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Genetic Profiling of Pediatric Patients with B-Cell Precursor Acute Lymphoblastic Leukemia

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Abstract

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a heterogeneous leukemia subgroup. It has multiple sub-types that are likely to be classified by prognostic factors. Following a systematic literature review, this study analyzed the genes correlated with BCP-ALL prognosis (*IKZF1, PAX5, EBF1, CREBBP, CRLF2, JAK2, ERG, CXCR4, ZAP70, VLA4, NF1, NR3C1, RB1, TSLP, ZNRF1,* and *FOXO3A)*, specifically their nucleotide variations and expression profiles in pediatric BCP-ALL samples. The study included 45 pediatric BCP-ALL patients with no cytogenetic anomaly and a control group of 10 children. The selected genes' hot-spot regions were sequenced using next-generation sequencing, while Polymorphism Phenotyping v2 and Supplemental Nutrition Assistance Program were used to identify pathogenic mutations. The expression analysis was performed using quantitative real-time polymerase chain reaction. The mutation analysis detected 328 variants (28 insertions, 47 indels, 74 nucleotide variants, 75 duplications, and 104 deletions). The most and least frequently mutated genes were *IKZF1* and *CREBBP*, respectively. There were statistically significant differences between patients and controls for mutation distribution in eight genes (*ERG, CRLF2, CREBBP, TSLP, JAK2, ZAP70, FOXO3A,* and *NR3C1*). The expression analysis revealed that *JAK* and *ERG* were significantly overexpressed in patients compared with controls (respectively, $p = 0.004$ and $p = 0.003$). This study combined genes and pathways previously analyzed in pediatric BCP-ALL into one dataset for a comprehensive analysis from the same samples to unravel candidate prognostic biomarkers. Novel mutations were identified in all of the studied genes.

Keywords
- pediatric BCP-ALL
- mutation
- gene expression
- NGS
- biomarker

Introduction

The frequencies and structures of individual anomalies among B-cell precursor acute lymphoblastic leukemia (BCP-ALL) subtypes differ significantly. BCP-ALL is disrupted because of genetic changes [t(12;21)(p13;q22) ETV6-RUNX1, t(1;19)(q23;p13) TCF3-PBX1, t(4;11)(q21;q23) KMT2-A/AFF1, and t(9;22)(q34;q11.2) translocations, such as

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BCR-ABL1, hyperdiploidy, and hypodiploidy] in two out of three normal lymphoid maturation processes.[1–5] However, these genetic anomalies are insufficient to explain the biological basis of leukemia, differences in treatment responses, or causes of leukemia in individuals without cytogenetic anomalies. Therefore, the identification of new molecular markers that may play a role in disease pathogenesis and can, thus, be used for diagnosis and treatment is very important for BCP-ALL. In modern treatment protocols, this is achieved through risk-adapted therapy, reflecting the probability of treatment failure. For this purpose, prognostic factors are used to predict an individual patient's risk of relapse and to adjust the required treatment intensity by classifying patients into different therapeutic risk groups, for instance, standard/low, medium/average, high risk, or very high.

In recent years, the advent of microarray and next-generation sequencing (NGS) technologies, gene expression, DNA copy number, and mutation analyses has enabled the identification of many genetic abnormalities in tumor suppressors, cell cycle control genes, transcription factors and co-activators, and the genes involved in the development, proliferation, and signaling of B-cell lines.[1,3,6,7] More specifically, the use of NGS technologies in leukemia has changed the perspective of leukemia pathogenesis because it is possible to examine genetic abnormalities with massively parallel analysis and deep sequencing ability.[2,4,7–10]

In this study, mutation and expression analyses of genes considered to be genetic prognostic factors (associated with disease-free survival, overall survival time, risk of relapse, and treatment response) in independent studies were analyzed in our study group.[2,4,9–12] Our data can help identify candidate diagnostic and/or prognostic biomarkers for pediatric BCP-ALL, a heterogeneous leukemia subgroup. The novelty of the study is its parallel analysis of genes selected after a systematic literature review of independent published studies with NGS and quantitative real-time polymerase chain reaction (qRT-PCR) in pediatric BCP-ALL cases.

Table 1 Pathological and clinical characteristics of the pediatric BCP ALL patients

Clinical features	All cases (*n*:45)
Sex	
Male: female	25:20
Age	
Median (min–max)	7.5 (1–15)
WBC, 10^9/L	23525
Median (min–max)	(600–131,000)
Peripheral blood blast rate (%)	38.8 (5–100)
Bone marrow blast	75.7
rate (%)	(43–100)
LDH, U/L	723
Median (min–max)	(222–3,768)
Risk group, *n* (%)	
SRG	16 (36)
MRG	20 (44)
HRG	9 (20)
Relapse, *n* (%)	
Yes	1 (2)
No	44 (98)
Steroid response, *n* (%)	
Yes	41 (91)
No	4 (9)
Last status, *n* (%)	
Alive	43 (96)
Exitus	2 (4)
Status of treatment during the study, *n* (%)	
Ongoing treatment	12 (27)
Post-treatment	33 (73)

Abbreviations: ALL BFM, acute lymphoblastic leukemia Berlin-Franfurt-Munster; CNS, central nervous system; HRG, high-risk group; LDH, lactate dehydrogenase; max, maximum; min, minimum; MRG, medium-risk group; SRG, standard risk group; WBC, white blood cells.

Material and Methods

Study Group

The study group consisted of peripheral blood ($n = 25$) or bone marrow ($n = 20$) taken from 45 individuals (25 females and 20 males) between the age of 1 and 15 years old, who had been diagnosed as BCP-ALL at the LÖSANTE Child and Adult Hospital and had no cytogenetic abnormalities. The patient group's demographic and clinical data are given in ►**Table 1**. Patients were treated according to the acute lymphoblastic leukemia Berlin-Frankfurt-Münster (ALL BFM 95) ($n = 36$), ALL-IC BFM 2009 ($n = 8$), and CCG 1961 ($n = 1$) protocols in which they were grouped (standard, medium, and high) according to the risk classification based on treatment and follow-up features and which determined the clinical course of the disease. The patient samples were also analyzed in two groups: ongoing treatment group ($n = 12$) and post-treatment group ($n = 33$). Each patient was classified according to their clinical characteristics at the time of diagnosis (age, number of white cells, number of blasts, presence of cytogenetic and/or molecular genetic anomalies, 8-day steroid response [<1,000/μL blast count in peripheral blood], and bone marrow evaluation on 15th and 33rd days). In addition, a healthy control group consisting of 10 children (six boys and four girls), aged between 1 and 15 years old was included in the study. The healthy control group was selected from individuals without any hematological disease, who came to the hospital for check-ups. An informed consent form was completed by the parents of the individuals whose blood and bone marrow samples were taken, while approval permission was obtained from Ankara University Clinical Research Ethics Committee on September 22, 2013, decision number 14-543-13.

