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Authors

Singh, Jay
Benjamin, Mercilena
Pandey, Avanish
et al.

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Original Article

Prognostic relevance of surface expression of cytokine receptor-like factor 2 in pediatric B-lineage acute lymphoblastic leukemia

Jay Singh[1], Mercilena Benjamin[1], Avanish Kumar Pandey[1], Sarita Kumari[1], Mohammad Shadab Ali[2], Jayanth Kumar Palanichamy[3], Sameer Bakhshi[4], Amitabh Singh[5], Deepam Pushpam[4], Akash Kumar[4], Rachna Seth[6], Inder Singh[7], Pranay Tanwar[1], Amar Ranjan Singh[1], Gunjan Sharma[3], Mohit Arora[3], Anita Chopra[1]

[1]Laboratory Oncology, AIIMS, New Delhi, India; [2]Department of Pulmonary Medicine, AIIMS, New Delhi, India; [3]Department of Biochemistry, AIIMS, New Delhi, India; [4]Department of Medical Oncology, AIIMS, New Delhi, India; [5]Department of Pediatrics, Safdarjung Hospital, New Delhi, India; [6]Department of Pediatrics, AIIMS, New Delhi, India; [7]Department of Neurology, AIIMS, New Delhi, India

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Abstract: Overexpression of cytokine receptor-like factor 2 (CRLF2) resulting from its genomic rearrangement is the most frequent genetic alteration found in Philadelphia chromosome-like (Ph-like) B-cell acute lymphoblastic leukemia (B-ALL), a high-risk leukemia. Detection of CRLF2 expression by multiparameter flow cytometry has been proposed as a screening tool for the identification of Ph-like B-ALL. However, the prognostic relevance of flow cytometric expression of CRLF2 in pediatric B-ALL is not very clear. Additionally, its association with common copy number alterations (CNA) has not been studied in detail. Hence, in this study, we prospectively evaluated the flow cytometric expression of CRLF2 in 256 pediatric B-ALL patients and determined its association with molecular features such as common CNAs detected using Multiplex ligation-dependent probe amplification and mutations in *CRLF2*, *JAK2* and *IL7RA* genes. Further, its association with clinicopathological features including patient outcome was assessed. We found that 8.59% (22/256) pediatric B-ALL patients were CRLF2-positive at diagnosis. Among CNAs, CRLF2 positivity was associated with presence of *PAX5* alteration (P=0.041). *JAK2* and *IL-7R* mutations were found in 9% and 13.6% CRLF2-positive patients, respectively. *IGH::CRLF2* or *P2RY8::CRLF2* fusions were each found in 1/22 individuals. CRLF2-positive patients were found to have inferior overall (hazard ratio (HR) =4.39, P=0.006) and event free survival (HR=2.62, P=0.045), independent to other clinical features. Furthermore, concomitant CNA of *IKZF1* in CRLF2 positive patients was associated with a greater hazard for poor overall and event free survival, compared to patients without these alterations or presence of any one of them. Our findings demonstrate that the surface CRLF2 expression in association with *IKZF1* copy number alteration can be used to risk stratify pediatric B-ALL patients.

Keywords: Leukemia, CRLF2, pediatric malignancies, B-ALL, copy number alterations, prognosis

Introduction

Acute lymphoblastic leukemia (ALL) is the most frequent pediatric malignancy. The prognosis of this malignancy has improved remarkably, particularly in developed countries, with the emergence of risk-adapted therapy [1]. However, the outcomes remain poor in approximately 15-20% of patients due to frequent relapse of the disease [2-5]. Comprehensive genomic profiling of B-ALL, including gene expression signature, mutations, fusion transcripts and copy number alterations (CNA) have been widely utilized to segregate B-ALL into clinically relevant subtypes [6]. Notable amongst these is Philadelphia-like (Ph-like) B-ALL, which is characterised by overexpression of Cytokine Receptor-Like Factor 2 (CRLF2) in 50% of cases, in addition to other molecular genetic alterations [7-9]. It was first discovered by Russell et al. (2009) when they reported deregulated CRLF2 expression due to its cryptic rearrangement in B-ALL [10]. Soon afterwards, Mullighan et al. identified a recurrent deletion resulting in the aberrant activation of CRLF2 under the control of the promoter of the

G-protein purinergic receptor (P2RY8) gene in patients with B-ALL, and Down syndrome-associated ALL using SNP array technology [11].

Copy number alterations (CNAs) are common in B-ALL and are associated with clinical outcomes. Gain of chromosome Xq26, which includes the *CRLF2* gene and is found in approximately 15-20% of B-ALL cases [11, 12]. While some CNAs have been shown to be associated with poor prognosis, such as *CRLF2* gene, others may have no impact on prognosis or may even be associated with a favorable prognosis [11, 12]. Several studies have also suggested concomitant alterations in *CRLF2* and other genes to be prognostic values in B-ALL [13-15]. However, the precise relationship between CNAs in other genes and CRLF2 expression in B-ALL remains unclear, and further studies are needed to elucidate the underlying mechanisms. *CRLF2* gene encodes the thymic stromal lymphopoietin receptor (TSLPR) and resides within the pseudoautosomal region 1 (PAR1) of chromosomes Xp22.3 and Yp11.3. TSLPR forms a heterodimer with the interleukin 7 receptor alpha chain (IL-7R) after binding of the cytokine TSLP, which has crucial roles in regulating B-cell development [16]. Bound TSLP activates the JAK-STAT pathway in addition to the mTOR and MAPK signalling pathways [16].

In addition to its copy number alterations, gain of function mutations in *CRLF2*, *IL7RA*, and *JAK* genes have been observed in 54.54% of B-ALL cases with CRLF2 overexpression [15]. Furthermore, two major genomic rearrangements of CRLF2 have been reported in B-ALL. A recurrent and cryptic deletion in the PAR1 area juxtaposes the promoter of *P2RY8* gene with the coding region of *CRLF2*, sculpting the *P2RY8::CRLF2* fusion transcript, frequently occurring in children with B-ALL [17]. Non-high-risk B-ALL patients with *P2RY8::CRLF2* gene rearrangement exhibit escalated risk of relapse which further strengthens the argument that characterization of *CRLF2* rearrangements in leukemia holds great promise in deducing a patient's prognosis [18]. The second type of rearrangement places *CRLF2* near the immunoglobulin heavy chain locus (*IGH*) as a result of anomalous V-D-J recombination, often found in adolescents and young adults [1]. Both of these rearrangements increase the expression of the CRLF2 multi-fold via the activation of the JAK-STAT pathway that balances cell proliferation and apoptosis [19]. B-ALL patients with high CRLF2 expression frequently bear concomitant *IKZF1* gene deletions [20].

CRLF2 expression and alterations in B-ALL can be investigated by various methods including mRNA quantification by real-time PCR (RQ-PCR), fluorescent in situ hybridisation (FISH) for detecting rearrangements, and CRLF2 protein expression by flow cytometry (FCM). The prognostic relevance of the aberrancies in CRLF2 is not clear due to variations in detection methodologies, age groups, inclusion criteria, and treatment regimens used in different studies [14]. In this prospective study, we have assessed CRLF2 expression using multiparameter FCM and determined its association with clinical characteristics, CNAs, and outcomes in a cohort of 256 pediatric B-ALL patients.

