









Original article

Multiparametric flow cytometry directing the evaluation of CRLF2 rearrangements and JAK2 status in pediatric B cell precursor acute lymphoblastic leukemia

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ABSTRACT

Introduction: This study aimed to determine whether cytokine receptor-like factor 2 (CRLF2) antigen expression evaluated using multiparametric flow cytometry (MFC) could predict the genotype of CRLF2 and Janus kinase 2 (JAK2) status for application in the diagnosis of pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL).

Methods: A total of 321 BCP-ALL bone marrow samples were collected, 291 at diagnosis and 13 at first relapse, while 17 samples were excluded due to low cellular viability. The CRLF2 antigen expression was evaluated using flow cytometry (percentage of positivity and median fluorescence intensity [MFI]). The CRLF2 transcript levels were assessed via quantitative reverse transcription polymerase chain reaction using SYBR Green. The CRLF2 rearrangements (CRLF2-r) were identified using the CRLF2 break-apart probe via fluorescence in situ hybridization. Sanger sequencing was performed to identify the JAK2 exon 16 mutations.

Results: We observed that 60 of the 291 cases (20.6%) presented CRLF2 antigen positivity, whereas the CRLF2 transcript overexpression was found in 19 of 113 cases (16.8%). The JAK2 mutation was found in four out of 116 cases (3.4%), all of which had CRLF2 $\geq 10\%$ of positive cells and intermediate or high MFI ($p < 0.0001$). In addition, in the 13 cases with the CRLF2-r, a positive correlation was found with the CRLF2 antigen intermediate (61.5%) MFI ($p = 0.017$). Finally, the CRLF2-positive antigen was identified in the BCP-ALL subclones.

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Conclusion: The identification of the CRLF2 antigen using the MFC, based on the percentage of positivity and MFI values, is a useful tool for predicting JAK2 mutations and CRLF2-r.

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Introduction

Childhood acute lymphoblastic leukemia (ALL) harbors genetic abnormalities regarded as established diagnostic and/or prognostic markers (approximately 70% of cases).¹ Integrating cytogenetic and genomic aberrations, such as hyperdiploidy, *ETV6-RUNX1*, *TCF3-PBX1*, *KMT2A-r*, *BCR-ABL1*, *iAMP21*, hypodiploidy and complex karyotypes, can define the prognosis of B-cell precursor ALL (BCP-ALL).² Patients without one of these abnormalities (approximately 30%) are referred to as B-other ALL and remain the subject of investigation due to high relapse rates and poor prognoses.³ Several techniques, from karyotyping to genomic landscape, have been used to assess biomarkers to unravel the B-other ALL genetic profiles. Among these biomarkers, special attention has been focused on BCP-ALL with cytokine receptor-like factor 2 (CRLF2) abnormalities and mutations in the Janus kinase (*JAK*)1/2 and interleukin 7 receptor alpha (*IL7R α*) genes.

The CRLF2, together with the *IL7R α* unit (CD127), forms a heterodimeric receptor for the cytokine thymic stromal lymphopoietin. Among the high-risk BCP-ALLs, the aberrant expression of the CRLF2/*IL7R α* and coexistence of mutations in genes of the *JAK-STAT* signaling pathway, such as the *JAK1/2* and *IL7R α* genes, can be found.^{4,5} Clinical trials have investigated the potential of JAK inhibitors in combination with chemotherapy in BCP-ALL with CRLF2-r/*JAK* pathway mutations.^{6–8}

The CRLF2 deregulation occurs in approximately 5 to 10% of the BCP-ALLs.² The CRLF2-r is associated with the CRLF2 antigen surface overexpression, rendering it suitable for detection using multiparametric flow cytometry (MFC).⁹ This technique allows for the identification of the CRLF2 antigen in subclones of BCP-ALL associated with disease relapse. Molecular tests to identify the CRLF2-r and mutations in the *JAK2* are time-consuming and expensive. The establishment of efficient parameters to define the CRLF2 cellular antigen overexpression could be an important improvement in the diagnosis with consequences in clinical practice.

