

## Study of Gene Expression Signatures for the Diagnosis of Pediatric Acute Lymphoblastic Leukemia (ALL) Through Gene Expression Array Analyses

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### Abstract

**Background:** Acute lymphoblastic leukemia (ALL) as the most common malignancy in children is associated with high mortality and significant relapse. Currently, the non-invasive diagnosis of pediatric ALL is a main challenge in the early detection of patients. In the present study, a systems biology approach was used through network-based analysis to identify the key candidate genes related to ALL development and relapse.

**Materials and methods:** In this systems biology (experimental) study, main and validating datasets were retrieved from a gene expression omnibus (GEO). Gene expression analyses were done using a bioinformatics array research tool (BART) and ExAtlas. Gene ontology and pathway enrichment analysis were also performed via Database for Annotation, Visualization and Integrated Discovery (DAVID). Furthermore, the Search Tool for the Retrieval of Interacting Genes (STRING) and cytoscape V.3.9.1 were used to network construction and analysis. The MCODE and NCMine Plugin of cytoscape were applied to find clusters and a functional module in the network. The Kaplan Myer curve was applied in order to survival analysis of the validated candidate genes. A P-value of < 0.05 was considered as significant.

**Results:** A total of 671 differentially expressed genes (DEGs) mainly involved in transporter/channel activity functions, cell communication/signaling processes and fatty acid transport/PPAR signaling/eicosanoid metabolism pathways were identified (P-value < 0.05). The main cellular compartments were plasma membrane, cell periphery and cell surface (P-value < 0.05). The network analysis revealed 68 hub genes, 29 of which were candidate genes. Five candidate genes were also validated in two independent experiments. These genes were considered as key candidate genes, and three of them (BCL2L11, IGF1, PDE5A) were predictors of pediatric ALL patients survival (P-value < 0.05).

**Conclusion:** BCL2L11, IGF1 and PDE5A genes, as key candidate genes, are potentially good diagnostic biomarkers and therapeutic targets for pediatric ALL.

**Keywords:** Acute lymphoid leukemia, Gene cluster, Gene ontology, Protein-protein interaction network, Survival analysis

### Introduction

Acute lymphoblastic leukemia (ALL) is a hematological disorder of bone marrow (1). Its incidence peak occurs between 2 and 5 years of age, and it is more prevalent in males than in females (2, 3). This disease is the most common cancer among children, taking a high toll of lives every

year. ALL arises from the uncontrolled proliferation of undifferentiated/unmatured B cells (80-85%) or T cells (20-25%) resting in bone marrow (4). Owing to the application of advanced treatment regimens and supportive cares, a 5-year event-free survival has improved to over 90% for children with ALL (5).

Nevertheless, relapses still happen in nearly 20% of pediatric ALL patients. Relapsed ALL patients have poor prognosis with an overall survival rate of 30-40% even when treated with multi-agent chemotherapy (6). Also, the clinical application of the present diagnosis methods for ALL is limited due to their expensiveness as well as invasive and inconvenient nature (7). The diagnosis of ALL is typically based on the aspiration of patients' bone marrow for histopathological examination including molecular, immunologic, and cytogenetic characteristics (8). Therefore, it is necessary to develop new non-invasive diagnostic methods to overcome the existing limitations. The identification of the molecular mechanisms involved in ALL and its recurrence helps to detect effective molecular markers and therapeutic targets (9). Microarray has been extensively used to reveal potential biomarkers and therapeutic targets by the simultaneous study of thousands of gene expressions at the transcriptomic level (10, 11). However, the traditional methods of analyzing microarray data only find individual genes, whereas genes interact in complex biological networks. Thus, the interaction among genes (proteins) is, indeed, important in microarray data analysis and should be considered in the use of systems biology approaches and network-based analyses (12, 13). In a network analysis, genes (proteins) are considered as nodes, and highly connected nodes have been discovered through the analysis of biological networks. These nodes mainly are involved in studied diseases or conditions and can be used as biomarkers or therapeutic targets (14, 15). The aim of the present research is to identify the key genes involved in pediatric ALL development and/or relapse by the network-based analysis of microarray data.