Materials and methods

Study subjects

In this study, we recruited 256 newly diagnosed pediatric B-ALL patients from the Department of Medical Oncology, and Department of Pediatrics, AIIMS New Delhi and Department of Pediatrics, Safdarjung Hospital, India. B-ALL was diagnosed based on the clinical, morphological, cytochemical and immunophenotypic findings. Enrolled patients were looked for the presence of lymphoid blasts (≥20%) in the morphological and cytochemical examination. Further the patients were investigated for the B-lymphoid blast using CD marker (CD45, CD34, CD10, CD19, CD79a) in the immunophenotyping. The patients were also analysed for the presence of recurrent genetic translocations (*BCR::ABL1*, KMT2A rearrangements, *TCF3::PBX1*, and *ETV6::RUNX1*) and aneuploidies, using conventional cytogenetics, multiplex RT-PCR, and/or fluorescent in situ hybridization (FISH). The study received ethical approval from the institutional ethics committee, and prior to participation, informed consent was obtained from the caregivers of all participants, as well as assent from all participants aged 8 years or older in accordance with the Declaration of Helsinki. The workflow of the study is depicted in **Figure 1**.

Detection of surface CRLF2 expression by multiparameter flow cytometry

Immunophenotyping was performed on the BM/PB samples obtained from the patients at

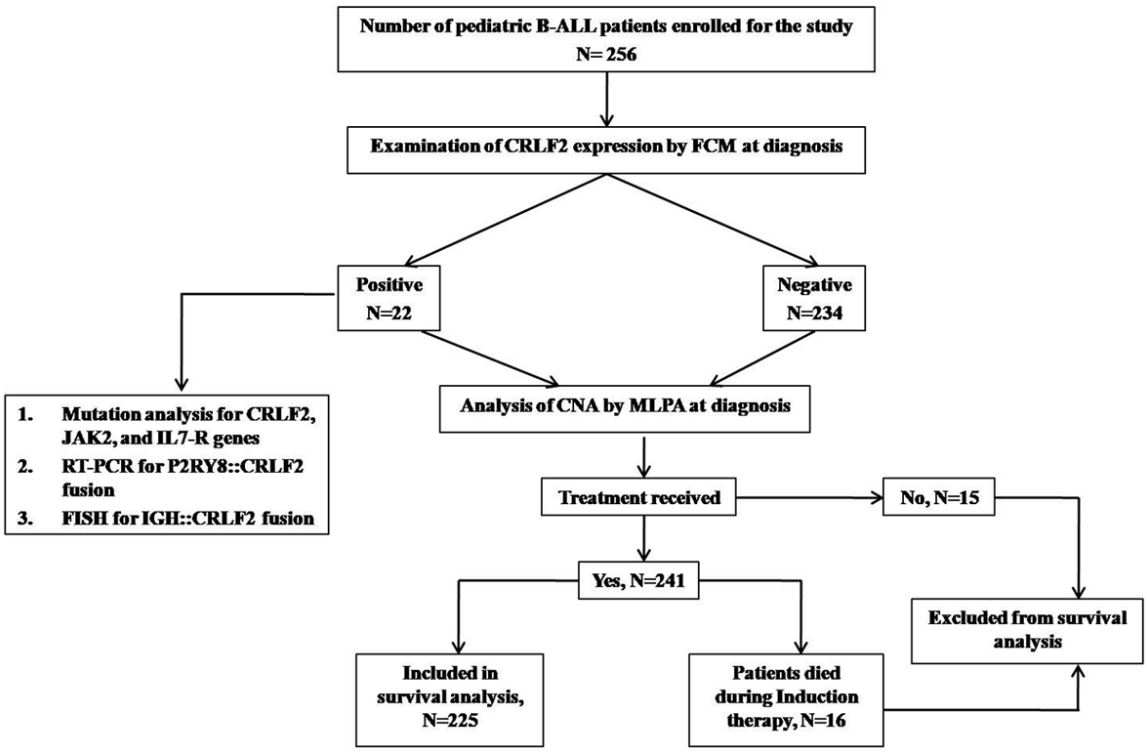


Figure 1. Flowchart showing the overall strategy of the study conducted in pediatic B-ALL patients.

the time of diagnosis, in vials containing EDTA. For FCM analysis, at least 1 million events were acquired in all the cases. Multiparameter FCM (Gallios, Beckman Coulter, USA) was done using antibodies - CRLF2 phycoerythrin (PE) (TSLP Receptor Monoclonal Antibody, Clone: eBio1A6, PE, eBioscience, Waltham, MA, USA), CD34 PE-Texas red (ECD), CD10 PE-Cyanine 5.5 (PC5.5), CD45 PE-Cyanine 7 (PC7), and CD19 Allophycocyanin (APC) (BC, Hialeah, FL, USA). All the samples were processed for immunophenotyping by the stain-lyse-wash protocol. The CRLF2 expression on B-lymphoblasts was determined by evaluating the percentage of leukemic blasts positive for CRLF2 surface expression. The cut-off for positivity was set at >5% beyond the negative control level of the mature B-cells [1]. Data were analysed using the Kaluza analysis software (Beckman Coulter Inc., CA, USA).

Mutation testing of CRLF2, JAK2, IL7-R genes in CRLF2-positive cases

We also tested CRLF2-positive cases for the presence of mutations in *CRLF2*, *JAK2* and *IL7RA* genes. Genomic DNA was extracted from BM/PB samples using a silica column-based DNA extraction kit (ReliaPrep Blood genomic DNA miniprep system, Promega Corporation, WI, USA). The quality and quantity of the genomic DNA were assessed by spectrophotometer (Nanodrop One, ThermoFisher Scientific, MA, USA). The hotspot regions for genetic mutations were analysed using Sanger sequencing. The primers used were: exon 6 of *CRLF2* gene (Forward primer [FP]: 5'-AGGGAGACTGGTTAGGGATGA-3', and Reverse Primer [RP]: 5'-TGGGCATTGTATGGAAACTG-3'), exon 16 of *JAK2* gene (FP: 5'-TGTTTTGGGGGCTTGAACATAC-3', and RP: 5'-CAACATGCCCTTTACACCACTG-3'), exon 5 (FP: 5'-TGGGACTAAAGGAATCCCAATTGAA-3', and RP: 5'-GCTCCCACACTTTGACATGC-3') and exon 6 (FP: 5'-TGCATGGCTACTGAATGCTC-3', and RP: 5'-CCCACACAATCACCCTCTTT-3') of *IL-7RA* gene. PCR reaction (total volume of 25 µl) contained 1 × PCR buffer, 0.2 mM dNTPs, 0.75 mM $MgCl_2$, and 2.5 U of Taq DNA polymerase (ThermoFisher Scientific, MA, USA), 0.4 µM FP and 0.4 µM RP per reaction. PCR cycling conditions were: denaturation at 94°C × 30 sec, annealing at 56°C × 30 sec and extension at 72°C × 1 minute, and a final extension at 72°C × 5 minutes for 35 cycles. The PCR prod-

ucts were purified using GeneJET PCR Purification Kit (ThermoFisher Scientific, MA, USA). All purified PCR products were then sequenced bidirectionally using Big Dye Terminator v3.1 (Life Technologies, ThermoFisher Scientific, MA, USA) dye chemistry using an ABI 3500 instrument (Applied Biosystems, WA, MA, USA). Sequencing data was analyzed using FinchTV software version 1.5.0 (Geospiza Inc., WA, USA).

Molecular testing for CRLF2 rearrangements

To assess *P2RY8::CRLF2*, RNA was extracted from the CRLF2-positive B-ALL samples using the TRIzol reagent (Ambion Life Technologies, Thermo Fisher Scientific, MA, USA). RNA was reverse transcribed to cDNA using random hexamers, RNase inhibitor, dNTPs, and M-MuLV reverse transcriptase enzyme (Fermentas, USA). The *P2RY8::CRLF2* fusion was detected by RT-PCR. The primers used were FP: 5'-AAGCGTTGCATCCTGTTACC-3', RP: 5'-GCCTCATCACCGTTGAATCT-3'. The amplified PCR products were purified using PCR purification kit (GeneJET PCR Purification Kit, Thermo Fisher Scientific, MA, USA); sequenced afterwards using Big Dye Terminator v3.1 (Life Technologies, Thermo Fisher Scientific, MA, USA) dye chemistry and run on ABI 3500 sequencing platform (Applied Biosystems, WA, MA, USA). Electropherograms were analysed using FinchTV software version 1.5.0. (Geospiza Inc., WA, USA).

