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ORIGINAL ARTICLE

Characterization of CRLF2 Expression in Pediatric B-Cell Precursor Acute Lymphoblastic Leukemia

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SUMMARY

Background: **Acute lymphoblastic leukemia (ALL) is a heterogeneous disease with several underlying genetic abnormalities. Several studies have tried to elucidate the prognostic significance of cytokine receptor-like factor 2 (CRLF2) overexpression in pediatric B-cell precursor (BCP)-ALL; however, it is still controversial.**
Methods: **CRLF2 expression was assessed by flow cytometry in 87 newly diagnosed BCP-ALL pediatric patients, and 80 age and gender-matched control group. *Janus Kinase2* (*JAK2)* (R683) mutation analysis was also performed in those identified to have CRLF2 overexpression with adequate DNA samples by direct sequencing.**
Results: **CRLF2 overexpression was identified in 26/87 (29.9%) of our patients with cutoff set at mean fluorescence intensity (MFI = 3.8) using the Receiver Operating Characteristic (ROC) curve. There were no significant differences in the clinical and laboratory features between patients with high and low-CRLF2 expression, apart from thrombocytopenia which showed statistically significant association with the low-expression group (p = 0.041). Sequence analysis of samples with high CRLF2 expression (n = 23) revealed that 2/23 (8.7%) cases harbored the mutation *JAK2* (R683). CRLF2 levels did not have a significant impact on either overall survival (OS) or disease free survival (DFS) (p = 0.601; p = 0.212, respectively).**
Conclusions: **CRLF2 overexpression was not an adverse parameter in pediatric BCP-ALL patients. However, patients with CRLF2 overexpression may harbor the *JAK2* mutation presenting a group that can benefit from targeted therapy by kinase inhibitors. The usage of CRLF2 expression to monitor minimal residual disease of BCP-ALL would be an area of interest for further evaluation.**
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KEY WORDS

BCP-ALL, CRLF2, *JAK2*, flow cytometry, direct sequencing

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is considered one of the most successful stories in cancer therapy with long term overall survival rates around 85%. However, relapse remains the leading cause of death in the remaining pediatric ALL patients [1]. The key for improved survival is the precise risk stratification of patients according to well-known prognostic parameters, then the treatment was adjusted accordingly [2,3].

Philadelphia chromosome like or BCR-ABL1 like B-ALL (that lacks the BCR-ABL1 translocation) shows a gene expression profile similar to that seen in ALL harboring the BCR-ABL1 translocation [4]. It accounts for around 7 - 25% of newly diagnosed B-ALL [5-8]. This distinct entity involves some genetic alterations in the signaling pathways of kinases or cytokine receptors and these changes may lead into targeted therapy of the affected pathways [9].

The cytokine receptor-like factor 2 (CRLF2) gene lies in the pseudoautosomal region1 (PAR1) of gender chromosomes at Xp22.3/Yp11.3 and encodes thymic stromal lymphopoietin receptor (TSLP R) that heterodimerizes with interleukin 7 receptor (IL-7 R) [10]. CRLF2 protein plays an important role in the regulation of proliferation and apoptosis of B cells, besides its effect on the growth and inflammatory responses of T and dendritic cells [11-13].

High CRLF2 expression arises from two main mechanisms, either due to a cryptic translocation with immunoglobulin heavy chain (IGH) locus [14] or an interstitial deletion resulting in fusion with the purinergic receptor P2Y, G-protein coupled, 8 (P2RY8) promoter [15]. Rarely, it may occur due to F232C mutation [16, 17].

Flow cytometry is considered a rapid, reliable, and inexpensive tool for the detection of CRLF2 expression [18]. CRLF2 overexpression is insufficient to cause overt leukemia [19]. The Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathway plays an important role in mediating signals from hematopoietic cytokine receptors [20]. Around 50% of pediatric cases with deregulated CRLF2 harbor mutations in the JAK/STAT pathway members [9,21] resulting in constitutive activation of CRLF2/ JAK/STAT signaling contributing to the process of leukemogenesis [22,23]. In addition, abnormal phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) signaling pathway has also been involved in leukemogenesis [24]. Thus, further assessment of JAK2 mutation in this group helps to categorize patients who can benefit from individualized therapy by JAK2 inhibitors [13].

Previous studies have addressed the prognostic significance of CRLF2 overexpression in pediatric B-cell precursor (BCP)-ALL [15,25-29]. Nonetheless, controversial reports still have been documented which may be attributed to different treatment regimens, characteristics of studied cohorts and other prognostic parameters which may co-exist. For further study, we aimed to assess CRLF2 expression in pediatric BCP-ALL by flow cytometry and to detect *JAK2 (*R683) mutation in cases with CRLF2 overexpression by direct sequencing to evaluate their impact on the outcome of those patients.

MATERIALS AND METHODS

Materials

This prospective study included 87 de novo pediatric BCP-ALL patients in the period from June 2016 to August 2018. All patients presented to the Pediatric Oncology outpatient clinic of the National Cancer Institute (NCI), Cairo University. Patients proved to be positive for BCR-ABL1 t(9;22) were excluded from our study. All patients´ guardians signed an informed consent. Eighty potentially healthy age and gender matched children were enrolled as a control group, with male to female ratio nearly 1:1 and their age ranged from 6 months to 17 years. The study protocol was approved by the Ethical Committee at the NCI, following the Declaration of Helsinki.

Therapy

All our patients received total XV protocol (modified from St Jude total XV protocol). It consists of three phases; induction of remission, consolidation, and maintenance [30].

Methods

Routine work-up was done at diagnosis for all patients including morphological examination of peripheral blood (PB) and bone marrow (BM) smears, cytochemistry, conventional cytogenetic analysis, and molecular studies. PB or BM samples were analyzed by flow cytometry using a wide panel of monoclonal antibodies as part of the routine immunophenotyping workup for acute leukemia. Morphological assessment and flow cytometric analysis (according to availability of samples and presence of leukemia-associated immunophenotype (LAIP)) of bone marrow samples at day 15 and day 42 post-induction were performed in order to evaluate treatment response.

Flow cytometric detection of CRLF2 expression

For the assessment of CRLF2 surface expression on blast cells, an extra tube containing a combination of CD45 fluorescein isothiocyanate (FITC)/TSLPR (human TSLPR phycoerythrin (PE)-conjugated antibody, Cat. No. FAB981P, clone #147036, R&D systems) /CD19 Phycoerythrin-Cyanin7 (PE cy7) was employed. Surface expression of TSLPR on blast cells was determined by gating on CD19 positive, CD45 dim population and calculation of mean fluorescence intensity (MFI) of expression of TSLPR on this population.

