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ORIGINAL ARTICLE

Panel-based next-generation sequencing identifies prognostic and actionable genes in childhood acute lymphoblastic leukemia and is suitable for clinical sequencing

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Abstract

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children. Although the cure rate of ALL has greatly improved, a considerable number of patients suffer from relapse of leukemia. Therefore, ALL remains the leading cause of death from cancer during childhood. To improve the cure rate of these patients, precisely detecting patients with high risk of relapse and incorporating new targeted therapies are urgently needed. This study investigated inexpensive, rapid, next-generation sequencing of more than 150 cancer-related genes for matched diagnostic, remission, and relapse samples of 17 patients (3 months to 15 years old) with relapsed ALL. In this analysis, we identified 16 single-nucleotide variants (SNVs) and insertion/deletion variants and 19 copy number variants (CNVs) at diagnosis and 28 SNVs and insertion/deletion variants and 22 CNVs at relapse. With these genetic alterations, we could detect several B cell precursor ALL patients with high-risk gene alterations who were not stratified into the highest-risk group (5/8, 62.5%). We also detected potentially actionable genetic variants in about half of the patients (8/17, 47.1%). Among them, we found that one patient harbored germline *TP53* mutation as a secondary finding. This inexpensive, rapid method can be immediately applied as clinical sequencing and could lead to better management of these patients and potential improvement in the survival rate in childhood ALL.

Keywords Leukemia · Pediatric · ALL · Molecular genetics · Precision medicine

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children [1]. With risk-adapted, multi-agent chemotherapy, more than 80% cases of newly diagnosed childhood ALL are cured. However, up to 20% of patients with childhood ALL suffer from relapse of leukemia. The cure rate of these patients is less than 40%. Therefore, childhood ALL still remains the main cause of death from cancer in children [1–3].

Several risk factors of relapse at initial diagnosis have been reported. These factors include age and white blood cell count at diagnosis. Additionally, a limited range of genetic alterations as gene rearrangement or chromosomal events, such as *ETV6-RUNX1* or hyperdiploidy, have been reported to be associated with a favorable outcome, and *BCR-ABL1*, *KMT2A*-rearrangement, hypodiploidy, intrachromosomal amplification of chromosome 21, and *TCF3-HLF* have been reported to be associated with an inferior outcome. Adding to

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these factors, minimal residual disease (MRD) measured by sensitive molecular tests, which reflects treatment response, is an emerging strong prognostic factor for pediatric ALL. Modern treatment protocols integrate these genetic alterations and MRD to stratify patients into several risk groups [1]. Generally, the highest-risk group receives maximally intensive treatment, including hematopoietic stem cell transplantation (HSCT), and the lowest-risk group receives less intensified multi-agent chemotherapy. Many patients who do not have high-risk features or low-risk features are classified as the intermediate-risk group. This group contains heterogeneous patients, and thus, one uniform therapy is not suitable for this group. While a considerable number of the lowest-risk group of patients are expected to be cured with less intensive chemotherapy, the cure rate of the intermediate- and highest-risk group of patients remains unsatisfactory. Therefore, to improve the overall cure rates of childhood ALL, precisely detecting patients with high risk of relapse or detecting new treatment targets in the intermediate- and high-risk group of patients is important for personalized precision medicine.

Recently, many gene deletions at diagnosis were reported to be associated with an inferior outcome in childhood ALL. These genes include transcriptional regulators of lymphoid development (*IKZF1* and *EBF1*), tumor suppressors and cell cycle regulation (*TP53*, *RB1*, *MSH2*, and *CDKN2A/B*), and epigenetic regulators (*CREBBP*) [4–14]. These adverse prognostic factors have been reproduced in multiple cohorts, but they have not been incorporated into prospective treatment studies.

Fewer prognostic factors for childhood ALL have been applied in the relapse setting compared with the setting of initial diagnosis. Among them, the time to relapse, the site of relapse, and immunophenotype are the most widely accepted and applied in many clinical trials [15–17]. According to these risk factors, patients with relapsed ALL are stratified into the low-risk group or high-risk group. Many patients receive multi-agent chemotherapy and subsequent HSCT. However, the overall survival rate of patients with relapsed ALL still remains poor and is low as 40%, despite these intensive treatments [2, 3]. With limited success of the conventional strategy of maximally intensified cytotoxic chemotherapy and HSCT, relapsed ALL might be a good candidate for recent targeted therapy [2] or cancer immunotherapy [18].

In recent years, development of the high-throughput next-generation sequencing (NGS) method has allowed detection of a large number of genetic alterations. NGS can detect single-nucleotide variants (SNVs), small insertions and deletions (in/dels), and gene copy number variants (CNVs) at the same time. Therefore, NGS will be used in the clinical setting, namely as clinical sequencing, in the near future.

This study aimed to detect genetic alterations, including single SNVs, small in/dels, and CNVs with the NGS method for matched diagnostic, remission, and relapse samples of 17 patients with relapsed ALL. In this retrospective study, we found that many relapsed patients had genetic alterations with adverse prognostic value at diagnosis. We also found that most patients had actionable variants, including the germline *TP53* mutation, which suggested that this method could work well as clinical sequencing and potentially improve management of patients.

Materials and methods

Patients Seventeen children aged 0 to 18 years who were diagnosed with relapsed ALL between 2000 and 2017 in Okayama University Hospital and Hokkaido University Hospital were enrolled in this study. Treatment protocols were diverse, as Japan Association of childhood Leukemia Study (JACLS) ALL97 and ALL02 protocol [19, 20], Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) Ph-04, MLL-03, MLL-10, and T-11 protocol [21, 22]. The results of JPLSG MLL-10 and T-11 protocol have not been published. These are listed in Table 1. Patients with molecular relapse were excluded from this study. We have already reported *ABL1* gene mutation analysis [23] and NGS analysis [24] for Ph + ALL (UPN 10 and 11) and the NGS analysis for infant ALL (UPN 13 and 14) [25]. The results of NGS analysis for infant ALL are slightly different between our previous report [24] and the present study. This is due to the changes in analytic pipeline; we used only two databases (dbSNP and 1000gp) to remove SNP in the previous study, but we use three databases (dbSNP, 1000gp, and Human Genetic Variation Database) in the present study as noted below. This study was approved by the institutional ethics committee in Okayama University Hospital and informed consent was obtained for each patient from the legal guardian of the patients and/or themselves. All methods as follows were performed in accordance with the relevant guidelines and regulations.

DNA isolation Somatic DNA was obtained from bone marrow samples at diagnosis and each episode of relapse, while germline DNA was obtained from a buccal swab or peripheral blood in complete remission (CR) status. DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and quantified using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit® 2.0 Fluorometer (Thermo Fisher Scientific) according to the manufacturer's instructions.

Targeted NGS approach We performed targeted sequencing of more than 150 cancer-related genes for DNA as previously reported [25]. Targeted gene lists are shown in Supplementary Table S4, and allocation of patients is shown in Supplementary Table S1. These gene panels were generated using an online