The *IGH::CRLF2* fusion was determined using fluorescence in situ hybridization (FISH) using CRLF2 break-apart FISH probe kit (Cytotest, CT-PAC114-10-OG) in CRLF2 positive patients. The cells were fixed in acetic acid and methanol solution in a 3:1 ratio and stored at -80°C until use. A total of 100 interphase nuclei were considered for analysis.

Analysis of copy number alterations

CNAs were identified using multiplex ligation-dependent probe amplification (MLPA) assay for the following genes: *EBF1, IKZF1, JAK2, CDKN2A, CDKN2B, PAX5, ETV6, BTG1, RB1, SHOX, CRLF2, CSF2RA, IL3RA,* and *P2RY8* in 256 B-ALL cases. CNAs in genomic DNA were analysed using the SALSA MLPA Probemix P335 ALL-IKZF1 semi-quantitative assay (MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. MLPA reaction was performed using the Bio-Rad T-100 thermal cycler (Bio-Rad Laboratories, Inc., CA, USA). The PCR amplicons were segregated using capillary electrophoresis on ABI 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Coffalyser.Net version v.210226.0000 software (MRC-Holland, Amsterdam, The Netherlands) was used to analyse the MPLA results. The final probe ratio lying above 0.8 and below 1.2 was regarded as the normal range. For homozygous deletion, a probe ratio of 0 was taken. For heterozygous deletion, a probe ratio $0 < FR < 0.65$ was considered. Probe ratios >1.3 and <1.65 indicated heterozygous duplication. Homozygous duplication was inferred if the final probe ratio value was >1.5 and <2.15. Oncoprint chart for result of copy number analysis was generated using cBioPortal web server [21].

Treatment regimen

Out of 256 B-ALL patients, 241 patients were treated uniformly by the Indian Childhood Collaborative Leukemia Group protocol [22], rest 15 patients did not receive treatment. All the patients were administered with steroids for 7 days and examined for the presence of blast cells in peripheral blood on day 8. Out of 241 patients, 16 patients died during the induction therapy. In the remaining 225 patients bone marrow was investigated for the presence of residual blast cells and minimal residual disease (MRD) measured at day 28 to monitor the remission status.

Outcome measures and statistical analysis

Comparison of the baseline clinical variables and the copy number altered genes with CRLF2 positivity was done using Fisher's exact test. A *p*-value ≤0.05 (two-sided) was considered significant. Achievement of complete remission (CR) was assessed after completion of induction chemotherapy and the presence of platelet count >100,000/µl, absolute neutrophil count >1000/µl, hemoglobin >10 gm/dl, BM blasts <1% and absence of any extramedullary leukemia with no blasts in PB. Overall Survival (OS) was calculated as the time (in months) between the date of diagnosis and the date of last follow

up or death. Event Free Survival (EFS) was defined as the time difference between the date of diagnosis and the date of the first event (date of relapse or death or last follow up). Patients lost to follow-up were censored at the last date of contact. The last follow-up was carried out in November 2022. The probabilities of the EFS, and OS were calculated by the Kaplan-Meier method, and the differences were compared using a two-sided log-rank test and univariate Cox regression analysis. To evaluate the independent prognostic impact of CRLF2-positivity status, univariate analysis was followed by multivariate Cox regression analysis considering the covariates-age, sex, TLC, NCI risk, cytogenetics, MRD status, remission status and IKZF1 alteration. Fisher's exact test was applied using the SPSS statistical software package, and survival analysis was performed using STATA software version 11 and SPSS version 20.0. Patients who did not receive the treatment were excluded from the survival analysis.

Results

Patients' characteristics

A total of 256 patients (171 males; 85 females) were enrolled in the study. The median age of the patients was 5 years (range 0.5-17 years). The median hemoglobin, total leucocyte count (TLC), and platelet count were 6.8 g/dl (range 1.7-14.3 g/dl), 15 × 10^3/μL (range 7.9-673 × 10^3/μL), and 35 × 10^3/μL (range 2 × 10^3-460 × 10^3/μl), respectively. On cytogenetic analysis, we found *BCR::ABL1* in 21 (8.20%), ETV6::RUNX1 in 31 (12.1%), TCF3::PBX1 in 17 (6.64%) and *KMT2A-rearrangement* in 8 (3.12%), hyperdiploid in 3 (1.17%) and hypodiploid in 1 (0.39%) and no sentinel translocation in 169 (66%) patients. Cytogenetic data was not available for 6 (2.34%) patients. Considering the National Cancer Institute (NCI) risk category, 107 patients (41.79%) were placed into the standard risk and 149 (58.20%) into the high-risk category. Out of 256 patients, 241 patients (94.14%) received treatment. Sixteen (6.25%) patients died during induction therapy. Out of 241 patients, 198 patients (82.15%) achieved complete remission. Post-induction MRD was positive in 59 (24.08%) patients. Out of 256 patients, 34 relapsed (13.28%).

CRLF2 expression by multiparameter flow cytometry

Flow cytometric analysis showed that 22/256 (8.59%) patients expressed CRLF2 on the leukemic blasts (**Figure 1**). Out of these, 15 (68.1%) were males and 7 (31.8%) were females. The median age of the positive patients was 7.5 years (range 2.5-15 years). The median TLC, hemoglobin, platelet count, and BM blast percentage were 10 × 10^9/L, 6.6 gm/dl, 33 × 10^3/μL, and 95%, respectively, in CRLF2-positive patients (**Table 1**). On cytogenetic analysis, 19/22 (86.36%) patients were negative for all known sentinel chromosomal aberrations. Patients who tested positive for CRLF2 expression differed in expression patterns as reported by Pastorczak et al. (2018) [1, 19]. Four patients displayed bimodal dim expression of CRLF2 with median fluorescence intensity (MFI) of 19.48 whereas three patients showed bimodal, strong expression with MFI of 25.88. Heterogenous low expression, showing negative to a dim expression of CRLF2, was present in 5 patients (MFI: 2.18, 3.21, 9.33, 8.76, 3.09 respectively). Similarly, dim homogenous expression was also present in 7 patients (median MFI: 4.56). Only three patients showed a strong homogenous expression pattern of CRLF2 (MFI 90.97, 53.94, 64.12). **Figure 2** shows different CRLF2 expression patterns in CRLF2-positive cases. We did not find any association between CRLF2 expression and age, sex, TLC at diagnosis, NCI risk, cytogenetics, BM remission and post-induction MRD (**Table 1**).

Identification of CRLF2 fusions

Sanger sequencing of CRF2 positive patients revealed P2RY8::CRLF2 to be present in 1/22 (4.54%). Similarly, analysis of FISH revealed only 1/22 (4.54%) samples to exhibit *IGH::CRLF2* fusion.

