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ORIGINAL ARTICLE

Copy number abnormality of acute lymphoblastic leukemia cell lines based on their genetic subtypes

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Abstract

In this study, we performed genetic analysis of 83 B cell precursor acute lymphoblastic leukemia (B-ALL) cell lines. First, we performed multiplex ligation-dependent probe amplification analysis to identify copy number abnormalities (CNAs) in eight genes associated with B-ALL according to genetic subtype. In Ph^+ B-ALL cell lines, the frequencies of *IKZF1, CDKN2A/2B, BTG1*, and *PAX5* deletion were significantly higher than those in Ph^- B-ALL cell lines. The frequency of *CDKN2A/2B* deletion in *KMT2A* rearranged cell lines was significantly lower than that in non-*KMT2A* rearranged cell lines. These findings suggest that CNAs are correlated with genetic subtype in B-ALL cell lines. In addition, we determined that three B-other ALL cell lines had *IKZF1* deletions (YCUB-5, KOPN49, and KOPN75); we therefore performed comprehensive genetic analysis of these cell lines. YCUB-5, KOPN49, and KOPN75 had *P2RY8-CRLF2, IgH-CRLF2*, and *PAX5-ETV6* fusions, respectively. Moreover, targeted capture sequencing revealed that YCUB-5 had JAK2 R683I and KRAS G12D, and KOPN49 had JAK2 R683G and KRAS G13D mutations. These data may contribute to progress in the field of leukemia research.

Keywords Copy number abnormality · Acute lymphoblastic leukemia cell line · *CDKN2A* · *CDKN2B* · *BTG1* · *IKZF1*

Introduction

Genetic alterations in leukemic cells play major roles in leukemogenesis and drug sensitivity. Thus, better characterization of leukemia genetics will be critical to the improvement of therapeutic strategies. Recent progress in technologies used for biological investigations has enabled characterization of patient primary samples to generate sufficient genetic data to facilitate in vitro and in vivo experiments [1–3]. Thus, knowledge of the genetic alterations present in leukemia cell lines is essential for these cells to be used in place of primary leukemia samples for in vitro and in vivo studies.

The aim of this study was to characterize genetic alterations in a large panel of B cell precursor acute

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lymphoblastic leukemia (B-ALL) cell lines. For this purpose, we investigated copy number abnormalities (CNAs) of genes involved in B-ALL leukemogenesis in a large number of B-ALL cell lines.

It is also important to identify cell lines harboring specific clinically relevant genetic alterations, including Philadelphia chromosome-like acute lymphoblastic leukemia (Ph-like ALL), comprising patients who exhibit a gene expression profile similar to that of Ph + B-ALL [4, 5]. In this study, we also identified putative Ph-like ALL cell lines, performed detailed genetic analysis of them.

Materials and methods

Samples and multiplex ligation-dependent probe amplification

Genomic DNA samples from 83 B-ALL cell lines stocked in the department of pediatrics Yamanashi University were analyzed using the SALSA multiplex ligation-dependent probe amplification (MLPA) kit, P335-B2 (MRC Holland, Amsterdam, the Netherlands) to detect the copy number abnormality of eight genes, namely *IKZF1, PAX5, CDKN2A, CDKN2B, ETV6, RB1, BTG1*, and *EBF1*, according to the manufacturer's instructions, as previously described [6–17]. These cell lines included 15 Philadelphia chromosome positive (Ph+) ALL, 11 *KMT2A*-rearranged (*KMT2A*+) ALL, 17 *t*(1;19)(q23;p13)/*TCF3-PBX1*-ALL, 4 *t*(17;19)(p22;q13)/*TCF3-HLF*-ALL, four *t*(12;21)(p13.1;q22)/*ETV6-RUNX1*-ALL, and 33 other B-ALL (without the above five translocations, classified as "B-other") cell lines. No sister cell line was included in this analysis.

