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ORIGINAL ARTICLE

Gene Expression Profiling of Acute Lymphoblastic Leukemia in Children with Very Early Relapse

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Background and Aims. Acute lymphoblastic leukemia (ALL) is the most common childhood cancer worldwide. Mexican patients have high mortality rates, low frequency of good prognosis biomarkers (i.e., *ETV6-RUNX1*) and a high proportion is classified at the time of diagnosis with a high risk to relapse according to clinical features. In addition, very early relapses are more frequently observed than in other populations. The aim of the study was to identify new potential biomarkers associated with very early relapse in Mexican ALL children through transcriptome analysis.

Methods. Microarray gene expression profiling on bone marrow samples of 54 pediatric ALL patients, collected at time of diagnosis and/or at relapse, was performed. Eleven patients presented relapse within the first 18 months after diagnosis. Affymetrix Human Transcriptome Array 2.0 (HTA 2.0) was used to perform gene expression analysis.

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Annotation and functional enrichment analyses were carried out using Gene Ontology, KEGG pathway analysis and Ingenuity Pathway Analysis tools.

Results. *BLVRB, ZCCHC7, PAX5, EBF1, TMOD1* and *BLNK* were differentially expressed (fold-change >2.0 and p value <0.01) between relapsed and non-relapsed patients. Functional analysis of abnormally expressed genes revealed their important role in cellular processes related to the development of hematological diseases, cancer, cell death and survival and in cell-to-cell signaling interaction.

Conclusions. Our data support previous findings showing the relevance of *PAX5*, *EBF1* and *ZCCHC7* as potential biomarkers to identify a subgroup of ALL children in high risk to relapse.

Key Words: Acute lymphoblastic leukemia, Gene expression profiling, Children, Very early relapse, Biomarkers.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer worldwide (1). Mexico has one of the highest ALL incidence rates reported (2) and it is one of the few countries in which mortality has not been reduced despite of using the same chemotherapy regimens than in developed countries (3,4). In previous studies, we reported that at the time of diagnosis, almost half of ALL Mexican children are classified as having high risk of relapse according to clinical criteria (5,6) and $<20\%$ are identified as positive for one of the four most common gene rearrangements (*ETV6-RUNX1, TCF3-PBX1, BCR-ABL1* and *MLL* rearrangements) associated with ALL prognosis (5). On the other hand, in developed countries, only one third of patients are classified as having a high risk of relapse at the time of diagnosis (7) and in 32% one of the four most common gene rearrangements mentioned above is detected (8).

Moreover, relapse is one of the main obstacles for achieving better survival rates in our population. Relapses occur in 26.2% of Mexican ALL pediatric patients (9). Noteworthy, they frequently occur in patients of standard risk group (55%) and very early during treatment (9), highlighting the importance of improving current clinical and molecular prognostic stratification in Mexican children with ALL.

Global gene expression profiling has been used in other populations to identify new potential genetic biomarkers associated with relapse in ALL pediatric patients (10–12). This methodology has also revealed possible mechanisms involved in relapse (13,14). *FLT3, XIAP, CCNB2, IKBKG, LIMS1, TEGT, DEFA1-3, SH3, BP5, TOSO, survivin, TOP2A, cyclin B1*, etc. are some examples of genes that have been reported as abnormally expressed in relapsed ALL (15–17). The aim of this study was to perform a transcriptome analysis in very early relapsed ALL children in order to identify new potential biomarkers associated with this outcome in our population.

Materials and Methods

Patients

Mexican Inter-Institutional Group for the Identification of Causes of Childhood Leukemia (MIGICCL) conducted a multicenter cohort study of patients aged <17 years with newly diagnosed ALL between August 1, 2014 and July 30, 2016 treated in Mexico City public hospitals. Diagnosis of ALL was based on the morphologic and immunophenotypic features of leukemic cells. Bone marrow samples (BMS) at the time of diagnostic confirmation and/or at the time of very early relapse (VER) were gathered.

Clinical Data Collection

Information regarding gender, age at diagnosis, white blood cell count (WBC), percentage of leukemic blasts in bone marrow, immunophenotype, and dates of ALL diagnosis, treatment initiation, last visit, death, and relapse was collected from the patients' clinical charts.

Risk classification at the time of diagnosis was based on the National Cancer Institute (NCI) risk criteria. Patients between 1 and 10 years old and with a leukocyte count $<50 \times 10^9$/L were classified as NCI standard-risk, whereas those aged ≥ 10 years or with a leukocyte count $\geq 50 \times 10^9$/L were classified as NCI high-risk, as previously was described (6).

Very early bone marrow relapse was defined when a patient who reached first complete remission (CR) presented $\geq 25\%$ lymphoblasts in a bone marrow aspirate within the first 18 months after diagnostic confirmation. Very early central nervous system (CNS) relapse was characterized as the presence of morphologically identified lymphoblasts on smears of cerebrospinal fluid (CSF) cytocentrifuged preparations with a CSF mononuclear cell count $>5/\mu l$ or as the evidence of tumor infiltration in the CNS following the first CR during the first 18 months after ALL diagnosis (18).

Approval by the National Scientific Research and Ethics Committee was obtained with the number R-2013-785-068. Written informed consent was obtained from the child's parents and assent was obtained from patients ≥ 8 years of age.

