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Research paper

Prognostic gene alterations and clonal changes in childhood B-ALL

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ABSTRACT

Genomic profiles of leukemia patients lead to characterization of variations that provide the molecular classification of risk groups, prediction of clinical outcome and therapeutic decisions. In this study, we examined the diagnostic (n = 77) and relapsed (n = 31) pediatric B-cell acute lymphoblastic leukemia (B-ALL) samples for the most common leukemia-associated gene variations *CRLF2, JAK2, PAX5* and *IL7R* using deep sequencing and copy number alterations (CNAs) *(CDKN2A/2B, PAX5*, *RB1, BTG1, ETV6, CSF2RA, IL3RA* and *CRLF2)* by multiplex ligation proximity assay (MLPA), and evaluated for the clonal changes through relapse. Single nucleotide variations SNVs were detected in 19% of diagnostic 15.3% of relapse samples. The CNAs were detected in 55% of diagnosed patients; most common affected genes were *CDKN2A/2B*, *PAX5,* and *CRLF2*. Relapse samples did not accumulate a greater number of CNAs or SNVs than the cohort of diagnostic samples, but the clonal dynamics showed the accumulation/disappearance of specific gene variations explained the course of relapse. The *CDKN2A/2B* were most frequently altered in relapse samples and 32% of relapse samples carried at least one CNA. Moreover, *CDKN2A/2B* alterations and/or *JAK2* variations were associated with decreased relapse-free survival. On the other hand, *CRLF2* copy number alterations predicted a better survival rate in B-ALL.

These findings contribute to the knowledge of *CDKN2A/2B* and *CRLF2* alterations and their prognostic value in B-ALL. The integration of genomic data in clinical practice will enable better stratification of ALL patients and allow deeper understanding of the nature of relapse.

1. Introduction

B-cell acute lymphoblastic leukemia (B-ALL), the most common cancer in children and young adults, is a genetically heterogeneous disease that originates from the malignant transformation of B cell progenitors [1,2]. Five-year survival rate exceeded 90% in children in

Abbreviations: B-ALL, B-cell acute lymphoblastic leukemia; CNAs, copy number alterations; MLPA, multiplex ligation proximity assay; SNVs, single nucleotide variations; iAMP21, intra-chromosomal amplification of chromosome 21; MRD, minimal residual disease; NGS, next-generation sequencing; BM, bone marrow; FAB, French–American–British; BFM-ALL, Berlin–Frankfurt–Munster-ALL; IRON, Interlaboratory RObustness of Next generation sequencing Consortium; OS, overall survival; RFS, relapse-free survival; WBC, white blood cell; Hb, hemoglobin; Plt, platelet; LAP, lymphadenopathy; CNS, central nervous system; MAF, minor allele frequency

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high-income countries, whereas in Turkey, five-year overall survival rate remained 73% in childhood ALL. Moreover, survival following a relapse in childhood ALL is still poor [3,4].

Chromosomal abnormalities and genetic alterations are the hallmark of B-ALL, several of which have demonstrated to be important prognostic factors including *MLL-AF4B, TEL-AML1, E2A-PBX1* and *BCR-ABL* [2,5]. Moorman et al. integrated the copy number alteration (CNA) data into existing cytogenetic classification of B-ALL; patients with any deletion of *IKZF1*, *PAR1*, *EBF1* or *RB1* classified in poor risk group, patients with isolated deletions of *ETV6*, *PAX5*, *BTG1* or patients who did not carry deletion of *IKZF1*, *CDKN2A/2B*, *PAR1*, *BTG1*, *EBF1*, *PAX5*, *ETV6*, *RB1* were subgrouped in good risk [6]. Recent advances in next-generation sequencing techniques allow for more comprehensive approaches to profile genomic variations in B-ALL and several genetic variations (*CRLF2, JAK2, IKZF1, TP53* etc.) have been implicated in current risk classification of ALL and also used monitoring minimal residual disease (MRD) [1,7–11]. Patients harboring hypodiploidy (< 44 chromosome), intra-chromosomal amplification of chromosome 21 (iAMP21), t(9;22)(q34;q11.2) or *CRLF2* overexpression have poor response when treated with standard chemotherapy [2].

Although successful treatment protocols, nearly 30% of the ALL cases present poor prognosis due to early relapse. Limited prognostic factors such as relapse time, the site of relapse, and immunophenotype are important in ALL relapse management [3,12,13]. At diagnosis, there is significant genetic heterogeneity with clonal evolution throughout disease progression. The relapse clone was found related to the diagnostic clone, with shared common CNAs in most childhood ALL cases. However, relapse clone may also acquire new CNAs or lose some of the CNAs, which present at diagnosis. The studies also displayed that the relapse clone was already present at diagnosis as a minor clone in most of ALL cases and after the elimination of the major clone at diagnosis by therapy favored the expansion of the remaining clones [14,15].

Integration of genomic data into ALL clinics together with the minimal residual disease is important [16]. Herein, we aimed to find out the prevalence of genetic alterations in B-ALL related genes, including CNAs and point variations in our cohort and to define their clinical correlation. Within this context, diagnosis and relapsed childhood B-ALL cases were examined for the hotspot mutation regions of the most common leukemia-associated genes, *JAK2, IL7R, PAX5,* and *CRLF2* by using amplicon-based next-generation sequencing (NGS). Moreover, CNAs were evaluated for the gain and loss of mutations by multiplex ligation-dependent probe amplification (MLPA) analysis to characterize the clonal changes in B-ALL.