Real-time quantitative-PCR analysis

Total RNA was extracted from leukemic cells using an RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. The cDNA for reverse transcription (RT)-PCR was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Real-time quantitative-PCR (q-PCR) was performed on human ALL cell lines to determine the expression levels of *CRLF2* using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green 1 (Takara Bio, Tokyo, Japan). Relative expression levels of target mRNA molecules were determined using the comparative threshold (ΔCT) method. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as an internal control.

Cell culture and flow cytometric analysis

Three ALL cell lines, KOPN49, KOPN75, and YCUB-5, were cultured in suspension in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (10 mg/ml) at 37 °C in a 5% CO_2 humidified atmosphere.

Cells (1.0×10^6) were harvested, washed twice with PBS, incubated for 20 min with PE anti-human TSLPR (TSLP-R) antibody (BioLegend, San Diego, CA, USA), and analyzed using a FACS Calibur cytometer (BD Biosciences) with FlowJo software (Treestar, SanCarlos, CA, USA). Immunophenotypic analysis was also performed for cell lines with *IKZF1* deleted.

Reverse transcription-PCR and mRNA sequencing

To determine unknown genetic alterations and identify Ph-like ALL-specific fusion genes, reverse transcription-PCR (RT-PCR) was performed in three B-other cell lines harboring *IKZF1* deletions, according to previously reported protocols [18]. The primer pairs are listed in supplementary Table 1 (sTable 1). Messenger RNA sequencing (mRNA-seq) was performed using KOPN49 and KOPN75 cell samples. An NEBNext® mRNA Library Prep Reagent Set for Illumina was used for construction of sequencing libraries, according to the manufacturer's instructions, and then sequenced using a HiSeq2500 (Illumina Inc., San Diego, CA, USA). Fusion transcripts were detected using Genomon-fusion (http://genomon.hgc.jp/rna/).

Targeted capture sequencing

Targeted sequencing of 163 genes known to be mutated in hematological diseases, customized to detect cancer-related genes, as previously described [19], was performed on a MiSeq system (Illumina, San Diego, CA) using HaloPlex custom panels (Agilent, Santa Clara, CA). Read alignment and variant calling were performed using the MiSeq reporter (Illumina), and variants were annotated using SureCall software (Agilent). Synonymous or noncoding variants were excluded, and single nucleotide polymorphisms (SNPs) reported to dbSNP, unless they were found in the COSMIC database. Among the remaining variants, with allele frequencies > 0.1, recurrently mutated genes were considered candidate genes and validated by conventional Sanger sequencing. In addition, *IgH* rearrangements were captured using 662 baits tiling the entire *IgH* enhancer locus and sequenced using the HiSeq2500 platform. Detection of structural variations was performed using Genomon-SV (https://github.com/Genomon-Project/GenomonSV).

Briefly, Genomon-SV uses information from chimeric reads (containing breakpoints) and discordant read pairs. For each candidate structural variation, reads are realigned to the assembled contig sequence containing the structural variation breakpoint (variant sequence).

Statistical analyses

Statistical analysis was performed using the Student's *t* test. *P* values < 0.05 were considered statistically significant. Other comparisons were performed using the 2 × 2 Chi square test or Fisher's exact test, as appropriate.

Results

Copy number abnormalities in 83 B-ALL cell lines

To identify CNAs of eight genes related to B-ALL leukemogenesis, including *IKZF1, PAX5, CDKN2A, CDKN2B, ETV6, RB1, BTG1*, and *EBF1*, in 83 B-ALL cell lines we performed MLPA analysis. The CNA results for the 83 cell lines are summarized in Fig. 1 and sTable 2, according to genetic subgroups. Overall, 68 of the 83 cell lines had at least one CNA. No CNAs of the eight genes were determined in 4 of 11 (36%) *KMT2A*(+) ALL and 8 of 17 (47.1%) *t*(1;19) positive ALL cell lines. In contrast, all of the Ph + B-ALL and 29 of 33 (87.9%) B-other cell lines harbored at least one CNA of the eight genes included in this analysis. Deletion of *CDKN2A* and/or *CDKN2B* was the most frequent CNA (60 of 83, 72.3%), while *EBF1* and *RB1* deletions were rarely detected (2 of 83, 2.4%, and 5 of 83, 6.0%, respectively). Interestingly, MLPA analysis also revealed that three of the B-other ALL cell lines, YCUB-5, KOPN49, and KOPN75, had *IKZF1* deletions (YCUB-5, deletion of exons 2–8; KOPN49, deletion of exons 2–3; and KOPN75, deletion of exons 1–8), indicating that they may be derived from Ph-like ALL patient samples.