In this context, this study aimed to explore whether the CRLF2 antigen expression tested using the MFC could predict the genotype status of the CRLF2 and *JAK2* in a reproducible and reliable manner and whether the CRLF2 antigen expression pattern could be applied as a first-line diagnostic setting for therapeutic decisions in BCP-ALL.

Materials and methods

Patients

A total of 321 bone marrow (BM) samples from 308 patients (0 – 18 years old) with BCP-ALL were collected. They were sent

to the Pediatric Hematology-Oncology Research Program, Research Center at the Instituto Nacional de Cancer, Rio de Janeiro, Brazil, as part of a multi-institutional collaborative research project (EMiLI, described in detail in the Supplemental Material). As described in Supplemental Figure 1, samples were collected at diagnosis prior to any oncological treatment ($n = 291$) and 13 sequential samples were collected according to the clinical treatment for ALL relapses. Samples with cellular viability of less than 70% were excluded ($n = 17$). Patients diagnoses were established according to the World Health Organization (WHO) classification using morphological, immunophenotypic, and cytogenetic molecular evaluations.¹⁰

Immunophenotyping CRLF2 staining

The immunophenotyping procedures were performed according to the modified EuroFlow Consortium.^{11,12} The specifications of the monoclonal antibody (MoAb) clones are shown in Supplemental Table 1.

The expression of the CRLF2 and CD127 in the following combination of MoAb was used: CD10 FITC/CD127 PE/CD45 PerCP-CY5.5/CD19 PE-CY7/CRLF2 APC. The BM samples (50 μ L) were incubated for 20 min at room temperature (RT) with the surface MoAb, according to previously defined titers. Subsequently, 2mL of the FACS Lysing Solution[®] (BD Biosciences, CA, USA) diluted 1:10 in Milli-Q water was added to the samples that were then incubated for 10 min at RT. The cells were then washed twice in phosphate-buffered saline (PBS)/azide 0.1% and the pellet was suspended in the PBS. At least 100,000 events per tube were acquired on the BD FACSCanto II[™] (BD Biosciences, CA, USA) and analyzed using the Infinicyt[™] software 1.8 (Cytognos, Salamanca, Spain).

Regarding the CRLF2 staining, the negative control was initially defined as the mature lymphocytes of the BM samples analyzed. Negative controls for the CD127 were established according to a previous study (Supplemental Figure 2a, b).^{11,12} A cutoff of 10% of positive cells for the CRLF2 and CD127 expression was used to define positive samples, as recommended by Dworzak et al.¹³ Subclones were defined as a minor subpopulation of blast cells with the CRLF2 expression clearly shifted to the right above the negative control, as exemplified in Figure 1b and according to a previous publication.⁹

Therefore, the CRLF2 median fluorescence intensity (MFI) of the positive cells was calculated for each sample as the difference between the MFI of CRLF2-positive blast cells and the MFI of total mature lymphocytes. The MFI was evaluated in samples determined as positive by the cutoff 10% CRLF2 ($\geq 10\%$), as well as in samples with a minor population with cellular expression of CRLF2 ($< 10\%$). We established three patterns of CRLF2 expression (low, intermediate or high) based on the frequency distribution of our cases, as shown in Supplemental Figure 2c. Briefly, samples with MFI values less