2. Materials and methods

2.1. Patient cohort

Childhood B-ALL diagnostic patients (n = 77) and 31 bone marrow (BM) relapse samples (n = 15 diagnosed–relapsed match samples) were included in the study. The patients were diagnosed and followed up at the Pediatric Hematology Departments of Cerrahpasa Medical Faculty, Istanbul Medical Faculty, Kocaeli Medical Faculty, and Istanbul Sisli Hamidiye Etfal Education and Research Hospital. Clinical characteristics of the diagnostic and relapsed patients are shown in supplemental Table 1. The patients were diagnosed according to FAB (French–American–British) classification criteria and treated with the BFM (Berlin–Frankfurt–Munster)-ALL or COG protocols. The patients were treated uniformly according to the ALL-REZ BFM 2002 relapse protocol after first BM relapses. BM samples were obtained from the patients with a minimum tumor load of 90% at diagnosis and > 50% at relapse were included in the study. *MLL-AF4B (n = 3), TEL-AML1 (n = 3)* and *BCR-ABL (n = 3)* fusion genes were identified in the diagnostic patient cohort. All samples were obtained with a protocol approved by the Ethical Board of Istanbul University. Informed consent was provided for each patient/parent.

2.2. Deep sequencing and data analysis

Total nucleated BM cells were isolated from aspirate samples after Ficoll-Paque separation [17]. Total RNA was isolated using RNeasy mini kit (Qiagen, Germany) and total DNA was isolated using QIAamp DNA mini kit (Qiagen, Germany).

Hotspot mutation domains of *CRLF2* (exon 6), *IL7R* (exon 6), *JAK2* (exons 12–16) and *PAX5* (exons 2–3) genes were amplified using FastStart High Fidelity PCR System (Roche Applied Science, Penzberg, Germany). In total, 19 primer pairs including a 10-base molecular identifier barcode sequence (MID) were designed and validated by IRON (Interlaboratory RObustness of Next generation sequencing) Consortium. The amplicons (size range 304–431 bp) were purified Ampure XP beads (Beckman Coulter, Krefeld, Germany) and libraries were quantified by Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA). Ninety-six well plates were designed for the panels that allowed us to multiplex patients per sequencing lane on GS Junior instrument (Roche Applied Science, Germany). Amplicon library preparation protocols; pooling, purification, emulsion PCR and sequencing which were designed by the IRON-II study group were followed. Bidirectional Sanger sequencing was performed for validation of pathogenic variants by using the same primers that were used in the amplicon sequencing. We performed a data quality assessment using default amplicon pipeline settings of the GS FLX Sequencer Instrument software version 2.3 (Roche Applied Science, Germany). Then the amplicons were aligned to the reference sequencing and searched for variations by using GS Amplicon Variant Analyzer software version 2.5.3 (Roche Applied Science, Germany). In-silico tools, Mutation Taster, PolyPhen and SIFT were used to interpret the clinical association and functional predictions of the variants [18–20].

2.3. Multiplex ligation proximity assay (MLPA) analysis

CNAs in *PAX5, RB1, CDKN2A, CDKN2B, BTG1, EBF1, ETV6* and Xp22.33 region (PAR region; *SHOX* area, *CRLF2, CSF2RA, IL3RA* and *P2RY8* genes) with 44 different probes were analyzed by MLPA using the ALL-*IKZF1* probe set P335 from MRC-Holland (Amsterdam, the Netherlands) according to the manufacturer's instructions. The *P2RY8-CRLF2* fusion identified through assessment of *CSFRA* and *IL3RA* deletions. MLPA amplification products were separated by capillary electrophoresis using an ABI 3730 XL DNA Analyzer and used 500 LIZ as internal size standard (both Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). Data were normalized using the Coffalyser.NET software provided by MRC-Holland (Amsterdam, the Netherlands). Ten DNA samples from healthy blood donors were used as reference DNA.

2.4. Statistical analysis

Proportional differences between the groups were analyzed by chi-square ($\chi 2$) or Fisher exact tests. Overall survival (OS) was defined by the interval from the date of diagnosis to the date of death or last follow-up. Relapse-free survival (RFS) was the duration from the date of complete remission to last follow up or to the first event (failure to achieve remission (early death or resistant leukemia), relapse or death in complete remission). The Kaplan–Meier method and COX regression analysis were used to estimate survival rates. $P \leq 0.05$ (two-sided) was considered statistically significant. Differences were compared with the two-sided log rank test. All statistical analyses were done by SPSS for windows 21.0 (IBM SPSS Data Editor Inc., Chicago, IL, USA).

Table 1
Genetic variations in B-ALL.