For the control group, PB samples were used for the measurement of CRLF2 expression on mature lymphocytes. A tube containing a combination of CD45 (FITC) /TSLP R (PE)/CD3 (cy5.5)/CD19 (PE cy7) was used for validation. CRLF2 expression was determined by gating on CD19 positive population and CD3 positive population and calculation of MFI of expression of TSLP R on mature B cells and mature T cells, respectively. Expression was negative on CD19 positive mature B cells, and low expression was detected on mature

T cells and compared to the expression on lymphoblasts. We further analyzed 10 bone marrow samples with a significant number of hematogones and they were negative for CRLF2 expression (Figure 1). The cutoff for CRLF2 expression to discriminate patients from controls was set at MFI = 3.8 using the receiver operating characteristic curve (ROC) (sensitivity 29.9%, specificity 100%, positive predictive value (PPV) 100%, negative predictive value (NPV) 56.7%, and accuracy 63.5%) (Figure 2). This expression was statistically significant compared to the control group ($p < 0.001$) (Table 1).
Analysis was carried out on Navios 6 color Flow cytometer (Beckman Coulter Incorporation, Hialeah, Florida, USA) using Navios software following the quality control procedures.

Detection of *JAK2* (R683) mutation
Direct sequencing was performed to detect point mutations of *JAK2* exon 16 (R683) for cases that were identified by flow cytometry to have CRLF2 overexpression. Genomic DNA was extracted using Invisorb Spin Blood Mini Kit (Cat. No. 1031100200, STRATEC Molecular GmbH) following the manufacturer´s instructions. Assessment of DNA concentration and purity was conducted using Nanodrop Spectrophotometer. The sequence of primers used for the amplification of *JAK2* exon 16 was: forward primer
GTCAGCTCCCATCCAGAAAC;
reverse primer
ACAACATGCCCTTTACACCA [31].
Amplification was carried out using *Hot FirePol DNA polymerase* (Cat. No. 01-02-00500 Solis BioDyne) with an initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 60 seconds and extension at 72°C for 60 seconds. Using a 2% agarose gel, electrophoresis was performed followed by visualization under ultraviolet transilluminator for the verification of amplified products at 498 base pairs. Purification of the amplicon was done using PureLink® PCR Purification Kit (Invitrogen Cat. No. K310001). Big Dye Terminator v3.1 Cycle Sequencing Kit (Cat. No. 4336917 Applied biosystems) was used following the manufacturer´s instructions to perform direct sequencing in both directions on 3500 Genetic Analyser (Applied Biosystems). Sequence analysis was carried out using the SeqA6.0 software (Applied Biosystems). Sequences were compared to the published reference sequence of the *JAK2* gene, NCBI (National Centre of Biotechnology Information): (NG 009904) by BLAST (Basic Local Alignment Search Tool).

Statistical methods
Overall survival (OS), complete remission (CR), and disease-free survival (DFS) were calculated according to Cheson BD et al. [32]. Numerical data were expressed as mean and standard deviation or median and range as appropriate. Pearson's Chi-square test or Fisher's exact test was performed to examine the degree of association between categorical variables and the Mann-Whitney U test for continuous variables. Correlation between numerical variables was conducted using the Spearman's Rho method. Kaplan-Meier method was employed for survival analysis and the survival curves were compared using the log-rank test. The receiver operating characteristic (ROC) curve was used for the prediction of the cutoff value of CRLF2. All tests were two-tailed. A p-value < 0.05 was considered statistically significant. Analyses were carried out using IBM SPSS® Statistics version 22.

RESULTS

Patients´ characteristics
Our study included 87 de novo BCP-ALL pediatric patients, 53 males and 34 females, with a median age of 5 years (2 months - 17 years). The clinical and laboratory features of the studied ALL patients are illustrated in Table 2.

CRLF2 expression
CRLF2 expression was detected by flow cytometry in our study group of BCP-ALL cases, and 80 age and gender matched, potentially healthy control subjects. No correlations were found between CRLF2 expression and other numerical parameters including total leucocytic count (TLC), hemoglobin, platelets, PB blasts percentage, and BMA blasts percentage for the whole group of studied ALL patients.
CRLF2 overexpression (OE) was identified in 26/87 patients (29.9%) (Figure 3), and low expression was detected in 70.1% (Figure 4). By comparing the clinical and laboratory features of patients with high and low CRLF2 expression, thrombocytopenia showed statistically significant association with the low-expression group as compared to the high-expressing one (74.3% vs. 25.7%, $p = 0.041$), respectively. However, no significant differences regarding other parameters were observed, Table 3. The detailed characterization of each case within the CRLF2 OE group ($n = 26$) is provided in Table 4.

***JAK2* mutation (R683)**
Sequence analysis was done for 23/26 patients (adequate DNA samples) with high CRLF2 OE. It revealed that 2/23 (8.7%) carried a mutation at *JAK2* R683. One patient had a mutation in c.2047 A>G (p.R683G) (Figure 5). The patient was a male with (pre-B) phenotype, CD34 positive, National Cancer Institute standard risk (NCI-SR), hyperdiploidy and in complete remission until the end of our study (MRD day 15 was 0.03%; MRD day 42 was < 0.01%). The second patient had a mutation in c.2049 A>T (p.R683S) (Figure 6). The patient was a male with (common-ALL) phenotype, CD34 positive, NCI-high risk (HR), normal DNA index and achieved complete remission on day 15 (MRD day 15

Table 1. Comparison of CRLF2 expression between ALL group and control group.

CRLF2 expression (MFI)	Mean	Median	Range	p-value
ALL group (n = 87)	3.4	2.8	1.0 - 10.6	< 0.001
Control group (n = 80)	2.2	2.1	1.3 - 3.7	

CRLF2 - Cytokine receptor like factor2, MFI - Mean fluorescence intensity.

Table 2. Clinical and laboratory features of the studied ALL patients.

Parameter	Patients (n = 87)
Age (years) *	5 (2 months - 17 years)
< 10 **	65 (74.7%)
≥ 10 **	22 (25.3%)
Gender **	
Males	53 (60.9%)
Females	34 (39.1%)
NCI risk classification **	
Standard risk (SR)	54 (62%)
High risk (HR)	33 (38 %)
Hemoglobin *** (gm/dL)	8.1 ± 2.1 (3.5 - 13.8) 7.9
≥ 10 g/dL **	16 (18.4)
< 10 g/dL **	71 (81.6)
TLC *** (x 10^3/mm^3)	55.6 ± 108.2 (0.7 - 719) 19.7
≥ 50 (x 10^3/mm^3) **	21 (24.1)
< 50 (x 10^3/mm^3) **	66 (75.9)
Platelets *** (x 10^3/mm^3)	53.0 ± 58.4 (5 - 267) 29
≥ 100 (x 10^3/mm^3) **	13 (14.9)
< 100 (x 10^3/mm^3) **	74 (85.1)
CD34 expression ** (n = 84)	
Negative	31 (36.9)
Positive	53 (63.1)
DNA index **	
≥ 1.16	16 (18.4)
< 1.16	71 (81.6)
Molecular findings **	
No recurrent translocation	72 (82.8)
t(12;21)	9 (10.3)
t(1;19)	4 (4.5)
t(4;11)	2 (2.2)

TLC - Total leucocytic count, NCI - National Cancer Institute.
*** - Shown as the median value with the range in between brackets.**
**** - Shown as number of cases with percentages in between brackets.**
***** - Shown as mean ± SD (range).**

Table 3. Comparison of the clinical and laboratory features between high and low-CRLF2 expression groups.

