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RECEIVED 10 July 2023

ACCEPTED 23 October 2023

PUBLISHED 07 November 2023

CITATION

Lin D, Yang K, Yu L, Huang L, Lai X, Wu L,
Xia X, Zhang J, Zheng Q and Yang L (2023)
Poor outcome of pediatric B-cell acute
lymphoblastic leukemia associated with
high level of *CRLF2* gene expression in
distinct molecular subtypes.
Front. Oncol. 13:1256054.
doi: 10.3389/fonc.2023.1256054

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Poor outcome of pediatric B-cell acute lymphoblastic leukemia associated with high level of *CRLF2* gene expression in distinct molecular subtypes

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Background: Overexpression of the cytokine receptor-like factor 2 (*CRLF2*) gene is the most common feature in the Philadelphia chromosome (Ph)-like subtype of B-cell acute lymphoblastic leukemia (B-ALL). However, the predictive value of *CRLF2* overexpression for the prognosis of pediatric B-ALL patients remain controversial. The molecular mechanisms that upregulate *CRLF2* expression level in patients has not been fully elucidated.

Methods: In this study, the prognostic impact of *CRLF2* expression level on molecular types of B-ALL in pediatric patients from Zhujiang Hospital (n = 111) was retrospectively analyzed. Youden index analysis was used to categorize *CRLF2* expression into 3 groups, and these categories more precisely described the differences in the prognosis of patients with varying expression levels of *CRLF2* in both the Zhujiang Hospital cohort and the TARGET cohort.

Results: We used the Zhujiang Hospital cohort as a discovery cohort to determine the cutoff value of *CRLF2* expression. *CRLF2*-high patients accounted for approximately 6%. In addition, the percentage of bone marrow blast cells and initial white blood cell count in *CRLF2*-high patients were higher than those in *CRLF2*-low patients, and MRD turned negative slower. The results were validated in the TARGET cohort and indicated that *CRLF2* overexpression could be subdivided by *CRLF2* expression levels into 2 categories: *CRLF2*-high with a poor survival and *CRLF2*-medium with a good OS and EFS. Such heterogeneity was attributed to the different molecular mechanisms leading to

CRLF2 upregulation, where the *CRLF2* overexpression level was high in Ph-like B-ALL and medium in high hyperdiploid B-ALL.

Conclusion: This study highlights the importance of the molecular mechanisms of the upregulation of *CRLF2* expression in predicting the prognosis of pediatric B-ALL patients.

KEYWORDS

B-ALL, *CRLF2*, Ph-like, pediatric, leukemia

Introduction

The cytokine receptor-like factor 2 (*CRLF2*) gene, located on chromosome Xp22.3 or Yp11.3, encodes cytokine receptor-like factors (CRLFs), which form heterodimers with IL-7Ra that receive and transmit signals from thymic stromal lymphopoietin and upregulated *CRLF2* expression activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) or phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) pathway to promote the occurrence and progression of leukemia (1–4). *CRLF2* overexpression has been reported as a major feature in the Philadelphia chromosome (Ph)-like subtype of B-cell acute lymphoblastic leukemia (B-ALL), and approximately 50–60% of patients with Ph-like B-ALL have *CRLF2* overexpression (1–4). Nonetheless, whether *CRLF2* overexpression can be employed to predict the prognosis of B-ALL patients is controversial. The majority of previous studies have demonstrated that *CRLF2* overexpression is associated with the poor prognosis of B-ALL patients (1–4). However, some studies have revealed that *CRLF2* overexpression is not an independent prognostic factor for B-ALL (4, 5). The definition of *CRLF2* overexpression varies in these studies, and the proportion of patients with *CRLF2* overexpression among unscreened patients ranges from 4.7 to 25% (2, 4–7). Different criteria for *CRLF2* overexpression may cover a variety of molecular mechanisms leading to the upregulation of *CRLF2* expression; therefore, it is difficult to precisely characterize the factors driving disease progression (7–10).

In this study of Chinese pediatric patients, we identified varying levels of *CRLF2* expression among different molecular subtypes. New cutoff value for *CRLF2* overexpression associated with molecular types were proposed. Moreover, the prognoses of patients with different *CRLF2* expression levels were validated using the B-ALL patient cohort from the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) public database. Finally, the corresponding cutoffs for *CRLF2*-high in real-time quantitative polymerase chain reaction (RQ-PCR) were also determined, thereby providing a basis for the rapid identification of B-ALL patients with a potentially poor prognosis via *CRLF2* expression level.

Materials and methods

Zhujiang Hospital cohort and sample collection

One hundred and eleven patients with B-ALL treated at the Department of Pediatrics, Southern Medical University, Zhujiang Hospital, between May 2020 and February 2022 were enrolled in this study; the final follow-up date was August 25, 2023. Children diagnosed before 1 year of age (n=5) were treated with the Inerfant-06 protocol (11). Among children diagnosed after 1 year of age, 1 was initially treated intermittently at another hospital, 1 was treated using the Guangdong Children's Leukemia Group-ALL-2008 (GD-ALL-2008) protocol (12), and the others were treated using the South China Children's Leukemia Group-ALL-2016 (SCCLG-ALL-2016) protocol (12) (n=104). The detailed clinical characteristics are shown in Table 1. Bone marrow (BM) samples were collected from patients at initial diagnosis. This study was approved by the central ethics committee of Zhujiang Hospital, Southern Medical University, in accordance with the Declaration of Helsinki (Approval number: 2022-KY-195-01).

The TARGET cohort public data collection and preprocessing

Data for the TARGET cohort were initially generated for the Therapeutically Applicable Research to Generate Effective Treatments (<https://ocg.cancer.gov/programs/target>) initiative (TARGET Phase II, phs000464). The data used for this analysis are available at (<https://portal.gdc.cancer.gov/projects>). Clinical and survival data were downloaded from cBioportal (<http://www.cbioportal.org>).

The TARGET cohort included 144 patients diagnosed with B-cell lymphoblastic leukemia and with diagnostic tumor RNA samples. FPKM values were calculated using the gene expression counts matrix from the RNA sequencing. The clinical characteristics are provided in Supplementary Table 2.

TABLE 1 Baseline characteristics of the Zhujiang Hospital cohort.

