS or OS (Figure 3, Table 3). A significantly greater proportion of patients with *PTPN11* mutations received SCT (Supplementary Table S4); in addition, 5 of 11 patients with events were rescued by SCT (Figure 1). We consider that AML patients with *PTPN11* mutations tended to have a high frequency of relapse or induction failure, and some of these patients were successfully rescued by SCT.

Consistent with a previous report,⁴⁹ *NRAS* mutations were significantly more frequently detected in *CBFB-MYH11* (Figure 1, Supplementary Table S4). Previous studies have found inconsistent evidence of the clinical significance of *NRAS* mutations.^{8,9} In the present study, *NRAS* mutations were associated with favorable prognosis. This seemed attributable to the characteristics of patients with *NRAS* mutations, i.e., high frequency of *CBFB-MYH11* with no other poor prognostic factors.

11q-UPD was detected in only one patient with *CBL* missense mutation, which might be consistent with a previous study reporting that somatically acquired *CBL* deletions are frequently heterozygous, whereas most missense

mutations are homozygous as a consequence of 11q-UPD.⁵⁰ We summarized previous reports on *CBL* mutations in AML in Supplementary Table S6. *CBL* mutations were previously shown to be associated with CBF-AML.¹³ In the present study, three out of six patients with *CBL* mutations had CBF-AML; however, there was no significant association in this respect (Figure 1, Table 2). Owing to the low incidence of *CBL* mutation, its prognostic significance is not well characterized.^{10,12,13} Although we did not observe any significant prognostic impact of *CBL* mutations in our cohort, all three patients without CBF experienced relapse and died (Table 2). These results might suggest that non-CBF patients with *CBL* mutation show poor prognosis.

RAS pathway alterations are also a major cause of JMML; in addition, each of these alterations are of prognostic relevance in patients with JMML.^{51,52} In previous studies, JMML patients with *PTPN11* and *NF1* mutations showed significantly poor prognosis.^{51,52} On the other hand, JMML patients with *NRAS* mutations exhibited favorable outcomes.^{51,52} In our study, the prognostic impact of *NF1*, *PTPN11*, and *NRAS* was similar to that observed in JMML. However, we are unable to explain this similarity because the transformation of JMML to AML is rare.⁵³

There may be some possible limitations in this study. First, we analyzed *PTPN11*, *CBL*, *NRAS* and *KRAS* mutations by Sanger sequencing because the mutation hotspots of these genes were well known. Although the frequency of these mutations was similar to the previous reports by Sanger sequencing,⁶⁻⁹ it appears to be lower than that of the recent pediatric report by targeted deep sequencing.¹ Next, there were a small number of patients harboring *NF1* alterations. Further investigation is needed to determine the clinical significance of *NF1* alterations in pediatric AML. Since there have been few reports on *NF1* alteration, especially in pediatric AML (Supplementary Table S5), our results might be valuable for future analysis. Lastly, we could not analyze germline alterations because of the lack of non-hematopoietic cells. Congenital alterations of RAS pathway genes are known as RASopathies predisposing to hematological malignancies.⁵⁴ Especially for *NF1* and *CBL*, it is difficult to distinguish between somatic and germline mutations because the mutation hotspots overlap. While it is sometimes difficult to diagnose RASopathy because of minor clinical symptoms, patients with distinct clinical features of AML predisposing diseases, such as neurofibromatosis, Noonan syndrome, or CBL syndrome were excluded from the AML-05 trial according to its eligibility criteria. Also, we estimated that

most of *NF1* and *CBL* mutations might be somatic from online databases and previous reports. Since there have been few reports of detailed analysis on *NF1* and *CBL* alterations in pediatric AML (Supplementary Table S5 and S6), further analyses are needed.

In conclusion, *NF1* alteration was possibly a poor prognostic factor and *NRAS* mutation was a favorable prognostic factor in pediatric patients with AML. Pediatric AML patients with *PTPN11* mutations may show a greater tendency for relapse and induction failure. Detailed analysis of RAS pathway alterations may enable a more accurate prognostic stratification of pediatric AML and may provide novel therapeutic molecular targets related to this signal transduction pathway.

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Table 1. Summary of characteristics of pediatric AML patients with *NF1* alteration.

UPN	Nucleotide change*	Amino acid change*	VAF	Copy number	Start to end	Gender	Age, y	WBC, ×10 ⁹ /L	Cytogenetics	Additional genetic aberrations	CR	Relapse	Event	SCT	Prognosis
50	c.G4681T	p.E1561X	0.94	-	-	M	13.7	19.9	45,XY,-7[13]/46,XY[7]	<i>KIT</i>	-	-	+	+	Death
262	c.2027 dupC	p.I679Dfs21X	0.28	-	-	M	12.3	159.3	46,XY,inv(16)(p13q22)[20]	<i>CBL, NRAS</i>	+	-	-	-	Alive
	c.6862_6863 insCG	p.P2289Rfs10X	0.26	-	-	M	7	9.9							
367	c.966_967 insGA	p.A323Efs54X	0.28	-	-	M	7	9.9	47,XY,+11[18]/54,ide m,+x,+10,+11,+13,+14,+20,+21[1]/46,XY[1]	<i>PTPN11</i>	+	-	-	-	Alive
	c.2027 dupC	p.I679Dfs21X	0.08	-	-	M	7	9.9							
57	c.C3721T	R1241X	0.83	1.16	1225849-29422297	M	15.2	69.0	#1	<i>RUNX1, BCORL1</i>	-	-	+	+	Death
				0.99	29485961-30325657										
				1.02	27009658-29588669										
105	-	-	-	0.29	29626467-29679186	M	10.8	15.5	#2	<i>ASXL1</i>	+	+	+	+	Death
				0.95	29683418-30325657										
415	-	-	-	1.26	1225849-29422297	F	12.3	1.9	45,XX,ins(1;?)(q21;?), add(4)(q12),add(7)(q36), der(17;18)(q10,q10)[20]	<i>PTPN11</i>	+	+	+	+	Death
				1.04	29485961-30325657										
333	-	-	-	0.22	29485961-29588669	F	9.8	4.1	46,XX,t(8;12)(q11.2;p11.2)[20]	<i>BCORL1</i>	+	-	-	-	Alive
				0.94	29626467-30325657										

UPN, unique patient number; VAF, variant allele frequency; WBC, white blood cell count; CR, complete remission; SCT, stem cell transplantation

*NCBI reference sequence; NM_00267

#1 47,X,-Y,add(3)(q11.2),+6,add(6)(p21)x2,+7,del(8)(q24)der(8)t(1;8)(q11;q24),del(11)(q?),add(17)(p11.2)[7]/48,sl,+22[6]/47,sl,-14,+mar1[2]

#2 46,XY,+Y,add(1)(p11),del(2)(q?),del(5)(q?),add(8)(p11.2),-9,-9,-11,-17,add(18)(q21),-19,add(22)(q11.2),+del(?)t(?;11)(?;q13),+mar1,+mar2,+mar3[2]/88,sl,x2,-3,-del(5)x2,-6,+9,-20,-20,-21,-mar1,-mar3x2,+5 mar[1]/47,XY,+Y[9]

Table 2. Summary of characteristics of pediatric AML patients with *PTPN11* and *CBL* mutations.