Table 1 Clinical information of analyzed patients in this study

Disease type	UPN	Age at diagnosis	Sex	chimera gene	Karyotyping at diagnosis	initial WBC	PSL response	initial therapy[a]
BCP	1	5	M	*ETV6-RUNX1*	46, XY	4.87 × 10^3	PGR	JACLS ALL02 SR
	2	12	M	[−]	46, XY	13.3 × 10^3	PGR	JPCLS ALL02 HR
	3	15	M	[−]	46, XY	8.64 × 10^3	PPR	JACLS ALL02 ER
	4	13	F	[−]	47, XX, +?10 [3/3]	265 × 10^3	PGR	JACLS ALL02 F
	5	4	M	*ETV6-RUNX1*	46, XY	7.87 × 10^3	PGR	JACLS ALL02 HR
	6	6	M	*TCF3-PBX1*	46, XY	19.4 × 10^3	PPR	JACLS ALL02 ER
	7	5	M	*ETV6-RUNX1*	46,XY [16/20]	4.7 × 10^3	PGR	JACLS ALL97 SR
	8	4	M	[−]	52, XY, +X, +6, +14, +17, +21, +21 [15/20]	29.1 × 10^3	PGR	JACLS ALL02 HR
	9	10	F	*TCF3-PBX1*	45, XX, del(6)(q?), −7, −13, der(19)?(1;19)(q23;p13), −21, +2mar [1/5]	34.8 × 10^3	PGR	JACLS ALL02 HR
Ph	10	5	M	*mBCR-ABL*	46, XY, del(5)(q?), t(9;22)(q34;q11.2) [10/20] 46, sl, +5, −del(5), del(7)(p?) [4/20] 46, sl, +5, −del(5), −20, +marl [6/20]	525 × 10^3	PPR	JPLSG Ph-04
	11	4	M	*mBCR-ABL*	50, XY, +X, +5, t(9;22)(q34;q11.2), +13, +der(22)t(9;22) [3/20]	53.5 × 10^3	PGR	JPLSG Ph-04
	12	11	M	*mBCR-ABL*	47,XY,+5,t(9;22)(q34;q11) [1/20]	4.4 × 10^3	PGR	JACLS ALL F97
infant	13	3 m	F	*KMT2A-MLLT1*	46, XX, t(11;19)	833 × 10^3	PPR	JPLSG MLL-10
	14	4 m	M	*KMT2A-AFF1*	46, XY, t(4;11)(q21:q23)	77.6 × 10^3	PGR	JPLSG MLL-03
	15	4 m	M	*KMT2A-AFF1*	46, XY, t(4;11)(q21;q23)	167 × 10^3	PGR	JPLSG MLL-10
T	16	11	M	[−]	46, XY, del(11)(q?) [13/20]	158 × 10^3	PGR	JPLSG T-11
	17	3	M	[−]	71, XY, −X, add(1)(p32), +10, −15, +17, +20, +21, +mar [1/20] 46, XY [19/20]	5.6 × 10^3	PGR	JACLS ALL02 T

Disease type	transplantation at first remission	site of relapse	timing of relapse	karyotype at relapse	Outcome	institute
BCP	no	BM + CNS	1 mo after treatment completion	46, Y, add(X)(p11.2), add(9)(p11) [2/20]	dead	OU
	no	BM	11 mo after treatment completion	46, XY	alive	OU
	no	BM	8 mo after treatment completion	46, XY	dead	OU
	yes	BM	7 mo after BMT	46, X, −X, or -Y, add(1)(p32), add(3)(p21), −7, −10, −14, −15, add(16)(p11), −17, −19, −20, +8mar [1/20] 46, XY [19/20]	dead	OU
	no	BM	1y after treatment completion	46, XY	alive	OU
	no	BM	3 mo after treatment completion	46, X, -Y, add(2)(p21), del(6)(q?), add(9)(p13), add(19)(p13), +marl [4/20]	alive	OU
	no	BM	19 mo after treatment completion	45, XY, der(1;3)(p10;p10), der(6)add(6)(p21)add(6)(q13), der(9)t(9;11)(q34;q13), add(11)(q13), der(12)t(3;12)(q21;p12), der(13;19)(q10;p100, del(14)(q?), +marl [2/20] 46, idem, +8 [10/20] 46, XY [8/20]	dead	HU
	no	BM	on therapy(maintenance)	52, XY, +X, +6, t(7;11), +14, +17, +21, +21 [10/20]	dead	HU

Table 1 (continued)

	no	BM	3 mo after treatment completion	92, XXXX, +add(1)(p11), +add(1)(p11), −3, add(4)(q21), −9, −9, add(11)(q13), −15, −19, der(19)t(1;19)(q21;p13), add(21)(p12), +add(21)(p12), +22, +mar [4/10] 67, XXX, −1, add(4)(q21), add(6)(q12), −7, −8, −9, +12, +12, −15, +16, +18, der(19)t(1;19)(q21;p13), −20, −21, +22, +mar [1/10] 46, XX [4/10]	dead	HU
Ph	yes	BM	6 mo after BMT	46, XY, −2, −8, t(9;22)(q34;q11.2), −10, −15, +4 mar [1/20]	dead	OU
	no	BM	on therapy (re-induction)	na	alive	OU
	yes	BM	8 mo after BMT	46, XX	dead	HU
infant	yes	ascite	14 mo after CBT	na	alive	OU
	yes	BM	1 mo after CBT	48, XY, +X, +1, t(4;11)(q21:q23), inv(7)(q22q32) [18/20]	dead	OU
	yes	combined (BM, CNS, extramedullary)	11 mo after CBT	46, XY [1/20] 46, XX [19/29]	alive	HU
T	no	BM	on therapy (maintenance)	46, XY	alive	OU
	no	combined (BM, mediastinum)	6 mo after treatment completion	na	alive	HU

WBC white blood cell, *PSL* predonisolone, *JACLS* Japan Association of childhood Leukemia Study, *JPLSG* Japanese Pediatric Leukemia/lymphoma Study Group, *BM* bone marrow, *CNS* central nervous systems, *BMT* bone marrow transplantation, *CBT* cord blood transplantation, *OU* Okayama University Hospital, *HU* Hokkaido University Hospital, *na* not available, *mo* months

[a] The details about treatment protocols are shown in references [16–20]

design tool for HaloPlex (Agilent Technologies) and target enrichment was performed using the HaloPlex standard protocol. Samples were then sequenced by MiSeq (Illumina, Inc., San Diego, CA, USA). Read alignment to the hg19 reference genome was performed by the Burrows–Wheeler Aligner and variant calling was performed using SureCall software (Agilent Technologies).

Variant prioritization and assessment of pathogenicity We excluded synonymous or non-coding variants, and single-nucleotide polymorphisms reported with a frequency of 1% or higher in single-nucleotide polymorphism databases (dbSNP, 1000gp, and Human Genetic Variation Database). Variant bases that had ≥ 5 reads in each sample proceeded to the next step. Genetic variations that were constantly detected from diagnostic, remission, and relapse samples with a VAF ≥ 0.2 were regarded as candidate germline alterations. Genetic alterations that were not or rarely detected from diagnostic samples, but were detected from diagnostic and/or relapse samples with a VAF ≥ 0.05, were regarded as candidate somatic alterations. Differences in VAF between normal and diagnostic/relapse samples were assessed by Fisher's exact test and regarded as significant with a threshold of ≤ 0.01. Finally, for germline and somatic alterations, the read quality was checked on IGV software (Broad Institute, Cambridge, MA, USA). Germline mutations detected as secondary findings were validated by Sanger sequencing after informed consent was obtained from each patient, again.

In the same single sequencing run, we could detect not only SNV, but also CNV in the targeted genes. CNV was identified with a read depth-based method using SureCall v3.0 software. CNVs were predicted based on the log ratio of the normalized sample read depth to the reference sample read depth using default parameters. The patients' CR samples were used as the reference sample. Base sequences with a log ratio below/above − 0.6/0.3 were classified as deletion/duplication, respectively. In CNV analysis, higher scores were considered more reliable and the maximum value was 1, which was calculated with an original algorithm of Agilent Technologies. We previously reported the utility of this method [24]. For childhood ALL, gene deletions are often reported as prognostic factors, and gene amplifications are less frequently reported as prognostic factors. Therefore, we selected deletions of previously reported genes (*IKZF1*, *PAX5*, *RB1*, *CDKN2A/B*, *ETV6*, *EBF1*, *BTG1*, *CREBBP*, *SH2B3*, *TP53*, and *MSH2*) [4, 5, 7–10, 13, 14, 26]. *BCL11B*, *NOTCH1*, and *FBXW7* deletions in T cell ALL [27, 28], *RUNX1* deletion in *ETV6-RUNX1* ALL, *TCF3* deletion in *TCF3-PBX1* ALL, *ABL1* deletion in *BCR-ABL1* ALL, and *KMT2A* deletion in *KMT2A*-rearranged ALL were also analyzed. We selected deletions among these genes mentioned above with a score of ≥ 0.5 and deletions detected in two or more consecutive regions.

We reported the gene deletions only when minimum 100 reads are aligned on the regions of reference samples.

Analysis for clonal changes between diagnosis and relapse We also checked the clonal relationship of diagnostic and relapse samples in ALL by analyzing SNVs, small insertion/deletions, and CNVs on matched diagnostic and relapse samples. For CNVs, we included not only gene deletions, but also gene gains. We used only highly reliable genetic alterations. Therefore, we chose SNVs and in/dels with a VAF ≥ 0.1, and CNVs with 100 or more reads aligned on the reference samples with a score of ≥ 0.5 and detection in two or more consecutive regions. If CNVs detected at diagnosis or relapse were also detected in matched relapse or diagnostic samples with narrowed regions and a sufficient score, the genes are not determined as changed. Four cases (UPN 9, 10, 12, and 17) were excluded from this analysis because they were not analyzed with the same gene panels among diagnostic, CR, and relapse samples. Finally, clonal changes were categorized into four patterns (A: genetically distinct leukemia, B: same as a diagnostic clone, C: clonal evolution from a diagnostic clone, D: clonal evolution from ancestral clones), as previously reported [29].