## Materials and Methods

### Microarray datasets

The Gene Expression Omnibus (GEO) database of NCBI was searched for suitable gene expression datasets to compare the expression of genes between pediatric ALL patients and healthy controls and between the patients relapsing from a previously treated relapse and those with complete remission. Four datasets were used in this study including GSE101425 for the main analysis with 86 samples (control = 16, leukemic = 35, remission = 29, relapse = 6), GSE7440 for validation with 99 samples (rapid responder = 23, slow responder = 17, V1, remission = 28, relapse = 31, V2), GSE19143 for validation (V3) with 52 samples (treatment-sensitive = 27, treatment-resistant = 25), and GSE28460 for validation (V4) with 98 samples (diagnosis = 49, relapse = 49).

### Differentially expressed genes (DEGs)

Gene expression analysis and data normalization were done with a bioinformatics array research tool (BART) (16) and ExAtlas (17) web-based applications. The differentially expressed genes in the samples were identified and selected according to the criteria of  $|\text{Fold change}| \geq 2$ ,  $P\text{-value} < 0.05$ . In this respect, there were only protein coding genes to select. Pseudogenes as well as unannotated and uncharacterized genes were removed. Overlapping genes with differential expressions in different conditions were selected using a Venn diagram (<http://bioinformatics.psb.ugent.be/webtools/Venn/>).

### Gene ontology and pathway enrichment analysis

Gene ontology (i.e., molecular functions, biological processes and cellular compartments) annotation and WikiPathways analysis were performed with the Database for Annotation, Visualization and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/>). Top ten enriched terms with the  $P\text{-value}$  of  $< 0.05$  and a minimum of 10 DEGs were considered as significant enriched terms.

### Protein-protein interaction network (PPIN) analysis

In this study, two software programs including the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) and Cytoscape V. 3.9.1 were used to analyze the protein-protein interaction network of the selected DEGs (with the minimum interaction score of  $\geq 0.4$  <https://string-db.org/>).

Top 150 nodes with the highest network parameters of degree, closeness centrality, betweenness centrality and radiality were selected. The overlapping nodes in these network parameters were identified via a Venn diagram and considered as hub genes. The degree parameter referred to the number of the edges connected to a node, the closeness centrality denoted the average shortest distance from each node to another node, and betweenness centrality represented the extent to which a node lies on the shortest paths between other nodes.

### Network clusters and functional module

The MCODE and NCMine Plugin of cytoscape served to find highly interconnected regions (clusters) and cooperatively working genes (functional module) in the network, respectively. The criteria for the MCODE analysis included cluster node number  $> 8$ , maximum depth = 100, cluster score  $> 6$ , k-core = 2, node score cutoff = 0.2, and degree cutoff = 2. The criteria for the NCMine analysis were size threshold = 3, cliqueness threshold = 0.6, dcliqueness threshold = 0.2, and merge threshold = 0.6.

### Survival analysis

Kaplan Meyer curve analysis was performed to find the relationship between the overall survival of the ALL-suffering children and the expression level of the validated candidate genes. The survival data were obtained from the TARGET (Therapeutically Applicable Research to Generate Effective Treatments) project

related to the office of cancer genomics ([www.xenabrowser.net](http://www.xenabrowser.net)). The samples included 207 pediatric patients with ALL. Log-rank test Statistics was used to compare the Kaplan–Meier curves, and P-value  $< 0.05$  was considered as significant. The significant validated candidate genes were considered as the key candidate genes.

### Ethical statement

This systems biology (experimental) study was approved by the ethics committee of Hamadan University of Medical Sciences [Ethics certificate number: IR.UMSHA.REC.1399.732].

