Investigation of Potential Prognostic Biomarkers in Pediatric Acute Lymphoblastic Leukemia

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer and identifying prognostic biomarkers for pediatric ALL is critical for improving outcomes. In this study, we aimed to investigate CXCR4, PDGFR-β, RANTES, TWIST1, and VEGFR2 genes as candidate prognostic biomarkers associated with pediatric ALL immunophenotypes. Bone marrow and peripheral blood mononuclear cells (PBMCs) of 46 pediatric ALL patients and healthy controls were collected. The expression of CXCR4, PDGFR-β, RANTES, TWIST1, and VEGFR2 genes, considered candidate prognostic biomarkers, was analysed using quantitative reverse transcription polymerase chain reaction (qRT-PCR). RANTES expression was upregulated in pre-B-ALL, pro-B-ALL, and T-ALL patients compared to healthy controls ($p= 0.015$). The expression of all targeted genes, except CXCR4, was elevated in patients with trisomies of chromosomes 1, 6, 8, 12, 17, 21, and 22 ($p< 0.05$). Increased CXCR4 and RANTES expression was observed in patients with the t(9;22) translocation ($p= 0.039$, $p= 0.017$, respectively). High PDGFR-β and RANTES expression was associated with prolonged remission duration ($p= 0.007$, $p= 0.015$, respectively). Additionally, CXCR4 expression was highest in the high risk (HR) group ($p= 0.039$). The results of this study indicate that RANTES and PDGFR-β may be potential prognostic biomarkers in pediatric ALL in the presence of common clinical features. Monitoring RANTES and PDGFR-β expressions could be a novel approach for determining and managing prognosis in pediatric ALL. The main limitation of the study is the collection of healthy bone marrow samples due to ethical concerns, which may require confirmation of our findings in larger cohorts.

Keywords: Pediatric ALL, RANTES, PDGFR-β, Prognosis, Biomarker

INTRODUCTION

Pediatric acute lymphoblastic leukemia (ALL) is the most common type of cancer in childhood, and it is a highly heterogeneous disease with prognostic indicators such as age of onset, immunophenotype, post-treatment bone marrow (BM) status, minimal residual disease (MRD), and cytogenetic abnormalities.[1] In the pathophysiology of ALL, malignant differentiation of precursor and progenitor lymphoblastic cells such as B-cell acute lymphoblastic leukemia (B-ALL) or T-cell acute lymphoblastic leukemia (T-ALL) is observed, and its leads to hematopoietic dysfunction in the BM. Pediatric ALL is mostly in the form of B-ALL (>80%) and has high cure rates (90%).[2,3]; nevertheless, 10-20% of patients experience resistance or relapse to treatment after the first complete remission (CR), and the prognosis may worsen and even results in death.[4] Subtypes of ALL are defined according to the French American British (FAB) morphological criteria and the World Health Organization (WHO) classification for the clinical management of the disease.[5] Pediatric ALL also encompasses several subtypes, and genetic or cytogenetic alterations cause significant heterogeneity in treatment regimen and survival rates.[6,7]

Thus far, risk stratifications have been defined by various study groups for the outcome after the first relapse.[8,9] The direct association of malignant cellular differentiation with human developmental stages, especially in pediatric ALL, highlights the need for biomarkers.[10] Nowadays, new generation platforms that emerged in parallel with technological developments have accelerated studies on the identification of new molecules associated with pediatric ALL[6]; in spite of that ethical concerns cause significant limitations in methodology, such as sample size and type, which are important in human research. Experimental or clinical studies involving bone marrow biopsy in children are classified as high-risk studies.[11] Although, ethical concerns regarding sample collection are a major constraint in preclinical and clinical research of pediatric diseases, it also offers researchers the opportunity to move beyond these limits by developing ex vivo models such as organoids and three dimensional (3D) tissues, or by using technologies such as artificial intelligence and in silico.

