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# Circulating microRNA expression profile in B-cell acute lymphoblastic leukemia

Claudia Maribel Luna-Aguirre[a], Margarita de la Luz Martinez-Fierro[b], Fermín Mar-Aguilar[c], Idalia Garza-Veloz[b], Víctor Treviño-Alvarado[d], Augusto Rojas-Martinez[a,e], Jose Carlos Jaime-Perez[f], Guadalupe Ismael Malagon-Santiago[c], Cesar Homero Gutierrez-Aguirre[f], Oscar Gonzalez-Llano[f], Rosario Salazar-Riojas[f], Alfredo Hidalgo-Miranda[g], Herminia Guadalupe Martinez-Rodriguez[a], David Gomez-Almaguer[f] and Rocio Ortiz-Lopez[a,e,*]

[a]*Departamento de Bioquímica y Medicina Molecular, Facultad de Medicina, Universidad Autónoma de Nuevo León, Monterrey NL, México*
[b]*Laboratorio de Medicina Molecular, Unidad Académica de Medicina Humana y Ciencias de la Salud, Universidad Autónoma de Zacatecas, Zacatecas, México*
[c]*Departamento de Biología Celular y Genética, Facultad de Ciencias Biológicas, Monterrey NL, México*
[d]*Departamento de Ciencias Computacionales, ITESM, Campus Monterrey, Monterrey NL, México*
[e]*Centro de Investigación y Desarrollo en Ciencias de la Salud, Universidad Autónoma de Nuevo León, Monterrey, México*
[f]*Servicio de Hematología. Hospital Universitario Universidad Autónoma de Nuevo León, Monterrey NL, México*
[g]*Instituto Nacional de Medicina Genómica, México D.F. Periférico Sur, México*

**Abstract.**
**BACKGROUND:** Acute lymphoblastic leukemia (ALL) is a highly diverse disease characterized by cytogenetic and molecular abnormalities, including altered microRNA (miRNA) expression signatures.
**AIM:** We perform and validate a plasma miRNA expression profiling to identify potential miRNA involved in leukemogenesis
**METHODS:** MiRNA expression profiling assay was realized in 39 B-ALL and 7 normal control plasma samples using TaqMan Low Density Array (TLDA) plates on Applied Biosystems 7900 HT Fast Real-Time PCR System. MiRNA validation was done for six miRNA differentially expressed by quantitative real-time PCR.
**RESULTS:** Seventy-seven circulating miRNA differentially expressed: hsa-miR-511, -222, and -34a were overexpressed, whereas hsa-miR-199a-3p, -223, -221, and -26a were underexpressed (p values $< 0.005$ for both sets). According to operating characteristic curve analysis, hsa-miR-511 was the most valuable biomarker for distinguishing B-ALL from normal controls, with an area under curve value of 1 and 100% for sensitivity, and specificity respectively.
**CONCLUSIONS:** Measuring circulating levels of specific miRNA implicated in regulation of cell differentiation and/or cell proliferation such as hsa-miRNA-511, offers high sensitivity and specificity in B-ALL detection and may be potentially useful for detection of disease progression, as indicator of therapeutic response, and in the assessment of biological and/or therapeutic targets for patients with B-ALL.

Keywords: Biomarkers, hematological malignancies, qRT-PCR, signaling pathway

## 1. Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disorder of lymphoid progenitor cells characterized by chromosomal abnormalities [1]. Treatment strategies using risk-adapted chemotherapy cure more than 80% of children with ALL; however, the disease relapses in

*Corresponding author: Rocio Ortiz-Lopez, Facultad de Medicina. Universidad Autónoma de Nuevo León, Av. F. I. Madero s.n. Colonia Mitras Centro. Monterrey, Nuevo León, 64349, México. Tel.: +52 81 3404370; Fax: +52 81 83337747; E-mail: rortizlopez@gmail.com.

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20–30% of patients, who require intensive treatment that associated with serious adverse events, including death [2,3]. Besides, the survival rate for adults with ALL remains below 40%. Although ALL can originate from either B or T lymphocyte progenitors, the B-lineage is present in 85% and 75% of children and adult cases of ALL, respectively [2,4].