RNA Isolation and Gene Rearrangement Detection

White blood cells from bone marrow were treated with TRizol reagent (Invitrogen Life Technologies) and stored at $-80°C$. Total RNA was extracted and purified using standard protocols. With a conventional RT-PCR, chimeric

gene detection (*ETV6-RUNX1, TCF3-PBX1, BCR-ABL1*) was performed based on protocols previously published (5).

RNA Preparation for Microarrays

RNA integrity was evaluated by capillary electrophoresis using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Only those BMS with a RNA integrity number (RIN) >7.0 were included in microarray analysis (MA). MA was conducted according to manufacturer protocols (Affymetrix Inc., Santa Clara, CA). Briefly, the first-strand cDNA, was synthetized from 200 ng of RNA and using Superscript II reverse transcriptase primed with a poly (T) oligomer that incorporated T7 promoter. To obtain cRNA, the second strand cDNA synthesis was followed by *in vitro* transcription. This cRNA was used as template for a second cDNA synthesis cycle with incorporated dUTPs into the new strand. After cDNA fragmentation using uracil-DNA glycosylase and purin-pyrimidin endonuclease; fragments were biotin-labeled (hybridization 45°C for 16 h), stained (streptavidin-phycoerythrin conjugate), washed and scanned also following Affymetrix HTA 2.0 chips protocol. All GeneChips were visually inspected for irregularities. The global method of scaling, or normalization, was applied to all GeneChips. Quality measures, likewise the percentage of present genes and the ratio of endogens genes, indicated a high overall quality of samples and assays.

Gene Expression Profiling

Gene expression analysis (GEA) was done using the high-resolution array of Affymetrix GeneChip Human Transcriptome Array 2.0 (HTA 2.0), which evaluates the expression of 67,528 different genes. This array was designed to interrogate all transcript isoforms in the human transcriptome with >6 million probes targeting coding transcripts, exon-exon splice junctions, and non-coding transcripts (19). Sample processing, labeling and hybridization were performed using the Affymetrix GeneChip WT PLUS with the WT Terminal Labeling Kit, according to the manufacturer's guidelines (Affymetrix). Scanning and data extraction of the microarray were followed by the transformation of fluorescence data into CEL files employing the Affymetrix GeneChip Command Console (AGCC) software.

Data Normalization and Analysis Plan

Background correction, probe set signal integration, and quantile normalization were performed through Robust Multichip Analysis (RMA) algorithm, which is implemented in Affymetrix Expression Console (ECS) software (20).

Unsupervised analysis. To identify a set of genes that might define molecular subtypes profiles and potential genes that might constitute evidence for VER in ALL patients, we performed an unsupervised clustering analysis of gene expression using R software (Bioconductor package), without considering any clinical features or molecular information. Those genes whose levels of expression highly differed (up- or downregulated) from the geometric mean were selected for a further cluster analysis. Afterwards, pre-treatment chip files were also analyzed by Affymetrix Transcriptome Analysis Console (TAC) software to detect differentially expressed genes (DEGs).

Supervised analysis. This approach was conducted to identify differentially expressed genes from:

i) BMS at the time of diagnosis from patients who eventually relapsed within the period of study vs. those who did not.
ii) BMS at the time of diagnosis, indistinctly if patients relapsed or not vs. BMS at relapse.
iii) BMS from patients who relapsed, matching BMS from diagnosis with their corresponding BMS at relapse.

Genes whose fold-change (FC) between each comparative group was ≥ 2.0 (with a p value cut-off of <0.01) were selected. In addition, false discovery rate (FDR) was applied for multiple hypotheses testing using Benjamini-Hochberg correction (21). Genes with a FDR-adjusted p value (adjusted p value) ≤ 0.05 were accepted. Enrichment analysis on gene sets was performed using Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/tools.jsp) (free available web tools).

The pathways and functional analyses were generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) in order to characterize the cellular and molecular functions and to identify the enriched canonical pathways/networks for the selected candidate gene list.

Validation of Microarray Data

Microarray results were validated using quantitative real-time reverse transcription PCR (qRT-PCR). For this purpose, we determined the level of gene expression of DEGs differentially expressed (FC >2, $p<0.01$, adjusted p value ≤ 0.05) between diagnosis of BMS of relapsed and non-relapsed patients (1:2), matched based on NCI risk group. Absolute quantification (AQ) was performed using the standard curve method and the average result was reported.

Probes of Human Universal ProbeLibrary and target-specific PCR primers were selected using the ProbeFinder assay design software v.2.50 (http://www.universalprobelibrary.com) (Supplementary Table 1). The comparative cycle of quantification (Cq) method was used to determine gene expression levels. FC for each gene was calculated using delta Ct′ ($\Delta\Delta$Ct) values. Reactions were performed using standard protocols. cDNA was prepared from 250 ng of total RNA for each sample using random primers and the first strand cDNA Synthesis Kit (Roche). Reactions were performed in

a final volume of 20 µl under the following conditions: at 95°C for 10 min, 45 cycles at 95°C for 10 s, 60 cycles for 30 s and 72°C for 1 sec in a LightCycler 480 using the LightCycler probe master reagent (Roche) (22). *HPRT* gene was used as a control reference gene.