Several genes that may be related with the clinic of pediatric ALL appear to be of interest for biomarker research with their genetic profiles have not yet been clarified. C-X-C chemokine receptor type 4 (CXCR4) expressed in haematopoietic cells, and altered expression levels has been observed with B-ALL and T-ALL in pediatric and adult populations[12-16]; however, studies provide conflicting clues.[17-19] Aberrant rearrangement of Platelet-derived growth factor receptor beta (PDGFR-β) gene is involved particularly in B-ALL[20-22], and Philadelphia-like ALL.[23,24] Furthermore, a high PDGFRβ-positive finding is associated with a poor prognosis in pediatric ALL cases.[25] C-C motif chemokine ligand-5 (RANTES/CCL5) is a chemokine that plays a pivotal role in the process of hematopoietic regeneration by enhancing cell survival and proliferation following irradiation.[26] Increased RANTES expression has been shown to reduce T-cell differentiation, while RANTES knockout has been observed to result in decreased myeloid HSCs but increased lymphoid HSCs and T cells.[27] Remarkably, overexpression of RANTES' receptor C-C chemokine receptor type 5 (CCR5) has been reported in ALL.[28] Its multifaceted roles suggest that RANTES may significantly influence the prognosis of ALL. Twist family BHLH transcription factor 1 (TWIST1) as one of the epithelial-mesenchymal transition (EMT) transcription factors is associated with drug resistance, and overexpressed in different type of cancer such as gastric cancer, bladder cancer, acute myeloid leukemia (AML), and chronic myelogenous leukemia (CML). In contrast, a normalization of TWIST1 expression was observed in patients with ALL.[29,30] A recent study has also shown that suppression of TWIST1 and Snail Family Transcriptional Repressor 2 (SNAI2) genes in an ex vivo co-culture model leads to drug resistance in adherent ALL cells[31]; therefore, TWIST1 may have prognostic significance for pediatric ALL and further studies are needed to clarify its role. Vascular endothelial growth factor (VEGF) and its receptors are one of the signaling networks targeted in cancer therapy, including AML, CML, and ALL.[32-34] A limited number of studies have reported that the VEGFR-1/VEGFR-2(KDR) pathway important for the proliferation and adherence of acute leukemias[35,36], and VEGFR-1 / VEGFR-2 are expressed in childhood pre-B-ALL.[37] Given that leukemia is an angiogenesis-dependent malignancy, determining the genetic profile of VEGFR2 in pediatric ALL patients may contribute to the clinical management of the disease.

It is important to discover new prognostic molecules to improve the survival rate in pediatric ALL patients. In this study, we aimed to investigate CXCR4, PDGFR-β, RANTES, TWIST1, and VEGFR2 genes, which are associated with haematological malignancies as candidate prognostic biomarkers associated with pediatric ALL immunophenotypes.

PATIENTS AND METHODS

Study Design

Patients diagnosed with pediatric ALL according to ALL IC-BFM 2009 criteria[8] were recruited from Akdeniz University Faculty of Medicine, Department of Pediatrics, Pediatric Haematology. According to International and Institutional Guidelines, blood and bone marrow samples obtained from children and adolescents younger than 18 years old diagnosed with ALL (n= 46) and healthy

Table 1. General characteristics of pediatric ALL patients

Characteristics	Number of cases (%)
Sex	
Female	24 (52,2%)
Male	22 (47,8%)
Age	
0 to 6	24 (52,2%)
7 to 17	22 (47,8%)
Immunophenotype	
pre-B-ALL	28 (60,86%)
pro-B-ALL	9 (19,57%)
T-ALL	9 (19,57%)
FAB Classification	
L1	30 (65,2%)
L2	5 (10,9%)
L1+L3	8 (17,4%)
L1+L2	3 (6,5%)

Abbreviations: ALL= acute lymphoblastic leukemia; FAB= French American British; L1= small uniform/homogenous blast cells; L2= large varied/heterogenous blast cells; L3= blasts large varied cells with vacuoles (bubble-like features/Burkitt's lymphoma type); pre-B-ALL= precursor B-cell ALL; pro-B-ALL= progenitor B-cell ALL; T-ALL= T-cell ALL

control group (n= 6) collected at the Center for Genetic Diseases and Diagnosis were analyzed. General information regarding the clinical characterization of patients included in this study was collected (Table 1). The cases were between 2-17 years of age, 52.2% (n= 24) were ≤6 years, and 47.8% (n= 22) were >6 years of age. Patients had pre B-ALL (n= 28), pro B-ALL (n= 9), and T-ALL (n= 9) immunophenotypes.