Association of CRLF2 expression and CNA

MLPA assay was done in all 256 patients. CNAs were found in 115 (44.92%) patients. We classified CNAs as amplification (heterozygous triplication/homozygous duplication), gain (heterozygous duplication), shallow deletion (heterozygous deletion) and deep deletion (homozygous deletion). *PAX5* gene was most frequently altered in 18.73% patients, followed

Table 1. Clinical characteristics of pediatric B-ALL patients according to CRLF2 expression

Clinical parameters	CRLF2-positive patients	CRLF2-negative patients	*p*-value
Number of patients, n (%)	22 (8.59)	234 (91.41)	
Age (in years) median (range)	7.5 (2.5-18)	5 (0.5-18)	0.112
Sex			1
Males	15 (68.18)	156 (66.67)	
Females	7 (31.81)	78 (33.33)	
Hemoglobin (gm/dL) median (range)	6.65 (2.1-11)	6 (1.7-14.0)	0.528
Platelet (× 10^9/L) median (range)	33000 (4000-214000)	35000 (2000-673000)	0.554
TLC at diagnosis (× 10^9/L) median (range)	10515 (7.9-457510)	15510 (410-673000)	0.11
NCI risk, n (%)			1
Standard risk	9 (40.91)	98 (41.88)	
High risk	13 (59.09)	136 (58.12)	
Cytogenetics, n (%)			0.33
BCR::ABL1	0	21 (8.97)	
TCF3::PBX1	0	17 (7.26)	
ETV6::RUNX1	2 (9.09)	29 (12.39)	
MLL-rearranged	0	8 (3.42)	
Hyperdiploid/hypodiploid	0	4 (1.71)	
B-other	19 (86.36)	150 (64.1)	
Not available	1 (4.54)	5 (2.14)	
BM remission, n (%)			0.441
Yes	15 (68.18)	183 (78.20)	
No	4 (18.18)	23 (9.82)	
Not available*	3 (13.63)	28 (11.96)	
Post-induction MRD status, n (%)			0.55
Positive	7 (31.81)	52 (22.22)	
Negative	10 (45.45)	130 (55.55)	
Inevaluable*,$	5 (22.72)	52 (22.22)	

*15 patients did not receive treatment and 16 patients died during induction therapy. $Bone marrow was hemodiluted in 21 patients.

by *CDKN2A*, *CDKN2B* and *IKZF1* genes which showed alterations in 14.45%, 15.62% and 12.48% patients, respectively. Gene amplification was observed in the genes located in the PAR1 region such as *SHOX, CRLF2, CSF2RA, IL3RA, and P2RY8*, whereas other commonly altered genes such as *PAX5, CDKN2A, CDKN2B, IKZF1, ETV6, RB1 JAK2, EBF1,* and *BTG1* were more often found to be deleted. A detailed summary of all the gene alterations is given in **Table 2**. An Oncoprint chart listing these CNAs in the entire patient cohort is depicted in **Figure 3**. Further, Fisher exact test was used to assess the association between frequencies of CRLF2 positivity with the above-mentioned CNAs (**Table 3**). This revealed CRLF2 positivity to be associated with presence of *PAX5* alteration (36.36 vs. 17.09%, P=0.041).

Mutational analysis of CRLF2, JAK2 and IL-7R genes

Mutation status of *CRLF2*, *JAK2* and *IL7R* in CRLF2-positive samples has been listed in **Table 4**. Two (9.09%) patients had mutations in *JAK2* exonic region 16 (p.N673fs, p.G261P) and three (13.63%) patients exhibited mutations in *IL7R* exon 6 (p.T224I). None of the patients showed any alterations in exon 6 of the *CRLF2* gene.

Survival analysis

The patients were followed-up for a maximum duration of 56.97 months. Kaplan-Meier survival plot along with univariate analysis revealed significantly poor OS of CRLF2-positive patients, compared to others (HR 2.81, 95% confidence

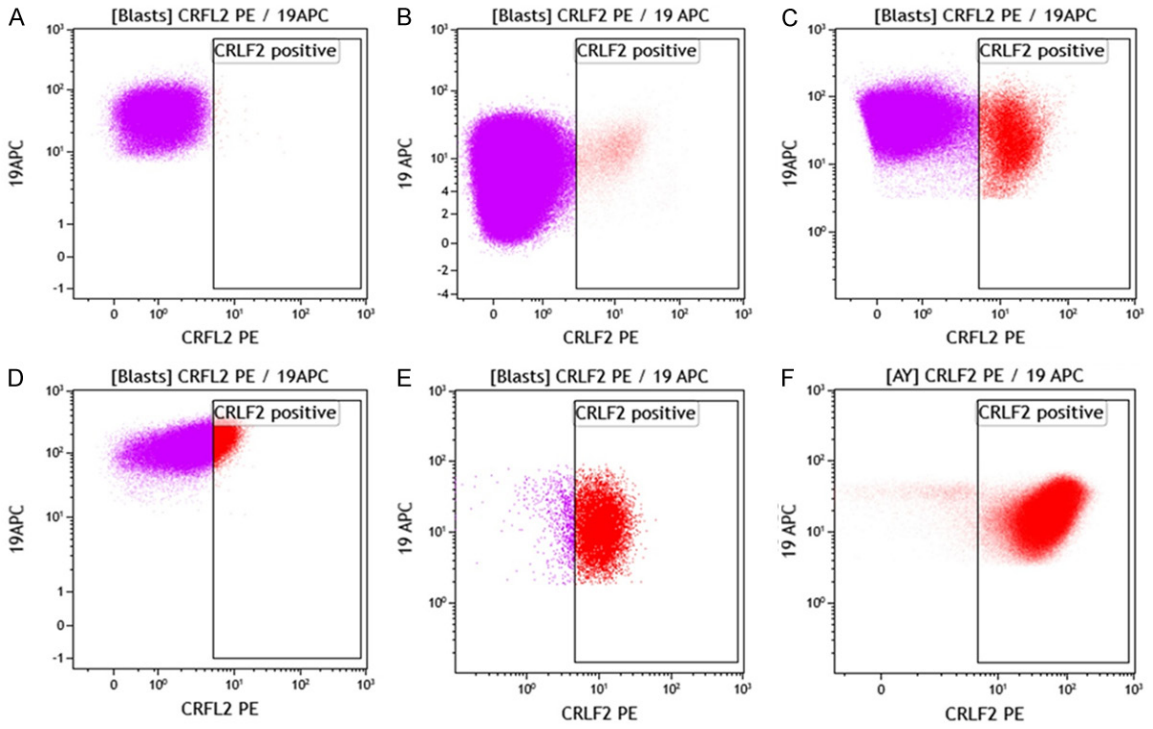


Figure 2. Flow cytometric analysis displaying various degrees of CRLF2 expression on blasts in pediatric B-ALL. CD19 versus CRLF2 dot plots displaying: A. Negative expression; B. Bimodal dim expression; C. Bimodal strong expression; D. Hetrogeneous dim expression; E. Homogeneous dim expression; F. Strong homogenous expression of CRLF2 protein. CRLF2-PE, Cytokine Receptor-Like Factor 2-phycoerythrin; APC, allophycocyanin.

Table 2. Summary of commonly altered genes in pediatric B-ALL patients (n=256)

Gene	Alterations n (%)				Noalterations n (%)
	Amplification	Gain	Shallow deletion	Deep deletion	
PAX5	13 (5.07)	4 (1.56)	31 (12.1)	0	208
IKZF1	0	1 (0.39)	30 (11.7)	1 (0.39)	224
ETV6	1 (0.39)	2 (0.78)	16 (6.25)	5 (1.95)	232
RB1	0	1 (0.39)	3 (1.17)	3 (1.17)	249
SHOX	0	33 (12.89)	0	0	223
CRLF2	0	32 (12.50)	0	0	224
CSF2RA	0	28 (10.93)	2 (0.78)	0	226
IL3RA	0	32 (12.50)	2 (0.78)	0	222
P2RY8	0	31 (12.10)	2 (0.78)	0	223
CDKN2A	0	2 (0.78)	19 (7.42)	16 (6.25)	219
CDKN2B	0	1 (0.39)	21 (8.20)	18 (7.03)	216
JAK2	0	2 (0.78)	9 (3.51)	0	245
EBF1	1 (0.39)	1 (0.39)	3 (1.17)	0	251 (98.04)
BTG1	0	1 (0.39)	3 (1.17)	0	252 (98.43)

interval [CI] 1.08-7.30, P=0.03) (**Figure 4B**; **Table 5**). A similar trend in association of CRLF2 expression with the EFS was observed (HR 2.16, 95% CI 0.92-5.06, P=0.077), however not statistically significant (**Figure 4A**). Among clinicopathological features, complete remission status were associated with EFS and OS of the patients. Furthermore, multivariate analysis revealed that CRLF2 expression was associated with worse OS (HR 4.39, 95% CI 1.53-12.60,