Characteristics		Total (n = 111)	
Sex (n, %)	Female	44	40%
	Male	67	60%
Age (n, %)	≤6	81	73%
	>6	30	27%
Karyotype (n, %)	Abnormal	35	32%
	Normal	61	55%
	Unknown	15	14%
Blasts in BM (%) (n=109)	median (IQR)	86	(76-93.25)
Initial WBC ($\times 10^9/L$)	median (IQR)	13.44	(5.23-43.5)
RNA-seq Subtype (n, %)	DUX4	4	4%
	ETV6-RUNX1	12	10%
	ETV6-RUNX1-like	2	2%
	High hyperdiploid	38	33%
	KMT2A Group	9	8%
	Low hypodiploid	1	1%
	MEF2D	4	4%
	NUTM1	2	2%
	PAX5 P80R	1	1%
	PAX5alt	5	5%
	Ph	4	4%
	Ph-like	5	5%
	TCF3-PBX1	11	10%
	ZNF384 Group	1	1%
	Unclassified	13	12%
Risk (n, %)	SR	13	12%
	IR	36	32%
	HR	61	55%
	Unknown	1	1%
Treatment (n, %)	Interfant 2006	5	5%
	SCCLG ALL 2008	1	1%
	SCCLG ALL 2016	104	94%
	Others	1	1%
CRLF2 FPKM group (n, %)	CRLF2-high	6	5.4%
	CRLF2-medium	47	42.3%
	CRLF2-low	58	52.3%
IKZF1 ^{plus} (n, %)	IKZF1 ^{plus}	7	6%
	IKZF1 deletion	7	6%
	No IKZF1 deletion	97	87%

BM, bone marrow; IQR, interquartile range; WBC, white blood cell; SR, standard risk; IR, intermediate risk; HR, high risk; SCCLG, South China Children's Leukemia Group.

DNA target sequencing and mutation analysis

Genomic DNA was obtained from BM at diagnosis and subjected to hybridization-based capture by using a KAPA HyperPlus Kits (Roche Diagnostics, Basel, Switzerland) and then captured using a NimbleGen SeqCap EZ Library SR (Roche Diagnostics) kit probing the exon regions of 339 genes relevant to hematologic malignancies, followed by high throughput sequencing on an Illumina NextSeq 550 platform to produce PE 150 bp. Somatic variant calling was performed with the Broad Institute's Genome Analysis Toolkit (GATK) v4.0.4.0 and Vardict v1.12 and annotated by Annovar v2018-04-16 (13–18). Selected pathogenic variants were manually inspected using the Integrative Genomics Viewer (IGV) software v2.8.0 (19).

mRNA sequencing and B-ALL subtype classification

Total RNA was extracted from the BM samples using a Cowin RNAPure Blood kit (Cowin Bio., Jiangsu, China) and quantified using a Nanodrop one (Thermo Fisher). Stranded mRNA sequencing libraries were constructed using a VAHTS Stranded mRNA-seq Library Prep Kit for Illumina (Vazyme, Nanjing, China). The polyadenylated mRNAs were then purified and fragmented, followed by the generation of double-strand cDNA. The double stranded cDNAs were ligated to the VAHTS RNA Adapters (N803/N804, Vazyme, Nanjing, China) following end repair and A tailing. The purified ligation products (size 200-500 bps) were selected and quantified, followed by sequencing on an Illumina Novaseq 6000 to produce $\geq 30M$ 150 base pair paired-end reads. After performing quality control on the sample's raw FASTQ data, reads were trimmed using fastp v0.19.4 to remove sequencing adaptors and low-quality bases or reads (13). The preserved clean reads were then mapped to the hg19 human reference genome using Hisat2 v2.2.1 and STAR v2.7.8a (20, 21). Gene fusions were detected using Arriba v2.3.0 and CICERO v1.7.0, with suspected pathogenic fusions confirmed by PCR and Sanger sequencing (22, 23). The read numbers for each gene were counted using HTSeq tool v0.13.5 (24). The gene expression counts for Zhujiang Hospital cohort samples and the TARGET cohort samples were input into ALLSorts (28 Jul 2021) (25). ALLSorts B-ALL subtype classifier predicted 18 subtypes and 5 meta-subtypes.

Multiplex ligation-dependent probe amplification assay

The MLPA assay was performed for all samples using 2 kits: Salsa MLPA Probemix P335 ALL-IKZF1 (MRC-Holland, Amsterdam, The Netherlands) to characterize the copy number alterations of the *IKZF1*, *PAX5*, *ETV6*, *RBI*, *BTG1*, *EBF1*, *CDKN2A*, *CDKN2B* genes and *PARI* region (*SHOX* area, *CRLF2*, *CSF2RA*, *IL3RA* and *P2RY8* genes) and Salsa MLPA Probemix P327 iAMP21-ERG (MRC-Holland) to identify the copy number alterations of the *RUNX1* and *ERG* genes.

Based on the MLPA results, patients were classified into 3 groups: No *IKZF1* deletion, *IKZF1* deletion (*IKZF1del* only) and *IKZF1^{plus}*. *IKZF1^{plus}* was defined as *IKZF1del* plus *PAX5*, *CDKN2A*, *CDKN2B* and/or *CRLF2* with *ERG* not deleted (26).

Detection of *CRLF2* expression using real-time quantitative PCR

RQ-PCR analysis of *CRLF2* expression was performed following the reverse transcription of RNA samples using a RevertAid First Strand cDNA Synthesis Kit (K1622) (Thermo Fisher). RQ-PCR was performed using an ABI 7500 Real-Time PCR System (Thermo Fisher). The housekeeping gene *GUS* was used as an internal control. The primers and probes used for RQ-PCR analysis are listed in Table 2. PCR was performed in 20 μ L reactions consisting of 1 \times TaqManTM Fast Advanced Master Mix (Thermo Fisher), 300 nM primer, 200 nM probe and 5 μ g of complementary DNA. The thermal cycling conditions were 50°C for 2 min and 95°C for 2 min, followed by 45 cycles of 95°C for 3 s and 60°C for 30 s. The ratio of *CRLF2* was calculated using *CRLF2* copies normalized to *GUS* copies.

Multi-parameter flow cytometry analysis

MFC for assessing MRD was performed on whole BM specimens obtained at the specified time intervals using a standard stain-lyse-wash procedure. A total of 1 \times 10⁶ cells were stained per analysis tube, acquired on a Navios flow cytometer using Navios software (Beckman Coulter) and analyzed using Kaluza (Beckman Coulter) or FCS Express (*De Novo* Software).

A 2-tube 7-colour panel was run. Tube A contained CD58-FITC (cLone: AICD58, Beckman Coulter), CD34-PE (cLone: 581, Beckman Coulter), CD10-PE-Cy7 (cLone: HI10a, BD), CD19-APC (cLone: SJ25C1, BD), CD38-eFluor 450 (cLone: HB7, eBioscience), CD45-BV510 (cLone: H130, BD) and 7AAD Viability Dye (Beckman Coulter). Tube B contained CD66c-FITC (cLone: B6.2/CD66, BD), CD13-PE (cLone: L138, BD), CD33-PE (cLone: D3HL60.251, Beckman Coulter), CD34-PerCP-Cy5.5 (cLone: 581, Biologend), CD10-PE-Cy7 (cLone: HI10a, BD), CD19-APC (cLone: J25C1, BD), and CD45-BV510 (cLone: H130, BD).