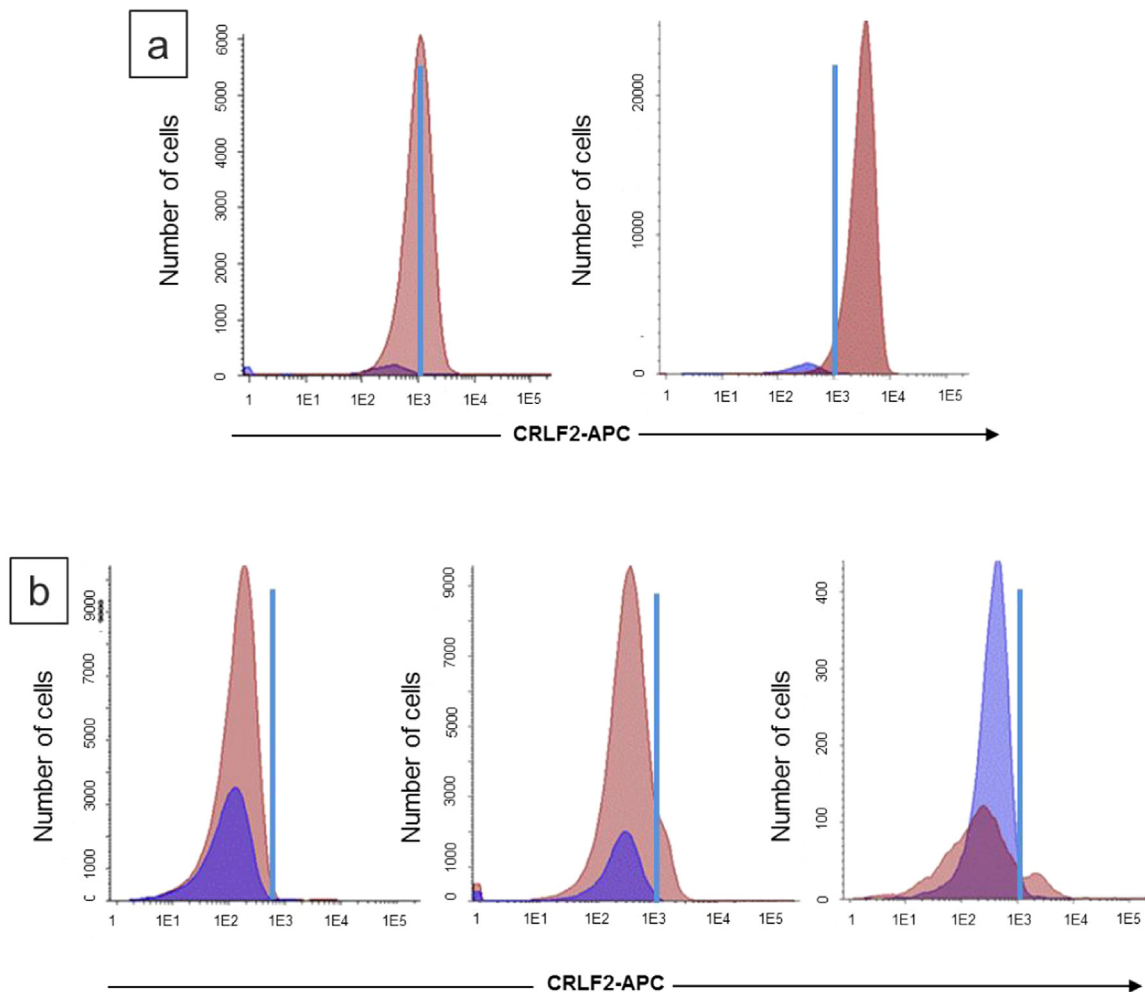


Figure 1–Main profiles of CRLF2 antigen expression in BCP-ALL blast cells. **(a)** Representative histograms of CRLF2 positive cases ($\geq 10\%$): moderately positive expression (left) and strongly positive expression (right). **(b)** Representative histograms of CRLF2-negative cases ($< 10\%$): completely negative curve (left), subclones with a shift to the right curve (middle) or a clear peak definition (right). The pink curves represent leukemic blasts; the blue curves represent the lymphocytes, used as negative controls.

than 1,500 were defined as low, those between 1,500 and 2,500 were defined as intermediate and those over 2,500 were defined as high.

Cytogenetic molecular tests

The BCP-ALL molecular subtypes, including *ETV6-RUNX1*, *TCF3-PBX1*, *KMT2A* rearrangement (*KMT2A-r*), and *BCR-ABL1*, were evaluated as previously described.¹⁴ Conventional karyotyping and/or DNA index (DNA-i) were performed to identify cell ploidy. DNA-i was determined by MFC and used as a proxy for hyperdiploidy, according to previous studies.¹⁵

The fluorescence in situ hybridization (FISH) was performed with the CRLF2 break-apart probe following the manufacturer's recommendations (CytoCell, Cambridge, UK) as the gold standard method. Using this probe, it was not possible to identify the genes involved in the translocation of the CRLF2.

