Received: 23 February 2018 | Revised: 10 July 2018 | Accepted: 23 October 2018

DOI: 10.1002/hon.2567

ORIGINAL RESEARCH ARTICLE

WILEY

MicroRNA expression profiles discriminate childhood T- from B-acute lymphoblastic leukemia

Renata Santos Almeida[1] | Matheus Costa e Silva[2] | Luiz Lehmann Coutinho[3] | Renan Garcia Gomes[1] | Francisco Pedrosa[4] | Juliana Doblas Massaro[2] | Eduardo Antônio Donadi[2] | Norma Lucena-Silva[1,4]

[1] Laboratory of Immunogenetics, Department of Immunology, Aggeu Magalhães Institute, Recife, Brazil

[2] Division of Clinical Immunology, Department of Medicine, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil

[3] Animal Biotechnology Laboratory, Animal Science Department, University of São Paulo (USP)/Luiz de Queiroz College of Agriculture (ESALQ), Piracicaba, Brazil

[4] Pediatric Oncology, Instituto de Medicina Integral Professor Fernando Figueira (IMIP) Hospital, Recife, Brazil

Correspondence
Dr Norma Lucena-Silva, Laboratory of Immunogenetics, Department of Immunology, Aggeu Magalhães Institute/Oswaldo Cruz Foundation, Avenida Professor Moraes Rego, s/n, Cidade Universitária, 50.740-465 Recife, Pernambuco, Brazil.
Email: norma.lucena@hotmail.com

Funding information
CNPq, Grant/Award Number: 310364/2015-9; CAPES, Grant/Award Number: PROCAD-177382/2013; FACEPE, Grant/Award Number: APQ-0040-4.00/13 APQ-1044-4.01/15

Abstract

MicroRNAs (miRNAs) play a critical role on biological and cellular processes; the search for functional markers may be of importance for differential diagnosis, prognosis, and development of new therapeutic regimens. In this context, we evaluated the bone marrow miRNA profile of Brazilian children exhibiting T- or B-cell acute lymphoblastic leukemia (T-ALL or B-ALL), using massive parallel sequencing, using the HiSeq 2500 platform (Illumina). The differential expression analysis was conducted considering a leave-one-out approach and FDR $\leq$ 0.05. Machine learning algorithms were applied to search for the disease subset biomarkers. Target prediction, functional enrichment, and classification of biological categories were also performed. Sixteen miRNAs were differentially expressed between T- and B-ALL, of which 10 (miR-708-5p, miR-497-5p, miR-151a-5p, miR-151b, miR-371b-5p, miR-455-5p, miR-195-5p, miR-1266-5p, miR-574-5p, and miR-425-5p) were downregulated and six (miR-450b-5p, miR-450a-5p, miR-542-5p, miR-424-5p, miR-629-5p, and miR-29c-5p) were upregulated in childhood T-ALL. These miRNAs may be used for distinguishing childhood lymphoblastic leukemia subtypes, since it provided the clear separation of patients in these two distinct groups. Six relevant biological pathways were identified according to their role in leukemia, namely, viral carcinogenesis, cell cycle, and B-cell receptor signaling pathways for induced miRNAs and TGF-beta signaling, apoptosis, and NF-kappa B signaling for the repressed miRNAs, of which several miRNA gene targets participate in cell differentiation and hematopoiesis processes. Machine learning analysis pointed out miR-29c-5p expression as the best discriminator between childhood T- and B-ALL, which is involved in calcium signaling, critical for B-cell lymphocyte fate. Further studies are needed to assure the role of the 16 miRNAs and miR-29c-5p on acute lymphoblastic leukemia subtypes and on disease prognosis.

KEYWORDS

acute lymphoblastic leukemia, B-ALL, biomarker, microRNAs, miRNome sequencing, T-ALL

1 | INTRODUCTION

MicroRNAs (miRNAs) play a critical role on biological and cellular processes, starting from the development of an organism until the development of diseases of diverse origins, including malignant transformation.[1] The expression profile of miRNAs has been extremely useful for the classification of hematological malignancies, since sets of miRNAs have been associated with genetic subtypes defined by

chromosomal translocations, ploidy of blasts, or both.[2-4] Specific miRNAs have also been identified as potential biomarkers of disease severity and disease prognosis, such as miR-155, whose expression has been associated with complete response in patients with chronic lymphocytic leukemia.[5] Besides the availability of immunological and genetic markers, the search for functional markers may be of importance for differential diagnosis, prognosis, and development of new therapeutic regimen. In this context, the miRNA profile of children exhibiting T- or B-cell acute lymphoblastic leukemia (T-ALL or B-ALL) was determined using massive parallel sequencing, which may disclose previously described and novel sets of miRNAs.

2 | METHODS

2.1 | Patients

We studied eight children (aged 4-16 y; seven boys and one girl) exhibiting T-ALL (one early T-cell precursor-ALL, four pre-T-ALL, one cortical T-ALL, and two mature T-cell) and eight patients (aged 3-17 y; seven boys and one girl) presenting B-ALL (five pre-pre-B-ALL and three pre-B-ALL) referred to and treated at the Pediatric Oncology Service of the Instituto de Medicina Integral Professor Fernando Figueira (IMIP) Hospital (Recife, Brazil). The study protocol was approved by the local Oswaldo Cruz Foundation Ethics Committee (Protocol#13296913.3.0000.5190), and informed consent was obtained from children guardians prior to sample collection.

2.2 | Identification and characterization of T- and B-cell leukemia

ALL diagnosis was performed evaluating bone marrow or peripheral blood blasts that were morphologically classified according to the French-American-British cooperative group.[6] Samples were submitted to cell separation using Ficoll-Paque gradient (GE Healthcare, Little Chalfont, UK), and the isolated mononuclear cell fraction was used for immunophenotyping analysis. T-cell lineage leukemia was confirmed by labeling mononuclear cells with fluorochrome-conjugated antibodies targeted to cell surface and cytoplasm antigens, including the lineage-specific markers CD3, CD7, CD5, CD2, CD4, CD8, CD1a, and CD99 and the immaturity markers terminal deoxynucleotidyl transferase (TdT), CD34, and HLA-DR (Becton Dickinson Biosciences, San Jose, California). B-cell lineage leukemia was determined by labeling blast cells with the following markers: CD10, CD34, CD19, CD22, CD79a, kappa and lambda light chains, and IgM (cytoplasmic and surface) (BD Biosciences). Fluorescence signals were acquired using FACS Calibur flow cytometer (BD Biosciences) and analyzed with BD CellQuest ProSoftware (BD Biosciences). The presence of each of these antigens was positive when expressed in 25% or more cells, as described previously.[7]

Patient bone marrow cells were also tested to detect the genetic errors, encompassing the *HOX11L2* and *SIL/TAL* alterations,[8] common in T-cell leukemia, and the t(1;19), t(4;11), t(9;22), and t(12;21) genetic alterations, described for B-lineage leukemia. These genetic alterations were determined according to prior study.[7]

2.3 | RNA extraction, quantitation, and integrity assessment

Ficoll-Paque (GE Healthcare, USA) isolated bone marrow or peripheral blood (used only in quantitative real-time PCR) cells from untreated patients were submitted to total RNA extraction using the TRIzol reagent (Invitrogen, Carlsbad, California), according to manufacturer's instructions. All RNA samples were verified for purity using Nanodrop spectrophotometer (Thermo Scientific, Waltham, Massachusetts), choosing only RNAs without protein contamination. RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, California), considering only RNA samples with RIN $\geq$ 8.0. Prior to library construction, quantitation of all samples was performed using Qubit fluorometer (Invitrogen) and Qubit RNA BR Assay Kit (Invitrogen).