MRD was identified using both the leukemia-associated immunophenotype (LAIP) and different-from-normal (DFN) method. To distinguish residual tumor B cells from normal B-

progenitor cells, we used B-lymphocyte lineage and stage differentiation associated antigens such as CD19, CD10, CD34 and CD45, as backbone antibodies. Tumor B cells exhibit strong expression of CD10, CD19 and CD34, low expression of CD45, cross-line expression of myeloid antigens such as CD66c, CD13 and CD33, overexpression of CD58 and low expression of CD38.

Efficacy assessment

The short-term efficacy assessment indicators included the results for the response to prednisone and the results for the 15th and 33rd days of BM flow cytometry minimal residual disease (FCM-MRD). Response to prednisone is an independent prognostic indicator of acute lymphocyte leukemia. Prednisone good response (PGR) is considered the absolute blast cell count of peripheral blood on the 8th day \leq 1000/ μ L (27). This study uses the scrag-al-2016 risk-layered system and the results of the response to prednisone, with the 15th- and 33rd-day BM FCM-MRD less than 1% and the 33rd-day FCM-MRD less than 0.01% (28). Complete remission (CR, Blasts cells in bone marrow <5% and no measurable extramedullary leukemia) was defined according to the U.S. National Comprehensive Cancer Network (NCCN) guidelines on day 33 (29).

Statistical analysis

Statistical analyses were conducted using R version 3.6.2 and IBM SPSS statistics 25. Data are presented as the median and interquartile range (IQR). The Wilcoxon rank-sum test and Kruskal-Wallis test were applied to assess differences between 2 groups and more than 2 groups. Receiver operating characteristic curves (ROCs) and the Youden index were used to identify optimal cutoff values for gene expression (30). Fisher's exact test was used to analyze the clinical baseline of patients with different *CRLF2* expression levels. Multivariate Cox regression, Kaplan-Meier curves and the log-rank test were used to analyze OS and EFS via the 'survival' and 'survminer' packages in R. $P < 0.05$ was considered significant for two-sided tests.

Data availability

The data from Zhujiang Hospital cohort used in this study can be found in Supplementary Table 1. The datasets analyzed in the current study are publicly available from the ALL dataset of the TARGET database (TARGET Phase II, phs000464) [<https://portal.gdc.cancer.gov/projects>], and the clinical data were downloaded from cBioPortal [https://www.cbioportal.org/study/clinicalData?id=all_phase2_target_2018_pub]. The data for the Zhujiang Hospital cohort are available upon reasonable request.

Results

Basic information on study cohorts

A total of 111 newly diagnosed pediatric B-ALL patients admitted to and treated in the Department of Pediatric Hematology, Zhujiang

TABLE 2 List of RQ-PCR primers and probes.

Gene	Type	5'-3'
<i>GUS</i>	F. Primer	GGAATTTTGCCGATTCATGA
<i>GUS</i>	R. Primer	CCGAGTGAAGATCCCCTTTT
<i>GUS</i>	Probe	AACAGTCACCGACGAGAG
<i>CRLF2</i>	F. Primer	GCAAGTCGCTGGATGGTTTATT
<i>CRLF2</i>	R. Primer	CCACGAAAATCTCACGTGCTT
<i>CRLF2</i>	Probe	CCTGAAACCCAGTTCC

Hospital, Southern Medical University, China, from May 2020 to February 2022 were enrolled in the Zhujiang Hospital cohort. The last follow-up was conducted on August 25, 2023, with a median follow-up time of 28.6 months (95%CI: 27.6-32.1 months). There were 67 (60%) males and 44 (40%) females, with a median age of 4 years (range: 0-14 years), including 81 patients (73%) aged ≤ 6 years and 30 patients (27%) aged > 6 years. On the basis of the South China Children's Leukemia Group-ALL-2016 (SCCLG-ALL-2016) risk stratification (28), 13 patients (12%) were standard risk, 36 (32%) were intermediate risk, 61 (55%) were high risk, and 1 (1%) had an uncompleted risk assessment due to the abandonment of treatment. At the initial visit, for all 111 patients, the median white blood cell count was $13.33 \times 10^9/L$ [interquartile range (IQR): $5.23-43.5 \times 10^9/L$], and 24 patients had a white blood cell count $\geq 50 \times 10^9/L$. Moreover, the median proportion of bone marrow blast cells was 86% (IQR: 76-93.25%) at the initial visit among the 109 patients with records (Table 1). Next, molecular typing of B-ALL was implemented using ALLSorts, with the following results: DUX (4, 4%), ETV6-RUNX1 (12, 10%), ETV6-RUNX1-like (2, 2%), high hyperdiploid (38, 33%), KMT2A group (9, 8%), low hypodiploid (1, 1%), MEF2D (4, 4%), NUTM1 (2, 2%), PAX5 P80R (1, 1%), PAX alt (5, 5%), Philadelphia chromosome positive (Ph+, 4, 4%), Ph-like (5, 5%), TCF3-PBX1 (11, 10%), ZNF384 (1, 1%), and unclassified (13, 12%). Six patients had the Ph-like subtype, carrying somatic mutations of *KRAS* (n=1), *FLT3* (n=1), *PAX5* (n=1), and *PHF6* (n=1) (Supplementary Figure 1A) as well as *CRLF2*-associated fusion (n=2) and *EBF1-PDGFRB* fusion (n=1).

CRLF2 overexpression was associated with the Ph-like and high hyperdiploid genetic subtypes

All the 111 treatment-naïve B-ALL patients in the Zhujiang Hospital cohort underwent RNA-seq using pre-treatment bone marrow samples, and fragments per kilobase of transcript per million (FPKM) were calculated through bioinformatics analysis. The expression level of the *CRLF2* gene was mainly within the interval of $FPKM < 1$, i.e., generally low in the bone marrow samples of the treatment-naïve B-ALL patients. Some patients also manifested very high *CRLF2* expression, with a maximum FPKM of 75.1 (Figure 1A, left). Patients with high *CRLF2* expression mainly had the following B-ALL subtypes: KMT2A group, Ph+, Ph-like and high hyperdiploid (Figure 1A, right).

Subsequently, the *CRLF2* expression level was used to analyze the differences among the Ph+, Ph-like and high hyperdiploid subtypes. The expression level of the *CRLF2* gene was not the same between the Ph+ and Ph-like subtypes despite similar expression profiles. In addition, the expression level of the *CRLF2* gene in patients with the Ph-like and high hyperdiploid subtypes was significantly higher than that in other patients [Figures 1C, D, Ph-like: area under curve (AUC)=0.81, 95% confidence interval (CI): 0.611-1.000, $P=0.021$, high hyperdiploid: AUC=0.81, 95% CI: 0.728-0.894, $p < 0.001$]. No correlation was found between the Ph+ subtype and *CRLF2* expression level (Figure 1B, $p=0.72$). The Youden indexes of *CRLF2* expression in predicting the Ph-like and high hyperdiploid subtypes were subsequently calculated (Figure 1E) (30), and the corresponding FPKM quantiles of

CRLF2 expression were set as the cutoffs (Ph-like cutoff point=94%, high hyperdiploid cutoff point=52%). *CRLF2* was assigned as a three-classification variable, namely *CRLF2*-high, *CRLF2*-medium and *CRLF2*-low (Figure 1E). Patients with *CRLF2*-high, *CRLF2*-medium and *CRLF2*-low accounted for 6% (Ph-like, n=3, high hyperdiploid, n=1, Ph+, n=1, KMT2A group, n=1), 42% (high hyperdiploid, n=31, Ph-like, n=1, other subtypes, n=15) and 52% (other subtypes, n=48, high hyperdiploid, n=6, Ph+, n=3, Ph-like, n=1), respectively, of all the patients (Figure 1F).