Statistical Analysis

Statistical analyses were performed by using SPSS IBM (Statistical Package for the Social Sciences, Inc., v.21, Chicago, IL). χ^2 or Fisher exact tests were calculated when appropriate to compare demographic, clinical and molecular characteristics between groups (VER/not VER); p values <0.05 were considered statistically significant. Student t-test and ANOVA were used to compare gene expression levels between subgroups. Adjusted p values <0.05 were considered as statistically significant.

Results

Study Population

Samples of 54 patients diagnosed with novo ALL were analyzed in the present study. Thirty patients (55.6%) were male and had a median age of 89 months (7.4 years) with a range of 2−195 months. Eleven patients belonged to the group of very early relapse and the remaining 43 to non-relapse group. No statistically significant differences between these groups regarding gender, age at diagnosis, leukocyte count, immunophenotype, risk classification and/or frequency of gene rearrangements studied were observed (Table 1). However, mortality was higher in the VER group in comparison with those patients who did not develop VER in the first 18 months of treatment (Table 1).

Isolated bone marrow (iBM) relapses occurred in nine patients and only two patients had isolated CNS relapse.

Table 1. Demographic and clinical characteristics of Mexican acute lymphoblastic leukemia children included

	ALL children				
	No relapse		Very early relapse		
	Group		Group		
	n = 43		*n* = 11		
Clinical features	***n***	**%**	***n***	**%**	***p****
Gender					
Male	23	53.5	7	63.6	0.39
Female	20	46.5	4	36.4	
Age at diagnosis in months					
Median (min-max)	80 (2-195)		152 (44-187)		
Age group (years)					
<1	2	4.7	—	—	0.07
1-9.99	26	60.5	3	27.3	
≥10	15	34.9	8	72.7	
WBC at diagnosis (x10^9/L)					
Median (min-max)	23.37 (2.88-670)		11.18 (0.81-52.17)		
<10	14	32.6	5	45.5	0.57
10-49.99	12	27.9	1	9.1	
50-99.99	5	11.6	2	18.2	
≥100	12	27.9	3	27.3	
Immunophenotype					
Pre-B Cell	43	100	10	91	0.20
T Cell	—	—	1	9.1	
BM blast at diagnosis (%)					
<90	15	34.9	3	27.3	0.46
≥90	28	65.1	8	72.7	
NCI risk group					
High risk	26	60.5	9	81.8	0.16
Standard risk	17	39.5	2	18.2	
Gene rearrangement					
ETV6-RUNX1	8	18.6	2	18.2	0.40
TCF3-PBX1	6	14.0	—	—	
BCR-ABL1	3	7.0	—	—	
Not Detected	26	60.5	9	81.8	
Death					
Yes	7	16.3	5	45.5	0.03

WBC, white blood cell count; BM, bone marrow; NCI, National Cancer Institute; SR, standard risk; HR, high risk.
*Chi square or Fisher exact test when appropriate.

On average, relapses occurred 10.6 months after diagnosis. In two patients, the first relapse occurred within the fourth month after treatment initiation; they relapsed to bone marrow and were classified with a pre-B immunophenotype and as high risk according to the criteria of the NCI at diagnosis. One of the patients died 2 months after relapse occurred (Table 2).

Patients who relapsed and died ($n = 5$) presented isolated bone marrow relapse. Survival after relapse was 3 months (range 0–8 months) for this group. Noteworthy, a subgroup of two patients who had been classified as standard risk relapsed, and even *ETV6-RUNX1* gene rearrangement was detected in one of them (Table 2).

Quality Control Assessment

From BMS analyzed at the time of diagnosis, four did not pass quality control assessment, leaving a total of 50 BMS to be considered for microarray expression analysis. These four corresponded to patients who relapsed.

From BMS analyzed at relapse, only 9/11 approved quality control examination. Therefore, five pairs of samples for the expression analysis at diagnosis and relapse were available (Table 2).

Gene Expression Data and Molecular Subtypes

An unsupervised hierarchical cluster analysis was performed in 50 BM samples at the time of diagnosis that passed quality control. The aim for this was to identify groups within the data set without pre-assigning labels. However, no gene expression profile that corresponded to molecular leukemia subtypes (*ETV6-RUNX1, TCF3-PBX1, BCR-ABL1* group or undetected gene rearrangement) was observed.

GEA for Potential Biomarkers Associated with Very Early Relapse Recognition

The first supervised analysis conducted to compare gene expression profiles was done between BMS at the time of diagnosis from patients who eventually relapsed ($n = 7$) within the period of study and those who did not ($n = 50$); 87 coding genes were differentially expressed (FC > 2, $p < 0.01$), finding 23 downregulated and 68 upregulated. The most significant upregulated coding genes were *DOCK5, KIT, ALAS2, TFP1, SLC4A1*, and *SLC25A37* and downregulated significantly expressed genes were *PAX5, EBF1, CD22, UBASH3B*, *BANK1, CD79A, ZCCHC7* and *BLK* (Table 3). Nevertheless, none of these genes passed the FDR correction test, but *BLVRB* and *TMOD1* (FC = 4.05 and 2.24; adjusted p value = 0.0077 and 0.048, respectively) did. Gene expression functional enrichment analysis elucidated that these DEGs were mainly involved in the development of hematological diseases ($p = 0032$) and cancer ($p = 2.07E-07$). Apoptosis, B-cell