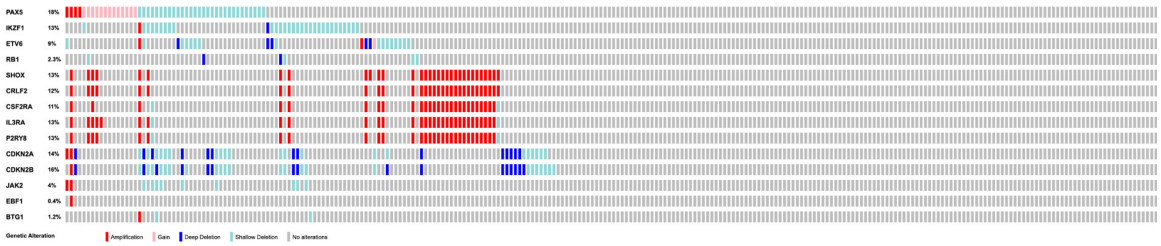


Figure 3. OncoPrint diagram providing overview of mutational frequencies and copy number alterations in 14 genes analyzed using MLPA. Each gene is depicted in single row, and patient samples in columns. The high level amplification mutation, low level gain, deep deletion, and shallow deletions, are shown as red, pink, blue and cyan color respectively.

P=0.006) and EFS (HR 2.62, 95% CI 1.01-6.77, P=0.045) (**Table 6**). Univariate analysis suggested that *IKZF1* CNA was associated with worse EFS (HR 3.00, 95% CI 1.63-5.53, P<0.001, **Figure 4C**) and OS (HR 2.75, 95% CI 1.29-5.87, P=0.009) (**Figure 4D**). Multivariate analysis also revealed association between presence of *IKZF1* CNA with worse EFS (HR 5.1, 95% CI 2.41-10.77, P<0.001), and OS (HR 5.47, 95% CI 2.18-13.6, P<0.001) of the patients (**Table 6**).

We further analyzed the combined effects of CRLF2 expression by FCM and *IKZF1* deletion on the patient outcome. The patients were divided into 4 groups: Group 1, CRLF2-negative and IKZF1 WT (wild type); Group 2, CRLF2-positive and IKZF1 WT; Group 3, CRLF2-negative and IKZF1 ALT (altered); and Group 4, CRLF2-positive and IKZF1 ALT (**Figure 5**). The group 4 patients had the worst EFS (HR 8.22, 95% CI 1.09-61.88, P=0.041) whereas the group 2 and 3 patients had the intermediate EFS (HR 3.07, 95% CI 1.62-5.81, P=0.001 and HR 2.30, 95% CI 0.90-5.89, P=0.080, respectively) as compared to group 1 patients. Similarly, the group 4 patients had worst OS (HR 9.96, 95% CI 1.30-76.25, P=0.027) compared to group 1, while group 2 and group 3 had intermediate OS (HR 2.82, 95% CI 1.25-6.32, P=0.012 and HR 2.93, 95% CI 1.00-8.52, P=0.049).

Discussion

In the present study, we investigated the incidence and prognostic relevance of CRLF2 expression, as assessed by multiparameter FCM, in pediatric B-ALL patients. We also showed an association of CRLF2 expression with CNAs of commonly altered genes. In addition, we analysed the impact of *IKZF1* deletion along with CRLF2 overexpression.

The overall frequency of CRLF2 positivity reported in B-ALL ranges from 7.51-42.7% [1, 8, 9, 19, 23-32]. Such variability can be explained by the fact that various research groups have used different methodologies (mRNA estimation, FISH, and FCM), cut-off values and inclusion criteria for the detection of CRLF2 dysregulation in B-ALL patients. We have chosen multiparameter FCM for the detection of CRLF2 expression as it seems more plausible for its applicability, ready availability and cost effectiveness in routine diagnostic work-up of B-ALL patients [31]. We found CRLF2 overexpression on leukemic blasts in 8.59% pediatric B-ALL patients. Our results are similar to those reported in a previous study by Pastorczak et al. (2018) [1], who found that 7.51% of 386 pediatric B-ALL patients were CRLF2-positive on FCM. Bugarin et al. (2015) [19] also reported a similar frequency of 8.8% in 421 pediatric patients using FCM. Schmäh et al. (2017) [30] and Yamashita et al. (2013) [32] found overexpression of CRLF2 in 91/927 (10%) and 15/141 (11%), respectively, of children with B-ALL by RQ-PCR. Similarly, Chen et al. (2012) [24], Yano et al. (2014) [9], Hassan et al. (2022) [27], and Dou et al. (2017) [25] estimated CRLF2 expression in pediatric patients by RQ-PCR and found frequency to be 17.5%, 18%, 18.5%, and 19%, respectively. Yoda et al. (2010) [33] used a combination of RQ-PCR, immunohistochemistry, and gene expression profiling to assay CRLF2 expression in adult B-ALL samples from DanaFarber Cancer Institute (DFCI; n=97) and Gruppo Malattie Ematologiche Dell'Adulto (GIMEMA; n=157) cohorts and found CRLF2 overexpression in 12.5% adult B-ALL cases. They further reported that the CRLF2 overex-

Table 3. Association of copy number alterations with CRLF2 expression

CNAs	CRLF2 positive patients n (%)	CRLF2 negative patients n (%)	*p*-value
Number of patients	22	234	
EBF1			0.364
Altered	1 (4.55)	4 (1.71)	
Unaltered	21 (95.45)	230 (98.29)	
IKZF1			0.744
Altered	3 (13.64)	29 (12.39)	
Unaltered	19 (86.36)	205 (87.61)	
JAK2			0.606
Altered	0	11 (4.70)	
Unaltered	22 (100.00)	223 (95.30)	
CDKN2A			0.536
Altered	4 (18.18)	33 (14.10)	
Unaltered	18 (81.82)	201 (85.90)	
CDKN2B			0.758
Altered	4 (18.18)	36 (15.38)	
Unaltered	18 (81.82)	198 (84.62)	
PAX5			0.041
Altered	8 (36.36)	40 (17.09)	
Unaltered	14 (63.64)	194 (82.91)	
ETV6			0.240
Altered	0	24 (89.74)	
Unaltered	22 (100.0)	210 (10.26)	
BTG1			0.303
Altered	1 (4.55)	3 (1.28)	
Unaltered	21 (95.45)	231 (98.72)	
RB1			1.000
Altered	1 (4.55)	3 (1.28)	
Unaltered	21 (95.45)	231 (98.72)	
SHOX			0.501
Altered	4 (18.18)	29 (12.39)	
Unaltered	18 (81.82)	205 (87.61)	
CRLF2			0.495
Altered	4 (18.18)	28 (11.97)	
Unaltered	18 (81.82)	206 (88.03)	
CSF2RA			1.000
Altered	2 (9.09)	28 (11.97)	
Unaltered	20 (90.91)	206 (88.03)	
IL3RA			0.508
Altered	4 (18.18)	30 (12.82)	
Unaltered	18 (81.82)	204 (87.18)	
P2RY8			0.501
Altered	4 (18.18)	29 (12.39)	
Unaltered	18 (81.82)	205 (87.61)	

pression highly correlated between assays in their study, suggesting the results of these methods can be compared and used interchangeably. A recent study from India has reported an incidence of CRLF2 positivity to be 14.6% [31]. They determined CRLF2 expres-

Table 4. CRLF2 expression intensity details and copy number aberrations using MLPA on leukemic blasts of B-ALL patients

