Translational Oncology 15 (2022) 101291

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon

Original Research

IKZF1 deletions associate with *CRLF2* overexpression leading to a poor prognosis in B-cell precursor acute lymphoblastic leukaemia

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ARTICLE INFO

Keywords:
CRLF2, IKZF1, Gene expression
Gene deletions
Outcome
B-ALL

ABSTRACT

Cytokine Receptor-Like Factor 2 (*CRLF2*) overexpression occurs in 5-15% of B-cell precursor acute lymphoblastic leukaemia (B-ALL). In ~50% of these cases, the mechanisms underlying this dysregulation are unknown. IKAROS Family Zinc Finger 1 (*IKZF1*) is a possible candidate to play a role in this dysregulation since it binds to the *CRLF2* promoter region and suppresses its expression. We hypothesised that *IKZF1* loss of function, caused by deletions or its short isoforms expression, could be associated with *CRLF2* overexpression in B-ALL. A total of 131 paediatric and adult patients and 7 B-ALL cell lines were analysed to investigate the presence of *IKZF1* deletions and its splicing isoforms expression levels, the presence of *CRLF2* rearrangements or mutations, *CRLF2* expression and *JAK2* mutations. Overall survival analyses were performed according to the *CRLF2* and *IKZF1* subgroups. Our analyses showed that 25.2% of patients exhibited *CRLF2* overexpression (*CRLF2*-high). *CRLF2*-high was associated with the presence of *IKZF1* deletions (*IKZF1*del, $p = 0.001$), particularly with those resulting in dominant-negative isoforms ($p = 0.006$). Moreover, *CRLF2* expression was higher in paediatric samples with high loads of the short isoform IK4 ($p = 0.011$). It was also associated with the occurrence of the *IKZF1* plus subgroup ($p = 0.004$). Furthermore, patients with *CRLF2*-high/*IKZF1*del had a poorer prognosis in the RELLA05 protocol ($p = 0.067$, 36.1 months, 95%CI 0.0-85.9) and adult cohort ($p = 0.094$, 29.7 months, 95%CI 11.8–47.5). In this study, we show that *IKZF1* status is associated with *CRLF2*-high and dismal outcomes in B-ALL patients regardless of age.

Introduction

CRLF2 (cytokine receptor factor 2) overexpression (*CRLF2*-high) is a marker of poor prognosis in acute lymphoblastic leukaemia (ALL). Previous studies demonstrated that patients with *CRLF2*-high have worse event-free survival with a higher cumulative incidence of relapse

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https://doi.org/10.1016/j.tranon.2021.101291
Received 9 August 2021; Received in revised form 29 October 2021; Accepted 19 November 2021

in both B-cell precursor (B-ALL) and T-cell ALL (T-ALL) [1,2]. *CRLF2*-high occurs in 5-15% of B-ALL and is enriched in the B-other subgroup (cases lacking commonly found genetic abnormalities) [3]. CRLF2 and IL7 receptor (IL7R) are subunits of a heterodimeric complex that binds its ligand, thymic stromal lymphopoietin (TSLP). Upon stimulation, the receptor changes its conformation and activates JAK auto-phosphorylation, mainly JAK2. The cascade is then activated, and it culminates with STATs transcriptionally regulating growth and apoptotic target genes. *CRLF2*-high, therefore, contributes to leukaemic transformation via JAK-STAT pathway dysregulation [4].

The mechanisms underlying *CRLF2* overexpression are not yet fully elucidated. Some genomic lesions involving this gene have already been described, such as *IGH-CRLF2, P2RY8-CRLF2*, PAR1 region deletions and the *CRLF2* F232C mutation. However, only half of the *CRLF2*-high cases harbour at least one of these known genetic abnormalities [5–8]. Thus, for the other half of *CRLF2*-high cases, the mechanisms accounting for this gene's upregulation are still unknown. A previous study described that IKZF1 (IKAROS) binds to the *CRLF2* promoter suppressing its expression through recruitment of chromatin methylators, which specifically increase trimethylation of histone 3 lysine 9 (H3K9me3) [9]. IKZF1 is a transcription factor that physiologically represses or activates genes involved in lymphoid differentiation through chromatin remodelling (nucleosome and deacetylase complex). Exons 4-6 of *IKZF1* code DNA-binding zinc finger domains, while exon 8 codes a zinc finger dimerization domain. Alternative splicing of *IKZF1* mRNA generates at least 13 different variants, including both long (IK1-IK3) and short (IK4-IK10) isoforms [10]. Those isoforms play different roles depending on their DNA-binding ability. Genome-wide studies have identified *IKZF1* gene deletions (*IKZF1* del) in ~15% of paediatric and 40%-50% of adult B-ALL cases [11]. These deletions give rise to the same isoforms generated by splicing and have been consistently associated with adverse outcomes [12]. Intragenic deletions affecting exons 4–7 impair IKZF1 DNA-binding activity and it results in a dominant-negative effect, while deletions of the whole gene, as well as deletions of exons 1,2, lead to the gene haploinsufficiency. Of note, it has been shown that ~50% of patients with *CRLF2* overexpression also carry some *IKZF1*-associated abnormalities [5]. In the present study, we hypothesised that the role of *IKZF1* on *CRLF2* overexpression (even in the absence of genomic lesions involving *CRLF2*) could involve either the presence of *IKZF1* somatic deletions or *IKZF1* short isoforms in B-ALL.

