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Sequential Approach to Improve the Molecular Classification of Childhood Acute Lymphoblastic Leukemia

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Identification of specific leukemia subtypes is a key to successful risk-directed therapy in childhood acute lymphoblastic leukemia (ALL). Although RNA sequencing (RNA-seq) is the best approach to identify virtually all specific leukemia subtypes, the routine use of this method is too costly for patients in resource-limited countries. This study enrolled 295 patients with pediatric ALL from 2010 to 2020. Routine screening could identify major cytogenetic alterations in approximately 69% of B-cell ALL (B-ALL) cases by RT-PCR, DNA index, and multiplex ligation–dependent probe amplification. *STIL-TAL1* was present in 33% of T-cell ALL (T-ALL) cases. The remaining samples were submitted for RNA-seq. More than 96% of B-ALL cases and 74% of T-ALL cases could be identified based on the current molecular classification using this sequential approach. Patients with Philadelphia chromosome–like ALL constituted only 2.4% of the entire cohort, a rate even lower than those with *ZNF384*-rearranged (4.8%), *DUX4*-rearranged (6%), and Philadelphia chromosome–positive (4.4%) ALL. Patients with *ETV6-RUNX1*, high hyperdiploidy, *PAX5* alteration, and *DUX4* rearrangement had favorable prognosis, whereas those with hypodiploid and *KMT2A* and *MEF2D* rearrangement ALL had unfavorable outcomes. With the use of multiplex ligation–dependent probe amplification, DNA index, and RT-PCR in B-ALL and RT-PCR in T-ALL followed by RNA-seq, childhood ALL can be better classified to improve clinical assessments. *(J Mol Diagn 2022, 24: 1195–1206; https://doi.org/10.1016/j.jmoldx.2022.08.001)*

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Childhood acute lymphoblastic leukemia (ALL) is one of the most curable cancers, with a 5-year event-free survival (EFS) and a 5-year overall survival (OS) exceeding 80% and 90%, respectively, in many developed countries.[1−7] This remarkable achievement is partly attributed to the more precise risk stratification of patients based on the identification of genetic abnormalities with prognostic and therapeutic implications and the evaluation of early treatment response using the minimal residual disease (MRD) test.[8,9] The mapping of the human genome and subsequent advances in DNA and RNA sequencing (RNA-seq) techniques and bioinformatic analysis pipelines have revolutionized understanding of the genomic landscape of ALL.[10−22] Using RNA-seq, molecular classification can be performed for nearly 97% of childhood B-cell ALL (B-ALL) cases into 23 subtypes, some with prognostic significance and others with targetable lesions.[23,24] In T-cell ALL (T-ALL), RNA-seq can provide provisional classifications according to transcriptional factor gene expression profiles or dysregulation of targetable functional pathways.[25−27]

In childhood B-ALL, conventional genetic analyses can identify *ETV6-RUNX1* and high hyperdiploidy, the two most common subtypes, in 40% to 45% of patients. Other common fusions, such as *BCR-ABL1* and *TCF3-PBX1,* account for 5% to 7% of patients.[28,29] Several novel subtypes of ALL that are not evident through conventional genetic analysis but can be identified by transcriptome analysis include Philadelphia chromosome (Ph)−like ALL; *DUX4*-, *MEF2D*-, and *ZNF384*-rearranged B-ALL; and *KMT2A*-like and *ETV6−RUNX1*−like B-ALL.[13,16,17,19,20,22] These new ALL subtypes have distinct clinical and biological characteristics. Unlike common recurrent ALL subtypes, such as *ETV6-RUNX1*, *TCF3-PBX1*, and *BCR-ABL1*, these subtypes have diverse genetic alterations that involve different partner genes, breakpoints, or signaling pathways, making routine screening by simple RT-PCR almost impractical. Some have no fusions but similar gene expression to known subtypes, which requires gene expression profiles for a precise diagnosis.[24] Several molecular methods, such as RT-PCR, DNA index (DI), and multiplex ligation−dependent probe amplification (MLPA), can identify common genetic subtypes of B-ALL, including *ETV6-RUNX1*, high hyperdiploidy, hypodiploidy, *BCR-ABL1*, and *TCF3-PBX1*. This approach can identify approximately 60% to 70% of subtypes of B-ALL[30]; RNA-seq can be applied to the remaining samples with negative findings to determine subtypes.

In the case of T-ALL, half have aberrant transcriptional factor expression, and whole genome sequencing (WGS) may be required to identify the precise breaking point that led to aberrant expression.[25] Gene expression defined by RNA-seq can identify some important genetic translocations and classify the basic T-ALL subtype by transcriptional factors. Although transcriptome analysis is vital to the comprehensive and precise classification of ALL, cost is a major hurdle to its widespread clinical use in a resource-limited country. In this study, a sequential approach was designed to identify childhood ALL genetic profiles using RT-PCR, DI, and RNA-seq to improve molecular classification while saving costs.

Materials and Methods

Patients and Protocols

Between May 2010 and December 2020, a total of 340 children with newly diagnosed ALL treated at the National Taiwan University Hospital were enrolled in two consecutive Taiwan Pediatric Oncology Group (TPOG) protocols: 2002 protocol (n = 103) and 2013 protocol (n = 237) (Figure 1).[31−33] Diagnostic bone marrow or peripheral blood samples were available for 295 children, and clinical features are described in Supplemental Table S1. Cases with insufficient material or a leukocyte count <70% were excluded from further analysis. The diagnosis of ALL was based on bone marrow morphology, and the immunophenotype of leukemic cells was determined using flow cytometry. Conventional cytogenetic analysis was performed using G-banding as part of the routine workup. Although both protocols used clinical and biological characteristics and remission induction response for risk assignment, TPOG 2013 incorporated the level of MRD on day 15 and the end of remission induction (between day 35 and day 42) to direct the intensity of treatment. The institutional review board of the National Taiwan University Hospital approved the study (201510016RIND), and written informed consent was obtained from parents, guardians, or patients in accordance with the Declaration of Helsinki.