2.4 | miRNA sequencing and data analysis

miRNA library construction was performed starting from 1 μg of each RNA sample using the TruSeq Small RNA Library Prep kit (Illumina, San Diego, California), following manufacturer's instructions. After library validation, quantitative PCR was conducted using KAPA Library Quantification kit (KAPA Biosystems, Foster City, California) for further dilution of library pools. HiSeq Rapid Cluster Kit v2 (Illumina) was used on HiSeq 2500 sequencing system (Illumina) for miRNA transcriptome sequencing. We performed sequencing runs of 36 cycles in a single-end read mode.

2.5 | Bioinformatic tools for target prediction, functional enrichment analysis, and biomarker identification

Data from miRNA sequencing were analyzed for reads quality and contamination using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Cutadapt[9] softwares, respectively. Only sequences exhibiting *Q* score $\geq$ 30 and length $\geq$ 17 nucleotides were examined.[10] Bowtie (http://bowtie-bio.sourceforge.net/index.shtml) was used for human reference genome indexing (hg38), available at UCSC Genome Browser (https://genome.ucsc.edu/), applying the miRDeep2 2.0.0.8 program[11] for sequence alignment. miRNA identification was performed by miRDeep2 using data from miRBase release 21 (http://www.mirbase.org/).[12,13] A standard differential expression analysis (DEA) was carried out to compare B-ALL versus T-ALL group. This analysis was performed using edgeR[14] package in the R environment (https://cran.r-project.org/), considering upper quantile normalization and the presence of at least three reads counts in a minimum of six analyzed samples, a cutoff point of 0.05 for false discovery rate (FDR), and 1 or −1 for logfold change (logFC). In addition, another strategy was applied based on a leave-one-out approach, usually employed for data mining validation procedures. All sets of differentially expressed miRNAs detected by this

approach were merged into a file and used to find intersections between comparisons using R environment. To confirm the data obtained above, machine learning algorithms were applied using the WEKA 3.8 software (http://www.cs.waikato.ac.nz/ml/weka/) and the random forest algorithm. miRNA target prediction was performed using 11 prediction tools integrated into the miRWalk 2.0 database (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/). Only targets predicted by at least three algorithms or those validated by miRTarBase 7.0 (http://mirtarbase.mbc.nctu.edu.tw/) database were kept for analyses. DAVID v.6.8 (https://david.ncifcrf.gov/) and KEGG pathway enrichment were used for functional annotation of target genes, considering significant P values ≤ 0.05 after Benjamini correction. Graphs with most significant KEGG pathways were constructed using GraphPad Prism v.5.01 (GraphPad Software, Inc). Venn diagram analyses with enriched KEGG pathways were also performed using Venny v.2.1 software (http://bioinfogp.cnb.csic.es/tools/venny/index.html) to identify exclusively pathways related to targets of downregulated and upregulated miRNAs in T-ALL. The GO-Slim biological process, included in the PANTHER classification system v.13.0 (http://www.pantherdb.org/), was applied for sets of genes belonging to selected biological pathways. The Cancer Gene Census, included in the COSMIC v.83 database[15] (http://cancer.sanger.ac.uk/cosmic/), was also used for identification of miRNA gene targets associated with genetic alterations in cancer/hematological malignancies.

2.6 | Validation by quantitative real-time PCR

For confirmation of miRNA-Seq data, the differentially expressed miRNA with the best result for discrimination of disease subtype according to machine learning approach was used in quantitative real-time PCR (qRT-PCR) experiments. In total, 20 pediatric B-ALL (pre-pre-B-ALL, n = 10; pre-B-ALL, n = 10) and 19 childhood T-ALL (ETP, n = 2; pre-T, n = 4; cortical T, n = 7; mature T, n = 6) samples were considered, all of which were derived from bone marrow (BM). Additionally, six T-ALL samples (one pre-T, two cortical T, and three mature T) derived from peripheral blood were used for comparison with BM group. Reverse transcription reactions were performed with 10 ng of RNA in a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, California), and TaqMan miRNA assays (rno-miR-29c* assay; Life Technologies, Foster City, California) were used for qRT-PCR experiments considering technical duplicates. RT-PCR reactions were performed in a QuantStudio 5 Real-Time System (Applied Biosystems) according to manufacturer's instructions.

For miRNA relative quantification, the small noncoding RNA U6 was used as endogenous control. miRNA expression was estimated based on delta cycle threshold (Ct) ($Ct_{miRNA\ (target)} - Ct_{U6\ RNA\ (control)}$)[16] and Ct values. Since Ct and delta Ct are inversely proportional to miRNA expression level, lower Ct and delta Ct values were associated with higher miRNA expression. Differences in expression between T- and B-ALL were evaluated by Mann-Whitney U and Kruskal-Wallis tests in GraphPad Prism v.5.01 (GraphPad Software, Inc). P value- 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Acute lymphoblastic leukemia patient's characteristics

T-ALL patients exhibited whole blood leukocyte counts between 11 000 and 423 000 (median = 135 000) cells/mm^3, and none of the eight patients presented the *HOX11L2* and *SIL/TAL* genetic alterations. Only one of the eight (12.5%) T-ALL patients died. In B-ALL group, whole blood leukocyte counts ranged from 2140 to 158 640 (median = 14 510) cells/mm^3. Regarding genetic errors, five B-ALL patients (62.5%) did not present any of the alterations studied, two (25%) exhibited t(1;19), and one (12.5%) showed t(12;21). Two of the eight (25%) B-ALL patients died.

3.2 | miRNA-Seq expression profiles in childhood acute lymphoblastic leukemia

Bioinformatic analysis identified 1642 miRNAs, of which 1440 were nonredundant mature miRNAs. After reads count screening, 331 miRNAs were retained based on different precursor, of which were 33 differentially expressed miRNAs exhibiting FDR ≤ 0.05. The leave-one-out approach revealed 16 differentially expressed miRNAs, potentially predictors of disease subtype, of which 10 were downregulated and six were upregulated in childhood T-ALL compared with B-ALL (Table 1). The dendrogram and the heatmap shown in Figure 1 display the hierarchical clustering of T-ALL and B-ALL samples, based on the 16 differentially expressed miRNAs.