We categorized three subgroups of BCP-ALL cases according to the presence of recurrent cytogenetic molecular

alterations (RCMA): (i) RCMA-negative: cases without hyperdiploidy (HeH), *ETV6-RUNX1*, *TCF3-PBX1*, *KMT2A-r*, *BCR-ABL1*, hypodiploidy and *iAMP21*; (ii) RCMA-positive cases characterized by the presence of one of the major aberrations, and; (iii) RCMA not defined: cases with missing information on cytogenetic molecular subtypes owing to insufficient material or technical failure.

CRLF2 transcript levels

The CRLF2 transcript levels were assessed in cDNA via the quantitative reverse-transcription polymerase chain reaction (RT-qPCR) using SYBR Green (Applied Biosystems, CA, USA), as previously described.¹⁶ Quantitative values were expressed using the mean $\Delta\Delta CT$. A melting curve was used to assess the reaction specificity. The relative gene expression (RGE) (indicated as fold change) was quantified using the $2^{-\Delta\Delta CT}$ method, as described by Palmi et al. 2012.¹⁷ The cutoff value to define high RGE was determined based on the outliers, with high CRLF2 RGE values

considered 10-fold above the median of all analyzed cases.

Sanger direct sequencing

The genomic DNA was obtained using a QIAamp® DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany), as recommended by the manufacturer. The JAK2 mutations (exon 16, R683) were detected using Sanger sequencing, as previously described.¹⁸

Statistical analyses

The Fisher's exact and chi-square tests were used to evaluate the distribution of categorical variables. The Mann–Whitney U test was used to compare continuous variables. Analyses were performed using the SPSS 18.0 (SPSS, Chicago, IL, USA) and PRISM software (PRISM GraphPad, CA, USA) and a minimum significance level of 5% was considered for all tests.

Results

Patient characteristics

At the initial diagnosis of BCP-ALL, 291 cases were evaluated and selected for analysis according to the inclusion and exclusion criteria. The demographic and clinical features of pediatric BCP-ALL patients according to the study design are shown in [Supplemental Figure 1](#).

Immunophenotyping CRLF2 antigen

We observed distinct CRLF2 antigen expression patterns in blast cells ([Figure 1](#)). In CRLF2 positive cases ($\geq 10\%$), two

patterns of expression were observed: (i) a moderate positivity pattern, in which a partial overlap with the negative control showed a shift to the right curve ([Figure 1a](#), left histogram), and; (ii) a strong CRLF2 positivity, in which most cells were above the negative control ([Figure 1a](#), right histogram). Additionally, three patterns of CRLF2 negative cases ($< 10\%$) were found: (i) blast cell curves overlapping the negative control ([Figure 1b](#), left histogram); (ii) a small proportion of blast cells with a shift to the right curve ([Figure 1b](#), middle histogram), and; (iii) the positive blast cell curve, with a clear peak definition ([Figure 1b](#), right histogram). Subclone blast cells expressing CRLF2 were observed in the last two patterns described above.

Given that the CD127 (IL7R α chain receptor) forms a heterodimeric receptor with the CRLF2, this receptor was also evaluated ([Supplemental Figure 2](#)). Overall, 74 of the 138 (53.6%) cases were CD127 positive. We did not observe a correlation between CD127 and CRLF2 expressions or statistically significant differences in the CD127 expression in the blast cells according to clinical and biological characteristics (data not shown).

Immunophenotyping genotyping association

The CRLF2 antigen expression according to the demographic, clinical and cytogenetic molecular characteristics of patients with BCP-ALL are summarized in [Table 1](#). Overall, 60 of the 291 cases (20.6%) were CRLF2 positive ($\geq 10\%$). Considering the distinct CRLF2 expression patterns, we also analyzed the MFI results. We found that 79 of the 291 cases (27.1%) presented an intermediate MFI and 25 of the 291 (8.6%) displayed a high MFI, but we did not find any statistical association with clinical-demographic or laboratory data.