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A Meta-Literature Search for Selection of Candidate Genes

PubMed was searched for combinations of the following words in article titles and abstracts: "BCP-ALL"(Title/Abstract) AND "GENE"(Title/Abstract) AND "PEDIATRIC" between 2003 and 2019. This search identified 60 candidate articles from which 66 genes were selected for further analyses. We then restricted our focus to 10 genes studied at the DNA level, 43 at the gene transcription level, and 13 at both levels. Since our study group consisted of individuals without cytogenetic abnormalities, 20 genes were excluded because they were also associated with cytogenetic abnormalities. According to the literature, 22 of the remaining 46 genes are not statistically significant prognostic biomarkers for BCP-ALL. Finally, eight genes were excluded because their relationship with the B-cell line was either unclear or indirect when classified according to ontological and biological pathway analyses of 24 genes, performed using the KEGG (*https://www.genome.jp/kegg/*) bioinformatics database.

Our study analyzed 16 genes in total, of which eight showed changes in both DNA and expression levels. That is, DNA sequence changes were analyzed in 14 genes (zeta chain of T-cell receptor-associated protein kinase 70 [*ZAP70*], thymic stromal lymphopoietin [*TSLP*], janus kinase 2 [JAK2], cytokine receptor-like factor 2 [*CRLF2*], IKAROS family zinc finger 1 [*IKZF1*], paired box 5 [*PAX5*], EBF transcription factor 1 [*EBF1*], CREB binding protein [*CREBBP*], forkhead box O3 [*FOXO3A*], retinoblastoma 1 [*RB1*], zinc and ring finger 1 [*ZNRF1*], nuclear receptor subfamily 3 group C member 1 [*NR3C1*], neurofibromin 1 [*NF1*], and ETS transcription factor ERG [*ERG*]), while expression level differences were examined in 11 genes (*ZAP70*, *JAK2*, *CRLF2*, *IKZF1*, *PAX5*, *EBF1*, *NR3C1*, *CXCR4*, *VLA4*, *NF1*, and *ERG*). For the NGS study, the gene regions with the highest mutation frequency in the articles were included in the study plan. The flow chart for the study selection process is shown in ►**Fig. 1**.

Candidate genes were divided into four different groups according to the cellular pathways in which they were present: cellular activities (*NF1, ERG, VLA4, CXCR4*); signal transduction (*ZAP70, TSLP, JAK2, CRLF2*); gene expression control (*IKZF1, PAX5, EBF1*); and treatment response (*NR3C1, CREBBP*).

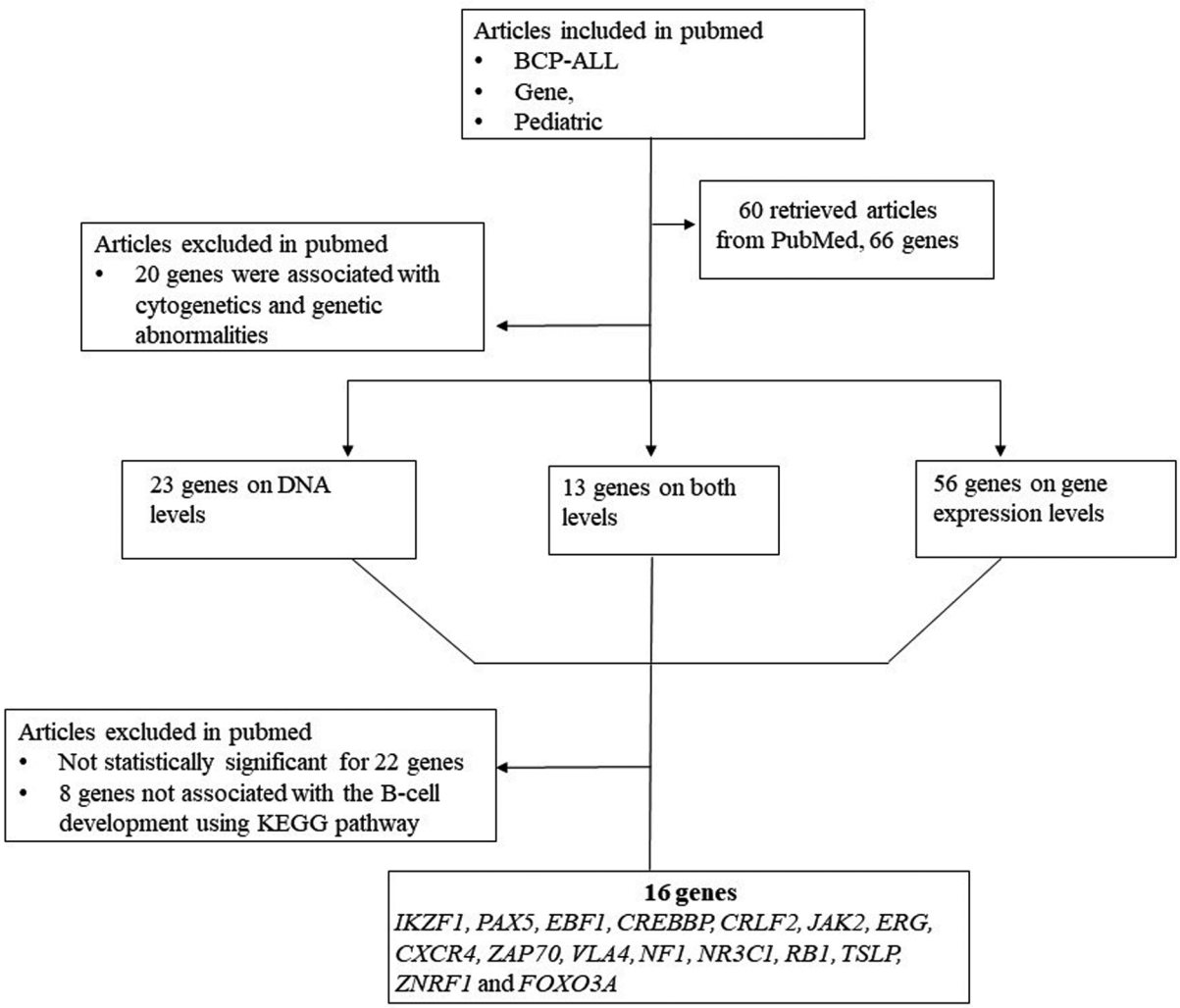

Fig. 1 Flow diagram of the study selection process.