Parameter	Low (n = 61)	High (n = 26)	p-value
Age (years) *	5 (0.2 - 17)	6 (1 - 17)	0.367
≤ 5 years **	37 (77.1)	11 (22.9)	0.346
6 - 9 years **	10 (58.8)	7 (41.2)	
10 - 14 years **	9 (69.2)	4 (30.8)	
15 - 18 years **	5 (55.6)	4 (44.4)	
Gender **			
Males	38 (71.7)	15 (28.3)	0.687
Females	23 (67.6)	11 (32.4)	
NCI risk classification **			
Standard risk (SR)	39 (72.2)	15 (27.8)	0.583
High risk (HR)	22 (66.7)	11 (33.3)	
TLCX $10^3/mm^3$ *	19.8 (0.7 - 719)	14.2 (1.7 - 187)	0.610
< 50 (x $10^3/mm^3$) **	47 (71.2)	19 (28.8)	0.692
≥ 50 (x $10^3/mm^3$) **	14 (66.7)	7 (33.3)	
Hb g/dL *	7.9 (3.5 - 12.2)	7.9 (5.1 - 13.8)	0.633
< 10 g/dL **	50 (70.4)	21 (29.6)	0.895
≥ 10 g/dL **	11 (68.8)	5 (31.3)	
PLT (x $10^3/mm^3$) *	28 (7 - 267)	37.5 (5 - 240)	0.875
< 100 (x $10^3/mm^3$) **	55 (74.3)	19 (25.7)	0.041
≥ 100 (x $10^3/mm^3$) **	6 (46.2)	7 (53.8)	
DNA index **			
1 - 1.15	52 (73.2)	19 (26.8)	0.180
≥ 1.16	9 (56.3)	7 (43.8)	
ETV6-RUNX1 fusion **	7 (77.8)	2 (22.2)	[a]
E2A-PBX1 fusion **	4 (100)	0 (0)	[a]
MLL/AF4 fusion **	1 (50)	1 (50)	[a]

NCI - National Cancer Institute, TLC - total leucocytic count, Hb - haemoglobin, PLT - platelets.
*** - Shown as the median value with the range in between brackets.**
**** - Shown as number of cases with percentages in between brackets.**
***** - Shown as mean ± SD (range) median.**
[a] - No p-value due to small number of cases within subgroup.

was 0.03%). The patient relapsed in week 10 and another induction cycle was started, but the patient passed away in week 12 of treatment, as shown in Table 4.

Treatment outcome

Response to induction treatment

In our study, 75/ 87 patients (86.2%) completed 15 days of therapy. Ten patients passed away before day 15 of induction and 2 patients were treated outside our institute. Of the patients, 90.7% (68/75) achieved CR while 7/75 (9.3%) did not. The response of the studied cases to induction treatment with respect to CRLF2 expression level is demonstrated in Table 5. There was no significant difference in the CR rate between the two groups (p = 1.0).

Minimal residual disease (MRD)

Day 15

The response to treatment was assessed on day 15 by flow cytometry for 55/75 (73.3%) according to the availability of samples and the presence of LAIPs detected at diagnosis. The MRD response on day 15 for the high and low CRLF2-expressing groups is illustrated in Table 6. There was no significant difference in MRD response on day 15 between the two groups (p = 0.588).

Day 42

MRD on day 42 was done for 48/55 patients (according to the availability of samples). The distribution of MRD response on day 42 is provided in Table 6. There was

Table 4. Detailed clinical characteristics of cases with CRLF2 over-expression (n = 26).

Serial	MFI	Age years	TLC (x 10^3/mm^3)	NCI risk	CD34	Recurrent cytogenetic abnormality	MRD d 15 %	MRD d 42 %	JAK2 R683	DFS months	OS months
1	3.8	10	183.6	HR	positive	no	NA	NA	Wild	32.5	33 m+
2	4.6	2	46.7	SR	positive	no	NA	NA	Wild	3 d, death in CR	19 d
3	4	1.5	134.2	HR	positive	no	= 0.01	NA	Wild	8 d, death in CR	26 d
4 *	5.4	12	68	HR	positive	no	NA	NA	Wild	NA, death	21 d
5 **	3.9	15	4.2	HR	positive	no	NA	NA	Wild	31.5 m+	32.5 m+
6	5.4	13	24	HR	positive	no	38	NA	Wild	Not in CR, death	26 d
7	6.7	8	5.4	SR	positive	hyperdiploidy	NA	NA	ND	NA	NA
8	4.3	8	6.7	SR	positive	hyperdiploidy	NA	= 0.02	Wild	16.5 m, death in CR	17 m
9	10.3	2	41.7	SR	positive	hyperdiploidy	< 0.01	< 0.01	Wild	28 m+	29 m+
10	6.8	5	14.3	SR	positive	hyperdiploidy	< 0.01	< 0.01	Wild	23 m+	23.5 m+
11	9	4	2.6	SR	negative	t(12;21)	= 0.04	< 0.01	Wild	13 m+	15 m+
12	8.1	2	5.7	SR	negative	no	NA	NA	Wild	NA, death	7 d
13	3.8	2	3	SR	negative	no	= 0.2	= 0.07	Wild	13 m+	14 m+
14	8.2	4	75.6	HR	positive	no	= 2.6	< 0.01	Wild	13 m+	14 m+
15 ^	3.8	3	2.2	SR	positive	no	= 0.07	< 0.01	Wild	13 m+	14 m+
16	4.7	5.5	7.9	SR	negative	no	= 0.12	< 0.01	Wild	13 m+	13.5 m+
17	4.3	2.5	103	HR	positive	hyperdiploidy	= 0.1	NA	Wild	11 d, death in CR	1.5 m
18	5.4	6	3.6	SR	negative	t(12;21)	= 0.3	< 0.01	Wild	12.5 m, relapse	13.5 m+
19	10.6	17	3.5	HR	positive	no	NA	NA	Wild	11 m+	12 m+
20	5.5	6	5.7	SR	positive	hyperdiploidy	= 0.03	< 0.01	c.2047A>G P.R683G	11.5 m+	12 m+
21	4.7	7	14.1	SR	positive	hyperdiploidy	= 3.5	= 0.05	ND	10 m+	10.5 m+
22	5	16	1.7	HR	positive	no	= 1.06	= 0.01	ND	9.5 m+	10 m+
23	4.8	7	29.2	SR	positive	t(4;11)	= 0.57	NA	Wild	8.5 m+	9 m+
24	6.5	1	37.4	SR	positive	no	= 0.06	< 0.01	Wild	8 m+	8.5 m+
25 **	3.8	13	187	HR	positive	no	= 0.49	= 0.03	Wild	7.5 m+	8 m+
26	5.7	17	53.5	HR	positive	no	= 0.03	NA	c.2049A>T P.R683S	2.5 m relapse, death	3 m

*** - Aberrant CD13 expression.**
**** - Aberrant CD33 expression.**
^ - Down syndrome.
NCI - National Cancer Institute, SR - standard risk, HR - high risk, TLC - total leucocytic count, MFI - mean fluorescence intensity, MRD - minimal residual disease, OS - overall survival, DFS - disease free survival, d - days, m - months, NA - not available.

