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Clinical significance of RAS pathway alterations in pediatric acute myeloid leukemia

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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%) 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 in the RAS pathway were mutually exclusive also together 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 biallelic 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]

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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 the *Online Supplementary Appendix* and the *Online 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 as previously described.[9,17,18] All coding exons of the *NF1* 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 real-time polymerase chain reaction (qRT-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 reported[34] 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 reports,[15] 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 (CI). For all analyses, two tailed *P*-values <0.05 were considered indicative of statistical significance.

Results

Frequencies of RAS pathway alterations in 328 pediatric acute myeloid leukemia 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 non-hematological or remission samples. The clinical characteristics of patients with available samples (n=328) and those with no available samples (n=115) are summarized in the *Online 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 (*Online 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, Tables 1 and 2 and the *Online Supplementary Tables S2* and *S3*, respectively.

We detected six *NF1* mutations in four patients; all of these were frameshift or nonsense mutations (Figures 1 and 2). Two patients concomitantly had two types of mutations, respectively (Table 1). In addition, we also detected four patients with a microdeletion within chromosome 17q containing *NF1* (Table 1; *Online Supplementary Figure S2*). One patient had both an *NF1* mutation and CN alteration and *NF1* alterations were detected in seven (2.1%) patients (Figure 1; Table 1). Two

patients: unique patient number (UPN) 57 and UPN 415 also had a heterozygous deletion. Additionally patient UPN 57 with variant allele frequency (VAF) 0.83 had nonsense mutations in the remaining allele, while UPN 50 with VAF 0.94 had 17q uniparental disomy (UPD) (*Online Supplementary Figure S2*). UPN 105 and UPN 333 had two or three different CN regions in *NF1*, respectively with partially homozygous deletions. Other two patients (UPN 262 and 367) had two types of mutations each. However, it was not clear whether these alterations were monoallelic 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, UPN 57, UPN 105, and UPN 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 is around 0.5.[36] We considered mutations in UPN 262 (VAF 0.28 and 0.26) and UPN 367 (VAF 0.28 and 0.08) as somatic mutations. Regarding UPN 50 (VAF 0.94) and UPN 57 (VAF 0.83), it was impossible to predict whether these mutations were somatic or germline because their VAF were high owing to their co-existence 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 non-hematological 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 observed,[39] codon 76 represented a mutational hot spot (four of 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 SNP on any database such as COSMIC v90, ClinVar, mutations taster, Ensembl GRCh37, or db SNP (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 identified 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 SNP 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 a missense mutation (UPN 97) by CN analysis (*Online Supplementary Figure S2*).