Material and methods

Patients and cell lines

We evaluated 91 paediatric (0-17 years old) and 40 adult (18-67 years old) B-ALL patients retrospectively collected from six cancer centres in Brazil (INCA-RJ, IPPMG/UFRJ-RJ, IMIP-PE, HEMORIO-RJ, ProntoBaby-RJ and Hospital Amaral Carvalho-SP). Mononuclear cells from bone marrow (BM) samples at diagnosis were isolated using Ficoll-Paque (Sigma-Aldrich) and screened for common B-ALL abnormalities, such as *ETV6-RUNX1, BCR-ABL1, TCF3-PBX1* and *KMT2A (MLL)* rearrangements, following standard procedures [13]. The local Ethics and Research Committee approved this study (CAEE #87793418.0.0000.5274 and #33709814.7.0000.5274). The main demographic and clinical characteristics of B-ALL patients are summarised in Table 1.

To compare and confirm our findings regarding patients' samples, we characterized B-ALL cell lines with available data on Cancer Cell Line Encyclopedia (CCLE) database (https://sites.broadinstitute.org/ccle/datasets) according to *CRLF2* expression to obtain a more representative panel of cell lines with *CRLF2*-high and -low. Based on the *CRLF2* status, we included 3 cell lines with *CRLF2*-high (MUTZ5, MHH-CALL4 and KOPN-8) and 4 cell lines with *CRLF2*-low (RS4;11, NALM6, SEM and REH) in our analysis. DNAs and cDNA aliquots of these same cell lines were kindly provided by Dr Anthony M. Ford (The Institute of Cancer Research, London, UK), Prof Rolf Marschalek (Goethe-University of Frankfurt, Germany) and Dr Wendy Stock (University of Chicago Medical Center, USA). Thus, we evaluated *CRLF2* and *IKZF1* genomic lesions and expression levels on these cells (Fig. S1).

Table 1
Demographic, clinical and molecular characterisation of paediatric and adult B-ALL patients.

Paediatric B-ALL Variable	n (%)	Adult B-ALL Variable	*n* (%)
Age (years)		**Age (years)**	
<5	33 (36.2)	18-40	25 (62.5)
5-10	31 (34.1)	41-60	11 (27.5)
11-17	27 (29.7)	61-67	4 (10.0)
Sex		**Sex**	
Female	28 (30.7)	Female	19 (47.5)
Male	63 (69.3)	Male	21 (52.5)
WBC count (x10^9/L)		**WBC count (x10^9/L)**	
<50	69 (75.8)	<50	26 (65.0)
>50	22 (24.2)	>50	14 (35.0)
Common abnormalities*		**Common abnormalities***	
ETV6-RUNX1	11 (12.5)	*ETV6-RUNX1*	-
Hyperdiploidy	13 (14.8)	Hyperdiploidy	3 (8.6)
B-other	40 (45.5)	B-other	11 (31.4)
BCR-ABL1	9 (10.2)	*BCR-ABL1*	17 (48.6)
KMT2A-rearranged	4 (4.5)	*KMT2A*-rearranged	2 (5.7)
TCF3-rearranged	10 (11.4)	*TCF3*-rearranged	2 (5.7)
Hypodiploidy	1 (1.1)	Hypodiploidy	-
***IKZF1* status**		***IKZF1* status**	
Wild-type	61 (74.4)	Wild-type	16 (45.7)
Deleted	21 (25.6)	Deleted	19 (54.3)
***IKZF1* plus**		***IKZF1* plus†**	
No	14 (66.7)	No	8 (50.0)
Yes	7 (33.3)	Yes	8 (50.0)
Total	**91 (100)**	**Total**	**40 (100)**

WBC, white blood cell count.
* In 3 paediatric and 4 adult cases it was not possible to define the molecular group.
† In 3 *IKZF1* deleted cases it was not possible to distinguish *IKZF1* plus from non-plus.

Nucleic acids extraction and cDNA synthesis

Genomic DNA and total RNA were isolated from BM samples at diagnosis using TRIzol™ reagent (Invitrogen, Carlsbad, California, USA) following the manufacturer's protocol. Subsequently, to wash away any remaining salt residues and to concentrate nucleic acids, both purified DNA and RNA were precipitated using a standard ethanol-isopropanol protocol. For some samples, DNA was alternatively isolated using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Quantification was performed by measuring absorbance in the 260nM range using NanoDrop™ (Thermo Scientific, Waltham, Massachusetts, USA).

An initial concentration of 1μg of RNA was treated with Ambion™ DNase I (Invitrogen, Carlsbad, California/USA) and then used to synthesize the cDNA with SuperScript™ II Reverse Transcriptase kit (Invitrogen, Carlsbad, California/USA). To confirm cDNA integrity, a fragment of *GAPDH* was amplified using the following primers: *GAPDH* Forward: 5' - TGACCCCTTCATTGACCTCA - 3'; *GAPDH* Reverse: 5' - AGTCCTTCCACGATACCAAA - 3'.

CRLF2 and IKZF1 gene expression analyses using reverse transcription quantitative real-time PCR

CRLF2 transcript levels were analysed by reverse transcription quantitative real-time PCR (RT-qPCR). The commercial TaqMan gene expression assays Hs00845692_m1 and Hs00939627_m1 (Applied Biosystems, Foster City, CA) were used to target *CRLF2* and the reference gene *GUSB*, respectively. cDNA samples were tested in duplicates using TaqMan Fast Advanced Master Mix protocol (Applied Bio-systems,

Foster City, CA). All experiments were performed in the 7500 Real-Time PCR System (Applied Biosystems, Foster City, California/USA). We tested the efficacy of three different housekeeping genes (*B2M, GAPDH* and *GUSB*) and *GUSB* was the most suitable reference gene for our expression analysis. The threshold cycle (Ct) value was determined by the average between duplicates for both *CRLF2* and *GUSB*. Subsequently, we calculated the ΔCt (CT_{CRLF2} - CT_{GUSB}) value. Relative gene expression was defined as fold change quantified by the $2^{-\Delta\Delta Ct}$ method referred to the median ΔCt of all samples [14]. All samples were categorised into quartiles according to the fold change value (quartile 1 to 4), and the samples located in the 4th quartile were categorised as *CRLF2*-high (Fig. 1).