Samples	CRLF2 expression	Pattern of CRLF2 expression	MFI	% positivity	Type of CNA	Mutation/CRLF2 rearrangements	Cytogenetics
1	Positive	Heterogenous low	2.18	11.64	PAX5 deletion	JAK2 mutation p.G261P	Normal B-other
2	Positive	Homogenous dim	4.56	90.21	SHOX amp, CRLF2 amp, CSF2RA amp, IL3RA amp, P2RY8 deletion	P2RY8::CRLF2 fusion	Normal B-other
3	Positive	Heterogenous low	3.21	90	No alteration	None	Hyperdiploidy
4	Positive	Bimodal dim	43.62	8.5	IKZF1 del	JAK2 mutation p.N673fs; IL7 mutation p.T224I	Normal
5	Positive	Homogenous dim	10.59	66.74	SHOX amp, CRLF2 amp, CSF2RA amp, IL3RA amp, P2RY8 amp	None	NA
6	Positive	Strong homogenous	90.97	33.86	CDKN2A del, CDKN2B del, PAX5 del	IL7 p.T224I	ETV6::RUNX1
7	Positive	Heterogenous low	9.33	7.32	No alteration	None	Normal
8	Positive	Homogenous dim	2.87	2.07	PAX5 amp	None	Normal
9	Positive	Strong homogenous	53.94	96.38	CDKN2A del, CDKN2B del, PAX5 del	None	Normal
10	Positive	Heterogenous low	8.76	17.09	IKZF1 deletion, PAX5 amp	None	Normal
11	Positive	Homogenous dim	8.94	19.53	No alteration	No pellet	Normal
12	Positive	Homogenous dim	4	1.31	No alteration	None	Normal
13	Positive	Homogenous dim	9.94	80.86	IKZF1 del, CDKN2A del, CDKN2B del, PAX5 del, SHOX amp, CRLF2 amp, IL3RA amp, P2RY8 amp	None	Normal
14	Positive	Bimodal strong	27.26	75.6	PAX5 amp, SHOX amp, CRLF2 amp, IL3RA amp, P2RY8 amp	IL7 p.T224I	Normal
15	Positive	Bimodal dim	2.2	2.42	No alteration	None	Normal
16	Positive	Bimodal strong	25.88	2.39	EBF1 amp	None	Failed
17	Positive	Homogenous strong	64.12	95.01	No alteration	IGH::CRLF2 fusion	Normal
18	Positive	Heterogenous dim	3.09	8.68	No alteration	None	Normal
19	Positive	Bimodal dim	34.0	Very high as it was acquired new FCM	No alteration	None	Normal
20	Positive	Bimodal dim	4.95	4.46	IKZF1 Het Del, BTG1 Het Del	None	Normal
21	Positive	Homogenous dim	4.25	11.57	No alteration	None	Normal
22	Positive	Bimodal strong	5.5	1.92	No alteration	None	Normal

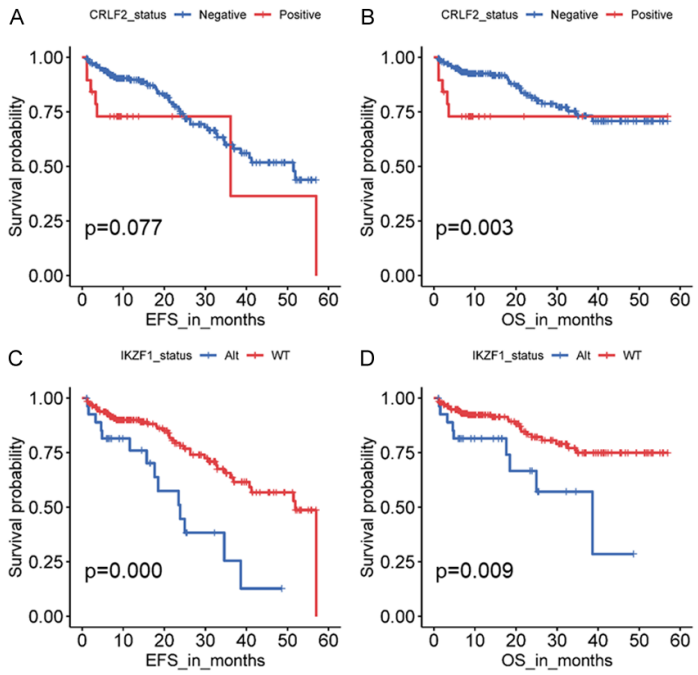


Figure 4. Kaplan-Meier survival plots showing (A) Event free survival and (B) overall survival in pediatric B-ALL based on the status of CRLF2 expression by multiparameter flow cytometry, and (C) Event free survival and (D) overall survival in pediatric B-ALL based on the status of IKZF1 alteration by MLPA (IKZF1 WT, wild type and IKZF1 Alt, altered).

sion by FCM in both pediatric and adult B-ALL patients. Maciel et al. (2022) [28] estimated CRLF2 mRNA expression and reported its positivity in 25.2% of pediatric and adult patients. Konoplev et al. (2017) [8] and Rasekh et al. (2021) [29] both determined CRLF2 expression by FCM and reported a positivity rate in 27% of adult and 29.9% in pediatric B-ALL patients, respectively. The highest frequency of 42.7% in pediatric B-ALL patients has been reported by Cui et al. (2021) using RQ-PCR [20]. Akin to Pastorczak et al. (2018) [1], and Virk et al. (2021) [31], we used a cut-off value of 5% positivity for the detection of CRLF2 expression by FCM. Konoplev et al. (2017) [8] used any positivity beyond the level of expression of hematogones (normal B-precursors) to define CRLF2 positivity. In a study by Bugarin et al. (2015) [19], a very low cut-off level of 1% positivity was used, however, using such a low cut-off level is not desirable as there are chances of getting false positive results on FCM due to non-specific staining, especially in the samples processed 24 hours after collection from the patients [1, 34]. A study from Egypt used a cut-off set at mean fluorescence intensity of 3.8 using the Receiver Operating Characteristic curve [29]. In view of these observations, it is evident that standardization of the method for determining CRLF2 positivity is highly warranted [27]. In addition to the above-mentioned factors, the prevalence of CRLF2 overexpression in B-ALL has also been shown to be influenced by the ethnicity of the patients. Hispanics/Latino have been reported to have higher frequency of CRLF2 overexpression compared to non-Hispanic/Latino [8, 11, 35]. Similarly, Konoplev et al. (2017) [8] reported an absence of CRLF2 expression, as assessed by FCM in Asian patients. Similar to previous studies, we found heterogenous patterns of CRLF2 positivity in our patients, suggesting the presence of subclones of leukemic cells harbouring CRLF2 dysregulation in B-ALL [1, 19, 36]. The clinical significance of the fraction of these subclones as detected by FCM needs to be ascertained in future functional studies.

We did not find any association between TLC at diagnosis and CRLF2 overexpression, similar to that reported by other research groups [1, 8, 18, 31, 37]. However, several previous reports suggest contradictory evidence regarding association of TLC with CRLF2 positivity. While some studies have reported higher TLC at diagnosis, others have found lower TLC in CRLF2 overexpressing B-ALL patients [25, 27, 38]. In concordance with previous reports, we did not find a significant difference between CRLF2-positive and negative patients with respect to age, sex, hemoglobin and platelet count [1, 8, 27, 31].

