ORIGINAL ARTICLE

Comprehensive genetic analyses of relapsed B-cell precursor acute lymphoblastic leukemia in children

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Abstract The causes for individual relapse in children with B-cell precursor acute lymphoblastic leukemia (BCP-ALL) remain incompletely understood. Here, we performed comprehensive genetic analyses of 20 pediatric BCP-ALL paired diagnosis-relapse samples. Copy number variation analysis of *IKZF1*, a gene for which deletions are associated with increased relapse risk, revealed deletions at both diagnosis and at relapse (n=5). Targeted next generation sequencing showed that RAS pathway genes including *KRAS* (n=4), *NRAS* (n=2) and *PTPN11* (n=2) were the most common mutations among the sequencing targets at diagnosis and/or relapse. In 7 of 20 cases (35%), mutations in *PTPN11*, *KRAS*, *TP53*, *CTCF*, *NCOR1*, *WHSC1*, *TUSC3*, *ERG* and *NT5C2* were detected only at relapse. Two cases with *TP53* mutations showed fatal outcomes. In two of the four cases with high hyperdiploid BCP-ALL, associated with favorable prognosis, *PTPN11* mutations were detected only at relapse. Targeted RNA sequencing identified a rare subtype of BCP-ALL with a *NUP214-ABL1* fusion that could benefit from tyrosine kinase inhibitors. Together with recent reports that RAS mutations confer sensitivity to MEK inhibitors, these results suggest that comprehensive genetic analysis will contribute to the optimal treatment for relapsed BCP-ALL.

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Key words: acute lymphoblastic leukemia; relapse; childhood; RAS pathway genes.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer[1,2]. The advance of risk-stratified chemotherapy for ALL has dramatically improved event-free survival, and aggressive salvage therapies involving allogeneic stem cell transplant have improved overall survival. Despite these improvements, relapse still occurs in around 10-20% of patients and is the leading cause of ALL fatal treatment failure. Recent genomic studies have identified relapse-specific mutations that induce clonal evolution in pediatric ALL[3-5]. However, there have been a limited number of studies on relapsed cases with pediatric B-cell precursor ALL (BCP-ALL) in Japan. In this study, we performed a comprehensive genetic analysis including a copy number analysis for Ikaros family zinc finger protein 1 (*IKZF1*)[6] using droplet digital polymerase chain reaction (ddPCR), targeted next generation sequencing covering 25 genes, and targeted RNA sequencing in 20 paired samples at diagnosis, relapse, and remission. Our findings clarify the molecular pathogenesis of childhood relapsed BCP-ALL.

Patients and methods

Patients

Between 1988 and 2016, a total of 20 pediatric patients with BCP-ALL were enrolled in this study. The patients were treated with various protocols including JACLS ALL-02 and JPLSG

ALL B-12 in the Hirosaki University Hospital. The study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee at Hirosaki University Graduate School of Medicine.

Copy number variation assay by ddPCR to detect *IKZF1* deletion

Copy Number Variation (CNV) assays for *IKZF1* were conducted using PrimePCR™ ddPCR™ Copy Number Assay primer sets (Bio-Rad, Hercules, USA) and a QX200™ Droplet Digital PCR System(Bio-Rad). The reaction mixtures for ddPCR consisted of the following components: 20-50 ng of gDNA, 11 µL 2 x ddPCR Supermix (Bio-Rad), 1 µL of each IKZF1 and EIF2C1 probes, in a final volume of 20 µL. The PCR reaction mixture was prepared in duplicated and loaded into an eight-channel disposable droplet generator cartridge (DG8 cartridge Bio-Rad), with 70 µL of droplet generation oil, into the QX200 Droplet Generator. The generated droplets in a volume of 40 µL were transferred into a 96-well PCR plate and then amplified using optimized following condition: 95°C for 10 minutes, then 43 cycles of 94°C for 30 seconds, 60°C for 60 seconds, and 98°C for 10 minutes. An absolute quantification of each target molecule was analyzed by QuantaSoft Analysis Pro v1.0 software. The chromosomal position of each targeted amplification was previously validated and are as follows: IKZF1(hg19 7:50444255-50444377) and EIF2C(hg19 1: 36359351-36359473). The IKZF1 primer, labeled with FAM dye, targets *IKZF1* exon 4, a region deleted in 98% of acute lymphoblastic leukemia with *IKZF1* deletion[7]. The EIF2C primer, labeled with HEX dye, was used as a reference molecule control. Copy number variation was calculated as the ratio of target molecule to reference molecule concentrations[8]. A total of 29 bone marrow samples in complete remission were analyzed to provide constitutional DNA and supplied a reference mean copy number and standard deviation. We set the threshold of CNV as 1.828–2.104 (1.967+–4SD, 99.99%, n=29). Data obtained from fewer than 10,000 droplets were excluded.