RT-PCR for Fusion Genes

Total RNA was isolated from bone marrow or blood samples using NucleoZOL (Macherey-Nagel, Düren, Germany). cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). A total of 1 μg of RNA was used for cDNA synthesis according to the manufacturer's instructions. The prepared reaction mix was incubated at 25°C for 10 minutes, followed by 60°C for 30 minutes; then the reaction was terminated by heating at 85°C for 5 minutes. PCR was performed using MyTaq HS Mix (Bioline, London, UK), and thermocycling was performed as follows: 95°C for 60 seconds, followed by 38 cycles at 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 30 seconds, followed by final extension at 72°C for 5 minutes. Primers used for RT-PCR are listed in Supplemental Table S2. The PCR products were visualized using agarose gel electrophoresis. Suspected bands were purified using the FavorPrep GEL/PCR Purification Kit (Favorgen, Ping-Tung, Taiwan). Sanger sequencing was performed using an ABI 3730XL DNA analyzer (Thermo Fisher Scientific). Sequencing results were analyzed with SnapGene software version 4.1.3 (GSL Biotech, San Diego, CA) (*https://www.ncbi.nlm.nih.gov/nuccore*; accession number NM_000546.6 and *https://www.ncbi.nlm.nih.gov/nuccore*; accession number NM_001987).

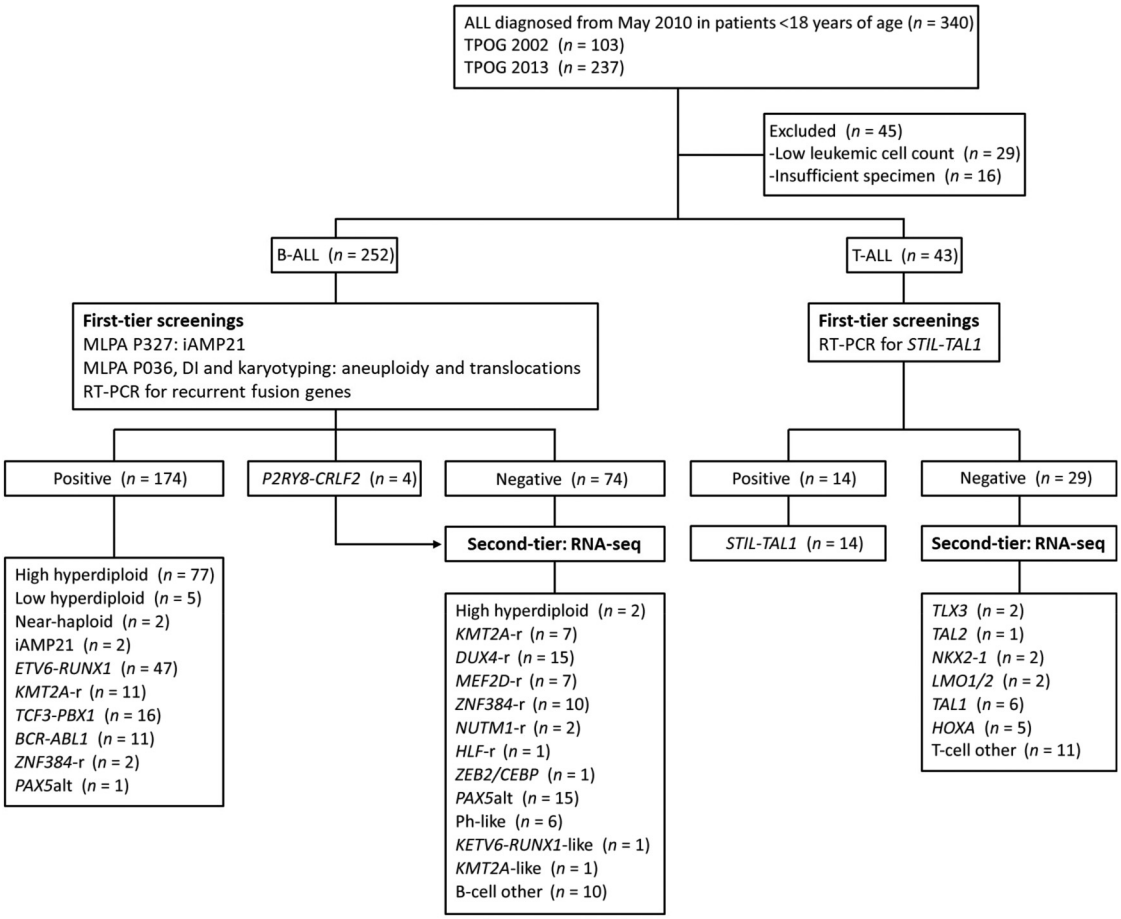

Figure 1 A flowchart of patients with the genetic diagnosis of acute lymphoblastic leukemia (ALL) enrolled in this study. ALL, acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia; DI, DNA index; MLPA, multiplex ligation–dependent probe amplification; T-ALL, T-cell acute lymphoblastic leukemia; TPOG, Taiwan Pediatric Oncology Group.

Ploidy Status Analysis

The ploidy status was evaluated using SALSA MLPA Probemix P036 Subtelomeres Mix 1 (MLPA P036) (MRC-Holland, Amsterdam, the Netherlands) and DI, as previously reported.[30] Briefly, DI was used to detect DNA aneuploidy, and MLPA P036 was used to identify individual chromosome gain or loss. The cases were suspected of masked hypodiploidy based on a hypodiploidy-like chromosome pattern.[34] These cases were further analyzed by single-nucleotide polymorphism array or short tandem repeat typing to confirm chromosomal loss of heterozygosity.