MicroRNAs (miRNA) are non-coding, single-stranded RNAs of 20–23 nucleotides in length that constitute a class of gene regulators. They bind their mRNA target counterparts to induce translational repression, or to decrease their stability. MiRNA have shown to control several biological processes [5], and some of them possess oncogenic or tumor-suppressor activity [6]. MiRNA genome-wide expression studies have been conducted to study deregulated miRNA in ALL. These studies have shown that malignant cells of ALL patients have different miRNA expression patterns from those in normal hematopoietic cells [7–10]. High expression of miR-33, miR-215, miR-369-5p, miR-496, miR-518d, and miR-599 has been associated with an unfavorable prognosis whereas high expression of a different set of miR-genes: miR-10a, miR-134, miR-214, miR-484, miR-572, miR-580, miR-624 and miR-627 is associated with favorable prognosis in pediatric ALL. The combined profile of these 14 miR-genes may predict the long-term clinical outcome in children with ALL [11]. Some reports suggest that these miRNA signatures combined with the analyses of recurrent cytogenetic/molecular abnormalities are helpful to classify subgroups and to define clinical outcomes for this disease [12]. Despite the great contributions and advances in the ALL miRNA research, most of the studies have been conducted in bone marrow samples and peripheral blood mononuclear cells, while the role of the circulating miRNA remains unclear. The aim of this study was to evaluate the presence of a general circulating miRNA expression profile in plasma samples from Mexican B-ALL patients and controls to define differentially expressed miRNA with potential diagnostic use, and to define their ability to predict potential biological targets.

## 2. Materials and methods

### 2.1. Samples collection

The study was approved by the Ethics Committee of the Hospital Universitario, Universidad Autónoma de Nuevo León, and written informed consent was obtained from patients and subjects (Protocol ID BI08-009). Peripheral blood samples were collected in tubes with EDTA from 7 healthy donors (controls) and 39 patients with recently diagnosed and untreated ALL-B at Servicio de Hematología, of Hospital Universitario, Universidad Autónoma de Nuevo León (Monterrey, Mexico). Plasma was separated from cellular fractions and collected after centrifugation at 2,500 rpm for 5 minutes. An additional centrifugation round at 14,000 rpm for 10 min at room temperature to eliminate cellular debris was also performed. Obtained plasma samples were used to prepare aliquots of 250 $\mu$l and stored at $-80$°C until further processing.

### 2.2. RNA isolation from human plasma samples

Total RNA for the miRNA profile was isolated as follows: plasma samples were thawed on ice and total RNA (including miRNA) was isolated from 200 $\mu$l of plasma using 700 $\mu$l of QIAzol Lysis Reagent and RNeasy Mini spin columns of a commercially available Qiagen miRNeasy Mini Kit (Qiagen, UK), according to manufacturer's instructions, and eluted with 30 $\mu$l of elution buffer. Total RNA used in the validation stage was extracted from 200 $\mu$l of plasma with 600 $\mu$l of Trizol LS Reagent (Invitrogen, Carlsbad, CA) coupled to precipitation with ethanol, according to the manufacturer's protocol and was resuspended in 20 $\mu$l of nuclease-free water. Quality and RNA concentration were determined by measuring the absorbance at 260 nm and 260/280 ratio, respectively, using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., USA).

### 2.3. miRNA profiling using TaqMan low density array (TLDA) plates

The expression analysis of the miRNA was performed in three steps: reverse transcription, preamplification step and real-time PCR, using Megaplex Pools that consist of primers pools and microfluidic plates of TaqMan Human MicroRNA Array set (v2.0) to analyze 667 highly characterized human miRNA (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. In brief, synthesis of cDNA for mature miRNA was performed on 30–1000 ng of total RNA from 2 controls, 1 pool of 3 controls (with the same quantity of RNA), and 13 ALL-B samples using the TaqMan miRNA reverse transcription kit and Megaplex-RT Primers Human Pool A and B (10X), which are pre-defined pools of 381 and 294

RT primers respectively. The 7.5 $\mu$l RT reactions were incubated at 16°C for 2 min, 42°C for 1 min, 50°C for 1 sec, 40 cycles; 85°C for 5 min and then held at 4°C. Subsequently, in order to enhance the ability to detect low expressed miRNA, we pre-amplified the RT product using 12.5 $\mu$l TaqMan preAmp Master Mix (2X), 2.5 $\mu$l Megaplex preAmp Primers Human Pool A and B (10X), respectively, 7.5 $\mu$l nuclease-free water and 2.5 $\mu$l cDNA. The 25 $\mu$l reactions were incubated at 95°C for 10 min, 55°C for 2 min and 72°C for 2 min, followed by 12 cycles at 95°C for 15 sec and 60°C for 4 min. The thermal cycler used for synthesis of cDNA and pre-amplification step was GeneAmp PCR System 9700 (Applied Biosystem). Finally, for the real-time PCR, the 25 $\mu$l pre-amplified product was diluted with 25 $\mu$l 0.1X Tris-EDTA buffer, take 9 $\mu$l of this dilution and mixture with 450 $\mu$l of TaqMan Universal PCR Master Mix No AmpErase UNG (2X) and 441 $\mu$l of nuclease-free water. Next, 100 $\mu$l of reaction mixture was dispensed into each port of the TLDA, and the cards were centrifuged and sealed. Quantitative real-time PCR was run on Applied Biosystems 7900 HT Fast Real-Time PCR System using default cycling conditions. One sample and one control were run in duplicate. Quantification cycle (Cq) values were calculated using the SDS Relative Quantification software v2.3 and RQ manager 1.2 (Applied Biosystems) using automatic baseline settings and assigned threshold of 0.2.