It is known that chromosomal abnormalities in the form of translocation and trisomy are associated with prognosis and survival in ALL.[6] Pediatric ALL cases were classified as having trisomy (n= 8) or not (n= 38); and for translocations they were further grouped as t(9;22), t(12;21), t(6;11), t(1;19) and none (n= 3, n= 17, n= 3, n= 7, n= 6, respectively). Remission and relapse have a direct effect on treatment success[38] in pediatric ALL. Patients were grouped according to their remission times (8th, 15th, 33rd and 36th days; n= 5, n= 18, n= 15, n= 8, respectively).

Complete remission (CR) was identified on day 33 of induction therapy, as fewer than 5% blast cells in bone marrow, the absence of leukemic blasts in blood and cerebrospinal fluid (CSF), and no evidence of local disease. According to the Berlin-Frankfurt-Münster (BFM) stratification, early relapses refer to relapses occurring between 18 and 30 months after remission, and late relapses refer to relapses occurring ≥ 30 months after remission.[39] The distribution of early relapse cases included a total of 7 patients for 15th (n= 3), 33rd (n= 2), and 36th (n= 2) days. Late relapse cases were observed on 8th (n= 1), 15th (n= 2), 33rd (n= 7) and 36th (n= 4) days in a total of 14 patients. Two patients were identified as part of the 33rd and 36th day relapse group. No relapse was observed in 23 patients.

Risk groups (standard risk (SR), intermediate risk (IR) and high risk (HR) in pediatric ALL are important in response to treatment and relapses.[5] The cases were divided into 3 groups according to presentation features, karyotype information, and treatment response: standard risk (SR), intermediate risk (IR) and high risk (HR). Risk group assignments of standard-risk group (SR); peripheral blood (PB) on day 8: < 1.000 blasts/μL, Age ≥1 yr – <6 yr., initial WBC < 20.000/μL, if available FC MRD < 0.1% or M1/ M2 marrow on day 15 and no M 2/3 marrow on day 33 (All criteria must be fulfilled). High-risk group (HR); **a.** IR and, if available FC MRD > 10% or M3 marrow on day 15, **b.** SR if available FC MRD > 10%. **c.** PB on day 8: ≥ 1.000 blasts/μL, **d.** M2 or M3 marrow on day 33, Translocation t(9;22) [BCR/ABL], **e.** t(4;11) [MLL/AF4], **f.** Hypodiploidy ≤ 44 (At least one criterion must be fulfilled). Intermediate-risk group (IR); patients who have not been stratified to SR or HR are classified as intermediate risk. Conventional cytogenetic analysis of the entire karyotype was performed, with high-resolution G-banding serving as the gold standard. To investigate prognostically significant fusion genes, molecular genetic methods (via RT-PCR) were employed at the Center for Genetic Diseases and Diagnosis at Akdeniz University. Assessment for MRD by flow cytometric analysis was applied in BM at day 15 and day 33. BM status (% Blasts), M1; < 5, M2; ≥5 – <25, and M3; ≥ 25. The assessment of early treatment response is facilitated by the absolute blast count (ABC) in the PB on day 8 following a 7-day period of prednisone pre-phase. Patients exhibiting an ABC on day 8 of less than 1.000/μL PB are categorized as prednisone-good responders, while

those with ≥ 1.000 blasts/μL PB are designated as prednisone-poor responders.

RNA Isolation and cDNA Synthesis

RNA was extracted from bone marrow using the PAX gene Bone Marrow RNA kit (Qiagen, Hilden, Germany). cDNA synthesis was performed with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Massachusetts, USA).