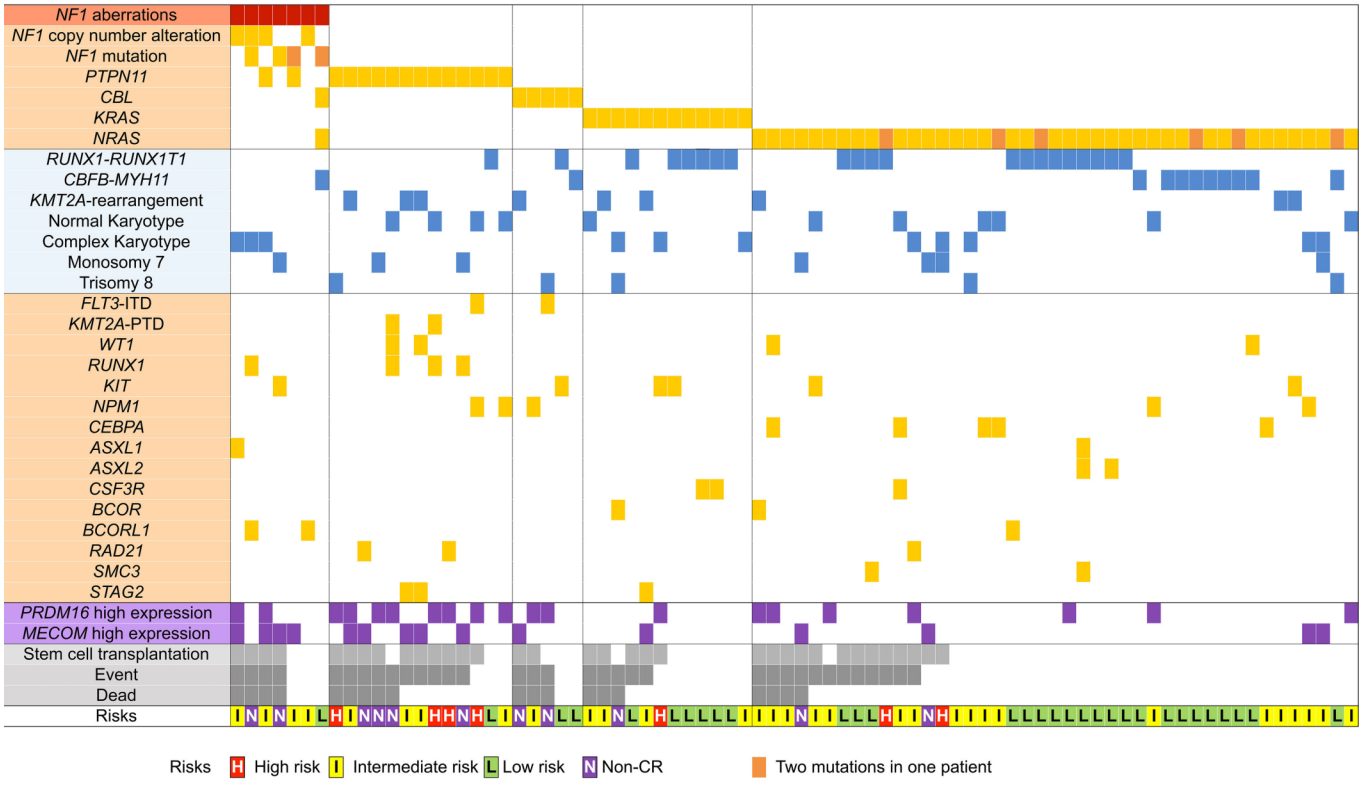

Figure 1. Molecular and cytogenetic aberrations in 80 pediatric acute myeloid leukemia patients with RAS pathway alterations. Each column displays the cytogenetic aberration pattern and clinical status of an individual sample. Orange indicates RAS pathway and other genetic alterations. Blue indicates 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.