Definition of potential actionable genes Among gene alterations we detected, we selected several alterations as "potentially actionable genes" based on several previous larger studies [30–32]. These potentially actionable genes include gene alterations that could lead to a newer molecular-targeted therapy, a change in selection of drugs, or a change in patient or family counseling and management by identifying germline cancer-predisposing gene alterations. We exclude gene alterations which were detected only in diagnostic samples but not in relapsed samples, because targeting these alterations could not avoid subsequent relapse events.

Fluorescence in situ hybridization FISH for detection of *TP53* deletion was performed with commercially available probes (Vysis TP53/CEP 17 FISH Probe Kit; Abbot, Chicago, IL, USA) and samples were counterstained with DAPI according to manufacturer's instructions.

Results

Patients' characteristics Clinical information of the analyzed patients is shown in Table 1. Sixteen patients were Japanese and one patient (UPN 10) was Egyptian. A relatively high ratio of boys to girls (14:3) was observed in our cohort, and the median age at diagnosis was 5 years (3 months to 15 years). Our study included nine patients with B cell precursor ALL (BCP-ALL; in this study, this terminology excludes Philadelphia chromosome-positive ALL or infant ALL), three patients with Philadelphia chromosome-positive ALL (Ph + ALL), three patients with infant ALL, and two patients with T cell ALL (T-ALL). Among the nine patients with BCP-ALL, only one (UPN 4) received HSCT at her first remission. All 17 patients received HSCT after the first relapse.

Descriptive results from sequencing runs The average number of total reads was 1,874,078 (range: 1,080,771–4,221,238) with an average read length of 112–136. The read depth in analyzable target regions per sample was between 210 and 928×. A total of 87.63–98.3% of analyzable regions were covered at least 20 reads, 83.63–97.34% were covered at least 50 reads, and 74.55–95.84% were covered at least 100 reads. These quality metrics data were obtained from analysis by SureCall v3.0 software (Agilent Technologies, Santa Clara, CA, USA), and the details are shown in Supplementary Table S1.

The results of NGS are shown in Table 2. We identified total 16 SNVs and in/dels at diagnosis, and 28 at relapse. For CNV, 19 genes were deleted at diagnosis and 22 were deleted at relapse. The total number of CNVs was mainly increased in BCP-ALL.

Germline variations In this study, we analyzed triplicate samples of diagnosis, remission, and relapse for childhood ALL, and this approach enabled us to detect germline variations as secondary findings. While candidates of germline variations were detected in six patients, most of them were not regarded as pathogenic or likely pathogenic according to published recommendations [33–35]. Only one variation, *TP53* R248Q detected in UPN 2, is widely known as a pathogenic mutation. This mutation was heterozygous in the germline sample with a variant allele frequency (VAF) of 0.498. However, the VAF was elevated to 0.744 and 0.698 in diagnostic and relapse leukemia samples, respectively. We also performed FISH analysis for these samples. While remission sample shows only normal signal patterns with two red (*TP53*) and green (centromere of chromosome 17), diagnostic and relapsed leukemic samples include leukemia cells with one red signal (Supplementary Figure). This finding suggested that loss of heterozygosity occurred due to the deletion of *TP53* and led to progression of leukemia, which is a hallmark of tumorigenesis of *TP53* mutations. Other candidate genes that were detected from germline samples are listed in Supplementary Table S2.

Genetic alterations at diagnosis All but one (UPN 2) of the patients had at least one genetic alteration at diagnosis, and all 17 patients had at least one genetic alteration.

This study included nine patients with BCP-ALL. Only one (UPN 4) of these patients was classified into the highest-risk group, and this patient received HSCT at first remission. However, among eight patients with BCP-ALL who were not classified into the highest-risk group, three

Table 2 Detected gene alterations at diagnosis and relapse

Disease type	UPN	germline SNV/ indels	SNV at diagnosis (VAF)	Deletion at diagnosis	SNV at relapse (VAF)	Deletion at relapse
BCP	1	[−]	[−]	*CDKN2A/BPAX5BTG1*	[−]	*CDKN2A/B* *PAX5*
	2	*TP53* R248Q	[−]	[−]	[−]	*RB1*
	3	[−]	*GATA1* P195L (0.0559)	*EBF1* *ETV6*	[−]	*ETV6*
	4	[−]	[−]	*EBF1* *IKZF1*	[−]	*EBF1* *IKZF1* *RB1*
	5	[−]	[−]	*EBF1* *ETV6*	[−]	*EBF1* *ETV6*
	6	[−]	*TCF3* G257C (0.233) *EZH2* D659G (0.204)	*TCF3*	*CREBBP* R1378Q(0.38) *CRLF2* Y96H (Y 0.369, X 0.352) *KIT* V532I (0.275) *TCF3* G257C(0.198) *TERT* Q53R (0.18) *ASXL1* E537K (0.0714) *SMARCA4* R1336H (0.0667)	*MSH2* *CDKN2A/B* *PAX5* *TCF3*
	7	[−]	[−]	*ETV6*	*CREBBP* E1562D (0.239) *DNMT1* T1519 L(0.155) *DNMT1* F1290 L (0.0874) *MECOM* P433T (0.0615)	*ETV6* *RB1*
	8	[−]	*KRAS* G12D (0.388) *CREBBP* R1446H (0.538)	[−]	*KRAS* G12D (0.465) *CREBBP* R1446H (0.97)	[−]
	9	[−]	*IKZF3* I99fs (0.327)	*TCF3*	*IKZF3* I99fs (0.333) *KIT* R122H (0.0988)	*MSH2* *TCF3*
Ph	10	[−]	*IKZF1* Q446* (0.101)	*EBF1* *IKZF1* *RB1*	*IKZF1* Q446* (0.474) *ABL1* F359C (0.399)	[−]
	11	[−]	[−]	*IKZF1*	*DOT1L* Q745P (0.165)	*IKZF1*
	12	[−]	*FANCG* S383F (0.0583) *TP53* T377P (0.0548) *SMARCC2* c.231-232insGG (0.209)	*IKZF1* *RB1*	*FANCG* S383F (0.0581) *TP53* T377P (0.0309) *SMARCC2* c.231-232insGG (0.176)	*IKZF1*
infant	13	[−]	*KRAS* G12D (0.358)	[−]	*KRAS* G12D (0.468)	*KMT2A*
	14	[−]	*TP53* R175H (0.363) *FLT3* A680V (0.22)	[−]	*TP53* R175H (0.33) *TP53* G245S (0.3) *FLT3* A680V (0.314)	[−]
	15	[−]	*KRAS* G12D (0.368)	*IKZF1*	[−]	*IKZF1*
T	16	[−]	*WT1* A358P (0.425) *JAK3* E958K (0.421) *NOTCH1* L1574P (0.355)	*FBXW7* *CDKN2A/B*	*WT1* R363G (0.443) *KMT2A* S3640R (0.172)	*CDKN2A/B*
	17	[−]	*NOTCH1* L1600P(0.152) *NOTCH1* L1574P (0.0364)**	*BCL11BCREBBP*	*NOTCH1* L1574P(0.263)	*CDKN2A/B* *BCL11B* *CREBBP*

SNV single-nucleotide variation, *VAF* variant allele frequency

patients (UNP 1, 3, and 5) had CNVs with unfavorable prognostic value previously reported [4, 5, 8–12, 36]. UPN 4, who was regarded as the highest-risk patient, also had unfavorable risk CNVs. UPN 4, who was regarded as the highest-risk patient, also had a cytogenetic risk factor. With regard to the remaining five patients, UPN 8 had a hyperdiploidy karyotype and well-known *KRAS* and *CREBBP* mutations, which are associated with a higher risk of relapse [26, 37, 38]. UPN 2 also had the germline *TP53* mutation, as mentioned above, and was thought to be a high-risk patient [39]. Therefore, among a total of nine patients with BCP-ALL, six had unfavorable prognostic genetic alterations at diagnosis. And, five out of eight BCP-ALL patients who were not stratified into the highest-risk group had these alterations at diagnosis. Many of these risk factors were also detected at relapse of each patient, and only one patient (UPN 3) lost his cytogenetic risk factor

