Panel-based Next-generation Sequencing Identifies Prognostic and Actionable Genes in Childhood Acute Lymphoblastic Leukemia and is Suitable for Clinical Sequencing

Hisashi Ishida¹, Akihiro Iguchi², Michinori Aoe³, Takahide Takahashi³, Kosuke Tamefusa¹, Kiichiro Kanamitsu¹, Kaori Fujiwara¹, Kana Washio¹, Takehiro Matsubara⁴, Hirokazu Tsukahara¹, Masashi Sanada⁵, and Akira Shimada†∗

¹Department of Pediatrics/Pediatric Hematology and Oncology, Okayama University Hospital, Okayama, Japan
²Department of Pediatrics, Hokkaido University Hospital, Hokkaido, Japan
³Division of Medical Support, Okayama University Hospital, Okayama, Japan
⁴Department of BioBank, BioRepository/BioMarker Analysis Center, Okayama University Hospital, Okayama, Japan
⁵Clinical Research Center, National Hospital Organization, Nagoya Medical Center, Nagoya, Aichi, Japan
Corresponding author: Akira Shimada, Department of Pediatrics, Okayama University Hospital, Okayama, 2-5-1, Shikatacho, Kitaku, Okayama, 700-8558, Japan

Tel: +81-86-235-7249, fax: +81-86-221-4745, e-mail: pajj236e@okayama-u.ac.jp

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Abstract

Acute lymphoblastic leukaemia (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 leukaemia. 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 –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 harboured 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

Leukaemia, paediatric, ALL, molecular genetics, precision medicine
Introduction

Acute lymphoblastic leukaemia (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 leukaemia. 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-RUNXI or hyperdiploidy, have been reported to be associated with a favourable 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 these factors, minimal residual disease (MRD) measured by sensitive molecular tests, which reflects treatment response, is an emerging strong prognostic factor for paediatric 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), tumour 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 has not been published. These are listed in Table1. 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 is 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 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 ≥ five 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], RUNXI deletion in ETV6-RUNXI ALL,

TCF3 deletion in TCF3-PBX1 ALL, ABL1 deletion in BCR-ABL1 ALL, and KMT2A deletion in KMT2A-

rearranged ALL were also analysed. 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 analysing 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

analysed with the same gene panels among diagnostic, CR, and relapse samples. Finally, clonal changes

were categorized into four patterns (A: genetically distinct leukaemia, 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 genes 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). 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 analysed 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 analysable target regions per sample was between 210 and 928×. A total of 87.63%–98.3% of analysable 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 analysed 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 leukaemia 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 leukaemia cells with one red signal (Supplementary Figure). These finding suggested that loss of heterozygosity occurred due to the deletion of TP53 and led to progression of leukaemia, 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
This study included nine patients with BCP-ALL. Only one (UPN 4) of these patients were 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 patients (UNP 1, 3, and 5) had CNVs with unfavourable prognostic value previously reported [4, 5, 8–12, 36]. UPN 4, who was regarded as the highest risk patient, also had unfavourable 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 hyperdiploid karyotype and well-known KRAS and CREBBP mutations, which are associated with a higher risk of relapse [37–39]. UPN 2 also had the germline TP53 mutation, as mentioned above, and was thought to be a high-risk patient [40]. Therefore, among a total of nine patients with BCP-ALL, six had unfavourable 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 (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 unfavourable outcome [41]. 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 signalling 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, 42]. These two patients showed a WTI 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 [43].

Patterns of relapse. To clarify the clonal origins of relapsed samples, we analysed the differences in SNVs, in/dels, and CNVs between matched diagnostic and relapse samples, and classified them into four groups (A: genetically distinct leukaemia, 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, 8 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 [44]. 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 [45, 46].

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 $\geq 30\%$ [47]. 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 [47].

Recently, SPI1 fusions were newly reported for paediatric T-ALL, and this subgroup is associated with a poor outcome [48]. While we detected many recurrent genetic alterations for T-ALL, a small number of genetic alterations with adverse prognostic value have been reported [49]. 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, 50]

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 [51]. 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 [52]. 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 [53], but about one-third of patients with germline TP53 pathogenic variants did not have typical hypodiploid karyotype [54].

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 [55].

Patients with LFS have a substantial lifetime risk of developing cancer, and a clinical surveillance protocol enables early detection of tumours and improves long-term survival with reduced treatment-related morbidity and mortality [56]. 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 are 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 detecting 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.

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

Author 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.

Compliance with Ethical Standards
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.

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