Gene	UPN	Nucleotide change*	Amino acid change*	Gender	Age, y	WBC, ×10 ⁹ /L	Cytogenetics	Additional genetic aberrations	CR	Relapse	Event	SCT	Prognosis
<i>PTPN11</i>	45	A227T	E76V	M	4.8	33.9	45,-7[1]/45,sl,t(3;12)(q26;p13)[18]/46,XY[1]	-	-	+	+	+	Death
	52	G133C	V45L	F	14.1	16.5	46,XX[20]	<i>WT1</i> , <i>KMT2A</i> -PTD	-	-	+	-	Death
	113	C215T	A72V	F	10.3	17.8	46,XX,add(12)(p11)[12]/46,XX[8]	<i>CBL</i> , <i>KRAS</i> , <i>KMT2A</i> -ELL, <i>WT1</i> , <i>STAG2</i>	+	+	+	+	Alive
	127	C218T	T73I	F	0.4	17.1	47,XX,t(7;12)(q36;p13),+19[20]	<i>RAD21</i>	+	+	+	+	Alive
	142	G1508C	G503A	M	6.9	190.5	N/A	<i>KMT2A</i> -MLLT3	+	+	+	+	Death
	156	C215A	A72D	F	11.5	4.5	46,XX[20]	<i>FLT3</i> -ITD, <i>NPM1</i>	+	-	-	+	Alive
	177	A227G	E76G	M	2.9	25.2	45,XY,-7[1]/45,sl,t(11;21)(q13;q22)[19]	-	-	-	+	+	Death
	249	G179T	G60V	F	11.8	60.1	46,XX[20]	<i>NRAS</i> , <i>KMT2A</i> -PTD, <i>RUNX1</i>	+	+	+	+	Alive
	300	C1478T	T493I	M	4.2	4.6	46,XY,t(8;21)(q22;q22)[2]/46,sl,del(9)(q?) [7]/46,XY[11]	-	+	-	-	-	Alive
	367	G226A	E76K	M	7	9.9	47,XY,+1[18]54, idem,+x,+10,+11,+13,+14,+20,+21[1]/46,XY[1]	<i>NF1</i>	+	-	-	-	Alive
	375	G181T	D61Y	M	1.9	16.1	46,XY,-7,+mar[17]/46, idem, del(6)(q?) [3]	<i>RUNX1</i>	-	-	+	+	Alive
	415	G1508C	G503A	F	12.3	1.9	45,XX,ins(1;?)(q21;?),add(4)(q12),add(7)(q36),der(17;18)(q10;q10)[20]	<i>NF1</i>	+	+	+	+	Death
	417	G1508C	G503A	M	5.6	51.7	46,XY,t(11;19)(q23;p13.1)[17]/47, idem,+8[1]/46,XY[2]	<i>KMT2A</i> -ELL, <i>STAG2</i>	+	+	+	+	Alive
	425	G205A	E69K	M	9.8	73.2	46,XY[20]	<i>NPM1</i>	+	-	-	-	Alive
438	A227T	E76V	F	13.6	161.0	49,XX,+8,+10,+12[20]	<i>FUS</i> - <i>ERG</i>	+	+	+	+	Death	
<i>CBL</i>	2	c.1174_1181delins TTATCATCCTTATCATT ATCACAGGT	p.392-394 delinsLSSLSSLSQV	M	2.3	172.0	46,XY,t(9;11)(p22;q23)[16]/46,XY[4]	<i>KMT2A</i> -MLLT3	-	+	+	+	Death
	67	c.A1405G	p.M469V	M	7.4	168.1	47,XY,+8[20]	-	-	+	+	-	Death
	97	c.T1248G	p.C416W	F	11.6	38.2	47,XX,+18[1]/46,XX[19]	<i>NPM1</i>	+	+	+	+	Death
	167	c.1096-75_1218 delinsAAAGGCT	p.366_406del	M	9.9	20.5	46,XY,t(8;21)(q22;q22)[17]/45,X,-Y,t(8;21)(q22;q22)[3]	<i>KIT</i>	+	-	-	-	Alive
	184	c.1183_1227+27del	p.395_409del	M	15.1	54.2	47,XY,inv(16)(p13.1q22),+22[20]	-	+	-	-	-	Alive
	262	c.1096-40_1227+35del	p.366_409del	M	12.3	159.3	46,XY,inv(16)(p13q22)[20]	<i>NRAS</i> , <i>NF1</i>	+	-	-	-	Alive

UPN, unique patient number; WBC, white blood cell count; CR, complete remission; SCT, stem cell transplantation; N/A, not applicable

*NCBI reference sequence; *PTPN11*, NM_002834; *CBL*, NM_005188

Table 3. Univariate and multivariate Cox regression analyses of overall survival and event-free survival.

	Univariate analysis				Multivariate analysis				
	HR	95%CI		p-value	HR	95%CI		p-value	
		Inferior	Superior			Inferior	Superior		
Overall survival									
<i>NF1</i>	4.104	1.492	11.29	0.006	4.109	1.471	11.48	0.007	
<i>PTPN11</i>	2.027	0.880	4.670	0.097	0.694	0.260	1.851	0.466	
<i>CBL</i>	2.145	0.676	6.800	0.195	2.617	0.794	8.630	0.114	
<i>NRAS</i>	0.305	0.111	0.833	0.021	0.309	0.112	0.849	0.023	
<i>KRAS</i>	1.201	0.379	3.808	0.756	2.064	0.618	6.892	0.239	
<i>RUNX1/RUNX1T1</i>	0.173	0.075	0.398	<0.001	0.250	0.106	0.590	0.002	
<i>CBFB/MYH11</i>	0.000	0.000	Inf	0.995	0.000	0.000	Inf	0.995	
Monosomy 7	1.655	0.522	5.250	0.392	2.617	0.781	8.775	0.119	
Complex karyotype	2.230	1.270	3.916	0.005	1.812	0.991	3.312	0.054	
<i>FLT3-ITD</i>	3.051	1.833	5.076	<0.001	1.853	0.985	3.486	0.056	
5q-	2.442	0.339	17.60	0.376	1.627	0.207	12.82	0.644	
<i>FUS-ERG</i>	10.19	3.671	28.26	<0.001	6.007	2.096	17.22	0.001	
<i>NUP98-NSD1</i>	5.232	2.605	10.510	<0.001	2.941	1.366	6.331	0.006	
<i>PRDM16</i> high expression	3.427	2.203	5.331	<0.001	1.921	1.165	3.168	0.010	
Event-free survival									
<i>NF1</i>	1.794	0.664	4.852	0.249	1.621	0.588	4.469	0.351	
<i>PTPN11</i>	2.142	1.157	3.965	0.015	1.239	0.616	2.494	0.548	
<i>CBL</i>	1.215	0.387	3.813	0.739	1.527	0.471	4.948	0.480	
<i>NRAS</i>	0.506	0.280	0.914	0.024	0.530	0.293	0.961	0.037	
<i>KRAS</i>	0.966	0.396	2.359	0.940	1.084	0.432	2.725	0.863	
<i>RUNX1/RUNX1T1</i>	0.466	0.306	0.708	<0.001	0.659	0.420	1.035	0.070	
<i>CBFB/MYH11</i>	0.422	0.186	0.956	0.039	0.603	0.255	1.427	0.250	
Monosomy 7	1.539	0.569	4.161	0.395	2.275	0.786	6.589	0.130	
Complex karyotype	1.926	1.222	3.037	0.005	1.810	1.111	2.948	0.017	
<i>FLT3-ITD</i>	2.250	1.460	3.469	<0.001	1.236	0.716	2.133	0.447	
5q-	5.587	1.366	22.86	0.017	4.441	1.009	19.54	0.049	
<i>FUS-ERG</i>	4.179	1.533	11.39	0.005	3.191	1.144	8.903	0.027	
<i>NUP98-NSD1</i>	8.056	4.180	15.53	<0.001	5.017	2.463	10.220	<0.001	
<i>PRDM16</i> high expression	2.797	1.990	3.931	<0.001	2.172	1.489	3.167	<0.001	

HR, hazard ratio; CI, confidence interval

Figure legends

Figure 1. Molecular and cytogenetic aberrations in 80 pediatric AML patients with RAS pathway alterations. Each column displays the cytogenetic aberration pattern and clinical status of an individual sample. Orange indicates RAS pathway and other genetic alterations. Blue indicates chromosomal aberrations. Purple indicates gene expression. Gray indicates clinical outcome. Blanks indicate the absence of the chromosomal aberration, genetic alteration, or prognostic event. CR, complete remission

Figure 2. Gene diagrams depicting RAS pathway mutations in pediatric patients with AML. (A) *NF1* mutations (NCBI reference sequence; NM_000267); (B) *PTPN11* mutations (NCBI reference sequence; NM_002834); (C) *CBL* mutations (NCBI reference sequence; NM_005188); (D) *NRAS* mutations (NCBI reference sequence; NM_002524); (E) *KRAS* mutations (NCBI reference sequence; NM_004985).

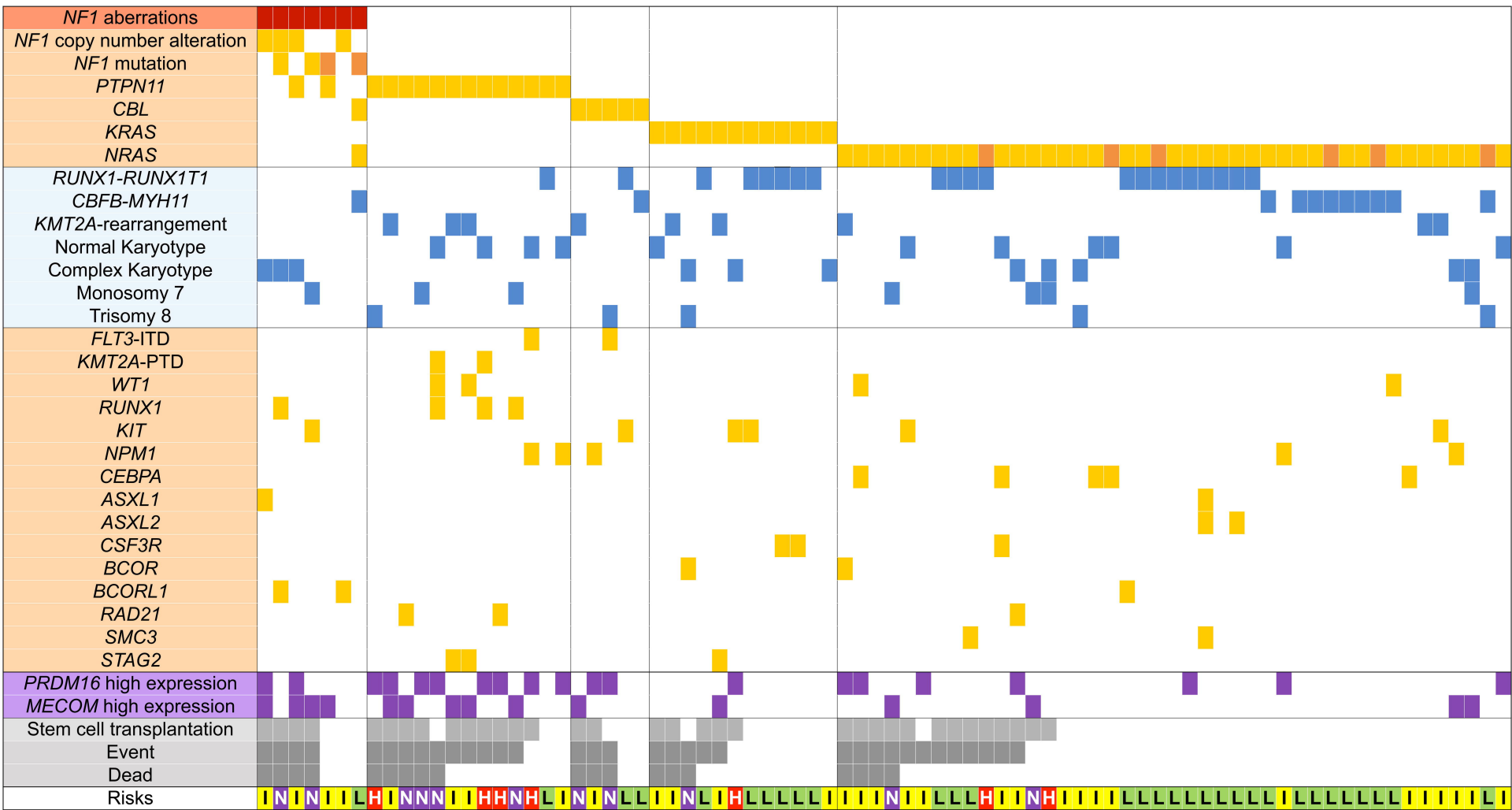
Figure 3. Prognostic significance of *NF1*, *PTPN11*, and *NRAS* alterations in pediatric patients with AML. (A), (C), and (E) show Kaplan–Meier curves of