## Results

### Differentially expressed genes

Generally, 415 DEGs (including 229 upregulated and 186 downregulated genes) were identified in the comparison of the leukemic and control patients, so were 295 DEGs (including 72 upregulated and 223 downregulated genes) as the relapse and remission patients were compared (Figures 1a and 1b). There were thirty-nine overlapping DEGs in the leukemic/control and relapse/remission groups (Figure 1c). The heatmap for the overlapping DEGs is shown in Figure 1d.

### Gene enrichment analysis

Gene enrichment analysis were performed for all the 671 DEGs in DAVID. Ten top enriched terms are shown in Figure 2. The DEGs were significantly enriched in terms of the molecular functions of transporter activity, channel activity, growth factor activity, metal binding, etc. The main enriched cellular compartments were the intrinsic/integral component of plasma membrane, cell leading edge, cell periphery, ion channel complex, cell surface, and cytoplasmic membrane vesicle. The DEGs were mostly involved in cell communication, developmental process, regulation of response to stimulus, ion transport and localization biological processes. The main enriched pathways were fatty acid transport pathway, PPAR signaling pathway, eicosanoid metabolism

via cyclooxygenase, glucocorticoid receptor pathway, prostaglandin synthesis and regulation, PD-1 signaling and pluripotent stem cell differentiation.

### Protein-protein interaction network and hub genes

The two primary networks (i.e., leukemic/control and relapse/remission networks, Figures 3a and 3b) were merged to construct a main network. The constructed network (Figure 3c) had significantly more interactions than expected ( $P$ -value  $< 10^{-16}$ , number of edges = 1570, average node degree = 4.81, average local clustering coefficient = 0.351). Through network analysis, 68 hub genes were obtained with the highest degree, closeness centrality, betweenness centrality and radiality (Figure 4a).

### Network clusters and functional module

Twenty four hub genes contributed to network clusters and/or functional module (Figures 4b and 4c and Figure 5). Also, 3 and 2 of the overlapping DEGs in leukemic/control and relapse/remission patients overlapped the hub genes and network clusters, respectively (Figures 1d and Figure 5). These 29 genes were considered as candidate genes (Table I).

### Validation and survival analysis

Five of 29 candidate genes (including BCL2L11, IGF1, PTPRC, PDE5A and KITLG) were validated in two of four independent experiments (Table I). These five genes were assessed to predict the survival of the ALL-suffering children, three of which (namely BCL2L11, IGF1 and PDE5A) had significant relationships with the patients' survival (Figure 6) ( $P$ -value  $< 0.05$ ). These three genes were considered as key candidate genes.