### 2.4. Validation stage by qRT-PCR

RT was performed using the TaqMan miRNA RT-kit and miRNA-specific primers (Applied Biosystems). The 7.5 $\mu$l reaction mixture consisted of 0.75 $\mu$l of Reverse-transcription buffer (10X), 0.095 $\mu$l of RNase inhibitor (20 units/$\mu$l), 0.075 $\mu$l of dNTPs (100 mM) with dTTP, 0.5 $\mu$l of Multi-Scribe reverse-transcriptase (50 units/$\mu$l), 1.5 $\mu$l of primer, 100 ng of RNA, and nuclease-free water. RT was performed in a PTC-100 Peltier Thermal Cycler (MJ Research) at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min.

Quantitative real-time PCR was performed in triplicate and included non-template negative controls. For the 10 $\mu$l reactions, we combined 3.5 $\mu$l of nuclease-free water, 5 $\mu$l of TaqMan Universal PCR Master Mix Gene Expression (2X), 0.5 $\mu$l of the TaqMan miRNA assay mix and 1 $\mu$l of cDNA. Amplification was performed on a 7500 Fast Real-Time Systems (Applied Biosystems) at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

### 2.5. Data analysis

High throughput data generated from TLDA plates were analyzed using DataAssist v2.0 software (Applied Biosystems). To select the candidate reference genes in the array plates, we performed quantile normalization with SAM software to identify the miRNA with lowest standard deviation between all samples, including controls, and their stability was determined by DataAssist v2.0 software (Applied Biosystems). The miRNA with the lowest standard deviation and better stability were selected for use in the posterior validation experiments, focusing on a selection of deregulated miRNA. Relative expression of miRNA was determined employing multiple reference gene normalization. Coefficient of variation, Spearman's correlation coefficient, similitude index and stability (M) were determined for the generated data. In the validation stage, relative gene expression of miRNA was determined from RT-qPCR data by the $2^{-\Delta\Delta Ct}$ method, using the selected reference gene normalizers, and the results obtained from samples and controls. $U$ of Mann-Whitney or Student's $t$ test were used to compare the patients with ALL and healthy controls. The diagnostic properties of miRNA deregulated were evaluated using Receiver Operating Characteristic curve (ROC) analysis. In all the comparisons the distribution of continuous data was determined using the D'Agostino-Pearson K2 test and the significance level ($\alpha$) of 0.05 was considered. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Software, USA) and Sigma plot software v12. In the prediction of signaling pathways B-ALL involved, according to the miRNA expression profile identified, 19 miRNA that showed more differences in their expression levels between study groups and the most significant p values for these differences were considered. Pathways analysis was realized online using multiple miRNA analysis on DIANA miRPath v2.0 software and KEGG PATHWAY Database (http://www.genome.jp/kegg/pathway.html). Additionally in gene target prediction for hsa-miR-511 and hsa-miR-199a miRanda software was used (http://www.microrna.org/microrna/getGeneForm.do).

## 3. Results

### 3.1. Patients

Demographic and clinical characteristics of patients and controls are summarized in Table 1. Nineteen pa-

Table 1
Clinical and biological characteristics of study population

| Characteristic | | Cases group ($n = 39$) | Controls ($n = 7$) |
|---|---|---|---|
| Gender | Male | 21 (53.8 %) | 2 (28.6%) |
| | Female | 18 (46.2 %) | 5 (71.4%) |
| Age (years) | Pediatric | 19 (mean age: 7) (range: 1–17) | 0 |
| | Adult* | 17 (median age: 36) (range: 18–66) | 7 (median age: 26) (range: 25–31) |
| MIC classification | Early precursor B | 1 (2.56 %) | – |
| | Common ALL | 13 (33.33 %) | – |
| | Pre-B ALL | 11 (28.22 %) | – |
| | B-cell ALL | 14 (35.89 %) | – |

*Age data for three adult B-ALL patients were not available.

tients were pediatric and 17 were adult (child-adult ratio, 1.1), 21 were male and 18 were female (male-female ratio, 1.2). Median ages for pediatric and adults were 7 and 36 years, respectively. According to the morphologic, immunologic, and cytogenetic (MIC) classification [13], 1 patient (2.56%) was classified as early B-precursor ALL, 13 (33.33%) as common ALL, 11 (28.22%) as pre-B ALL, and 14 (35.89%) as B-cell ALL.