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DNA Isolation and Next Generation Sequencing

For the mutation analysis, 2 mL of peripheral blood or bone marrow samples were collected from patients and healthy individuals in tubes containing 2 mL of 2% ethylenediaminetetraacetic acid (Becton Dickinson, United States). DNA isolation was performed according to the manufacturer's protocol using MagNa Pure Automated Isolation System and Magna Pure Compact Nucleic Acid Isolation Kit I from blood taken from the patients and healthy individuals. NGS was used to detect mutations in *ZAP70, TSLP, JAK2, CRLF2, IKZF1, PAX5, EBF1, CREBBP, FOXO3A, RB1, ZNRF1, NR3C1, NF1,* and *ERG.* The target genes' hotspot regions were selected from the literature and sequenced. The 49 hotspot exons were amplified. The amplicon lengths ranged between 241 and 345 base pairs along with adapter arrays. The primer sequences used in accordance with the NGS analysis device operating system were designed using the www.idtdna.com database by including 26-base adapter A and B-series, the 4-key sequence (TCAG), and, after the adapter array, multiplex identifiers sequences. The heterodimer and homodimer analyses of the candidate primers were performed using the www.idtdna.com/oligoanalyzer database. Primer sequences can be given if requested. Exons were amplified using the FastStart High Fidelity PCR System and GC-RICH PCR System kits (Roche Applied Science, Germany). Amplicons were purified with Ampure XP beads (Beckman Coulter, Krefeld, Germany), while the libraries were quantified by Quant-iT PicoGreen dsDNA Reagent (Invitrogen, Carlsbad, CA, United States). Targeted sequencing was performed on a Roche FLX GS Junior (454-Life Sciences, Branford, CT, United States) according to the manufacturer's instructions. [13] After data quality assessment, variant detection analyses were performed using AVA software (GS Amplicon Variant Analyzer software version 2.5.3, Roche Applied Science, Germany). The mutation analysis was performed using Sequence Pilot Module SeqNext Patient software, while the nucleotide exchange, insertion, deletion, and indel detection were done with Sequence Pilot Module SeqNext Patient software. Coverage below 5% was considered as a device read error.

RNA Isolation, c-DNA Synthesis, and Quantitative RT-PCR

For gene expression analysis, 2.5 mL peripheral blood or bone marrow samples were taken into PaXGene Blood RNA tubes. RNA isolation was performed using BiOstic Stabilized Blood RNA Isolation Kit according to the manufacturer's recommended protocol.

Quantitative RT-PCR was used to calculate the expression rates of the ribosomal protein lateral stalk subunit P0 (*RPLP0)* reference gene with the *ZAP70, JAK2, CRLF2, IKZF1, PAX5, EBF1, NR3C1, CXCR4, VLA4, NF1,* and *ERG* target genes. The primers were designed using the Primer 3 program. Primer sequences can be given if requested. c-DNA synthesis was performed using a First Strand Transducer c-DNA synthesis kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. m-RNA expression levels were detected using a SYBR Green I Master Mix (Roche Diagnostics GmbH) and Light Cycler 480 II (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. qRT-PCR reactions were set up in triplicate, while m-RNA expression levels were calculated according to Pfaffl's method.

Statistical Analysis

All analyses were performed using IBM SPSS 20.0 program with $p < 0.05$ being considered statistically significant.[14,15] The chi-square test was used to determine the effects of the mutations on the prognosis of pediatric BCP-ALL ($p < 0.05$ using the margin of error of 5%, 0.05). This test was used to calculate the comparative expression of the samples in the measurement of relative quantitation. Mann–Whitney U tests were used to compare the patient and control group m-RNA levels. Kruskal–Wallis tests were used to analyze whether there was a statistically significant difference between the pediatric BCP-ALL patient risk groups in terms of gene expression.

Results

Demographic and Clinical Analyses of Studied Groups and Characterization Analysis of Detected Mutations

►**Table 1** presents the patient and control groups' demographic, clinical, and diagnostic parameters. Age in both groups varied between 1 and 15 years, with a median age of 7.5 years for the patient group and 8.5 years for the control group, and this difference was not statistically significant ($p = 0.231$). Of the patient group ($n = 45$), 55% ($n = 25$) were female and 44% ($n = 20$) male, whereas, for the control group ($n = 10$), 60% ($n = 6$) were female and 40% ($n = 4$) male. All patients were cytogenetically normal. The percentage of peripheral blood blasts was between 5 and 100% and the average blast percentage in peripheral blood was 39%. The percentage of bone marrow blast was between 27 and 100%, and the average blast percentage in bone marrow was 75%.

When the final status of the patients was examined, treatment of 32 cases was completed (post-treatment group), the treatment of 12 cases was reported to continue (ongoing treatment group), and no information about one case could be found. For this reason, blood samples from 32 patients were taken after the treatment, while blood samples from 12 patients were taken during the treatment phase.

Since it was not possible to obtain germline tissue from patients, mutation analysis was performed with somatic tissues. Targeted NGS was used to screen the 45 pediatric BCP-ALL patients for hotspot regions in *ZAP70, TSLP, JAK2, CRLF2, IKZF1, PAX5, EBF1, CREBBP, FOXO3A, RB1, ZNRF1, NR3C1, NF1,* and *ERG*. The 328 detected missense mutations are categorized in ►**Table 2**. The minimum mutation coverage for all studied genes was minimum 5% (►**Fig. 2A**). There were 28 insertions, 47 indels, 74 nucleotide variants, 75 duplications, and 104 deletions (►**Fig. 2B**), of which 15 had previously been recorded in Human Gene Mutation Database (HGMD) and 313 were novel. The most and least frequently mutated genes were, respectively, *CRLF2* (47 variants) and *TSLP* (6 variants) (►**Fig. 3A**). From the most mutated gene to the least mutated gene, it was CRFL2 (14.3%), NR3C1 (12.8%),

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Table 2 Missense mutations detected in pediatric BCP-ALL patients

No.	Gene	Nt alteration	Accession number	Localization	AA position	Number of patients carrying changes	Possible pathogenity		Risk groups
							PolyPhen2 (score)	SNAP (score)	
C-1	NR3C1	c.35A > T	Novel	E-2/ NTD domain	p.E12V	4	Benign 0.18	Effect 5	2 SRG/1 MRG/1 HRG
C-2	NR3C1	c.212G > T	Novel	E-2/ NTD domain	p.S71I	2	Probably Damaging 0.98	Effect 31	1SRG/1 MRG
C-3	NR3C1	c.366G > A	Novel	E-2/ NTD domain	p.L122T	7	Probably Damaging 1.00	Effect 62	4 SRG/1 MRG/2 HRG
C-4	NR3C1	c.1237A > G	rs145046100	E-3/ NTD domain	p.T413A	2	Benign 0.00	Neutral −20	2 MRG
C-5	PAX5	c.342C > T	Novel	3′UTR	–	4	NA	NA	1 SRG/2 MRG /1 HRG
C-6	PAX5	c.356T > C	Novel	E-3/ PD domain	p.L119P	1	Probably Damaging 0.99	Effect 75	1 MRG
C-7	IKZF1	c.709T > A	Novel	E6-Dimerization domain	p.Y237N	17	Benign 0.33	Effect 64	5 SRG/9 MRG/3HRG
C-8	IKZF1	c.704C > G	Novel	E6-Dimerization domain	p.T235R	2	Benign 0.00	Neutral −6	2 SRG
C-9	IKZF1	c.703 A > T	Novel	E6-Dimerization domain	p.T235*	1	Possibly Damaging 0.87	Neutral −13	1 SRG
C-10	IKZF1	c.700 G > T	Novel	E6-Dimerization domain	p.G234C	1	Possibly Damaging 0.88	Neutral −9	1 SRG
C-11	IKZF1	c.691 G > A	Novel	E6-Dimerization domain	p.G231S	1	Benign 0.020	Neutral −55	1 SRG
C-12	IKZF1	c.686G > A	Novel	E6-Dimerization domain	p.S229N	1	Benign 0.024	Neutral −46	1 SRG
C-13	IKZF1	c.673A > T	Novel	E6-Dimerization domain	p.N225Y	1	Possibly Damaging 0.99	Effect 23	1 SRG
C-14	IKZF1	c.643T > C	Novel	E6-Dimerization domain	p.S215P	1	Probably Damaging 0.97	Effect 12	1 SRG
C-15	ZNRF1	c.320G > A	Novel	E1	p.G107D	4	Benign 0.008	Effect 61	1 SRG/3 MRG
C-16	ZNRF1	c.224C > G	Novel	E1	p.A75G	8	Benign 0.16	Effect 13	7 MRG/1 HRG
C-17	CRLF2	c.337A > G	Novel	E3	p.M113V	1	Benign 0.00	Neutral −28	1 MRG