TABLE 1 miRNAs differentially expressed between childhood B-ALL and T-ALL revealed as possible biomarker of disease subtype

miRNA	LogFC	*P* Value	FDR
Downregulated in T-ALL			
hsa-miR-708-5p	−8.2108	0.0000	0.0000
hsa-miR-497-5p	−5.7028	0.0000	0.0000
hsa-miR-151a-5p	−5.3482	0.0000	0.0000
hsa-miR-151b	−5.3477	0.0000	0.0000
hsa-miR-371b-5p	−4.8908	0.0000	0.0000
hsa-miR-455-5p	−4.3943	0.0000	0.0010
hsa-miR-195-5p	−4.2802	0.0000	0.0000
hsa-miR-1266-5p	−4.0427	0.0000	0.0000
hsa-miR-574-5p	−2.8515	0.0000	0.0010
hsa-miR-425-5p	−1.6899	0.0002	0.0051
Upregulated in T-ALL			
hsa-miR-450b-5p	3.6456	0.0000	0.0011
hsa-miR-450a-5p	3.6078	0.0000	0.0011
hsa-miR-542-5p	3.4120	0.0000	0.0011
hsa-miR-424-5p	2.9686	0.0008	0.0138
hsa-miR-629-5p	2.1316	0.0003	0.0052
hsa-miR-29c-5p	1.8817	0.0003	0.0051

Abbreviations: LogFC, fold change in log (base 2); FDR, false discovery rate < 0.01.

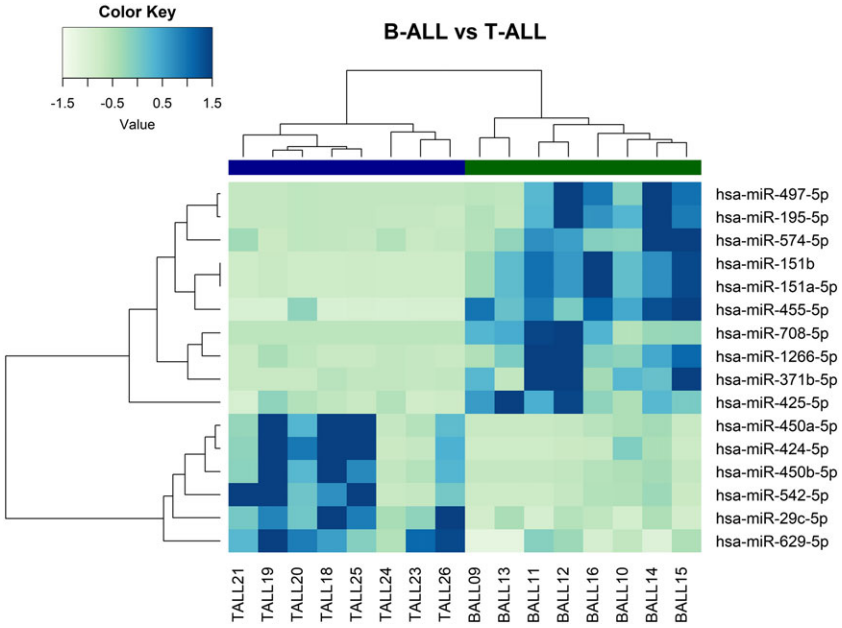

FIGURE 1 Heatmap of 16 differentially expressed miRNAs in childhood T-ALL compared with B-ALL revealed by leave-one-out analysis. In blue, miRNAs with positive regulation; in green, miRNAs with negative regulation

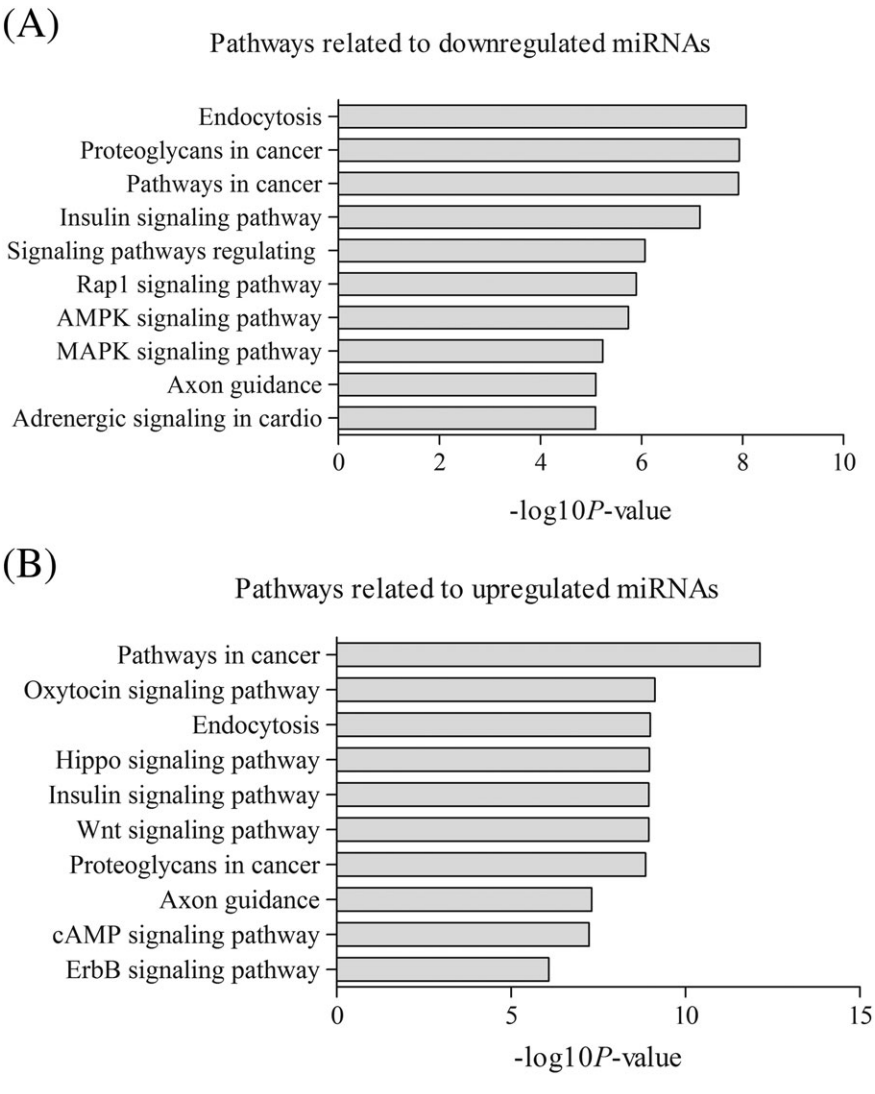

FIGURE 2 Most significant KEGG pathways related to targets of 16 differentially expressed miRNAs in T-ALL. Pathways related to A, downregulated and B, upregulated miRNAs in T-ALL

3.3 | Target prediction and enrichment of functional categories for selected miRNAs

Target genes obtained by in silico prediction using the miRWalk and miRTarBase databases were submitted to enrichment analyses of biological pathways. Eighteen significant pathways were associated to genes targeted by overexpressed miRNAs in T-ALL, whereas 93 pathways were shared by targets for overexpressed and underexpressed miRNAs and 13 were unique pathways for underexpressed miRNAs. The most significant pathways of each analysis are illustrated in Figure 2. Among the unique pathways in each group, some were selected according to their well-known role in leukemia, namely, viral carcinogenesis, cell cycle, and B-cell receptor signaling pathways for induced miRNAs and TGF-beta signaling, apoptosis, and NF-kappa B signaling for the repressed miRNAs. The amount of shared and specific gene targeted by the differentially expressed miRNAs is illustrated in Venn diagrams shown in Figure 3.