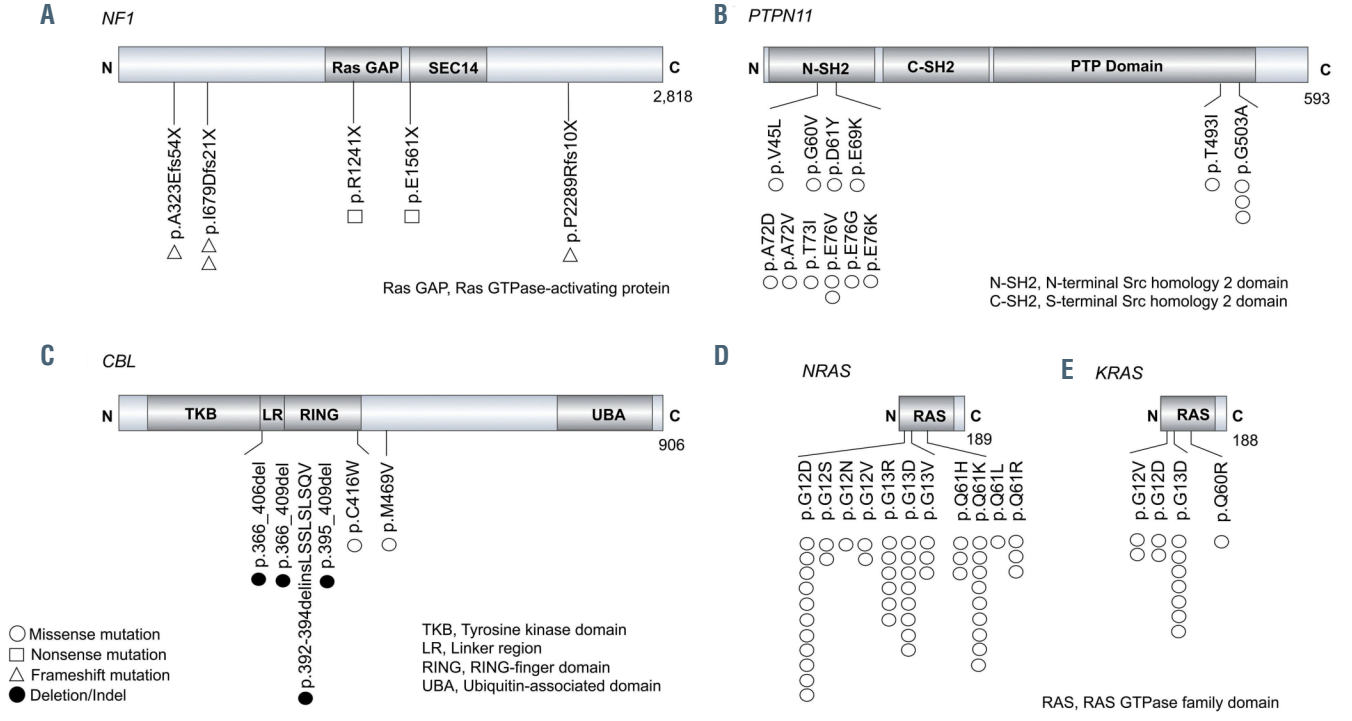

Figure 2. Gene diagrams depicting RAS pathway mutations in pediatric patients with acute myeloid leukemia. (A) *NF1* mutations (NCBI reference sequence; NM_000267); (B) *PTPN11* mutations (NCBI reference sequence; NM_002834); (C) *CBL* mutations (NCBI reference sequence; NM_005188); (D) *NRAS* mutations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

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 the *Online Supplementary Table S4*. Patients with RAS pathway alterations showed a 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, sex, 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 ($P=0.026$; *Online Supplementary Table S4*; *Online 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; *Online 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; *Online 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; *Online 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; *Online 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; *Online Supplementary Table S4*). Although there was no significant difference, three of six patients with *CBL* mutations were identified in CBF-AML ($P=0.411$) (Figure 1; *Online 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; *Online 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 3A and 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 3C and 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 3E and F). The presence of *CBL* or *KRAS* mutations showed no significant impact on prognosis (*Online*

Table 1. Summary of characteristics of pediatric acute myeloid leukemia patients with *NF1* alteration.

UPN	Nucleotide change*	Amino acid change*	VAF	Copy number	Start to end	Sex	Age, y	WBC, ×10³/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]	*KIT*	-	-	+	+	Death
262	c.2027dupC c.6862_6863insCG	p.I679Dfs21X p.P2289Rfs10X	0.28 0.26	-	-	M	12.3	159.3	46,XY,inv(16)(p13q22)[20]	*CBL*, *NRAS*	+	-	-	-	Alive
367	c.966_967insGA c.2027dupC	p.A323Efs54X p.I679Dfs21X	0.28 0.08	-	-	M	7	9.9	47,XY,+11[18]/54, idem,+X,+10,+11,+13,+14, +20,+21[1]/46,XY[1]	*PTPN11*	+	-	-	-	Alive
57	c.C3721T	R1241X	0.83	1.16 0.99	1225849-29422297 29485961-30325657	M	15.2	69.0	#1	*RUNX1*, *BCORL1*	-	-	+	+	Death
105	-	-	-	1.02 0.29 0.95	27009658-29588669 29626467-29679186 29683418-30325657	M	10.8	15.5	#2	*ASXL1*	+	+	+	+	Death
415	-	-	-	1.26 1.04	1225849-29422297 29485961-30325657	F	12.3	1.9	45,XX,ins(1;?)(q21;?), add(4)(q12), add(7)(q36), der(17;18)(q10;q10)[20]	*PTPN11*	+	+	+	+	Death
333	-	-	-	0.22 0.94	29485961-29588669 29626467-30325657	F	9.8	4.1	46,XX,t(8;12)(q11.2;p11.2)[20]	*BCORL1*	+	-	-	-	Alive

UPN: unique patient number; VAF: variant allele frequency; WBC: white blood cell count; CR: complete remission; SCT: stem cell transplantation; M: male, F: female; y: years; 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,+5mar[1]/47,XY,+Y[9]

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 (*Online 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% 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). (*Online Supplementary Figure S5*)

Discussion

In this study, we detected RAS pathway alterations in 80 (24.4%) 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 the *Online 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 two of the 71 AML patients with *KMT2A* rearrangement, one 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]