Gene mutation analysis

Genomic DNA was isolated from paired samples (bone marrow or peripheral blood) at diagnosis, remission, and relapse using the Qiagen Blood mini DNA Kit (Qiagen, Hilden, Germany). We first performed capture-based targeted sequencing covering 23 genes using genomic DNA by the HaloPlex Target Enrichment System (Agilent, Santa Clara, CA, USA) on a MiSeq platform (Illumina, San Diego, CA, USA)[3]. The custom gene lists are shown in Table 1. CLC Genomic Workbench was used for sequencing alignment to the reference human genome (hg19) and for variant detection. We identified only non-synonymous variants with the following cutoffs: a variant allele frequency (VAF) of >10%, a minimum bi-directional coverage of >10, and an allele frequency of <5% in the normal population (1000 Genomes Project). In addition, we performed mutational analysis of *WHSC1* (*NSD2*)[9] and *NT5C2*[4,5] by amplicon-sequencing using custom-designed primers and next generation sequencing for the Miseq. Target amplicons were amplified by KOD plus Neo (TOYOBO, Japan) using optimized parameters: 94°C for 2 minutes, then 30 cycles of 98°C for 10 seconds, 63°C for 30 seconds, and 72°C for 60 seconds. Each library was ligated to an index sequence and subsequently sequenced on the Miseq platform. The primer sequences are shown in Table 2. To eliminate germline variants, we performed sequencing on bone marrow samples in complete remission as a control.

Targeted RNA sequencing

Total RNA was extracted from bone marrow or blood using the Qiagen Blood RNA Kit (Qia-

Table 1. Gene list for targeted sequencing

gene	function
KRAS	
NRAS	
PTPN11	RAS pathway
NF1	
FLT3	
JAK1	
JAK2	
JAK3	Cell signaling
CRLF2	
IL7R	
PAX5	
ERG	
SPI1	Transcription factors
TCF4	
CTCF	
TP53	
TUSC3	
CREBBP	Transcriptional coactivator
NCOR1	
EZH2	
HIST1H2BG	Epigenetics
ASMTL	
WHSC1	
THADA	Apoptosis
NT5C2	Drug resistance

gen, Hilden, Germany). RNA-Seq libraries were prepared using the TruSight™ RNA Pan-Cancer Panel kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The quality of isolated total RNA and cDNA library was analyzed using the Bio-Rad Experion system (Bio-Rad Laboratories, Hercules, CA, USA). An input of 50-100 ng of total RNA was converted to cDNA and then sequenced using the MiSeq platform (Illumina, San Diego, CA, USA). We analyzed the data using the UCSC hg19 reference genome aligners STAR (STAR_2.5.0a) or TopHat (Version 2.0.5), RefSeq annotation, and fusion calling with RNA-Seq alignment software (Illumina, San Diego, CA, USA). Fusion genes were identified as those with a calling score greater than 0.7.

Results

Tested patients

To evaluate genetic alterations in evolutional process, characteristics of 20 patients with relapsed BCP-ALL were collected and are summarized in Table 3 and Table 4. The male to female ratio was 0.82:1, and the median age at diagnosis was 6.8 years (range, 0.75–16 years), and the median time to relapse was 34 months (range, 4–69 months). Clinical risk, according to the National Cancer Institute (NCI) risk criteria, consisted of 12 cases with high risk and 8 cases with standard risk. Recurrent cytogenetic abnormalities were found in 10 patients (50%): four with high hyperdiploidy, four with t (12;21) (p13;q22), and two with 11q23 rearrangement.

