Multiparametric flow cytometry in the determination of protein expression and gene transcription of CRLF2 in B cell precursor acute lymphoblastic leukemia

Priscilla Moniz S. Ferreira, Elda Pereira Noronha, Franciane Gomes de Andrade; Caroline Barbieri; Ingrid Sardou Cesar; Bruno Alves de Aguiar Gonçalves; Carolina da Paz Zampier; Etel Rodrigues Pereira Gimba; Eugênia Terra Granado Pina; Maria do Socorro Pombo-de-Oliveira

INSTITUTO NACIONAL DO CÂNCER – CPQ

PROGRAMA DE HEMATOLOGIA E ONCOLOGIA PEDIÁTRICO

INTRODUCTION

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) presents classical cytogenetic molecular biomarkers with well-defined prognosis. These primary abnormalities are important initiating events, often used in diagnostic testing algorithms and patient risk stratification. Secondary alterations affect crucial pathways involving lymphoid differentiation, cell cycle regulation, cell proliferation and survival, besides affecting transcription cofactor. Together, these primary and secondary alterations lead to the process of leukemogenesis. Thirty percent of these cases do not have any of these abnormalities and are referred to as BCP-ALL negative and 50% of this subgroup has a BCR-ABL1 similar gene expression profile, then being called *BCR-ABL1-like.* This subgroup has characteristic genetic differences as mutations in the genes *JAK1 / 2, IL7Rα*, and *CRLF2*, as well as increased gene expression of the latter. CRLF2 forms a heterodimer with the CD127 molecule forming the TSLP cytokine receptor. This molecule is involved wth cell proliferation and survival, being more frequently associated with the BCP-ALL negative group and less frequent in the iAMP21 group. Studies involving the CRLF2 have presented controversial results due to the use of non-standardized techniques for their detection.

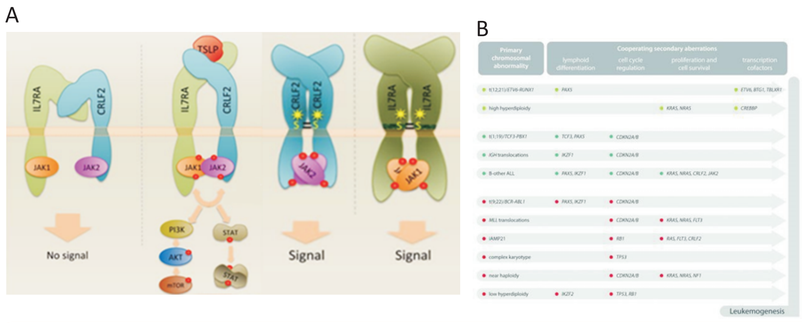


Figure 1: TSLP signaling pathways e prognostics of primary and secondary alterations affecting crucial pathways according to the primary molecular cytogenetic characteristics in BCP-ALL. (Adapted, Moorman, 2016)

OBJECTIVE

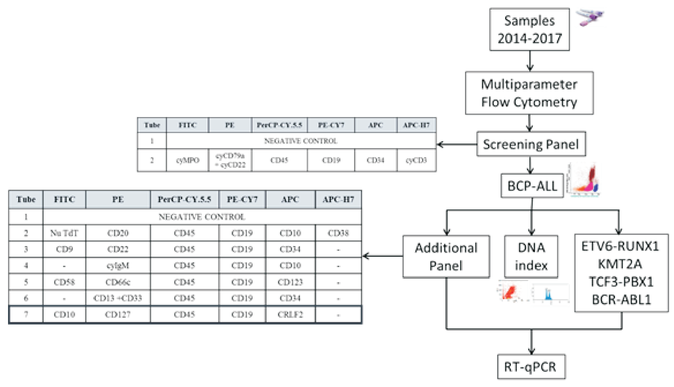
General Objective

To establish a test algorithm for combined immunophenotypic and molecular tests capable of predicting the status of CRLF2 in samples of children with BCP-ALL.

