Panel-based next-generation sequencing facilitates the characterization of childhood acute myeloid leukemia in clinical settings

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Abstract. Acute myeloid leukemia (AML) accounts for ~20% of pediatric leukemia cases. The prognosis of pediatric AML has been improved in recent decades, but it trails that of most other types of pediatric cancer, with mortality rates of 30-40%. Consequently, newer more targeted drugs are required for incorporation into treatment plans. These newer drugs selectively target AML cells with specific gene alterations. However, there are significant differences in genetic alterations between adult and pediatric patients with AML. In the present study, inexpensive and rapid next-generation sequencing (NGS) of >150 cancer-related genes was performed for matched diagnostic, remission and relapse (if any) samples from 27 pediatric patients with AML. In this analysis, seven genes were recurrently mutated. *KRAS* was mutated in seven patients, *NRAS* was mutated in three patients, and *KIT, GATA1, WT1, PTPN11, JAK3* and *FLT3* were each mutated in two patients. Among patients with relapsed AML, six harbored *KRAS* mutations at diagnosis; however, four of these patients lost these mutations at relapse. Additionally, two genetic alterations (*FLT3*-ITD and *TP53* alterations) were detected among patients who eventually relapsed, and these mutations are reported to be adverse prognostic

factors for adult patients with AML. This panel-based, targeted sequencing approach may be useful in determining the genetic background of pediatric AML and improving the prediction of treatment response and detection of potentially targetable gene alterations. RAS pathway mutations were highly unstable at relapse; therefore, these mutations should be chosen as a target with caution. Incorporating this panel-based NGS approach into the clinical setting may allow for a patient-oriented strategy of precision treatment for childhood AML.

Introduction

Acute myeloid leukemia (AML) accounts for ~20% of all cases of pediatric leukemia (1). Although the prognosis of pediatric AML has improved in recent decades, it trails that of other types of pediatric cancer, as 30-40% of children with AML eventually succumb to the disease (1-3). The treatment strategies for pediatric AML include intensive multimodal chemotherapy with or without stem cell transplantation, and cytarabine and anthracyclines have remained the primary choices of chemotherapy for >30 years (1,2,4,5). As clinical outcomes have not improved, even with intensive contemporary chemotherapeutic regimens and/or stem cell transplantation, newer targeted drugs are required for incorporation into treatment plans (5,6).

Recently, various newer targeted therapies have emerged, most of which target AML cells with specific genetic alterations (7). Among them, FLT3 inhibitors, such as midostaurin and gilteritinib, and IDH inhibitors, such as enasidenib and ivosidenib have already been approved for use in clinical settings (7). These newer drugs selectively target AML cells with specific features; hence, the genomic characterization of AML cells is becoming increasingly important in the clinical

setting. Using next-generation sequencing (NGS), several studies have reported on the value of performing NGS for adult patients with AML (8-12). However, there are comparatively fewer reports focusing on childhood AML, particularly in cases of relapsed AML (13,14).

In the present study, panel-based, targeted NGS for the molecular characterization of AML cells from pediatric patients was retrospectively performed. The objective of this study was to determine whether it was possible to obtain clinically useful information for children with AML through this NGS approach.

Materials and methods

Patients. A total of 27 children aged 0-18 years who were diagnosed with AML between January 2000 and December 2017 in Okayama University Hospital, Kochi Health Sciences Center, St. Marianna University School of Medicine Hospital or Hokkaido University Hospital were enrolled in the present study. The ratio of boys to girls was 17:10 in the present cohort and the median age at diagnosis was 6 years (range, 0 months to 15 years). The treatment protocols were diverse, including those from the Japanese Pediatric Leukemia/Lymphoma Study Group AML-99 (15), AML-05 (16) and AML-12 studies, and are listed in Table I. The standard chimeric fusion gene screening varied has changed over time in Japan. AML-05 protocol included the PCR-based detection of eight frequent chimeric gene fusions; *RUNX1-RUNXT1*, *CBFB-MYH11*, *KMT2A-MLLT3*, *KMT2A-MLLT4*, *KMT2A-MLLT1*, *FUS-ERG*, *NUP98-HOXA9* and *PML-RARA*, and the AML-12 protocol included eight gene fusions; *RUNX1-RUNXT1*, *CBFB-MYH11*, *KMT2A-MLLT3*, *KMT2A-MLLT4*, *BCR-ABL*, *FUS-ERG*, *NUP98-NSD1* and *PML-RARA*. In our previous study, the clinical courses of UPN 6 and UPN 25 was reported (17,18). The present study was performed in accordance with the Declaration of Helsinki and other applicable guidelines (19-21). The present study was approved by the Institutional Ethics Committee of Okayama University Hospital, and informed consent was obtained the patients and/or their legal guardians.