The six major selected pathways (three associated with induced and three with repressed miRNAs in T-ALL) encompassed 45 genes that were shared by induced and repressed miRNAs; 210 genes were only targeted by overexpressed miRNAs, and 143 genes were only targeted by underexpressed miRNAs. The sets of genes exclusively associated with induced or repressed miRNAs were classified by the PANTHER database yielding several biological categories, of whom we selected cell differentiation (GO: 0030154) and hemopoiesis (GO: 0030097) processes that were related to leukemia pathogenesis.

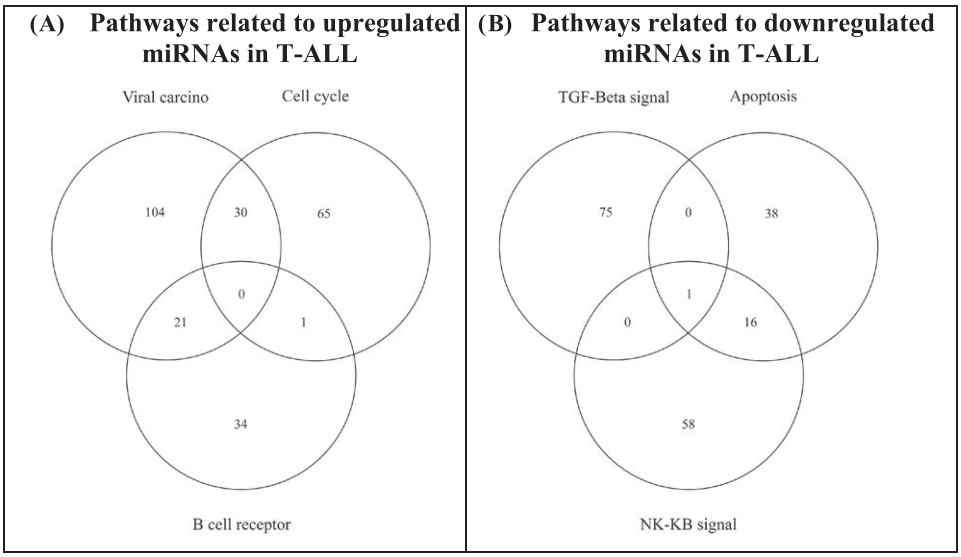

FIGURE 3 Venn diagram analysis of KEGG pathways related to leukemia and their genes targeted by different modulated miRNAs in T-ALL. Pathways related to A, upregulated and B, downregulated miRNAs in T-ALL. Viral carcino refers to viral carcinogenesis pathway; B-cell receptor, B-cell receptor signaling pathway, and NF-KB signal, NF-KB signaling pathway

TABLE 2 Targets of miRNAs differentially expressed between childhood B-ALL and T-ALL involved in cell differentiation and hemopoiesis

	Gene Name	Panther Protein Class
Targets of upregulated miRNAs in T-ALL		
ABL1	Tyrosine-protein kinase ABL1	Nonreceptor tyrosine protein kinase
GRB2	Growth factor receptor-bound protein 2	Nonreceptor tyrosine protein kinase
IL6ST	Interleukin-6 receptor subunit beta	Cytokine; defense/immunity protein
JAK1	Tyrosine-protein kinase JAK1	Nonreceptor tyrosine protein kinase
JAK3	Tyrosine-protein kinase JAK3	Nonreceptor tyrosine protein kinase
JUN	Transcription factor AP-1	Nucleic acid binding; transcription factor
LYN	Tyrosine-protein kinase Lyn	Nonreceptor tyrosine protein kinase
SRC	Proto-oncogene tyrosine-protein kinase Src	Nonreceptor tyrosine protein kinase
Targets of downregulated miRNAs in T-ALL		
BMP2	Bone morphogenetic protein 2	Growth factor
BMP4	Bone morphogenetic protein 4	Growth factor
BMP5	Bone morphogenetic protein 5	Growth factor
BMP6	Bone morphogenetic protein 6	Growth factor

(Continues)

TABLE 2 (Continued)

	Gene Name	Panther Protein Class
BMP7	Bone morphogenetic protein 7	Growth factor
BMP8A	Bone morphogenetic protein 8A	Growth factor
BMP8B	Bone morphogenetic protein 8B	Growth factor
CD40LG	CD40 ligand	Tumor necrosis factor family member
CXCL12	Stromal cell-derived factor 1	
FASLG	Tumor necrosis factor ligand superfamily member 6	Tumor necrosis factor family member
GDF5	Growth/differentiation factor 5	Growth factor
GDF6	Growth/differentiation factor 6	Growth factor
IL3RA	Interleukin-3 receptor subunit alpha	Cytokine; defense/immunity protein
INHBA	Inhibin beta A chain	Growth factor
INHBB	Inhibin beta B chain	Growth factor
INHBC	Inhibin beta C chain	Growth factor
INHBE	Inhibin beta E chain	Growth factor
LCK	Tyrosine-protein kinase Lck	Nonreceptor tyrosine protein kinase
LEFTY1	Left-right determination factor 1	Growth factor
LEFTY2	Left-right determination factor 2	Growth factor
LTB	Lymphotoxin-beta	Tumor necrosis factor family member
NGF	Beta-nerve growth factor	Neurotrophic factor
NODAL	Nodal homolog	Growth factor
TNFSF10	Tumor necrosis factor ligand superfamily member 10	Tumor necrosis factor family member
TNFSF11	Tumor necrosis factor ligand superfamily member 11	Tumor necrosis factor family member
TNFSF14	Tumor necrosis factor ligand superfamily member 14	Tumor necrosis factor family member
ZAP70	Tyrosine-protein kinase ZAP-70	Nonreceptor tyrosine protein kinase

This approach revealed that eight out of 210 genes and 27 out of 143 genes were targeted by induced or repressed miRNAs, respectively, in T-ALL. The nonreceptor tyrosine kinase was the major class of proteins modulated by the upregulated miRNAs, and growth factors and the family of tumor necrosis factors were the major actors for repressed miRNAs (Table 2).

3.4 | Machine learning and miRNA expression validation

The machine learning analysis revealed that, among the 16 differentially expressed miRNAs observed in the study, hsa-miR-29c-5p was the best predictor for leukemia subtypes, exhibiting an accuracy of 95% and a ROC area of 0.953. This miRNA participates in four enriched pathways, namely, calcium signaling pathway, insulin resistance, dopaminergic synapse, and transcriptional misregulation in cancer. miR-29c-5p targeted 19 genes of the calcium signaling pathway, three of them were related to some type of cancer, and from the 17 targeted genes related to the transcriptional misregulation in cancer, nine were previously associated with cancer according to Cancer Gene Census, particularly, with hematological malignancies (multiple myeloma; leukemia). These results are demonstrated in Table 3.