Detection of IKZF1 transcripts using semi-quantitative reverse transcription PCR

We evaluated cDNA sequences from *IKZF1* exons 1-8 by semi-quantitative reverse transcription PCR (RT-PCR) following previously described procedures [15–17]. In summary, samples were denatured at 94 °C for 3 min, followed by 35 cycles of 30 s at 94 °C, 60 s at 63 °C, and 60 s at 72 °C, with a final extension at 72 °C for 10 min.

PCR products obtained were separated by electrophoresis using a 2% agarose gel stained with GelRed (Uniscience, Osasco, SP, Brazil) and semi-quantified with ChemiDoc XRS+ System (BioRad, Hercules, California, USA). *IKZF1* relative expression was determined by gel band densitometry using ImageJ software (ImageJ, US National Institutes of Health, Bethesda, MD, USA, https://imagej.nih.gov/ij/) with *GAPDH* as the reference gene expression using the same primer sequence listed in the "Nucleic acids extraction and cDNA synthesis" section. Band intensity was represented as relatively arbitrary units established by the software. Samples were categorised as low load expression and high load expression based on amplicons' patterns as shown in Fig. S2A. The presence of the expected isoforms was confirmed by Sanger sequencing (Fig. S2B). *IKZF1* isoforms expression level was categorised into three groups: absent/undetectable, bands not seen in the electrophoresis; low load, bands showing low intensity; high load, bands presenting high strength in the electrophoresis. Additionally, to separate patients between low and high load, we used the median value of the expression obtained in the semi-quantitative RT-PCRs.

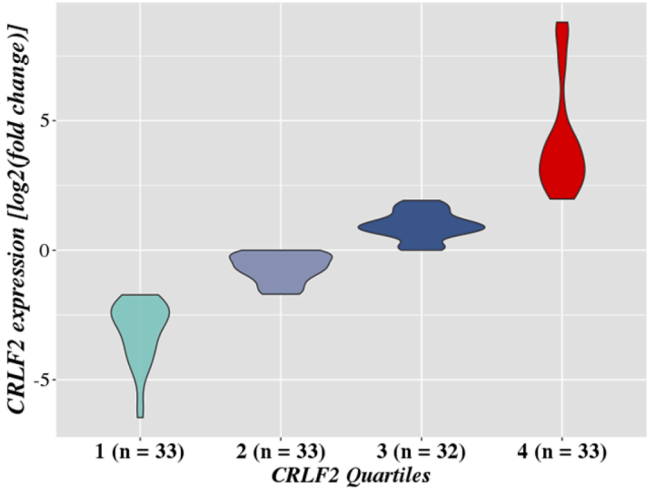

Fig. 1. *CRLF2* gene expression data. Patients were categorised according to the quartiles of *CRLF2* expression value. Samples located in the fourth quartile showed higher levels of *CRLF2* expression and were considered *CRLF2*-high. *CRLF2* expression was measured by 2-ΔΔCt and represented by the log2 (fond change).

Mutational screening of CRLF2 and JAK2

CRLF2 and *JAK2* mutations were evaluated by PCR and direct sequencing of the mutational hotspot regions of these genes, i.e. exons 6 (*CRLF2*) and 16 (*JAK2*), according to previously published methods [18, 19]. PCR products were purified with Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Chicago, Illinois, USA) and sequenced on both strands using the BigDye Terminator Cycle Sequencing kit v. 3.1 (Life Technologies, Carlsbad, California, USA). Sequencing was performed in the 3130 Genetic Analyzer (Life Technologies, California, USA) and analysed with BioEdit 7.0.9 and Mutation Surveyor. The following reference sequences were used for mutational analyses: NG_034237.1 (*CRLF2*) and NG_009904.1 (*JAK2*).

Copy number alterations analyses

The presence of CNAs in *IKZF1, CDKN2A/B, PAX5, EBF1, ETV6, BTG1, RB1*, and the pseudoautosomal region 1 (PAR1) was evaluated by multiplex ligation-dependent probe amplification (MLPA) using SALSA MLPA P335-C1 kit (MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's recommendations. The different types of *IKZF1* deletions were confirmed by long-distance multiplex-PCR or the SALSA MLPA P202 B1-2 kit, as previously described [20,21]. *ERG* deletion was detected by SALSA MLPA P327-C1 and/or digital MLPA D007 ALL kit following manufacturer's instructions (Benard-Slagter A et al, 2017). Conventional MLPA data were analysed using Gene Marker 2.2.0 software (Soft Genetics LLC, State College, PA). Relative peak ratios between 0.8 and 1.2 were considered normal, while values below or above indicated losses or gains, respectively. Deletion of the PAR1 region was defined as the presence of a deletion in at least *CSF2RA* and *IL3RA* genes. *IKZF1* deletions that co-occurred with *CDKN2A/B, PAX5* or PAR1 deletion in the absence of *ERG* deletion were classified as *IKZF1* plus. *CSFRA* and *IL3RA* deletions and retention of the *CRLF2* probe were considered as *P2RY8-CRLF2* fusion [22].

Detection of CRLF2 rearrangements using fluorescence in situ hybridization

Fluorescence *in situ* hybridization was performed in fixed cells (methanol and acetic acid solution, 3:1) using the commercial probe CRLF2 Breakapart LPH 085-S (Cytocell, Cambridge, UK). A total of 12 samples previously identified by RT-qPCR as *CRLF2*-high were analysed. The experiments were performed according to the manufacturer's instructions (Cytocell, Cambridge, UK). Cases were designated as *CRLF2*-rearranged (*CRLF2*-r) when one red (R), one green (G) and one fusion (F) pattern (1R1G1F) were observed in less than or equal to 5% of interphase nuclei, considering a total of 100 nuclei.