Specific objectives

- To test a panel of monoclonal antibodies through multiparametric flow cytometry, including CD66c and CD127, and to identify cases of BCP-ALL with differential cell expression of CRLF2 on the cell surface;
- To evaluate the DNA index of the BCP-ALL samples, to correlate the expression of CRLF2 and / or the most frequent genetic alterations associated with the different levels of cellular ploidy;
- To correlate the expression of the CRLF2 gene with its levels of protein expression and different immunophenotypic profiles of

METHODS



Tube	FITC	PE	PerCP-CY-5.5	PE-CY7	APC	APC-H7
1	NEGATIVE CONTROL					
2	cyMPO	cyCD79a + cyCD22	CD45	CD19	CD34	cyCD3

Tube	FITC	PE	PerCP-CY-5.5	PE-CY7	APC	APC-H7
1	NEGATIVE CONTROL					
2	Nu TdT	CD20	CD45	CD19	CD10	CD38
3	CD9	CD22	CD45	CD19	CD34	-
4	-	cyIgM	CD45	CD19	CD10	-
5	CD58	CD66c	CD45	CD19	CD123	-
6	-	CD13 +CD33	CD45	CD19	CD34	-
7	CD10	CD127	CD45	CD19	CRLF2	-

RESULTS

In the period of the study 138 samples were included for analysis, with 128 cases at diagnosis, including 5 cases of Down's Syndrome. In addition to 10 samples of sequential relapses. For the evaluation of the CRLF2 and CD127 markers the results were expressed as percentage of positive cells and as median fluorescence intensity (MFI) of the positive cells. For CRLF2, lymphocytes were used as the negative control, whereas for CD127, the unlabeled tube was used.

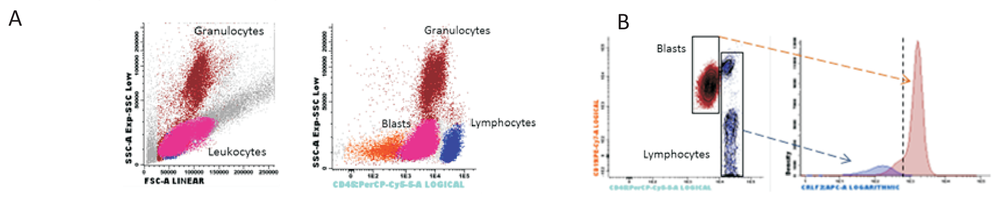


Figure 2: Analysis scheme for the evaluation of CRLF2 and CD127. (A) Viable cells defined in the first FSC x SSC dotplot. Leukocyte populations defined by the internal complexity and CD45 staining (CD45 x SSC) and B lineage lymphoid blasts defined by the CD45 and CD19 staining. (B) In blue the lymphocytes (used as negative control) and in lilac the leukemic blasts. The MFI of the leukemic blasts was measured from the positive cells shown in dotted lines on the histogram.

It was observed that the CRLF2 presents different expression profiles, thus, it was defined strongly positive, moderately positive and negative cases, this analysis was used to define groups with different levels of CRLF2 expression. The cases with low expression (named in the histogram below as negative) represent percentage less than 20%. We observed that 21 cases have this expression profile, but that they have intermediate or high MFI.

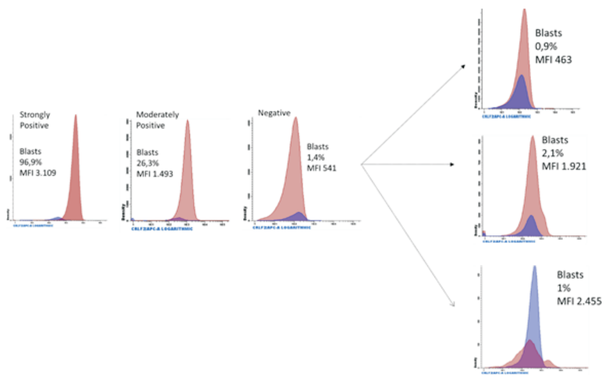
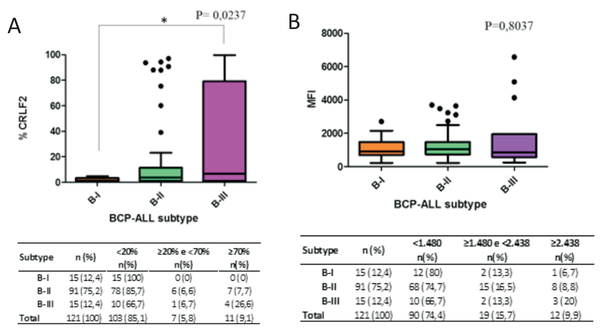


Figure 3: CRLF2 expression profile. On the left representative histograms with CRLF2 expression in lymphoid blasts, showing the strongly positive, moderately positive and negative profiles. Histograms on the right representing the cases with a low percentage of CRLF2, that present low MFI (first histogram on the right), an intermediate MFI (middle histogram), and those with high MFI, showing a minor clone (shown in the last right histogram). In lilac the leukemic blasts and in blue the lymphocytes used as negative control.