DNA isolation. Somatic DNA was obtained from bone marrow samples at diagnosis and each episode of relapse, whereas germline DNA was obtained from a buccal swab or peripheral blood in CR status. DNA was extracted using a QIAamp DNA Blood Mini kit (Qiagen, Inc.) and quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Inc.) and a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Inc.) according to the manufacturers' protocol.

Targeted NGS approach. Targeted sequencing of >150 cancer-related genes was performed as described previously (22-24). The targeted gene lists are shown in Table SI and patient allocation is shown in Table SII. These gene panels were generated using an online design tool for HaloPlex (Agilent Technologies, Inc.), and target enrichment was performed using the HaloPlex standard protocol. Samples were then sequenced using MiSeq (Illumina, Inc.). Read alignment to the hg19 reference genome was performed using Burrows-Wheeler Aligner (bio-bwa.sourceforge.net)

and variant calling was performed using SureCall version 3.0 (Agilent Technologies, Inc.).

Variant prioritization and assessment of pathogenicity. Synonymous or non-coding variants and single nucleotide polymorphisms reported with a frequency of >1% in various databases (dbSNP, 1000gp and Human Genetic Variation Database) were excluded. Variant bases that had >5 reads in each sample were used for the next step. Genetic variations that were constantly detected from diagnostic, remission and relapse samples with a variant allele frequency (VAF) ≥0.2 were regarded as candidate germline alterations. Genetic alterations that were rarely detected, or not detected at all, from remission samples but were detected from diagnostic and/or relapse samples with a VAF ≥0.05 were regarded as candidate somatic alterations with reference to a previous study (25). To exclude the possibility of false-positive findings, differences in VAF between normal and diagnostic/relapse samples were assessed using a Fisher's exact test. P<0.01 was considered to indicate a statistically significant difference. Finally, for germline and somatic alterations, the read quality was checked using IGV software version 2.3 (Broad Institute).

Results

Patient characteristics. The clinical information of the analyzed patients is shown in Table I. All patients were Japanese. The present cohort included one patient with Down-syndrome (UPN 8) and no other patients had a known underlying congenital condition. Remission samples were obtained by buccal smear from patient No. 2 and the sample collection was performed during remission. All relapsed patients lost their first remission within 24 months; 6 out of 15 patients experienced their relapse within 12 months, which is considered to be an adverse prognostic factor for survival (26).

Descriptive results from sequencing runs. The average number of total reads was 1,917,277 (range, 998,341-4,333,332) and the average read length was 116-136. The read depth in analyzable target regions per sample ranged from 168-757x. In total, 70.49-97.51% of analyzable regions were covered by at least 20 reads, 66.72-95.24% were covered by at least 50 reads and 58.15-91.05% were covered by at least 100 reads. These quality metrics data were obtained from analysis using SureCall version 3.0 software (Agilent Technologies, Inc.) and the details are shown in Table SII.

The results of NGS are shown in Table II. A total of 26 single nucleotide variations (SNVs) and insertions/deletions (indels) were identified at diagnosis, and 22 SNVs and indels at relapse for 15 patients with relapsed AML, as well as 12 SNVs and indels for the leukemia samples of 12 patients without relapse.

Somatic genetic alterations at diagnosis and AML subtypes. In the present study, seven genes were recurrently mutated. *KRAS* was mutated in 7 patients, *NRAS* was mutated in 3 patients, and *KIT*, *GATA1*, *WT1*, *PTPN11*, *JAK3* and *FLT3* were each mutated in 2 patients. As previously reported, *KIT* was mutated in patients with core-binding factor AML (UPN 19

Table I. Clinical information of the analyzed patients.