Statistical analysis

Differences in the distribution of patients' subsets were analysed using the Chi-square or Fisher's exact test for categorical variables and the Kruskal-Wallis and Mann-Whitney test for continuous variables. The statistical tests were performed in the RStudio software version 1.2 with the package Ggpubr version 0.1.6 (CRAN-R) and *p*-values less than 0.05 were considered statistically significant. Ggplot2 version 3.5.1 was used to generate the graphs.

Overall survival (OS) was defined as the time in months from diagnosis to outcome (survival, last follow-up, or death). OS was estimated using the Kaplan-Meier method and differences found between tested variables were compared with the log-rank test. The variables tested were sex, white blood cell count (WBC), glucocorticoid response, common B-ALL abnormalities, *IKZF1* status, *CRLF2* expression, and *IKZF1* isoforms. Multivariate Cox analysis was performed with variables associated with *p*-values of less than 0.10 in univariate analysis. OS analyses were performed using SPSS Statistics version 18.0 software

(IBM Corp., Armonk, NY, USA). For the survival analyses, patients were grouped according to age and treatment strategy used. Adult patients were treated with similar therapeutic protocols and called "Adult cohort". On the other hand, paediatric patients were separated in "RELLA05 cohort" - patients homogeneously treated with RELLA05 protocol - and "Paediatric cohort" - remaining patients treated with other paediatric protocols. RELLA05 is a reduced-intensity treatment scheme developed to decrease treatment-related mortality in children diagnosed with B-ALL at very low risk of relapse. This classification is based on favourable features such as age (age greater than or equal to 1 and less than 10 years), white blood cell count less than 50×10^9/L, lack of extramedullary leukaemia, and minimal residual disease levels less than 0.01% on day 19 of induction [23,24].

Results

A total of 131 samples, 91 children and 40 adults, obtained from patients diagnosed with B-ALL were evaluated in this study. The main demographic and laboratory characterisation of these samples are described in Table 1.

To define the *CRLF2*-high group, patients were separated into quartiles according to *CRLF2* expression data obtained from the B-ALL samples. Those located in the upper quartile were considered *CRLF2*-high cases (Fig. 1).

Thirty-three patients showed *CRLF2*-high, of which 18% presented either *CRLF2*-r or *CRLF2* F232C mutation as the mechanisms leading to this gene deregulation. We have also assessed potential associations among different *CRLF2* expression groups, *CRLF2*-low cases (Group A, blue), *CRLF2*-high wild-type (wt, Group B, light pink) and *CRLF2*-high with *CRLF2*-r and/or F232C mutation (Group C, dark pink) (Fig. 2A). Interestingly, *CRLF2* expression differed significantly in these groups, even when comparing groups B and C ($p < 0.001$). As expected, patients in Group C had a significantly higher expression when compared to Group B ($p = 0.003$). Intriguingly, all patients allocated in group C were adults (greater than or equal to 18 years). In addition, we observed that only three patients harboured *JAK2* mutations (R683G/R683T, grey dots). They presented *CRLF2*-r and were all part of Group C, accounting for 50% of this group. We also detected other molecular alterations in the *CRLF2* region, such as V249M mutation and PAR1 gain (a green square and an orange triangle, respectively), both occurring in Group A and B (Fig. 2A).

We investigated possible associations between *CRLF2* gene expression and demographic-clinical variables, such as central nervous system (CNS) involvement and glucocorticoid response, but no significant results were observed (Table S1). Nevertheless, when comparing children and adults, we showed that paediatric patients had significantly higher *CRLF2* expression levels (Fig. S3A; $p = 0.049$). Aiming to integrate the most relevant genomic investigations performed herein, we assembled an OncoPrint (Fig. 3) displaying both B-ALL patients and cell lines grouped according to their *CRLF2* gene expression profile (high *versus* low). Additionally, patients were classified as paediatric or adult. For the *CRLF2*-high patients, 65.4% were classified as B-other, 30.8% as *BCR-ABL1* and only one as hypodiploid. For the cell lines analyses, we had different molecular groups of B-ALL represented (B-other, *KMT2A*-r,