Quantitative Reverse Transcription PCR

qRT-PCR was performed according to the protocol for the SensiFAST™ SYBR® No-ROX Kit (Bioline by Meridian Bioscience, Ohio, USA) and was run on the Applied Biosystem QuantStudio 3 (Applied Biosystem, Massachusetts, USA). Specific primer sequences designed for the CXCR4 (F: 5'-CATCCTCATCCTGGCTTTCTT-3', R: 5'-CACACCATTGCTTGATGATTTC-3'), PDGFR-β (F: 5'-GCTCACCATCATCTCCCTTATC-3',R:5'-CTCACAGACTCAATCACCTTCC-3'), RANTES (F: 5'-CTTTGCCTACATTGCCCGCC-3', R: 5'-TGCTGTCCCTCTCTCTTTGGC-3'),TWIST1 (F:5'-GCACCATCCTCACACCTCT-3',R:5'-TGGCACGACCTCTTGAGAAT-3'), and VEGFR2 (F: 5'-AGCAGGATGGCAAAGACTAC-3', R: 5'-TACTTCCTCCTCCTCCATACAG-3') genes. ACTB (F: 5'-ACAGAGCCTCGCCTTTGCC-3', R: 5'-TGGGGTACTTCAGGGTGAGG-3') gene product was measured as an internal control for normalization of the mRNA data. Water was used instead of cDNA as both the RNA-negative extraction control and the non-template control (NTC).

PPI Analysis of Targeted Gene Set

Network analysis of protein-protein interactions (PPIs) of CXCR4, PDGFR-β, RANTES, TWIST1, and VEGFR2 genes was performed for Homo sapiens organism in STRING database version 11.5.[40] It was determined which molecular processes the genes of interest shared.

Ethical Approval: All protocols and researches of our study were approved by Ethical Review Board of the Istanbul Medical Faculty (dated 12.09.2019; decree no: 808). Written informed consent was obtained from each participant's parent or legal guardian. The study was conducted in accordance with the 1964 Declaration of Helsinki with its later amendments.

Statistical Analysis

Analysis and visualization of qRT-PCR data were performed using the programs Jamovi 1.6.15, GraphPad Prism 8.0.2 and SPSS 25 (Statistical Package for the Social Sciences). In this study, Independent Sample T-test, Mann Whitney U Test, Chi-Square Test and Shapiro Wilk-W Test were applied for parametric and non-parametric data comparison, qualitative data correlation and distribution determination of gene expressions. In addition, values with a p value less than 0.05 ($p< 0.05$) were considered as statistically significant. One-Way ANOVA Test was performed for analysis of variance between gene expressions and clinical characteristics of pediatric ALL patients. Games-Howell multiple comparisons test was applied.

RESULTS

Network Analysis of Targeted Genes at the Protein Level

In the STRING database, we analysed the protein-level associations of the candidate biomarker set containing the CXCR4, PDGFR-β, RANTES (CCL5), TWIST1, and VEGFR2 (KDR) genes, which have been associated with haematological malignancies in the literature. Protein-protein interaction (PPI) enrichment result (p= 0.000216), were showed that the proteins could be related to each other at the molecular level (Figure 1). All targeted genes were associated with positive regulation of cell motility, cell migration, regulation of protein phosphorylation, regulation of programmed cell death, regulation of intracellular signal transduction, positive regulation of molecular function, cell surface receptor signaling pathway, cellular response to organic substance, positive regulation of nitrogen compound metabolic process, positive regulation of cellular metabolic process, response to stress, and positive regulation of macromolecule metabolic process (Gene Ontology (GO): 2000147, GO:0016477, GO:0001932, GO:0043067, GO:1902531, GO:0044093,

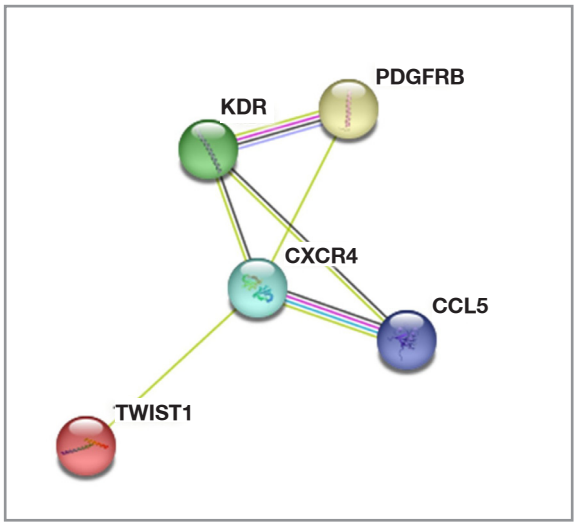

Figure 1. STRING interaction network of target genes at protein level in pediatric ALL. Known interactions (turquoise, magenta), text mining (yellow), protein homology (lilac), and co-expression (grey) connections in the database are shown. Network statistics include; 5 nodes, 6 edges, 2.4 average node degree, and PPI enrichment p-value (p= 0.000216)