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Table 2 (*Continued*)

No.	Gene	Nt alteration	Accession number	Localization	AA position	Number of patients carrying changes	Possible pathogenity		Risk groups
							PolyPhen2 (score)	SNAP (score)	
C-18	CRLF2	c.133A > G	Novel	E3	p.T45A	1	Probably Damaging 0.99	Effect 46	1 MRG
C-19	CRLF2	c.110T > C	Novel	E2	p.F37S	1	Possibly Damaging 0.99	Effect 53	1 SRG
C-20	CRLF2	c.101T > C	Novel	E2	p.I34T	2	Possibly Damaging 0.99	Effect 70	1 SRG/1 HRG
C-21	CRLF2	c.124G > A	Novel	E2	p.V42M	1	Probably Damaging 0.99	Neutral −24	1 MRG
C-22	CRLF2	c.343T > C	Novel	E3	p.Y115H	1	Benign 0.002	Neutral −13	1 MRG
C-23	CRLF2	c.271C > T	Novel	E3	p.R91*	1	Probably Damaging 0.97	Effect 40	1 SRG
C-24	CRLF2	c.257T > C	Novel	E3	p.L86P	1	Benign 0.01	Neutral −53	1 SRG
C-25	CRLF2	c.617T > C	Novel	E3/ FN3 Domain	p.V206A	1	Probably Damaging 0.99	Effect 2	1 SRG
C-26	CRLF2	c.322A > G	Novel	E3	p.T108A	1	Benign 0.009	Effect 11	1 SRG
C-27	CRLF2	c.301A > G	Novel	E3	p.N101D	1	Possibly Damaging 0.93	Effect 15	1 SRG
C-28	CRLF2	c.299G > A	Novel	E3	p.R100K	1	Benign 0.005	Effect 8	1 SRG
C-29	CRLF2	c.127C > A	Novel	E2	p.Q43K	1	Possibly Damaging 0.87	Effect 7	1 SRG
C-30	NF1	c.2546G > A	Novel	E21-GR Domain	p.G885K	1	Benign 0.05	Neutral −64	1 SRG
C-31	NF1	c.2474 C > G	Novel	E21-GR Domain	p.S825C	3	Possibly Damaging 0.89	Neutral −40	2 SRG/1MRG
C-32	NF1	c.2461A > C	Novel	E21-GR Domain	p.S821R	3	Benign 0.41	Effect 13	2 SRG/1MRG
C-33	ERG	c.68A > C	Novel	E2	p.Y53S	3	Possibly Damaging 0.97	Effect 88	3 MRG

(*Continued*)

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Table 2 (*Continued*)

No.	Gene	Nt alteration	Accession number	Localization	AA position	Number of patients carrying changes	Possible pathogenity		Risk groups
							PolyPhen2 (score)	SNAP (score)	
C-34	ERG	c.46 C > G	Novel	E2-PNT Domain	p.Q16E	2	Possibly Damaging 0.86	Effect 79	2 MRG
C-35	FOXO3A	c.13C > G	rs757315159	E1	p.P5A	2	Benign 0.00	Neutral −36	1 MRG/1 HRG
C-36	RB1	c.1949T > C	Novel	E19-A Domain	p.F650S	1	Probably Damaging 1.00	Effect 69	1 SRG
C-37	RB1	c.1867A > G	Novel	E19-A Domain	p.N623D	1	Benign 0.20	Neutral −13	1 SRG
C-38	RB1	c.1959A > C	Novel	E19-A Domain	p.K653N	1	Benign 0.02	Neutral −16	1 HRG

Abbreviations: AA, amino acid; C, change; E, exon; FN3, fibronectin type-III; GRD, GAP-related; HRG, high-risk group; MRG, medium-risk group; NA, not applicable; NTD, N-terminal domain; PNT, pointed; SRG, standard risk group; UTR, untranslated region.

RB1 (9.7%), IKZF1 (9.1%), PAX5 (8.8%), EBF1 (7.9%), JAK2 (6.0%), CREBBP (5.4%).

Missense mutations were detected in *NR3C1*, *PAX5*, *IKZF1*, *CRLF2*, *RB1*, *FOXO3A*, *ZNRF1*, *NF1*, and *ERG*, while nonsense mutations were detected in *FOXO3A*, *IKZF1*, and *CRLF2*. Two *in silico* prediction tools—Poly-Phen2 Database Program (http://genetics.bwh.harvard.edu/pph2) and Supplemental Nutrition Assistance Program (SNAP) (https://www.rostlab.-org/services/SNAP/)—were used to evaluate the functional effects on target genes of the identified missense variants. This indicated that 19 of the 38 missense mutations in ►**Table 2** may have a pathogenic feature (damage likely) because their pathogenic scores were close to 1. However, the second analysis using SNAP indicated that four missense mutations were neutral, with scores between 0 and 100.

In addition, a comparative, cross-species analysis of amino acid sequences affected by missense mutations was performed using the multiple sequence alignment option in PolyPhen2. This showed that the following proteins have been conserved across species during evolution, from *Xenopus tropicalis* to *Homo sapiens*: NR3C1 p.S71I and p.L122T; PAX5 p.L119P; IKZF1 p.Y237N, p.G231S, p.S229N, p.N225Y, and p.S215P; ZNRF1 p.G107D and p.A75G; CRLF2 p.T45A, p. F37S, p.I34T, p.V42M, p.Q43K, p.R100K, p.N101D, p.T108A, p. M113V, and p.V206A; NF1 p.S825C; ERG p.Y23S and p.Q16E; RB1 p.F650S.

The 5′ UTR variants were also found in *EBF1, FOXO3A, PAX5,* and *CRLF2*. Variants in *EBF1*, *FOXO3A*, and *CRLF2* lay in the 5′flanking promoter region, whereas variants in *PAX5* were found in the 3′UTR. There was no statistically significant difference between risk stratification and mutation distribution.