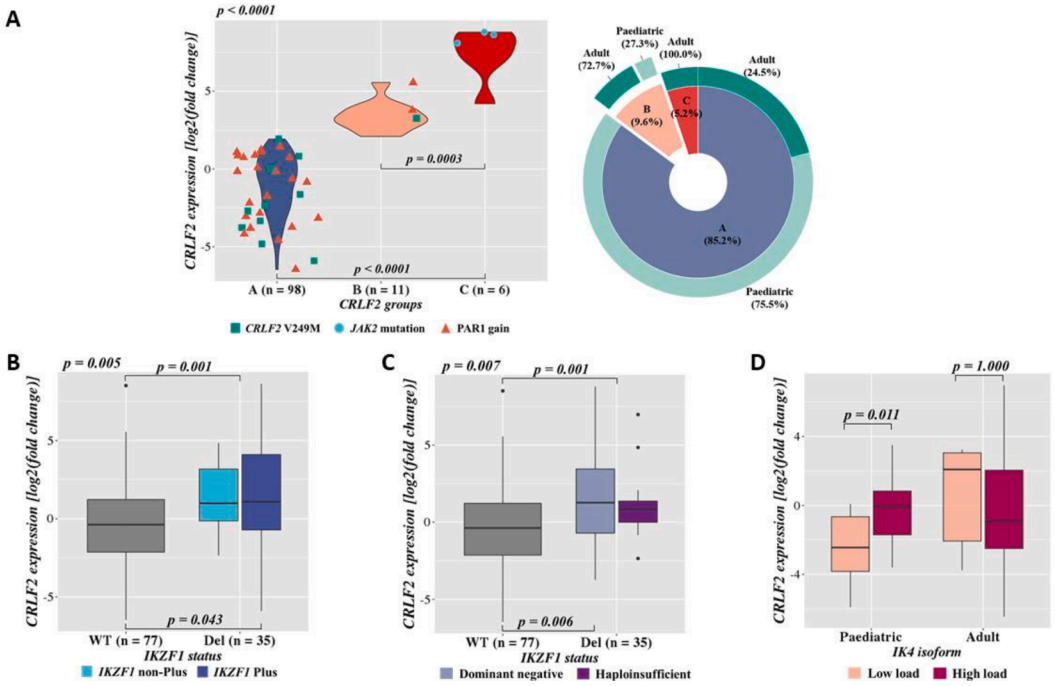

Fig. 2. *CRLF2* gene expression data. **A.** *CRLF2* expression according to different *CRLF2* groups and the presence of aberrations associated with *CRLF2*-high. *CRLF2* groups are Group A, cases with *CRLF2*-low (blue); Group B, cases with *CRLF2*-high in absence of *CRLF2* genomic aberrations (*CRLF2*-r and/or mutation, light pink); and Group C, cases with *CRLF2*-high and harbouring *CRLF2*-r and/or F232C (dark pink). Patients with *CRLF2* V249M mutation are represented by green squares and those presenting *JAK2* mutations and PAR1 gain by a blue dot and orange triangle, respectively. On the right side, the multilevel doughnut chart represents the distribution of these *CRLF2* groups in the age cohorts (light green for paediatric patients and dark green for adults). **B.** and **C.** *CRLF2* gene expression according to *IKZF1* copy number alterations. *IKZF1* wt is represented in a grey box. The top *p-value* inside the chart ($p = 0.001$) represents the comparison of *CRLF2* overexpression in *IKZF1* wild type *vs* deleted samples, and the *p-value* outside the Kruskal Wallis test comparing the three groups demonstrated. **B.** *CRLF2* expression according to the presence of *IKZF1* plus. *IKZF1* wt, deleted (*IKZF1* non-Plus, light blue) and *IKZF1* plus (*IKZF1* Plus, dark blue), defined as *IKZF1* deletions with *CDKN2A/B, PAX5* or PAR1 deletion and *ERG* wt [22]. **C.** Association between *CRLF2* expression and *IKZF1* status according to the functional consequence of *IKZF1* deletions. Deletions involving the following exons 2-7, 4-7, 4-8 and 5-6 were considered as dominant-negative (light purple) and those in 1-7, 1-8 and 2-8 exons as haploinsufficient (dark purple). **D.** *CRLF2* expression according to IK4 isoform. Patients are categorised by age in paediatric and adult cohorts. The status of the *IKZF1* short isoform, IK4, is represented in light pink, low load, and dark pink, high load. **A-D.** *CRLF2* expression was measured by $2^{-\Delta\Delta Ct}$. Statistical analyses were performed using Kruskal Wallis and Mann-Whitney tests (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

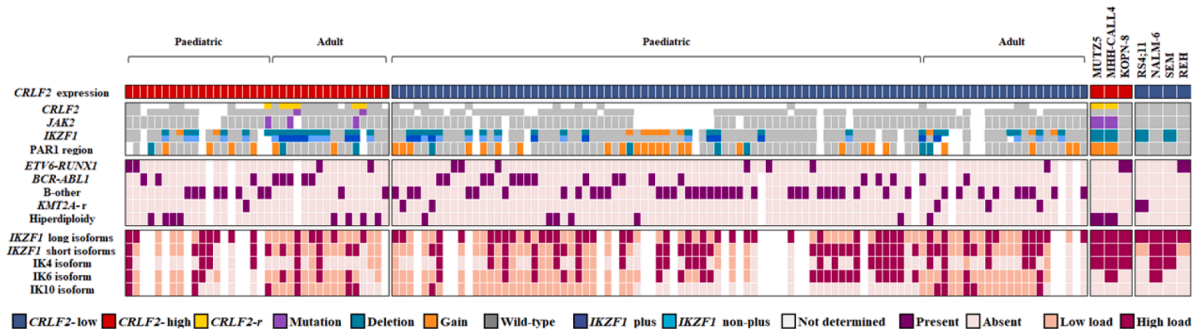

Fig. 3. OncoPrint showing the molecular characterisation of B-ALL samples — paediatric and adult patients and cell lines — according to *CRLF2* gene expression status. Each column represents a patient or a cell line included in the study. *CRLF2*-r, *CRLF2* rearrangement; PAR1 deletion, deletion in at least *CSF2RA* and *IL3RA* genes; *IKZF1* plus, patients with *IKZF1* deletions co-occurring with *CDKN2A/B, PAX5* or PAR1 deletion in the absence of *ERG* deletion. *CRLF2*-r, *JAK2, IKZF1* and PAR1 molecular features of cell lines were obtained from the Cancer Cell Line Encyclopedia (CCLE) database.

ETV6-RUNX1, etc) with two out of three presenting *CRLF2*-high and *CRLF2*-r. On the other hand, none of them, regardless of the *CRLF2* expression group, exhibited an *IKZF1* plus profile. All cell lines expressed *IKZF1* long isoforms and five out of six expressed the short ones (particularly IK4) with a high load profile.