Table 3 Detected actionable findings and potential actions for them

Disease type	UPN	Potentially actionable findings	Potential actions based on sequencing results
BCP	1	*CDKN2A/B* deletion(diagnosis and relapse)	CDK inhibitors
	2	germline *TP53* mutation	cancer screening for LFS
	3	[−]	[−]
	4	[−]	[−]
	5	[−]	[−]
	6	[−]	[−]
	7	[−]	[−]
	8	*KRAS* mutation(diagnosis and relapse)	MEK inhibitors
	9	[−]	[−]
Ph	10	*ABL1* mutation(relapse)	higher generation TKI
	11	[−]	[−]
	12	[−]	[−]
infant	13	*KRAS* mutation(diagnosis and relapse)	MEK inhibitors
	14	*FLT3* mutation(diagnosis and relapse)	FLT3 inhibitors
	15	[−]	[−]
T	16	*CDKN2A/B* deletion(diagnosis and relapse)	CDK inhibitors
	17	*NOTCH1* mutation (diagnosis and relapse)*CDKN2A/B* deletion(relapse)	NOTCH1 inhibitorsCDK inhibitors

CDK cycline-dependant kinase, *LFS* Li-Fraumeni syndrome, *VD* vincristine and dexamethasone, *HDAC* histone deacetylase, *MEK* mitogen-activated protein kinase/extracellular signal-regulated kinase, *TKI* tyrosine kinase inhibitor

(*EBF1* deletion) at relapse. Three patients did not have any prognostic genetic alterations, including two patients who had *TCF3-PBX1*-positive ALL, and both of them had *TCF3* gene deletions. UNP 7 did not show any prognostic genetic alteration at diagnosis.

All three patients with Ph + ALL were accompanied by *IKZF1* deletions, which are associated with an unfavorable outcome [40]. While two of three patients with Ph + ALL had *RB1* deletions, both of them lost *RB1* deletions at relapse.

This study included three patients with infant ALL. All of the three patients showed mutations in tyrosine kinase-PI3K-RAS signaling pathways. The mutations in these pathways are frequently reported as activating mutations, and VAFs were relatively high (0.22–0.368, Table 2). These gain of function mutations are candidates for new target treatment, as mentioned below.

Two patients with T-ALL had sequencing performed, and both patients had *NOTCH1* mutations at diagnosis. The prognostic significance of *NOTCH1* alterations are not consistent, but are reported to be good prognostic factors [28, 41]. These two patients showed a *WT1* mutation, *JAK3* mutation, *FBXW7* deletion, *CDKN2A/B* deletion, *BCL11B* deletion, and *CREBBP* deletion, and all of these genetic alterations are frequently involved in T-ALL [42].

Patterns of relapse To clarify the clonal origins of relapsed samples, we analyzed the differences in SNVs, in/dels, and CNVs between matched diagnostic and relapse samples and classified them into four groups (A: genetically distinct leukemia, B: same as a diagnostic clone, C: clonal evolution from a diagnostic clone, D: clonal evolution from ancestral clones), as previously reported [29]. We used genetic alterations that passed a higher threshold, as described in the "Materials and methods" section below. Four cases were excluded from analysis because gene panels that were used for these patients were not consistent among triplicate samples (see Supplementary Table S1). The results are shown in Supplementary Table S3. In most cases (9/13, 69.2%), relapsed samples acquired new lesions and lost some lesions that were present at diagnosis, which showed their clonal evolution from ancestral clones. Four (30.7%) cases showed direct clonal evolution from diagnostic clones. No relapsed ALL clones were classified into genetically distinct or identical clones as diagnostic clones.

Actionable genetic alterations In this study, eight patients (46.1%) had several potentially actionable genetic alterations according to the definition noted above. These detected genetic alterations and potential actions for these are shown in Table 3. These genetic alterations included not only genes that could be therapeutic targets, but also genes that are predictive markers of resistance to specific therapy (*ABL1* mutation and tyrosine kinase inhibitor).

Discussion

This study showed the results of NGS for matched diagnostic, remission, and relapse samples of childhood ALL. We showed the utility of NGS to clarify new prognostic genetic alterations and many targetable genetic alterations, including cancer-predisposing, germline genetic alterations in the clinical setting.

In patients with BCP-ALL, many genetic alterations have been reported to be associated with an adverse prognosis [1]. In this study, we detected adverse prognostic genetic alterations in 6/9 (66.7%) patients with BCP-ALL, and most of them were not classified into the highest-risk group at diagnosis. Among three patients without any prognostic genetic alterations, two patients with *TCF3-PBX1* showed a *TCF3* deletion, but their prognostic value is unknown. Relapse of childhood ALL occurs owing to a variety of reasons, such as older age, a less sophisticated treatment protocol, and patients' adherence to therapy besides genetic prognostic factors [43]. Therefore, some relapsed patients should be free from any genetic risk factors. The total number of CNVs was mainly increased in BCP-ALL, as previously shown [29].

IKZF1 deletion is an adverse prognostic factor in any type of BCP-ALL [8–10]. Detecting *IKZF1* deletions in Ph + ALL is also important because Ph + ALL without *IKZF1* deletions has a better prognosis than that with *IKZF1* deletions, and it could potentially avoid HSCT. Addition of vincristine-steroid pulses during maintenance therapy may specifically benefit patients with non-Ph + BCP-ALL with *IKZF1* deletions [10], but this benefit has not been confirmed for all patients with Ph + ALL. Therefore, optimal treatment modifications in patients with Ph + ALL and *IKZF* deletions are under investigation [40, 44].

Remarkably, all of the patients with infant ALL in our study had tyrosine kinase-PI3K-RAS pathway-activating mutations. In a larger study, a trend toward inferior survival and an increased relapse rate in patients with these mutations were reported, and this effect was emphasized when VAF of these mutations was ≥ 30% [45]. These mutations are also new candidates of target treatment. This targeted therapy should not be used alone but should be added to conventional chemotherapy. This is because tyrosine kinase-PI3K-RAS pathway-activating mutations in infant ALL are sometimes lost at relapse [45].

Recently, *SPI1* fusions were newly reported for pediatric T-ALL, and this subgroup is associated with a poor outcome [46]. While we detected many recurrent genetic alterations for T-ALL, a small number of genetic alterations with adverse prognostic value have been reported [47]. Patients with T-ALL have many non-coding genetic alterations or genetic rearrangement, such as *SPI1* fusions. Therefore, the method used in our study might not be appropriate for patients with T-ALL.

We checked the patterns of relapse events for our patients. Our patients showed clonal evolution from a diagnostic clone or clonal evolution from ancestral clones. These distributions are comparable to the previous report [29, 48].

One remarkable finding of this study was frequent detection of actionable genetic alterations at diagnosis and relapse. A total of 8/17 (47.1%) patients showed actionable alterations, and these genetic alterations were detected, irrespective of ALL subtype. The frequency is comparable to that in a previous report [30, 31]. Among these alterations, many alterations dominantly presented at diagnosis of patients with T-ALL and one tyrosine kinase-PI3K-RAS pathway-activating mutation in a patient with infant ALL was lost at relapse. These alterations should be chosen with caution as target treatment.

Another interesting finding in this study was detection of a germline variation as secondary findings. The larger childhood cancer case series find that 8.5% of their patients conferred a pathogenic mutation among cancer predisposition genes they selected for analysis [49]. And among patients with candidate gene mutations, only 40% of patients had a family history of cancer. Moreover, another large study shows that panel-based gene sequencing is associated with increased detection of individuals with germline gene mutations over the predicted yield of targeted germline testing based on clinical guidelines [50]. These observations have illustrated the importance of genetic testing for selected patients even when they do not have a family history of cancer.

Among cancer predisposition syndromes, Li-Fraumeni syndrome (LFS) is one of the most aggressive and investigated conditions. Patients with LFS suffered from wide spectrums of cancers such as brain tumors, adrenocortical carcinoma, soft tissue and bone tumors, hematologic malignancies, or breast cancer. Germline *TP53* mutations are the primary underlying genetic alteration that predisposes individuals to the development of these cancers. As for ALL, low-hypodiploid type is known to be associated with germline *TP53* mutations [51], but about one-third of patients with germline *TP53* pathogenic variants did not have typical hypodiploid karyotype [39].