Detection of *IKZF1* deletion by ddPCR

IKZF1 deletions, which are associated with increased relapse risk and poor prognosis[6], were detected in 5 of 20 samples both at diagnosis and at relapse (Figure 1 and Table 5).

Mutational analysis

Genetic analysis results for our cohort are summarized in Figure 1. Each somatic mutation is listed in Table 6. The mean coverage of deep sequencing of Haloplex analysis was x781, and >95% of target regions were covered over 100-fold (Figure 2A). The mean coverage of tailed PCR-based deep sequencing was x13,980 (Figure 2B). We detected 28 somatic mutations in total, including 5 onset-specific mutations. Mutations in RAS pathway genes and in *TP53* were detected in 7/20 (35%) and 2/20 (10%) cases, respectively. The RAS pathway genes were the most common mutations among the sequencing targets *KRAS* (n=4), *NRAS* (n=2) and *PTPN11* (n=2), and were mutated at diagnosis and/or relapse. These mutations were mutually exclusive. We identified 11 relapse-specific mutations, affecting 7/20 (50%) cases, occurring in genes *PTPN11* (n=2), *TP53*

Table 2. Primer sets and amplicon target regions for tailed PCR

Gene	Target regions	Amplicon size	Oligo sequence (5'-3')	
WHSC1 Exon 20	chr4 1962715_1962957	243bp	forward	attggtcagcacgctttttgtcatgg
			reverse	caaagtccagttctacaggtgaccatc
WHSC1 Exon 21	chr4 1976543_1976781	239bp	forward	acatgcgattgctaacacttgaccga
			reverse	actctgcccccatagccaggagccc
NT5C2 Exon 10	chr10 104856997_104857269	340bp	forward	agtgcaaaattctgaaatcagctaacc
			reverse	taggccaaaataattccactgcataaa
NT5C2 Exon 14	chr10 104852848_104853091	311bp	forward	ctcattgttcactctgttggttcaggt
			reverse	gctggtcatttagatatataaattacacc
NT5C2 Exon 15	chr10 104851272_104851508	304bp	forward	ggtaactgtacactcagcatgttagtc
			reverse	aagtgattcctgagtaagtttttgggga
NT5C2 Exon 16	chr10 104850491_104850717	294bp	forward	cagttccatccagagacgtattaaggt
			reverse	gcgaaacaggcttcccatcatcccata

Table 3. Clinical and biological features

Total, n	20
Sex, male, n (%)	9 (45)
Age at relapse, years, n (%)	
Median, range	6.8 (0.75-16)
<1	1 (5)
1-9	11 (55)
>10	8 (40)
Clinical risk group, n (%)	
High	12 (60)
Standard/intermediate	8 (40)
Genetic subgroup, n (%)	
High hyperdiploidy	3 (15)
11q23 rearrangement	2 (10)
t(12;21)(p13;q22)	4 (20)
Time to first relapse (month), n (%)	
Median, range	34 (4-69)
Extremely early	5 (25)
Early	3 (15)
Late	12 (60)
Site of first relapse, n (%)	
Isolated BM	18 (90)
Isolated CNS	0
Extramedullary	1 (5)
Combined	1 (5)
Stem cell transplantation, n (%)	
Yes	18 (90)
No	2 (10)
Outcome of induction therapy, n (%)	
Induction failure	4 (20)
Induction death	0
Second CR	16 (80)
Outcome of patients achieving a second remission, n (%)	
Relapsed	5 (25)
Died	7 (35)
Continuing second CR	11 (55)

Abbreviation: CR, complete remission, CNS, central nervous system

(n=2), and *KRAS*, *ERG*, *TUSC3*, *CTCF*, *NCOR1*, *WHSC1* and *NT5C2* (one case each). In case 3, we detected two different *NRAS* mutations, G12S at diagnosis, and G12D at relapse (Table 6). However, careful analysis of the onset sample sequence data revealed that the patient had a low burden of *NRAS* G12D mutation (VAF: 0.0818) at diagnosis, and an increase in VAF at onset higher than the detection cutoff.