Time point	Sample ID	Gene	Position	AA Change	Variation type	Variant frequency (%)	Read deep	Database	Mutation taster	Polyphen	SIFT
Diagnose	P#1	*PAX5*	NM_016734.1:c.68 T > G	L23R	Missense	91	2362	COSM5986425	DC	PD	D
Diagnose	P#47	*PAX5*	NM_016734.1:c.68 T > G	L23R	Missense	3	2911	COSM5986425	DC	PD	D
			NM_016734.1:c.70 G > T	G24W	Missense	3	2911	COSM303871	DC	PD	D
Diagnose	P#81	*PAX5*	NM_016734.1:c.97C > T	L33F	Missense	84	2188	NOVEL	DC	PD	D
Diagnose	P#71	*PAX5*	NM_016734.1:c.97C > T	L33F	Missense	3	1630	NOVEL	DC	PD	D
Diagnose	P#12	*PAX5*	NM_016734.1:c.317A > G	N106S	Missense	5	1843	NOVEL	DC	PD	D
Diagnose	P#81	*JAK2*	NM_004972.3:c.2047A > G	R683G	Missense	35	1277	rs1057519721	DC	PD	D
			NM_004972.3:c.2049A > T	R683S	Missense	6	1277	COSM29302	DC	PD	D
Diagnose	P#113	*JAK2*	NM_004972.3:c.2047A > G	R683G	Missense	3	1146	rs1057519721	DC	PD	D
Diagnose	P#104	*JAK2*	NM_004972.3:c.1542 G > A	T514T	Splice site*	43	714	COSM1109352 (rs565502628)	DC	–	–
Relapse of P#104	P#105	*JAK2*	NM_004972.3:c.1542 G > A	T514T	Splice site*	41	1541	COSM1109352 (rs565502628)	DC	–	–
Diagnose	P#74	*JAK2*	NG_009904.1:g.89837 T > C	–	Intronic	98	432	NOVEL	–	–	–
Diagnose	P#116	*CRLF2*	NG_034237.1:g.20998 G > A	–	Intronic	41	304	NOVEL	–	–	–
Diagnose	P#3	*IL7R*	NG_009567.1:g.22664 T > C	–	Intronic	50	1657	rs202114203	–	–	–
Diagnose	P#57	*IL7R*	NG_009567.1:g.22664 T > C	–	Intronic	50	906	rs202114203	–	–	–
Relapse of P#55	P#101	*IL7R*	NG_009567.1:g.22664 T > C	–	Intronic	8	964	rs202114203	–	–	–
Diagnose	P#69	*PAX5*	NG_033894.1:g.18643C > T	–	Intronic	26	2070	rs377355229	–	–	–

Abbreviations: *HSF (Human Splicing Finder) potential alteration of splicing, DC; disease causing, PD; probably damaging, D; damaging, B; benign T; tolerated; AA, amino acid.

3. Results

3.1. Somatic variations at diagnostic and relapse B-ALL samples

The samples were screened for hot spot regions of *PAX5, JAK2*, *CRLF2* and *IL7R* by amplicon deep sequencing. Minimum sequencing depth was set to 500X, ensuring detection of sub clones. In total 76 samples (n = 63 diagnose, n = 13 relapse B-ALL) passed the quality control and filtering steps for the variant analysis and 19.2% of the patients (19% of diagnose, 15.3% of relapse samples) showed at least one variation for the selected amplicons. The variant allele frequency ranged from 3% to 98% and all the variants that may contribute to disease pathology are listed in Table 1 and benign variants were shown in Supplement Table 2.

PAX5 (n = 6 diagnose) and *JAK2* (n = 4 diagnose, n = 1 relapse) were the most frequently mutated genes in our cohort. Three of the *PAX5* mutant carriers developed relapse and two of them died after relapse. P#1 had low-level p.L23R and P#47 carried low-level compound p.L23R and p.G24W mutations at diagnoses in *PAX5*. Both patients had early relapse however, mutations vanished in the matched relapsed sample of P#47. Moreover, novel *PAX5* variant p.L33 F was detected in two patients and p.N106S was detected in P#12 which were classified as disease-causing according to *in silico* analysis. As a novel variant p.L33F constituted a major clone in P#81 while it was detected at a low level in P#71. A total of 2 of the 4 *JAK2* mutation positive patients carried p.R683S/G, 1 patient showed splice site variation p.T514T both in diagnosis (P#104) and relapse (P#105). Interestingly, P#81 acquired p.R683G pathogenic variant as a dominant clone and R683S as a minor clone who also carried the p.L33F variant in *PAX5* (Table 1). Both of these patients (P#81 and P#104) developed early relapse and P#104 died after relapse.

Beside the known alterations, P#74 acquired a novel intronic variant (NG_009904.1:g.89837T > C) in *JAK2* and P#116 carried an intronic variant for *CRLF2* (NG_034237.1:g.20998 G > A) (Table 1). *IL7R* was present in 3.1% of diagnosed, 7.6% of relapsed; *CRLF2* was present in 1.5% of diagnosed patients and none in the relapsed cohort. Diagnostic samples of P#3 and P#57 and relapse sample P#101 showed the same rare intronic variant for *IL7R* (rs202114203). In addition to the pathogenic variants, previously identified benign/likely benign variants in *PAX5, CRLF2* and *IL7R* genes were also detected in our cohort (Supplement Table 2).