In this study, we detected well-known *TP53* R248Q mutation from germline sample of UNP2. He was initially diagnosed as ALL with normal karyotype and did not have family history of cancer at the first diagnosis. Thus, he was not suspected as having any underlying cancer predisposition conditions. However, after his first relapse, his younger sister suffered from pontine glioma, and subsequently, he developed skin tumor (Bowen disease). Therefore, he was diagnosed with LFS according to Chompret criteria [52].

Patients with LFS have a substantial lifetime risk of developing cancer, and a clinical surveillance protocol enables early detection of tumors and improves long-term survival with reduced treatment-related morbidity and mortality [53]. As shown for UPN2, panel-based genetic analysis, but not

targeted germline testing based on clinical information, would be beneficial for better management of patients with underlying cancer predisposition syndromes at the first diagnosis. However, the survival benefit of surveillance protocol has not been verified in the most cancer predisposition syndromes. Moreover, interpretations of detected variants and reporting them in the clinical setting should be performed with caution, usually in accordance with published guidelines [33–35].

Time and cost required are another advantage of the approach used in our study. This method requires relatively low throughput of NGS, and thus, less time and cost are required. Currently, MRD is the most powerful prognostic factor. However, MRD is usually measured at the end of induction and at the end of consolidation therapy, which are several months after diagnosis. We can obtain an informative result from this approach within a few days, and we might possibly be able to adjust treatment intensity at an earlier phase than MRD in a future study. However, this should be confirmed in the prospective larger study.

Our study has several limitations. This study was retrospective and only included relapsed patients. We detected several poor prognostic factors among our patients, but it is unclear whether we could stratify patients according to these factors. Prospective study of a greater number of patients is needed to confirm true usefulness of this method for stratifying patients with high-risk ALL. Moreover, targeted therapy should be considered with caution, because actionable genetic alterations would be detected in patients who do not relapse with conventional chemotherapy. For these patients, actionable genetic alterations might possibly be used to reduce chemotherapeutic burden, but we could not elucidate this point because this study includes only patients with high-risk ALL.

In summary, we used the NGS method for patients with relapsed ALL and detected SNVs, in/dels, and CNVs with prognostic value, including a well-known germline mutation. We precisely detect patients with high risk of relapse and could also detect actionable targets in most patients with this approach. Incorporating this inexpensive, rapid method into the clinical setting will enable a patient-oriented, precision strategy for childhood ALL.

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Availability of data The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contribution H.I, A.I, and A.S wrote the manuscript. H.I, M.A, T.T, T.M, M.S., and A.S performed the genetic analysis and interpreted the result. H.I, K.T, K.K, K.F, K.W, H.T, and A.S did the patient's care and collected the clinical data. All authors reviewed the manuscript.

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Compliance with ethical standards

This study was approved by the institutional ethics committee in Okayama University Hospital and informed consent was obtained for each patient from the legal guardian of the patients and/or themselves. All methods were performed in accordance with the relevant guidelines and regulations.

Conflict of interests The authors declare that they have no conflict of interest.

Ethical approval The institutional Review Board of Okayama University Hospital approved this study.

Informed consent Informed consent was obtained for each patient from the legal guardian of the patients and/or themselves.

References

1. Hunger SP, Mullighan CG (2015) Acute lymphoblastic leukemia in children. N Engl J Med 373:1541–1552. https://doi.org/10.1056/NEJMra1400972
2. Bhojwani D, Pui C-H (2013) Relapsed childhood acute lymphoblastic leukaemia. Lancet Oncol 14:e205–e217. https://doi.org/10.1016/S1470-2045(12)70580-6
3. Goto H (2015) Childhood relapsed acute lymphoblastic leukemia: its biology and recent progress of treatment. Pediatr Int 57:1059–1066. https://doi.org/10.1111/ped.12837
4. Schwab CJ, Chilton L, Morrison H, Jones L, al-Shehhi H, Erhorn A, Russell LJ, Moorman AV, Harrison CJ (2013) Genes commonly deleted in childhood B-cell precursor acute lymphoblastic leukemia: association with cytogenetics and clinical features. Haematologica 98:1081–1088. https://doi.org/10.3324/haematol.2013.085175
5. Ribera J, Morgades M, Zamora L, Montesinos P, Gómez-Seguí I, Pratcorona M, Sarrà J, Guàrdia R, Nomdedeu J, Tormo M, Martínez-Lopez J, Hernández-Rivas JM, González-Campos J, Barba P, Escoda L, Genescà E, Solé F, Millá F, Feliu E, Ribera JM, for the Spanish PETHEMA Group and the Spanish Society of Hematology (2015) Prognostic significance of copy number alterations in adolescent and adult patients with precursor B acute lymphoblastic leukemia enrolled in PETHEMA protocols. Cancer 121:3809–3817. https://doi.org/10.1002/cncr.29579
6. Messina M, Chiaretti S, Fedullo AL, Piciocchi A, Puzzolo MC, Lauretti A, Gianfelici V, Apicella V, Fazi P, te Kronnie G, Testi AM, Vitale A, Guarini A, Foà R (2017) Clinical significance of recurrent copy number aberrations in B-lineage acute lymphoblastic leukaemia without recurrent fusion genes across age cohorts. Br J Haematol 178:583–587. https://doi.org/10.1111/bjh.14721
7. Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD, Girtman K, Mathew S, Ma J, Pounds SB, Su X, Pui CH, Relling MV, Evans WE, Shurtleff SA, Downing JR (2007) Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature 446:758–764. https://doi.org/10.1038/nature05690
8. Mullighan CG, Su X, Zhang J, Radtke I, Phillips LA, Miller CB, Ma J, Liu W, Cheng C, Schulman BA, Harvey RC, Chen IM, Clifford RJ, Carroll WL, Reaman G, Bowman WP, Devidas M, Gerhard DS, Yang W, Relling MV, Shurtleff SA, Campana D, Borowitz MJ, Pui CH, Smith M, Hunger SP, Willman CL, Downing JR, Children's Oncology Group (2009) Deletion of