A, Relapsed AML

UPN	Age	Sex	Disease subtype	Chimeric gene	Karyotyping	Initial therapy[a]	SCT at CR1	Duration of CR1, months	Karyotype at relapse	Outcome	Institute
1	12 years	M	FAB M5	KMT2A-MLLT3	46, XY, add(11)(q23), der(21)t(1;21)(q11;q11.2) [9/20], 46, sl, der(19)t(11;19)(q13;p13) [11/20]	AML 05	No	24	47, XY, add(11)(q23), +marl	Alive	OU
2	4 years	M	FAB M6 (RAEB)	None	46, XY, inv(9)(p11q13)	Other	Yes	7	46, XY, t(2;5)(q33;q31), add(6)(q21), t(6;12)(q13;p13), inv(9)(p11q13)	Alive	OU
3	5 months	M	FAB M7 (non-Down-syndrome)	None	49, XY, +4, ?t(4;5)(q21;q15), del(12)(p?), +19, +22	AML 12	No	13	50, XY, +4, ?t(4;5)(q21;q15, +8, del(12)(p?), +19, +22	Alive	OU
4	10 years	M	FAB M5	None	48, XY, +16, +19	AML 05	No	9	46, XY, add(7)(q32), ?t(10;11)(p12;q23)	Succumbed to disease	OU
5	4 years	F	FAB M7 (non-Down-syndrome)	None	46, XX, add(5)(p13), del(6)(q), add(12)(p11.2)	Other	No	22	46, XX, add(5)(p13), del(6)(q), add(12)(p11.2)	Alive	OU
6	12 years	F	FAB M2	None	46, XX	AML 05	Yes	7	NA	Succumbed to disease	KHSC
7	7 years	F	FAB M2	None	46, XX	AML 99	No	10	46, XX	Succumbed to disease	HU
8	1 year	M	FAB M7 (Down-syndrome)	None	47, XY, +21	Other	No	12	45, XY, -10, der(17)t(17;18)(p11;q11), -18, +21	Dead (CR)	HU
9	13 years	M	FAB M4	NA	46, XY, t(6;11)(q27;q23)	AML 99	No	18	46, XY, t(6;11)(q27;q23)	Succumbed to disease	HU
10	1 year	M	FAB M5b	NA	47, XY, t(9;11)(p22;q23), +mar1	AML 99	No	6	NA	Alive	HU
11	14 years	M	FAB M1	None	46, XY, del(9)(q?)	AML 05	No	18	46, XY	Alive	HU

Table I. Continued.

A, Relapsed AML

UPN	Age	Sex	Disease subtype	Chimeric gene	Karyotyping	Initial therapy[a]	SCT at CR1	Duration of CR1, months	Karyotype at relapse	Outcome	Institute
12	11 years	F	T/Myeloid	None	46, XX, t(1;2) (p31;p16)	AML 05	No	13	46, XX, t(1;2)(p31;p16)	Alive	HU
13	5 years	M	FAB M7 (non-Down-syndrome)	None	47, XY, del(9) (q12q34), del(12)(p12), +21	Other	Yes	19	47, XY, +8, del(9)(q12q34), t(10;17)(q11.2;q25), del(12)(p12)	Succumbed to disease	HU
14	5 years	F	FAB M4	None	46, XX, der(2) t(11;10;2)(q21; q11.2;q37), der(10) add(10)(p11.2) t(11;10;2), der(11) t(11;10;2)	AML 05	No	24	46, XX	Succumbed to disease	HU
15	14 years	M	T/Myeloid	None	47, XY, del(4)(q?), +22, inc	AML05	No	4	47, XY, del(4)(q?), 22	Alive	SMU

B, Non-relapsed AML

UPN	Age	Sex	Disease subtype	Chimeric gene	Karyotyping	Initial therapy[a]	SCT at CR1	Duration of CR1, months	Karyotype at relapse	Outcome	Institute
16	10 years	M	FAB M3	PML-RARA	46, XY, t(15;17) (q22;q11~21)	AML99	No	-	-	Alive	OU
17	9 years	F	FAB M3	PML-RARA	46, XX, t(15;17) (q22;q12)	AML 99	No	-	-	Alive	OU
18	8 months	F	FAB M5a	KMT2A-MLLT10	46, XX, t(10;11) (p12;q23)	AML 12	Yes	-	-	Alive	OU
19	13 years	M	FAB M4Eo	CBFB-MYH11	47, XY, +8, inv(16)(p13.1q22)	AML 05	No	-	-	Alive	OU
20	7 years	M	FAB M2	RUNX1-RUNXT1	45, X, - Y, t(8;21) (q22;q22)	AML 05	No	-	-	Alive	OU

Table I. Continued.