Characteristics at diagnosis and minimal residual disease status during induction therapies among patients with different CRLF2 expression levels

The clinical characteristics of the 3 groups of patients were analyzed. There were no significant differences in the incidence rates of concomitant testicular infiltration, central nervous system leukemia, intracranial hemorrhage, hepatosplenomegaly, and bone pain at the diagnosis of the disease in patients with different *CRLF2* expression levels (Supplementary Table 1).

The proportion of bone marrow blast cells was not significantly different among the 3 groups of patients at diagnosis but was slightly higher in patients with *CRLF2*-high than in those with *CRLF2*-low (*CRLF2*-high vs. *CRLF2*-low, $p=0.036$, Kruskal-Wallis, $p=0.06$, Figure 2A). Similarly, the white blood cell count at initial visit was significantly higher in patients with *CRLF2*-high than in patients with *CRLF2*-low and *CRLF2*-medium (*CRLF2*-high vs. *CRLF2*-low, $p=0.016$, *CRLF2*-high vs. *CRLF2*-medium, $p=0.016$, Kruskal-Wallis, $p=0.042$, Figure 2B).

The CR rate on 33rd day was significantly lower in patients with *CRLF2*-high (33%) compared to those with *CRLF2*-medium (80%, $p=0.03$) and *CRLF2*-low (76%, $p=0.046$, global $p=0.062$, Figure S2). Furthermore, the MRD status of the patients at different treatment time points was dynamically monitored by means of flow cytometry. The time points for monitoring were set as the 15th day and 33rd day after the start of induction therapies as well as before the start of consolidation therapy. The proportion of tumor cells in flow cytometry MRD decreased rapidly during induction therapy in patients with *CRLF2*-medium, and MRD remained negative by the time of assessment before consolidation therapy (a total of 43 patients available for assessment via flow cytometry MRD $< 0.1\%$). However, the patients with *CRLF2*-high and *CRLF2*-low had varying degrees of residual MRD, and the proportion of positive patients before consolidation therapy was 33.3% (n=2) and 5.5% (n=3), respectively (Figure 2C).

Prognostic impact of CRLF2-high in the TARGET cohort

To further validate the association between the *CRLF2* expression level and poor prognosis, the publicly available TARGET cohort data were used as an externally independent

validation cohort. The stratified cutoffs (quantiles) for the *CRLF2* expression level obtained from the Zhujiang Hospital cohort were employed to stratify the FPKM data of *CRLF2* in the TARGET cohort, followed by a survival analysis.

Because only pediatric patients aged ≤ 15 years were recruited for the Zhujiang Hospital cohort and the age range of patients in the TARGET cohort was 2-30 years, age was included as a stratification factor in the multivariate Cox regression analysis (Figures 3A, B). Both age and *CRLF2* FPKM expression stratification served as independent prognostic factors for both event-free survival (EFS) and overall survival (OS) (EFS: C-index = 0.61, $p < 0.001$, OS: C-index = 0.64, $p = 0.03$). Age > 15 years was a risk factor for both EFS and OS [EFS: hazard ratio (HR) = 1.79, 95% CI: 1.03-3.10, $p = 0.037$,

OS: HR=2.74, 95% CI: 1.18-6.4, $p = 0.019$]. However, *CRLF2* expression stratification of *CRLF2*-medium was not a risk factor for EFS (HR=0.52, 95% CI: 0.35-0.78, $p = 0.002$), and *CRLF2*-high was a distinct risk factor for OS (HR=4.33, 95% CI: 1.20-15.6, $p = 0.025$).

The effects of the *CRLF2* expression level on EFS and OS were further validated when plotting K-M curves. Without considering the influence of age on prognosis, patients with *CRLF2*-high and *CRLF2*-low had notably shorter EFS than those with *CRLF2*-medium (*CRLF2*-high vs. *CRLF2*-medium, $p = 0.0048$, *CRLF2*-low vs. *CRLF2*-medium, $p = 0.0012$, global $p = 0.0013$, Figure 3C). The patients with *CRLF2*-high manifested an overtly shorter OS than did those with *CRLF2*-low and *CRLF2*-medium (*CRLF2*-high vs.