overall survival of patients with and without *NF1*, *PTPN11*, and *NRAS* alterations.

(B), (D), and (F) show Kaplan–Meier curves of event-free survival of patients

with and without *NF1*, *PTPN11*, and *NRAS* alterations.

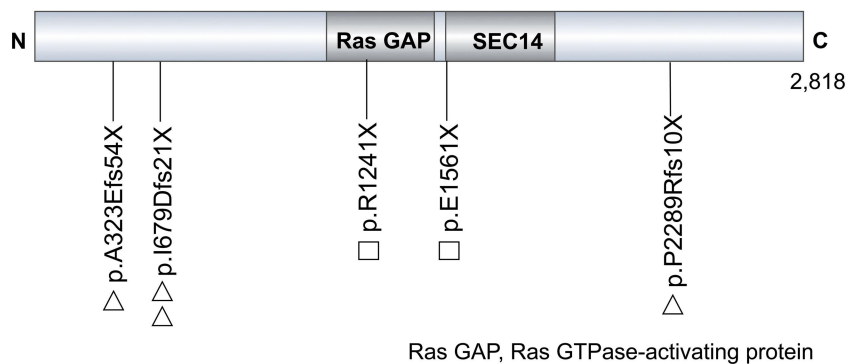
Figure 1



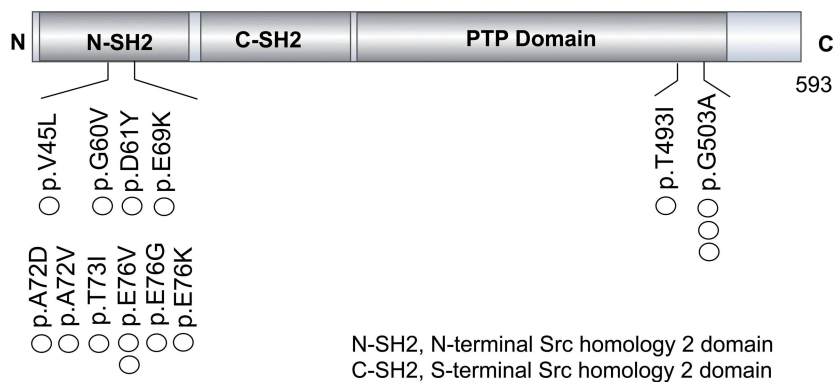
Risks **H** High risk **I** Intermediate risk **L** Low risk **N** Non-CR **■** Two mutations in one patient

Figure 2

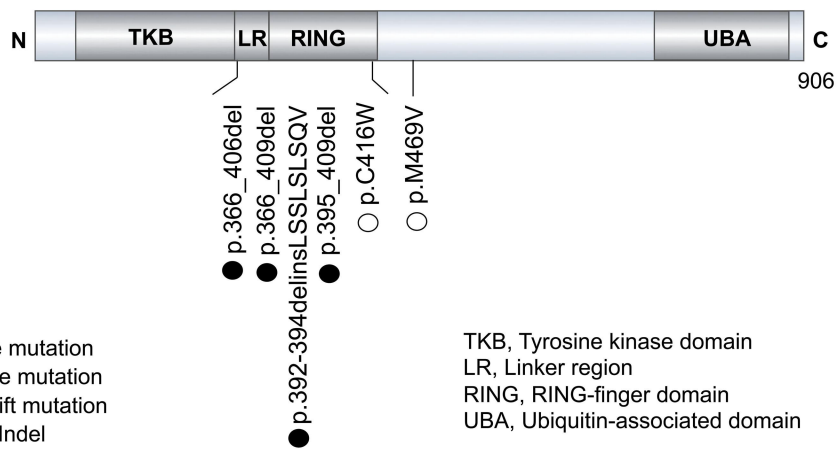
(A) *NF1*



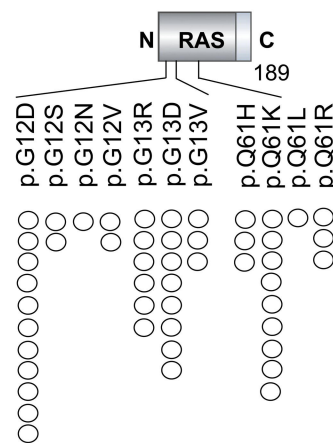
(B) *PTPN11*



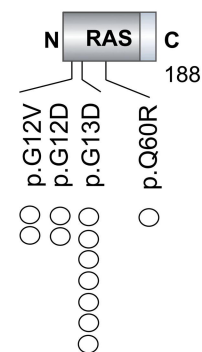
(C) *CBL*



(D) *NRAS*



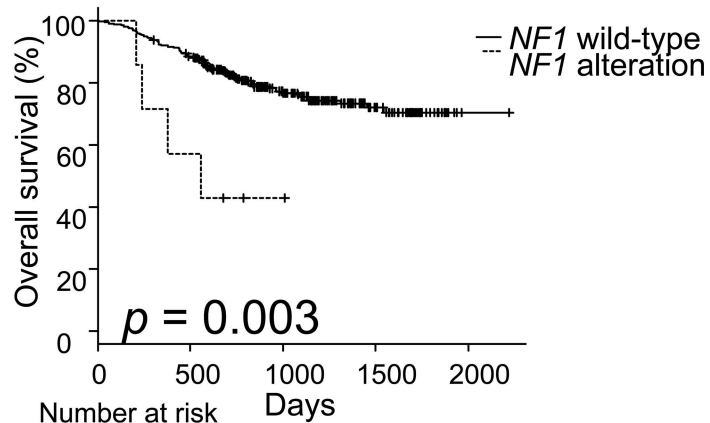
(E) *KRAS*



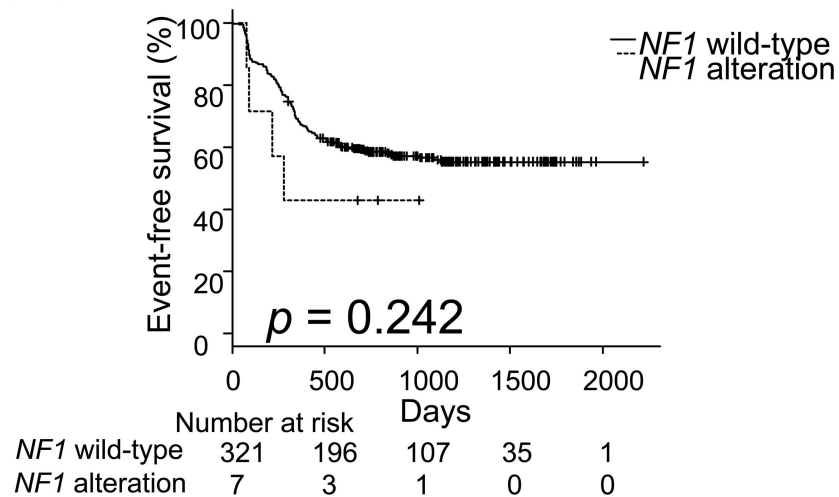
RAS, RAS GTPase family domain

Figure 3

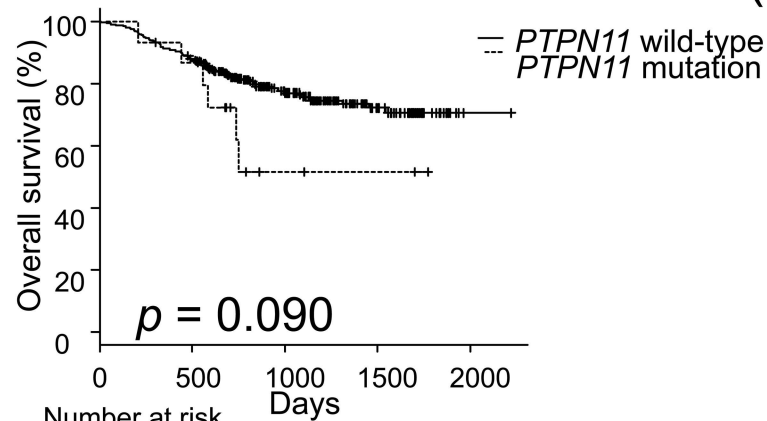
(A)