B, Non-relapsed AML

UPN	Age	Sex	Disease subtype	Chimeric gene	Karyotyping	Initial therapy[a]	SCT at CR1	Duration of CR1, months	Karyotype at relapse	Outcome	Institute
21	15 years	M	AML with myelodysplasia-related changes	None	46, XY	Other	Yes	-	-	Dead (CR)	OU
22	6 years	F	FAB M2	CBFB-MYH11	46, XX, inv(16)(p13.1q22)	AML 05	No	-	-	Alive	OU
23	5 years	F	FAB M2	RUNX1-RUNXT1	46, XX, t(8;21)(q22;q22)	AML 05	No	-	-	Alive	OU
24	3 years	M	FAB M2	RUNX1-RUNXT1	46, XY, t(1;21;8)(p36;q22;q22), del(9)(q13q22)	AML 99	No	-	-	Alive	OU
25	0 months	F	FAB M5	KAT6A-CREBBP	t(8;16)(p11;p13)	AML 99	No	-	-	Alive	OU
26	11 months	M	FAB M5	KMT2A-MLLT3	46, XY	AML 99	No	-	-	Alive	OU
27	1 year	M	FAB M2	RUNX1-RUNXT1	46, XY, t(8;21)(q22;q22), del(9)(q?), add(11)(q23)	AML 99	No	-	-	Alive	OU

[a]Details regarding treatment protocols are described in references (15,16). UPN, unique patient number; AML, acute myeloid leukemia; SCT, stem cell transplantation; CR1, first complete remission; OU, Okayama University Hospital; HU, Hokkaido University Hospital; SMU, St. Marianna University School of Medicine Hospital; KHSC, Kochi Health Sciences Center.

Table II. Gene alterations detected at diagnosis and relapse.

A, Relapsed AML

UPN	Disease subtype	SNVs at diagnosis (VAF)	SNVs at relapse (VAF)
1	FAB M5	*KRAS*p.G12V (0.296)	None
2	FAB M6 (RAEB)	*PTPN11*p.G60V(0.83) *PTPN11* p.V45L (0.10)	*PTPN11*p.G60V(0.44)
3	FAB M7 (non-Down-syndrome)	*KRAS* p.A146T (0.09)	None
4	FAB M5	None	None
5	FAB M7 (non-Down-syndrome)	*GATA1*p.R270W (0.43) *KRAS* p.G12A (0.43) *IKZF1* p.F154Y (0.38)[a] *IKZF1*p.L161H (0.38)[a]	*GATA1*p.R270W(0.23) *KRAS* p.G12A (0.19) *IKZF1*p.F154Y(0.17)[a] *IKZF1*p.F161H(0.17)[a]
6	FAB M2	*FLT3*-ITD (0.572)	*FLT3*-ITD (0.689)
7	FAB M2	*PTPN11* p.I479F (0.40) + c.1473-1481del[a]	*PTPN11*p.I479F (0.35) + c.1473-1481del[a]
8	FAB M7 (non-Down-syndrome)	*GATA1* p.P50fs (0.30)	*GATA1* p.P50fs (0.05)
9	FAB M4	*KRAS* p.G12V (0.41) *SMARCA4*p.R979Q (0.06)[a]	*MLH1*p.A586S (0.06)[a]
10	FAB M5b	*KRAS* p.G13D (0.32)	*RUNX1*p.Q390fs(0.2)
11	FAB M1	*GATA2* p.R362Q (0.11) *CEBPA*p.Q312HR (0.99)[a]	*CEBPA*p.D63fs(0.37)[a] *CEBPA*p.Q312HR (0.39)[a]
12	T/Myeloid	*JAK3* p.L857P (0.10) *NOTCH1*p.V1721E (0.33) *STAT5B*p.N642H(0.11) *NF1* p.L2317H (0.05)[a]	*JAK3* p.L857P (0.13) *NOTCH1*p.V1721E (0.33) *IL7R* p.L243GTARCV (0.10)[a] *VHL* p.L85fs (0.11)
13	FAB M7 (non-Down-syndrome)	None	None
14	FAB M4	*U2AF1* p.R35L (0.53) *KRAS* p.G12D (0.37)	*KRAS* p.G12D (0.23)
15	T/Myeloid	*TP53* p.K164E (0.998) *PHF6* p.R274fs (0.724) *NPM1* p.L287fs (0.579)	*TP53* p.K164E (0.858) *PHF6*p.R274fs (0.611) *NPM1* p.L287fs (0.53) *MPL*p.A486V(0.064)[a]