►**Table 3** summarizes the distribution of detected mutations by risk groups, while ►**Fig. 3B** shows the mutation distribution by risk classification. The most nucleotide changes were detected in *NR3C1*, with 42, including 23 frame-shift mutations. *NR3C1* was one of the four most frequently mutated genes in all risk groups, whereas *CREBBP* had the lowest mutation frequency in all risk groups. Relapse occurred in only one patient in our study group. In this patient, mutations were detected in the *CRF2*, *FOXO3*, *IKZF1*, *JAK2*, *NF1*, *NR3C1*, *PAX5*, *ZAP70*, *ZNRF1* genes.

There were 42 nucleotide changes in *IKZF1*, with all nine patients in the high-risk group having *IKZF1* variants. These were detected from the nucleotide sequences encoding the DNA-binding domain (DBD) and dimerization domain (DD), which form the splice region at the end of Intron 5 and the 3′ UTR region. *IKZF1* deletions were detected in 24 (53%) of 45 patients. In five patients, two nonsense mutations were detected in *IKZF1*, specifically in codons 137 and 106. *CRLF2-JAK2* mutations were detected in 15 patients, *CRLF2-TSLP* mutations in 14 patients, and *CRLF2-JAK2-TSLP* mutations in seven patients. ►**Table 4** presents all the variant types detected in pediatric BCP-ALL patients, while ►**Supplementary Figs. S1**, **S2**, **S3**, and **S4** (available in the online version only) provide schematic representations of the candidate genes' domain architectures and detected mutations.

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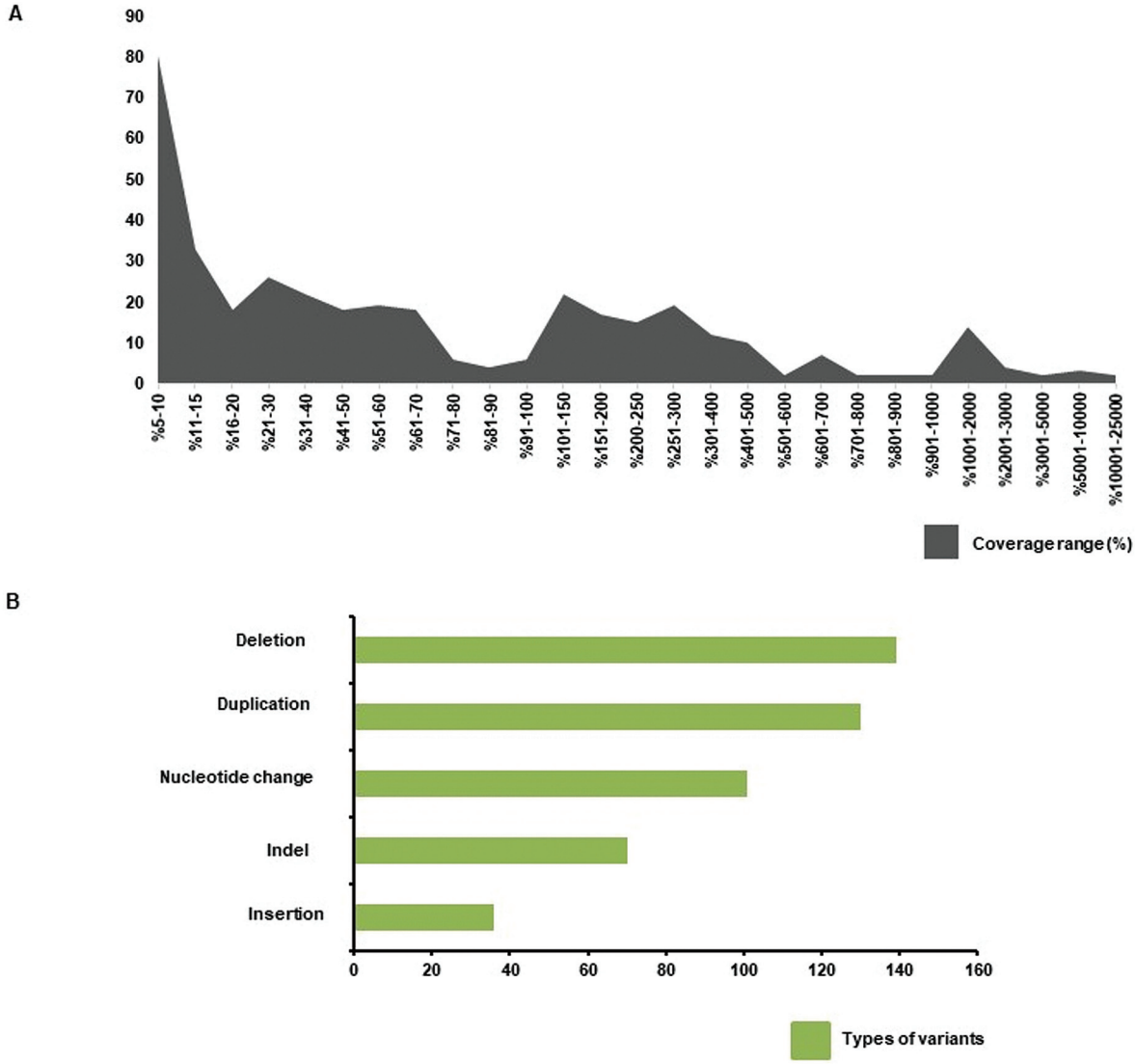

Fig. 2 (A) Number of detected variants in the cover range. (B) Distribution of determined variants according to structural characteristics.

The chi-square tests were performed to evaluate the association between the diagnosis and treatment parameters of the patients and the mutation status of the 14 genes. The results indicated that the patient group were significantly more likely to have missense mutations in *ERG* ($p = 0.004$), silent mutations in *CRLF2* ($p = 0.016$), an indel variant in *CREBBP, FOXO3A, TSLP, JAK2* ($p = 0.006$), and deletions in *ERG, CREBBP, CRLF2* ($p = 0.004$, $p = 0.016$, and $p = 0.006$, respectively). Patients were also significantly more likely to have a duplication in *FOXO3A* ($p = 0.000$), *NR3C1* ($p = 0.005$), and/or insertions in *FOXO3A* ($p = 0.008$), *ZAP70* ($p = 0.001$), and/or *JAK2* ($p = 0.001$). The frequency of *ERG* variants indicates that pediatric BCP-ALL patients in the ongoing treatment group were 5.25 times more likely to carry gene mutations than patients in the post-treatment group (confidence interval 95% =1.18–23.2, [$p = 0.022$]) (►**Tables 5** and **6**). There were no statistically significant between-group differences in diagnosis, protocols, and follow-up parameters for any other genes those mentioned above.

Results of Gene Expression Analysis

For gene expression analysis, qRT-PCR was used to determine the expression levels of *ZAP70, JAK2, CRF2, IKZF1, PAX5, EBF1, NR3C1, CXCR4, VLA4, NF1, ERG* and the reference gene *RPLP0* in 45 pediatric BCP-ALL patients and 10 healthy controls. ►**Fig. 4** shows the m-RNA expression results. Only *JAK2* was overexpressed in the patient group compared with the control group ($p = 0.004$). *ERG* was overexpressed in the ongoing treatment group compared with the treatment group ($p < 0.05$). Finally, risk stratification and gene expression levels were not correlated for any of the genes analyzed.