Genomic DNA Extraction

Lymphoblasts were purified from bone marrow or peripheral blood specimens using the Ficoll-Paque centrifugation method, according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ). Genomic DNA was extracted from leukemic cells using standard phenol/chloroform-based methods. Briefly, 1×10^6 cells were lysed in 10 mmol/L Tris hydrochloride, 10 mmol/L sodium chloride, 10 mmol/L EDTA, 20 μg proteinase K, and 0.5% SDS by incubating at 37°C for 16 hours. Total RNA was further removed by adding 500 μg PureLink RNase A (Thermo Fisher Scientific) and incubating for 10 minutes at 37°C. An equal volume of phenol-chloroform-isopropanol (25:24:1) was added to the lysates and mixed by vigorous shaking, followed by centrifugation at 16,100 × *g* at 4°C for 5 minutes. The upper aqueous phase was transferred to a fresh tube; genomic DNA was then precipitated by adding 2× 100% ethanol stored at −80°C. The DNA pellet was washed with 75% ethanol and rehydrated with Tris-EDTA buffer. The concentration of DNA was determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

MLPA Analysis

Genomic DNA was analyzed using the SALSA MLPA kit (MRC-Holland), according to the manufacturer's instructions, as described in a previous study.[30] SALSA MLPA Probemix P335 ALL-IKZF1 was used for the detection in the alterations of the *IKZF1*, *PAX5*, and *ETV6* genes. SALSA MLPA Probemix P327 iAMP21-ERG was used for detecting alterations in *ERG* and the iAMP21 subtype.

Mutation Analysis of *TP53* and *ETV6*

Genomic DNA was used for mutation analysis. For sequencing analysis, the coding regions were amplified using Phusion Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific). The primers are listed in Supplemental Table S2. Thermocycling was performed as follows: 98°C for 30 seconds, then 38 cycles of 98°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. The PCR products were confirmed using 2% agarose gel electrophoresis. DNA bands were purified using the FavorPrep GEL/PCR Purification Kit. Sanger sequencing was performed using an ABI 3730XL DNA analyzer. The sequencing results were aligned to the National Center for Biotechnology Information (NCBI) GenBank (*http://www.ncbi.nlm.nih.gov*; GenBank accession number NM_000546 or NM_001987) using SnapGene software version 4.1.3.

Transcriptome Sequencing and Bioinformatic Analysis

RNA-seq was performed using the TruSeq Library Prep Kit and the HiSeq 2000 Sequencing System (Illumina, San Diego, CA). All sequence reads were paired end and analyzed using total RNA-seq (100−base pair reads). Default parameters were used for all software programs in a high-performance computing environment, and fastq files were mapped to the GRCh37 human genome reference using STAR version 2.5.3a (*https://github.com/alexdobin/STAR/releases*). Gene annotation software downloaded from the Ensembl website (*http://www.ensembl.org*, last accessed January 1, 2021) was used for STAR mapping and the read count evaluation.[35] The bioinformatic tools used included STAR-Fusion version 1.8.1 (*https://github.com/STAR-Fusion/STAR-Fusion*), FusionCatcher version 1.2.0 (*https://github.com/ndaniel/fusioncatcher*), Squid version 1.5 (*https://github.com/Kingsford-Group/squid*), Pizzly version 0.37 (*https://github.com/pmelsted/pizzly*), arriba version 1.2.0 (*https://github.com/suhrig/arriba*), and Pindel version 0.2.0 (*https://github.com/genome/pindel*), which specifically search for internal tandem duplication.[36] The primers used for RNA-seq validation are listed in Supplemental Table S2.

Evaluation of Gene Expression Level from RNA-Seq Data

The HTSeq package version 0.11.2 (*https://htseq.readthedocs.io/en/release_0.11.1/count.html*) was used to evaluate gene expression levels, and the DESeq2 version 1.32.0 Bioconductor R package (*https://bioconductor.org/packages/release/bioc/html/DESeq2.html*) was used to perform gene expression level normalization and differential expression analysis. A regularized log-transformed value was calculated using DESeq2 to evaluate the digital gene expression levels. To correct the batch effect introduced by different library preparation strategies and sequencing lengths, the ComBat function in the sva R package version 3.40.0 (*https://bioconductor.org/packages/release/bioc/html/sva.html*) was used.RNA-seq from 1268 reference samples with clear ALL subtypes predefined was included in the analysis to increase accuracy.[24] With the log-transformed gene expression level, the R package Rtsne version 0.15 (*https://cran.r-project.org/web/packages/Rtsne/index.html*) was used to map the samples to a 2-dimensional *t*-distributed stochastic neighbor embedding (tSNE) plot with the 1000 most variable genes (based on the median absolute deviation), and the tSNE perplexity parameter was set to 30. Different gene numbers (200, 500, 1000, and 2000) and tSNE parameters (perplexity of 20, 30, 40, and 50) were explored and stable clusters were observed.[24] The R package umap version 0.2.7.0 (*https://cran.r-project.org/web/packages/umap/index.html*) was used to map the T-ALL samples to a two-dimensional Uniform Manifold Approximation and Projection (UMAP) plot with the top 1000 median absolute deviation genes, and the number of the nearest neighbors was set to 15. Gene signature analysis was also performed using DESeq2 with default parameters to evaluate differentially expressed genes.

Statistical Analysis

The EFS was calculated from the date of diagnosis to the first major adverse event, including induction failure, relapse, development of a second malignancy, or death from any cause. The OS was calculated from the date of diagnosis to death of any cause. The time was censored at the date of last contact if no event occurred. The EFS and OS were estimated according to the Kaplan-Meier method and compared using the log-rank test. Univariate and multivariate Cox regression analyses were performed to evaluate hazard ratios and 95% CIs of risk factors. *t*-tests were performed for surface marker expression. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the SAS software version 9.4 (SAS Institute, Cary, NC).