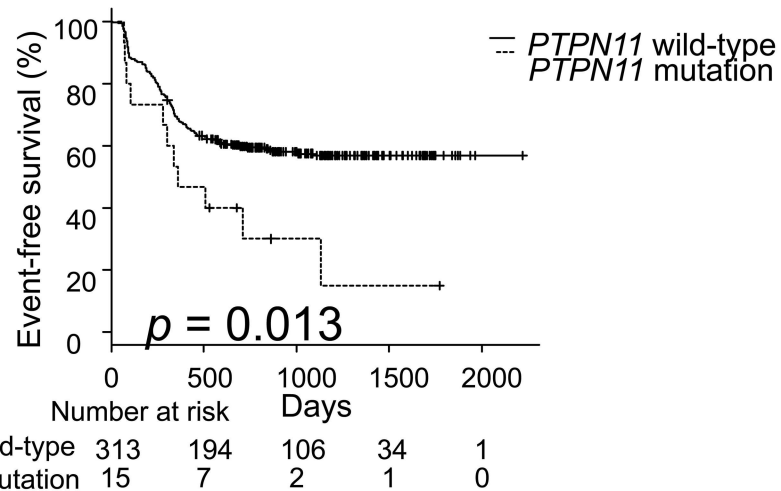
(B)



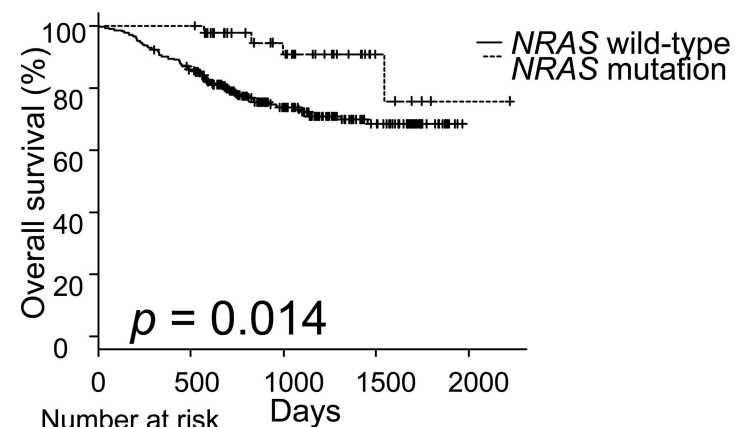
(C)



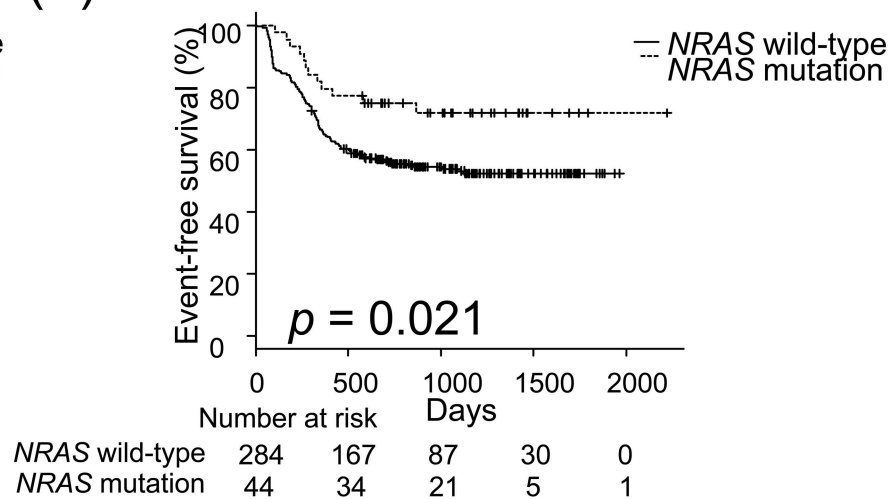
(D)



(E)



(F)



Supplementary Methods

Patients

The present study enrolled patients with de novo AML who participated in the Japanese AML-05 trial conducted by the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG). The AML-05 trial is registered with the UMIN Clinical Trials Registry [UMIN-CTR, (URL: <http://www.umin.ac.jp/ctr/index.htm>), number UMIN00000511]. Patients diagnosed with acute promyelocytic leukemia, Down syndrome-associated AML, and secondary AML were excluded from this study. Details pertaining to the diagnosis, and risk stratification have been previously reported in different studies.^{1,2} The treatment protocols and procedures for data and sample collection were approved by the institutional review boards of each participating institution; written informed consent was obtained from all patients or their parents/guardians.

Treatment

After the second induction course, the patients were stratified to one of the three risk groups according to their cytogenetic characteristics and treatment responses following the induction therapy. All the patients received three

additional intensified chemotherapeutic courses. Patients who had failed to achieve a complete remission after the second induction course were identified with induction failure and removed from the study. Allogeneic hematopoietic stem cell transplantation was indicated for all the high-risk patients after three or more treatment courses (Supplementary Figure S1).¹

Data Collection

Every 6 months, data forms were forwarded to the JPLSG data coordination center at the National Center of Child Health and Development. These data were reviewed for internal consistency and face validity and transferred into an Excel database (Microsoft Corporation, Redmond, WA, USA). The clinical data of patients in each risk group were followed until December 2013 (censored for 3 years from the date of final registration). The JPLSG performed a central review of morphologic classification and karyotyping based on the World Health Organization Classification and cytogenetic analysis using conventional G-banding.

Supplementary Tables

Table S1.

Clinical characteristics of the patients between 328 available samples and 115 unavailable samples

	Unavailable (n = 115)	Available (n = 328)	p-value
Age median at diagnosis, y (range)	7.3 (0.1-16.7)	8.0 (0.0-17.9)	0.645
Gender, Male, n (%)	61 (53)	177 (54)	0.914
WBC median, $\times 10^9/L$ (range)	7.6 (0.80-380.5)	21.8 (0.62-985.0)	<0.001
Stem cell transplantation, n (%)	59 (51)	151 (46)	0.385
Risk classification, n (%)			
Low risk	27 (23)	111 (34)	0.046
Intermediate risk	63 (55)	126 (38)	0.003
High risk	13 (11)	42 (13)	0.745
Induction failure	12 (10)	49 (15)	0.272
Cytogenetic feature n (%)			
Normal karyotype	24 (21)	61 (19)	0.585
Complex karyotype	17 (15)	36 (11)	0.316
<i>RUNX1-RUNX1T1</i>	29 (25)	93 (28)	0.546
<i>CBFB-MYH11</i>	5 (4)	27 (8)	0.211
<i>KMT2A</i> -rearrangement	22 (19)	49 (15)	0.303
Prognosis			
2-year Overall survival (%)	83.2	81.5	0.633
2-year Event-free survival (%)	70.1	58.5	0.642

WBC, white blood cell count

Table S2. Summary of characteristics of pediatric AML patients with *NRAS* mutation.