We found 81.81% (18/22) patients with CRLF2 overexpression had no recurrent cytogenetic abnormalities. In contrast, 80/234 (34.18%) patients with no overexpression of CRLF2 had recurrent cytogenetic abnormalities. However, we did not observe statistically significant relation between cytogenetics and CRLF2 expression. Our results are in concordance with previous studies which have reported the presence of recurrent cytogenetic abnormalities in CRLF2 overexpressing patients [8, 14, 18, 31]. Others

Table 5. Univariate analysis of EFS and OS probability in pediatric B-ALL patients

Variables	Event free survival			Overall survival		
	HR	*p*-value	95% CI	HR	*p*-value	95% CI
Age (in years)						
<10	Ref			Ref		
≥10	2.24	0.003	1.31-3.84	1.2	0.50	0.60-2.72
Sex						
Male	Ref			Ref		
Female	1.58	0.109	0.90-2.76	1.83	0.080	0.93-3.60
TLC (× 10^9/L)						
<50	Ref			Ref		
≥50	1.29	0.380	0.73-2.29	1.25	0.520	0.62-2.60
NCI risk						
Standard	Ref			Ref		
High	0.67	0.132	0.40-1.12	0.54	0.007	0.28-1.05
Cytogenetics						
B-other	Ref			Ref		
BCR:ABL1	1.39	0.500	0.55-3.5	0.91	0.89	0.21-3.85
ETV6::RUNX1	1.67	0.180	0.78-3.65	1.91	0.16	0.78-4.72
TCF3::PBX1	2.27	0.090	0.86-5.93	2.46	0.10	0.83-7.27
MLL:: rearranged	1.25	0.820	0.17-9.17	1.71	0.59	0.23-12.78
Hyper/Hypodiploidy	4.5e-19	1.000	0-	2.2e-19	-	-
Inevaluable	4.5e-19	1.000	0-	2.2e-19	-	-
MRD						
Negative	Ref			Ref		
Positive	1.14	0.650	0.63-2.07	1.16	0.166	0.80-3.5
Inevaluable	3.11	0.003	1.47-6.61	4.74	0.000	2.01-11.15
CR						
In remission	Ref					
Not in remission	2.35	0.006	1.27-4.35	3.00	0.001	1.57-5.72
CRLF2 expression						
Negative	Ref			Ref		
Positive	2.16	0.077	0.92-5.06	2.81	0.03	1.08-7.30
IKZF1 Altered						
Wild type	Ref			Ref		
Altered	3.00	<0.001	1.63-5.53	2.75	0.009	1.29-5.87

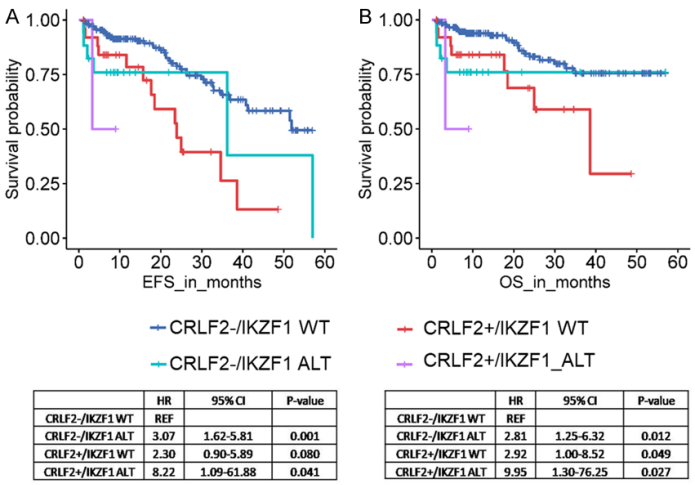
have reported that all CRLF2-positive pediatric B-ALL patients were negative for the recurrent cytogenetic translocations [27, 37].

Several reports have revealed association between other CNAs in B-ALL and CRLF2 expression [28, 39, 40]. CRLF2 expression was associated with several other CNAs in B-ALL, including deletions of the *PAX5* gene on chromosome 9p and the *EBF1* gene on chromosome 5q, as well as gains of the *JAK2* gene on chromosome 9p [11, 40]. In addition, a study by Harvey and colleagues (2010) identified several additional CNAs associated with CRLF2 overexpression in B-ALL, including deletions of the *BTG1* gene on chromosome 12q and the *CDKN2A/B* locus on chromosome 9p, as well as gains of the *IGH*α locus on chromosome 14q and the *CRLF2* gene itself on chromosome Xq26 [12]. Thus, there is growing evidence that CRLF2 expression in B-ALL is associated with a complex network of CNAs affecting multiple genes, highlighting the need for a comprehensive analysis of CNAs in this disease. A study by Mullighan and colleagues (2009) found that CRLF2 overexpression and *IKZF1* deletions

Table 6. Multivariate analysis of EFS and OS probability in pediatric B-ALL patients

Variables	Event free survival			Overall survival		
	HR	*p*-value	95% CI	HR	*p*-value	95% CI
Age (in years)						
<10	Ref			Ref		
≥10	3.29	0.000	1.75-6.18	1.85	0.161	0.78-4.38
Sex						
Male	Ref			Ref		
Female	1.24	0.472	0.68-2.25	1.35	0.413	0.65-2.81
TLC (× 10^9/L)						
<50	Ref			Ref		
≥50	0.91	0.790	0.46-1.70	0.85	0.725	0.36-2.02
NCI risk						
Standard	Ref			Ref		
High	0.5	0.030	0.28-0.94	0.48	0.063	0.22-1.03
Cytogenetics						
B-other	Ref			Ref		
BCR:ABL1	0.92	0.870	0.32-2.59	0.63	0.573	0.13-3.05
ETV6::RUNX1	3.65	0.030	1.53-8.70	3.13	0.031	1.10-8.87
TCF3::PBX1	5.18	0.002	1.81-14.8	6.55	0.003	1.91-22.4
MLL:: rearranged	3.50	0.239	0.43-28.6	6.23	0.097	0.71-54.4
Hyper/Hypodiploidy	3.98e-20	-	-	2.70e-16	1	0-
Inevaluable	1.45e-19	-	-	3.69e-16	1	0-
MRD						
Negative	Ref			Ref		
Positive	0.97	0.931	0.50-1.88	1.61	0.257	0.70-3.70
Inevaluable	4.42	0.001	1.88-10.4	8.42	0.000	3.08-23.05
CR						
In remission	Ref					
Not in remission	2.42	0.052	0.99-5.93	2.19	0.129	0.79-6.06
CRLF2 expression						
Negative	Ref			Ref		
Positive	2.62	0.045	1.01-6.77	4.39	0.006	1.53-12.60
IKZF1 Altered						
Wild type	Ref			Ref		
Altered	5.1	<0.001	2.41-10.77	5.47	<0.001	2.18-13.6

were both independent predictors of a poor outcome in B-ALL, with patients carrying both abnormalities having a dismal prognosis [13]. Similarly, Harvey and colleagues (2010) demonstrated that B-ALL patients with CRLF2 overexpression and deletions of either *BTG1* or *CDKN2A/B* had a significantly worse overall survival compared to patients without these abnormalities [12]. In a study by Russell and colleagues (2017), B-ALL patients with CRLF2 overexpression and *JAK2* mutations were found to have a higher risk of relapse and poorer survival than patients without these abnormalities [39]. Overall, these studies suggest that the combination of CRLF2 overexpression with specific CNAs, such as deletions of *PAX5*, *IKZF1*, *BTG1*, or *CDKN2A/B*, or mutations in *JAK2*, may be particularly informative for risk stratification in B-ALL and could help guide treatment decisions. In this line, we also studied the concomitant effect of CRLF2 expression and *IKZF1* deletion in our patients. Although the number of patients were small in group 4 (CRLF2-positive and *IKZF1* altered), we found that these patients had the worst EFS and OS, compared to patients without altera-



	HR	95% CI	P-value
CRLF2-/IKZF1 WT	REF		
CRLF2-/IKZF1 ALT	3.07	1.62-5.81	0.001
CRLF2+/IKZF1 WT	2.30	0.90-5.89	0.080
CRLF2+/IKZF1 ALT	8.22	1.09-61.88	0.041