Availability of Data and Materials

The data sets used and/or analyzed during the current study are publicly available and have been deposited in the Gene Expression Omnibus (*https://www.ncbi.nlm.nih.gov/geo*; accession number GSE207057).

Results

RT-PCR and DI to Identify Samples with Recurrent Fusions and the Status of Ploidy of B-ALL and *STIL-TAL1* to Screen T-ALL

In this study, 252 patients with B-ALL were recruited (Figure 1). Because most B-ALL subtypes were mutually exclusive, RT-PCR was used to screen the samples for *ETV6-RUNX1*, *BCR-ABL1*, *KMT2A-AFF1*, *TCF3-PBX1*,

and *P2RY8-CRLF2* and DI to verify ploidy. Four cases of *P2RY8-CRLF2* and one case with hyperdiploidy and *BCR-ABL1* were further analyzed using RNA-seq. Two cases with iAMP21 were diagnosed using MLPA.[30] Two cases with *ZNF384-r* were identified by karyotyping and validated by RT-PCR. These approaches could identify specific subtypes in 173 of 252 patients (69%) with B-ALL. For T-ALL, only RT-PCR was used to screen for *STIL-TAL1* fusion and identified 14 cases (32.6%) among 43 patients studied. The results of RT-PCR, DI, and MLPA are available within 5 working days after diagnosis.

Integrative Genetic and Genomic Classification of B-ALL by RNA-Seq

The remaining cases without specific leukemia subtypes identified by conventional genetic analyses were analyzed using RNA-seq (Figure 1) and classified according to the criteria proposed by Jeha et al.[37] RNA-seq data were analyzed from 111 individuals with B-ALL (including 33 B-ALL samples with known subtypes, four samples with *P2RY8-CRLF2*, and one sample with hyperdiploidy status and *BCR-ABL1*) to obtain gene expression profiles analyzed using hierarchical clustering, tSNE analysis, prediction analysis of microarrays, and predictive modeling 17 using cases of known subtypes (Figure 2).[24] Of these, 10 patients harbored *ZNF384*-r, 15 *PAX5*alt, 7 *MEF2D*-r, 6 Ph-like, 15 *DUX4*-r, 7 *KMT2A*-r, 1 *BCR-ABL1*, 2 *NUTM1* fusion, 1 *TCF3-HLF*, and 1 *ZEB2/CEBP*. With the use of this approach, molecular classification could not be performed for only 10 cases (4%). The heatmap of the entire B-ALL cohort is shown in Supplemental Figure S1, and the final genetic subtypes of all patients are listed in Table 1.

IKZF1 Deletions in Patients with *DUX4* Rearrangements

Of the 15 cases with *DUX4* rearrangements, 14 had sufficient genomic material for further analysis. Two samples had somatic *TP53* mutations. Seven cases had *IKZF1* deletions [7 of 14 cases (50%) that could be evaluated], a rate higher than that in other reports (Table 2).[16,18,22] In contrast, the *ERG* deletions detected in 2 of 14 cases (14.3%) were less common compared with other studies. In line with previous studies,[16,37] CD2 expression was higher in patients with *DUX4*-r (Supplemental Figure S2). *IKZF1* deletions were not associated with inferior clinical outcomes in patients with *DUX4*-r (Supplemental Figure S3).

ETV6 Deletion May Be Enriched in the *ZNF384* Subtype

Several novel fusion partners in the *ZNF384* fusions were identified (Supplemental Figure S4). Like previous reports,[20,38,39] *ZNF384* fusion is associated with loss of CD10 and expression of CD33 and CD13 compared with other subtypes (Supplemental Figure S4, Supplemental Table S3). Similar to cases with *ETV6-RUNX1*, the *ETV6* alteration was also enriched in this cohort, a finding not identified in previous studies (Table 3).[20,38,39] Two patients with somatic *ETV6* mutations were identified. The Sanger sequencing of these two samples is shown in Supplemental Figure S5.

Pediatric Cases of B-ALL with Multiple Primary Alterations

Three patients with two primary alterations were identified in this cohort (Table 4). All samples were submitted for the screening of fusion genes, DI, and MLPA P036 to determine the status of the ploidy, and the analysis was stopped at this step if the samples had positive results. The three patients had hyperdiploidy status besides *BCR-ABL1* (case 1098) or *P2RY8-CRLF2* (cases 829 and 1019). To determine the exact molecular subtype, RNA-seq was performed for these cases, and they were classified as *BCR-ABL1* or high hyperdiploidy based on gene expression clustering.

Genomic Alterations in T-ALL

A total of 32 T-ALL samples were submitted for RNA-seq, including three samples with *STIL-TAL1*. An ensemble approach was used to identify the fusion transcripts in these samples. The gene expression profiles of the RNA-seq data were analyzed using hierarchical clustering (UMAP analysis), which revealed seven clusters of tumors, indicative of different subtypes of T-ALL (Figure 3). The most common subgroup was *TAL1* [20 of 43 (46.5%)]. Six samples were identified to have fusions, including *BCR-BAL1*, *KMT2A*-r, *ETV6-NCOA2*, *SFPQ-ZFP36L1*, and *PICALM-MLLT10*. The heatmap of T-ALL is presented in Supplemental Figure S6. Complete genetic alterations and molecular classification by RNA-seq and gene expression of the entire cohort are listed in Supplemental Tables S4 and S5.

Outcome Analysis by Genotyping

Patients with *ETV6-RUNX1*, high hyperdiploidy, *PAX5*alt, and *DUX4*-r had favorable outcomes, whereas those with hypodiploidy and *KMT2A* and *MEF2D* fusions had unfavorable outcomes based on EFS and OS (Figure 4, Supplemental Table S6).