We also found a positive association between high CRLF2 cell expression in patients older than 10 years (p = 0.0482), this result is interesting taking into account their high-risk classification according to NCI. In SD cases, it was found that 40% of the cases have a CRLF2 intermediate/high cellular expression.



Subtype	n (%)	<20% n(%)	≥20% e <70% n(%)	≥70% n(%)
B-I	15 (12,4)	15 (100)	0 (0)	0 (0)
B-II	91 (75,2)	78 (85,7)	6 (6,6)	7 (7,7)
B-III	15 (12,4)	10 (66,7)	1 (6,7)	4 (26,6)
Total	121 (100)	103 (85,1)	7 (5,8)	11 (9,1)

Subtype	n (%)	<1.480 n(%)	≥1.480 e <2.438 n(%)	≥2.438 n(%)
B-I	15 (12,4)	12 (80)	2 (13,3)	1 (6,7)
B-II	91 (75,2)	68 (74,7)	15 (16,5)	8 (8,8)
B-III	15 (12,4)	10 (66,7)	2 (13,3)	3 (20)
Total	121 (100)	90 (74,4)	19 (15,7)	12 (9,9)

Figure 4: Expression profile of CRLF2-positive cells in BCP-ALL subtypes. (A) In relation to the percentage, there was a statistical difference between Pro-B (B-I) and Pre-B (B-III) subtypes (* p = 0.0237) (B) There was no statistical difference when the MFI was analyzed in BCP-ALL subtypes. The horizontal bars in the boxplots represent median, minimum and maximum value, P-value calculated by the kruskal-Wallis test.

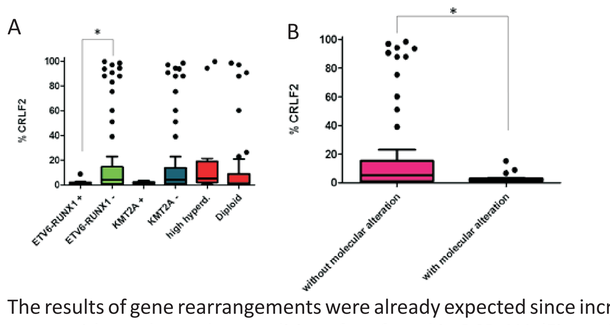
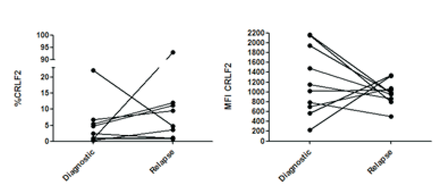


Figure 5: Comparison of the percentage of positive cells for CRLF2 in cases with the presence and absence of more frequent molecular alterations. (A) The cases were statistically significant when compared between the cases with presence and absence ETV6-RUNX1 (*P=0.0298). (B) Expression profile of CRLF2-positive cells in cases with absence and presence of *ETV6-RUNX1* alterations. *E2A-PBX1, KMT2* and *BCR-ABL1* (*P=0.0221). The horizontal bars in the Box plots represent median, minimum and maximum values; p-value calculated by the Mann Whitney test.

The results of gene rearrangements were already expected since increased expression of the CRLF2 gene has been observed within a group without these abnormalities often found in BCP-ALL (Figure 5). We analyzed 10 cases with relapses, so these samples were evaluated according to the percentage and MFI of cells positive for the CRLF2 molecule (Figure 6)



Cases	%		MFI	
	Diagnostic	Relapse	Diagnostic	Relapse
1	0,9	0,7	972	869
2	0,3	3,6	1.017	1.043
3	0,8	93	788	498
4	1	1,1	2.155	801
5	4,8	11,3	226	1.329
6	2,5	1	699	1.078
7	22,1	4,7	1.945	982
8	5,5	12	2.161	972
9	0,9	0,9	1.479	951
10	6,7	9,5	570	1.339

Figure 6: Analysis of CRLF2 expression in diagnostic and relapse samples in relation to expression levels in percentage and MFI. There was no correlation when analyzed by percentage and MFI. p-value calculated by the Wilcoxon test.

The correlation between the gene expression of CRLF2 and the percentage and MFI of the molecule, analyzed by flow cytometry, was also evaluated. A significant statistical value between gene expression vs percentage (P=0.0144) and gene expression vs MFI (P=0.0313) was observed through the correlation test. In the two correlations, according to the test used, a weak positive correlation was observed.