	HR	95% CI	P-value
CRLF2-/IKZF1 WT	REF		
CRLF2-/IKZF1 ALT	2.81	1.25-6.32	0.012
CRLF2+/IKZF1 WT	2.92	1.00-8.52	0.049
CRLF2+/IKZF1 ALT	9.95	1.30-76.25	0.027

Figure 5. Survival estimation in CRLF2-positive and CRLF2-negative pediatric B-ALL patients based on the IKZF1 altered *combinations*. The Kaplan-Meier curve shows (A) Event free survival (EFS), and (B) overall survival (OS) in CRLF2-positive plus IKZF1-altered and IKZF1-non-altered patients based on the detection using MLPA analysis. Additional table enumerating the *p*-values, and hazard ratios calculated from Cox regression analysis for the above categories of CRLF2 positive and CRLF2 negative patients.

tions in these genes. The HR was highest for this group compared to other groups having either CRLF2 positivity or *IKZF1* alterations. Our results are akin to Cui et al. (2021) [20], who did combined modelling and found that 7.8% of CRLF2-positive and *IKZF1* altered cases as of the highest risk and they had a HR greater that either of the 2 factors alone or in their absence. They further found that CRLF2-positive and *IKZF1* altered in combination can be helpful in guiding treatment in BCR-ABL1 negative intermediate risk group of patients. However, we could not do such analysis as the number of CRLF2-positive patients was less in our cohort. Pastorczak et al. (2018) [1] have also reported that *IKZF1* deleted and CRLF2-positive patients had worse relapse free survival compared to other risk groups. They concluded that surface expression of CRLF2 increases the risk of relapse in pediatric B-ALL patients having *IKZF1* deletions. *P2RY8::CRLF2* translocation was associated with poor prognosis in B-ALL in previous studies [12, 18, 37]. In our study, this translocation was positive in only 1 patient only. Similarly, *IGH::CRLF2* translocation was also present only in 1/22 CRLF2 patients. Hence, we could not study the impact of CRLF2 translocations on patient outcome.

CRLF2 overexpression is frequently accompanied by activating mutations in genes encoding Ikaros (*IKZF1*), Janus kinase 1 (*JAK1*) and Janus kinase 2 (*JAK2*), and/or the IL-7 receptor alpha chain (*IL7RA*) [17]. We also evaluated the mechanism of CRLF2 overexpression in B-ALL by testing mutations in *JAK2*, *CRLF2* and *IL7R*A genes. We found *JAK2* mutation in 2/22 (9.09%) patients. Our results are similar to those of earlier reports, where JAK2 mutations in CRLF2 positive patients have been reported to be 8.1% [28], 8.7% [18], and 19% [27]. Concomitant *JAK2* mutations have also been reported in ~50% of CRLF2-rearranged Ph-like B-ALL cases [11, 41, 42]. We found novel mutations (p.G261P and p.N673fs) in *JAK2* gene which have not been reported before to the best of our knowledge. CRLF2-positive/*JAK2* wild type patients have mutations in other genes like *IL7R*, *CRLF2*, *JAK1* and *JAK3*. We found *IL7R* mutation in 3/22 (13.63%) patients. All 3 patients had *IL7R* p.T224I mutation. This mutation has not been reported before. One patient had concomitant mutation in *JAK2* and *IL7R* genes. We did not find any mutation in *CRLF2* gene. *P2RY8::CRLF2* and *IGH::CRLF2* translocations were found in one patient each, contrary to the results from other studies [8, 19, 37]. This suggests that our paediatric B-ALL samples may have some other novel lesions which we have not investigated in the current study.

Previous studies reported frequent deletions of *CDKN2A*, *IKZF1*, and *PAX5* gene in CRLF2-positive B-ALL [39, 43]. Therefore, we looked for the presence of CNA in *EBF1, IKZF1, JAK2, CDKN2A, CDKN2B, PAX5, ETV6, BTG1, RB1, SHOX, CRLF2, CSF2RA, IL3RA,* and *P2RY8* genes using MLPA technique. A previous study estimated the frequencies of common CNAs in B-ALL, and reported *CDKN2A/B* deletions to be present in 41%, followed by *PAX5* aberrations (35%) [44]. Deletions of *RB1* (5.1%), *BTG1* (4.3%) and *EBF1* (1.7%) were much less common according to the same group and are similar to our findings [44]. The same group also confirmed *PAX5* alteration in 2/5 patients with *PAX5* amplification who relapsed, and suggested that *PAX5* aberration can act like a prognostic marker in B-ALL patients. Our study revealed

that CRLF2-positive patients characterized by different expression patterns have distinct CNA profiles. CRLF2 patients may have different prognoses based on these CNAs. *IKZF1* deletions were found in 13% of all B-ALL patients. This is consistent with previous studies which have reported overall frequency of *IKZF1* deletion to be ~15% [20, 45, 46]. Similar to earlier studies [1, 20, 24], we did find a significant association between *IKZF1* deletion and CRLF2 expression.

We did not find any significant difference in remission and post-induction MRD levels in CRLF2-positive and negative B-ALL patients. Our results are in concordance with those reported by Virk et al. (2021), who did not find any association between MRD levels and CRLF2 positivity [31]. In agreement with our results, Hassan et al. (2022) and van der Veer et al. (2013), reported that CRLF2 expression did not correlate with MRD in children with B-ALL [27, 47]. Chiarreti et al. (2016) [23] also reported no such association in adult patients. In contrast, other groups have reported a correlation between MRD positivity and CRLF2 overexpression [1, 20, 37]. This discordance can be explained by difference in the treatment protocols, time point for MRD estimation and ethnicity of the patients [27].

The prognostic relevance of CRLF2 overexpression in pediatric B-ALL remains unclear. While a few studies have reported a correlation between CRLF2 expression and prognosis, others did not find any association [1, 9, 14, 20, 23-25, 27]. We found that the CRLF2-positive patients had worse OS compared to CRLF2-negative patients. Similarly, Dou et al. (2017) [25] and Chiaretti et al. (2016) [23] have also identified CRLF2 overexpression as independent factor for poor prognosis in pediatric B-ALL patients [14, 19, 41].

To conclude, this study determined the frequency of CRLF2-positivity by multiparameter FCM in pediatric B-ALL patients and explored the associated genes which are commonly altered in B-ALL patients. In our study, only 8.59% of pediatric patients were found to be positive for aberrant CRLF2 expression, not specific to any cytogenetic subtype. Our population has shown various CRLF2 expression patterns indicating its sub-clonal properties. Also, our pediatric CRLF2-positive B-ALL patients were not majorly defined by the *P2RY8::CRLF2* and *IGH::CRLF2* fusions and were strongly associated with *PAX5* CNA which are commonly altered in B-ALL. The prognosis of the CRLF2-positive patients is worse compared with the CRLF2-negative B-ALL patients. While several important directions were observed in this study, some limitations remain to be addressed. The numbers of patients enrolled in the study were limited. The clinical utility of concurrent CRLF2 overexpression and *IKZF1* deletion needs to be validated in larger number of patients. Although the study found an association between CRLF2 expression and poor prognosis, the underlying biological mechanisms and pathways leading to this association were not explored. In future, it would be interesting to investigate the relationship between CRLF2 expression and response to different treatment regimens to determine the optimal treatment approach for CRLF2-positive patients. While this study focused on the association between CRLF2 expression and common CNAs, but it would be worthwhile to explore other genomic alterations in CRLF2-positive patients that may contribute to their poor prognosis. Further, this study only evaluated CRLF2 expression at diagnosis, and it would be useful to monitor changes in CRLF2 expression during treatment and disease relapse to assess its potential role as a biomarker for disease monitoring and treatment response.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Anita Chopra, Laboratory Oncology, Room No. 423, 4th Floor, Dr. BRAIRCH, AIIMS, Ansari Nagar, New Delhi 110029, India. Tel: +91-1129575415; Fax: +91-1126588663; E-mail: chopraanita2005@gmail.com

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