Discussion

In developing countries with limited resources, a major hurdle to the comprehensive use of RNA-seq for childhood ALL is the cost associated with it. With the use of conventional molecular techniques, such as RT-PCR, DI, and MLPA, approximately 70% of childhood B-ALL can be genetically classified. RT-PCR can aid in the identification of approximately 30% of patients with *STIL-TAL1* in the T-ALL cohort. The results of these molecular methods used in this study can be available within 5 working days, and the sequential approach decreases the number of samples

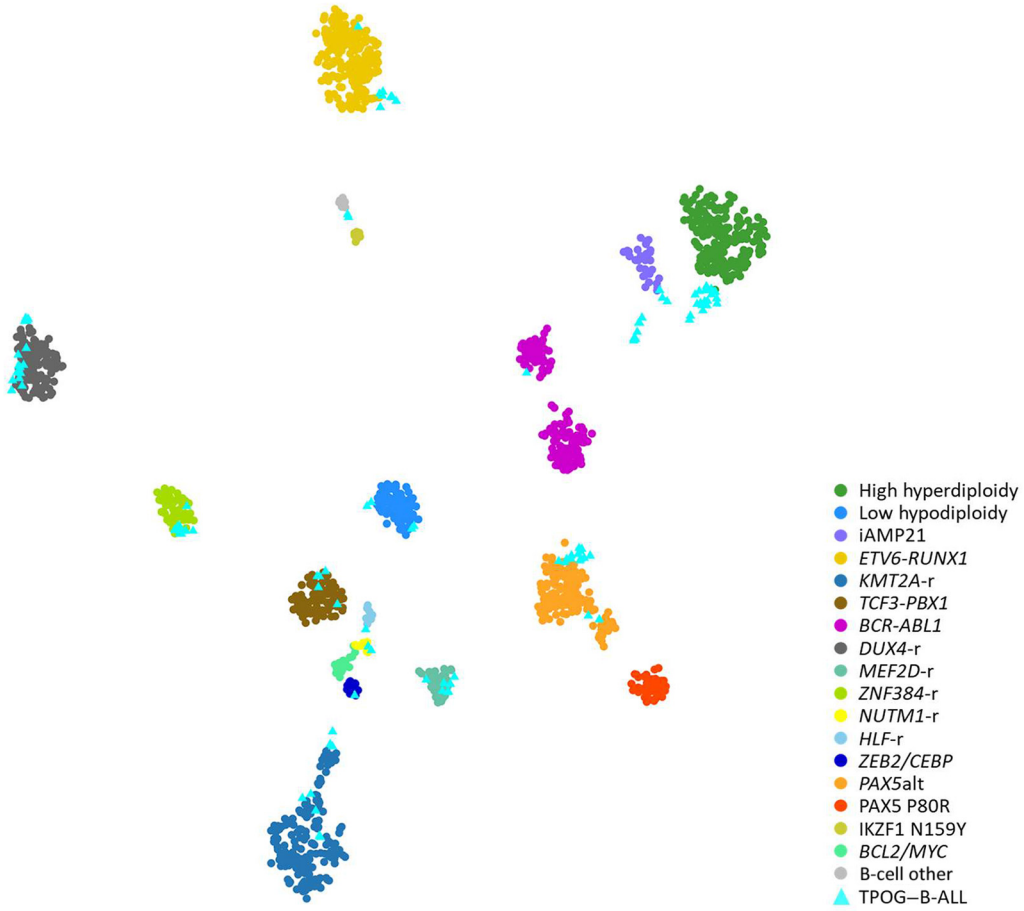

Figure 2 Integrative acute lymphoblastic leukemia (B-ALL) subtypes. Gene expression profiling of reference samples with clear B-ALL subtype predefined and Taiwan Pediatric Oncology Group (TPOG)—B-ALL samples shown in a two-dimensional t-distributed stochastic neighbor embedding plot. Each dot represents a sample. Major subtypes of reference samples are represented as circles in different colors and TPOG—B-ALL samples are represented as light blue triangles.

required for RNA-seq analysis. RNA-seq can provide information on targetable genetic fusions and molecular classification of transcriptional factors in T-ALL. The data showed that the prevalence rates of genetic alterations in childhood B-ALL in Taiwan differ from those reported in the United States and Europe, and the incidence of Ph-like ALL in Taiwan is much lower than that reported in White and Hispanic populations.[12,13,16,22,24,40,41]

Until now, most reports on the application of RNA-seq in childhood ALL are from White populations, whereas only a few RNA-seq studies have focused on Asian populations.[18,42,43] Although all leukemia subtypes occur in all racial or ancestral groups, there are substantial differences in the incidence rates of several subtypes of B-ALL. Ph-like ALL is a high-risk subtype of B-ALL, with a frequency of 10% to 25% in different age populations among White and Hispanic populations. However, in Japan, Imamura et al[42] identified 29 pediatric patients with Ph-like ALL by transcriptome and multiplex RT-PCR analyses among 373 Ph-negative B-ALL patients without recurrent genetic abnormalities. Another study in China that used RNA-seq showed that only 5% of children with ALL exhibited Ph-like subtype.[18] Using RNA-seq among children with ALL in Singapore and Malaysia, Ni Chin et al[43] found that the frequency of Ph-like ALL was only 2%, a rate similar to the rate [6 of 252 (2.4%)] in this report. When more sequencing studies are conducted in Asian populations, the true distribution of these genetic subtypes could become clearer in the future.