B, Non-relapsed AML

UPN	Disease subtype	SNVs at diagnosis (VAF)	SNVs at relapse (VAF)
16	FAB M3	None	-
17	FAB M3	None	-
18	FAB M5a	None	-
19	FAB M4Eo	*KIT* p.D816Y (0.28) *WT1* p.H448Y (0.22)[a] *NRAS* p.Q61K (0.08)	- - -
20	FAB M2	*JAK3* p.M511I (0.13)	-
21	AML with myelodysplasia-related changes	None	-
22	FAB M2	*NRAS* p.G13D (0.37) *WT1* p.D447N (0.34)	- -
23	FAB M2	*NRAS* p.Q61K (0.25)	-
24	FAB M2	*KRAS* p.G12D (0.52)	-

Table II. Continued.

B, Non-relapsed AML

UPN	Disease subtype	SNVs at diagnosis (VAF)	SNVs at relapse (VAF)
25	FAB M5	None	-
26	FAB M5	*FLT3* p.D839G (0.21)	-
		FLT3 p.Y591D (0.08)	-
		FLT3 p.D839N (0.07)	-
27	FAB M2	*KIT* p.N822K (0.43)	-

^aAlterations not reported in Catalogue of Somatic Mutations in Cancer. UPN, unique patient number; AML, acute myeloid leukemia; VAF, variable allele frequency; SNV, single nucleotide variation.

Table III. Prognostic genetic alterations at diagnosis according to the European LeukemiaNet guidelines.

UPN	Low-risk features	Intermediate risk features	High-risk features.
1	No	*KMT2A-MLLT3*	Complex karyotype
2	No	No	No
3	No	No	Complex karyotype
4	No	No	No
5	No	No	Complex karyotype
6	No	No	*FLT3*-ITD
7	No	No	No
8	No	No	No
9	No	No	t(6;11)(q27;q23)
10	No	t(9;11)(p22;q23)	No
11	*CEBPA* p.Q312HR	No	No
12	No	No	No
13	No	No	Complex karyotype
14	No	No	Complex karyotype
15	No	No	*TP53* p.K164E
16	*PML-RARA*	No	No
17	*PML-RARA*	No	No
18	No	No	*KMT2A-MLLT10*
19	*CBFB-MYH11*	No	No
20	*RUNX1-RUNXT1*	No	No
21	No	No	No
22	*CBFB-MYH11*	No	No
23	*RUNX1-RUNXT1*	No	No
24	*RUNX1-RUNXT1*	No	No
25	No	No	No
26	No	*KMT2A-MLLT3*	No
27	*RUNX1-RUNXT1*	No	No

UPN, unique patient number.

and UPN 27), and *GATA1* was mutated in patients with acute megakaryoblastic leukemia with or without Down-syndrome (UPN 5 and UPN 8) (27-29). The present cohort included 2 patients with mixed-phenotype acute leukemia, and these patients harbored mutations previously reported in a larger study (30). Other mutations were not apparently associated with a specific type of AML. Most detected mutations have been reported in the Catalogue Of Somatic Mutations In Cancer database (cancer.sanger.ac.uk/cosmic), but some alterations were not. These mutations were thought to be variants of
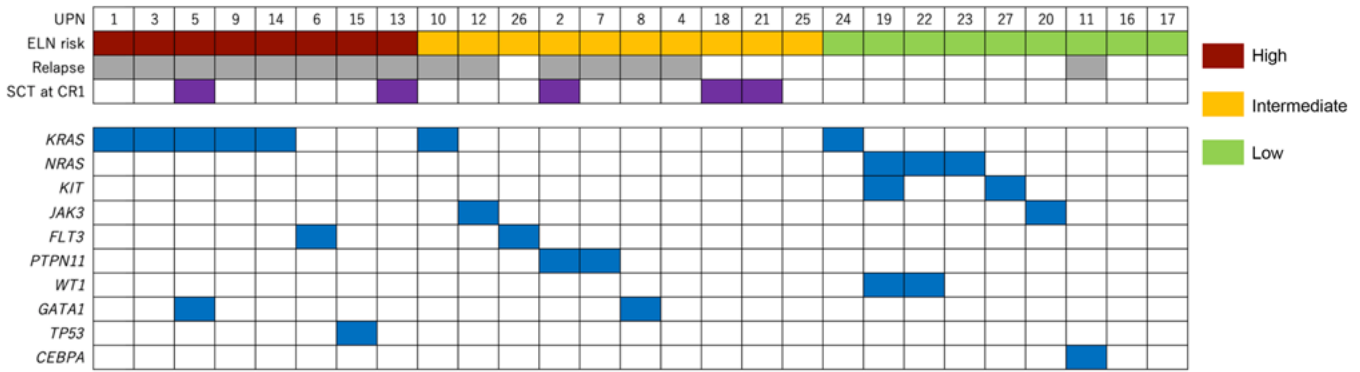
Figure 1. Gene alterations defined in ELN guidelines or recurrently detected in the present study. Distribution of relevant gene mutations. UPN, unique patient number; ELN, European LeukemiaNet; SCT, stem cell transplantation; CR1, first complete remission.