Fusion genes in relapsed BCP-ALL

We performed targeted RNA sequencing to identify oncogenic fusion genes using the TruSight™ RNA Pan-Cancer Panel kit. The panel targets 1385 cancer-associated genes, including 507 genes involved in fusion and > 850 genes either mutated or deregulated in cancers. At diagnosis, known fusions, including *ETV6-RUNX1* (n=4), *KMT2A-MLLT3* (n=2), *ARID1B-ZNF384* (n=1), *NUP214-ABL1* (n=1) and *TCF3-PBX1* (n=1), were detected in 9 of 20 cases (Figure 1). *ARID1B-ZNF384* and *NUP214-ABL1* fusions were validated by Sanger sequencing of reverse transcription PCR products (Figure 3 and Table 7). All of these fusion genes were retained at relapse.

Table 4. Patient characteristics of relapsed BCP-ALL

Case	Age at diagnosis (y, m)	Gender	WBC at diagnosis (/μL)	Time from diagnosis to relapse (m)	Time from therapy-off to relapse (m)	NCI risk	Phase of relapse	Prognosis	Recurrent cytogenetic abnormalities
1	3y6m	F	7710	31	5	SR	early	dead	t(12;21)(p13;q22)
2	0y9m	M	105770	24	16	HR	late	alive	11q23 rearrangement
3	10y3m	F	1130	60	33	HR	late	alive	
4	11y6m	F	305050	32	17	HR	late	dead	
5	8y0m	F	2300	69	39	SR	late	alive	
6	5y7m	F	1470	40	10	SR	late	alive	High hyperdiploid
7	4y1m	M	56860	39	9	HR	late	dead	t(12;21)(p13;q22)
9	12y1m	F	4500	16	(−)	HR	extremely early	dead	
10	14y8m	F	40800	47	23	HR	late	alive	
12	8y4m	M	1960	60	34	SR	late	alive	t(12;21)(p13;q22)
13	3y9m	M		65	40	SR	late	alive	High hyperdiploid
14	12y8m	M	132860	4	(−)	HR	extremely early	alive	
15	1y8m	F	95100	16	(−)	HR	extremely early	alive	11q23 rearrangement
18	11y2m	M	35600	9	(−)	HR	extremely early	dead	
20	1y0m	M	17700	18	(−)	HR	extremely early	dead	
21	1y10m	F	12500	44	15	SR	late	alive	t(12;21)(p13;q22)
23	11y0m	F	2100	32	3	HR	early	dead	
24	3y9m	F	7400	28	3	SR	early	alive	High hyperdiploid
25	15y7m	M	12620	35	9	HR	late	alive	
26	1y10m	M		37	11	SR	late	alive	High hyperdiploid

Abbreviation: y, year; m, month; F, female; M, male; WBC, white blood cell count ; NCI, National Cancer Institute; HR, high risk; SR, standard risk

Discussion

In this study, we performed a comprehensive genetic analysis of 20 paired diagnosis-relapse samples of childhood relapsed BCP-ALL in a single center. Analysis included targeted next generation sequencing covering 25 genes, CNV assays for *IKZF1* deletions by ddPCR, and targeted RNA sequencing. In 7 of 20 (35%) cases, we detected 11 mutations including *TP53*, *PTPN11*, *KRAS*, *ERG*, *TUSC3*, *CTCF*, *NCOR1*, *WHSC1* and *NT5C2* only at relapse. Two cases with multiple relapse specific-mutations including *TP53* resulted in a fatal outcome, consistent with a previous study[9]. The frequency of somatic mutations in relapsed childhood BCP-ALL in a Japanese cohort was similar to previous reports[3, 9].