IKZF1 and prognosis in acute lymphoblastic leukemia. N Engl J Med 360:470–480. https://doi.org/10.1056/NEJMoa0808253
9. Dörge P, Meissner B, Zimmermann M et al (2013) IKZF1 deletion is an independent predictor of outcome in pediatric acute lymphoblastic leukemia treated according to the ALL-BFM 2000 protocol. Haematologica 98:428–432. https://doi.org/10.3324/haematol.2011.056135
10. Clappier E, Grardel N, Bakkus M et al (2015) IKZF1 deletion is an independent prognostic marker in childhood B-cell precursor acute lymphoblastic leukemia, and distinguishes patients benefiting from pulses during maintenance therapy: results of the EORTC Children's leukemia group study 58951. Leukemia 29:2154–2161. https://doi.org/10.1038/leu.2015.134
11. Moorman AV, Schwab C, Ensor HM, Russell LJ, Morrison H, Jones L, Masic D, Patel B, Rowe JM, Tallman M, Goldstone AH, Fielding AK, Harrison CJ (2012) IGH@ translocations, CRLF2 deregulation, and microdeletions in adolescents and adults with acute lymphoblastic leukemia. J Clin Oncol 30:3100–3108. https://doi.org/10.1200/JCO.2011.40.3907
12. Olsson L, Castor A, Behrendtz M, Biloglav A, Forestier E, Paulsson K, Johansson B (2014) Deletions of IKZF1 and SPRED1 are associated with poor prognosis in a population-based series of pediatric B-cell precursor acute lymphoblastic leukemia diagnosed between 1992 and 2011. Leukemia 28:302–310. https://doi.org/10.1038/leu.2013.206
13. Diouf B, Cheng Q, Krynetskaia NF, Yang W, Cheok M, Pei D, Fan Y, Cheng C, Krynetskiy EY, Geng H, Chen S, Thierfelder WE, Mullighan CG, Downing JR, Hsieh P, Pui CH, Relling MV, Evans WE (2011) Somatic deletions of genes regulating MSH2 protein stability cause DNA mismatch repair deficiency and drug resistance in human leukemia cells. Nat Med 17:1298–1303. https://doi.org/10.1038/nm.2430
14. Gupta SK, Bakhshi S, Kumar L, Kamal VK, Kumar R (2017) Gene copy number alteration profile and its clinical correlation in B-cell acute lymphoblastic leukemia. Leuk Lymphoma 58:333–342. https://doi.org/10.1080/10428194.2016.1193855
15. Gaynon PS, Qu RP, Chappell RJ, Willoughby MLN, Tubergen DG, Steinherz PG, Trigg ME (1998) Survival after relapse in childhood acute lymphoblastic leukemia: impact of site and time to first relapse–the Children's Cancer group experience. Cancer 82:1387–1395
16. Parker C, Waters R, Leighton C, Hancock J, Sutton R, Moorman AV, Ancliff P, Morgan M, Masurekar A, Goulden N, Green N, Révész T, Darbyshire P, Love S, Saha V (2010) Effect of mitoxantrone on outcome of children with first relapse of acute lymphoblastic leukaemia (ALL R3): an open-label randomised trial. Lancet 376:2009–2017. https://doi.org/10.1016/S0140-6736(10)62002-8
17. Eckert C, Henze G, Seeger K, Hagedorn N, Mann G, Panzer-Grümayer R, Peters C, Klingebiel T, Borkhardt A, Schrappe M, Schrauder A, Escherich G, Sramkova L, Niggli F, Hitzler J, von Stackelberg A (2013) Use of allogeneic hematopoietic stem-cell transplantation based on minimal residual disease response improves outcomes for children with relapsed acute lymphoblastic leukemia in the intermediate-risk group. J Clin Oncol 31:2736–2742. https://doi.org/10.1200/JCO.2012.48.5680
18. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, Chew A, Gonzalez VE, Zheng Z, Lacey SF, Mahnke YD, Melenhorst JJ, Rheingold SR, Shen A, Teachey DT, Levine BL, June CH, Porter DL, Grupp SA (2014) Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med 371:1507–1517. https://doi.org/10.1056/NEJMoa1407222
19. Horibe K, Yumura-Yagi K, Kudoh T, Nishimura S, Oda M, Yoshida M, Komada Y, Hara J, Tawa A, Usami I, Tanizawa A, Kato K, Kobayashi R, Matsuo K, Hori H (2017) Long-term results of the risk-adapted treatment for childhood B-cell acute lymphoblastic leukemia. J Pediatr Hematol Oncol 39:81–89. https://doi.org/10.1097/MPH.0000000000000760
20. Sakamoto K, Imamura T, Kihira K, Suzuki K, Ishida H, Morita H, Kanno M, Mori T, Hiramatsu H, Matsubara K, Terui K, Takahashi Y, Suenobu SI, Hasegawa D, Kosaka Y, Kato K, Moriya-Saito A, Sato A, Kawasaki H, Yumura-Yagi K, Hara J, Hori H, Horibe K (2018) Low incidence of osteonecrosis in childhood acute lymphoblastic leukemia treated with ALL-97 and ALL-02 study of Japan Association of Childhood Leukemia Study Group. J Clin Oncol 36:900–907. https://doi.org/10.1200/JCO.2017.75.5066
21. Manabe A, Kawasaki H, Shimada H, Kato I, Kodama Y, Sato A, Matsumoto K, Kato K, Yabe H, Kudo K, Kato M, Saito T, Saito AM, Tsurusawa M, Horibe K (2015) Imatinib use immediately before stem cell transplantation in children with Philadelphia chromosome-positive acute lymphoblastic leukemia: results from Japanese pediatric leukemia/lymphoma study group (JPLSG) study Ph $^{+}$ ALL04. Cancer Med 4:682–689. https://doi.org/10.1002/cam4.383
22. Koh K, Tomizawa D, Moriya Saito A, Watanabe T, Miyamura T, Hirayama M, Takahashi Y, Ogawa A, Kato K, Sugita K, Sato T, Deguchi T, Hayashi Y, Takita J, Takeshita Y, Tsurusawa M, Horibe K, Mizutani S, Ishii E (2015) Early use of allogeneic hematopoietic stem cell transplantation for infants with MLL gene-rearrangement-positive acute lymphoblastic leukemia. Leukemia 29:290–296. https://doi.org/10.1038/leu.2014.172
23. Aoe M, Shimada A, Muraoka M, Washio K, Nakamura Y, Takahashi T, Imada M, Watanabe T, Okada K, Nishiuchi R, Miyamura T, Chayama K, Shibakura M, Oda M, Morishima T (2014) ABL kinase mutation and relapse in 4 pediatric Philadelphia chromosome-positive acute lymphoblastic leukemia cases. Int J Hematol 99:609–615. https://doi.org/10.1007/s12185-014-1565-3
24. Aoe M, Ishida H, Matsubara T, Karakawa S, Kawaguchi H, Fujiwara K, Kanamitsu K, Washio K, Okada K, Shibakura M, Shimada A (2018) Simultaneous detection of ABL1 mutation and IKZF1 deletion in Philadelphia chromosome-positive acute lymphoblastic leukemia using a customized target enrichment system panel. Int J Lab Hematol 40:1–10. https://doi.org/10.1111/ijlh.12805
25. Ishida H, Kanamitsu K, Washio K, Muraoka M, Sakakibara K, Matsubara T, Kanzaki H, Shimada A (2016) Relapsed infant MLL -rearranged acute lymphoblastic leukemia with additional genetic alterations. Pediatr Blood Cancer 63:2059–2060. https://doi.org/10.1002/pbc.26100
26. Mullighan CG, Zhang J, Kasper LH, Lerach S, Payne-Turner D, Phillips LA, Heatley SL, Holmfeldt L, Collins-Underwood JR, Ma J, Buetow KH, Pui CH, Baker SD, Brindle PK, Downing JR (2011) CREBBP mutations in relapsed acute lymphoblastic leukaemia. Nature 471:235–239. https://doi.org/10.1038/nature09727
27. Gutierrez A, Kentsis A, Sanda T, Holmfeldt L, Chen SC, Zhang J, Protopopov A, Chin L, Dahlberg SE, Neuberg DS, Silverman LB, Winter SS, Hunger SP, Sallan SE, Zha S, Alt FW, Downing JR, Mullighan CG, Look AT (2011) The BCL11B tumor suppressor is mutated across the major molecular subtypes of T-cell acute lymphoblastic leukemia. Blood 118:4169–4173. https://doi.org/10.1182/blood-2010-11-318873
28. Jenkinson S, Koo K, Mansour MR, Goulden N, Vora A, Mitchell C, Wade R, Richards S, Hancock J, Moorman AV, Linch DC, Gale RE (2013) Impact of NOTCH1/FBXW7 mutations on outcome in pediatric T-cell acute lymphoblastic leukemia patients treated on the MRC UKALL 2003 trial. Leukemia 27:41–47. https://doi.org/10.1038/leu.2012.176
29. Mullighan CG, Phillips LA, Su X, Ma J, Miller CB, Shurtleff SA, Downing JR (2008) Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. Science 322:1377–1380. https://doi.org/10.1126/science.1164266