Discussion

Pediatric BCP-ALL is a highly heterogeneous leukemia subset, in which the development of the B-cell line from the hematopoietic stem cell is regulated by multiple transcription factors, genes, and gene-associated pathways.[3–5,11,12,16,17] Although there are many identified BCP-

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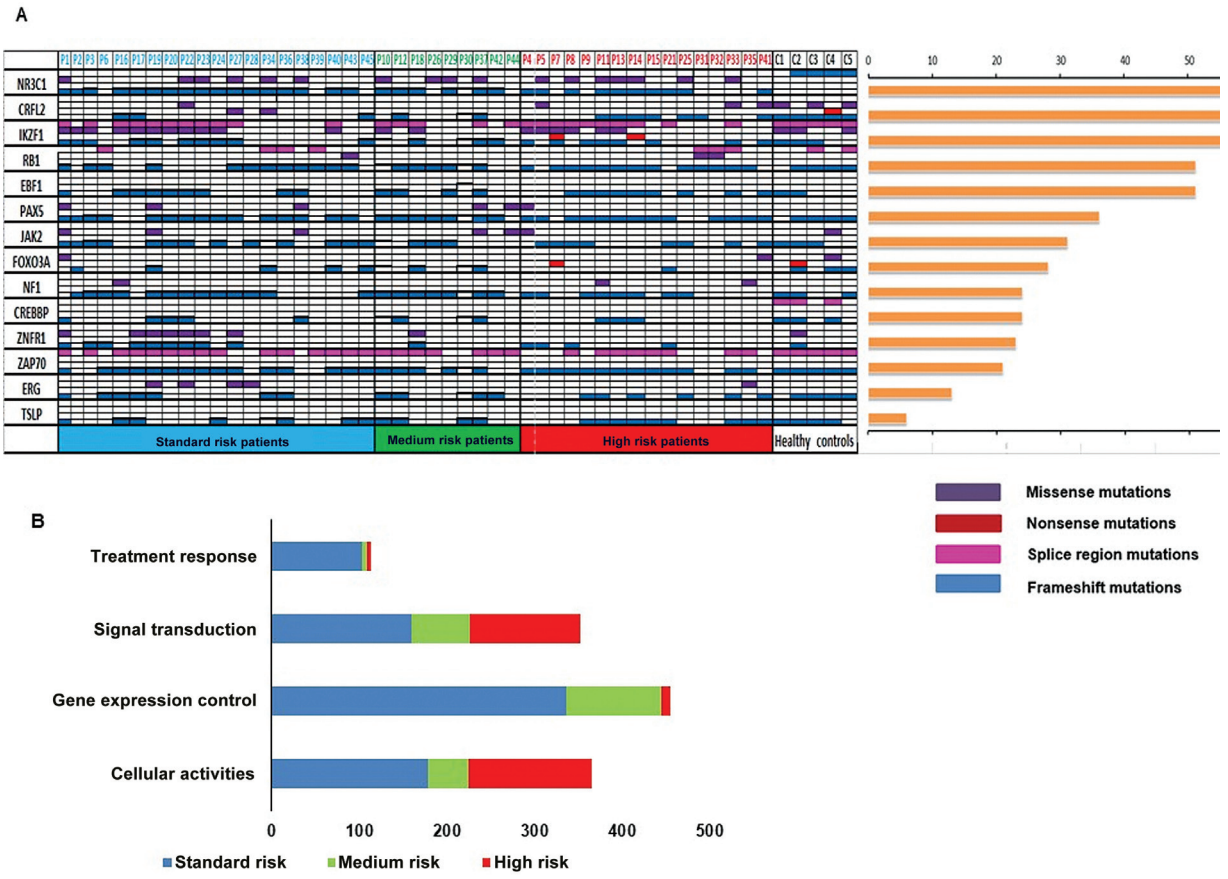

Fig. 3 (**A**) Landscape of mutations in 45 pediatric BCP-ALL patients. One gene is represented in each line and one patient in each column. Bars at the right represent the number of mutations present in each gene. For risk classification, blue boxes indicate standard-risk, green boxes indicate medium-risk, and red boxes indicate high-risk. (**B**) Distribution of the number of mutations detected by functional pathways in risk groups.

Table 3 The distribution of detected mutations according to risk groups in pediatric BCP-ALL patients

Genes	Standard risk (*n*:16)	Medium risk (*n*:20)	High risk (*n*:9)
IKZF1	16	17	9
NR3C1	13	19	8
PAX5	13	18	8
ZAP70	13	17	8
NF1	13	17	7
CRLF2	11	11	5
JAK2	9	15	2
EBF1	16	11	5
RB1	13	11	5
FOXO3A	10	12	4
ERG	8	10	3
TSLP	8	8	6
ZNFR1	5	11	1
CREEBP	3	6	1

ALL subtypes, there are still multiple, as yet unclassified BCP-ALL subtypes, which are likely to be distinguished by their molecular profile. Compared with research on adult leukemia, there have been few pediatric BCP-ALL studies. These studies have identified prognostic biomarkers for BCP-ALL from genes investigated in mutation and gene expression studies; however, research is still needed to address several key questions: What do the gene and gene products reported as prognostic biomarkers in these independent studies show in terms of expression and mutation assessment of patients with pediatric BCP-ALL? Do patients have different prognoses if they have anomalies in the same prognostic biomarkers? Do these prognostic biomarkers determine recurrence? Are these genes evaluated together in a single study?

The present study used targeted NGS and gene expression analysis performed with qRT-PCR to analyze mutations in *ZAP70, TSLP, JAK2, CRLF2, IKZF1, PAX5, EBF1, CREBBP, FOXO3A, RB1, ZNRF1, NR3C1, NF1, ERG, VLA-4,* and *CXCR4.* NGS has recently revolutionized the study of hematological malignancies, with remarkable success in characterizing the genetic basis of these disorders. Thus, we investigated whether these genes or gene-related pathways can be used as prognostic biomarkers for pediatric BCP-ALL cases,

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Table 4 Types of detected mutations in pediatric BCP-ALL patients

Gene	Deletion	Duplication	Indel	Insertion	Missense mutation	Nonsense mutation	Silence mutation	SNP
NR3C1	16	9	7	3	4	–	3	–
CREEBP	5	8	5	–	–	–	–	–
PAX5	15	6	3	2	2	–	1	–
EBF1	12	5	3	3	–	–	3	–
IKZF1	5	–	8	6	7	1	3	–
ZNFR1	2	2	5	–	4	1	–	–
TSLP	3	1	2	–	–	–	–	–
ZAP70	6	3	3	2	–	–	–	–
CRLF2	5	14	1	2	12	1	10	2
JAK2	7	9	–	2	–	–	1	–
NF1	6	–	1	1	3	–	3	–
ERG	5	2	3	1	2	–	–	–
FOXO3A	10	5	4	2	1	1	2	–
RB1	5	12	2	4	4	–	5	–

Table 5 Mutation distribution and gene expression difference between patients and controls