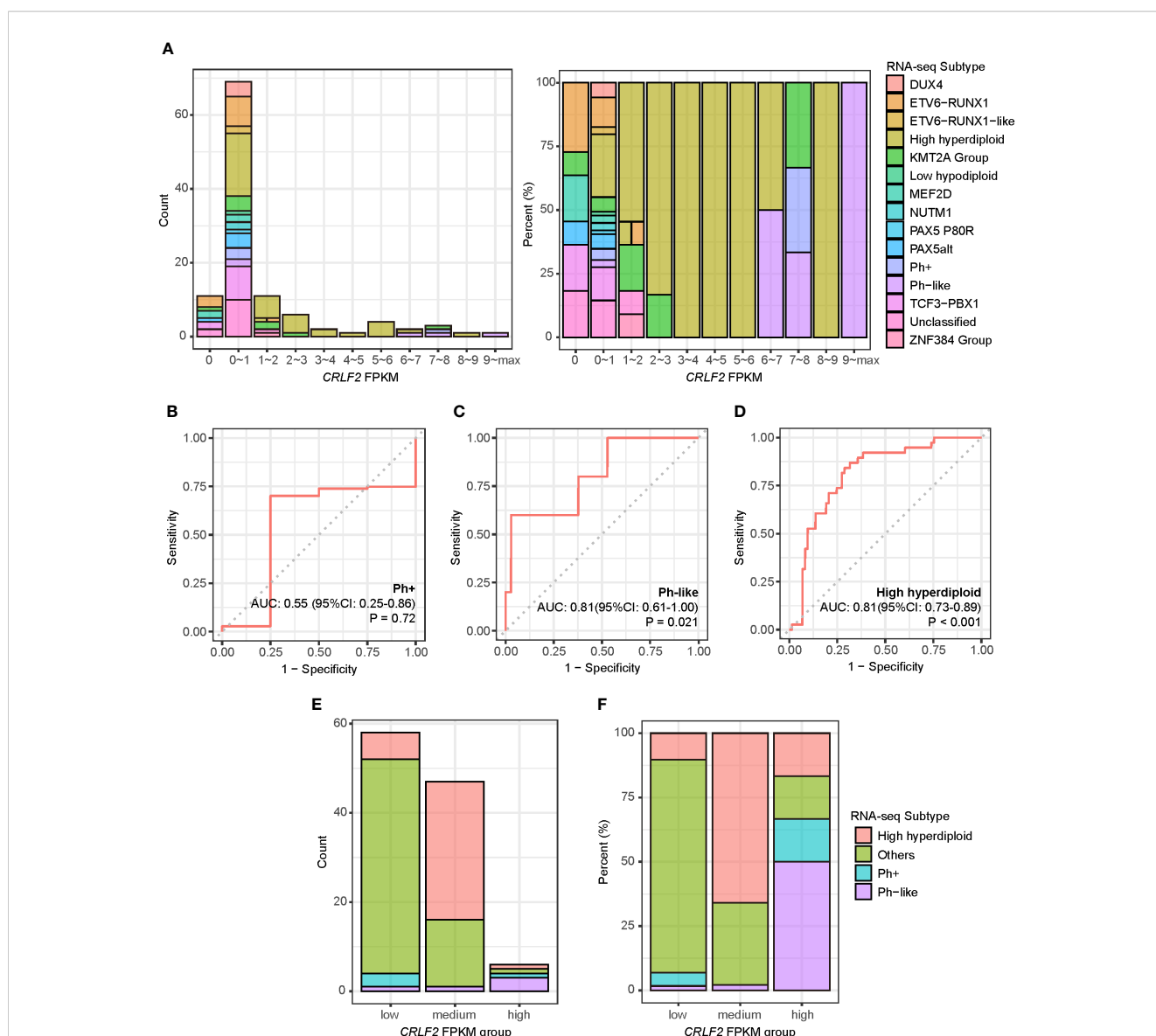


FIGURE 1

CRLF2 expression and B-ALL subtypes of pediatric B-ALL patients in the Zhujiang Hospital cohort. (A) *CRLF2* expression in 111 B-ALL patients with RNA-seq B-ALL subtypes. (B–D) ROC curves for the correlation of *CRLF2* expression with the Ph+, Ph-like and high hyperdiploid subtypes. (E, F) RNA-seq subtypes in *CRLF2* FPKM groups. The Wilcoxon rank-sum test was applied to assess differences between 2 groups. The cutoff point between high/medium levels and medium/low levels were determined using the Youden index for *CRLF2* expression with the Ph-like and high hyperdiploid subtypes.

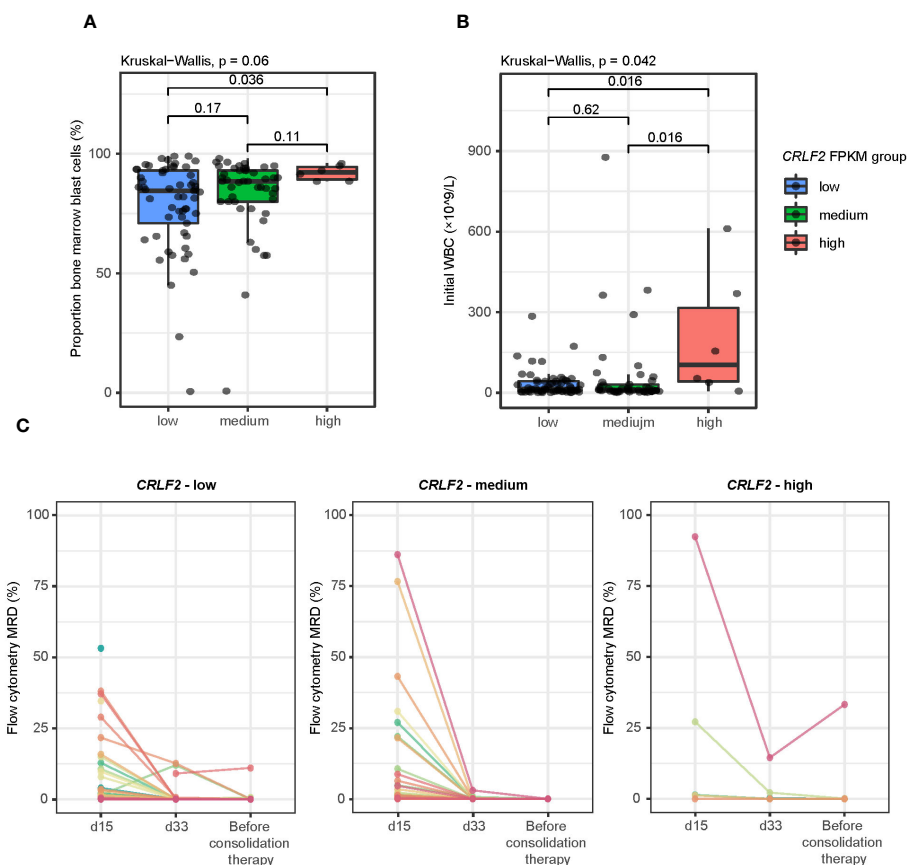


FIGURE 2

Association of *CRLF2* expression with blasts in BM, WBC, and flow cytometry MRD. (A) Proportion of bone marrow blast cells for patients with different *CRLF2* expression levels. (B) Initial WBC of patients with different *CRLF2* expression levels. (C) Flow cytometry MRD for different *CRLF2* expression levels. The Wilcoxon rank-sum test and Kruskal-Wallis test were applied to assess differences between 2 groups and more than 2 groups.

CRLF2-low, $p=0.035$; *CRLF2*-high vs. *CRLF2*-medium, $p=0.002$, global $p=0.023$, Figure 3D); there was no significant difference in OS between the patients with *CRLF2*-medium and *CRLF2*-low.

Then, patients in the TARGET B-ALL cohort were selected as a childhood group (age ≤ 15 years old). The age range for the children in the childhood group from the TARGET B-ALL cohort was consistent with that for the children in the Zhujiang Hospital cohort. In the childhood group, both EFS and OS were markedly superior for patients with *CRLF2*-medium, compared with those for patients with *CRLF2*-low and *CRLF2*-high (EFS: *CRLF2*-medium vs. *CRLF2*-high, $p=0.003$, *CRLF2*-medium vs. *CRLF2*-low, $p<0.0001$, global $p<0.0001$, Figure 3E, OS: *CRLF2*-medium vs. *CRLF2*-high, $p=0.014$, *CRLF2*-medium vs. *CRLF2*-low, $p=0.018$, global $p=0.015$, Figure 3F). The results are consistent with the MRD status observed in the Zhujiang Hospital cohort.

CRLF2 expression levels among pediatric patients with *P2RY8-CRLF2* fusion

In our cohort, fusion genes were also detected in 58 out of the 111 patients (53.3%). The fusion genes detected in patients with varying *CRLF2* gene expression levels are shown in Figure 4A.