Gain of function mutations in *NT5C2* induce resistance to 6-mercaptopurine and are selectively present in relapsed ALL[4, 5]. The *NT5C2* mutations are present in about 20% of relapsed pediatric T-cell ALL cases[5] and 3-45% of relapsed BCP-ALL[4, 5, 9]. We observed a slightly lower frequency of mutations in *NT5C2* in our cohort of Japanese BCP-ALL patients than the frequencies previously reported from western countries, although the data from both populations are based on low numbers of patients and values showing significant differences between populations are lacking.

In two of the four cases with high hyperdiploid BCP-ALL associated with favorable progno-

K. Kubo, *et al.*
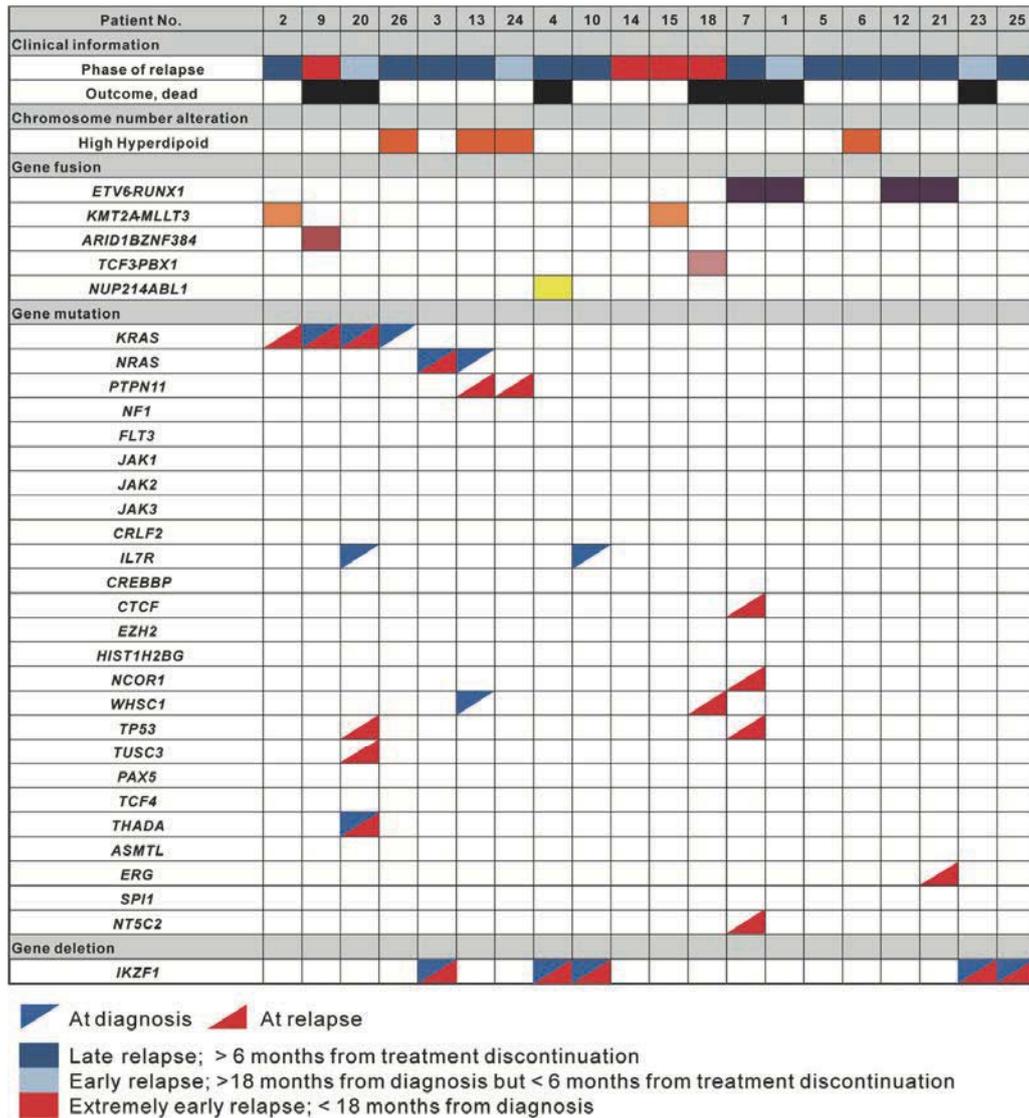
Figure 1 Genetic landscape of childhood BCP-ALL relapse cases.