Given the machine learning results, hsa-miR-29c-5p was selected for expression validation by qRT-PCR. We confirmed miRNA-Seq data and bioinformatic analysis and showed that miR-29c-5p expression is higher in T-ALL than in B-ALL (relative quantification, $P = 0.0002$, Figure 4A; comparison between Cts, $P = 0.0010$, Figure 4B). We also observed that miRNA expression did not differed between pre-pre-B- and pre-B-ALL ($P = 0.6842$, Figure 4C) or between T-ALL subtypes ($P = 0.4513$, Figure 4D). We showed no differences between less and more mature subtypes (ETP and pre-T-ALL versus cortical and mature T-ALL, $P = 0.6295$, Figure 4E) and also did not observe difference on the hsa-miR-29c-5p expression in bone marrow compared with peripheral blood samples ($P = 0.1713$, Figure 4F). These findings confirmed that the miR-29c-5p expression predicts ALL cell lineage, but it is not associated with a specific subtype within the cell lineage, nor maturation stage.

4 | DISCUSSION

Considering that (1) bone marrow is the focus of cellular and immunological dysregulation in leukemias, (2) miRNAs are very informative molecules used for the differentiation of human tumors,[17] including childhood acute leukemias,[2,3,17,18] and (3) the mononuclear component of each ALL subtype exhibits a large percentage of blasts (>70% of transformed cells), we compared the expression profiles of bone marrow mononuclear cell miRNAs obtained from patients with T-ALL and B-ALL, before the beginning of the specific chemotherapy.

Among the 33 differentially expressed miRNAs obtained from the comparison of the childhood T- and B-ALL cells, 16 modulated miRNAs (10 downregulated and six upregulated) in T-ALL may be used for distinguishing childhood lymphoblastic leukemia subtypes, since

TABLE 3 Targets of hsa-miR-29c-5p that belong to two selected pathways and are associated with cancer[a]

	Gene Name	Panther Protein Class	Cancer
hsa05202: transcriptional misregulation in cancer			
AFF1	AF4/FMR2 family member 1	Transcription factor	**Acute leukemia**
CSF1R	Macrophage colony-stimulating factor 1 receptor		**Myelodysplastic syndrome**; **chronic myeloid leukemia**; **acute myeloid leukemia**; hemangioblastoma; clear cell renal cell carcinoma
ELK4	ETS domain-containing protein Elk-4	Nucleic acid binding; signaling molecule; winged helix/forkhead transcription factor	Prostate
FLI1	Friend leukemia integration 1 transcription factor	Nucleic acid binding; signaling molecule; winged helix/forkhead transcription factor	Ewing sarcoma
KDM6A	Lysine-specific demethylase 6A	Transcription factor	Renal cell carcinoma; bladder carcinoma; esophageal squamous cell carcinoma; **multiple myeloma**; medulloblastoma; **T-ALL**; other tumor types
KMT2A	Histone-lysine N-methyltransferase 2A		**Acute myeloid leukemia; acute lymphocytic leukemia**
MAX	Myc associated factor X	Basic helix-loop-helix transcription factor; nucleic acid binding	Pheochromocytoma; endometrioid carcinoma; colon carcinoma
MYCN	N-myc proto-oncogene protein	Basic helix-loop-helix transcription factor; nucleic acid binding	Neuroblastoma
RUNX1T1	Protein CBFA2T1	Transcription cofactor	**Acute myeloid leukemia**
hsa04020: calcium signaling pathway			
ATP2B3	ATPase; Ca++ transporting; plasma membrane 3	Cation transporter; hydrolase; ion channel	Adrenal aldosterone producing adenoma
CACNA1D	Calcium channel; voltage-dependent; L type; alpha 1D subunit		Adrenal aldosterone producing adenoma
GRIN2A	Glutamate receptor; ionotropic; N-methyl D-aspartate 2A		Melanoma; colorectal carcinoma; gastric carcinoma; lung carcinoma

[a]Hematological cancers are marked in bold.

the hierarchical clustering of these miRNAs clearly separated patients in two distinct groups, as shown in Figure 1. The miR-151a-5p and miR-708-5p were modulated in childhood acute lymphoblastic leukemia exhibiting several genetic subtypes of B-ALL and T-ALL when compared with nonleukemic $CD34^+$ bone marrow cells, as detected by real-time quantitative PCR.[2] In addition, the expression of miR-708-5p, as evaluated by single miRNA expression, was also downregulated in T-ALL bone marrow compared with B-ALL bone marrow in Dutch children[3] and in bone marrow of Brazilian children with T-ALL when compared with B-ALL and to normal bone marrow.[19] miR-195-5p is reported to be upregulated in peripheral blood of adults with B-cell lymphocytic leukemia when compared with nonleukemic $CD19^+$ cells.[20] miR-497-5p has been associated to a higher overall survival in patients with diffuse large B-cell lymphoma, and it is related to patient's sensitivity to chemotherapy.[21] Considering the set of miRNAs that were downregulated in T-ALL as compared with B-ALL and considering that the canonical miRNA-mRNA interaction preconizes that genes targeted by downregulated miRNAs have high protein expression and increased activity of the miRNA-related pathways, as a corollary, neoplastic B-cell lineages may need the induction of these miRNAs for B-cell differentiation or to escape from the development of malignant T-cell lineages.

Pathways related to the downregulated miRNAs were primarily related to TGF-beta and NF-κB signaling pathways and to apoptosis. NF-κB signaling is a well-known pathway involved in T-ALL leukemogenesis due to pathway constitutive activation as well as through its antiapoptotic role.[22,23] TGF-beta signaling pathway is an important network involved on the control of hematopoiesis with a suppressing role in the growth of immune cells including T-cells, and positive effects on T-cell survival.[24-26] Its upregulation in T-ALL may be related to intracellular messages to proliferation, contributing to exacerbated growth of T-cells without normal apoptosis, processes that may be more pronounced in T-ALL. Regarding the upregulation prediction of apoptosis-related genes in T-ALL, it is possible that the higher cellular density observed in T-ALL compared with B-ALL is contributing to this pathway activation. Recently, Rozovski et al[27] demonstrated that cells from chronic lymphocytic leukemia (CLL) patients with high cell counts exhibited higher apoptosis rates than those cells from patients with low cell counts. In our previous study, we observed a correlation between high blood cell counts in T-ALL and increased soluble HLA-G levels, an immunologic factor induced by hypoxia,[28] and high cellular density has been shown to be associated with hypoxic environment.[29,30]

Many genes that present a relevant role in cell differentiation and hemopoiesis are ligands of TGF-beta superfamily or intracellular molecules of NF-κB signaling and seem to be overexpressed in T-ALL, possibly due to its posttranscriptional regulation by miRNAs. TGF-beta and NF-κB signaling pathways have already been reported to be