30. Mody RJ, Wu Y-M, Lonigro RJ, Cao X, Roychowdhury S, Vats P, Frank KM, Prensner JR, Asangani I, Palanisamy N, Dillman JR, Rabah RM, Kunju LP, Everett J, Raymond VM, Ning Y, Su F, Wang R, Stoffel EM, Innis JW, Roberts JS, Robertson PL, Yanik G, Chamdin A, Connelly JA, Choi S, Harris AC, Kitko C, Rao RJ, Levine JE, Castle VP, Hutchinson RJ, Talpaz M, Robinson DR, Chinnaiyan AM (2015) Integrative clinical sequencing in the Management of Refractory or relapsed Cancer in youth. JAMA 314:913–925. https://doi.org/10.1001/jama.2015.10080
31. Zehir A, Benayed R, Shah RH, Syed A, Middha S, Kim HR, Srinivasan P, Gao J, Chakravarty D, Devlin SM, Hellmann MD, Barron DA, Schram AM, Hameed M, Dogan S, Ross DS, Hechtman JF, DeLair DF, Yao JJ, Mandelker DL, Cheng DT, Chandramohan R, Mohanty AS, Ptashkin RN, Jayakumaran G, Prasad M, Syed MH, Rema AB, Liu ZY, Nafa K, Borsu L, Sadowska J, Casanova J, Bacares R, Kiecka IJ, Razumova A, Son JB, Stewart L, Baldi T, Mullaney KA, al-Ahmadie H, Vakiani E, Abeshouse AA, Penson AV, Jonsson P, Camacho N, Chang MT, Won HH, Gross BE, Kundra R, Heins ZJ, Chen HW, Phillips S, Zhang H, Wang J, Ochoa A, Wills J, Eubank M, Thomas SB, Gardos SM, Reales DN, Galle J, Durany R, Cambria R, Abida W, Cercek A, Feldman DR, Gounder MM, Hakimi AA, Harding JJ, Iyer G, Janjigian YY, Jordan EJ, Kelly CM, Lowery MA, Morris LGT, Omuro AM, Raj N, Razavi P, Shoushtari AN, Shukla N, Soumerai TE, Varghese AM, Yaeger R, Coleman J, Bochner B, Riely GJ, Saltz LB, Scher HI, Sabbatini PJ, Robson ME, Klimstra DS, Taylor BS, Baselga J, Schultz N, Hyman DM, Arcila ME, Solit DB, Ladanyi M, Berger MF (2017) Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. Nat Med 23:703–713. https://doi.org/10.1038/nm.4333
32. Gröbner SN, Worst BC, Weischenfeldt J, Buchhalter I, Kleinheinz K, Rudneva VA, Johann PD, Balasubramanian GP, Segura-Wang M, Brabetz S, Bender S, Hutter B, Sturm D, Pfaff E, Hübschmann D, Zipprich G, Heinold M, Eils J, Lawerenz C, Erkek S, Lambo S, Waszak S, Blattmann C, Borkhardt A, Kuhlen M, Eggert A, Fulda S, Gessler M, Wegert J, Kappler R, Baumhoer D, Burdach S, Kirschner-Schwabe R, Kontny U, Kulozik AE, Lohmann D, Hettmer S, Eckert C, Bielack S, Nathrath M, Niemeyer C, Richter GH, Schulte J, Siebert R, Westermann F, Molenaar JJ, Vassal G, Witt H, Lichter P, Weber U, Eils R, Korshunov A, Witt O, Pfister S, Reifenberger G, Felsberg J, von Kalle C, Schmidt M, Bartholomä C, Taylor M, Pfister S, Jones D, Lichter P, Jäger N, Buchhalter I, Korbel J, Stütz A, Rausch T, Radlwimmer B, Yaspo ML, Lehrach H, Warnatz HJ, Landgraf P, Borkhardt A, Brors B, Zapatka M, Eils R, Eils R, Eils J, Lawerenz C, Siebert R, Wagner S, Haake A, Richter J, Richter G, Eils R, Lawerenz C, Eils J, Kerssemakers J, Jaeger-Schmidt C, Scholz I, Bergmann AK, Borst C, Burkhardt B, Claviez A, Dreyling M, Eberth S, Einsele H, Frickhofen N, Haas S, Hansmann ML, Karsch D, Kneba M, Lisfeld J, Mantovani-Löffler L, Rohde M, Ott G, Stadler C, Staib P, Stilgenbauer S, Trümper L, Zenz T, Hansmann ML, Kube D, Küppers R, Weniger M, Hummel M, Klapper W, Kostezka U, Lenze D, Möller P, Rosenwald A, Ott G, Szczepanowski M, Ammerpohl O, Aukema SM, Binder V, Borkhardt A, Haake A, Hoell JI, Leich E, Lichter P, López C, Nagel I, Pischimariov J, Radlwimmer B, Richter J, Rosenstiel P, Rosenwald A, Schilhabel M, Schreiber S, Vater I, Wagener R, Siebert R, Bernhart SH, Binder H, Brors B, Doose G, Eils R, Hoffmann S, Hopp L, Hübschmann D, Kleinheinz K, Kretzmer H, Kreuz M, Korbel J, Langenberger D, Loeffler M, Rosolowski M, Schlesner M, Stadler PF, Sungalee S, Burkhardt B, Kratz CP, Witt O, van Tilburg CM, Kramm CM, Fleischhack G, Dirksen U, Rutkowski S, Frühwald M, von Hoff K, Wolf S, Klingebiel T, Koscielniak E, Landgraf P, Koster J, Resnick AC, Zhang J, Liu Y, Zhou X, Waanders AJ, Zwijnenburg DA, Raman P, Brors B, Weber UD, Northcott PA, Pajtler KW, Kool M, Piro RM, Korbel JO, Schlesner M, Eils R, Jones DTW, Lichter P, Chavez L, Zapatka M, Pfister SM (2018) The landscape of genomic alterations across childhood cancers. Nature 555:321–327. https://doi.org/10.1038/nature25480
33. Richards S, Aziz N, Bale S et al (2015) Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 17:405–423. https://doi.org/10.1038/gim.2015.30
34. Green RC, Berg JS, Grody WW, Kalia SS, Korf BR, Martin CL, McGuire A, Nussbaum RL, O'Daniel JM, Ormond KE, Rehm HL, Watson MS, Williams MS, Biesecker LG, American College of Medical Genetics and Genomics (2013) ACMG recommendations for reporting of incidental findings in clinical exome and genome sequencing. Genet Med 15:565–574. https://doi.org/10.1038/gim.2013.73
35. Kalia SS, Adelman K, Bale SJ, Chung WK, Eng C, Evans JP, Herman GE, Hufnagel SB, Klein TE, Korf BR, McKelvey KD, Ormond KE, Richards CS, Vlangos CN, Watson M, Martin CL, Miller DT (2017) Recommendations for reporting of secondary findings in clinical exome and genome sequencing, 2016 update (ACMG SF v2.0): a policy statement of the American College of Medical Genetics and Genomics. Genet Med 19:249–255. https://doi.org/10.1038/gim.2016.190
36. Moorman AV, Enshaei A, Schwab C, Wade R, Chilton L, Elliott A, Richardson S, Hancock J, Kinsey SE, Mitchell CD, Goulden N, Vora A, Harrison CJ (2014) A novel integrated cytogenetic and genomic classification refines risk stratification in pediatric acute lymphoblastic leukemia. Blood 124:1434–1444. https://doi.org/10.1182/blood-2014-03-562918.The
37. Inthal A, Zeitlhofer P, Zeginigg M, Morak M, Grausenburger R, Fronkova E, Fahrner B, Mann G, Haas OA, Panzer-Grümayer R (2012) CREBBP HAT domain mutations prevail in relapse cases of high hyperdiploid childhood acute lymphoblastic leukemia. Leukemia 26:1797–1803. https://doi.org/10.1038/leu.2012.60
38. Malinowska-Ozdowy K, Frech C, Schönegger A, Eckert C, Cazzaniga G, Stanulla M, zur Stadt U, Mecklenbräuker A, Schuster M, Kneidinger D, von Stackelberg A, Locatelli F, Schrappe M, Horstmann MA, Attarbaschi A, Bock C, Mann G, Haas OA, Panzer-Grümayer R (2015) KRAS and CREBBP mutations: a relapse-linked malicious liaison in childhood high hyperdiploid acute lymphoblastic leukemia. Leukemia 29:1656–1667. https://doi.org/10.1038/leu.2015.107
39. Qian M, Cao X, Devidas M, Yang W, Cheng C, Dai Y, Carroll A, Heerema NA, Zhang H, Moriyama T, Gastier-Foster JM, Xu H, Raetz E, Larsen E, Winick N, Bowman WP, Martin PL, Mardis ER, Fulton R, Zambetti G, Borowitz M, Wood B, Nichols KE, Carroll WL, Pui CH, Mullighan CG, Evans WE, Hunger SP, Relling MV, Loh ML, Yang JJ (2018) *TP53* germline variations influence the predisposition and prognosis of B-cell acute lymphoblastic leukemia in children. J Clin Oncol 36:591–599. https://doi.org/10.1200/JCO.2017.75.5215
40. van der Veer A, Zaliova M, Mottadelli F, de Lorenzo P, te Kronnie G, Harrison CJ, Cave H, Trka J, Saha V, Schrappe M, Pieters R, Biondi A, Valsecchi MG, Stanulla M, den Boer ML, Cazzaniga G (2014) IKZF1 status as a prognostic feature in BCR-ABL1-positive childhood ALL. Blood 123:1691–1698. https://doi.org/10.1182/blood-2013-06-509794
41. Petit A, Trinquand A, Chevret S, Ballerini P, Cayuela JM, Grardel N, Touzart A, Brethon B, Lapillonne H, Schmitt C, Thouvenin S, Michel G, Preudhomme C, Soulier J, Landman-Parker J, Leverger G, Macintyre E, Baruchel A, Asnafi V, French Acute Lymphoblastic Leukemia Study Group (FRALLE) (2017) Oncogenetic mutations combined with MRD improve outcome prediction in pediatric T-cell acute lymphoblastic leukemia. Blood 131:289–300. https://doi.org/10.1182/blood-2017-04-778829