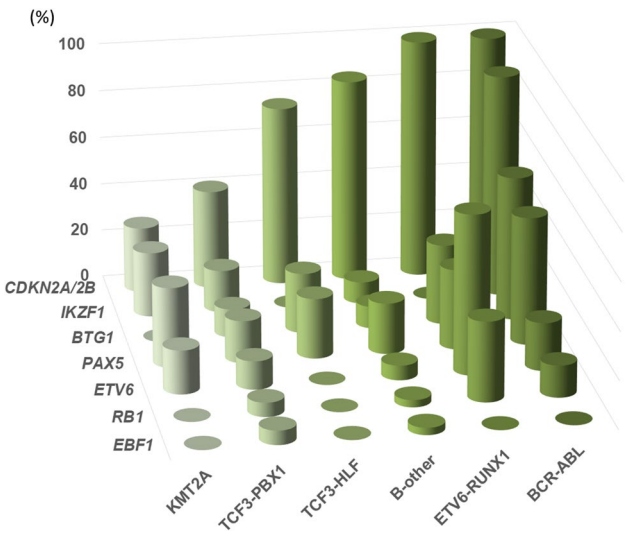

Fig. 1 Copy number abnormalities in 83 B-cell precursor acute lymphoblastic leukemia cell lines, according to genetic subtypes. The bar graph represents frequency of the deletion of eight genes, namely *IKZF1, PAX5, RB1, CDKN2A, CDKN2B, ETV6, EBF1* and *BTG1*, according to the six genetic subtypes, namely Ph+, *TCF3-PBX1* positive, *ETV6-RUNX1* positive, *KMT2A* rearranged, *TCF3-HLF* positive and B-others

Distinct pattern of CNA according to the genetic subtypes

Next, we compared CNAs in B-ALL cell lines according to their specific genetic abnormalities. In Ph + B-ALL cell lines, *IKZF1, CDKN2A/2B, BTG1*, and *PAX5* deletion were more enriched than in Ph negative B-ALL cell lines (Fig. 2). On the other hand, the frequency of *CDKN2A/2B* deletion in *KMT2A* rearranged cell lines was significantly lower than that in non-*KMT2A* rearranged cell lines (Fig. 2). These findings suggested that CNA was related to genetic subtype