Table 2. Clinical and molecular characteristics at the time of diagnosis and relapse of Mexican children with ALL who relapsed during the first 18 months of treatment

Patient ID	Gender	Age at diagnosis (months)	WBC at diagnosis ($\times 10^9$/L)	Immuno-pheno-type	BM blast at diagnosis (%)	NCI-risk group	Gene rearrangement detected at diagnosis	Time to relapse occurrence (months)	Site of relapse	BM blast at relapse (%)	WBC at relapse ($\times 10^9$/L)	Follow-up		BMS included in GEA[a]	
												Death	time (months)	BMS at diagnosis	BMS at relapse
1	M	187	62.8	Pre-B Cell	90	HR	ND	8	iBM	100	185.0	yes	9	yes	no
2	M	169	97.6	Pre-B Cell	96	HR	ND	4	iBM	85	685.0	yes	6	yes	no
3	F	173	52.1	Pre-B Cell	20	HR	ND	17	iBM	45	12.5	yes	25	yes	yes
4	M	44	8.9	Pre-B Cell	97	SR	*ETV6-RUNX1*	6	iBM	30	10.5	no	22	yes	yes
5	F	146	11.1	Pre-B Cell	74	HR	ND	17	iBM	26	0.8	no	25	yes	yes
6	M	51	8.2	Pre-B Cell	20	SR	ND	9	iCNS	0	2.6	no	10	no	yes
7	M	156	105.2	Pre-B Cell	98	HR	ND	16	iBM	90	15.8	yes	20	no	yes
8	F	152	6.1	T-Cell	100	HR	ND	15	iBM	25	10.5	no	17	yes	yes
9	M	143	119.4	Pre-B Cell	90	HR	ND	4	iBM	95	125.1	no	25	no	yes
10	M	60	521.8	Pre-B Cell	100	HR	ND	7	iCNS	3	3.2	no	9	no	yes
11	F	168	0.8	Pre-B Cell	100	HR	*ETV6-RUNX1*	14	iBM	76	36.0	yes	14	yes	yes

M, male; F, female; WBC, white blood cell count in peripheral blood; BMS, bone marrow samples; GEA, gene expression analysis; iBM, isolated bone marrow relapse; NCI, National Cancer Institute; SR, standard risk; HR, high risk; ND, non-detected; iCNS, isolated central nervous systems relapse.

[a]Because they approved quality control examination.

Table 3. Top-ranked differentially expressed genes by comparing BMS at the time of diagnosis from patients who eventually relapsed within the first 18 months of treatment and those who did not

Gene symbol	Description	Fold change	*p*	FDR-adjusted *p*
CP	Ceruloplasmin (ferroxidase)	2.2	3.97E-11	0.000003
CXCL12	Chemokine (C-X-C motif) ligand 12	4.44	1.85E-09	0.000021
RAB32	RAB32, member RAS oncogene family	10.16	7.15E-09	0.000051
FABP4	Fatty acid binding protein 4, adipocyte	2.05	1.57E-08	0.000088
MAPKAPK3	Mitogen-activated protein kinase-activated protein kinase 3	2.62	5.68E-08	0.000274
LTBP1	Latent transforming growth factor beta binding protein 1	3.85	1.58E-07	0.000592
VCAM1	Vascular cell adhesion molecule 1	4.43	3.40E-07	0.000958
NFE2	Nuclear factor, erythroid 2	5.44	4.54E-07	0.000958
PRSS57	Protease, serine, 57	8.77	4.77E-07	0.000977
TALDO1	Transaldolase 1	7.25	9.08E-07	0.001368
MIER3	Mesoderm induction early response 1, family member 3	−3.22	9.81E-07	0.00141
CD33	CD33 molecule	3.48	0.000001	0.001822
STON2	Stonin 2	3.19	0.000001	0.001822
TRAPPC8	Trafficking protein particle complex 8	−2.46	0.000002	0.002368
GGTA1P	Glycoprotein, alpha-galactosyltransferase 1 pseudogene	2.18	0.000003	0.003227
KLHL24	Kelch-like family member 24	−3.1	0.000003	0.003034
NID1	Nidogen 1	2.04	0.000003	0.003034
ZNF644	Zinc finger protein 644	−2.97	0.000003	0.003034
BLVRB	Biliverdin reductase B	4.96	0.000005	0.004127
GLB1	Galactosidase beta 1; transmembrane protein with metallophosphoesterase domain	2.25	0.000005	0.004127

GEPs, gene expression profiles; BMS, bone marrow samples.

and T-cell activation, EGF and FGF receptor signaling are the principal pathways in which these genes play a role. *GATA1* showed a low level of differential expression; however, this was one of the master regulator genes. Glutathione biosynthesis was also a highly affected canonical pathway ($p = 0.0122$).

Finding Candidate Biomarkers for VER

A second supervised analysis was performed by comparing BMS at the time of diagnosis, indistinctly if patients relapsed or not ($n = 50$), vs. BMS at relapse ($n = 9$); 970 differential expressed probes between groups (FC >2, $p = 0.01$) were observed of which 743 coding genes were tested. From these, 337 were abnormally expressed genes after FDR correction test was applied (adjusted p value <0.05). Upregulated genes were almost as many as downregulated genes (168 and 169, respectively) (data not shown). *ELANE* ($p = 0.000056$), *PRTN3* ($p = 0.000006$), *CTSG* ($p = 0.000012$), *PAX5* ($p = 0.000483$), *ZCCH7* ($p = 0.000139$) and *EBF1* ($p = 0.000173$) showed the highest FCs (Supplementary Table 2).