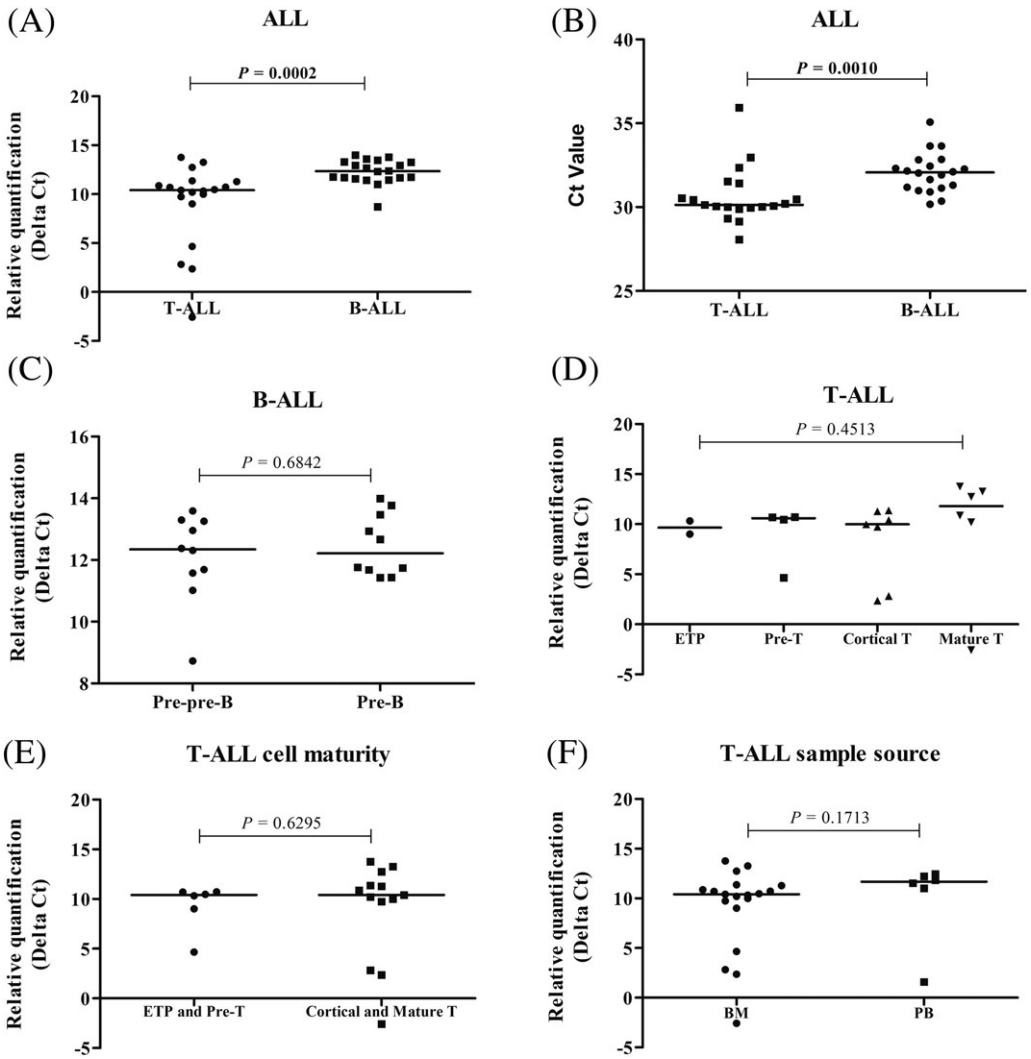

FIGURE 4 Quantitative real-time PCR confirmation of miR-29c-5p differential expression between childhood T- and B-ALL according to miRNA-Seq analysis. A and B, Differences according to delta Ct and Ct values, respectively. C and D, Comparison of miR-29c-5p relative expression between different subtypes of B- and T-ALL. E and F, Evaluation of miRNA expression according to T-ALL cell maturity and sample source. For both Ct and delta Ct, the higher the values, the lower the miRNA expression. A-C, E, and F, Mann-Whitney *U* test; D, Kruskal-Wallis test. ETP, early T-cell precursor leukemia; BM, bone marrow; PB, peripheral blood

dysregulated in hematological malignancies, including myelodysplastic syndromes (MDSs),[31,32] chronic myeloid leukemia,[33] acute lymphoblastic leukemia,[34] and T-cell leukemia.[35-37]

The genes targeted by upregulated miRNAs in T-ALL are primarily related to viral carcinogenesis, cell cycle, and B-cell receptor. Several lines of evidence support the role of virus infections on the pathogenesis of T-ALL, particularly in association with adult T-cell leukemia.[38,39] On the other hand, the most known evidence for previous Epstein-Barr virus infection is associated with the childhood B-ALL.[40] Cell cycle pathways are deregulated in both lymphocytic leukemias, but in this study, the more prominent posttranscriptional deregulation appeared to be in T-ALL. The induction of miRNAs related to B-cell receptor signaling in T-ALL is an interesting finding. According to the canonical miRNA-mRNA interaction, the upregulation of these post-transcriptional elements in T-ALL may promote the downregulation of important genes responsible for B-cell development. Indeed, several signal transducers of B-cell receptor have been associated with survival and proliferation of malignant cells in chronic lymphocytic leukemia.[41-44]

Interestingly, among all the differentially expressed miRNAs observed in this study, the most prominent putative biomarker of disease subtype was miR-29c-5p, which was a better discriminator than hsa-miR-708-5p, a reported miRNA with differential expression between childhood T- and B-ALL.[19,45] Enrichment analysis shows that miR-29c-5p is involved on transcriptional dysregulation in cancer and in pathways related to leukemia development, including T-ALL. Another important pathway, calcium signaling, may be upregulated in B-ALL due to downregulation of miR-29c-5p expression, which has a critical role in B-cell lymphocyte fate and defective signaling in chronic lymphocytic leukemia signaling.[46] miR-29c-5p was upregulated in T-ALL and does not have a defined role in leukemias; however, another member of the miR-29 family, miR-29c-3p, is modulated in many hematological malignancies, including acute and chronic leukemias,[47,48] and lymphomas.[49,50] On the other hand, miR-29c-5p has

been modulated in solid tumors, like gallbladder carcinoma[51] and breast cancer,[52] and its downregulation is associated with metastasis and cell proliferation.[51] Besides genetic abnormalities observed in B- and T-cell leukemias, distinct posttranscriptional regulation of leukemogenic genes may be present yielding different molecular mechanisms of growth and malignant transformation. Further and larger studies are needed to assure the role of miR-29c-5p on acute lymphoblastic leukemia subtype and to clarify whether its expression is related to a favorable leukemia prognosis, as reported in malignant pleural mesothelioma[53] and gallbladder carcinoma.[51]

5 | CONCLUSION

We report a set of 16 differentially expressed miRNAs that may distinguish childhood lymphoblastic leukemia subtypes in terms of posttranscriptional functional analysis and validate a novel leukemogenic marker, miR-29c-5p, that discriminates T-ALL from B-ALL.