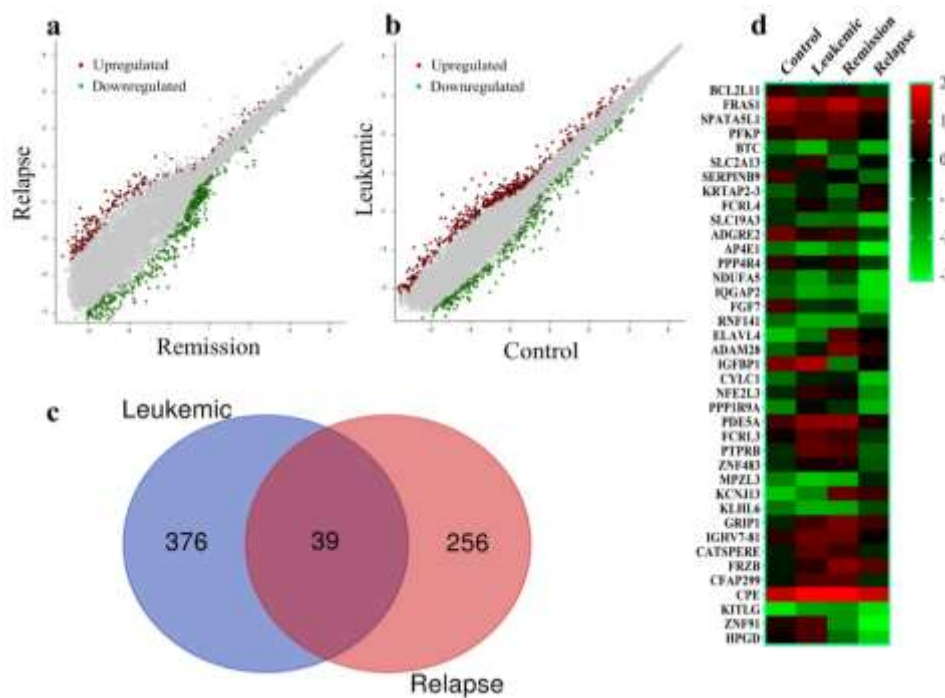


Figure 1. Identified differentially expressed genes. a) DEGs between leukemic and control patients, b) DEGs between relapse and remission patients, c) Venn diagram of overlapping DEGs between leukemic/control and relapse/remission patients, d) Heatmap of overlapping DEGs.

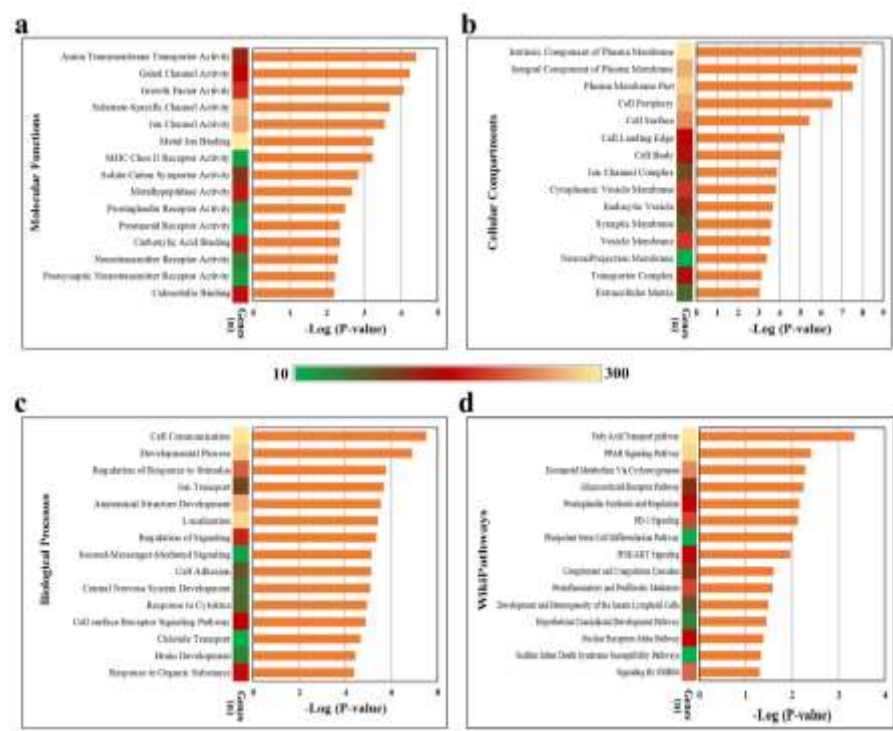


Figure 2. Gene ontology and pathway enrichment analysis. a) Molecular functions, b) Cellular compartments, c) Biological processes, d) WikiPathways.