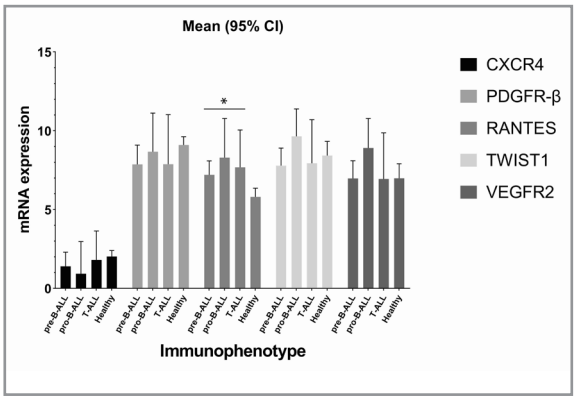

Figure 2. Expression plot of targeted genes in pediatric ALL patients by Immunophenotype. Interactions of patient group WHO immunophenotypes with targeted genes. CXCR4; C-X-C chemokine receptor type 4 (p= 0.415), PDGFR-β; Platelet derived growth factor receptor beta (p= 0.277), *RANTES; Chemokine (C-C motif) ligand 5 (p= 0.015), TWIST1; Twist family BHLH transcription factor 1 (p= 0.302), VEGFR2; Vascular endothelial growth factor receptor 2 (p= 0.235).

GO:0007166, GO:0071310, GO:0051173, GO:0031325, GO:0006950, and GO:0010604, respectively). The Benjamini-Hochberg multiple test results within each category were statistically significant ($p< 0.05$).

Association of Immunophenotype and RANTES in Pediatric ALL

In our study, CXCR4, PDGFR-β, RANTES, TWIST1, and VEGFR2 gene expression levels were evaluated in pediatric patients with pre-B-ALL, pro-B-ALL and T-ALL immunophenotypes. Increased expression levels of RANTES ($p= 0.015$) were positively correlated in all ALL cases compared to healthy controls (Figure 2). Post-hoc analysis confirmed the altered RANTES expression between pre-B-ALL cases and healthy control group ($p= 0.032$).

Expression Profile of Target Genes in the Presence of Trisomy or Translocation

High CXCR4 expression was found in patients with the t(9;22) translocation ($p= 0.039$). In post-hoc data, a difference was observed for the t(9:22) group between the t(1;19) and translocation-negative groups ($p= 0.073$, $p= 0.093$, respectively), although not statistically significant. RANTES expression was increased at the t(9;22) translocation compared to the t(1;19) translocation ($p= 0.017$, ($p= 0.028$)post-hoc) (Figure 3A). In patients with low hyperploidy was examined the relationship between "chr1, chr6, chr8, chr12, chr17, chr21 and chr22" trisomies and target genes. Analysis showed that PDGFR-β, RANTES, TWIST1 and VEGFR2 expressions ($p< 0.05$) were upregulated (Figure 3B) and the results were consistent with the multiple comparison test ($p< 0.05$).

Expression of PDGFR-β and RANTES Depending on the Remission Duration

The genetic contribution of target gene expression to the remission duration was investigated according to the remission status on the 8th, 15th, 33rd, and 36th days in patients receiving induction therapy. As a result of analysis, PDGFR-β and RANTES gene expressions were determined to be increased ($p= 0.007$, $p= 0.015$, respectively) in all patients who went into remission at 15th, 33rd, and 36th day compared to the relative early remission status at 8th day (Figure 4). Post-hoc analysis of PDGFR-β expression found a significant difference only remission status between 8th day and 15th day ($p= 0.010$).