ACKNOWLEDGEMENTS

The authors thank the financial support from Brazilian agencies FACEPE (APQ-0040-4.00/13 and APQ-1044-4.01/15) and CAPES (PROCAD-177382/2013 grant). N.L.-S. is the recipient of a fellowship from CNPq (CNPq 310364/2015-9) and R.S.A. from CAPES (PROCAD-177382/2013).

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ORCID

Norma Lucena-Silva https://orcid.org/0000-0002-6140-1794

REFERENCES

1. Peng Y, Croce CM. The role of microRNAs in human cancer. *Signal Transduct Target Ther*. 2016;1(1):15004. https://doi.org/10.1038/sigtrans.2015.4.
2. Schotte D, Chau JCK, Sylvester G, et al. Identification of new microRNA genes and aberrant microRNA profiles in childhood acute lymphoblastic leukemia. *Leukemia*. 2009;23(2):313-322. https://doi.org/10.1038/leu.2008.286
3. Schotte D, Moqadam FA, Chen C, Van Ijcken WFJ, Pieters R, Den Boer ML. Discovery of new microRNAs by small RNAome deep sequencing in childhood acute lymphoblastic leukemia. *Leukemia*. 2011;25(9):1389-1399. https://doi.org/10.1038/leu.2011.105
4. Wallaert A, Van Loocke W, Hernandez L, Taghon T, Speleman F, Van Vlierberghe P. Comprehensive miRNA expression profiling in human T-cell acute lymphoblastic leukemia by small RNA-sequencing. *Sci Rep*. 2017;7(1):7901. https://doi.org/10.1038/s41598-017-08148-x.
5. Ferrajoli A, Shanafelt TD, Ivan C, et al. Prognostic value of miR-155 in individuals with monoclonal B-cell lymphocytosis and patients with B chronic lymphocytic leukemia. *Blood*. 2013;122(11):1891-1899. https://doi.org/10.1182/blood-2013-01-478222
6. Bennett JM, Catovsky D, Daniel MT, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann Intern Med*. 1985;103(4):620-625. https://doi.org/10.7326/0003-4819-103-4-620.
7. Marques EALV, Neves L, Fonseca TC, Lins MM, Pedrosa F, Lucena-Silva N. Molecular findings in childhood leukemia in Brazil: high frequency of MLL-ENL fusion/t(11;19) in infant leukemia. *J Pediatr Hematol Oncol*. 2011;33(6):470-474. https://doi.org/10.1097/MPH.0b013e3181fb8f61
8. Zhu YM, Zhao WL, Fu JF, et al. NOTCH1 mutations in T-cell acute lymphoblastic leukemia: prognostic significance and implication in multifactorial leukemogenesis. *Clin Cancer Res*. 2006;12(10):3043-3049. https://doi.org/10.1158/1078-0432.CCR-05-2832
9. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnetjournal*. 2011;17(1):10. https://doi.org/10.14806/ej.17.1.200
10. Bartel DP. MicroRNAs. *Cell*. 2004;116(2):281-297. https://doi.org/10.1016/S0092-8674(04)00045-5
11. Friedländer MR, Chen W, Adamidi C, et al. Discovering microRNAs from deep sequencing data using miRDeep. *Nat Biotechnol*. 2008;26(4):407-415. https://doi.org/10.1038/nbt1394
12. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res*. 2006;34(suppl_1):D140-D144. https://doi.org/10.1093/nar/gkj112
13. Kozomara A, Griffiths-Jones S. MiRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res*. 2014;42(D1). https://doi.org/10.1093/nar/gkt1181
14. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139-140. https://doi.org/10.1093/bioinformatics/btp616
15. Forbes SA, Beare D, Boutselakis H, et al. COSMIC: somatic cancer genetics at high-resolution. *Nucleic Acids Res*. 2017;45(D1):D777-D783. https://doi.org/10.1093/nar/gkw1121
16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real- time quantitative PCR and the 2 Δ ⌬⌬ C T method. 2001;408:402-408. https://doi.org/10.1006/meth.2001.1262.
17. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature*. 2005;435(7043):834-838. https://doi.org/10.1038/nature03702
18. Marcucci G, Mrózek K, Radmacher MD, Garzon R, Bloomfield CD. The prognostic and functional role of microRNAs in acute myeloid leukemia. *Blood*. 2011;117(4):1121-1129. https://doi.org/10.1182/blood-2010-09-191312
19. de Oliveira JC, Scrideli CA, Brassesco MS, Yunes JA, Brandalise SR, Tone LG. MiR-708-5p is differentially expressed in childhood acute lymphoblastic leukemia but not strongly associated to clinical features. *Pediatr Blood Cancer*. 2015;62(1):177-178. https://doi.org/10.1002/pbc.25222
20. Zanette DL, Rivadavia F, Molfetta G a, et al. miRNA expression profiles in chronic lymphocytic and acute lymphocytic leukemia. *Braz J Med Biol Res*. 2007;40(11):1435-1440. https://doi.org/10.1590/S0100-879X2006005000179
21. Troppan K, Wenzl K, Pichler M, et al. miR-199a and miR-497 are associated with better overall survival due to increased chemosensitivity in diffuse large B-cell lymphoma patients. *Int J Mol Sci*. 2015;16(8):18077-18095. https://doi.org/10.3390/ijms160818077
22. Bongiovanni D, Saccomani V, Piovan E. Aberrant signaling pathways in T-cell acute lymphoblastic leukemia. *Int J Mol Sci*. 2017;18(9). https://doi.org/10.3390/ijms18091904
23. dos Santos NR, Ghezzo MN, da Silva RC, Fernandes MT. NF-κB in T-cell acute lymphoblastic leukemia: oncogenic functions in leukemic and in microenvironmental cells. *Cancers (Basel)*. 2010;2(4):1838-1860. https://doi.org/10.3390/cancers2041838
24. Blank U, Karlsson S. TGF-β signaling in the control of hematopoietic stem cells. *Blood*. 2015;125(23):3542-3550. https://doi.org/10.1182/blood-2014-12-618090
25. Akhurst RJ, Hata A. Targeting the TGFβ signalling pathway in disease. *Nat Rev Drug Discov*. 2012;11(10):790-811. https://doi.org/10.1038/nrd3810