Clinical information, chromosome number alteration, gene fusions, gene mutations, and deletion of *IKZF1* in 20 paired diagnosis-relapse samples of pediatric BCP-ALL. Somatic mutations at diagnosis and relapse are indicated by blue and red triangles, respectively. Phase of relapse are categorized into late onset, early relapse and extremely early relapse and are indicated by dark blue and right blue and red, respectively.

sis, relapse-specific mutations including *PTPN11* mutations（n=2）were detected. However, in two of four BCP-ALL cases with *ETV6-RUNX1* fusion, another favorable subtype, no additional genetic alterations related to poor prognosis were detected, indicating the potential evolutional process, which needs to be resolved.

RAS pathway mutations are a common genetic alteration in relapsed ALL and are associated with high-risk features and poor prognosis. In particular, *KRAS* mutations are associated with reduced overall survival[10, 11]. Indeed, two of four patients with *KRAS* mutations had a fatal outcome in our cohort. *RAS* mutations induce resistance to methotrexate but not to L-aspraginase, daunorubicin, or thiopurines. Furthermore, these mutations show improved response to vincristine[12, 13]. Targeted MEK inhibition with selumetinib showed the clear evidence to reduce leukemic cell

Table 5. Deletion gene copy number measurements of IKZF1 gene

case	status onset	CR	relapse	2nd relapse
1	1.96	2.03	2.02	1.99
2	2.09	1.99	2.01	
3	**1.05**	2.03	**1.66**	
4	**1.02**	2.11	**0.991**	
5	2.05	1.95	1.99	2.02
6	2.03	2.02	2.09	1.98
7	1.96	2.08	1.96	1.95
9	1.96	2.00	2.06	
10	**0.138**	2.08	**0.583**	
12	2.04	2.01	**2.59**	
13	1.98	2.03	**4.77**	
14	1.99	2.08	1.96	2.01
15	2.08	2.05	2.13	
18	2.04	2.02	2.02	
20	1.88	1.99	2.06	
21	1.98	2.02	2.04	
23	**1.73**	2.01	**1.67**	
24	1.97	2.06	2.01	
25	**1.08**	1.98	**1.27**	
26	1.95	1.91	1.98	1.85

Copy number measurements of the gene *IKZF1* by ddPCR on bone marrow samples at diagnosis (onset), in complete remission (CR), and at relapse. CNV values out of normal range are shown in bold.

growth in ALL with RAS pathway mutations, both *in vitro* and *in vivo*[11]. Furthermore, MEK inhibitors can synergize with prednisolone[14]. These findings suggest that patients with RAS pathway mutations could profit from intensification of these drugs and/or MEK inhibitors.

Targeted RNA sequencing identified rare subtypes of BCP-ALL with *ARID1B-ZNF384* (case 9)[15] and *NUP214-ABL1* (case 4) fusions[16]. The *NUP214-ABL1* fusion is a recurrent abnormality found in about 6% of T-ALL[16]. However, only a few cases with BCP-ALL cases with *NUP214-ABL1* fusion have been reported[17]. This subtype belongs to Philadelphia chromosome-like ALL (Ph-like ALL) and is associated with poor outcome. Deletions or mutations of *IKZF1* are a hallmark of both BCR-ABL-positive ALL and Ph-like ALL. Indeed, our patient (case 4) also harbored *IKZF1* deletion. Notably, Ph-like ALL could benefit from tyrosine kinase inhibitors[17, 18].

This study has the following limitations: limited total number of patients from a single center, limited target regions for genetic analysis, various treatments in both the first and second lines, and no data regarding minimal residual disease indicating treatment response.

In conclusion, this study identified a number of genetic alterations that are likely to be involved in the pathogenesis of treatment failure in BCP-ALL. These results suggest that comprehensive genetic analyses will contribute to the optimal treatment for relapsed BCP-ALL.