Parameters:*Patients vs. Controls*	Genes	*p*-Value
Missense mutation	*ERG*	0.003
Silent mutation	*CRLF2*	0.009
Indel	*CREBBP*	0.005
	TSLP	0.004
	FOXO3A	0.009
	JAK2	0.002
Deletion	*ERG*	0.004
	CRLF2	0.006
Duplication	*FOXO3A*	0.000
	NR3C1	0.005
Insertion	*FOXO3A*	0.008
	ZAP70	0.001
	JAK2	0.001
Gene expression	*JAK2*	0.004

Table 6 Mutation distribution and gene expression difference between treatment status

Parameters	Genes	*p*-Value
Status of treatment *n* (%)		
Ongoing 12 (27)	ERG mutation	0.022
Completed 33 (73)	ERG expression	0.003

especially those lacking cytogenetic abnormalities. Our candidate genes represented four different cellular pathways: cellular activities (*NF1*, *ERG*, *RB1*, *ZNFR1*, *FOXO3A*, *VLA4*, and *CXCR4*); signal transduction (*ZAP70*, *TSLP*, *JAK2*, and *CRLF2*); gene expression control (*IKZF1*, *PAX5*, and *EBF1*); and treatment response (*NR3C1* and *CREBBP*). These pathways are not separate with clear boundaries; rather, they are intertwined, especially in leukemia pathogenesis.

We detected 328 mutations, of which 15 have been previously identified in the HGMD database. We detected missense mutations in evolutionary conserved aminoacids of the proteins encoded by nine genes (*CRLF2, RB1, FOXO3A, ZNRF1, NF1, ERG, PAX5, IKZF1,* and *NR3C1*). Thus, these missense mutations might affect the function of the protein they belong to. In *ZAP70, RB1, IKZF1,* and *CREEBP*s, the variants in the splice region may cause abnormal splicing and affect the relevant protein's structure function.

A key feature of hematopoietic malignancies is alterations in the normal regulation of gene expression. Transcription factors, such as *IKZF1*, *PAX5*, and *EBF1*, are critical regulators of the genes responsible for lymphoid differentiation. *IKZF1* deletions are among the potential prognostic biomarkers that can be used for pediatric BCP-ALL risk classification.[18,19] Deletions and mutations are correlated with poor prognosis in *BCR-ABL1*-negative (*BCR-ABL1*-like) pediatric BCP-ALL cases.[20] *IKZF1* deletions are associated with 70% of *BCR-ABL1* positive B-ALL and are correlated with an increased risk of relapse and decreased lifetime in both groups.[19–21] Relapse occurred in only one patient in our study group. IKZF1 deletions were detected in this patient. Our results indicate that *IKZF1* was the most frequently mutated gene in the patient samples grouped by risk classification. Thus, *IKZF1* variants with mutations in the nucleotide sequences encoding the DBD and DD domains may be detrimental to the function of the IKAROS transcription factor produced by *IKZF1* gene. These domains control the ability of the transcription factor to activate other genes. At the same time, mutations detected in *IKZF1*'s splice site regions may make the IKAROS transcription factor non-functional in the cell by causing them to occur in different transcripts.

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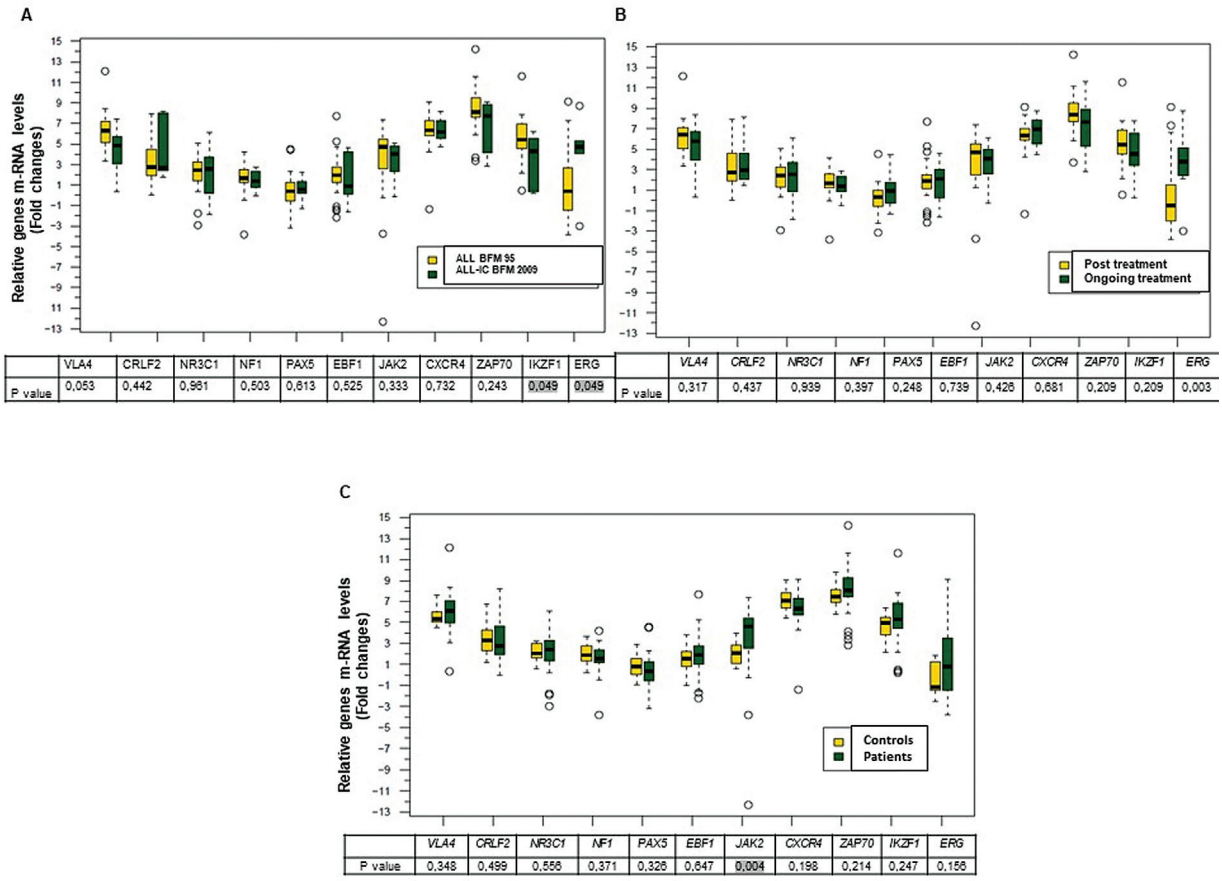

	VLA4	CRLF2	NR3C1	NF1	PAX5	EBF1	JAK2	CXCR4	ZAP70	IKZF1	ERG
P value	0,053	0,442	0,961	0,503	0,613	0,525	0,333	0,732	0,243	0,049	0,049

	VLA4	CRLF2	NR3C1	NF1	PAX5	EBF1	JAK2	CXCR4	ZAP70	IKZF1	ERG
P value	0,317	0,437	0,939	0,397	0,248	0,739	0,426	0,681	0,209	0,209	0,003

	VLA4	CRLF2	NR3C1	NF1	PAX5	EBF1	JAK2	CXCR4	ZAP70	IKZF1	ERG
P value	0,348	0,499	0,556	0,371	0,326	0,647	0,004	0,198	0,214	0,247	0,156

Fig. 4 (A) Comparison of normalized relative gene expression levels of targeted genes in patient samples according to the status of protocols. (B) Comparison of normalized relative gene expression levels of targeted genes in patient samples according to the status of treatment. (C) Comparison of normalized relative gene expression levels of targeted genes in patient and control groups. The green and yellow boxes indicate pediatric BCP-ALL patients and healthy individuals.