unknown significance and are indicated with a superscripted letter in Table II.

Prognostic genetic alterations detected at diagnosis. According to the European LeukemiaNet (ELN) guideline, several factors are associated with the prognosis of AML (31). Among these, two high-risk genetic alterations (*FLT3*-ITD in patient 6 and *TP53* alteration in patient 15) and one low-risk genetic alteration (*CEBPA* mutation in patient 11) were added to the known cytogenetic risk factors (Table III). According to the guidelines, *RUNX1* or *ASXL1* alterations are indicated not to be used as adverse prognostic markers if they are present concurrently with favorable-risk AML subtypes. However, *TP53* alterations are regarded as an independent adverse prognostic factor, thus UPN 15 was placed in the ELN high-risk group. The gene alterations which were defined in the ELN guideline or recurrently detected in the present study are summarized in Fig. 1.

Mutational changes between diagnosis and relapse. A total of 15 patients who experienced relapse were analyzed. Among these, six harbored *KRAS* mutations at diagnosis. However, four of the six patients lost these mutations at relapse. None of the patients gained new RAS pathway mutations at relapse.

UPN 11 had a *CEBPA* p.Q312HR insertion-type alteration at diagnosis, and this alteration appeared to be homozygous as its VAF was notably high (0.99). At relapse however, the VAF of *CEBPA* p.Q312HR alteration decreased, and a new p.D53fs alteration appeared, leading to a 'double hit' status.

Germline variations. In the present study, matched samples at diagnosis, remission and relapse (if any) among patients with childhood AML was analyzed, and this approach enabled detection of germline variations. Although candidates of germline variations were detected in 8 patients, none were regarded as pathogenic or likely pathogenic according to published recommendations (19,21). These candidate genes are listed in Table SIII.

Discussion

In the present study, matched samples obtained from pediatric patients with AML at diagnosis, remission and relapse (if any) were analyzed using a panel-based NGS method. Several studies have reported the utility of NGS for analysis of AML in adult patients (8-12). However, there are comparatively fewer reports focusing on childhood AML, particularly in cases of relapsed AML (13,14).

The utility of NGS should be discussed separately in adult and pediatric patients. As was shown in the present study, there are significant differences in genetic alterations between adult and pediatric patients with AML. Whereas mutations in epigenetic components or spliceosome complexes are common among adults with AML (11), these mutations were notably less common amongst children with AML, based on the results of the present study. However, large structural aberrations such as chromosomal translocations are more common amongst children with AML (11,14). As a result, disease stratification guidelines or newer drugs developed for adults with AML may not always be suitable for children.

The panel-based NGS strategy has been used several times in our previous studies for hematological malignancies, and the results of this method have been compared with conventional approaches, such as the multiplex ligation-dependent probe amplification or Sanger sequencing in our previous studies (22-24). Based on the panel-based NGS approach, several prognostic genetic alterations for patients with AML were detected in the present study. The ELN guidelines identified several cytogenetic alterations and a smaller number of genetic mutations as prognostic factors. In the present cohort, additional cytogenetic risk factors possessed prognostic implications than the genetic mutations. Using this approach, two high-risk genetic mutations were detected amongst patients who relapsed but did not possess any cytogenetic risk factors (UPN 6 and UPN 15). Conversely, none of the non-relapsed patients possessed adverse prognostic genetic mutations, and non-relapsed patients were enriched in low risk genetic alternations defined in the ELN guidelines. Thus, it may be possible to more accurately predict the prognosis of patients with AML using this approach. However, as noted above, there are significant differences in genetic alterations between adult and pediatric patients with AML. Previous larger studies suggested that current guidelines, including the ELN guidelines, are not adequate for children with AML (14,32,33), thus, there is a need for the development of a pediatric-specific guidelines for more precise stratification.