K. Kubo, **et al.**

Table 6. List of somatic mutations detected by target sequencing

Case	Status	Gene	Amino acid change	Allele frequency
2	1st relapse	KRAS	Q61R	0.146
3	onset	NRAS	G12S	0.271
3	onset	NRAS	G12D	0.0818
3	1st relapse	NRAS	G12D	0.143
3	2nd relapse (ethmoid sinuses)	NRAS	G12D	0.458
7	1st relapse	CTCF	Q131*	0.122
7	1st relapse	TP53	E154*	0.504
7	1st relapse	NCOR1	S2104*	0.513
7	2nd relapse	TP53	E154*	0.216
7	2nd relapse	NCOR1	S2104*	0.195
7	2nd relapse	NT5C2	D415A	0.184
9	onset	KRAS	G13D	0.194
9	1st relapse	KRAS	G13D	0.444
10	onset	IL7R	T244delinsADAISACD	0.309
13	onset	WHSC1	V1166G	0.156
13	onset	NRAS	G12D	0.212
13	1st relapse (testis)	PTPN11	E76V	0.359
18	relapse	WHSC1	T1150A	0.473
20	onset	THADA	V30M	0.455
20	onset	IL7R	L248-V258delinsPPYW	0.125
20	onset	KRAS	G12D	0.419
20	2nd relapse	THADA	V30M	0.529
20	2nd relapse	TUSC3	A13V	0.462
20	2nd relapse	KRAS	G12D	0.528
20	2nd relapse	TP53	R116Q	0.917
21	1st relapse	ERG	V127G	0.275
24	1st relapse	PTPN11	T507K	0.279
26	onset	KRAS	G13D	0.237

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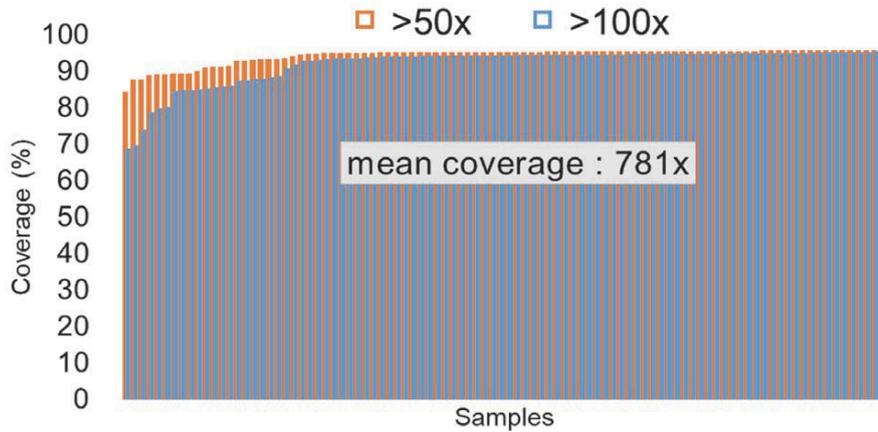
B. PCR based deep sequencing
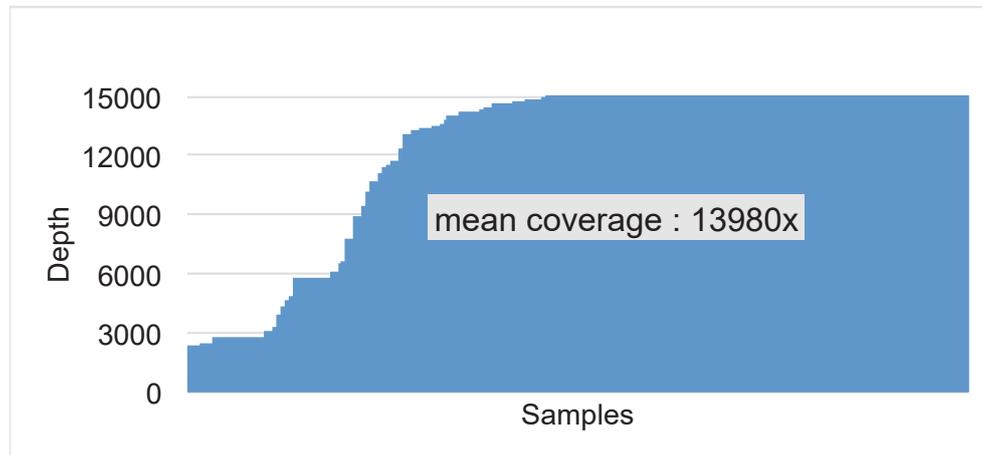
Figure 2 Mean coverage of targeted deep sequencing.
　（A）The mean coverage of deep sequencing of Haloplex analysis in total 96 samples. The coverage of coding regions is plotted for each sample.（B）The mean coverage of tailed PCR-based deep sequencing in the total 370 amplicons is shown.