3.2. Frequency of copy number alterations (CNAs) in B-ALL

To determine CNAs in childhood B-ALL, MLPA analysis was performed. In total 91 (60 diagnosed, 31 relapsed) samples achieved quality criteria for MLPA analysis and the results are summarized in Fig. 1. Overall, 55% (33 in 60) of diagnosed patients and 32% (10 in 31) of relapse samples carry at least one CNA. *CDKN2A/2B* alterations were the most common variants observed in both diagnosed (27%) and relapsed (19%) cases. Only two samples had heterozygous *CDKN2B* deletion without *CDKN2A* alteration, the rest of the patients carried dual *CDKN2A/B* gain or deletions. At diagnosis, the other common genetic alterations were *PAX5* (20%) and *ETV6* (13%) alterations, duplication of *CRLF2* (18%), *CSF2RA and/ or IL3RA* (12%). Deletions of *RB1* and *BTG1, P2RY8-CRLF2* fusion were detected in less than 10% at diagnosis

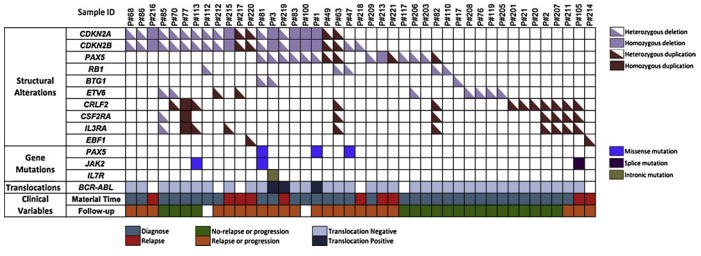

Fig. 1. CNAs in B-ALL. Heat-map representation of individual CNAs and variations in B-ALL cohort.

and none of the diagnosed patients harbored *EBF1* copy number changed. Furthermore, CNAs were observed in 10 samples at relapse: *PAX5* (n = 3), *EBF1* (n = 2), *ETV6* (n = 1), *CRLF2* (n = 1) and *CSF2RA* (n = 2), *IL3RA* (n = 2) (Fig. 1).

Two patients (P#1 and P#81) who carried homozygous *PAX5* mutations also had a heterozygous deletion of all exons of *PAX5*. The patients (P#12 and P#71), who presented with minority sub clones with *PAX5* mutations, had no CNAs in the MLPA analysis.

3.3. Clonal changes in the diagnose and relapsed B-ALL

In our paired diagnosed-relapse cohort; 11 patients developed early relapse and 1 patient had late relapse, and 84% (10 patients) of relapse patients died. To observe the clonal changes, we evaluated these 12 matched diagnose-relapse samples and showed relapse dynamics. Six of the cases showed CNVs and/or SNVs in their mutation repertoire.

In all paired samples common CNVs or SNVs were observed between samples indicating a clonal origin. Amplicon deep sequencing permits sensitive detection of minority clones and an identified clonal relationship for the selected amplicon. However, we cannot discriminate heterozygous clones at single cell resolution by these techniques. The single or multiple CNVs/ SNVs might be presented in the same cell or different cells.

A total of four of the matched samples developed different leukemia clones. Three patients (P#47, P#49, P#63) acquired CNAs at diagnosis, which had vanished in matched relapse samples. In addition, P#47 also demonstrated low-level p.L23R and p.G24W mutations in *PAX5* and in parallel to CNAs profile; these variants were lost at relapse time. Another patient, P#209 had heterozygous focal *PAX5* deletion Δe6-8 at the time of diagnosis and then he gained homozygous deletion of *PAX5* Δe2 at relapse. P#55 gained rs202114103 as a minor clone at relapse (Fig. 2).

P#104 showed a clear clonal relationship with diagnose and relapse time. She had p.T514T mutation in *JAK2* at the presenting diagnostic clone and acquired additional *CRLF2*, *CSF2RA and IL3RA* at relapsed time (Fig. 2). Similarly, P#218 had heterozygous deletions in the *CDKN2B* gene at first relapse, whereas in the second relapse (P#219), the deletion region was extended (*CDKN2A-2B-4A)* and had *PAX5* deletion, which is indicating evolution from a diagnosis clone (Fig. 2). On the other hand, six of 12 patients did not show any CNAs for the respective gene panel both in diagnose and relapse.

3.4. Association of genetic alterations with clinical characteristics and outcome

We examined the associations with identified variations and the clinical features such as age (0–2, 2–10 and > 10 years), gender (male and female), immune phenotype (pro, pre, common ALL), risk groups (high, medium, standard), white blood cell (WBC) count at diagnosis (> 50,000 vs. < 50,000), platelet (Plt) count at diagnosis (< 20,000, 20–100,000 and > 100,000), Hemoglobin levels (Hb) (> 10 dL vs. < 10 dL), lymphadenopathy (LAP) (yes vs. no), central nervous system involvement (CNS) (yes vs no), other organ involvement (yes vs. no), of pediatric B-ALL patients by using chi-square (Fisher's exact) test.

CDKN2A/2B variations were most commonly detected in patients who developed relapse ($p = 0.04$), whereas *CRLF2* copy number alterations were mostly seen in the non-relapsed group ($p = 0.05$) and less were observed in the deceased subgroup ($p = 0.02$). Higher induction failure was seen with *PAX5* alterations ($p = 0.03$). Higher WBC counts were seen with *CDKN2A/2B* ($p = 0.03$), and *BTG1* ($p = 0.04$) alterations. *CRLF2* and *PAX5* alterations were found more frequently in patients with high Hb levels ($p = 0.05$, $p = 0.05$ by Fisher's exact test) moreover, *PAX5* gene variations also showed significant association with high Plt count ($p = 0.03$) and LAP ($p = 0.03$). All the identified associations are given in Supplement Table 3.