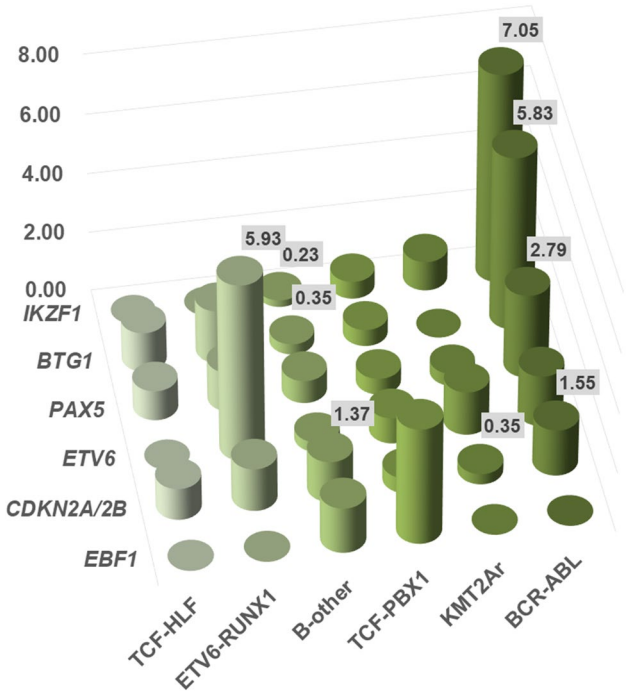

Fig. 2 Comparison between cell lines of copy number abnormalities of six genes, according to the genetic subtypes. The ratio of [CNA positive cell lines (%) with positive genetic marker]/[CNA positive cell lines (%) with negative genetic marker] is shown. In Ph + B-ALL cell lines, the frequencies of *IKZF1, CDKN2A/2B, BTG1*, and *PAX5* deletion were significantly higher than those in Ph negative B-ALL cell lines. The frequency of *CDKN2A/2B* deletion in *KMT2A* rearranged cell lines was significantly lower than that in non-*KMT2A* rearranged cell lines. *KMT2Ar* indicates KMT2A rearranged B-ALL cell lines

in B-ALL cell lines, which was similar to that in primary patients' samples [17, 20].

Molecular characterization of three B-other ALL cell lines harboring *IKZF1* deletions

As B-other ALL cell lines harboring *IKZF1* deletion are rare and considered putative Ph-like ALL cell lines [5, 21], we performed further molecular characterization of the three cell lines, YCUB-5, KOPN49, and KOPN75. Given that genetic alteration of *CRLF2* is the most frequent genetic event observed in *IKZF1* deleted B-other ALL [5, 21] and high expression of *CRLF2* is considered to be one of the surrogate makers for Ph-like ALL [22], we performed real-time qPCR to determine levels of *CRLF2* expression in these cell lines. Real-time qPCR analysis revealed high levels of *CRLF2* expression in YCUB-5 and KOPN49 cell lines, which was confirmed by FACS analysis (Fig. 3a, b).

Next, we performed multiplex PCR to determine whether any Ph-like ALL associated fusion transcripts were expressed in these three cell lines. The results revealed that YCUB-5 was positive for the *P2RY8–CRLF2* fusion transcript and this was confirmed by Sanger sequencing (Fig. 4a, b); however, no fusion transcripts were detected in KOPN49 or KOPN75 (Fig. 4a). To screen for other fusion transcripts in the KOPN49 and KOPN75 cell lines, we performed mRNA-seq. These investigations identified a *PAX5-ETV6* fusion transcript in KOPN75, which was confirmed by Sanger sequencing (Fig. 5a, b); however, no *CRLF2*-related fusion transcripts were identified in the KOPN49 cell line.

To further molecularly characterize these B-ALL cell lines, we performed targeted capture sequencing (TCS) of 163 genes established as mutated in hematological malignancies. TCS identified the following mutations: JAK2 R683I and KRAS G12D in YCUB-5, and JAK2 R683G and KRAS G13D in KOPN49. These findings suggest that both cell lines not only have activating mutations in the JAK-STAT pathway, but also in the RAS pathway (Table 1 and sTable 4). Finally, we performed TCS using 662 probes covering the entire *IgH* enhancer locus, resulting in detection of an *IgH-CRLF2* fusion in KOPN49 cells. The molecular and genetic characteristics of these three cell lines are summarized in Table 1.

Discussion

CDKN2A/2B are well-known tumor suppressor genes that map to chromosome 9p21. *CDKN2A* encodes p14/ARF and p16/INK4A, and *CDKN2B* encodes p15/INK4B [23]. All of these proteins regulate the cell cycle and apoptosis through RB or TP53. Deletion is the most common cause of inactivation of *CDKN2A/2B* in ALL. Although deletions of *CDKN2A/2B* are reported as associated with poor prognostic