Table 5. Response to induction treatment on day 15 based on CRLF2 expression (n = 75).

Criteria	CRLF2 high expression**	CRLF2 low expression**	p-value
CR	21 (30.9%)	47 (69.1%)	1.0
No CR	2 (28.6%)	5 (71.4%)	

CR - complete remission.
**** - Shown as number of cases with percentages in brackets.**

Table 6. Comparison between high and low CRLF2 expressing groups according to MRD status on day 15 and day 42.

Criteria	MRD day 15 ** (n = 55)	MRD day 42 ** (n = 48)
High CRLF2 expressing group	< 1%: 14 (31.1%) ≥ 1%: 4 (40%)	< 0.01%: 9 (32.1%) ≥ 0.01%: 5 (25%)
Low CRLF2 expressing group	< 1%: 31 (68.9%) ≥ 1%: 6 (60%)	< 0.01%: 19 (67.9%) ≥ 0.01%: 15 (75%)
p-value	0.588	0.591

MRD - minimal residual disease. ** - Shown as number of cases with percentages in between brackets.

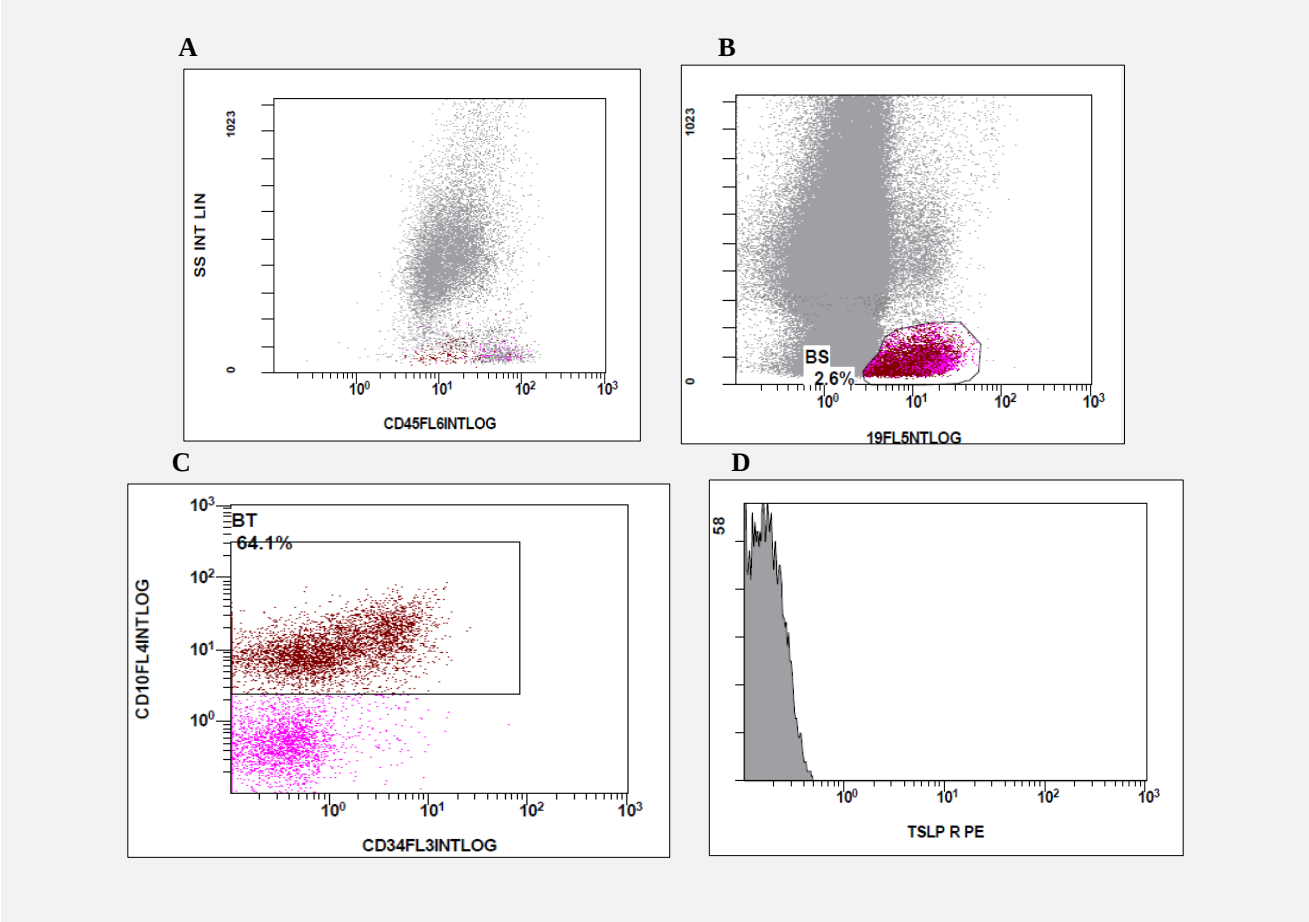

Figure 1. Demonstration of CRLF2 expression on hematogones in a case diagnosed as neuroblastoma.

The histograms illustrate (A) Side scatter versus CD45, (B) Side scatter versus CD19, (C) Sequential gating on CD10 & CD34 population, (D) CRLF2 expression gated on CD10, CD19 and CD34.

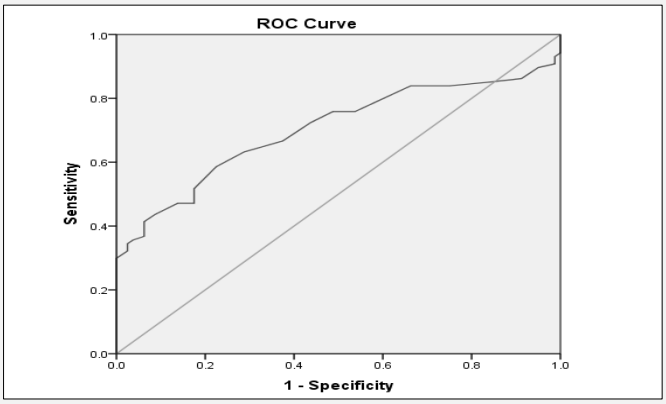

Figure 2. Receiver operating characteristic curve (ROC) curve analysis showing the diagnostic performance of MFI for discriminating patients from controls (area under curve = 0.703).