Median OS was 40 months (min 1-max 204 mounts) and median RFS was 31 months (0 min -max 145 months) among the patient cohort. According to the Kaplan-Meier analysis, patients carrying *CDNK2A/2B* alterations had a reduced OS (p = 0.053) and RFS ($p = 0.045$). Furthermore, *CDNK2A/2B* alteration and/or *JAK2* mutation carriers had worse OS ($p = 0.028$) and RFS ($p = 0.02$) rates than wild type cases. On the other hand, patients who had *CRLF2* copy number gain showed longer survival rates (for OS, $p = 0.043$ and for RFS, $p = 0.03$) (Fig. 3). The probabilities of OS and RFS did not differ with regard to single gene *PAX5*, *BTG1*, *RB1*, *ETV6*, *CSF2RA*, *IL3RA*, *EBF1* alterations, *ETV6* and/or one gene alteration combination. No notable differences were observed in the OS and RFS analysis of B-ALL patients with ≥3 gene deletions compared with the B-ALL patients who harbored less than 3 CNAs/no alterations. We further separated the patients into three subgroups based on the MLPA data: (i) patients with an alteration

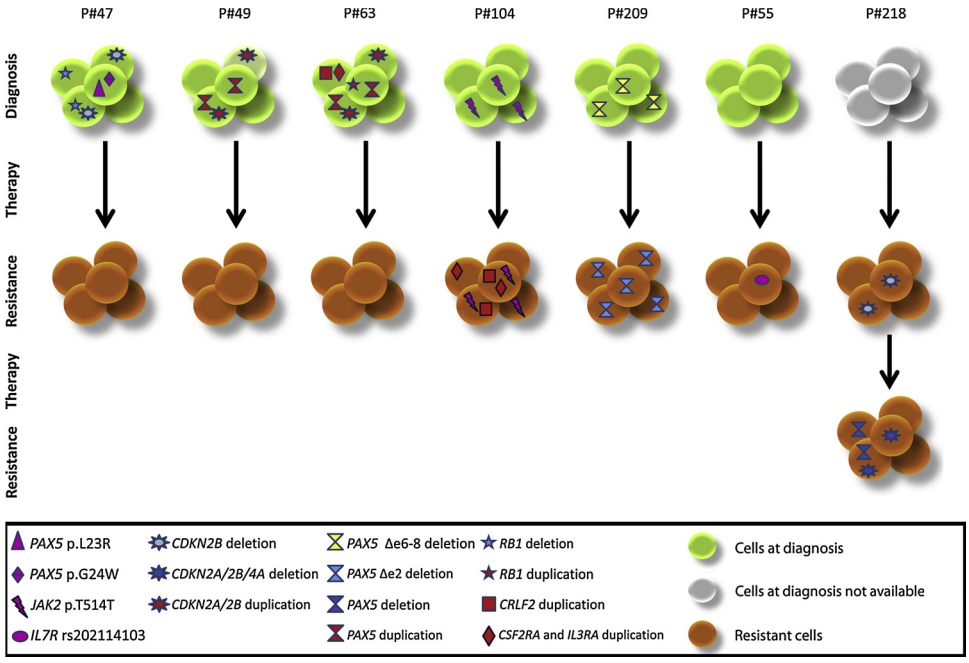

Fig. 2. Clonal changes in matched diagnose-relapsed B-ALL. Green circles represent cells at diagnosis and orange circles cells at relapse and circles with depicted alterations are not reflecting clonal ratio but represent probable clonal heterogeneity. Each cell circle represents probable clonal distribution of mutations and circle positioning was not intended to show same lineage consistency. Heterozygous CNAs detected with MLPA cannot distinguish clonal origins of mutations; so depicted cells are not necessarily indicating separate clones at diagnosis (#P47, #P49 and #P63). #P47 had low level compound *PAX5* mutations.