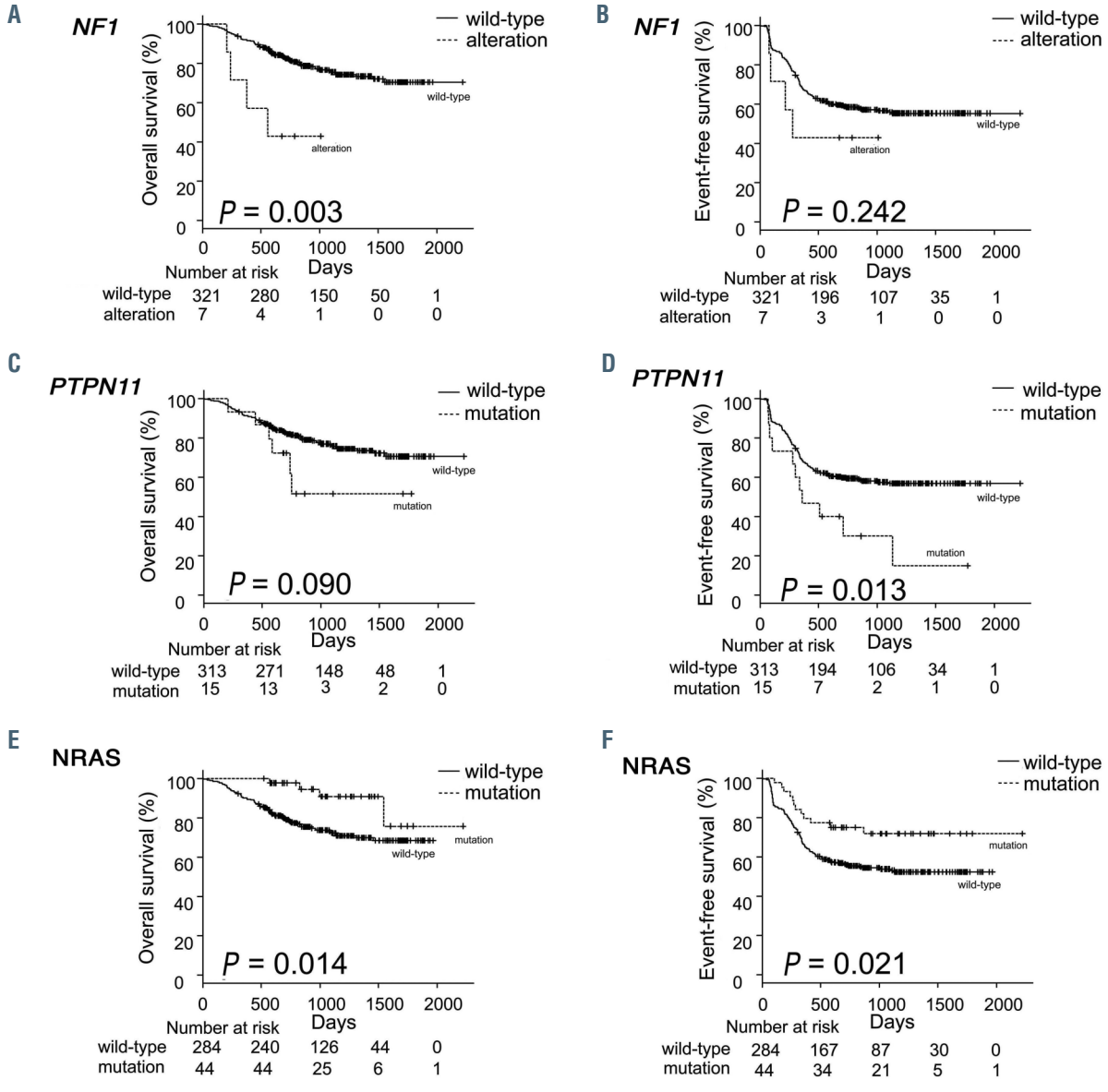

Figure 3. Prognostic significance of *NF1*, *PTPN11*, and *NRAS* alterations in pediatric patients with acute myeloid leukemia. (A), (C), and (E) show Kaplan–Meier curves of overall survival of patients with and without *NF1*, *PTPN11*, and *NRAS* alterations. (B), (D), and (F) show Kaplan–Meier curves of event-free survival of patients with and without *NF1*, *PTPN11*, and *NRAS* alterations.

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 a high frequency of *RUNX1* mutations, *MECOM* high expression, and *PRDM16* high expression which are strongly associated with poor prognosis (Figure 1; *Online 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 (*Online Supplementary Table S4*); in addition, five 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; *Online Supplementary Table S4*). Previous

Table 2. **Summary of characteristics of pediatric acute myeloid leukemia patients with *PTPN11* and *CBL* mutations.**