Although *IKZF1* deletions were found in all three groups (A, B and C), patients with these alterations showed higher *CRLF2* expression compared to the *IKZF1* wt ($p = 0.001$) (Fig. 2B and C). Moreover, *IKZF1* plus patients had a higher *CRLF2* expression compared to *IKZF1* wild-type cases (Fig. 2B, $p = 0.043$). Regarding the previous results demonstrating *IKZF1* regulation in *CRLF2* expression [9], we postulated that IKZF1 deletions or its unbalanced short isoforms expression would be associated with *CRLF2* expression (Fig. 4). We then analysed if the different types of *IKZF1* deletions were associated with *CRLF2* overexpression, and we observed that the occurrence of *IKZF1* deletions leading to a dominant-negative phenotype was associated with higher *CRLF2* expression (Fig. 2C, $p = 0.006$).

Considering our hypothesis (Fig. 4), we then evaluated if *CRLF2* expression was associated with different *IKZF1* isoforms. Upon categorization of patients in different age groups, paediatric patients exhibited a significantly higher *CRLF2* expression in the presence of the IK4 isoform, but not of the other short isoforms ($p = 0.011$) (Fig. 2D and Fig. S3B-E).

Finally, we performed overall survival (OS) analyses by grouping patients according to age and treatment protocol. The paediatric patients that uniformly received the RELLA05 protocol ($n = 32$) were analysed separately from the remaining paediatric cases ($n = 53$) (Tables S2 and S3). The 6-year OS for adults, RELLA05 and paediatric patients was 17.5 months, 72.0 months, and 36.2 months, respectively. Clinical and molecular features, such as high WBC count ($p < 0.001$, 42.3 months, 95%CI 25.6–59.0), presence of *BCR-ABL1* fusion ($p = 0.065$, 45.8 months, 95%CI 36.4–55.3) and poor glucocorticoid response ($p = 0.015$,53.0 months, 95%CI 41.1–64.9) were associated with dismal prognosis in the paediatric cohort. *KMT2A*-r and *CRLF2*-high were also associated with poorer outcomes in RELLA05 ($p = 0.064$, 39.6 months, 95%CI 0.0-84.5, and $p = 0.067$, 54.9 months, 95%CI 34.3–75.5, respectively) and adult cohorts ($p = 0.007$, 4.2 months, 95% CI 0.0-8.5, and $p = 0.072$, 26.5 months, 95%CI 11.5–41.6, respectively). The occurrence of *IKZF1* deletions was associated with an inferior outcome in adults, but not in paediatric patients ($p = 0.042$, 29.6 months, 95%CI 16.4-42.7). *IKZF1* deletions had a negative impact on the prognosis of the adult cohort regardless of the treatment received (either BFM or any other adult protocol; $p = 0.813$, 27.5 months vs 31.5 months). A combined analysis of *CRLF2/IKZF1* status revealed that *CRLF2*-high/*IKZF1* deleted patients had worse prognosis in RELLA05 ($p = 0.067$, 36.1 months, 95%CI 0.0-85.9) and adult cohorts ($p = 0.094$,

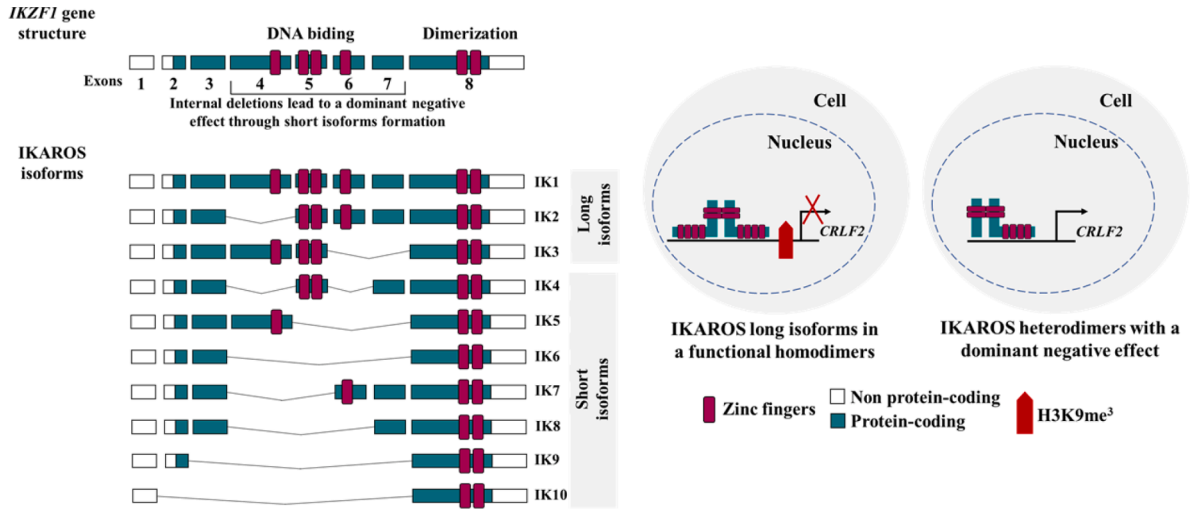

Fig. 4. Schematic representation of *IKZF1* isoforms and the proposed model for dominant-negative isoforms participating in *CRLF2* overexpression. Overview of the human family of IKAROS Family Zinc Finger 1 (*IKZF1*) gene and its isoforms (IK1 to IK10). *IKZF1* isoforms that retain at least three DNA binding zinc fingers (ZF, represented by the pink bar), maintain their functional effect and are called "long isoforms" (IK1 to IK3). On the other hand, the isoforms that lose two or more of these ZFs, are unable to bind DNA, but they retain their capability to form heterodimers with *IKZF1* long isoforms leading to a dominant-negative effect. Physiologically, IKZF1 directly binds to the *CRLF2* promoter and suppresses its expression through enrichment of H3K9me3 (represented by the red cross in the figure) [9, 10,26].