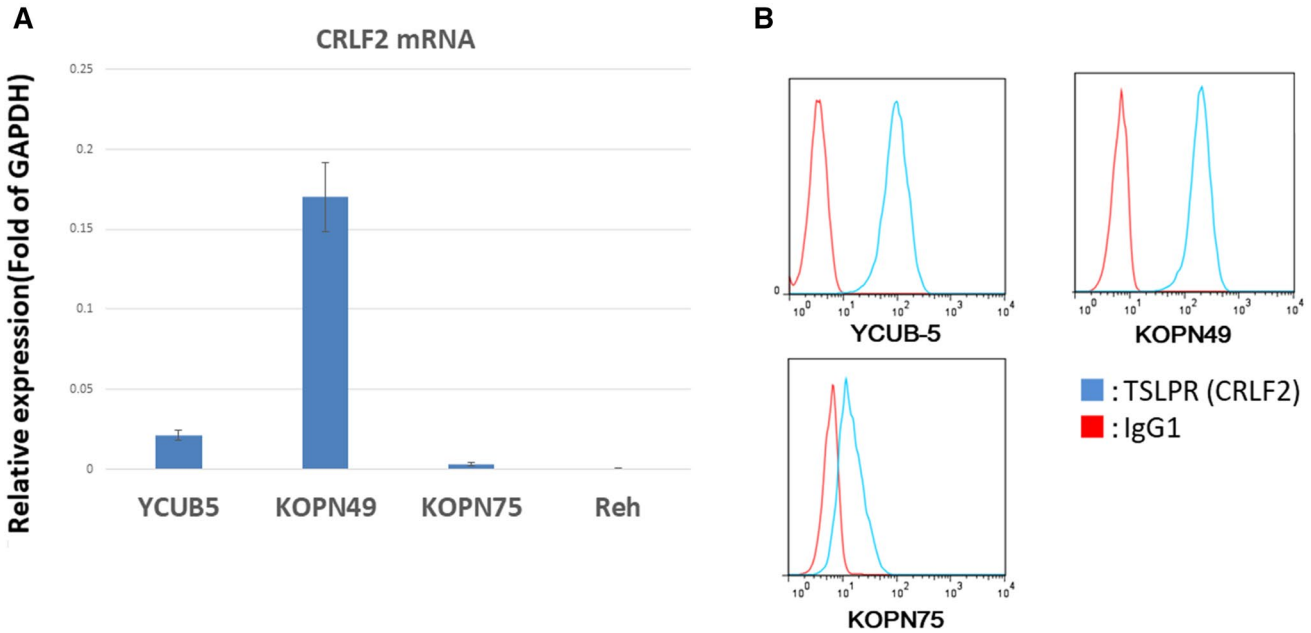

Fig. 3 *CRLF2* mRNA and protein expression levels in three *IKZF1* deleted B-other ALL cell lines. **a** *CRLF2* mRNA expression levels in three *IKZF1* deleted B-other ALL cell lines, YCUB-5, KOPN49, and KOPN75, determined by quantitative RT-PCR. Expression of *CRLF2* mRNA was detected in YCUB-5 and KOPN49, but not in KOPN75 cells. Reh cell lines carrying the *ETV6-RUNX1* fusion were used as a negative control. The bars indicate mean ± SD of duplicate PCR assays. **b** CRLF2 protein expression in the three cell lines, YCUB-5, KOPN49, and KOPN75, was determined by FACS analysis. CRLF2 was expressed on YCUB-5 and KOPN49 cells