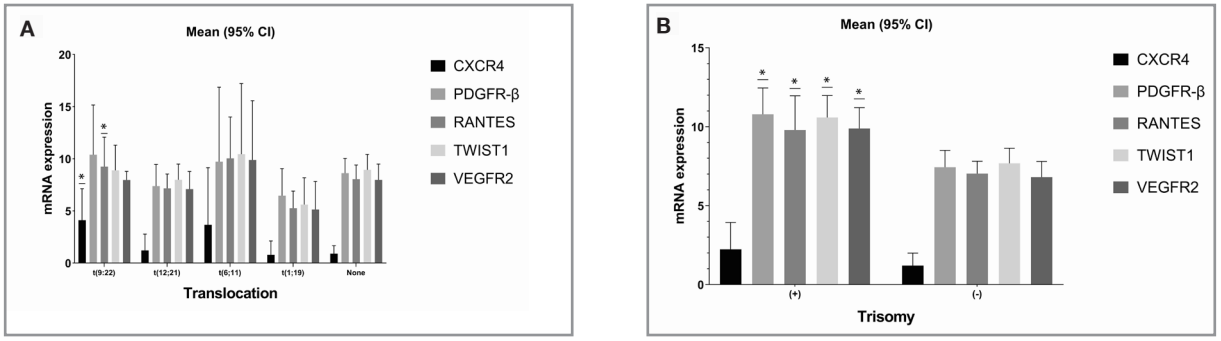

Figure 3. Expression changes of target genes by translocation and trisomy in pediatric ALL cases. **(A)** Profile of expression of target genes in patients with translocation types t(9;22), t(12;21), t(6;11) and t(1;19) compared to healthy controls; **(B)** Comparative analysis of gene expression plot in patients with (n= 8) and without (n= 38) trisomy. Results of Independent Sample T-test with Mann Whitney U Test and Student's t test; CXCR4 (p= 0.263), *PDGFR-β (p= 0,007), *RANTES (p= 0.005), *TWIST1 (p= 0.009), and *VEGFR2 (p= 0.008).

Risk Groups and CXCR4 Expression

The patients who are included in this study had a relatively homogeneous distribution in terms of risk groups. 30.4% (n= 14) of the cases were classified as SR group, 30.4% (n= 14) as IR group, and 39.2% (n= 18) as HR group. Among the risk groups, CXCR4 expression was observed to be upregulated in the HR group compared to SR (p= 0.039, (p= 0.043)post-hoc) (Figure 5).

DISCUSSION

Nowadays, clinical features, genetic alterations, and MRD factors are evaluated together in ALL risk classification.[5] Cytogenetic abnormalities and molecular changes are important as prognostic factors in ALL; however, only a few related biomarkers have been identified.[41] Although, survival rates are high in pediatric ALL, studies are continuing to reduce the effect of treatment on long-term mortality and morbidity.[42] In addition, drug resistance and relapse in pediatric ALL occur in a significant number of patients[43], and the need for the new prognostic biomarkers is increasing.

First of all, we evaluated the PPI interactions of targeted genes in the STRING database. Network analysis showed that the relevant gene proteins could be highly correlated in terms of biological process such as cell motility, cell migration, intracellular signal transduction, and cell surface receptor signaling pathway (p< 0.05). It is known that these processes also participate in the formation of haematological malignancy in pediatric ALL.[44] For this reason, we decided to study with a screening set of CXCR4, PDGFR-β, RANTES, TWIST1, and VEGFR2 genes as candidate prognostic biomarkers.

In our study, we investigated whether there is a relationship between RANTES gene expression and pediatric ALL immunophenotypes (pre B-ALL, pro B-ALL and T-ALL). As a result of our statistical analysis, we observed a significant increase in RANTES gene expression level in all ALL subgroups compared to the healthy control group (p= 0.015), especially between pre-B-ALL cases and healthy control group (p= 0.032)post-hoc. We suggest that, the data that we have obtained will shed light on further studies in terms of elucidating the relationship between pediatric pre-B-ALL immunophenotype and the RANTES gene. Translocation and trisomy type chromosomal abnormalities are considered as cytogenetic evidence in pediatric ALL. Our results have shown that the RANTES' modified expression for both types of abnomalies is remarkable. In pediatric ALL, downstream signaling pathways of RANTES (PI3K/AKT, RAS-ERK-MEK, JAK-STAT) are likely to be rearranged by altered gene expression.[45-47] Thus, expression profile of RANTES may be an indicator of changes in the pathogenesis of ALL, and may provide insight into prognosis in patients with certain cytogenetic abnormalities.

The duration of remission after the initiation of ALL-treatment is an important criterion for determining the treatment protocol and prognosis.