UPN	Amino acid change*	Gender	Age, y	WBC, ×10 ⁹ /L	Cytogenetics	Additional genetic aberrations	CR	Relapse	Event	SCT	Prognosis
17	p.Q61H	F	6.5	13.2	46,XX,t(8;21)(q22;q22)[4]/47,sl,add(15)(p11.2),+21[16]	-	+	+	+	+	Alive
24	p.Q61K	M	0.9	20.2	46,XY,add(5)(p11),add(7)(p11.2),?t(13;19)(q11;p13)[20]	<i>NPM1</i>	+	-	-	-	Alive
26	p.G13V	M	10.9	9.9	46,XY[20]	-	+	-	-	-	Alive
33	p.G12S, p.G13R	F	10.6	21.1	48,XX,+8,inv(16)(p13q22),+22,[7]	-	+	-	-	-	Alive
36	p.Q61K	M	16.2	100.7	46,XY[20]	<i>CEBPA</i> (biallelic)	+	-	-	-	Alive
48	p.G13R	M	11.8	56.8	47,XY,+21[3]/46,XY[17]	<i>CEBPA</i> (monoallelic)	+	-	-	-	Alive
61	p.G12S, p.G13R	F	10.1	64.1	46,XX,inv(9)(p12q13),inv(16)(p13.1q22)[20]	-	+	-	-	-	Alive
78	p.G12D	M	13.8	12	46,XY[10]	<i>KMT2A-MLL3</i>	+	-	-	-	Alive
83	p.G12D, p.G13V	F	12.1	10.3	46,XX[20]	<i>CEBPA</i> (biallelic)	+	-	-	-	Alive
95	p.G13D	F	6	14.7	45,X,-X,t(8;21)(q22;q22)[20]	-	+	-	-	-	Alive
107	p.Q61R	F	6.2	162.4	45,XX,t(8;21)(q22;q22)[20]	<i>ASXL1, ASXL2, SMC3</i>	+	-	-	-	Alive
111	p.G12D	M	5.6	547	46,XY,del(9)(q?) [20]	<i>NUP98-NSD1, WT1,</i> <i>CEBPA</i> (monoallelic)	+	+	+	+	Death
148	p.Q61R	M	8.6	121.6	47,XY,t(6;11)(q27;q23),+8[19]	<i>KMT2A-AFDN</i>	+	+	+	+	Death
151	p.G12D	M	4.1	38.6	46,XY,t(8;21)(q22;q22)[20]	-	+	-	-	-	Alive
152	p.Q61L	M	0.9	9.0	46,XY,-3,add(3)(p13),-7,-9,add(16)(q12.1),add(17)(p11.2),add(19)(p11), add(21)(q22),+r1,+mar1,+mar2[14]/46,XY[5]	-	+	-	-	+	Alive
173	p.G13D	M	12.3	17.2	45,X,-Y,t(8;21)(q22;q22),inv(9)(p12q13)[20]	-	+	-	-	-	Alive
190	p.Q61H	M	8.6	42.9	46,XY,add(7)(q11.2),t(8;21)(q22;q22)[18]/46,XY[2]	-	+	+	+	+	Alive
196	p.G12N	M	1.2	100.2	46,XY,inv(16)(p13.1q22)[20]	-	+	-	-	-	Alive
227	p.G12D	F	4.4	5.0	46,XX,t(16;21)(q24;q22)[4]/46,sl,t(1;16)(q32;p13.3)[16]	-	+	-	-	-	Alive
229	p.G13R	F	14.9	8.1	45,XX,inv(3)(q21q26.2),-7[20]	-	-	+	+	+	Death
255	p.G12D	M	11.7	18.6	46,XY,inv(16)(p13.1q22)[20]	-	+	-	-	-	Alive
262	p.G12D	M	12.3	159.3	46,XY,inv(16)(p13q22)[20]	<i>CBL, NF1</i>	+	-	-	-	Alive
268	p.G13D	F	15.3	9.1	46,XX[20]	<i>NPM1</i>	-	-	-	-	Alive
273	p.Q61K	M	1.5	43.3	47,XY,+8,t(14;20)(q11.2;q11.2),del(21)(q21q22)[20]	-	+	-	-	-	Alive
274	p.G13V	F	8.2	0.9	46,XX,?t(10;11)(p12;q14)[19]/46,XX[1]	-	+	+	+	+	Death

286	p.Q61K	F	5.8	16.4	46,XX,t(8;21)(q22;q22)[20]	-	+	-	-	-	Alive
289	p.G12V	F	2.3	49.9	46,X,-X,-2,-7,add(17)(q25),del(20)(q11.2),+r1,+mar1,+mar2[20]	-	+	-	-	-	Alive
292	p.Q61K	M	1	55.5	46,XY,t(8;21)(q22;q22)[9]/46,sl,add(22)(p11.2)[8]/46,XY[2]	-	+	+	+	+	Alive
322	p.G12D, p.G13D	M	13.2	91.7	46,XY,t(8;12;21)(q22;p13;q22)[8]/46,XY[1]	-	+	+	+	+	Alive
328	p.G13D	M	3.2	5.2	48,XY,+8,inv(16)(p13.1q22),+21[19]/48,XY,?	-	+	-	-	-	Alive
358	p.Q61R	F	4.3	10.4	46,XX,t(8;21)(q22;q22)[15]/46,XX[5]	ASXL1	+	-	-	-	Alive
362	p.G13D, p.Q61K	F	6.7	65.1	46,XX,t(8;21)(q22;q22)[20]	-	+	-	-	-	Alive
363	p.G13R, p.Q61K	M	1.3	119.2	46,XY,inv(16)(p13.1q22)[20]	-	+	-	-	-	Alive
379	p.Q61K	M	1.6	12.7	46,XY,-7[20]	-	+	-	-	+	Alive
384	p.G13D	M	9.3	5.8	45,X,-Y,t(8;21)(q22;q22)[18]/46,idem,+8[1]/46,XY[1]	-	+	-	-	-	Alive
390	p.Q61H	F	2.8	168.7	46,XX,del(16)(q22q24)[18]/47,XX,del(16)(q22q24),+22[2]	-	+	-	-	-	Alive
393	p.G13D	F	13.3	28.8	46,XX,?(5;6)(p15;q24)[6]/47,idem,+mar,inc[10]	-	+	+	+	-	Alive
399	p.Q61R	F	12.8	68.9	46,XX,inv(16)(p13.1q22)[10]/47,idem,+8[9]/48,idem,+8,+22[1]	-	+	-	-	-	Alive
410	p.G12D	F	11.2	43.9	46,XX,t(8;21)(q22;q22)[20]	-	+	-	-	-	Alive
422	p.G13R	F	3.2	56.2	46,XX,del(9)(q?),t(16;16)(p13.1;q22)[20]	WT1	+	-	-	-	Alive
423	p.Q61K	M	14.9	159.5	46,XY[20]	CEBPA (biallelic), CSF3R	+	+	+	+	Alive
430	p.G12D	F	11	430.1	46,XX[20]	KIT	+	+	+	+	Alive
436	p.G12D	M	10.2	29.9	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]	RAD21	+	+	+	+	Alive
437	p.G12V	F	0.6	19.3	46,XX,der(4)(4pter→4q33::11q21→11q23::19p13→19pter),der(11) t(4;11)(q33;q21),der(19)t(11;19)(q23;p13)[20]	KIT, KMT2A-rearrangement (partner undetermined)	+	-	-	-	Alive

UPN, unique patient number; WBC, white blood cell count; CR, complete remission; SCT, stem cell transplantation

*NCBI reference sequence; NM_002524

Table S3. Summary of characteristics of pediatric AML patients with *KRAS* mutation.

UPN	Amino acid change*	Gender	Age, y	WBC, ×10 ⁹ /L	Cytogenetics	Additional genetic aberrations	CR	Relapse	Event	SCT	Prognosis
100	p.G60R	F	10.9	25.2	46,XX,t(8;21)(q22;q22)[1]/45,sl,-X[18]/46,XX[1]	<i>KIT</i>	+	-	-	-	Alive
122	p.G13D	F	0.6	58.0	48,XX,+8,+18,-19,+20[19]/45,XX,-2,-17,+18,-19,+20[1]	<i>BCOR</i>	-	-	+	-	Death
194	p.G13D	F	7.8	33.1	46,XX,t(8;21)(q22;q22)[19]/46,XX[1]	<i>CSF3R</i>	+	-	-	-	Alive
203	p.G13D	F	16.8	20.8	46,XX,t(7;21;8)(q22;q22;q22)[20]	<i>CSF3R</i>	+	-	-	-	Alive
259	p.G13D	M	10.6	85.3	46,XY[20]	-	+	+	+	+	Death
297	p.G13D	F	10.8	128.9	46,XX,t(6;11)(p21;q23)[25]	<i>KMT2A</i> -rearrangement (partner undetermined)	+	+	+	+	Death
303	p.G12V	M	11.9	3.0	46,XY,t(8;21)(q22;q22)[17]/46,XY[3]	-	+	+	+	+	Alive
308	p.G12V	F	1.6	35.4	46,XX,add(2)(q31),t(16;21)(q24;q22)[5]/ 45,sl,+2,-add(2),-11,add(18)(p11.2)[12]/ 91,sl,x2,-11[2]	-	+	-	-	-	Alive
339	p.G13D	M	4.1	7.9	45,X,-Y,t(8;21)(q22;q22)[20]	-	+	-	-	-	Alive
360	p.G13D	F	10.3	6.7	46,XX,t(8;21)(q22;q22)[20]	-	+	-	-	-	Alive
394	p.G12D	F	2.2	60.0	51,XX,+X,+6,add(7)(p11.2),+8,del(12)(p?),+13,+19[19]	-	+	-	-	+	Alive
439	p.G12D	F	8.9	321.7	46,XX,t(11;19)(q23;p13.1)[20]	<i>KMT2A-MLL1</i>	+	+	+	+	Alive

UPN, unique patient number; WBC, white blood cell count; CR, complete remission; SCT, stem cell transplantation

*NCBI reference sequence; NM_004985

Table S4. Clinical characteristics between patients with or without RAS pathway alterations.