Table 1 – CRLF2 antigen expression analyzed via MFC according to demographic, clinical and molecular characteristics of patients with BCP-ALL, Brazil 2014-2019.

	%CRLF2			p-value	MFI CRLF2			p-value
	Total	< 10%	$\geq 10\%$		Low	Interm.	High	
Age (years)				0.098				0.761
<1	12 (4.1)	12 (5.2)	0		8 (4.3)	3 (3.8)	1 (4.0)	
1-9	224 (77)	179 (77.5)	45 (75)		148 (79.1)	58 (73.4)	18 (72)	
≥ 10	55 (18.9)	40 (17.3)	15 (25)		31 (16.6)	18 (22.8)	6 (24)	
WBC ($\times 10^9/L$)				0.072				0.264
< 50	231 (79.4)	178 (77.1)	53 (88.3)		146 (78.1)	62 (78.5)	23 (92.0)	
≥ 50	60 (20.6)	53 (22.9)	7 (11.7)		41 (21.9)	17 (21.5)	2 (8.0)	
Cytogenetic molecular				0.003				0.278
RCMA ^a -positive	107 (36.8)	93 (40.3)	14 (23.3)		71 (38.0)	29 (36.7)	7 (28.0)	
RCMA-negative	101 (34.7)	69 (29.9)	32 (53.3)		65 (34.8)	23 (29.1)	13 (52.0)	
RCMA not defined	83 (28.5)	69 (29.9)	14 (23.3)		51 (27.3)	27 (34.2)	5 (20.0)	
CRLF2 expression ^b				0.004				0.594
High	19 (16.8)	12 (12.2)	7 (46.7)		7 (13.2)	9 (19.1)	3 (23.1)	
Low	94 (83.2)	86 (87.8)	8 (53.3)		46 (86.8)	38 (80.9)	10 (76.9)	
JAK2 Status ^b				< 0.0001				0.186
Mutated	4 (3.4)	0	4 (22.2)		0	3 (6.2)	1 (6.2)	
WT	112 (96.6)	98 (100)	14 (77.8)		52 (100)	45 (93.8)	15 (93.8)	
Total	291 (100)	231 (79.4)	60 (20.6)		187 (64.3)	79 (27.1)	25 (8.6)	

^a RCMA – recurrent cytogenetic molecular alterations.

^b CRLF2 gene expression and mutations in JAK2 were not performed in all cases due to specimen availability; Interm. – intermediate; WBC – white blood cell count; MFI – median fluorescence intensity; RGE – relative gene expression; WT – wild type.

The CRLF2 expression and CRLF2 antigen was tested in 113 patients. High CRLF2 RGE was found in 16.8% of cases (19/113). Among the 94 patients with low CRLF2 RGE, 86 had low CRLF2 antigen levels, demonstrating positive concordant associations ($p = 0.004$). The screening for JAK2 mutations was performed in a subgroup of 116 patients with concomitant CRLF2 antigen testing. The frequency of this alteration was 3.4% (4/116) (Table 1).

Cytogenetic molecular tests and associations

The cytogenetic molecular results and CRLF2 antigen expression are described in Table 1. The most common cytogenetic subtype in BCP-ALL was represented by the RCMA-positive group with mutually exclusive cytogenetic molecular aberrations ($n = 107$). The RCMA-negative ($n = 101$) and RCMA not defined ($n = 83$) groups presented a higher chance of finding CRLF2 aberrations. Indeed, the CRLF2 antigen positivity ($\geq 10\%$) among the RCMA-negative cases was 53.3%, whereas in the RCMA-positive cases, the CRLF2 positivity was 23.3% ($p = 0.003$). Among these final 14 patients, six presented with HeH, six with ETV6/RUNX1, one with TCF3/PBX1 and one with KMT2A-r. Interestingly, an association between the CRLF2 antigen ($\geq 10\%$) and low RGE was observed in the HeH group.