Functional analysis exhibited these genes are involved in the hematological system development and function ($p = 0.0023$), lymphoid tissue structure and development ($p = 0.00188$) and in immune cell trafficking ($p = 0.00213$) process. Based on their molecular and cellular function, 204 genes were related with cell death and survival ($p = 0.035$); 104 with cellular development (0.00251); 201 linked to cellular growth and proliferation (0.0024); 147 with cell-to-cell signaling and interaction ($p = 0.0024$) and 119 genes associated with cellular movement (0.0021). Gene expression profile suggested that *CEBPA* ($p = 1.99E-0.09$), *TGM2* ($p = 2.59E-09$), *GATA1* ($p = 5.7E-07$) and *CCL5* ($p = 4.57E-05$) pathways were activated, whereas *SOX11* (1.98E-03) was inhibited. Notably, *PAX5* pathway also was observed as high-enriched gene signaling pathway, consequently to highly abnormal expression of *EBF1* and *BLNK* genes (Figure 1).

The third supervised approach included BMS from patients who relapsed, matching BMS from diagnosis with their corresponding BMS at relapse (five pairs). A total of 145 genes were differentially expressed (FC >2 and $p <$ 0.01) of which 103 were downregulated and 32 upregulated. None of these genes passed the FDR test (adjusted p value >0.05). Nevertheless, 15 upregulated genes and 34 downregulated genes clustered in relapsed samples (Figure 2). GO enrichment analysis showed that these altered genes are involved in metabolic processes ($p = 0.46$) and alternative splicing ($p = 0.0001.3$). Pathway analysis exhibited that most were involved in DNA replication ($p = 0.0473$) and B cell activation ($p = 0.00405$).

Validation of DEGs Associated with VER by Quantitative RT-PCR

To validate some DEGs associated with VER (*BLNK*, *EBF1* and *ZCCHC7*) we used qRT-PCR. These genes were also