	All RAS pathway genes			<i>NF1</i>			<i>PTPN11</i>			<i>CBL</i>			<i>NRAS</i>			<i>KRAS</i>		
	Wild-type	Alteration	<i>p</i> -	Wild-type	Alteration	<i>p</i> -	Wild-type	Mutation	<i>p</i> -	Wild-type	Mutation	<i>p</i> -	Wild-type	Mutation	<i>p</i> -	Wild-type	Mutation	<i>p</i> -
	(n=248)	(n=80)	value	(n=321)	(n=7)	value	(n=313)	(n=15)	value	(n=322)	(n=6)	value	(n=284)	(n=44)	value	(n=316)	(n=12)	value
Age median at diagnosis,	7.5	9.1	0.719	7.80	12.30	0.059	8.20	7.00	0.927	7.80	10.75	0.367	7.80	8.40	0.862	7.80	8.40	0.891
years (range)	(0.0-17.9)	(0.4-16.8)		(0.0-17.9)	(7.0-15.2)		(0.0-17.9)	(0.4-14.1)		(0.0-17.9)	(2.3-15.1)		(0.0-17.9)	(0.6-16.2)		(0.0-17.9)	(0.6-16.8)	
Gender, Male, n (%)	136 (54.8)	41 (51.2)	0.607	172 (53.6)	5 (71.4)	0.459	169 (54.0)	8 (53.3)	1.000	172 (53.4)	5 (83.3)	0.223	154 (54.2)	23 (52.3)	0.871	174 (55.1)	3 (25.0)	0.073
WBC median, ×10 ⁹ /L (range)	20.6 (0.62-985.0)	29.4 (0.90-430.1)	0.132	22.4 (0.62-985.0)	15.5 (1.9-159.3)	0.450	21.9 (0.62-985.0)	17.8 (1.9-190.5)	0.912	21.3 (0.62-985.0)	106.7 (20.5-172.0)	0.026	21.6 (0.62-985.0)	29.4 (0.90-430.1)	0.295	21.3 (0.62-985.0)	34.3 (3.0-321.7)	0.434
Stem cell transplantation, n	117	34	0.520	147	4	0.707	140	11	0.035	149	2	0.691	138	13	0.022	146	5	1.000
Relapse, n	84	28	0.892	110	2	1.000	104	8	0.161	109	3	0.414	100	12	0.393	108	4	1.000
Risk classification, n																		
Low risk	80	31	0.342	110	1	0.430	110	1	0.024	108	3	0.411	89	22	0.017	105	6	0.231
Intermediate risk	96	30	0.895	122	4	0.435	120	6	1.000	125	1	0.412	109	17	1.000	122	4	0.773
High risk	35	7	0.252	42	0	0.601	38	4	0.111	42	0	1.000	40	2	0.091	41	1	1.000
No complete remission	37	12	1.000	47	2	0.281	45	4	0.254	47	2	0.221	46	3	0.116	48	1	1.000
Cytogenetic features, n																		
Normal karyotype	50	11	0.248	61	0	0.356	57	4	0.493	61	0	0.598	55	6	0.414	60	1	0.704
Complex karyotype	25	11	0.410	33	3	0.031	35	1	1.000	36	0	1.000	31	5	1.000	33	3	0.134
<i>RUNX1-RUNX1T1</i>	72	21	0.671	93	0	0.198	92	1	0.076	92	1	1.000	80	13	0.858	87	6	0.106
<i>CBFB-MYH11</i>	16	11	0.058	26	1	0.455	27	0	0.622	25	2	0.080	17	10	0.001	27	0	0.609
<i>KMT2A</i> -rearrangement	40	9	0.368	49	0	0.600	46	3	0.477	48	1	1.000	46	3	0.116	47	2	0.696
Monosomy 7	1	7	<0.001	7	1	0.160	6	2	0.047	8	0	1.000	4	4	0.013	8	0	1.000
<i>FLT3</i> -ITD	38	2	0.001	40	0	1.000	39	1	1.000	39	1	0.545	40	0	0.005	40	0	0.374
<i>KIT</i> mutations	42	6	0.045	47	1	1.000	48	0	0.140	47	1	1.000	46	2	0.040	46	2	0.690
<i>WT1</i> mutations	20	4	0.464	24	0	1.000	22	2	0.301	24	0	1.000	22	2	0.754	24	0	1.000
<i>KMT2A</i> -PTD	9	2	1.000	11	0	1.000	9	2	0.085	11	0	1.000	11	0	0.372	11	0	1.000
<i>NPM1</i> mutations	11	5	0.552	16	0	1.000	14	2	0.162	15	1	0.261	14	2	1.000	16	0	1.000

<i>CEBPA</i> biallelic mutations	18	3	0.430	21	0	1.000	21	0	0.610	21	0	1.000	18	3	1.000	21	0	1.000
<i>RUNX1</i> mutations	4	4	0.103	7	1	0.160	5	3	0.004	8	0	1.000	8	0	0.604	8	0	1.000
<i>CSF3R</i> mutations	5	3	0.409	8	0	1.000	8	0	1.000	8	0	1.000	7	1	1.000	6	2	0.030
5q deletion	2	0	1.000	2	0	1.000	2	0	1.000	2	0	1.000	2	0	1.000	2	0	1.000
<i>FUS-ERG</i>	3	1	1.000	4	0	1.000	3	1	0.171	4	0	1.000	4	0	1.000	4	0	1.000
<i>NUP98-NSD1</i>	10	1	0.307	11	0	1.000	11	0	1.000	11	0	1.000	11	0	1.000	11	0	1.000
<i>ASXL1</i> mutations	6	2	1.000	7	1	0.160	8	0	1.000	8	0	1.000	7	1	1.000	8	0	1.000
<i>ASXL2</i> mutations	12	2	0.531	14	0	1.000	14	0	1.000	14	1	1.000	12	2	1.000	14	0	1.000
<i>BCOR</i> mutations	2	2	0.251	4	0	1.000	4	0	1.000	4	0	1.000	3	1	0.440	3	1	0.139
<i>BOCRL1</i> mutations	3	4	0.063	4	2	0.006	6	0	1.000	6	0	1.000	5	1	0.582	6	0	1.000
<i>RAD21</i> mutations	5	3	0.409	8	0	1.000	6	2	0.047	8	0	1.000	7	1	1.000	8	0	1.000
<i>SMC3</i> mutations	4	2	0.636	6	0	1.000	6	0	1.000	6	0	1.000	4	2	0.186	6	0	1.000
<i>STAG2</i> mutations	2	3	0.096	5	0	1.000	3	2	0.018	5	0	1.000	5	0	1.000	4	1	0.171
<i>PRDM16</i> high expression	57	20	0.762	75	2	0.669	68	9	0.002	75	2	0.628	70	7	0.253	76	1	0.307
<i>MECOM</i> high expression	36	15	0.377	47	4	0.013	44	7	0.004	50	1	1.000	47	4	0.268	50	1	0.700

WBC, white blood cell count

Table S5. Summary of references for *NF1* alterations in AML.

Reference	Number of patients	Frequency of deletion	Frequency of mutation	Prognosis	Other results	Somatic or germline
Eisfeld AK, et al. <i>Leukemia</i> (2018)	1021 de novo AML (adult)	(LOH was detected in patients with VAF>0.70)	5.2%	Poor in patients aged <60	Significant association with complex karyotype	NA
Boudry-Labis E, et al. <i>Am J Hematol.</i> (2013)	485 de novo AML (adult)	3.5%	1/5 (20%) patients with <i>NF1</i> deletion	No significant difference but tendency for lower CR rate and shorter OS	Significant association with unfavorable cytogenetics	One mutation (c.2027dupC) in a patient with <i>NF1</i> deletion was determined as a somatic mutation
Harferlach C, et al. <i>Leukemia</i> (2012)	1161 myeloid malignancies (adult)	23/315 (7.3%) in de novo AML	15/29 (51.7%) patients with myeloid malignancies and <i>NF1</i> deletion	NA	-	NA
Parkin B, et al. <i>Clin cancer res.</i> (2010)	95 AML* (adult)	10.5%	2/10 (20%) of patients with <i>NF1</i> deletion	NA	High frequency (7/10) in complex karyotype	Determined as somatic mutations
Harferlach C, et al. <i>Leukemia</i> (2010)	37 AML with <i>CBFB/MYH11</i> ** (adult)	16%	NA	NA	-	NA
Suela J, et al. <i>J Clin Oncol.</i> (2007)	120 de novo AML (adult)	5.8%	NA	NA	High frequency (5/7) in complex karyotype	NA
Balgobind BV, et al. <i>Blood</i> (2008)	71 AML with <i>KMT2A-r</i> (pediatric)	2.80%	1/2 (50%) of patients with <i>NF1</i> deletion	1 patient with <i>NF1</i> deletions relapsed	-	NA (Both patients with <i>NF1</i> deletion had no clinical characteristics of neurofibromatosis)

AML, acute myeloid leukemia; LOH, loss of heterozygosity; VAF, variant allele frequency; NA, not analyzed; CR, complete remission; OS, overall survival.

* Including de novo, relapse, primary, secondary, therapy-related AML

** Including de novo and therapy-related AML

Table S6. Summary of references for CBL alterations in AML.