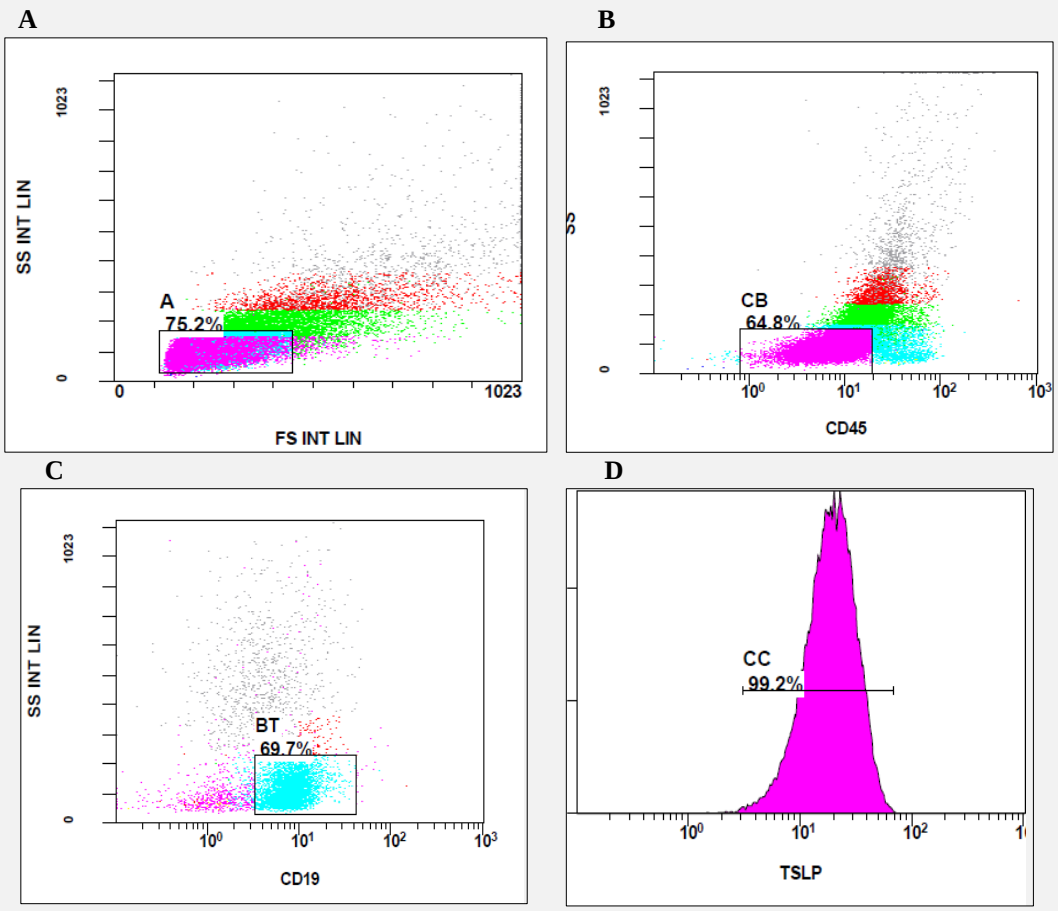

Figure 3. Flow cytometric analysis for a case with CRLF2 overexpression.

(A) Forward scatter versus side scatter, (B) Side scatter versus CD45 flow plot showing blast gate (dim CD45/low side scatter), (C) Side scatter versus CD19, (D) MFI of CRLF2 expression gated on CD45 dim & CD19 positive blasts = 10.6.

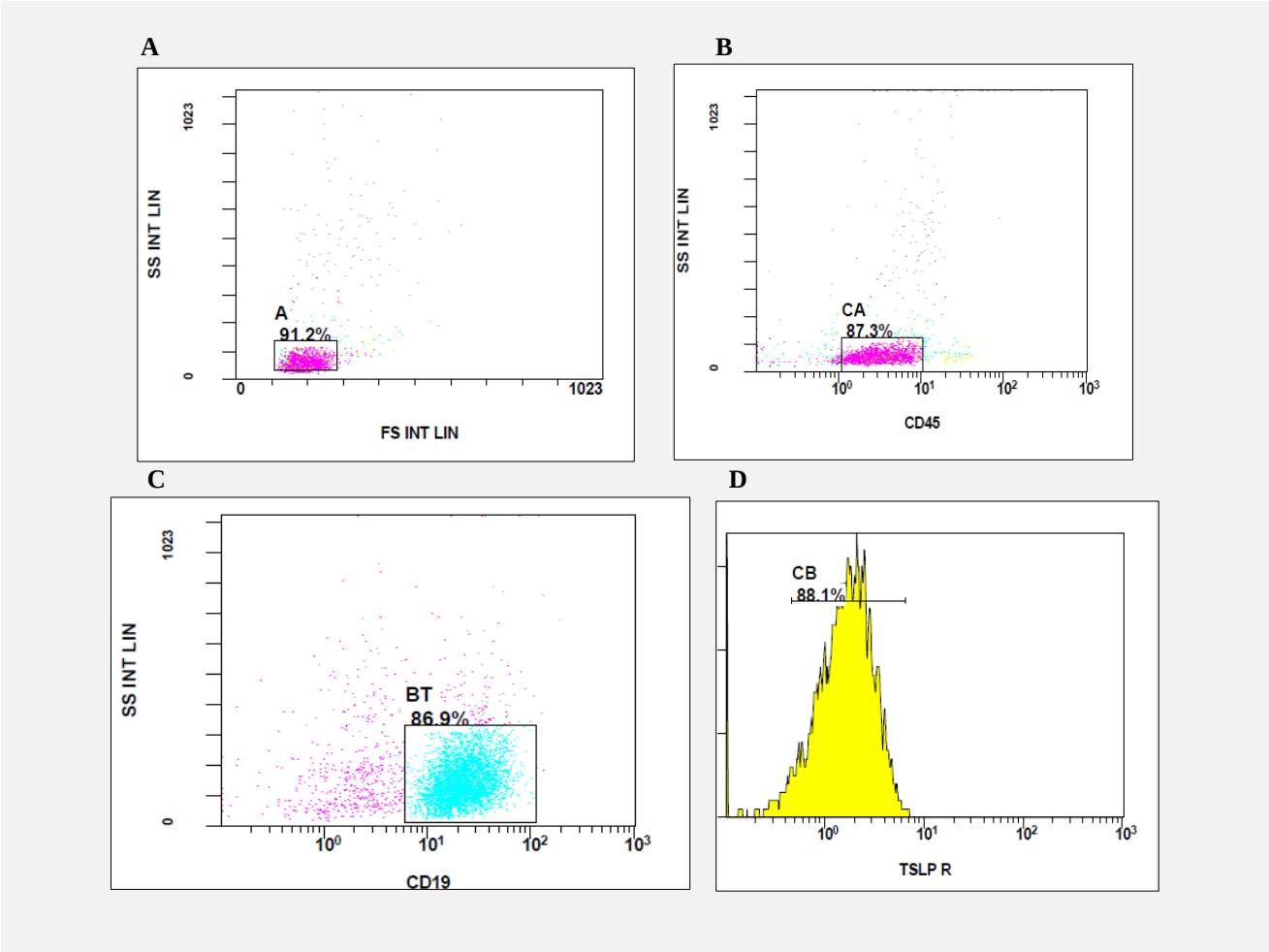

Figure 4. Flow cytometric analysis for a case with low CRLF2 expression.