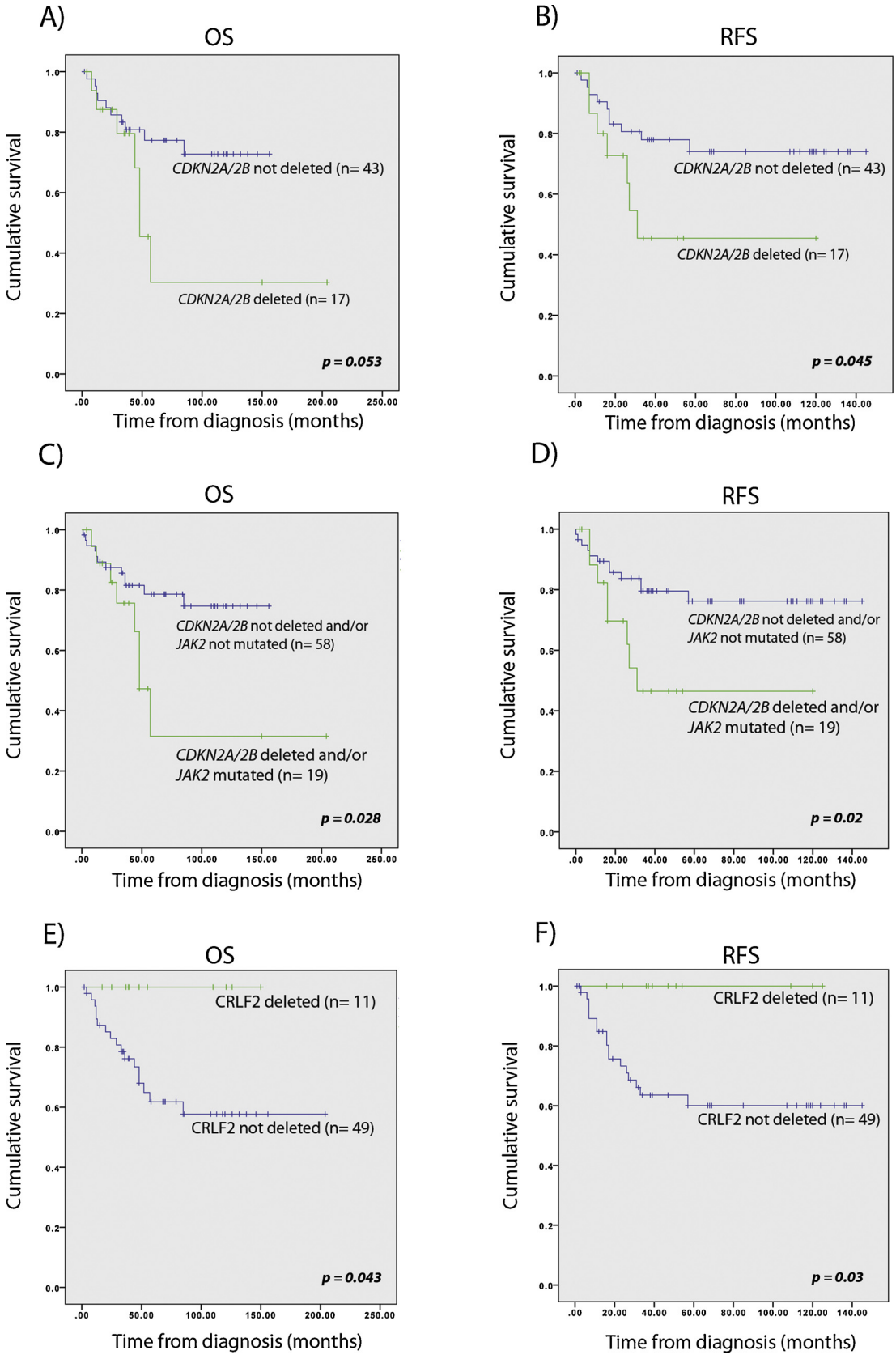

Fig. 3. (A): Kaplan-Meier analysis of *CDKN2A/2B* alterations and OS in childhood B-ALL. (B): Kaplan-Meier analysis of *CDKN2A/2B* deletions and not deleted and RFS in childhood B-ALL. (C): Kaplan-Meier analysis of *CDKN2A/2B* deleted and/ or *JAK2* mutated and *CDKN2A/2B* not deleted and/or *JAK2* not mutated and OS in childhood B-ALL. (D): Kaplan-Meier analysis of *CDKN2A/2B* deleted and/ or *JAK2* mutated and *CDKN2A/2B* not deleted and/or *JAK2* not mutated and RFS in childhood B-ALL. (E): Kaplan-Meier analysis of *CRFL2* not deleted and *CRLF2* deleted and OS in childhood B-ALL. (F): Kaplan-Meier analysis of *CRFL2* not deleted and *CRLF2* deleted and RFS in childhood B-ALL.

of both loci (*CDKN2A/2B* and *PAX5* CNV/SNV), (ii) patients with a *CDKN2A/2B* alteration only and (iii) patients with a *PAX5* CNA/SNV only. The EFS and OS did not significantly differ among subgroups ($p = 0.09$ and $p = 0.182$).

Association between the relapse risk, the therapy response (day8, day15 and day33) and genetic variations were also examined. Cox regression analysis did not identify an association between the mutation status and relapse or treatment response. Due to low frequency of the SNVs, clinical analysis was only performed for *PAX5* and higher WBC counts were found correlated with SNVs of *PAX5* ($p = 0.03$) but not with other clinical parameters or follow-up.

4. Discussion

B-ALL is characterized by genetic alterations that block differentiation, promotes proliferation of lymphoid precursor cells, and are important for risk stratification. Herein, we described the molecular heterogeneity of the patients with adverse prognosis in a group of pediatric B-ALL cases and mutation analysis showed the clonal change in the 12 matched diagnostic-relapsed samples. We have targeted the most frequently mutated exons of "B-ALL-related genes" (*CRLF2, JAK2, PAX5* and *IL7R*) and also analyzed CNAs for *CDKN2A/2B, PAX5*, *RB1, BTG1, ETV6, CSF2RA, IL3RA* and *CRLF2* genes in pediatric B-ALL samples.