Reference	Number of patients	Frequency of mutation	Mutation/splice variant	Prognosis	Other results	Somatic or germline
Sargin B, et al. <i>Blood</i> (2007)	150 de novo AML (adult)	1 (0.7%)	Missense mutation at exon 9	NA	Normal karyotype	Somatic
Caligiuri MA, et al. <i>Blood</i> (2007)	12 de novo AML (adult)	4 (30%)	1 exon 8 deleted variant 3 missense mutations at exon 8	NA	-	NA
Abbas S, et al. <i>Haematologica</i> (2008)	319 de novo AML additional 79 CBF-AML (adult)	2/319 (0.6%) in AML 3/79 in CBF-AML	5 exon 8 deleted variants	NA	Significant association with CBF-AML	1 of 5 patients was analyzed and determined as somatic
Sanada, et al. <i>Nature</i> (2009)	222 myeloid malignancies (adult)	18/222 in all patients 2/24 (8%) in AML with MRC	16 missense mutations at exon 8 or exon 9 2 exon 8 deleted variants	NA	15/18 in patients had 11q UPD 1/2 in AML with MRC had 11q UPD	3 of 18 patients were analyzed and determined as somatic.
Reindl C, et al. <i>Clin Cancer Res.</i> (2009)	279 AML/MDS (adult)	3 (1.1%)	2 exon 8 deleted variants 1 exon 8+9 deleted variants	NA	2 in patients with CBF-AML 1 in patients with 11q aberration	All mutations were determined as somatic
Coenen EA, et al. <i>Br J Haematol.</i> (2012)	277 de novo AML (pediatric)	2/277 (0.7%)	1 exon 8 deleted variants 1 missense mutation at exon 8-intron 8 splice site (not affected the splicing)	NA	Exon 8 deleted variant was detected in patient with <i>KMT2A-r</i>	Exon 8 deleted variant was determined as somatic. Missense mutation at splice site was determined as germline

AML, acute myeloid leukemia; NA, not analyzed; CBF, core binding factor; MRC, myelodysplasia-related changes; MDS, myelodysplastic syndrome; UPD, uniparental disomy

Supplementary Figures

Figure S1. Treatment schedule of the AML-05 study

ECM comprised etoposide (150 mg/m²/d on days 1–5), cytarabine (200 mg/m²/d via 12-h continuous intravenous [CIV] infusion on days 6–12), mitoxantrone (5 mg/m²/d on days 6–10), and an age-adjusted dose of triple intrathecal chemotherapy (TIT) on day 6. HCEI comprised high-dose cytarabine (HDCA; 3 g/m² every 12 h on days 1–3), etoposide (100 mg/m²/d on days 1–5), idarubicin (10 mg/m² on day 1), and TIT on day 1. HCE comprised HDCA (2 g/m² every 12 h on days 1–5), etoposide (100 mg/m²/d on days 1–5), and TIT on day 1. HCI comprised HCEI without etoposide. HCM comprised HDCA (2 g/m² every 12 h on days 1–5), mitoxantrone (5 mg/m²/d on days 1–3), and TIT on day 1. CR, complete remission; Allo-HSCT, allogeneic hematopoietic stem cell transplantation. Asterisks indicate patients in the intermediate-risk or high-risk groups who experienced Grade 4 infection during intensification course 1 with HCM and received HC for intensification course 3. (Modified from Tomizawa D, et al. *Leukemia*. 2013).

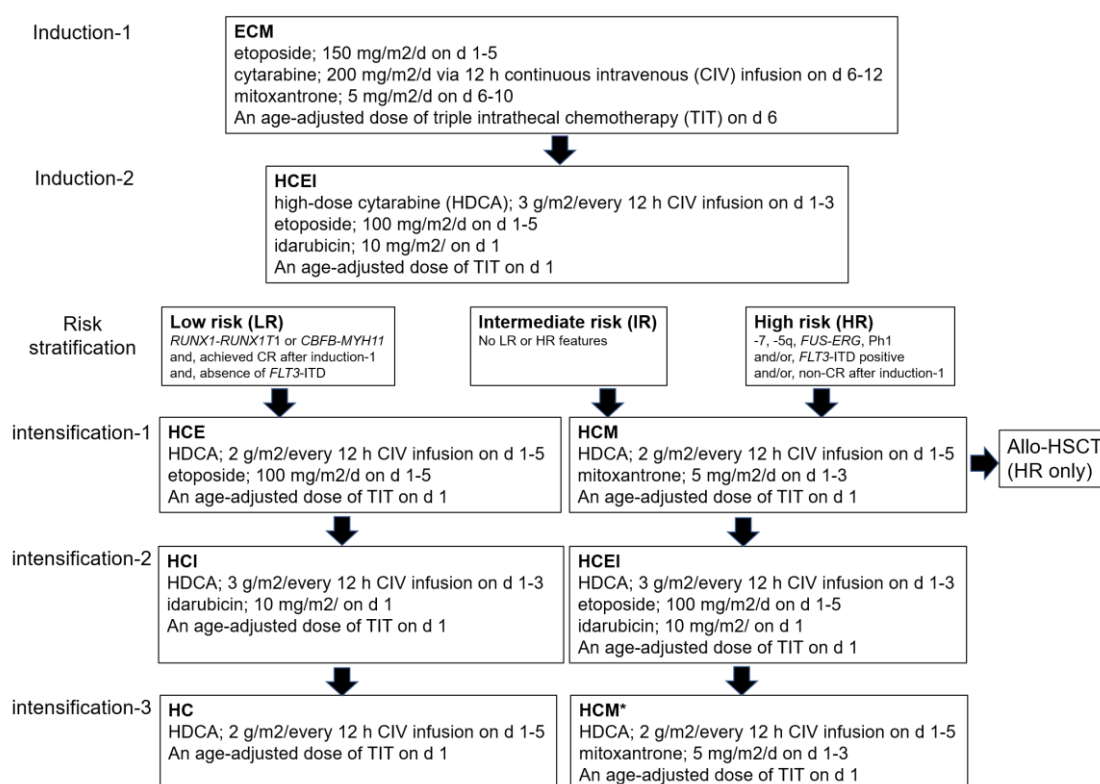
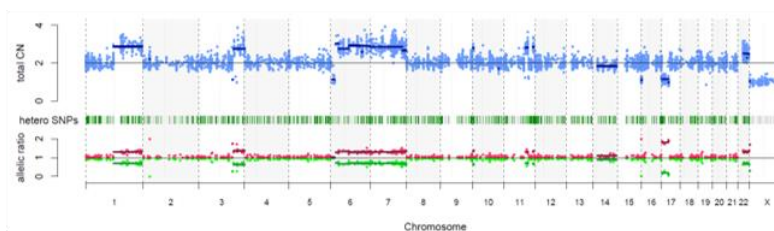


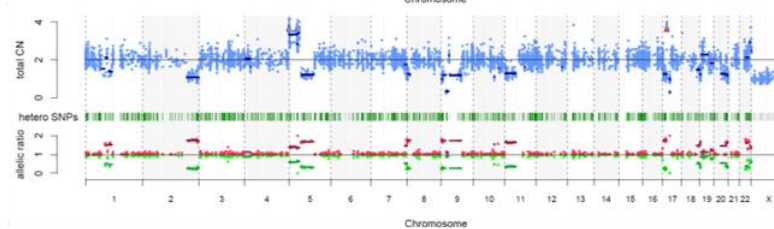
Figure S2. Copy number analysis by CNACS

Upper blue plots indicate total copy number (CN). Middle green bars indicate heterozygous single nucleotide polymorphisms used for CN analysis. Lower red and green plots indicate allelic ratio. (A)-(D) Results of patients with *NF1* deletions; (E) Results of UPN 50 having *NF1* mutation with 17q uniparental disomy (UPD); (F) Results of UPN 97 having *CBL* mutation with 11q UPD.

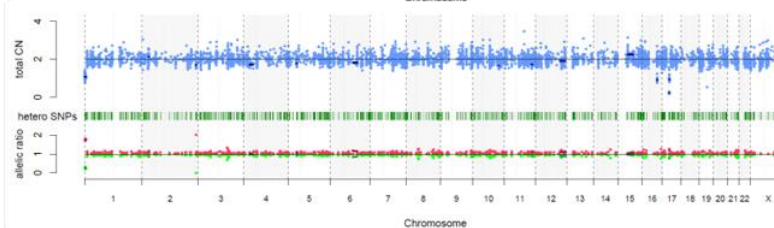
(A) UPN 57



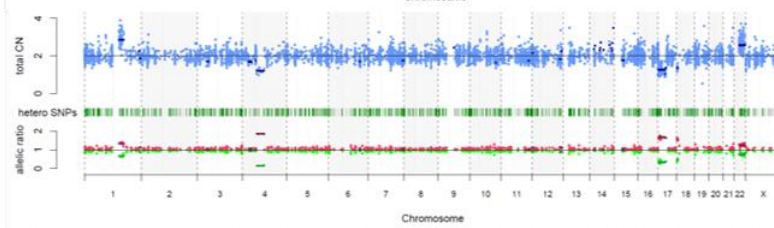
(B) UPN 105



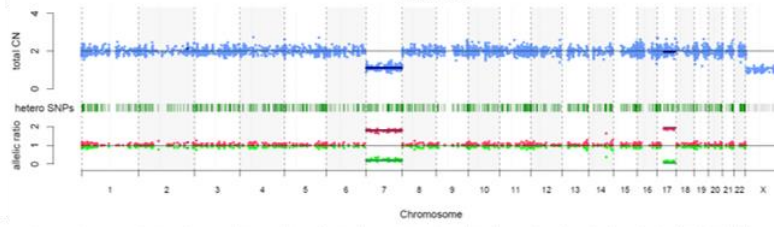
(C) UPN 333



(D) UPN 415



(E) UPN 50



(F) UPN 97

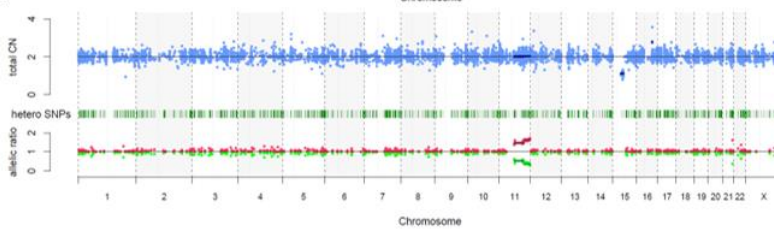


Figure S3. Comparison of white blood cell count at diagnosis

This figure shows white blood cell (WBC) count at diagnosis of patients with RAS pathway alterations. All RAS pathway genes, $29.4 (0.90\text{--}430.1) \times 10^9/\text{L}$; *CBL*, $106.7 (20.5\text{--}172.0) \times 10^9/\text{L}$; *NRAS*, $29.4 (0.90\text{--}430.1) \times 10^9/\text{L}$; *NF1*, $15.5 (1.9\text{--}159.3) \times 10^9/\text{L}$; *PTPN11*, $17.8 (1.9\text{--}190.5) \times 10^9/\text{L}$, *KRAS*, $34.3 (3.0\text{--}321.7) \times 10^9/\text{L}$. WBC count at diagnosis was significantly higher in patients with *CBL* mutations ($p = 0.026$).