(A) Forward scatter versus side scatter, (B) Side scatter versus CD45 flow plot showing blast gate (dim CD45/low side scatter), (C) Side scatter versus CD19, (D) MFI of CRLF2 expression gated on CD45 dim &CD19 positive blasts = 2.0.

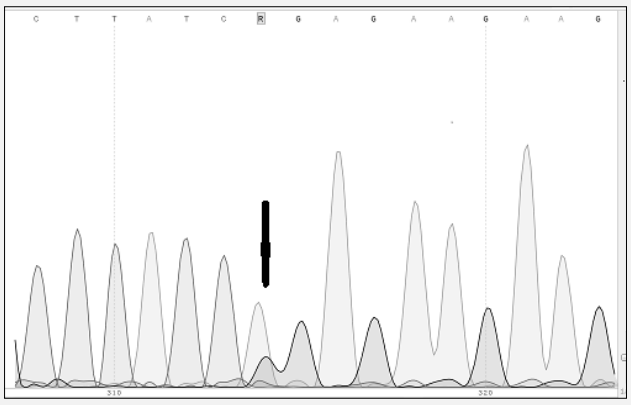

Figure 5. Sequence analysis showing mutation in c.2047 A>G (P.R683G).

The A to G substitution is shown by an arrow.

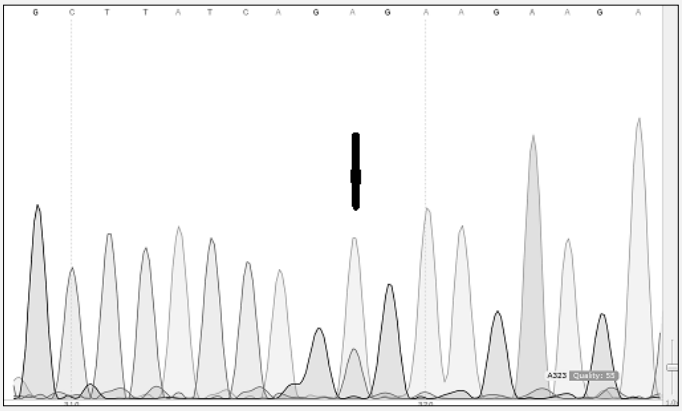

Figure 6. Sequence analysis showing mutation in c.2049 A>T (P.R683S).

The A to T substitution is shown by an arrow.

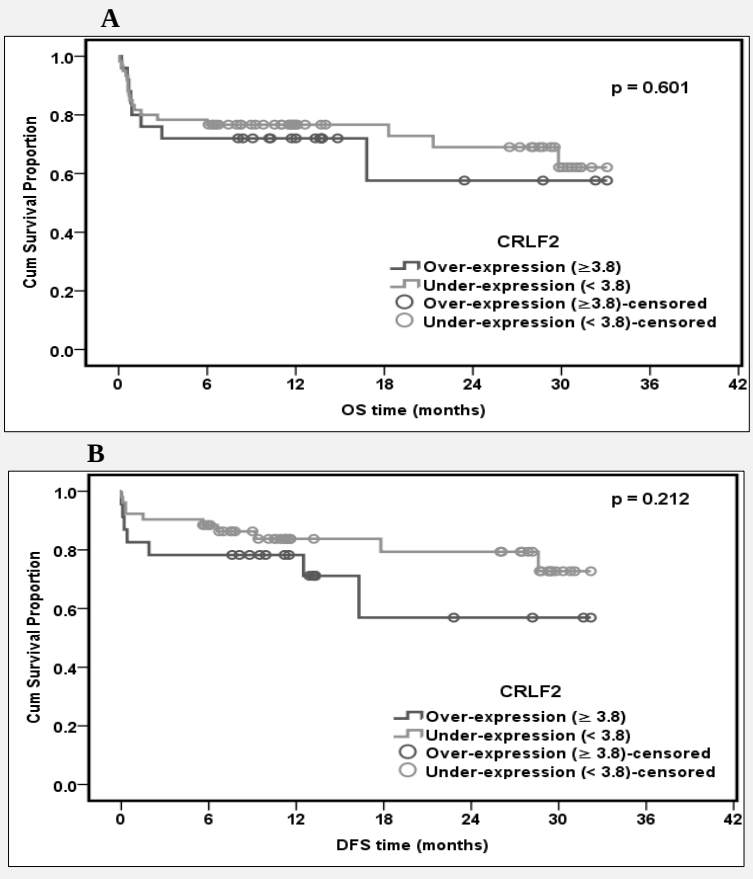

Figure 7. A. Comparison of overall survival (OS) in patients with high CRLF2 expression and low CRLF2 expression (p = 0.601), B. Comparison of disease-free survival (DFS) in patients with high CRLF2 expression and low CRLF2 expression (p = 0.212).

no significant association between MRD response on day 42 and CRLF2 expression status (p = 0.591).

Survival analysis

Overall survival (OS)

The overall survival analysis included 85 cases, as two patients were treated outside our institute. The median follow-up period was 11.5 months. The cumulative overall survival (COS) in 12 months was 75.3%, while it was 60.7% at the end of the study (33.1 months).

Mortality was reported in 25 of our patients; the early deaths (first 42 days of treatment) occurred in 18 patients mostly due to overwhelming sepsis. Upon comparison of OS in patients with high and low CRLF2 expression, no significant differences were observed in the COS status at 12 months between high and low expressing groups (72% vs. 76.7%; p = 0.601), respectively, as illustrated in Figure 7A.

Out of the remaining 7 deaths, two patients had CRLF2 overexpression. In one patient, relapse with disease progression was the main cause of death, and the other one died in remission with infection related mortality. The remaining 5 deaths occurred in the low CRLF2 expression group. Relapse with disease progression was the main cause of death in 2 patients while the other 3 patients died in remission due to septicemia.

Disease free survival (DFS)

The median follow-up period was 11.3 months. The DFS at 12 months was 82.2%, while at the end of the study (32.2 months) it was 67.7%. No significant differences were observed when comparing 1 year DFS of high and low CRLF2-expressing groups (78.1% vs. 83.8%; p = 0.212) respectively, as shown in Figure 7B. Out of the 75 patients with available data, relapse was reported in 5 patients, and two patients in the CRLF2 overexpression group had very early relapse (< 18 months from diagnosis), while the other three relapsed patients in the low CRLF2 expression group had very early relapse in two patients and early relapse (> 18 month - 36 month from diagnosis) in one patient.