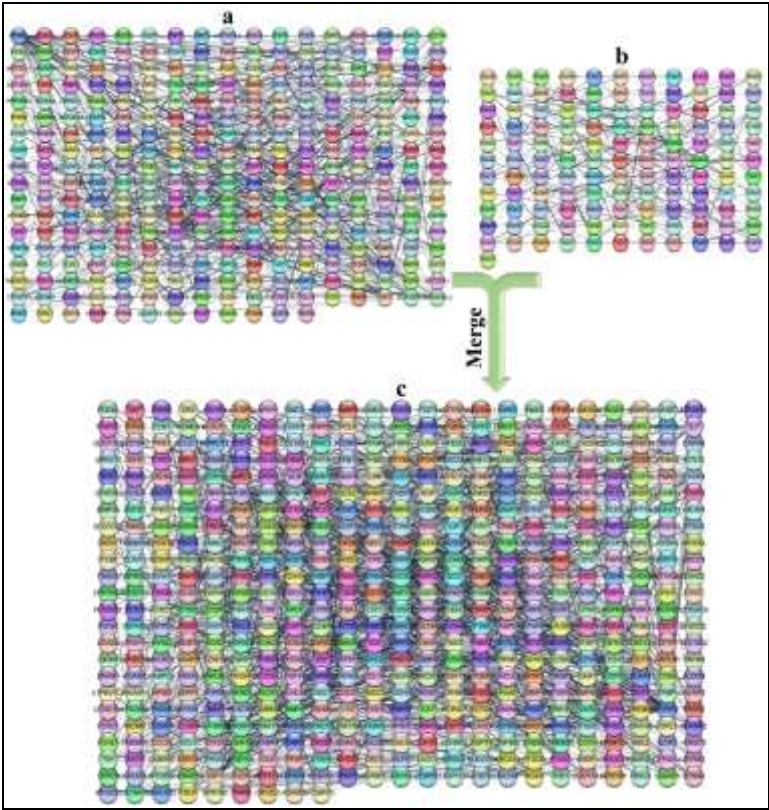


Figure 3. Protein-protein interaction networks. a) PPIN of DEGs between leukemic/control patients, b) PPIN of DEGs between relapse/remission patients, c) Merged PPIN of a & b networks. Non-connected nodes are hidden



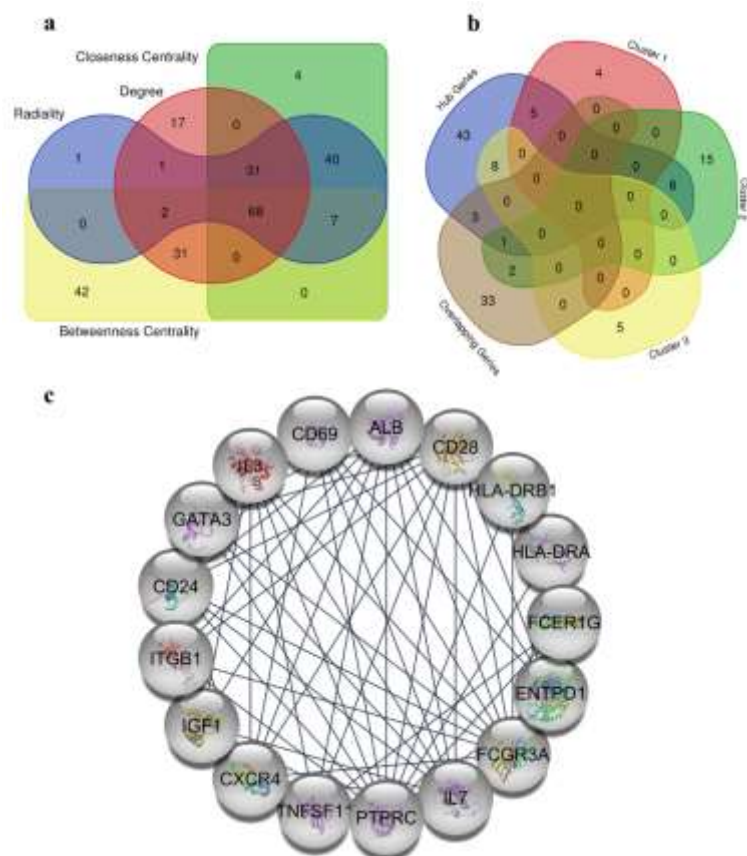


Figure 4. Venn diagram of overlapping genes and functional module of network. a) Venn diagram of overlapping genes in terms of network parameters, b) Venn diagram of overlapping genes between network clusters, hub genes and leukemic/control and relapse/remission overlapping genes, c) Functional module of the network: 17 nodes & 79 edges.