26. McKarns SC, Schwartz RH. Distinct effects of TGF-beta 1 on CD4+ and CD8+ T cell survival, division, and IL-2 production: a role for T cell intrinsic Smad3. *J Immunol*. 2005;174(4):2071-2083. https://doi.org/10.4049/jimmunol.174.4.2071
27. Rozovski U, Harris DM, Li P, et al. At high levels, constitutively activated STAT3 induces apoptosis of chronic lymphocytic leukemia cells. *J Immunol*. 2016;196(10):4400-4409. https://doi.org/10.4049/jimmunol.1402108
28. dos Almeida RS, de Ramos AML, Luna CF, Pedrosa F, Donadi EA, Lucena-Silva N. Cytokines and soluble HLA-G levels in bone marrow stroma and their association with the survival rate of patients exhibiting childhood T-cell acute lymphoblastic leukemia. *Cytokine*. 2018;102(July):94-101. https://doi.org/10.1016/j.cyto.2017.07.014
29. Sheta EA, Trout H, Gildea JJ, Harding MA, Theodorescu D. Cell density mediated pericellular hypoxia leads to induction of HIF-1α via nitric oxide and Ras/MAP kinase mediated signaling pathways. *Oncogene*. 2001;20(52):7624-7634. https://doi.org/10.1038/sj.onc.1204972
30. Tokuda Y, Crane S, Yamaguchi Y, Zhou L, Falanga V. The levels and kinetics of oxygen tension detectable at the surface of human dermal fibroblast cultures. *J Cell Physiol*. 2000. https://doi.org/10.1002/(SICI)1097-4652(200003)182:3<414::AID-JCP12>3.0.CO;2-5;182(3):414-420.
31. Bhagat TD, Zhou L, Sokol L, et al. MiR-21 mediates hematopoietic suppression in MDS by activating TGF-β signaling. *Blood*. 2013;121(15):2875-2881. https://doi.org/10.1182/blood-2011-12-397067
32. Zhou L, McMahon C, Bhagat T, et al. Reduced SMAD7 leads to overactivation of TGF-β signaling in MDS that can be reversed by a specific inhibitor of TGF-β receptor I kinase. *Cancer Res*. 2011;71(3):955-963. https://doi.org/10.1158/0008-5472.CAN-10-2933
33. Naka K, Hoshii T, Muraguchi T, et al. TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature*. 2010;463(7281):676-680. https://doi.org/10.1038/nature08734
34. Kordes U, Krappmann D, Heissmeyer V, Ludwig WD, Scheidereit C. Transcription factor NF-kappaB is constitutively activated in acute lymphoblastic leukemia cells. *Leukemia*. 2000;14(3):399-402. http://www.ncbi.nlm.nih.gov/pubmed/10720133
35. Kumar V, Palermo R, Talora C, et al. Notch and NF-kB signaling pathways regulate miR-223/FBXW7 axis in T-cell acute lymphoblastic leukemia. *Leukemia*. 2014;28(12):2324-2335. https://doi.org/10.1038/leu.2014.133
36. Vilimas T, Mascarenhas J, Palomero T, et al. Targeting the NF-kappaB signaling pathway in Notch1-induced T-cell leukemia. *Nat Med*. 2007;13(1):70-77. https://doi.org/10.1038/nm1524
37. Bellavia D, Campese A, Alesse E, et al. Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice. *EMBO J*. 2000;19(13):3337-3348. https://doi.org/10.1093/emboj/19.13.3337
38. Bazarbachi A, Suarez F, Fields P, Hermine O. How I treat how I treat adult T-cell leukemia/lymphoma. *Blood*. 2015;118(7):13-21. https://doi.org/10.1182/blood-2011-03-345702
39. Matsuoka M. Human T-cell leukemia virus type I (HTLV-I) infection and the onset of adult T-cell leukemia (ATL). *Retrovirology*. 2005;2. https://doi.org/10.1186/1742-4690-2-27(1):27.
40. Guan H, Miao H, Ma N, Lu W, Luo B. Correlations between Epstein-Barr virus and acute leukemia. *J Med Virol*. 2017;89(8):1453-1460. https://doi.org/10.1002/jmv.24797
41. Burger JA, Chiorazzi N. B cell receptor signaling in chronic lymphocytic leukemia. *Trends Immunol*. 2013;34(12):592-601. https://doi.org/10.1016/j.it.2013.07.002
42. Stevenson FK, Krysov S, Davies AJ, Steele AJ, Packham G. B-cell receptor signaling in chronic lymphocytic leukemia. *Blood*. 2011;118(16):4313-4320. https://doi.org/10.1182/blood-2011-06-338855
43. Gobessi S, Laurenti L, Longo PG, et al. Inhibition of constitutive and BCR-induced Syk activation downregulates Mcl-1 and induces apoptosis in chronic lymphocytic leukemia B cells. *Leukemia*. 2009;23(4):686-697. https://doi.org/10.1038/leu.2008.346
44. Muzio M, Apollonio B, Scielzo C, et al. Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. *Blood*. 2008;112(1):188-195. https://doi.org/10.1182/blood-2007-09-111344
45. Han BW, Feng DD, Li ZG, et al. A set of miRNAs that involve in the pathways of drug resistance and leukemic stem-cell differentiation is associated with the risk of relapse and glucocorticoid response in childhood ALL. *Hum Mol Genet*. 2011;20(24):4903-4915. https://doi.org/10.1093/hmg/ddr428
46. Debant M, Hemon P, Brigaudeau C, Renaudineau Y, Mignen O. Calcium signaling and cell fate: how can Ca2+ signals contribute to wrong decisions for chronic lymphocytic leukemic B lymphocyte outcome? *Int J Dev Biol*. 2015;59(7-8-9):379-389. https://doi.org/10.1387/ijdb.150204om
47. Garzon R, Volinia S, Liu C-G, et al. MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood*. 2008;111(6):3183-3189. https://doi.org/10.1182/blood-2007-07-098749
48. Calin GA, Ferracin M, Cimmino A, et al. A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med*. 2005;353(17):1793-1801. https://doi.org/10.1056/NEJMoa050995
49. Fang C, Zhu D-X, Dong H-J, et al. Serum microRNAs are promising novel biomarkers for diffuse large B cell lymphoma. *Ann Hematol*. 2012;91(4):553-559. https://doi.org/10.1007/s00277-011-1350-9
50. Zhao JJ, Lin J, Lwin T, et al. MicroRNA expression profile and identification of miR-29 as a prognostic marker and pathogenetic factor by targeting CDK6 in mantle cell lymphoma. *Blood*. 2010;115(13):2630-2639. https://doi.org/10.1182/blood-2009-09-243147
51. Shu YJ, Bao RF, Jiang L, et al. MicroRNA-29c-5p suppresses gallbladder carcinoma progression by directly targeting CPEB4 and inhibiting the MAPK pathway. *Cell Death Differ*. 2017;24(3):445-457. https://doi.org/10.1038/cdd.2016.146
52. Haakensen VD, Nygaard V, Greger L, et al. Subtype-specific microRNA expression signatures in breast cancer progression. *Int J Cancer*. 2016;139(5):1117-1128. https://doi.org/10.1002/ijc.30142
53. Pass HI, Goparaju C, Ivanov S, et al. hsa-miR-29c* is linked to the prognosis of malignant pleural mesothelioma. *Cancer Res*. 2010;70(5):1916-1924. https://doi.org/10.1158/0008-5472.CAN-09-3993

How to cite this article: Almeida RS, Costa e Silva M, Coutinho LL, et al. MicroRNA expression profiles discriminate childhood T- from B-acute lymphoblastic leukemia. *Hematological Oncology*. 2019;37:103–112. https://doi.org/10.1002/hon.2567