Table 7. Primer sets for RT-PCR analysis of fusion genes

Gene	Oligo	Sequence (5'-3')
ARID1B-ZNF384	forward primer	AGCCTTATATGTCGTCCTCAGC
	reverse primer	TAGGGTCGATGCTACCTTCTTG
NUP214-ABL1	forward primer	GTTTGGCAGCCCTCCTACTTTT
	reverse primer	ACTGTTGACTGGCGTGATGTAGT

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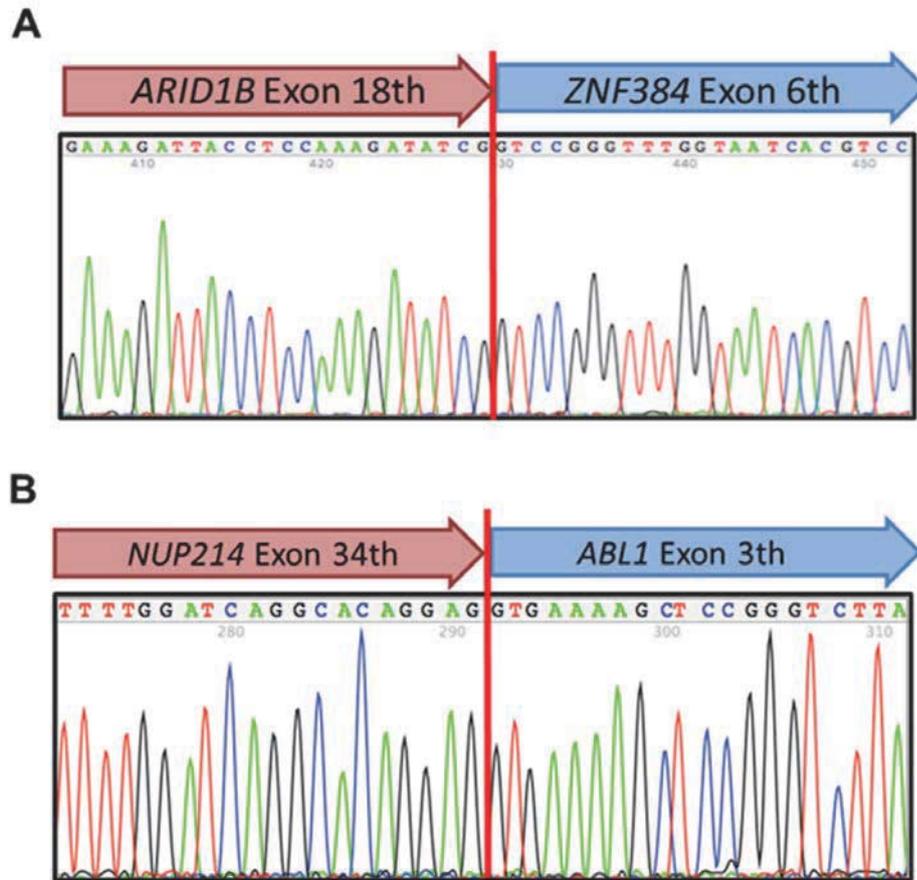
Figure 3   Rare subtypes of BCP-ALL with *ARID1B-ZNF384* and *NUP214-ABL1* fusion.
Sequencing analysis of RT-PCR products of *ARID1B-ZNF384*（A）and *NUP214-ABL1* fusions（B）

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