All 12 cases with the CRLF2 $< 10\%$ and high CRLF2 RGE (12.7%) found in the RCMA-positive group ($n = 5$) had aneuploidy (four HeH and one hypodiploid), whereas two and five cases were found in the RCMA-negative and RCMA not defined groups, respectively. Altogether, these data suggest that the CRLF2 is expressed in the HeH cytogenetic subtype at the protein and transcriptional levels, as shown in Supplemental Figure 3.

All cases with the JAK2 mutation presented the CRLF2 in $\geq 10\%$ of blast cells with an intermediate or high MFI. Furthermore, all mutated cases had high CRLF2 transcriptional expression and were RCMA-negative (Supplemental Table 2).

Additionally, we evaluated the CRLF2-r in a small subset of 30 cases that were randomly selected to evaluate the prediction of the CRLF2 antigen. The main characteristics of the

BCP-ALL with CRLF2-r are listed in Table 2. We prioritized FISH for the CRLF2-r status in samples with CRLF2 antigen expression, which was previously identified by the MFC. There were 23 ($\geq 10\%$) CRLF2 cases and 13 of the 30 (43.3%) cases had the CRLF2-r. The characteristics of the CRLF2 antigen were: eight cases with an intermediate MFI, three with a high MFI and two with a low MFI. Among the 30 cases selected for FISH, 10 were subjected to the RT-qPCR. Nine cases presented a high CRLF2 transcript expression, six being cases with the CRLF2-r.

Overlapping cytogenetic molecular aberrations were also observed in the HeH subgroup. These data, together with the previous results presented in this study, indicate the need to integrate the MFC results with the analysis of the RCMA before the FISH evaluation of the CRLF2 gene rearrangements.

Discussion

The interaction between immunophenotypic and genotypic characterization has defined distinct ALL subtypes with predictive values and therapeutic decisions, considering that chromosomal and genomic abnormalities in BCP-ALL are almost mutually exclusive.^{2–4} Therefore, interactive immunomolecular markers for diagnosis and MRD monitoring have become important in defining therapeutic targets and predicting relapses.¹⁹ Flow cytometry is the primary tool for the ALL diagnosis, before cytogenetic molecular analysis, arguing in favor of its use as a screening tool to direct the use of further laborious techniques. In this study, we have shown the results from a Brazilian series of childhood BCP-ALL cases, with a focus on the CRLF2 antigen expression (MFC), its association with the CRLF2 transcript level (qRT-PCR) and the gold standard CRLF2-r method using the FISH. Additionally, the JAK2 mutation status was also tested and the consistent result of the CRLF2 antigen status was useful in predicting BCP-ALL cases with JAK2 mutations. All cases with the JAK2 mutation were associated with the CRLF2 antigen with intermediate or high MFI expression patterns. In the same

Table 2 – The main characteristics of the BCP-ALL with CRLF2-r.

	Total n (%)	CRLF2 rearrangements n (%)	No rearrangements n (%)	p-value
% CRLF2				0.427
≥ 10	23 (76.7)	11 (84.6)	12 (70.6)	
< 10	7 (23.3)	2 (15.4)	5 (29.4)	
MFI CRLF2				0.017
High	6 (20)	3 (23.1)	3 (17.6)	
Intermediate	11 (36.7)	8 (61.5)	3 (17.6)	
Low	13 (43.3)	2 (15.4)	11 (64.7)	
Cytogenetic molecular				0.057
RCMA ^a -positive	8 (26.7)	1 (7.7)	7 (41.2)	
RCMA-negative	16 (53.3)	10 (76.9)	6 (35.3)	
RCMA not defined	6 (20)	2 (15.4)	4 (23.5)	
CRLF2 RGE ^b				0.400
High	9 (90)	6 (100)	3 (75)	
Low	1 (10)	0	1 (25)	
Total	30 (100)	13 (43.3)	17 (56.7)	

^a RCMA – recurrent cytogenetic molecular alterations.

^b CRLF2 gene expression was not performed in all cases due to specimen availability; RGE – relative gene expression; WT – wild type.

direction, the MFI values were predictive of aberrations involving the CRLF2, considering that most CRLF2-r cases had intermediate and high values. Thus, the MFI can aid in better screening potential patients with JAK2 mutations and the CRLF2-r.