Gene	UPN	Nucleotide change*	Amino acid change*	Sex	Age, y	WBC, $\times 10^9$/L	Cytogenetics	Additional genetic aberrations	CR	Relapse	Event	SCT	Prognosis
PTPN11	45	A227T	E76V	M	4.8	33.9	45,XY,-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]	*WT1, KMT2A*-PTD	-	-	+	-	Death
	113	C215T	A72V	F	10.3	17.8	46,XX,add(12)(p11)[12]/46,XX[8]	*CBL, KRAS, KMT2A-ELL, WT1, STAG2*	+	+	+	+	Alive
	127	C218T	T73I	F	0.4	17.1	47,XX,t(7;12)(q36;p13),+19[20]	*RAD21*	+	+	+	+	Alive
	142	G1508C	G503A	M	6.9	190.5	N/A	*KMT2A-MLLT3*	+	+	+	+	Death
	156	C215A	A72D	F	11.5	4.5	46,XX[20]	*FLT3*-ITD, *NPM1*	+	-	-	+	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]	*NRAS, KMT2A*-PTD, *RUNX1*	+	+	+	+	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,+11[18]/54,idem,+X,+10,+11,+13,+14,+20,+21[1]/46,XY[1]	*NF1*	+	-	-	-	Alive
	375	G181T	D61Y	M	1.9	16.1	46,XY,-7,+mar[17]/46,idem,del(6)(q?)[3]	*RUNX1*	-	-	+	+	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]	*NF1*	+	+	+	+	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]	*KMT2A-ELL, STAG2*	+	+	+	+	Alive
	425	G205A	E69K	M	9.8	73.2	46,XY[20]	*NPM1*	+	-	-	-	Alive
	438	A227T	E76V	F	13.6	161.0	49,XX,+8,+10,+12[20]	*FUS-ERG*	+	+	+	+	Death
CBL	2	c.1174_1181delins TTATCATCCTTATCAT TATCACAGGT	p.392-394 delins LSSLSLSQV	M	2.3	172.0	46,XY,t(9;11)(p22;q23)[16]/46,XY[4]	*KMT2A-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]	*NPM1*	+	+	+	+	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]	*KIT*	+	-	-	-	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]	*NRAS, NF1*	+	-	-	-	Alive

UPN: unique patient number; WBC: white blood cell count; CR: complete remission; SCT: stem cell transplantation; N/A: not applicable; M: male; F: female.; y: years. *NCBI reference sequence; *PTPN11*, NM_002834; *CBL*, NM_005188.

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 a *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 the *Online Supplementary Table S6*. *CBL* mutations were previously shown to be associated with CBF-AML.[13] In the present study, three 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

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								
NF1	4.104	1.492	11.29	**0.006**	4.109	1.471	11.48	0.007
PTPN11	2.027	0.880	4.670	0.097	0.694	0.260	1.851	0.466
CBL	2.145	0.676	6.800	0.195	2.617	0.794	8.630	0.114
NRAS	0.305	0.111	0.833	**0.021**	0.309	0.112	0.849	**0.023**
KRAS	1.201	0.379	3.808	0.756	2.064	0.618	6.892	0.239
RUNX1-RUNX1T1	0.173	0.075	0.398	**<0.001**	0.250	0.106	0.590	**0.002**
CBFB-MYH11	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
FLT3-ITD	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
FUS-ERG	10.19	3.671	28.26	**<0.001**	6.007	2.096	17.22	**0.001**
NUP98-NSD1	5.232	2.605	10.510	**<0.001**	2.941	1.366	6.331	**0.006**
PRDM16 high expression	3.427	2.203	5.331	**<0.001**	1.921	1.165	3.168	**0.010**
Event-free survival								
NF1	1.794	0.664	4.852	0.249	1.621	0.588	4.469	0.351
PTPN11	2.142	1.157	3.965	**0.015**	1.239	0.616	2.494	0.548
CBL	1.215	0.387	3.813	0.739	1.527	0.471	4.948	0.480
NRAS	0.506	0.280	0.914	**0.024**	0.530	0.293	0.961	**0.037**
KRAS	0.966	0.396	2.359	0.940	1.084	0.432	2.725	0.863
RUNX1-RUNX1T1	0.466	0.306	0.708	**<0.001**	0.659	0.420	1.035	0.070
CBFB-MYH11	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**
FLT3-ITD	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**
FUS-ERG	4.179	1.533	11.39	**0.005**	3.191	1.144	8.903	**0.027**
NUP98-NSD1	8.056	4.180	15.53	**<0.001**	5.017	2.463	10.220	**<0.001**
PRDM16 high expression	2.797	1.990	3.931	**<0.001**	2.172	1.489	3.167	**<0.001**

HR: hazard ratio; CI: confidence interval.

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* alterations, especially in pediatric AML (*Online 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 RASopthies 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 (*Online Supplementary Tables S5* and *S6*), further analyses are needed.

In conclusion, *NF1* alteration is possibly a poor prognostic factor and *NRAS* mutation is 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.

Disclosures

No conflicts of interest to disclose.

Contributions

TK, GY, NS, and YH designed and performed the research, analyzed the data, and wrote the paper; YHayashi designed the research, led the project, and wrote the paper; KY, YS, SM, and SO performed the research; KT performed the research and bioinformatics analysis; KO, MS, HA, HM, AS, TTabi, NK, DT, KH,

TTaga and SA provided patient samples and data. All authors critically reviewed and revised the manuscript.

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