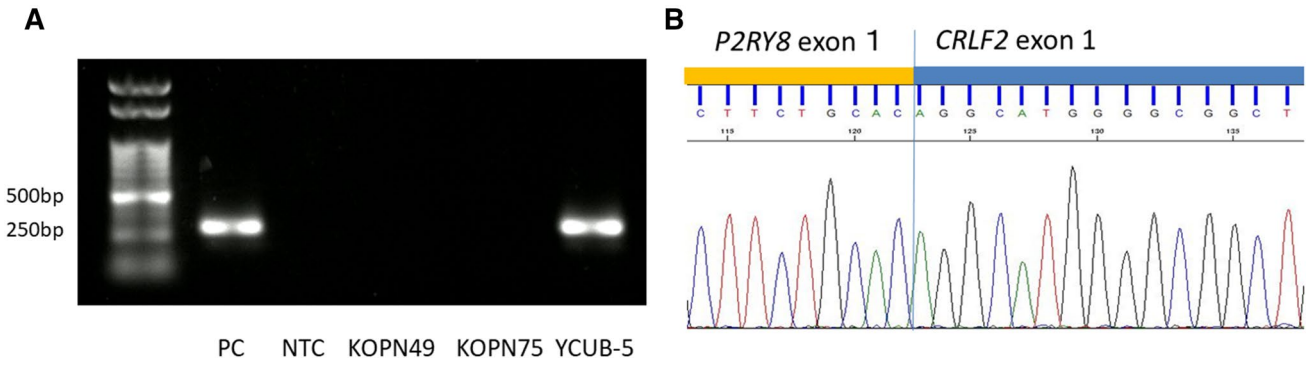

Fig. 4 The *P2RY8–CRLF2* fusion transcript was detected only in YCUB-5. **a** The *P2RY8-CRLF2* fusion transcript was detected only in YCUB-5 by reverse transcription polymerase chain reaction (RT-PCR). **b** Sanger sequencing of the RT-PCR product revealed that *P2RY8* exon 1 was fused in-frame to *CRLF2* exon 1. PC indicates positive control from patient's leukemic sample with *P2RY8–CRLF2* fusion transcript. *NTC* no template control

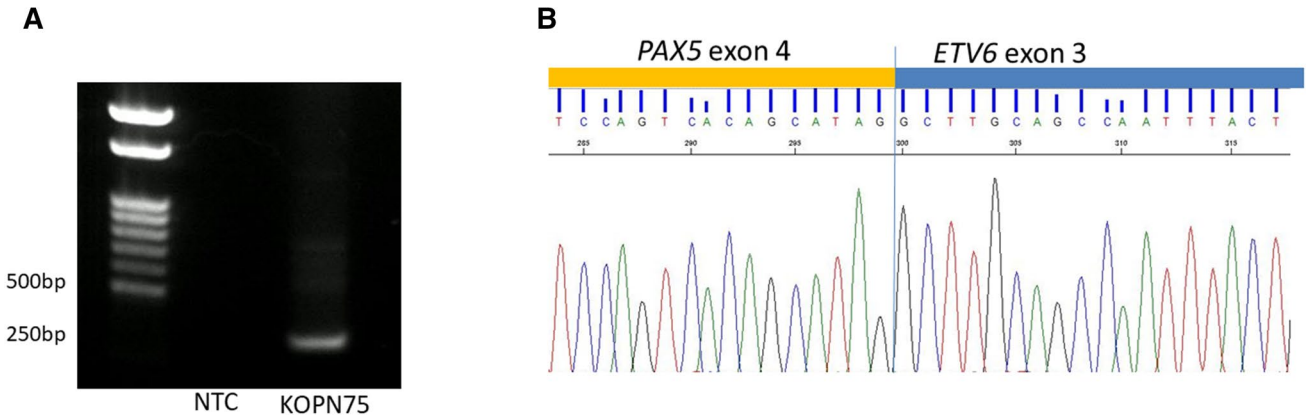

Fig. 5 The *PAX5-ETV6* fusion transcript was detected in KOPN75. **a** The *PAX5-ETV6* fusion transcript was detected by reverse transcription polymerase chain reaction (RT-PCR) amplification in KOPN75. *NTC* no template control. **b** Sanger sequencing of the RT-PCR product revealed that *PAX5* exon 4 was fused in-frame to *ETV6* exon 3. *NTC* no template control

Table 1 Summary of genetic analysis of B-other ALL cell lines with *IKZF1* deletion

Cell line	Karyotype	Fusion transcript	*IKZF1* del	*JAK2* mutation	RAS pathway mutation
YCUB-5	48,XY,+X,+21	*P2RY8-CRLF2*	+	JAK2 R683I	KRAS G12D
KOPN49	50,XY,+Y,+10,+18,der(1;3)(q25;q25)	*IgH-CRLF2*	+	JAK2 R683G	KRAS G13D
KOPN75	NE	*PAX5-ETV6*	+	ND	ND

NE not examined, *ND* not detected

features, such as high white blood cell count, older age at diagnosis, *IKZF1* deletion, and positive Philadelphia chromosome, the prognostic significance of this genetic alteration in B-ALL remains controversial [24, 25].