Although patients carried a wide range of fusion genes, *CRLF2*-related fusions were more prevalent in patients with *CRLF2*-high (2/6, 33.3%). In comparison, *P2RY8-CRLF2* fusion was the predominant *CRLF2*-related fusion (6/7, 85.7%). Among the patients carrying the *P2RY8-CRLF2* fusion, RQ-PCR was performed for 5 patients; the results are shown in Figure 4B. The FPKM of *CRLF2* was consistent with the RQ-PCR results (*CRLF2* ratio). The patient with the highest *CRLF2* expression level, namely, P067, had the Ph-like subtype, had a flow cytometry MRD of 0.1% as of consolidation therapy, and failed to achieve negative flow cytometry MRD. Patients P009, P081 and P082 with *CRLF2*-medium all had the high hyperdiploid subtype, and flow cytometry MRD was negative on the 33rd day of induction therapy except for 1 patient who had an MRD of 0.1%; no residual tumor cells were detected in flow cytometry MRD in all 3 patients before consolidation therapy. Patient P085, with the lowest *CRLF2* expression level, had the ETV6-RUNX1-like subtype and had negative flow cytometry MRD results on the 33rd day of induction therapy and thereafter. These results suggest that not all patients carrying the *P2RY8-CRLF2* fusion were *CRLF2*-high. Additionally, patients with the high hyperdiploid subtype and *P2RY8-CRLF2* fusion showed a better prognosis, as determined through flow cytometry MRD.

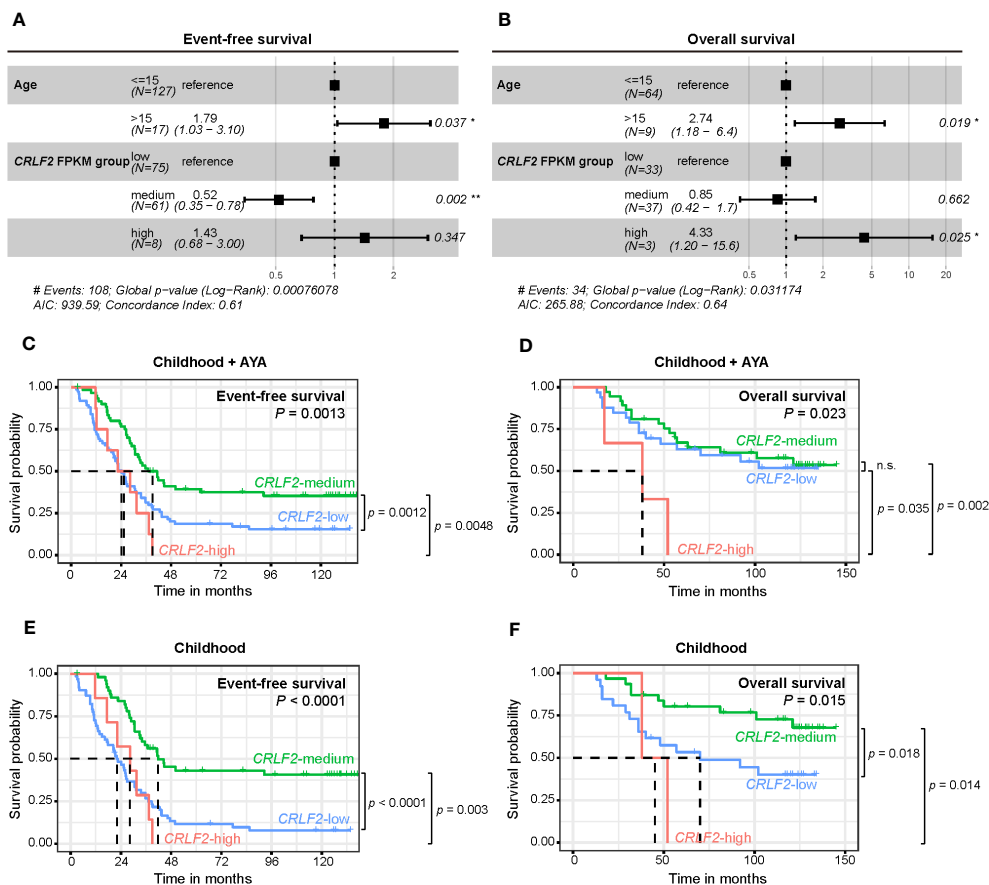


FIGURE 3

Survival analysis of pediatric B-ALL patients with *CRLF2* expression in the TARGET cohort. (A, B) Effect of *CRLF2* expression levels on event-free survival (EFS, A) and overall survival (OS, B) in the TARGET cohort. Estimates are based on multivariate Cox regression. (C, D) Kaplan-Meier plots of OS and EFS for patients with different *CRLF2* expression levels in the TARGET cohort. (E, F) Kaplan-Meier plots of OS and EFS for pediatric patients with different *CRLF2* expression levels in the TARGET cohort.

Determination of Youden index for quantitative cutoffs of *CRLF2* gene expression by RQ-PCR

A total of 49 patients in the Zhujiang Hospital cohort underwent *CRLF2* expression testing through RNA-seq and RQ-PCR, and relatively consistent RQ-PCR results for *CRLF2* (*CRLF2* ratio) were obtained ($R=0.79$, $p<0.0001$, Figure 5A). Moreover, the cutoff value for *CRLF2*-high in RQ-PCR was determined, and the point value corresponding to the Youden index was used as a cutoff point as well (AUC=0.988, 95% CI: 0.968-1.000, $p<0.001$, Youden index cutoff point =37.75%, sensitivity =100%, and specificity =97%, Figure 5B). Then, the *CRLF2* ratios were arranged in descending order and classified by virtue of the RQ-PCR cutoff point, and 7 patients were classified as *CRLF2*-high (RQ-PCR data). There were 2 patients with the Ph-like subtype, 1 with the Ph+ subtype, 3 with the high hyperdiploid subtype, and 1 with the KMT2A subtype (Figure 5C). The 3 patients with the high hyperdiploid subtype had poor prognostic factors. Specifically, 1 patient had prednisone-poor response (PPR) on the 8th day of prednisone treatment, 1 had flow cytometry MRD results that were positive by the 15th day, and 1

carried mutations in genes (*NRAS* p.G13D, *PTPN11* p.S502A) in the RTK-RAS pathway.

Discussion

CRLF2 overexpression is involved in the pathogenesis and progression of B-ALL through the JAK/STAT signaling pathway (1-4). However, it is still unknown whether the prognosis of B-ALL patients can be predicted based on *CRLF2* expression levels (5, 31, 32). The proportion of patients with *CRLF2* overexpression varies substantially in previously published studies, and the proportion of patients with *CRLF2* overexpression among unscreened patients ranges from a minimum of 4.7% to a maximum of 25% (2, 4-7), implying that *CRLF2* overexpression needs to be defined by a more suitable cutoff value.

B-ALL can be categorized into different subtypes on the basis of genomic features and driver gene mutations, and such classification is of vital significance in risk stratification, treatment guidance and prognosis prediction. In this study, ALLSorts, defined by Gu et al., and RNA-seq analysis were used to identify 18 molecular subtypes (5, 31-33).