FLT3-ITD has a prognostic impact in pediatric AML (34,35), and this alteration was detected using the panel-based NGS method. However, the clinical impact of FLT3-ITD

has been reported to be modulated by other sequence aberrations (*WT1* mutation, *NUP98-NSD1* or *NPM1* mutations for adult AML) (14,36). In this context, panel-based sequencing may be more useful than conventional approaches that detect only *FLT3*-ITD. However, the ELN guidelines recommend the use of DNA fragment analysis to determine the ratio of *FLT3*-ITD and prognosis. To confirm the usefulness of the NGS approach, a direct comparison of standard procedures and the NGS approach is required.

The number of genes that should be assessed has increased; however, one large study found that a limited number of genes are recurrently mutated in pediatric patients with AML (14); where several genetic analytical methods, including whole-genome and targeted DNA sequencing were performed. Mutations in only 5 genes (*FLT3*, *NPM1*, *WT1*, *CEBPA* and *KIT*) were present in >5% of patients, and <40 gene mutations were reported in >2% of patients. This previous study illustrated the need to focus on these 40 genes to detect recurrent gene mutations in AML cells obtained from pediatric patients, and that panel-based sequencing is an ideal approach in a clinical setting. Furthermore, Morita *et al* (37) reported that the clearance of somatic mutation at remission was associated with significantly improved survival and a lower risk of relapse. This strategy requires a sufficient read depth to detect mutations with a VAF <1%; contrarily, the approach used in the present study could not reach that read depth due to low throughput and relatively high number of targeted genes. Limiting the number of targeted genes to 40 recurrently mutated genes will increase the read depth and potentially enable detection of gene alterations with lower VAFs.

The panel-based approach used in the present study also offers potentially useful information regarding targetable genetic alterations. Cytotoxic chemotherapy primarily based on cytarabine and anthracyclines with or without stem cell transplantation has long been the mainstay of AML treatment (1). The curative rate of pediatric AML steadily improves with increasing doses of these drugs; however, this leads to substantial treatment-related complications in vulnerable pediatric populations (1,16,26). Hence, newer targeted therapies are desired. These targeted drugs include midostaurin, gilteritinib, enasidenib and ivosidenib, which target leukemic cells with specific genetic alterations such as *FLT3*, *KIT*, *MEK*, *DOT1L* or *BET* alterations (5,6); hence, genomic characterization of AML cells is becoming increasingly important in the clinical setting. Among these potentially targetable gene alterations, 6 patients had RAS pathway mutations at the time of AML diagnosis. However, in the present cohort, a notably high percentage of patients (four out of six cases, 66.7%) lost the *KRAS* mutations at relapse. Thus, RAS pathway mutations should be chosen with caution as treatment targets.

The present study has several limitations. First, the study was retrospective, and it was not possible to clarify whether dose increases in patients who had high-risk features would improve their prognoses. Second, the method used in the present study has a disadvantage of comparatively low throughput. Third, potential false positives were excluded, thus several relevant genetic alterations may have been missed. To detect minor clones with low variant frequencies at diagnosis, the number of genes to be targeted should be limited, as noted above. The genetic events including *TP53* loss of heterozygosity in UPN 15 should have also been illustrated using other experiments, such as fluorescent *in situ* hybridization,

but this could not be achieved due to the sample availability and quality. Relatively small patient numbers is another disadvantage of the present study. Furthermore, the ELN guidelines were established for adult patients; hence its validity in a larger cohort of pediatric patients with AML requires validation.

In summary, the panel-based targeted sequencing approach used in the present study may be useful for revealing the genetic background of pediatric AML, and may facilitate the precise prediction of patient prognosis and detection of druggable gene alterations. Incorporating this method into the clinical setting may enable a patient-oriented precision strategy for treatment of childhood AML.

Availability of data and materials

The datasets generated and/or analyzed during the present study could not be submitted to a public curated database as the informed consent obtained does not include unrestricted disclosure of sequencing data. Instead, these data are available from the corresponding author upon reasonable request.

Authors' contributions

HI and AS wrote the manuscript. HI, MA, TM, MS and AS performed the genetic analysis and interpreted the results. HI, AI, RN, DK and AS performed patient care and collected the clinical data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Institutional Review Board of Okayama University Hospital (Okayama, Japan). Informed consent was obtained from the patients and/or their legal guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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