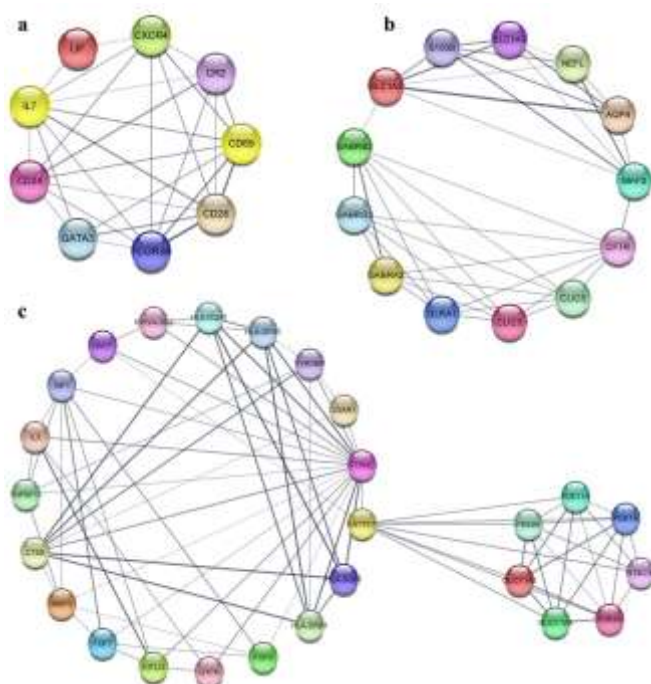


Figure 5. Significant clusters of the PPIN. a)Module 1: 9 nodes & 28 edges, b) Module 2: 13 nodes & 38 edges, c)Module 3: 26 nodes & 81 edges.

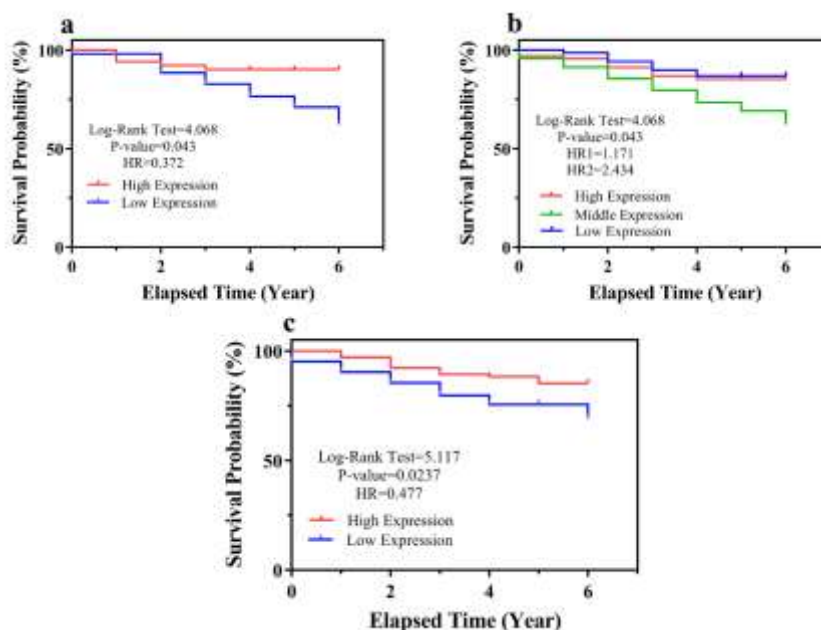


Figure 6. Survival analysis of validated candidate genes. a) BCL2L11, b) PDE5A, c) IGF1. Hazard ratio (HR) is the ratio of the hazard rates corresponding to the conditions described by two levels of an explanatory variable.

Table I. The list and network parameters related to candidate genes. Candidate genes those validated in 2 of 4 independent experiments are boded and considered as validated candidate genes.