NGS enabled identification of both low-level variants and co-occurrence of mutations. Herein, we have demonstrated the existence of low-level variants in B-ALL samples for the respective gene panel both in coding and non-coding regions. Nineteen percent of the diagnostic B-ALL cases were found to carry at least one SNV for the amplified gene regions, *PAX5* (9.5%) and *JAK2* (6.3%) were the most frequently mutated genes in our cohort. In addition to the SNVs, CNAs of *PAX5* were also seen in 20% of the diagnostic samples. On the other hand, *PAX5* alterations were detected less often at relapse time than diagnosed patients. Earlier studies demonstrated higher *PAX5* expression in B-ALL, and loss-of-function mutations either in CNVs, SNVs or fusion partner in translocations occurring in one-third of B-ALL [21–23]. Exon 2 and 3 are the hot spot regions of the *PAX5* gene and coding the "paired domain" part of the protein. Paired domain mutations of *PAX5,* also heterozygous mutations depending on haploinsufficiency, lead to impaired or absent DNA-binding activity, which causes reduced transcriptional activity [24]. In this study, we also identified two novel *PAX5* paired domain mutations p.L33 F and p.N106S in pediatric B-ALL patients, which were predicted as disease causing by *in-silico* tools. Moreover, homozygous missense mutations were accompanying heterozygous deletions of *PAX5* in two patients. However, with the exception of higher WBC counts, we did not observe any correlation between SNVs of *PAX5* and clinical findings.

Pseudokinase domain of *JAK2,* coded by 12–16 exons, was commonly found mutated in several hematologic malignancies [25,26]. Most of the observed *JAK2* mutations are thought to result in enhanced *JAK2* kinase activity. *JAK2* kinase activity was associated with a higher risk of relapse and mutations in *JAK2* were predicted as independent prognostic biomarkers in B-ALL [26–28]. We examined *JAK2* exons (exon 12–16) by amplicon sequencing and analyses revealed that 6% of the diagnosed B-ALL cases harbored *JAK2* mutations. Mutations involved amino acid residue R683 in two of four mutated cases, which is an important amino acid for the JH2 domain mediated negative autoregulation of *JAK2* activity [29]. The acquired *JAK2* mutation (p.R683S and p.R683G) is presumed to be a biomarker for B-ALL [26]. In our study, one patient consisted of two independent clones, major clone harbored p.R683 G and a minor clone carried p.R683S variant. The same clonal heterogeneity was detected in two patients in a previous study although the biological relevance of clonal heterogeneity at R683 residue is not clear [27]. Beside the missense mutations, a splice site mutation p.T514 T was determined in both the patients' diagnosed and relapsed samples, and a novel intronic variant (NG_009904.1:g.89837 T > C) was detected in another case.

IL7R and *CRLF2* genes SNVs were rarely observed in our cohort in concordance with previous studies. Overall, the incidence and frequency of SNVs detected in our study were similar to observations made in previous studies [8,27,30–32]. Furthermore, relapse samples mutation frequency for the respected gene panel was relatively low compare to the diagnose samples. Due to the low frequency of SNVs in the individual gene level, we could not perform further clinical statistics. However, mutational screening of B-ALL patients would be important for the targeted therapy selection in coming protocols.

CNAs were detected more frequently (53%) than the SNVs in our pediatric cohort for the selected genes. Herein, the most common CNAs were found in *CDKN2A/2B, PAX5* and *CRLF2* genes in parallel to previous studies [33,34]. *CDKN2A/2B* are important regulators of cell growth regulation and apoptosis, and are also well-known for secondary acquired deletion in childhood ALL. The deletion of both *CDKN2A* and *CDKN2B* leads to uncontrolled cell cycle progression. The frequency of *CDKN2A/2B* from the reports range from 11 to 41% in B-ALL with controversial relevance to disease outcome due to the heterogeneity of 9p21 alterations [35–39]. In this study, *CDKN2A/2B* alterations were found in 27% of diagnosed and 19% of relapsed B-ALL, which is in concordance with previous reports [33,34,40,41]. The patients who had a relapse feature acquired *CDKN2A/2B* alterations more often than the patients who did not develop relapse. Moreover, the CNAs of *CDKN2A/2B* were associated with shorter RFS rates and higher WBC counts in our cohort. Several studies found that deletion of *CDKN2A/2B* associated with inferior event-free and RFS [38,42]. Herein, not only *CDKN2A/2B* deletions were found correlated with reduced survival, but a copy number amplification of *CDKN2A/2B* were also detected in four patients; two of them were diagnosed patients who developed relapse in their follow-up, and the other two were relapsed patients. The majority of the patients with *CDKN2A/2B* alterations also harbored other CNVs and SNVs, which may indicate chromosomal instability. When we assessed *CDKN2A/2B* copy number alterations together with SNVs profile, *CDKN2A/2B* and/or *JAK2* mutations were found significantly correlated with decreased OS and RFS.

PAX5 alterations were the second common CNAs among the cases (20% of diagnosed patients) and most patients with *PAX5* alterations (68.3%, 7/12 in diagnosed B-ALL cohort) had *CDKN2A/2B* alterations. In contrast to *CDKN2A/2B* alterations, *PAX5* alterations were not found associated with the outcome although *PAX5* alterations were significantly correlated with higher induction therapy failure. *PAX5* is an important determinant of B-lineage commitment and deletion of *PAX5* leads to incomplete B-cell development. Accompanying alterations of *CDKN2A/2B* and *PAX5* may contribute to leukemogenesis however; *CDKN2A/2B* together with *PAX5* alterations did not observe significant differences for OS and RFS.