DISCUSSION

In this study, we have reported on the incidence and prognostic value of CRLF2 overexpression in pediatric BCP-ALL patients lacking *BCR-ABL* fusion transcript. CRLF2 overexpression was detected in 26/87 (29.9%) of our patients using MFI set at 3.8. However, lower frequencies have been reported by previous studies [33, 29] which can be attributed to different methods used to set the cutoff of CRLF2 overexpression by flow cytometry. Previous reports [26,28,34] have detected (17.5%; 18%;19%) frequencies of CRLF2 overexpression, respectively, in pediatric BCP-ALL patients using quantitative RT-PCR. A standardized method for setting the cutoff of CRLF2 overexpression is necessary for the upcoming studies.

In agreement with previous studies [15,27-29], we did not demonstrate any significant differences in terms of age, gender, or NCI risk groups between high and low CRLF2 expressing patients. On the other hand, Dou et al. [34] reported that the median age among patients with high CRLF2 expression was younger than those with low CRLF2 expression in their whole unselected cohort.

In our study, thrombocytopenia showed statistically significant association with low CRLF2 expression as compared to the high expression group (74.3% vs. 25.7%, p = 0.041, respectively). In contrast, Jiang et al. [35] reported that there was no significant difference in the platelet count among children with low and high CRLF2 expression.

We did not find any significant differences regarding TLC count and hemoglobin between the two groups and this goes in concordance with previous studies [28,29]. In the same context, Jiang et al. [35] reported that hemoglobin concentration was similar between children with low and high CRLF2 expression. However, TLC was different between these two groups (p < 0.0001). On the contrary, Dou et al. [34] reported that the median TLC count was lower in patients with CRLF2 overexpression compared to those with low CRLF2 expression (6.2×10^3/mL vs. 8.6×10^3/mL, p = 0.009, respectively) in their whole unselected cohort.

Among the high CRLF2 expression group (n = 26), one patient (3.8%) was positive for t(4;11) and 2/26 (7.7%) patients were t(12;21) positive. This goes in the same line with Pastorczak et al. [29]. On the contrary, other studies [27,28] reported that all high CRLF2 pediatric patients were negative for the recurrent chromosomal translocations t(4;11) and t(12;21).

In our cohort, 7/26 (26.9%) patients with CRLF2 overexpression had high hyperdiploid karyotype (DNA index ≥ 1.16). This is in agreement with Palmi et al. [27] who reported that 5/22 (22.7%) high CRLF2 patients were classified as high hyperdiploid. However, Pastorczak et al. [29] found that among CRLF2 positive pediatric patients, 13.8% had high hyperdiploid karyotype.

As a part of this study, we assessed *JAK2* R683 mutations for 23 patients with high CRLF2 expression. Direct sequence analysis showed that only 2/23 (8.7%) carried the mutant *JAK2* R683. One patient had a mutation in c.2047 A>G (p.R683G), and the other one had a mutation in c.2049 A>T (p.R683S). In concordance with our results, Cario et al. [15] detected *JAK2* R683G mutation in 1/49 patients (2%) and *JAK2* R683S in 3/49 (6.1%) pediatric patients with high CRLF2 expression. Further, Schmäh et al. [36] detected *JAK2 R*683G mutation in 9 (9.9%) cases and *JAK2* R683S mutation in 3 (3.3%) cases, out of 91 high CRLF2 expressing cases. Conversely, Dou et al. [34] did not detect *JAK2* mutations in the whole Chinese cohort of 271 children.

Regarding response to induction chemotherapy, we did not find any significant difference in achieving complete remission on day 15 between high and low CRLF2 expressing patients (p = 1.0) and this goes in line with a

previous report [37] that did not find a difference in response to prednisolone on day 8 between the two groups. Moreover, no correlation was detected between MRD levels on day 15 and day 42 and CRLF2 expression level. This is in agreement with van der Veer et al. [5]. In contrast to our results, Pastorczak et al. [29] found that CRLF2 positive patients showed lower median MRD on day 15 than CRLF2 negative patients (0.01% vs. 0.45%, respectively, p = 0.001). However, the median MRD on day 33 was similar for both groups. Palmi et al. [27] and van der Veer et al. [5] reported that patients with CRLF2 overexpression were more sensitive to treatment based on MRD levels.

In the present study, we made clear that CRLF2 overexpression did not have an adverse effect on the outcome of our patients regarding OS and DFS which is consistent with previous studies [5,28]. On the contrary, the US Children's Oncology Group (COG) study demonstrated a poor prognosis in high risk B-ALL with CRLF2 overexpression [26]. Also, Dou et al. [34] identified CRLF2 overexpression as an independent prognostic parameter for shorter survival in unselected pediatric B-ALL.

In conclusion, our study showed that the levels of CRLF2 expression was not an adverse prognostic parameter in pediatric BCP-ALL. However, patients with CRLF2 overexpression may harbor JAK2 mutations, presenting a subset that can benefit from specific targeted approaches thus paving the way towards individualized therapy. In addition, the usage of CRLF2 expression in the monitoring of minimal residual disease seems to be an area of interest for further evaluation.

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Declaration of Interest:
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References:

1. Hunger S, Mullighan C. Acute Lymphoblastic Leukemia in Children. N Engl J Med 2015;373(16):1541-52 (PMID: 26465987).

2. Schmiegelow K, Forestier E, Hellebostad M, et al. Long term results of NOPHO ALL-92 and ALL-2000 studies of childhood acute lymphoblastic leukemia. Leukemia 2010;24(2):345-54 (PMID: 20010622).

3. Biondi A, Cazzaniga G. Novel clinical trials for pediatric leukemias: lessons learned from genomic analyses. Hematology Am Soc Hematol Educ Program. 2013;2013:612-9 (PMID: 24319240).

4. Borowitz MJ, Chan JKC, Downing JR, et al. B-lymphoblastic leukemia/lymphoma with recurrent cytogenetic abnormalities. In: Swerdlow SH, Campo E, Harris NL, et al, eds. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Revised 4th ed. Lyon, France: IARC; 2017:208.

5. van der Veer A, Waanders E, Pieters R, et al. Independent prognostic value of BCR-ABL1-like signature and IKZF1 deletion, but not high CRLF2 expression, in children with B-cell precursor ALL. Blood 2013;122(15):2622-9 (PMID: 23974192).

6. Herold T, Baldus CD, Gökbuget N. Ph-like acute lymphoblastic leukemia in older adults. N Engl J Med 2014;371(23):2235 (PMID: 25470705).

7. Boer JM, Marchante JR, Evans WE, et al. BCR-ABL1-like cases in pediatric acute lymphoblastic leukemia: a comparison between DCOG/Erasmus MC and COG/St. Jude signatures. Haematological 2015;100(9):e354-7 (PMID: 26045294).

8. Tasian SK, Loh ML, Hunger SP. Philadelphia chromosome-like acute lymphoblastic leukemia. Blood 2017;130(19):2064-72 (PMID: 28972016).

9. Roberts KG, Li Y, Payne-Turner D, et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. N Engl J Med 2014; 371(11):1005-15 (PMID: 25207766).