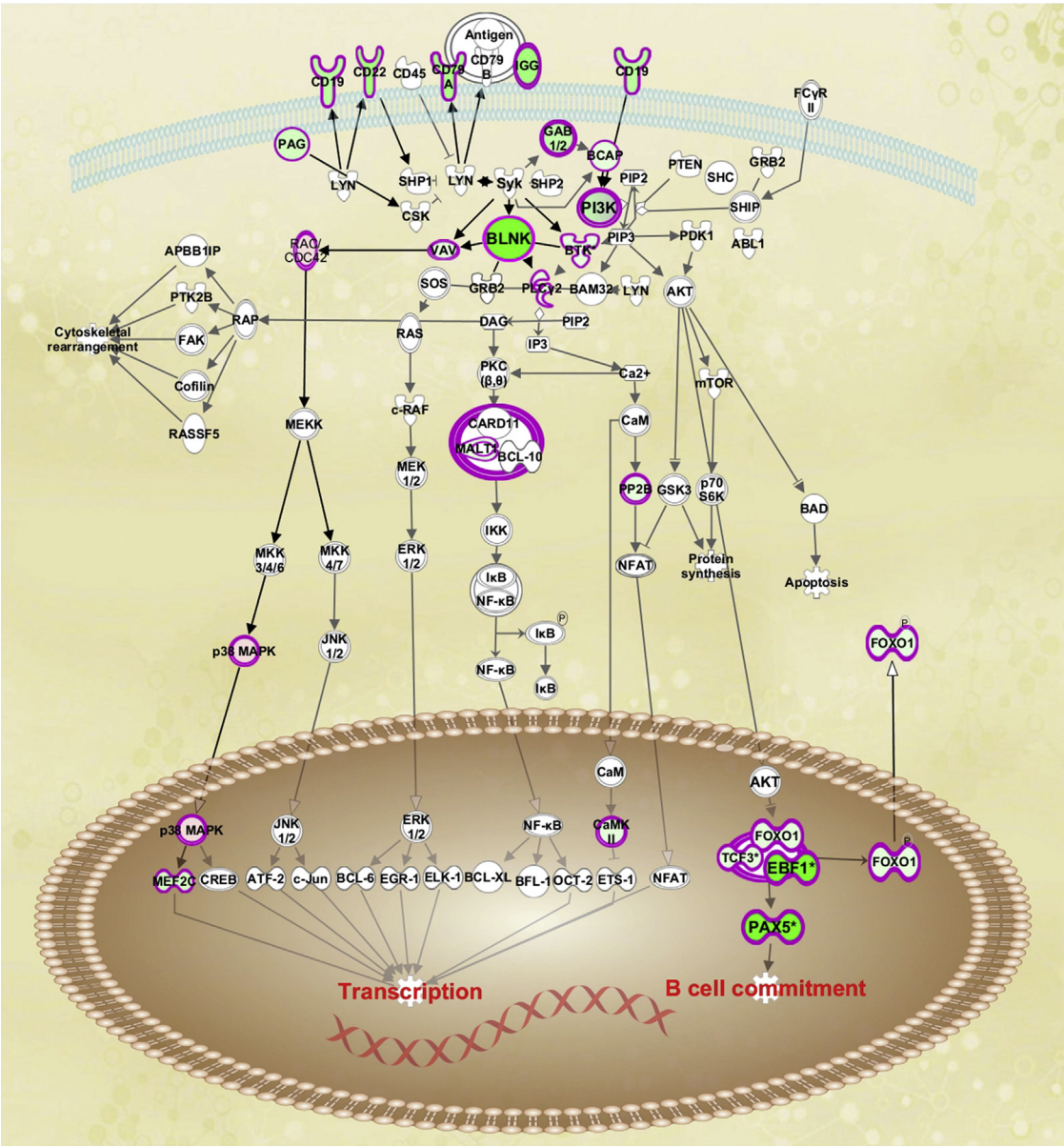

Figure 1. B-cell receptor signaling pathway. *PAX5*, *EBF1* and *BLNK* were among the highest differentially expressed genes in Mexican acute lymphoblastic leukemia children with very early relapse. Green and red colors signify downregulated and upregulated genes, respectively; color intensity reflects the level of gene expression. This figure was generated through the use of QIAGEN's Ingenuity Pathway Analysis.

included in this validation analysis based on previous data reporting abnormal expression in childhood ALL (23). Gene expression directions change for those selected for validation, consistent with results obtained in microarray analysis. *BLNK, EBF1* and *ZCCHC7* expressions were downregulated in patients with VER (Figure 3).

Discussion

To the best of our knowledge, this study is the first reporting results from a transcriptomic analysis in Mexican ALL pediatric patients in order to identify new biomarkers associated with VER.

In developed countries, 5-year event-free survival is ~90% (24,25). In contrast, Mexico has one of the highest mortality rates not only in Latin America but also around the world (3,4) and relapses occur in 26.2% of patients (9).

Currently, in public Mexico City hospitals where ALL pediatric patients are treated, the prognostic stratification and allocation treatment are carried out considering clinical factors such as age at diagnosis, leukocyte count in the peripheral blood, the presence or absence of CNS infiltration, immunophenotype, presence of any of the four