Cytokine receptors are involved in the pathogenesis of B-ALL. *CRLF2* copy number amplifications were detected in 18% of our diagnosed cases. Moreover, B-ALL patients with *CRLF2* copy number alterations showed significantly longer OS and increased RFS compared to non-altered patients. We detected a higher frequency of *CRLF2* gain in the non-recurrence B-ALL subgroup. Previous studies did not report directly a significant correlation between *CRLF2* copy number gain and outcome. However, in some studies *CRLF2* expression is associated with activating the JAK-STAT pathway and poor outcome in ALL. Most particularly, deregulated expression of *CRLF2* is observed in 27–50% of BCR-ABL1-like B-ALL patients [32,43]. On the other hand, van der Veer et al. reported that a BCR-ABL1-like gene expression signature and *IKZF1* deletion were independent adverse prognostic factors for RFS in childhood B-ALL. They also found that *CRLF2* overexpression was not an adverse prognostic factor for RFS [44]. Similar to this Dutch study, in terms of the prognostic impact of *CRLF2* overexpression, Yano et al. did not find a significant effect in the Japanese group [45]. Deregulated *CRLF2* expression may be caused by the *P2RY8/CRLF2* fusion, *IGH-CRLF2* translocations, extra X/Y chromosomes and/or amplification of the *CRLF2* locus, mutations of other kinase genes such as *JAK2*, whereas

the reason for *CRLF2* high expression is still unknown for a proportion of cases [43,46–48]. In this study, we only evaluated the patients for CNVs/SNVs of *CLRF2* and therefore, we cannot make any assessment for *CRLF2* expression.

The pseudo autosomal region, PAR1, deletion was reported as a poor prognostic factor in pediatric B-ALL. *P2RY8-CRLF2* fusion occurs in 1–8% of childhood B-ALL and often carries additional alterations in JAK/STAT and RAS pathway genes [40,45,46]. Due to low patient numbers, we were unable to ascertain any impact of *P2RY8-CRLF2* on the prognosis of these patients. Only one patient had this fusion in addition to *CDKN2A/2B* deletion and did not develop treatment resistance during the three year follow-up. Out of 11 diagnosed patients with *CLRF2* copy number amplification; seven cases had the gain of *CSF2RA* and *IL3RA*. Duplication of PAR1 containing *CRLF2*, or increased copy number of the *CRLF2* locus alone was observed both in high and low *CRLF2* expressing ALL subgroups in previous studies [46,47]. Moreover, ALL cases with *CRLF2* high expression associated with increased *CRLF2* gene copy were characterized by rare additional deletions and hyperdiploid karyotype, which is associated with a good therapy response [47]. In concordance with these findings, *CDKN2A/2B* and/or *PAX5* and/or *RB1* alterations were identified in 45% of *CRFL2* copy number positive cases and *CRLF2* locus amplification was found associated with longer survival rates. However, more detailed functional analyzes of *CRLF2* copy number gain are needed to understand further implications.

Our cohort of relapsed B-ALL samples did not exhibit a greater number of CNAs or SNVs than the cohort of diagnostic samples. Thirty-one relapsed samples were included in the study and 32% of samples harbored CNVs and rarely carried SNVs. *CDKN2A/2B* alterations followed by *PAX5* alterations were the most frequent CNVs. Paired diagnostic and first relapse samples from 15 patients were available for SNVs analysis, and 12 patients for CNVs analysis in our cohort. ALL have clonal diversity, and this diversity evolves over time. Mullighan et al. determined that 34% of relapse clone evolved from the diagnostic clone and 52% of relapse clones shared some, but not all CNAs identified in the diagnostic clone. In parallel to these findings, three patients had *CDKN2A/2B*, *PAX5* and/or PAR1 alterations at diagnosis and were lost in relapse or became a minor clone that could not be detected by MLPA analysis. The other three patients gained additional alterations showing a clonal relationship between diagnosed and relapsed clones. Only paired first and second relapse samples were available in one individual, and the patient also showed a clonal relationship at both relapse times. Ribera et al. observed increased homozygous deletion at adult relapsed B-ALL, however, we did not have this result in our childhood cohort [41].

Several genetic alterations were found together with diagnosed and relapsed B-ALL, suggesting that several pathways are concomitantly altered and so it is hard to evaluate their prognostic impact in a limited cohort. Based on our findings, *CDNK2A/2B* alterations and/or *JAK2* variations were associated with decreased progression-free survival and commonly found at relapse. These alterations might contribute to therapy resistance; however, *CDKN2A/2B* alterations did not always show clonal dominancy at relapse time. Moreover, the gain of *CRLF2* locus was found to be associated with longer RFS, and rarely observed in relapse samples. Larger cohorts will verify these results. The integration of CNA and cytogenetic risk groups in clinical practice will enable better stratification of ALL patients and allow deeper understanding of the nature of relapse.

Compliance with ethical standards

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

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YE, SF equally contributed to the study, performed the experiments, analyzed data, created figures, tables, and assisted in manuscript writing. SM, OT, SS performed the next-generation sequencing experiments and analyzed data. OHN, UO and MS designed the study. MS analyzed data, created tables and assisted in manuscript writing. SK, TTJ, ZK, EZ, NS, and ZYY contributed patient data.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2019.05.009.

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