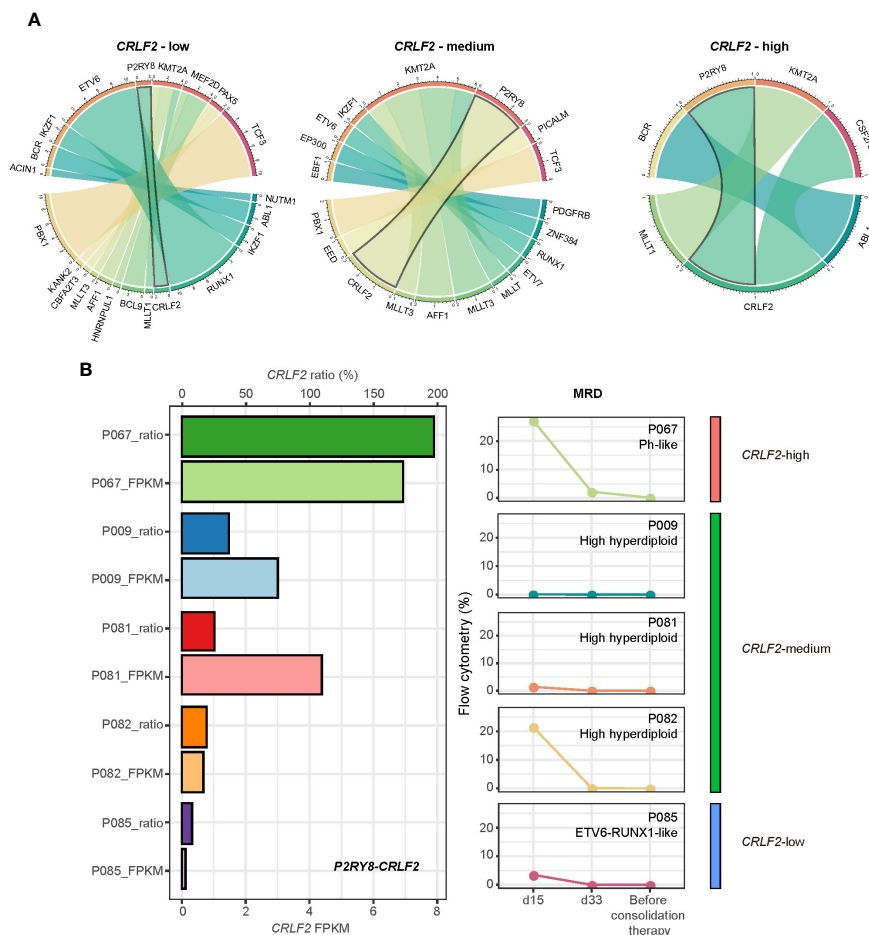
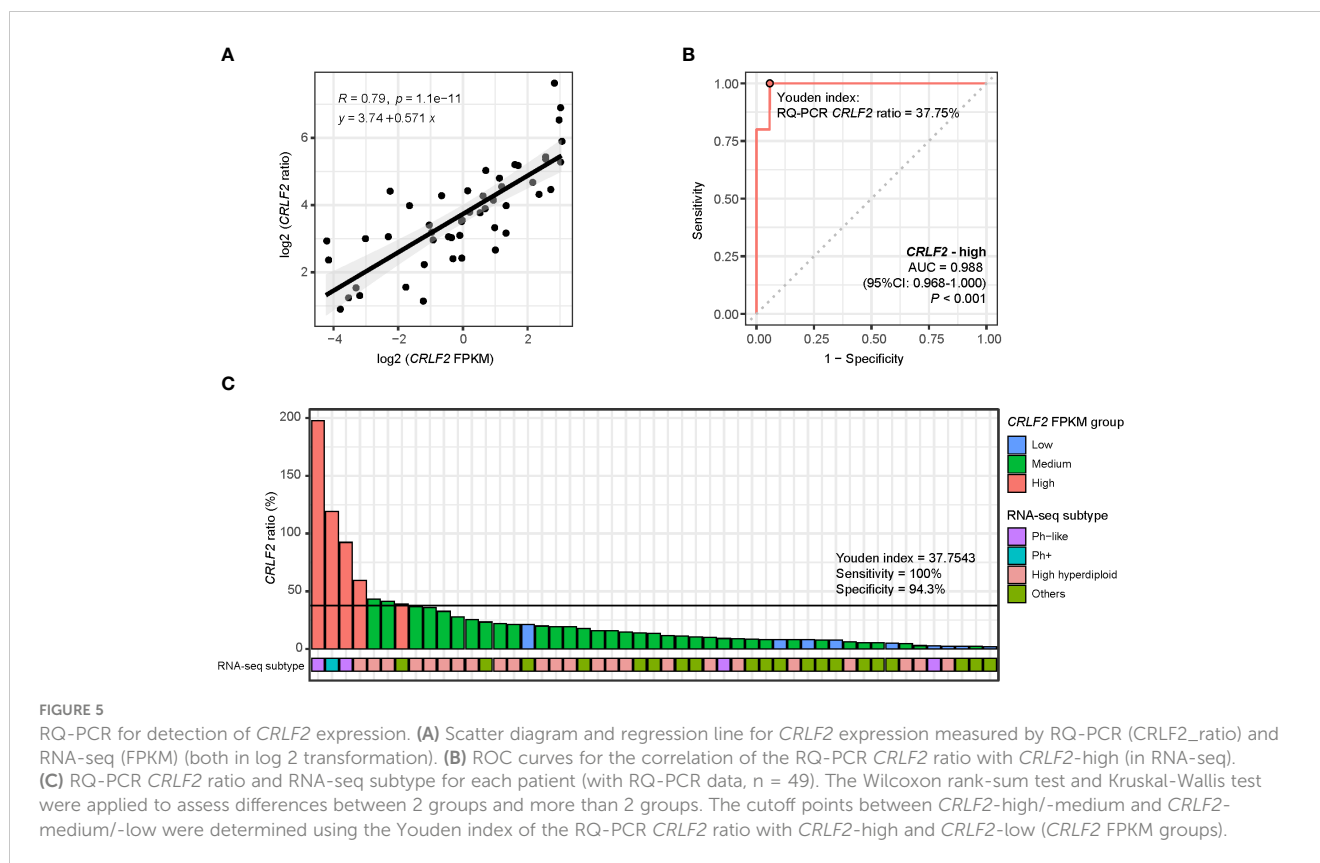


FIGURE 4 Association of *CRLF2* expression and *P2RY8-CRLF2* fusion. **(A)** Circos plot shows fusions in each *CRLF2* expression level. **(B)** RNA-seq (FPKM) and RQ-PCR (ratio) of *CRLF2* expression and dynamic changes in flow cytometry MRD for each *P2RY8-CRLF2* positive patient (n = 5).