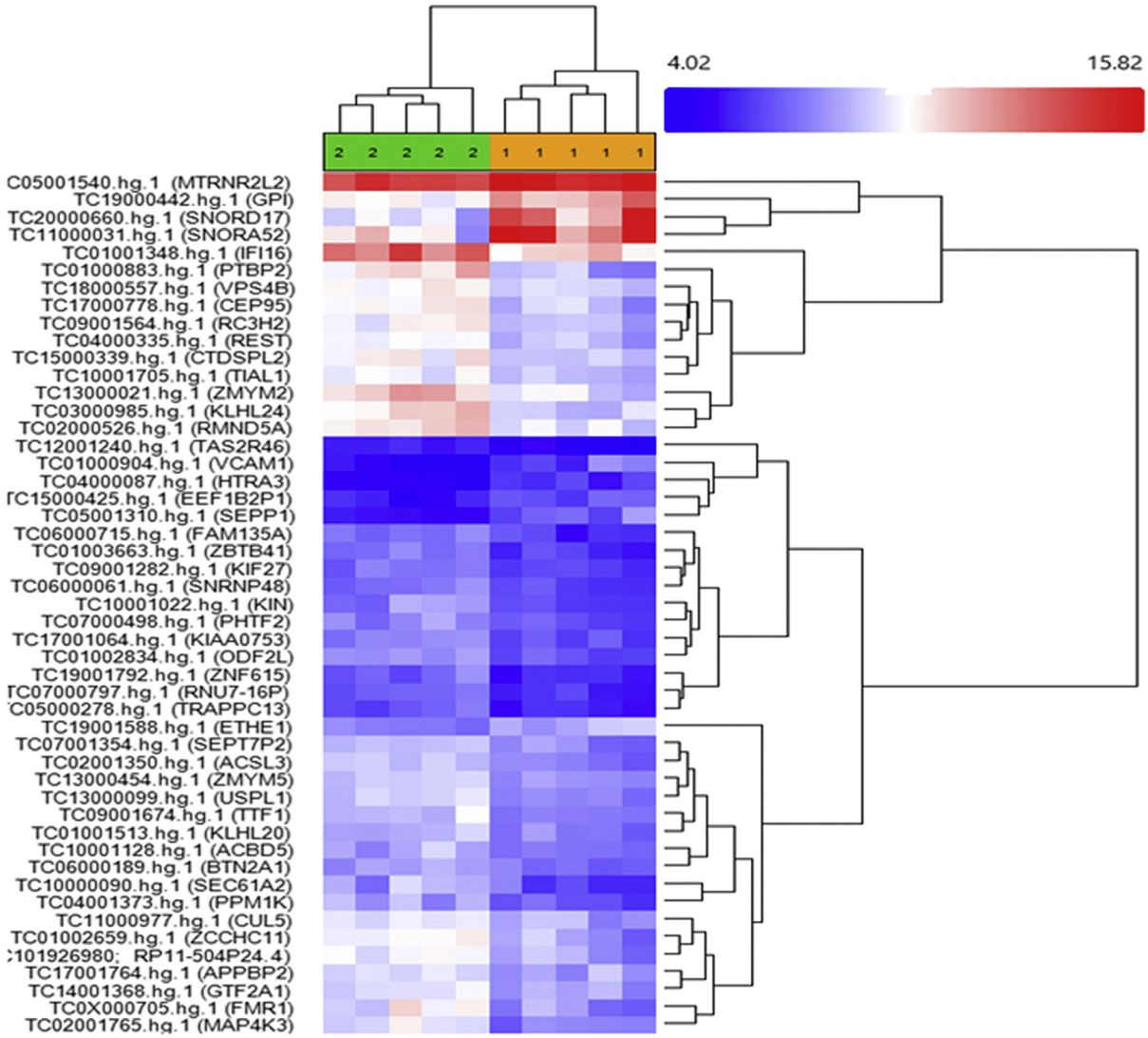

Figure 2. Gene expression profiling of very early relapse ALL patients. Heat map displaying the top 15 upregulated genes and top 34 downregulated genes from patients who relapsed, matching BMS from diagnosis (green color) with their corresponding BMS at relapse (orange color). Blue and red colors indicate downregulated and upregulated genes, respectively; color intensity reflects the level of gene expression.

gene rearrangements most frequently associated with ALL prognosis (*ETV6-RUNX1, TCF3-PBX1, BCR-ABL1* and *MLL-r*) as well as response to prednisone. However, risk stratification seems insufficient to predict which patients will relapse during early stages of treatment because, according to a previous study by our research group (MIGICCL), relapse occurring within the first year after diagnosis is approximately three-fold higher than reported in developed countries in ALL children (13.4 vs. 4.5%, respectively) (26).

It is well known that prognosis of patients who relapse is worse than those who do not. Furthermore, extremely low survival rates are reported in specific subgroups, those relapsing during the first 18 months after diagnosis, those who present iBM and those occurring in NCI high-risk patients (27−30).

In the present study, VER patients died in a significantly higher proportion than patients in the relapse-free group. In addition, five relapsed patients who died presented iBM recurrent disease and had been classified as NCI high-risk patients. Such deaths occurred within the first 8 months after relapse and two died during the first 15 days of reinduction chemotherapy. This highlights once again the need to identify specific biomarkers for our population in order to predict these events and reduce mortality in our children with ALL.

Microarray technology has proven to be a useful approach to identify genes, pathways, and systems involved in leukemogenic processes and also to discover prognosis-related biomarkers (31,32). In the present study we used cDNA microarrays in order to identify gene expression signatures related to a high risk of VER in Mexican ALL

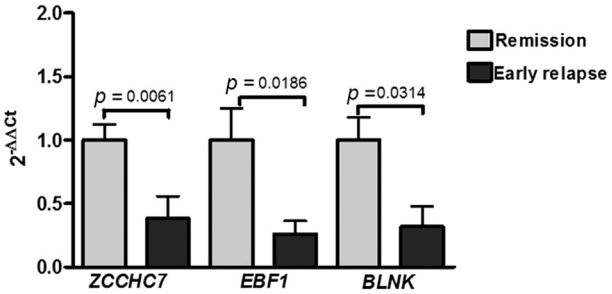

Figure 3. Real-time PCR validation of some DEGs associated with VER (*EBF1*, *BLNK*, and *ZCCH*) in BMS of patients in remission (without relapse) and relapse. mRNA levels were calculated by the 2−ΔΔCt. *HPRT* was used as the reference gene. Graphs are presented as mean (±SD) and differences between groups were evaluated by unpaired *t*-test; *p* value was determined.

pediatric patients. We included in this analysis the most common clinical and molecular subgroups of ALL children, except for MLL rearrangements (*MLL-r*) subgroup.

It has also been reported that GEA, using high-density microarrays, is a powerful tool for diagnosing and classifying ALL subtypes (33). However, we observed an absence of clustering among BM diagnostic samples regarding molecular subgroups. These findings could be explained by heterogeneity due to a small sample size. Moreover, a variety of other risk-related factors such as gender, age, and leukocyte count might have prevented a distinct signature from being identified.

When we looked for a set of genes associated with a high risk of VER we identified 87 DEGs, which are key molecules related to oncogenesis, apoptosis, B-cell activation and angiogenesis. *BLVRB* was one of the two most significantly abnormally expressed genes at diagnosis of BM samples of patients with VER. This is a cell surface membrane receptor, which participates in the heme-group catabolism (34). Loss-of-function mutations result in exaggerated reactive oxygen species accumulation as a putative metabolic signal leading to differential hematopoietic lineage commitment (35). Likewise, abnormal expression of *BLVR* appeared to be important because it has been identified as a major cryoprotectant. Data in leukemia HL60 cells have shown that overexpression of this gene correlates with prednisolone therapy resistance (12,35).

TMOD1 was also significantly overexpressed in this analysis. This gene codifies for an erythrocyte membrane protein and is expressed in differentiated cells. In spite of this, direct evidence that *TMOD1* is a leukemogenic mediator has not been documented (36). However, its role as a relevant participant in ALL relapse could be related with cancer cell proliferation because this gene regulates thin filament lengths (37) and abnormal expression of specific isoforms have been associated with a transformed phenotype (38).