Some differences in genetic alterations have been observed between subtypes. In this cohort, 14.3% of patients with *DUX4*-r had *TP53* somatic mutations, which have not been previously reported.[16,22] One *DUX4-r* case with the *TP53* mutation had tetraploidy (Supplemental Table S4), which might have resulted from somatic *TP53* mutations.[44] The significance of somatic *TP53* mutations was unclear because of the low sample number in this study. Besides samples with *ETV6-RUNX1* fusion, *ETV6* alterations were enriched in patients with *ZNF384* fusions, which were not previously identified.[20,38,39]

The prevalence rates of these novel subtypes might affect the diagnosis strategy. Unlike the higher incidence rate of Ph-like ALL in White and Hispanic populations,[13,40,45,46] the incidence rate in this cohort is <3%. Several methods

Table 1 Details of the Entire Cohort

Subtype	No. (%)
B-cell acute lymphoblastic leukemia (n = 252)	
High hyperdiploidy	79 (31.3)
Low hypodiploidy	5 (2.0)
Near-haploid	2 (0.8)
iAMP21	2 (0.8)
ETV6-RUNX1	47 (18.7)
KMT2A-r	18 (7.1)
TCF3-PBX1	16 (6.3)
BCR-ABL1	11 (4.4)
DUX4-r	15 (6.0)
MEF2D-r	7 (2.8)
ZNF384-r	12 (4.8)
NUTM1-r	2 (0.8)
HLF-r	1 (0.4)
ZEB2/CEBP	1 (0.4)
*PAX5*alt	16 (6.3)
Ph-like	6 (2.4)
ETV6-RUNX1—like	1 (0.4)
KMT2A-like	1 (0.4)
B-cell other	10 (4.0)
Total	252 (100)
T-cell acute lymphoblastic leukemia (n = 43)	
TAL1	20 (46.5)
TAL2	1 (2.3)
TLX3	2 (4.7)
LMO1/2	2 (4.7)
NKX2-1	2 (4.7)
HOXA	5 (11.6)
T-cell other	11 (25.6)
Total	43 (100)

are used to screen for Ph-like ALL because of the availability of possible targeted treatment options after its diagnosis.[13,40,45,47] The very low prevalence rate of Ph-like ALL may make its diagnosis by RNA-seq easier in Asian populations, especially for patients who receive risk-directed therapy. RNA-seq alone can identify all cases with targetable lesions. However, *DUX4*-r accounts for many of the patients in this study, and its diagnosis requires RNA-seq.[16,22,48] Some subtypes, such as the *ETV6-RUNX1*—like and *KMT2A*-like subtypes, had no specific fusions but had gene expression profiles similar to those of known subtypes.[22,24] In such cases, RNA-seq can provide the required information and be helpful for risk-directed therapy. Several samples using gene expression profiles were identified in this cohort.

Liu et al[25] used WGS and RNA-seq to identify genetic alterations in T-ALL. So far, this is the largest T-ALL cohort with comprehensive genome sequencing data. Unlike B-ALL, only 50% of the samples had in-frame fusions in T-ALL; the remaining samples could be classified using gene expression profiles, although specific breaking points that result in overexpression should be identified using WGS instead of RNA-seq.[49–51] However, RNA-seq can identify targetable kinase fusions suitable for treatment with tyrosine kinase inhibitors.[25] A patient with T-ALL with *BCR-ABL1* using RNA-seq was identified, although the t(9;22)(q34;q11)

Table 3 *ETV6* Alterations Were Enriched in *ZNF384*-r ALL

Alteration	TPOG, no. (%) (n = 252)	Zhang et al[16] (n = 1261)		Liu et al[18] (n = 204)	
		No. (%)	P*	No. (%)	P*
ZNF384-r ALL	12 (4.8)	31 (2.5)	0.06	8 (3.9)	0.82
ETV6					
Alteration	7 (58.3)	8 (32.0)	0.16	0 (0)	0.04
Wild type	5 (41.7)	17 (68.0)		8 (100)	

*P values were calculated using the Fisher exact test between this study and previous studies.[16,18]

ALL, acute lymphoblastic leukemia; TPOG, Taiwan Pediatric Oncology Group.

Table 2 *IKZF1*, *ERG*, and *TP53* Alterations in *DUX4*-r ALL

Alteration	TPOG, no. (%) (n = 252)	Zhang et al[16] (n = 1743)		Lilljebjörn et al[22] (n = 195)		Liu et al[18] (n = 94)	
		No. (%)	P*	No. (%)	P*	No. (%)	P*
DUX4-r ALL	15† (6.0)	134 (7.7)	0.37	8 (4.1)	0.52	6 (6.4)	>0.99
IKZF1							
Deletion	7 (50)	27 (23.3)	0.02	1 (12.5)	0.07	3 (50.0)	>0.99
Wild type	7 (50)	89 (76.7)		7 (87.5)		3 (50.0)	
ERG							
Deletion	3 (21.4)	68 (59.1)	0.002	6 (75.0)	0.008	4 (66.7)	>0.99
Wild type	11 (88.6)	47 (40.9)		2 (25.0)		2 (33.3)	
TP53							
Mutation	2 (14.3)	2 (3.3)	0.04	0 (0.0)	0.27	0 (0.0)	0.52
Wild type	12 (85.7)	59 (96.7)		8 (100.0)		6 (100.0)	

*P values were calculated using the Fisher exact test between this study and previous studies.[16,18]

†Material was not available for one case for genetic alteration analysis.

ALL, acute lymphoblastic leukemia; TPOG, Taiwan Pediatric Oncology Group.

Table 4 Pediatric B-Cell Acute Lymphoblastic Leukemia Cases with Multiple Primary Alterations

ID	Molecular subtype	Fusion gene	Cytogenetics
1098	*BCR-ABL1*	*BCR-ABL1*	58,XX,+X,+4,+4,+5,+6,+9,t(9;22)(q34;q11),+10,+14,+18,+20,+21,+21,der(22)t(9;22)
829	High hyperdiploidy	*P2RY8-CRLF2*	57,XY,+X,+X,+4,+8,+9,+10,+11,+14,+14,+21,+21
1019	High hyperdiploidy	*P2RY8-CRLF2*	52,XY,del(3)(q13q2?7),+X,+8,+10,der(16)t(7;16)(q11;p11),add(16)(p11),+21,+21,+22

translocation could not be identified by cytogenetic analysis. However, other translocations can be identified using gene expression analysis.