Based on the review of the literature, our results are the first to show an association between the CRLF2 antigen expression and different BCP-ALL cytogenetic subtypes, demonstrating that the HeH subtype is associated with the presence of the CRLF2. Previous studies have demonstrated the occurrence of high CRLF2 transcriptional expression in HeH cases.^{20–22} without describing the pattern of the CRLF2 at immunophenotypic diagnosis. Schmah *et al.* (2017) have demonstrated throughout the multiplex-ligation probe amplification a concomitant occurrence of the CRLF2 amplification and the HeH chromosomal abnormality in a subset of patients.²⁰ Because the HeH subtype is associated with a good prognosis, it is very important to determine the impact of CRLF2 aberrations on these BCP-ALL patients.

In the natural history of leukemia, it is essential to identify the occurrence of cell subtypes at different time points and the immunophenotypic analysis has proven to be a handling method for such technical sensitivity to predict the relapse risk.¹³ In this context, the identification of CRLF2 subclones, especially at the time of diagnosis and relapse, is relevant to the understanding of the role of the CRLF2 in therapeutic resistance and relapse. As shown in [Supplemental Table 3](#), paired cases of diagnosis/relapse samples were tested and we identified four patients that presented subclones with the CRLF2 antigen at diagnosis and progressively maintained them at relapse (cases 1, 5, 6 and 8 from [Supplemental Table 3](#)). These data need further investigation in a well-designed clinical protocol to estimate the value of the CRLF2 antigen as a predictive relapse marker. Bulgarin *et al.* (2015) studied the JAK-STAT pathway and demonstrated that patients with the CRLF2 < 10%, with an intermediate CRLF2 expression level, had an activated phosphosignaling cascade, with a baseline increase in STAT5 phosphorylation, distinct from patients with a completely negative CRLF2 antigenic expression.⁹ They hypothesized that the low CRLF2-r detected via the FISH and RT-qPCR could be found throughout the MFC with visualized subclones.

The BCP-ALL patients with CRLF2-r respond poorly to chemotherapy and have high rates of relapse and mutations in JAK2 are more frequent in these patients.⁶ Therefore, alternative therapies, such as JAK kinase inhibitors, may be suitable for these patients. The development of screening algorithms to rapidly identify patients harboring these alterations is important in practical clinical settings, especially in low- and middle-income countries, where methodologies such as FISH and NGS are costly. In this manner, as a more accessible tool distributed along with many regional centers of treatment, the MFC can contribute to directing the cytogenetic molecular evaluations of BCP-ALL samples, decreasing the use of high-cost techniques.

This study has the limitations of being a retrospective analysis, such as the lack of aligned samples in all methods. Therefore, missing variables implied a low statistical association. However, the overall results rely on the applicability and accessibility of integrative techniques already recommended

by WHO to characterize acute leukemia with a predictive value.¹⁰ The data presented here suggest that CRLF2 alterations can be detected by complementing techniques, with the MFC as a support to rapidly identify patients who could benefit from new promising therapeutic approaches targeting the CRLF2/JAK/STAT pathway.^{23,24}

Therefore, based on our results, we suggest an algorithm of recommended tests that would be explored in ALL treatment protocols, as shown in [Figure 2](#). Initial MFC analyses, including the use of the CRLF2 antibody in the diagnostic panel, are the first step in the BCP-ALL characterization. Cases with CRLF2 \geq 10% of positive cells and/or intermediate or high MFI should be screened for JAK2 mutations and the CRLF2-r. The remaining cases should be investigated for such alterations when they are negative for the RCMA, except for the HeH.

Conclusion

In conclusion, the identification of the CRLF2 antigen expression is associated with the CRLF2 status, rendering the MFC an excellent tool for immunomolecular strategies for the BCP-ALL subtype characterization. The MFI pattern associated with the percentage of cells with the CRLF2 antigen diagnosis should be considered to determine leukemia subclones during the follow-up of the BCP-ALL treatment.

Author contributions

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Supervision of analysis and interpretation of molecular test results: FGA, ERPG.