Candidate Genes	Betweenness Centrality	Closeness Centrality	Degree	Radiality	Functional Module	Cluster Number	Overlapping Genes	V1	V2	V3	V4
<b>FGF7</b>	0.008770	0.305809	15	0.968472	N	3	N	N	N	N	N
<b>CD28</b>	0.033110	0.315697	24	0.969894	N	1	N	N	N	Y	Y
<b>CXCR4</b>	0.033350	0.327439	30	0.971472	N	1	N	N	N	N	Y
<b>CD69</b>	0.009434	0.308443	20	0.968860	N	1	N	Y	N	N	N
<b>FCGR3A</b>	0.028530	0.312391	34	0.969429	N	1	N	N	N	Y	N
<b>GATA3</b>	0.038319	0.322910	26	0.970877	N	1	N	N	N	N	N
<b>DNTT</b>	0.011099	0.309154	13	0.968963	N	3	N	N	N	N	N
<b>IGF1</b>	0.040675	0.334997	33	0.972429	Y	3	N	N	Y	N	Y
<b>ENTPD1</b>	0.031545	0.299498	16	0.967515	Y	3	N	N	N	N	Y
<b>TYROBP</b>	0.011748	0.290113	23	0.966015	N	3	N	N	N	N	Y
<b>TNFSF11</b>	0.013484	0.315326	18	0.969843	Y	3	N	Y	N	N	N
<b>HLA-DRB1</b>	0.007784	0.273700	14	0.963144	Y	3	N	N	N	N	Y
<b>MMP3</b>	0.018582	0.308266	15	0.968834	N	3	N	N	N	N	N
<b>PTPRC</b>	0.117201	0.345116	62	0.973645	Y	3	N	Y	Y	N	N
<b>GABRB2</b>	0.042921	0.293122	23	0.966506	N	2	N	N	N	N	N
<b>GABRA2</b>	0.008122	0.283228	14	0.964851	N	2	N	N	N	N	N
<b>AQP4</b>	0.018860	0.308090	18	0.968808	N	2	N	N	N	N	N
<b>MAP2</b>	0.011512	0.309867	18	0.969067	N	2	N	N	N	N	N
<b>CFTR</b>	0.047759	0.311485	19	0.969300	N	2	N	N	N	N	Y
<b>NEFL</b>	0.013973	0.309510	15	0.969015	N	2	N	N	N	N	N
<b>SLC1A2</b>	0.012486	0.306857	16	0.968627	N	2	N	N	N	N	N
<b>S100B</b>	0.009320	0.303733	13	0.968162	N	2	N	N	N	N	N
<b>BCL2L11</b>	0.015368	0.311666	13	0.969325	N	N	Y	Y	N	N	Y
<b>ELAVL4</b>	0.009264	0.304077	9	0.968213	N	N	Y	N	Y	N	N

<b>GRIP1</b>	0.010302	0.275526	8	0.96348	N	N	Y	N	N	N	N
<b>PDE5A</b>	0.004739	0.249188	10	0.958152	N	3	Y	N	Y	N	Y
<b>KITLG</b>	0.003948	0.297342	12	0.967179	N	3	Y	N	Y	N	Y
<b>ALB</b>	0.229504	0.377107	72	0.977059	Y	N	N	N	N	Y	N
<b>ITGB1</b>	0.039660	0.317564	24	0.970153	Y	N	N	N	N	N	N

N: no, Y: yes, V1: validation experiment 1, V2: validation experiment 2, V3: validation experiment 3, V4: validation experiment 4

## Discussion

Through a systems biology approach and network-based analyses, the present study focused on finding suitable diagnostic biomarkers for easy and non-invasive detection of pediatric ALL. The results showed that the main molecular function of DEGs is channel transporter/channel activity. In fact, membrane proteins have an essential role in moving nutrients and toxic compounds into and out of the cells, respectively (18). These proteins have a main role in cancer chemo-resistance and are potentially excellent therapeutic targets (19). The major enriched biological processes in this study were cell communication/signaling/localization. Cell communication and signaling play essential roles in cancer development not only in primary sites but also in distant locations (20). In the present study, the selected DEGs were mainly enriched in the fatty acid transport pathway, PPAR signaling pathway and eicosanoid metabolism pathway. The relationship between fatty acid metabolism and malignancies including acute myeloblastic leukemia (21) and ALL (22) has been reported in other studies. PPAR signaling has a critical role in cell growth and the survival of cancer cells, as in leukemia (23). The production of eicosanoids by cyclooxygenase-2 enzyme induces malignant cell proliferation and metastasis in various type of cancers (24). Five validated candidate genes (i.e., BCL2L11, IGF1, PTPRC, PDE5A and KITLG) were identified in the present study, three of which (i.e., BCL2L11, IGF1 and PDE5A) were able to predict pediatric ALL