10. Russell LJ, Capasso M, Vater I, et al. Deregulated expression of cytokine receptor gene, CRLF2, is involved in lymphoid transformation in B-cell precursor acute lymphoblastic leukemia. Blood 2009;114(13):2688-98 (PMID: 19641190).

11. Maude SL, Tasian SK, Vincent T, et al. Targeting JAK1/2 and mTOR in murine xenograft models of Ph-like acute lymphoblastic leukemia. Blood 2012;120(17):3510-8 (PMID: 22955920).

12. Chiaretti S, Brugnoletti F, Messina M, et al. *CRLF2* overexpression identifies an unfavourable subgroup of adult B-cell precursor acute lymphoblastic leukemia lacking recurrent genetic abnormalities. Leuk Res 2016;41:36-42 (PMID: 26754556).

13. Konoplev S, Lu X, Konopleva M, et al. CRLF2-Positive B-Cell acute lymphoblastic leukemia in adult patients: a single institution experience. Am J Clin Pathol 2017;147(4):357-63 (PMID: 28340183).

14. Moorman AV, Schwab C, Ensor HM, et al. IGH$^{@}$ translocations, CRLF2 deregulation, and microdeletions in adolescents and adults with acute lymphoblastic leukemia. J Clin Oncol 2012; 30(25):3100-8. (PMID: 22851563).

15. Cario G, Zimmermann M, Romey R, et al. Presence of the P2RY8-CRLF2 rearrangement is associated with a poor prognosis in non-high-risk precursor B-cell acute lymphoblastic leukemia in children treated according to the ALL-BFM 2000 protocol. Blood 2010;115(26):5393-7 (PMID: 20378752).

16. Yoda A, Yoda Y, Chiaretti S, et al. Functional screening identifies CRLF2 in precursor B-cell acute lymphoblastic leukemia. Proc Natl Acad Sci USA 2010;107(1):252-7 (PMID: 20018760).

17. Jain N, Roberts KG, Jabbour E, et al. Ph-like acute lymphoblastic leukemia: a high-risk subtype in adults. Blood 2017;129 (5):572-81 (PMID: 27919910).

18. Conant JL, Czuchlewski DR. BCR-ABL1-like B-lymphoblastic leukemia/lymphoma: Review of the entity and detection methodologies. Int J Lab Hematol 2019;41(Suppl.1):126-30 (PMID: 31069976).

19. Mullighan CG, Collins-Underwood JR, Phillips LA, et al. Rearrangement of CRLF2 in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia. Nat Genet 2009;41(11): 1243-6 (PMID: 19838194).

20. Stark GR, Darnell JE Jr. The JAK-STAT pathway at twenty. Immunity 2012;36(4):503-14 (PMID: 22520844).

21. Reshmi SC, Harvey RC, Roberts KG, et al. Targetable kinase gene fusions in high-risk B-ALL: a study from the Children's Oncology Group. Blood 2017;129 (25):3352-61 (PMID: 28408464).

22. Jain N, Roberts KG, Jabbour EJ, et al. High-Risk Subtype of Ph-like Acute Lymphoblastic Leukemia (ALL) in Adults: Dismal Outcomes of CRLF2+ ALL Patients Treated with Intensive Chemotherapy. Blood 2016;128(22):1082. (https://doi.org/10.1182/blood.V128.22.1082.1082).

23. Roberts KG, Gu Z, Payne-Turner D, et al. High frequency and poor outcome of Philadelphia chromosome-like acute lymphoblastic leukemia in adults. J Clin Oncol 2017;35(4):394-401 (PMID: 27870571).

24. Tasian SK, Doral MY, Borowitz MJ, et al. Aberrant STAT5 and PI3K/mTOR pathway signaling occurs in human CRLF2-rearranged B-precursor acute lymphoblastic leukemia. Blood 2012; 120:833-42 (PMID: 22685175).

25. Harvey RC, Mullighan CG, Chen IM, et al. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. Blood 2010; 115(26):5312-21 (PMID: 20139093).

26. Chen IM, Harvey RC, Mullighan CG, et al. Outcome modeling with CRLF2, IKZF1, JAK, and minimal residual disease in pediatric acute lymphoblastic leukemia: A Children's Oncology Group study. Blood 2012;119(15):3512-22 (PMID: 22368272).

27. Palmi C, Vendramini E, Silvestri D, et al. Poor prognosis for P2RY8-CRLF2 fusion but not for CRLF2 over-expression in children with intermediate risk B-cell precursor acute lymphoblastic leukemia. Leukemia 2012;26(10):2245-53 (PMID: 22484421).

28. Yano M, Imamura T, Asai D, et al. An overall characterization of pediatric acute lymphoblastic leukemia with CRLF2 overexpression. Genes Chromosomes Cancer 2014;53(10):815-23 (PMID: 24935070).

29. Pastorczak A, Sedek L, Braun M, et al. Surface expression of cytokine receptor-like factor 2 increases risk of relapse in pediatric acute lymphoblastic leukemia patients harboring IKZF1 deletions. Oncotarget 2018;9(40):25971-82 (PMID: 29899835).

30. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. N Engl J Med 2006;354(2):166-78 (PMID: 16407512).

31. Asai D, Imamura T, Suenobu S, et al. IKZF1 deletion is associated with a poor outcome in pediatric B-cell precursor acute lymphoblastic leukemia in Japan. Cancer Med 2013;2(3):412-9 (PMID: 23930217).

32. Cheson BD, Bennett JM, Kopecky KJ, et al. Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. J Clin Oncol 2003;21(24):4642-9 (PMID: 14673054).

33. Bugarin C, Sarno J, Palmi C, et al. Fine tuning of surface CRLF2 expression and its associated signaling profile in childhood B-cell precursor acute lymphoblastic leukemia. Haematologica 2015; 100(6):e229-32 (PMID: 25862705).

34. Dou H, Chen X, Huang Y, et al. Prognostic significance of P2RY8-CRLF2 and CRLF2 overexpression may vary across risk subgroups of childhood B cell acute lymphoblastic leukemia. Genes Chromosomes Cancer 2017;56(2):135-46 (PMID: 27637012).

35. Jiang M, Zou X, Lu L. Potential efficacy and prognosis of silencing the CRLF2-mediated AKT/mTOR pathway in pediatric acute B-cell lymphoblastic leukemia. Oncol Rep 2019;41(2):885-94 (PMID: 30535452).

36. Schmäh J, Fedders B, Panzer- Grümayer R, et al. Molecular characterization of acute lymphoblastic leukemia with high CRLF2 gene expression in childhood. Pediatr Blood Cancer 2017; 64(10). (PMID: 28371317).

37. Yamashita Y, Shimada A, Yamada T, et al. IKZF1 and CRLF2 gene alterations correlate with poor prognosis in Japanese BCR-ABL1-negative high-risk B-cell precursor acute lymphoblastic leukemia. Pediatr Blood Cancer 2013;60(10):1587-1592 (PMID: 23804397).