The exploration and analysis of the *CRLF2* expression level and molecular types of B-ALL revealed that the molecular types were heterogeneous in patients with different *CRLF2* expression levels. Patients with moderate upregulation of *CRLF2* mostly had the high hyperdiploid subtype, while those with *CRLF2* overexpression mainly had the Ph-like subtype. A close correlation between the Ph-like subtype and *CRLF2* expression has been reported. The studies by Martínez-Anaya et al. and Harvey et al. reported that more than 50% of patients with *CRLF2* overexpression have the Ph-like subtype (34, 35), a result that is consistent with the findings of this study. The high hyperdiploidy-associated upregulation of *CRLF2* expression primarily manifested as *CRLF2*-medium in our cohort, where more than half of this subgroup had the high hyperdiploid subtype. Such upregulation may be associated with the amplification of the X/Y chromosome where the *CRLF2* gene is located (Supplementary Figures 1B, C) (31, 36).

The majority of relevant studies have argued that *CRLF2* overexpression is correlated with the poor prognosis of B-ALL patients, especially those with high-risk B-ALL (2, 3, 31, 34, 37). However, some studies have argued that *CRLF2* overexpression has

no impact on patient prognosis (5, 36). According to the baseline analysis of this cohort, the patients with *CRLF2*-high had a higher proportion of bone marrow blast cells and initial white blood cell counts. A high initial white blood cell count in childhood ALL is a recognized high-risk factor (38). Additionally, flow cytometry MRD decreased slowly or persistently positive during induction therapy in patients with *CRLF2*-high, indicating poor treatment response for such patients. The survival outcome for patients in the Zhujiang hospital cohort could not be analyzed due to the limited follow-up time. The publicly available childhood B-ALL data from the TARGET database were used to further validate the relationship between the *CRLF2* expression level and prognosis. The *CRLF2* expression level was defined in the TARGET cohort using the same methodology as that in the present cohort. Among B-ALL patients (pediatric + AYA), the patients with *CRLF2*-high had worse EFS and OS. Furthermore, pediatric patients with *CRLF2*-medium manifested a better prognosis, which is consistent with the better prognosis of pediatric patients with high hyperdiploid and X/Y chromosome amplification particularly (39–41). These results confirm the hypothesized relationship between *CRLF2* expression



level and prognosis in this study: There is heterogeneity in patients with elevated *CRLF2* expression, that is, patients with *CRLF2*-high have a poor prognosis, and medium upregulation does not increase the prognostic risk.

CRLF2 rearrangement is the most studied class of genetic alteration leading to *CRLF2* overexpression, mainly consisting of *P2RY8-CRLF2* and *IGH-CRLF2*. The former alteration results from local deletion on pseudoautosomes, an event that is related to Down's syndrome-associated pediatric ALL (DS-ALL) (42). It was previously reported that *P2RY8-CRLF2* is mostly associated with a poor prognosis (2, 5, 37). Hence, the patients with *P2RY8-CRLF2* fusion in our cohort were also analyzed. In our cohort, *P2RY8-CRLF2* did not definitely lead to *CRLF2*-high. One patient with *P2RY8-CRLF2* fusion and *CRLF2*-high had the Ph-like subtype, and the flow cytometry MRD decreased slowly and persistently, suggesting a possible poor prognosis. However, most of the *P2RY8-CRLF2* patients with *CRLF2*-medium and *CRLF2*-low had the high hyperdiploid subtype and a better prognosis (3/4). It was speculated that *P2RY8-CRLF2* may be a minor subclone in such patients (43). *IGH-CRLF2* is another common type of *CRLF2* rearrangement. Russell et al. identified an immunoglobulin heavy chain gene (*IGH*) translocated to *CRLF2* at Xp22.3 or Yp11.3, forming the *IGH-CRLF2* fusion, in approximately 5% of BCP-ALL patients (44). In our cohort, *IGH-CRLF2* was found in only 1 (0.9%) patient. It has been reported that translocation patients (*IGH-CRLF2*) are older (median age: 16 years old), whereas deletion patients (*P2RY8-CRLF2*) are younger (median age: 4 years old)

(2, 5, 37). In this study, the overall young age of our cohort (median age: 4 years old) may have led to fewer *IGH-CRLF2* patients.

In the exploration of cutoffs for the 3 categories of *CRLF2* expression, RNA-seq was adopted to detect the *CRLF2* expression level (FPKM) and molecular types obtained from transcriptional profiling analysis. However, RNA-seq, as a research-based assay, is not practical to carry out in the clinic. In contrast, RQ-PCR is a more accessible assay in clinical practice. For detecting *CRLF2* expression levels, RQ-PCR is faster, less expensive and more standardized than RNA-seq. The RNA-seq and RQ-PCR results were compared, and the detected *CRLF2* expression levels were highly consistent. Furthermore, defining *CRLF2*-high using RQ-PCR *CRLF2* ratio $\geq 37.75\%$ as the cutoff yielded classification results that were consistent with those obtained using RNA-seq, thus laying a foundation for subsequent prospective studies on *CRLF2* overexpression for stratified treatment.

By analyzing the data from 111 Chinese pediatric B-ALL patients in our center, the classification of the *CRLF2* expression level into 3 categories more accurately described the prognosis and features of patients with various *CRLF2* expression levels. In the external validation cohort (TARGET cohort), patients with *CRLF2*-high had a significantly poor prognosis. The three-classification system for the *CRLF2* expression level detected by RQ-PCR in this study facilitates the rapid identification of patients with *CRLF2*-high in the clinic. For such patients, studies need to be conducted to further confirm and discover how to improve patient prognosis by intensifying chemotherapy or combining classic treatments with novel therapies.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary Material](#).

Ethics statement

The studies involving humans were approved by the central ethics committee of Zhujiang Hospital, Southern Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

DL: Data curation, Methodology, Resources, Writing – original draft. KY: Formal Analysis, Software, Visualization, Writing – original draft. LYu: Data curation, Resources, Writing – review & editing. LH: Data curation, Resources, Writing – review & editing. XL: Data curation, Resources, Writing – review & editing. LW: Data curation, Resources, Writing – review & editing. XX: Formal Analysis, Software, Writing – original draft. JZ: Investigation, Writing – original draft. QZ: Conceptualization, Methodology, Supervision, Writing – review & editing. LYa: Conceptualization, Methodology, Supervision, Writing – review & editing.

Acknowledgments

The authors would like to thank all the participating patients and their families.

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Conflict of interest

Author JZ is employed by Guangzhou KingMed Center for Clinical Lab. Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2023.1256054/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Genomic alterations in pediatric B-ALL patients in the Zhujiang Hospital cohort. (A). Gene mutation profiles for individual patients. (B, C). CRLF2-related structural variants (B) and X chromosome structural variation (C) with different CRLF2 expression levels.

SUPPLEMENTARY FIGURE 2

Survival and efficacy analysis in pediatric B-ALL patients in the Zhujiang Hospital cohort. CR rate in 33rd with different CRLF2 expression levels.

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