survival. BCL2L11 (BCL2-like 11) is a protein-coding gene located on chromosome 2q13 based on Ensembl and Entrez Gene data. BCL2L11 is also known as BIM. It acts as a pro-apoptotic gene and induces apoptosis in the cell by inactivating Bcl2 and activating Bax-Bak1. Furthermore, BCL2L11 as a mediator of pro-apoptotic signals can induce apoptosis by Atf4/CHOP-mediated UPR upon endoplasmic reticulum (ER) stress if excess ER stress conditions persist (25). As a pro-apoptotic member, its downregulation in gastric cancer has been reported by Zhang et al. (26). Also, its upregulation has been reported in ALL cells in response to dexamethasone treatment (27). As Jing et al. (28) reported, the expression level of BCL2L11 is upregulated in the pediatric acute lymphoblastic leukemia cells that are sensitive to glucocorticoids. In children and adults, higher IGF-1 levels are associated with a higher risk of developing many types of cancers (29). This gene encodes insulin-like growth factor (IGF) proteins and causes growth and development during fetal and postnatal life. In addition to contributing to overall growth, IGF-GH is essential for the embryonic-to-neonatal transition (30). In terms of genetic structure, IGF-1 is about 90 kilobytes of genomic DNA, which consists of six exons. After splicing, the exons produce multiple transcripts, each encoding a different IGF-I precursor protein with variable signal peptide leader sequences. All the transcript isoforms give rise to mature 70-amino acid IGF-I peptide that uses the same receptor (31). Al-



Azzawi et al. (32) showed that the expression levels of IGF-1 in pediatric ALL patients reduces after treatment. The reports on PDE5A expression in different cancer types are controversial. This gene encodes a specific phosphodiesterase from the cyclic nucleotide phosphodiesterase family. It specifically hydrolyzes cGMP to 5'-GMP, regulates the intracellular concentration of cyclic nucleotides and is required for smooth muscle relaxation in the cardiovascular system. Three transcript variants encoding distinct isoforms are yielded from its alternative splicing (33, 34). Arozarena et al. (35) showed that PDE5A is downregulated in metastatic melanoma. Moreover, Zhang et al. (36) reported the lower expression of the mRNA encoding PDE5A in chronic lymphocytic leukemia cells compared with normal PBMC. Its overexpression has also been reported in several types of cancers (37). Nowadays, circulating tumor cells (CTCs) are known as another type of cancer marker for many cancers. They are tumor cells that leave the primary tumor tissue and flow into the blood. Many studies have predicted the great potential of CTCs in clinical applications. Therefore, measuring the changes of the key candidate genes identified in CTCs can be a promising and non-invasive approach for the early diagnosis of pediatric ALL (38).

## Conclusion

In this study, a network-based analysis was performed on microarray datasets for pediatric ALL to identify diagnostic biomarkers. The candidate genes were validated in two independent experiments, and their ability to predict the survival of children patients with ALL was assessed. Finally, three key candidate genes, including BCL2L11, IGF1 and PDE5A, were selected as potential diagnostic and prognostic biomarkers in pediatric ALL. It is suggested that the serum level of mRNA be detected for these genes, for example in the CTCs of patients' samples, in order to

non-invasively identify pediatric cases with ALL. Also, the gene signatures can potentially be used as therapeutic targets to treat pediatric ALL. To achieve defendable results, however, animal and clinical explorations are needed more than in vitro studies.

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## Conflict of interest

The authors declare no conflict of interest.

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