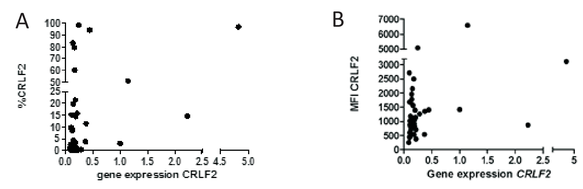


Figure 7: Correlation of genetic expression with the CRLF2 cell expression levels. (A) Correlation analysis between gene expression and percentage of positive cells showed a weak positive correlation (r=0.3626) and p significant value (p=0.0144). (B) Correlation analysis between gene expression and MFI of positive cells with weak positive correlation (r=0.3214) and p significant value (p=0.0313). The Spearman's test was used.

The CD127 or IL-7Rα molecule forms with CRLF2 a heterodimer that binds to the cytokine TSLP. Thus, along with the labeling of CRLF2 in the samples included in the present study, the labeling of the CD127 molecule was also performed. The correlation between the expression levels of the CRLF2 and CD127 markers was performed according to percentages and the MFI. There was no correlation between the two markers.

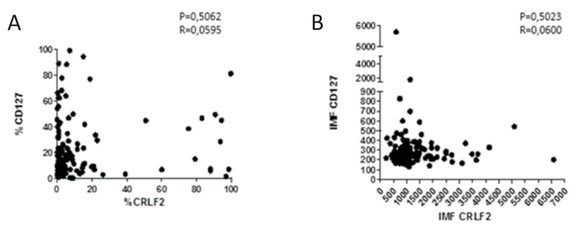
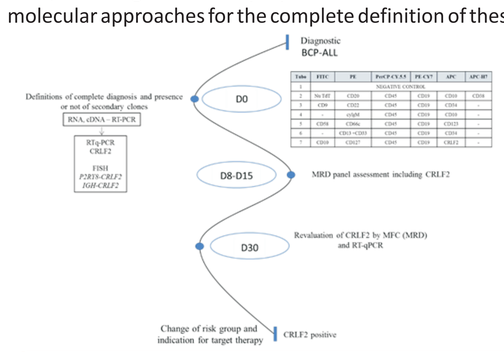


Figure 8: Correlation between expression of the CRLF2 and CD127 markers. (A) Correlation between percentages of cells labeled for CRLF2 and CD127. No statistically significant difference was observed (p=0.5062) and no correlation was found (r=0.0595). (B) Correlation between the MFI of CRLF2 and CD127 positive cells. No statistically significant difference was observed (p=0.5023) and no correlation (r=0.0600). For this analysis, the Spearman test was used.

Thus, a test algorithm was proposed with the insertion of immunophenotypic markers to characterize the BCP-ALL and important molecular approaches for the complete definition of these cases



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6	-	CD13 +CD33	CD45	CD19	CD34	-
7	CD10	CD127	CD45	CD19	CRLF2	-

Figure 9: Algorithm of tests for the identification of high expression of CRLF2. The immunophenotypic diagnosis is performed at D0 with the complete panel to define the subtype of BCP-ALL, subsequent to the sample processing RT-PCR will be performed for the most frequent alterations within this group (*ETV6-RUNX1*, *TCF3-PBX1*, *BCR-ABL1* and *r-KMT2A*). Given the importance of RT-qPCR, it will be performed to verify the differential expression of CRLF2 and FISH for the most common rearrangements involving this gene (*P2RY8-CRLF2* and *IGH-CRLF2*). Finally, the expression of CRLF2 by MFC and RT-qPCR in MRD D30 will be reassessed.

CONCLUSION

- We suggest a test algorithm using MFC and molecular methods (Figure 9) to identify markers whose predictive association in BCP-ALL subgroups will benefit from the introduction of target-molecular medication;
- It was possible to establish by MFC and RT-qPCR the force of association between the cellular and molecular expression of CRLF2 in patients with BCP-ALL. In addition, we identified minor cell clones among the heterogenicity of ALL in patients with low expression of the CRLF2 molecule;
- Strong association between age over 10 years and BCP-ALL with high CRLF2 cell expression when compared to patients less than 10 years of age;
- High CRLF2 cellular expression and positive correlation with BCP-ALL subtype B-III;
- No significant differences were found for expression of CD127 between the variables gender, ethnicity, leucometry, and no correlation was found with the cellular expression of CRLF2.

Projeto Gráfico: Setor de Edição e Informação Técnico-Científica / INCA