The gene expression profile may be another important determinant of subtype classification, especially in B-ALL, because it may be the only way to identify leukemia subtypes in some patients. Although MRD is the most powerful prognostic marker in childhood ALL, genotyping is also an important tool to determine whether patients are at high risk of relapse. In this sense, negative MRD could not preclude a high incidence of relapse among patients with certain unfavorable leukemia subtypes.[9,37,52] Furthermore, it is important to genotype all patients not only for prognostic value but also for possible targeted or novel therapy. With the development of next-generation sequencing, it will be ideal to apply tools such as whole exome sequencing and/or WGS and RNA-seq to all samples. However, application of these tools is difficult to achieve in a developing country. RNA-seq is currently the best approach to identify unknown subtypes and provides useful information for targeted

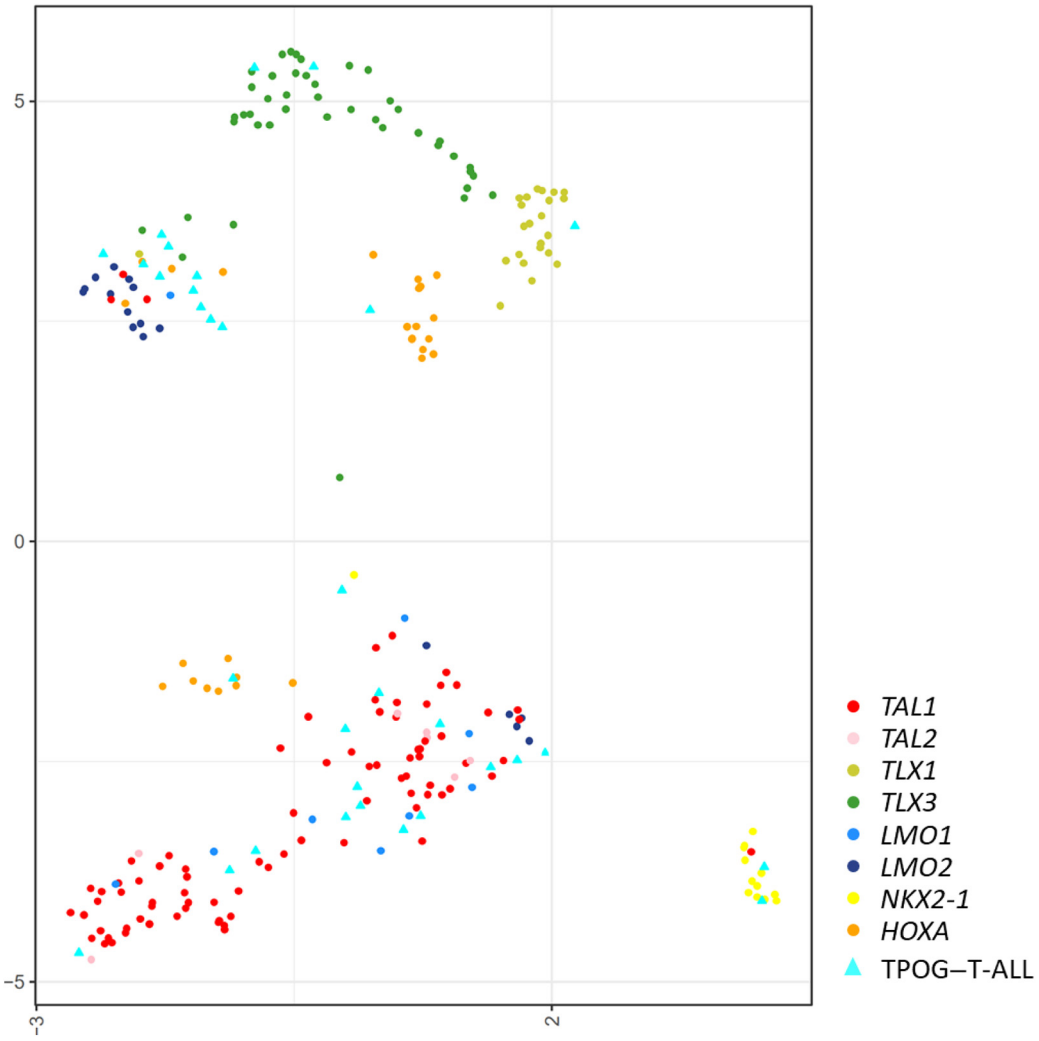

Figure 3 Integrative T-cell acute lymphoblastic leukemia (T-ALL) subtypes. Gene expression profiling of reference samples with clear T-ALL subtype predefined and Taiwan Pediatric Oncology Group (TPOG)—T-ALL samples shown in a two-dimensional uniform manifold approximation and projection plot. Each dot represents a sample. Major subtypes of reference samples are represented as circles in different colors and TPOG—T-ALL samples are represented as light blue triangles.