BLNK resulted as one of the main downregulated genes in VER patients (FC = 32.4, adjusted *p* value = 0.05) associated with PAX5 pathway (Figure 1). It is a pivotal adaptor protein in transduction B cell antigen receptor (BCR) signaling in apoptotic cell death processes (39). As a matter of fact, mutations in this gene completely block B-cell development in humans (40). Because *Blnk*-null mutant mice present a reduction of mature B-cells in the peripheral blood and a pre-B cell accumulation in the bone marrow, it has been proposed that deficient expression of *Blnk* has a primary role in the development of B-cell ALL in mice. Additionally, Hayashi et al. reported that 5–10% of *Blnk* knockout developed pre-B-cell ALL at 4–20 weeks of age (41). Although a lost BLNK protein expression has been documented in 50% of the childhood B-lineage ALL cases (42), this gene is not the only responsible gene in human ALL, taking into account that *BLNK* expression is influenced by other relevant genes in leukemia progression such as *EBF1* and *PAX5* (Figure 1) (43). In the present research, both *PAX5* and *EBF1* were among the group with the highest abnormally expressed genes in BMS from children who developed VER. Notably, *EBF1* expression is dependent on *PAX5* and it has been reported that *EBF1* deletions cooperate in leukemogenesis of Down syndrome ALL patients (44).

PAX5 gene is essential in the lymphopoiesis process because it controls the identification and development of B cells by repressing or activating specific genes in early and late stages. *PAX5* is maintained at a remarkably stable level throughout the life of a B-cell (45). Suppression of *PAX5* expression in hematopoietic compartment of mice induces B-ALL and the restauration of its endogenous expression induces normal B-cell differentiation (46). This was the highest downregulated gene in our relapsed patients. Molecular mechanisms by which *PAX5* is underexpressed in our ALL relapsed patients are unknown. In a recent study conducted by Rosales-Rodriguez et al. (2016, in press) in Mexican ALL children, a high frequency (16.67%) of *PAX5* somatic deletions was found. Therefore, we hypothesize that mono-allelic deletions could be an important mechanism for *PAX5* inactivation in our patients. However, further investigation is required to elucidate this. In addition, fusion translocation and point mutations that disrupt *PAX5* DNA binding or transcriptional regulators have also been reported (47–50). It is important to highlight that almost 50% of the *BCR-ABL1* and *BCR-ABL* like ALL patients (a subpopulation with high risk of relapse) carrier somatic mutations in *PAX5* (48,50).

Noteworthy, *PAX5* also regulates *FLT3* gene, whose increased expression results in a pronounced diminution in bone marrow B lymphopoiesis. Loss of *PAX5* enables early lymphoid and myeloid developmental potential to be influenced by other signals and thus the commitment to alternative lineages at the expense of B cells in an *FLT3* mediated way (51), which could explain the abnormal expression of myeloid lineage genes such as myeloperoxidase (*MPO*) gene in our patients. By definition, ALL blasts are negative for MPO; nevertheless, low level MPO positivity without expression of other myeloid markers has been detected in ALL cases, particularly in cases that entail *BCR-ABL1* translocation. Accordingly,

MPO expression has been suggested as an additional biomarker for minimal residual disease monitoring (52).

As expected, we observed that a set of genes were abnormally expressed (*PAX5, EBF1, ZCCCH37* and *BLK*) either at diagnosis or at relapse BMS of children with VER. In addition, statistically significant differences in their expression between diagnosis-relapse BMS were also observed, highlighting the importance for further evaluation of these genes as potential minimal residual disease biomarkers or as therapeutic targets in order to prevent relapses. As a matter of fact, *BLK* gene is a potential therapeutic target of drugs currently used in non-small lung cell cancer treatment, osimertinib, because *in vitro* these have shown the capacity of osimertinib to inhibit the activity of BLK at clinically relevant concentrations (53).

Finally, matched GEA between diagnosis and relapse samples in VER patients revealed a certain genetic profile with a small set of genes which, in the future, could provide additional information about the biological basis of VER in ALL children. We cannot identify a particular mechanism possibly involved in VER development. We do not rule out that relapse phenotype results in the selection of resistant-therapy minor clones present at diagnosis rather than being due to a direct adaptation of the original disease (54,55).

Our study still has limitations. We cannot disregard that several differentially expressed genes were actually derived from contaminating non-leukemic cells, particularly myeloid cells and T-lymphocytes. Therefore, purification of the ALL cells should be implemented to validate all potential biomarkers (54). Furthermore, additional validation on independent samples seems necessary.

The results of this study point to the need for biomarkers at the time of ALL diagnosis to be identified in Mexican children. Nowadays, these children are being misclassified, resulting in a poor outcome. Even though the high costs that routine genetic biomarker-seeking protocols may imply, cost-benefit assessment considering the implications in reducing mortality rates in our country is worthy of being acknowledged.

Therefore, it is likely that gene expression profiling may also be used in prognostic risk stratification schemes of Mexican ALL children. As a matter of fact, our data revealed some genes as potential predictive biomarkers associated with the development of VERs in ALL children.

To conclude, initial evaluation of children with ALL must include every available predictive tool so that better treatment results can be achieved. This is the best investment.

Mexican Inter-institutional Group for the Identification of the Causes of Childhood Leukaemia

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Supplementary Data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.arcmed.2016.12.005.

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