42. Liu Y, Easton J, Shao Y, Maciaszek J, Wang Z, Wilkinson MR, McCastlain K, Edmonson M, Pounds SB, Shi L, Zhou X, Ma X, Sioson E, Li Y, Rusch M, Gupta P, Pei D, Cheng C, Smith MA, Auvil JG, Gerhard DS, Relling MV, Winick NJ, Carroll AJ, Heerema NA, Raetz E, Devidas M, Willman CL, Harvey RC, Carroll WL, Dunsmore KP, Winter SS, Wood BL, Sorrentino BP, Downing JR, Loh ML, Hunger SP, Zhang J, Mullighan CG (2017) The genomic landscape of pediatric and young adult T-lineage acute lymphoblastic leukemia. Nat Genet 49:1211–1218. https://doi.org/10.1038/ng.3909
43. Bhatia S, Landier W, Shangguan M, Hageman L, Schaible AN, Carter AR, Hanby CL, Leisenring W, Yasui Y, Kornegay NM, Mascarenhas L, Ritchey AK, Casillas JN, Dickens DS, Meza J, Carroll WL, Relling MV, Wong FL (2012) Nonadherence to Oral Mercaptopurine and risk of relapse in Hispanic and non-Hispanic white children with acute lymphoblastic leukemia: a report from the Children's oncology group. J Clin Oncol 30:2094–2101. https://doi.org/10.1200/JCO.2011.38.9924
44. Boer JM, van der Veer A, Rizopoulos D, Fiocco M, Sonneveld E, de Groot-Kruseman HA, Kuiper RP, Hoogerbrugge P, Horstmann M, Zaliova M, Palmi C, Trka J, Fronkova E, Emerenciano M, do Socorro Pombo-de-Oliveira M, Mlynarski W, Szczepanski T, Nebral K, Attarbaschi A, Venn N, Sutton R, Schwab CJ, Enshaei A, Vora A, Stanulla M, Schrappe M, Cazzaniga G, Conter V, Zimmermann M, Moorman AV, Pieters R, den Boer ML (2016) Prognostic value of rare IKZF1 deletion in childhood B-cell precursor acute lymphoblastic leukemia: an international collaborative study. Leukemia 30:32–38. https://doi.org/10.1038/leu.2015.199
45. Andersson AK, Ma J, Wang J et al (2015) The landscape of somatic mutations in infant MLL-rearranged acute lymphoblastic leukemias. Nat Genet 47:330–337. https://doi.org/10.1038/ng.3230
46. Seki M, Kimura S, Isobe T, Yoshida K, Ueno H, Nakajima-Takagi Y, Wang C, Lin L, Kon A, Suzuki H, Shiozawa Y, Kataoka K, Fujii Y, Shiraishi Y, Chiba K, Tanaka H, Shimamura T, Masuda K, Kawamoto H, Ohki K, Kato M, Arakawa Y, Koh K, Hanada R, Moritake H, Akiyama M, Kobayashi R, Deguchi T, Hashii Y, Imamura T, Sato A, Kiyokawa N, Oka A, Hayashi Y, Takagi M, Manabe A, Ohara A, Horibe K, Sanada M, Iwama A, Mano H, Miyano S, Ogawa S, Takita J (2017) Recurrent SPI1 (PU.1) fusions in high-risk pediatric T cell acute lymphoblastic leukemia. Nat Genet 49:1274–1281. https://doi.org/10.1038/ng.3900
47. Jenkinson S, Kirkwood AA, Goulden N, Vora A, Linch DC, Gale RE (2015) Impact of PTEN abnormalities on outcome in pediatric patients with T-cell acute lymphoblastic leukemia treated on the MRC UKALL2003 trial. Leukemia 30:39–47. https://doi.org/10.1038/leu.2015.206
48. Ma X, Edmonson M, Yergeau D, Muzny DM, Hampton OA, Rusch M, Song G, Easton J, Harvey RC, Wheeler DA, Ma J, Doddapaneni HV, Vadodaria B, Wu G, Nagahawatte P, Carroll WL, Chen IM, Gastier-Foster JM, Relling MV, Smith MA, Devidas M, Auvil JMG, Downing JR, Loh ML, Willman CL, Gerhard DS, Mullighan CG, Hunger SP, Zhang J (2015) Rise and fall of subclones from diagnosis to relapse in pediatric B-acute lymphoblastic leukaemia. Nat Commun 6(6604):6604. https://doi.org/10.1038/ncomms7604
49. Zhang J, Walsh MF, Wu G, Edmonson MN, Gruber TA, Easton J, Hedges D, Ma X, Zhou X, Yergeau DA, Wilkinson MR, Vadodaria B, Chen X, McGee RB, Hines-Dowell S, Nuccio R, Quinn E, Shurtleff SA, Rusch M, Patel A, Becksfort JB, Wang S, Weaver MS, Ding L, Mardis ER, Wilson RK, Gajjar A, Ellison DW, Pappo AS, Pui CH, Nichols KE, Downing JR (2015) Germline mutations in predisposition genes in pediatric Cancer. N Engl J Med 373:2336–2346. https://doi.org/10.1056/NEJMoa1508054
50. Mandelker D, Zhang L, Kemel Y, Stadler ZK, Joseph V, Zehir A, Pradhan N, Arnold A, Walsh MF, Li Y, Balakrishnan AR, Syed A, Prasad M, Nafa K, Carlo MI, Cadoo KA, Sheehan M, Fleischut MH, Salo-Mullen E, Trottier M, Lipkin SM, Lincoln A, Mukherjee S, Ravichandran V, Cambria R, Galle J, Abida W, Arcila ME, Benayed R, Shah R, Yu K, Bajorin DF, Coleman JA, Leach SD, Lowery MA, Garcia-Aguilar J, Kantoff PW, Sawyers CL, Dickler MN, Saltz L, Motzer RJ, O'Reilly EM, Scher HI, Baselga J, Klimstra DS, Solit DB, Hyman DM, Berger MF, Ladanyi M, Robson ME, Offit K (2017) Mutation detection in patients with advanced cancer by universal sequencing of cancer-related genes in tumor and normal DNA vs guideline-based germline testing. JAMA J Am Med Assoc 318:825–835. https://doi.org/10.1001/jama.2017.11137
51. Holmfeldt L, Wei L, Diaz-Flores E, Walsh M, Zhang J, Ding L, Payne-Turner D, Churchman M, Andersson A, Chen SC, McCastlain K, Becksfort J, Ma J, Wu G, Patel SN, Heatley SL, Phillips LA, Song G, Easton J, Parker M, Chen X, Rusch M, Boggs K, Vadodaria B, Hedlund E, Drenberg C, Baker S, Pei D, Cheng C, Huether R, Lu C, Fulton RS, Fulton LL, Tabib Y, Dooling DJ, Ochoa K, Minden M, Lewis ID, To LB, Marlton P, Roberts AW, Raca G, Stock W, Neale G, Drexler HG, Dickins RA, Ellison DW, Shurtleff SA, Pui CH, Ribeiro RC, Devidas M, Carroll AJ, Heerema NA, Wood B, Borowitz MJ, Gastier-Foster JM, Raimondi SC, Mardis ER, Wilson RK, Downing JR, Hunger SP, Loh ML, Mullighan CG (2013) The genomic landscape of hypodiploid acute lymphoblastic leukemia. Nat Genet 45:242–252. https://doi.org/10.1038/ng.2532
52. Tinat J, Bougeard G, Baert-Desurmont S, Vasseur S, Martin C, Bouvignies E, Caron O, Bressac-de Paillerets B, Berthet P, Dugast C, Bonaïti-Pellié C, Stoppa-Lyonnet D, Frébourg T (2009) 2009 version of the Chompret criteria for li Fraumeni syndrome. J Clin Oncol 27:e108–e109
53. Villani A, Shore A, Wasserman JD, Stephens D, Kim RH, Druker H, Gallinger B, Naumer A, Kohlmann W, Novokmet A, Tabori U, Tijerin M, Greer MLC, Finlay JL, Schiffman JD, Malkin D (2016) Biochemical and imaging surveillance in germline TP53 mutation carriers with Li-Fraumeni syndrome: 11 year follow-up of a prospective observational study. Lancet Oncol 17:1295–1305. https://doi.org/10.1016/S1470-2045(16)30249-2