29.7 months, 95%CI 11.8–47.5) (Fig. 5). No significant difference in OS was observed for the expression of IK4, IK6, IK10 and *IKZF1* long isoforms. Nonetheless, the combination of low loading short isoforms with *CRLF2*-high profile had a negative impact in OS of RELLA05 paediatric and adult cohorts ($p < 0.001$ and $p = 0.006$, respectively). Furthermore, the inferior outcome observed in adults with *CRLF2*-high is maintained even when patients with *CRLF2*-r or F232C mutations are excluded ($p = 0.041$). A multivariate Cox analysis including the variables significantly associated with survival was performed to evaluate possible independent prognostic factors. We observed that *KMT2A*-r and *IKZF1* deletions impact the prognosis of adult patients independently of other variables ($p = 0.017$, HR = 61.3, 95%CI 2.1–1778.8 and $p = 0.022$, HR = 34.6, 95%CI 1.7–724.6, respectively), whereas for paediatric patients, only high WBC count remained significant in the multivariate analysis ($p = 0.017$, HR = 2.7, 95%CI 1.2–6.2).

Discussion

CRLF2 was overexpressed in 25.2% of cases included in our study and the majority of them were classified as either *BCR-ABL1* or B-other, in agreement with literature reports [3,4]. Surprisingly, molecular alterations previously associated with *CRLF2* overexpression, such as V249M mutation and PAR1 gain, were identified in patients with *CRLF2* low expression. This indicates that these alterations might not necessarily lead to *CRLF2* overexpression in B-ALL. Subsequently, characterising the type of *IKZF1* deletions, we demonstrated that those resulting in dominant-negative phenotypes were associated with higher expression of *CRLF2*. Aware that dominant-negative proteins sequester IKZF1 long isoforms, this result supports the assumption that the presence of these types of deletions would lead to a decreased IKZF1 binding to a *CRLF2* promoter, culminating in *CRLF2* overexpression (Fig. 4). However, functional experiments are necessary to confirm this hypothesis. The finding that *CRLF2* expression was higher in the *IKZF1* plus when compared to the *IKZF1* wt subgroup goes along with the idea of both being poor prognostic markers and more frequently found within the 'B-other' subgroup [2,9,10,13].

We then investigated the impact of different *IKZF1* isoforms on *CRLF2* expression. Here, we observed a positive association between the presence of IK4 short isoform and *CRLF2*-high in paediatric patients only, reinforcing our hypothesis pictured in Fig. 4. However, we did not find any association between IK6 and IK10 with *CRLF2* expression. Interestingly, we also identified a high load of IK4 in the B-ALL cell lines that harbour *CRLF2* overexpression, corroborating with the results obtained from the patients' samples. Nonetheless, we cannot completely rule out the role of the other *IKZF1* short isoforms, since CNA analyses revealed an association of *IKZF1* deletions that lead to the formation of dominant-negative isoforms, specifically IK6 and IK10. Moreover, our data resulted from a single experimental approach, which may present some intrinsic limitations. On the other hand, this semi-quantitative method was validated as a feasible test to detect *IKZF1* alterations as well as a practical risk prognostic tool [16,17]. Additionally, former studies have also shown a biological link between *IKZF1* and *CRLF2* in both B- and T-ALL [8,9,18,25].

Herein, we demonstrated for the first time the prognostic impact of the cooccurrence of *CRLF2*-high/*IKZF1*del in the RELLA05 Brazilian protocol. The RELLA05 Brazilian protocol for the treatment of children diagnosed with B-ALL was developed for patients with a low risk of relapse and managed to reduce the toxic effects of the therapy [24]. Therefore, our findings may contribute to further benefit the risk stratification of this therapeutic protocol.

Of note, we found that the low load of *IKZF1* short isoforms combined with *CRLF2* overexpression conferred a poorer prognosis. This effect seems to mainly reflect the impact of *CRLF2* high transcript levels, because of the small number of cases with a low load of *IKZF1* short isoforms. In addition, previous studies demonstrated the prognostic impact of *IKZF1*del/*CRLF2*-high in other series of cases, mainly in paediatric patients with high-risk B-ALL [6–8]. Our data revealed that the co-occurrence of these alterations culminates in a poor prognosis regardless of age, even in patients with a very low risk of relapse. Raca et al. [27] recently demonstrated that the occurrence of *IGH-CRLF2* and *IKZF1* deletions is more frequent in paediatric Hispanic and Latin populations with B-ALL, providing a biological rationale for the higher