In this study, we identified deletion of *CDKN2A/2B* as frequent in Ph + B-ALL cell lines. It is interesting that *CDKN2A* and *IKZF1* alterations in Ph + B- ALL are reported to synergize in promotion of leukemia development by conferring stem cell-like properties [26]. These findings could explain why *CDKN2A/2B* deletion is enriched in Ph + B-ALL cell lines.

We also identified deletion of *BTG1* was frequent in Ph + cell. BTG1 also has anti-proliferative effects and the deletion of *Btg1* was reported to increase the frequency of T cell acute lymphoblastic leukemia development in combination with *Ikzf1* alteration in a mouse model, suggesting *Btg1*

deletion might augment leukemogenesis of *Ikzf1* deletion [27, 28]. These findings also suggest that *BTG1* deletion contributes to the establishment of Ph + B-ALL cell lines via a similar mechanism to *CDKN2A/2B* deletion. Taking together with high frequency of deletion of *IKZF1* in Ph + B-ALL cell lines, alterations of *IKZF1, CDKN2A/2B*, and *BTG1* may be necessary for the acquired characteristics of Ph + leukemic cell lines, such as high proliferation and augmented self-renewal activity.

The limitation of this study is that the genetic alterations detected in the cell lines are not always events occurring in the patients' bone marrow. Due to the lack of data on CNA in the original patient samples from which cell lines were established, we were unable to determine whether deletion of *CDKN2A/2B* and *BTG1* was cell line-specific or the alterations were already present in the primary leukemic blasts. Analysis of CNA in leukemic blasts from the patients of origin would answer this question.

In this study, we identified three B-other cell lines with *IKZF1* deletion: KOPN75, KOPN49, and YCUB-5. As *IKZF1* deletion in B-other ALL is a surrogate marker for Ph-like ALL, we conducted a comprehensive genetic analysis of these three cell lines and mRNA-seq revealed that KOPN75 had *PAX5-ETV6* fusion transcript. *PAX5-ETV6* was first identified by Cazzaniga and colleagues in a sample from a patient with ALL carrying a *t*(9;12)(q11;p13) translocation [29]. This fusion protein has also been identified in ALL patients with the recurrent chromosomal abnormality dic(9;12)(q11;p13) [30]; however, *PAX5-ETV6* positive ALL is different from Ph-like ALL because, as the PAX5-ETV6 fusion protein retains the PAX5 paired-box domain and both the helix-loop-helix and ETS binding domains, hence this protein could act as an aberrant transcription factor. Consistent with this hypothesis, Fazio and colleagues determined that PAX5-ETV6 induces a B-cell development block via repression of genes activated by PAX5 [31]. Hence, KOPN75 should be a useful cell line for further investigation of the leukemogenic functions of *PAX5-ETV6*.

We determined that KOPN49 and YCUB-5 carry *IgH-CRLF2* and *P2RY8-CRLF2*, respectively. Considering that both cell lines have an *IKZF1* deletion, they can be considered Ph-like ALL cell lines. *JAK2* mutation is established as occurring in patients with Ph-like ALL with *CRLF2* rearrangement [5, 32]. In accordance with previous finding, TCS revealed *JAK2* activating mutation in both cell lines. Interestingly, TCS also revealed that activating mutation of *KRAS* was present in both cell lines. These findings are consistent with the previous report that describing some Ph-like ALL patients harbored activating mutations in both JAK2 and RAS pathways [5].

In conclusion, we examined genetic alterations in 83 B-ALL cell lines and identified enrichment for *CDKN2A/2B* and *BTG1* deletions particularly in Ph + B-ALL cell lines. We also identified two putative Ph-like ALL cell lines harboring *IKZF1* deletion, *CRLF2* rearrangement, and activating mutations of *JAK2* and *KRAS*. The genetic data characterizing alterations in these cell lines will be useful for the investigation of B-ALL.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. A summary of relevant information will be published with the manuscript.

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