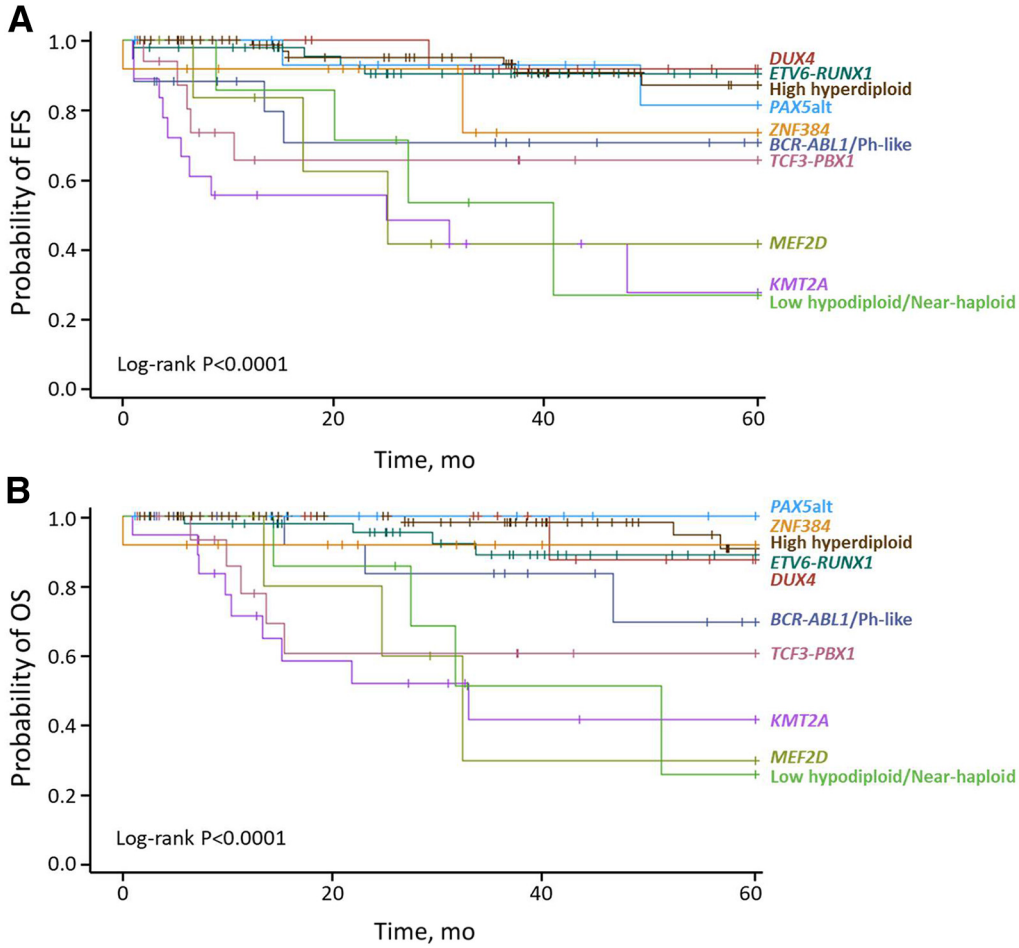

Figure 4 The 5-year event-free survival (EFS) (**A**) and 5-year overall survival (OS) (**B**) according to the risk groups by genetic subtypes. The EFS and OS were estimated using the Kaplan-Meier method, and *P* values were calculated using the log-rank test.

therapy to augment traditional chemotherapy. In countries with limited resources, MLPA and DI also provide important information on genetic alterations of B-ALL, if interpreted accurately.

This study had limitations. Although a comprehensive genome analysis requires RNA-seq, the prevalence rates for some B-ALL subtypes, such as *PAX5* P80R and *IKZF1* N159Y, are very low. This cohort comprised only 340 patients, and all rare subtypes could not be identified. The hypothesis of the sequential approach that the genetic subtypes in B-ALL are mutually exclusive may not be completely accurate. In this cohort, three patient samples had two genetic alterations, which had high hyperdiploidy. However, with the use of DI and fusion gene tests, samples with high hyperdiploidy and fusion genes could be identified. In addition, not all patients with *CRLF2* rearrangements were Ph-like. For patients with *P2RY8-CRLF2*, RNA-seq might be indicated to confirm Ph-like status. In the largest cohort in the St. Jude's report, 77 of 1261 patients (6.1%) had more than one genetic alteration. These subtypes included *KMT2A*-r, *PAX5*alt, *CRLF2*-r, and iAMP21. The sequential approach might have missed iAMP21, high hyperdiploidy, and *CRLF2*-r if these cases had more than one genetic alteration and had not been submitted for the RNA-seq.

In conclusion, RNA-seq, RT-PCR, MLPA, and DI could classify 96% of patients with B-ALL in this study. This approach might improve the risk-directed classification system in childhood ALL and help identify targetable and prognostic genetic lesions in patients with childhood ALL. RNA-seq could also provide information about gene expression classification and a few targetable fusions in T-ALL. This study showed that the distribution of genetic alterations in childhood B-ALL is different among various racial populations. More sequencing efforts are required to obtain a clearer picture of the genetic distribution of childhood ALL in Asian populations. The most important hurdle to the comprehensive use of RNA-seq is high cost. The approach described in this study may decrease the size of samples required for RNA-seq analysis, which can be saved for patients for whom molecular classification cannot be performed with traditional methods. Therefore, genetic classification of childhood ALL could be performed in an economical manner without compromising precision medicine in countries with limited resources.

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Author Contributions

C.-H.Y., G.W., J.J.Y., C.-H.P., S.-L.Y., and Y.-L.Y. conceived and designed the study; S.-L.Y. and Y.-L.Y. obtained funding; Y.-L.N. and S.-W.L. provided administrative support; S.-T.J., M.-Y.L., K.-H.L., S.-H.C., K.-H.W., F.-L.H., C.-N.C., H.-H.C., J.-L.W., H.-J.Y., M.-J.L., S.-W.C., D.-T.L., and Y.-L.Y. provided study material or patients; C.-T.H., Z.-S.L., and Y.-C.H. performed sequencing experiments; C.-H.Y., G.W., C.-C.C., and Y.-C.H. collected data; G.W., C.-C.C., D.H., Y.-C.H., C.-Y.L., and H.-Y.C. analyzed and interpreted data; all authors wrote, revised, and approved the manuscript for publication. All authors are accountable for all aspects of the work.

Supplemental Data

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