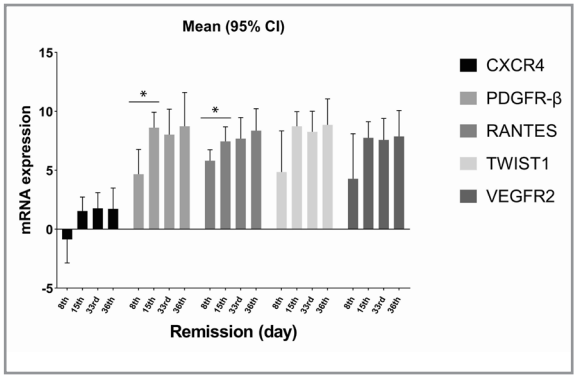

Figure 4. The relationship between remission duration and target genes in pediatric ALL cases. The target gene expression distributions of the patient cases are shown depending on the remission time (8th, 15th, 33rd and 36th days).

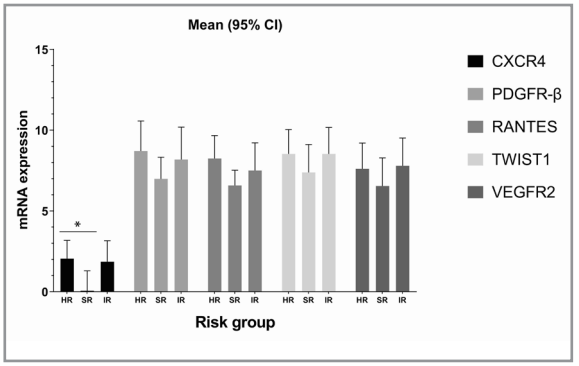

Figure 5. Plot of pediatric ALL risk groups to targeted genes. mRNA expression analysis results of targeted genes in HR (n=18), SR (n=14) and IR (n= 14) group patients. One-Way ANOVA (Welch's) test results: *CXCR4 (p= 0.039), PDGFR-β (p= 0.261), RANTES (p= 0.125), TWIST1 (p= 0.503), and VEGFR2 (p= 0.506).

Schwab et al. reported that PDGFR-β fusion gene (EBF1/PDGFR-β) expression caused patients to go into late remission.[48] In our study, when we investigated the interaction between the remission duration and the target genes, we found that the expressions of PDGFR-β and RANTES were increased in cases with late remission compared to cases with remission on the 8th day; however, in the multiple comparison test we performed for validation, we found significant results only for PDGFR-β (p= 0.010). We construed this result as the change in PDGFR-β expression due to a prolonged remission duration can be attributed to chromosomal abnormality, and we suggest that the remission period may vary according to the variety of fusion genes containing PDGFR-β as a potential area of research for further studies.

According to the results of our study, RANTES and PDGFR-β can be used as a biomarker in predicting the time of patients to go into remission and may lead to further studies. Moreover, heterogeneous CXCR4 expression levels in ALL, especially pre-B-ALL, have been reported in previous studies and have been associated with extramedullary organ infiltration (EOI) in pediatric cases. There is also an immune checkpoint clinical study which is targeting HR group patients in adult AML.[12,49] Hence, when we examined the distribution of CXCR4 expression in pediatric cases, we observed a change in the expression of CXCR4 directly proportional to the increased risk; however, it is necessary to examine in larger series to reach more significant results. In addition, our single-center study has the clear advantage of minimizing possible variations in clinical evaluations. For keeping authenticity, our study is a preliminary screening study limited to the evaluation of candidate genes that have not been clearly associated with ALL. Our sample size was one of the limitations of our study due to ethical issues in obtaining bone marrow, especially from a healthy pediatric population; however, we demonstrated the accuracy of our data with advanced statistical analyses such as multiple comparison tests.

Despite the high survival rates in pediatric ALL, relapse and treatment-related advanced complications stand out as an important threat in the lives of patients. Therefore, in our study, we investigated the potential of CXCR4, PDGFR-β, RANTES, TWIST1 and VEGFR2 genes, which we identified as candidate prognostic biomarkers in pediatric ALL. Our results indicated a causal relationship between pediatric ALL and RANTES, while demonstrating identifiable interactions of the disease with clinical risk factors. Based on our results, we recommend RANTES and PDGFR-β as prognostic biomarkers in pediatric ALL; however, we also point out the need to confirm our results with more extensive studies.

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