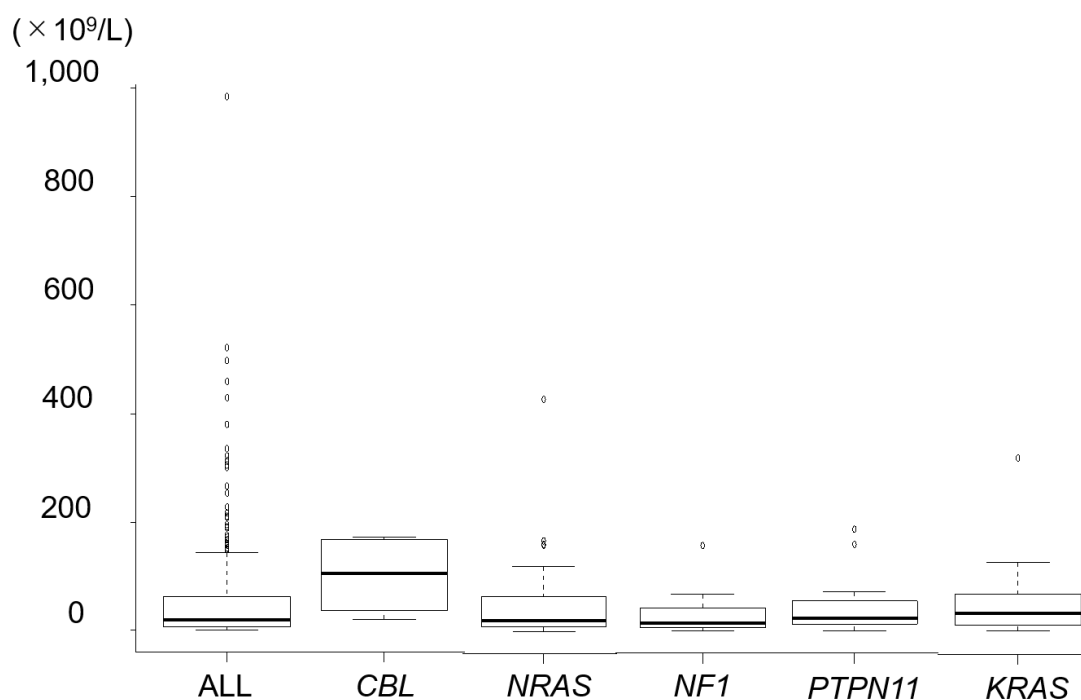


Figure S4. Prognostic significance of *CBL* and *KRAS* mutations in pediatric patients with AML

(A) and (C) show Kaplan–Meier curves of the overall survival of patients with and without *CBL* and *KRAS* alterations. (B) and (D) show the Kaplan–Meier curves of the event-free survival of patients with and without *CBL* and *KRAS* alterations.

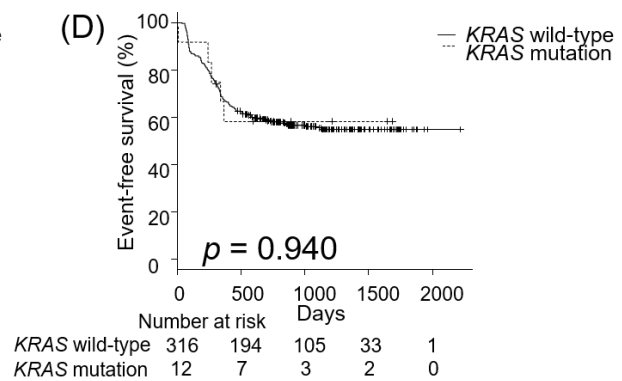
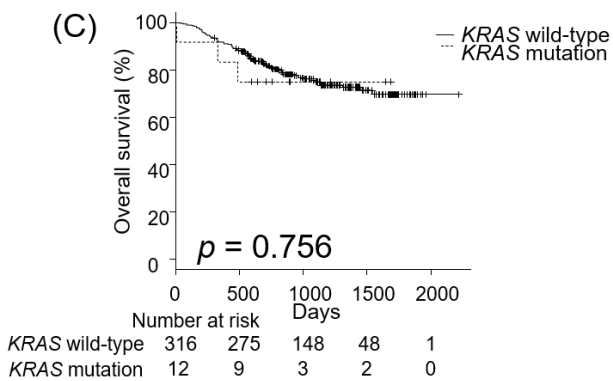
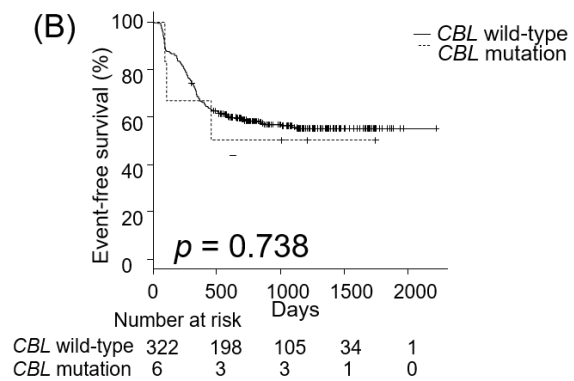
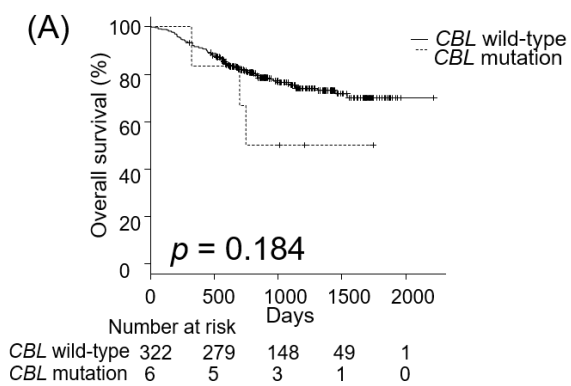
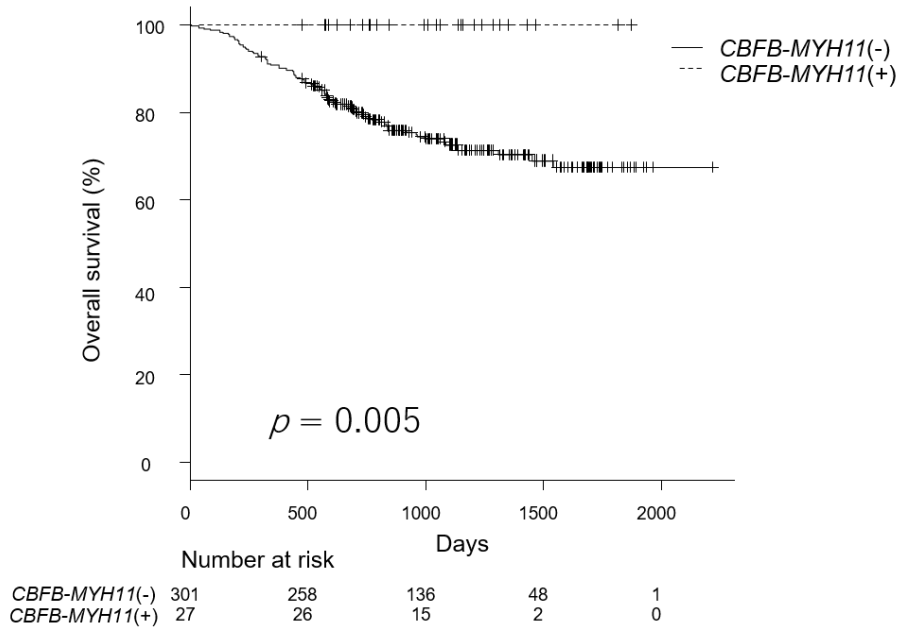


Figure S5. Kaplan–Meier curves of the overall survival of pediatric AML patients with and without *CBFB-MYH11*

Overall survival of patients with *CBFB-MYH11* was significantly better than that of patients without *CBFB-MYH11* ($p = 0.005$).



Supplementary References

1. Tomizawa D, Tawa A, Watanabe T, et al. Excess treatment reduction including anthracyclines results in higher incidence of relapse in core binding factor acute myeloid leukemia in children. *Leukemia*. 2013;**27**(12):2413-2416.
2. Kinoshita A, Miyachi H, Matsushita H, et al. Acute myeloid leukaemia with myelodysplastic features in children: a report of Japanese Paediatric Leukaemia/Lymphoma Study Group. *Br J Haematol*. 2014;**167**(1):80-86.