Original draft preparation: EPN, ETG, MSPO.

Draft revision: ERPG, FGA, EPN, MSPO, ETG.

Study supervision: ETG, MSPO.

All authors have read, contributed with intellectual content, and approved the manuscript writings.

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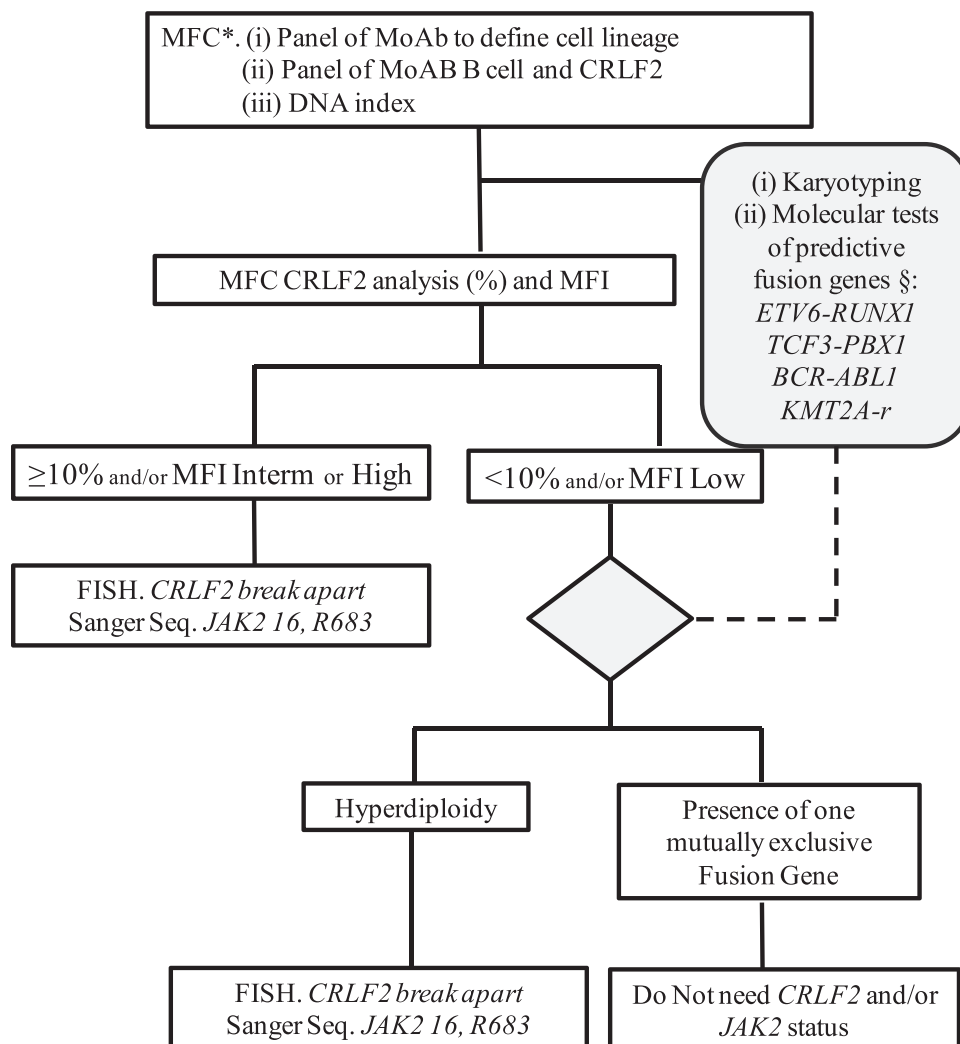


Figure 2 – Test algorithm to evaluate CRLF2 alterations in childhood BCP-ALL. (*) According to References 12 and 13; § According to Reference 11; Abbreviations: MFC – multiparametric flow cytometry; MoAb – monoclonal antibodies; MFI – median fluorescence intensity; Sanger Seq – Sanger sequencing method.

Conflicts of interest

The authors report no conflicts of interest in this study.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.htct.2022.06.008](https://doi.org/10.1016/j.htct.2022.06.008).

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