### 3.2. Establishment of reference genes for plasma samples

Expression of 667 miRNA was measured in the plasma from 13 B-ALL cases representing different stage of maturation of B-ALL and from 5 plasma control samples. Prior to analyzing the expression profile of circulating miRNA, the identification of internal reference miRNA that remain stably expressed for ALL in plasma was realized through quantile normalization. MiRNA that amplified in all samples with a Cq value $\leqslant 36$ were included in the analysis. One hundred forty eight miRNA with these characteristics were studied; ten of them were selected and their M values were determined. Four of these miRNA: hsa-miR-106a and hsa-miR-26b for the plate A, and hsa-miR-30a and hsa-miR-30d for the plate B, respectively, showed the lowest standard deviation values, high expression stability values (M values of 0.6603 and 0.8323 for the miRNA of the plate A, and M values of 1.1528 and 1.1108 for the miRNA of the plate B, respectively), therefore, they were selected and used as internal reference genes throughout the study.

### 3.3. Circulating microRNAs differentially expressed in B-ALL

Circulating miRNA differentially expressed between B-ALL cases and controls, were determined by data assist software using as reference endogenous hsa-miR-106a and hsa-miR-26b (plate A) and hsa-miR-30a and hsa-miR-30d (plate B). We identified 77 miRNA differentially expressed (p values $< 0.05$) in cases, with an expression level greater than two (cutoff of 2) compared to the healthy controls. Forty miRNA were overexpressed at a significant higher level and the remaining thirty-seven were underexpressed (Table 2). From these, 39 miRNA have been associated previously to leukemia and 38 miRNA have remained unpublished (Table 2). The highest mean of expression level for an overexpressed miRNA corresponded to hsa-miR-511, (159.5 fold change, $p = 0.002$) while the most underexpressed was hsa-miR-199a-3p ($-13.48$ fold change, $p < 0.001$).

### 3.4. Validation of abnormal miRNA expression

MiRNA-specific RT-qPCR was used to confirm the expression level of the most deregulated miRNA: three overexpressed (hsa-miR-511, hsa-miR-34a and hsa-miR-222), and three underexpressed (hsa-miR-26a, hsa-miR-221 and miR-223) were selected for the validation in 39 B-ALL samples (32 B-ALL cases) and 7 controls. The hsa-miR-106a and hsa-miR-26b were considered as reference endogenous genes. Figure 1 and Table S1, show concordance between the TLDA results and the validation using qRT-PCR for the six selected miRNA (p values $< 0.001$). Expression levels of hsa-miR-511, hsa-miR-34a, hsa-miR-222, hsa-miR-26a, hsa-miR-221 and hsa-miR-223 ranged from 3.32 to 12.98, 0.73 to 7.55, $-1.50$ to 3.12, $-1.63$ to $-9.17$, 1.1 to $-8.21$, and 0.31 to $-8.52$, respectively (Figs 1B and 1C); being the miRNA expression patterns independently confirmed in B-ALL cases and controls.

Table 2
B-ALL differential miRNA profile. Total RNA from 13 B-ALL samples and 5 health donors were included in the obtaining of miRNA differentially expressed by TLDA platform. Relative quantification (RQ) was obtained considering as reference endogenous hsa-miR-106a and hsa-miR-26b (plate A) and hsa-miR-30a and hsa-miR-30d (plate B) respectively (see material and methods for additional details). Reference column displays previous reports for the specific miRNA