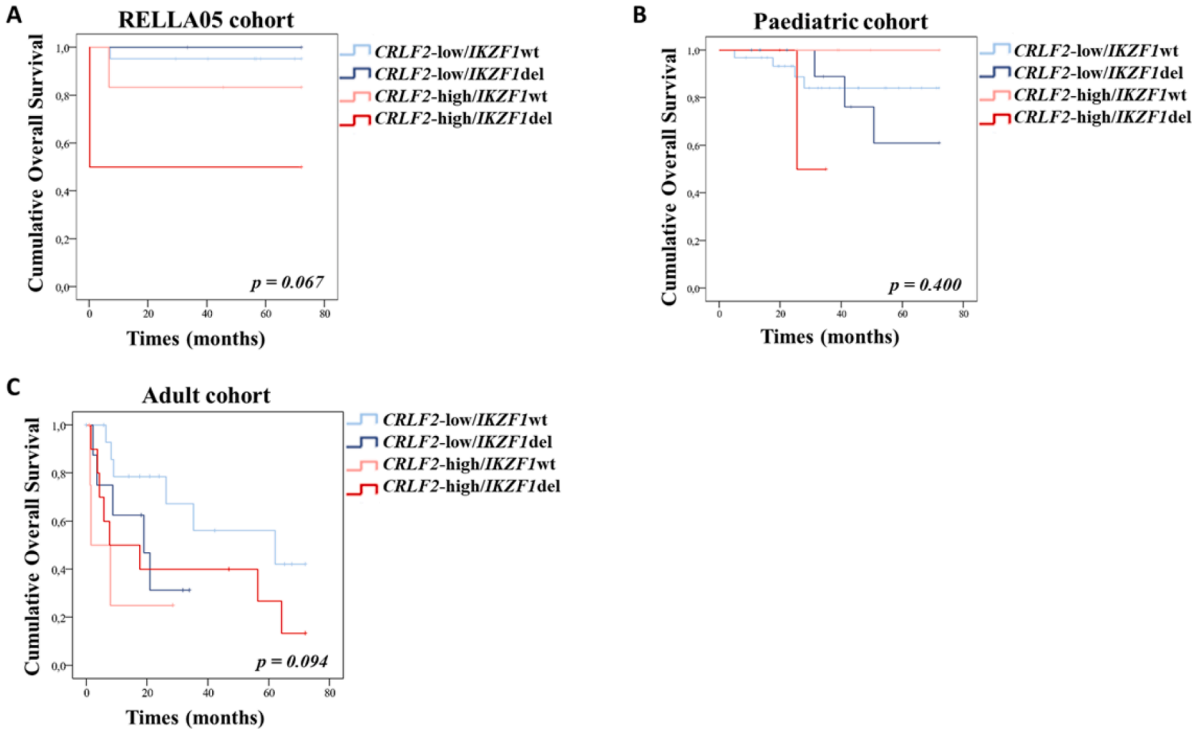

Fig. 5. Impact of *CRLF2* expression/*IKZF1* status in for on outcome of B-ALL patients. Overall survival according to the combination of *CRLF2* expression and *IKZF1* status in **A.** RELLA05 **B.** paediatric and **C.** adult cohorts. Wt, wild-type; del, deleted.

death rate in these patients. Following these results, the authors highlighted the importance of testing Hispanic/Latino children for these alterations as a strategy to improve the prognosis of B-ALL, as well as reducing social inequalities [27]. Indeed, in our study, we identified a higher frequency of *IKZF1* deletions in paediatric Brazilian patients than what is commonly found in other populations (25 vs 15%, respectively) [19]. We also demonstrated that the higher *CRLF2* transcript levels, independent of *CRLF2*-r or mutations, in concomitance with *IKZF1* deletions were associated with a worse outcome. Therefore, our data aligns with the proposal that the detection of these molecular abnormalities is important to improve the prognosis of these populations. Altogether, these data reinforce the need to develop and test therapeutic strategies which restore IKAROS function while targeting *CRLF2* signalling pathways, as a means to improve the prognosis of B-ALL in Brazilian patients with B-ALL.

Some pitfalls must be considered, such as the absence of event-free survival information and the limited number of patients after stratifications, which precluded us from performing more detailed prognosis analyses.

In summary, our findings demonstrate that *CRLF2*-high is associated with the occurrence of dominant-negative *IKZF1* deletions in B-ALL patients and confers a poor outcome in these patients. Ours and other data support the need for further exploring a functional regulation between those genes, as well as including these molecular biomarkers in protocols designed for these populations with known increased frequency.

CRediT authorship contribution statement

Ana Luiza Tardem Maciel: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Thayana da Conceição Barbosa:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Caroline Barbieri Blunck:** Methodology, Formal analysis, Investigation, Writing – original draft. **Karolyne Wolch:** Methodology, Formal analysis, Investigation, Writing – original draft. **Amanda de Albuquerque Lopes Machado:** Methodology, Formal analysis, Investigation, Writing – original draft. **Elaine Sobral da Costa:** Resources, Writing – review & editing. **Lavínia Lustosa Bergier:** Resources, Writing – review & editing. **Márcia Trindade Schramm:** Resources, Writing – review & editing. **Maura Rosane Valério Ikoma-Coltutato:** Resources, Writing – review & editing. **Mecneide Mendes Lins:** Resources, Writing – review & editing. **Thais Ferraz Aguiar:** Resources, Writing – review & editing. **Marcela Braga Mansur:** Conceptualization, Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Mariana Emerenciano:** Conceptualization, Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

CRediT authorship contribution statement

Ana Luiza Tardem Maciel: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Thayana da Conceição Barbosa:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Caroline Barbieri Blunck:** Methodology, Formal analysis, Investigation, Writing – original draft. **Karolyne Wolch:** Methodology, Formal analysis, Investigation, Writing – original draft. **Amanda de Albuquerque Lopes Machado:** Methodology, Formal analysis, Investigation, Writing – original draft. **Elaine Sobral da Costa:** Resources, Writing – review & editing. **Lavínia Lustosa Bergier:** Resources, Writing – review & editing. **Márcia Trindade Schramm:** Resources, Writing – review & editing. **Maura Rosane Valério Ikoma-Coltutato:** Resources, Writing – review & editing. **Mecneide Mendes Lins:** Resources, Writing – review & editing. **Thais Ferraz Aguiar:** Resources, Writing – review & editing. **Marcela Braga Mansur:** Conceptualization, Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Mariana Emerenciano:** Conceptualization, Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors have no competing interests.

Acknowledgments

The authors would like to thank the patients and their families who agreed to be involved in the study. ALTM has been supported by CAPES Foundation. TCB is supported by the Ministry of Health (INCA-Brazil). ME is supported by Brazilian National Counsel of Technological and Scientific Development-CNPq (PQ-311220/2020-7) and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro-FAPERJ (E_26/203.214-2017; E-26-010.101072-2018; and E-26/010.002187-2019) research grants. MBM was supported by the Ministry of Health (INCA-Brazil) and is currently funded by a John Goldman Fellowship from leukaemia UK (University of Oxford-UK).

Data availability statement

The data that support the findings of this study are available upon reasonable request to the corresponding authors.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101291.

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