PAX5 is the main target of somatic mutations in the formation of B-ALL. In 30% of BCP-ALL, *PAX5* contains monoallelic losses or point mutations. Genomic deletions were detected in 31.7% of BCR-ABL-negative high-risk BCP-ALL patients compared with 33% for the BCR-ABL-positive group. Rearrangements occurred at a frequency of 2 to 3%.[22,23] *PAX5* deletions are, thus, the main cause of PAX5 inactivation. These deletions cause PAX5 haploinsufficiency and/or impaired *PAX5* expression. PAX5 haploinsuffiency may cause ALL activation due to activation of STAT5 or *BCR-ABL1*.[22–24] In our study, 27 (60%) patients had *PAX5* deletions, of whom nine carried both *PAX5* deletions and *ZNRF1* mutations together. In the literature, there is no information available indicating that *ZNRF1* mutations are directly related to leukemia development, although studies indicate that the coexistence of *PAX5* deletions and *ZNRF1* mutations may be associated with poor prognosis and lead to the development of leukemia.[10]

Treatment stratification in current pediatric leukemia protocols is based on clinical and hematological parameters, cytogenetic abnormalities, and early response to therapy. Although these criteria normally allow patients at the risk of relapse to be distinguished from those at low risk, a critical genetically and clinically heterogenous proportion of patients remain unclassifiable. For instance, the mechanism of glucocorticoid (GC) resistance in pediatric ALL is still poorly understood, although genetic factors may play an important role. According to the ALL-BFM protocol, in the initial phase of the remission induction treatment of pediatric ALL, GC therapy is administered during the first 8 days. Its goal is to lower the number of blasts since GC has the ability to induce apoptosis in leukemic cells mediated through GC receptor (GR). The blast count on the 8th day is one of the stratification criteria important for therapy protocol and survival. Early response to GC treatment is accepted as important prognostic criteria.[25–27] GC response is determined by the blast count of the 8th day of the therapy. Therefore, it is crucial for better treatment of pediatric ALL to investigate, understand, and overcome the problems related to the pharmacogenomic profiles of those patients that respond poorly to initial GC treatment. Around 10% of pediatric ALL patients develop GC resistance during treatment.[25] NR3C1 is responsible for regulating the intracellular concentration of steroids and biological activity in the target tissue.[28] In our study, 42 variants were detected in *NR3C1* among which the most frequent were frameshift mutations. It is one of the first four genes according to the frequency of mutations in all risk groups in the analysis of risk groups. The

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detected variants had changes in the TAD and DBD regions. The former region contains the AF1 region responsible for the transcriptional activation of target genes. The loss of this activation site may be the result of these variants.[29,30] Mutations in *CREBBP*, which encodes acetyltransferases, have also been previously linked to GC resistance in ALL. All the *CREBBP* variants we detected had changed to the nucleotide sequences encoding the HAT domain, which impair expression of GR-responsive genes and are associated with relapse.[31,32] In our study group, four patients showed GC resistance but no expression level difference was detected in these children compared with the group with GC sensitivity. On the contrary, we found variable expression patterns in GRα expression in pediatric BCP-ALL patients when compared with controls (unpublished data). Unlike GRα, the increase of GRβ expressions was detected in the entire patient samples. Hence, GRα/β expression ratios for these patients were significantly low.

Cytokines manage lymphoid differentiation by binding to specific cell-surface receptors in a stage- and lineage-specific manner. Following the binding of a cytokine to its related receptor (such as *JAK2*, *CRLF2*, and *TSLP*), intracellular signal transduction pathways are activated, which accelerates the lymphoid differentiation steps.[33–35] The cytokine receptor and members of the JAK family have also gained great importance in B-ALL studies. *JAK* mutations are found in 10% of BCP-ALL cases.[33] In particular, *JAK2* mutations that may cause premature termination of polypeptides as a result of shifting of the reading frame have been identified. These mutations we identified in the *JAK2* gene in our study are found to be on the pseudokinase (JH2) domain. Frameshift mutations in this domain may cause stop codon formation and truncated protein formation.

The association of *IKZF1* and *CDKN2A/B* variants with *JAK* mutations, especially in BCR-ABL-like B-ALL, is associated with genetic lesions in genes involved in many cellular pathways, including lymphoid development, tumor suppression, and tyrosine kinase activation.[33–35] In our study, *JAK2* expression was significantly higher in the patient group than in the control group ($p = 0.004$). Increased *JAK2* expression due to mutations in its pseudokinase and kinase domains is a marker of poor prognosis, which confirms our findings.[33,36] In addition, as in our study, *JAK2* overexpression and *JAK2-CRLF2* mutations cause the transformation of hematopoietic cells and B-ALL formation.[33,35,37] We detected *CRLF2-JAK2* mutations in 15 patients (4 SRG/9 MRG/2 HRG), *CRLF2-TSLP* mutations in 14 patients (4 SRG/7 MRG/3 HRG), and *CRLF2-JAK2-TSLP* mutations in seven patients (2 SRG/4 MRG/1 HRG).

ERG expression is associated with poor prognosis in adult AML and T-ALL cases without cytogenetic anomaly.[38,39]*ERG* is required for normal hematopoiesis, but as a result of impaired *ERG* expression, it causes leukemia development as well as resistance to kinase inhibitors due to the disruption of kinase signaling pathways controlled by ERG in early leukemic cells.[38,40] Increased *ERG* expression in acute leukemia is known to be an independent prognostic risk factor. In our study, *ERG* expression was higher in the ongoing treatment group than in the post-treatment group. The detected variants had changes in the nucleotide sequences encoding the PNT domain, which functions as the gene's phosphorylation site. It is an important domain that performs heterodimerization with proteins that are other members of the ETS family. Mutations that might cause a loss of function of this domain may cause the loss of the gene's phosphorylation site, thereby preventing heterodimerization.

Our study combined genes and pathways previously analyzed separately in pediatric BCP-ALL into one dataset for a comprehensive approach, analyzing DNA sequencing and gene expression data from the same samples to unravel relevant pathway alterations. We detected novel variants in all the studied genes. However, the number of patients and controls involved in the study was limited, so the target genes should be analyzed in a larger study group to draw a definite conclusion about their role in leukemia pathogenesis and prognosis and to determine their biomarker potential.

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Conflict of Interest
None declared.

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