| miRNA overexpressed | RQ | p-value | Type of leukemia/Reference | miRNA underexpressed | RQ | p-value | Type of leukemia/Reference |
|---|---|---|---|---|---|---|---|
| hsa-miR-511 | 159.55 | 0.002 | | hsa-miR-199a-3p | −13.48 | < 0.001 | |
| hsa-miR-34a* | 98.56 | 0.015 | CLL [45] | hsa-miR-340* | −7.03 | < 0.001 | |
| hsa-miR-565 | 92.04 | 0.004 | | hsa-miR-151-3p | −5.74 | 0.002 | CLL [46] |
| hsa-miR-34a | 20.03 | 0.004 | CLL [45] | hsa-miR-335 | −4.94 | < 0.001 | AML [47] |
| hsa-miR-10b* | 19.37 | 0.039 | AML [48] | hsa-miR-99b | −4.51 | < 0.001 | |
| hsa-miR-630 | 15.11 | 0.029 | | hsa-miR-425* | −3.96 | 0.017 | |
| hsa-miR-610 | 13.64 | 0.035 | | hsa-miR-224 | −3.95 | 0.001 | AML [9] |
| hsa-miR-181a | 13.24 | 0.013 | CML, CLL, AML [45,49,50] | hsa-miR-221 | −3.89 | < 0.001 | CML, ALL, AML, CLL [37,51–53] |
| hsa-miR-181c | 9.26 | 0.014 | | hsa-miR-744 | −3.86 | < 0.001 | |
| hsa-miR-222 | 8.74 | 0.004 | CML, ALL, AML, CLL [35,51,53,54] | hsa-miR-15b | −3.71 | 0.008 | AML, APL [55,56] |
| hsa-miR-138-1* | 7.74 | 0.031 | CML [57] | hsa-miR-223 | −3.67 | < 0.001 | CLL, CML, ALL, AML, APL [58–62] |
| hsa-miR-363 | 7.44 | 0.006 | AML [63] | hsa-miR-26a | −3.67 | < 0.001 | AML, ALL [64,65] |
| hsa-miR-144* | 7.01 | 0.034 | CML [51] | hsa-miR-454* | −3.56 | 0.005 | |
| hsa-miR-451 | 5.94 | 0.008 | CML, ALL [35,66] | hsa-miR-452 | −3.55 | 0.001 | |
| hsa-miR-99a | 5.51 | 0.048 | CLL, ALL [11,67] | hsa-miR-491-5p | −3.39 | < 0.001 | |
| has-miR-155 | 5.14 | 0.026 | CLL, CML, ALL, AML [36,45,68,69] | hsa-miR-340 | −3.39 | 0.001 | |
| hsa-miR-886-3p | 5.02 | 0.031 | | hsa-miR-196b | −3.28 | 0.001 | ALL, AML [52,70] |
| hsa-miR-223* | 4.95 | 0.044 | CML, CLL, AML, ALL [58,60,71,72] | hsa-miR-301a | −3.23 | 0.005 | |
| hsa-miR-422a | 4.91 | 0.007 | | hsa-miR-324-5p | −3.21 | 0.047 | |
| hsa-miR-146a | 4.90 | 0.018 | ALL, AML, APL, CML, CLL [36,46,73,74] | hsa-miR-126* | −3.17 | 0.002 | CLL, AML, ALL [9,45,75] |
| hsa-miR-192 | 4.78 | 0.005 | | hsa-miR-152 | −3.00 | 0.005 | ALL [76] |
| hsa-miR-190b | 4.40 | 0.022 | | hsa-miR-330-3p | −2.92 | 0.041 | |
| hsa-miR-95 | 3.94 | 0.019 | | hsa-miR-652 | −2.77 | < 0.001 | |
| hsa-miR-140-3p | 3.82 | 0.013 | | hsa-miR-374b | −2.71 | < 0.001 | |
| hsa-miR-660 | 3.77 | 0.005 | CLL [45] | hsa-miR-148b | −2.59 | < 0.001 | |
| hsa-miR-886-5p | 3.60 | 0.021 | | hsa-miR-671-3p | −2.57 | 0.002 | |
| hsa-miR-25 | 3.41 | 0.019 | AML [36] | hsa-miR-18a | −2.52 | 0.046 | ALL [77] |
| hsa-miR-320 | 3.28 | 0.014 | AML [78] | hsa-let-7d | −2.44 | 0.003 | APL [56] |
| hsa-miR-30e | 3.18 | 0.024 | | hsa-miR-339-3p | −2.32 | 0.013 | |
| hsa-miR-16 | 2.84 | 0.015 | CLL, ALL, APL [62,79,80] | hsa-miR-126 | −2.32 | < 0.001 | CLL, AML, ALL [9,45,75] |
| hsa-miR-19b | 2.66 | 0.002 | ALL [65] | hsa-miR-30b | −2.30 | < 0.001 | CLL [45] |
| hsa-miR-500 | 2.58 | 0.046 | | hsa-miR-148b* | −2.25 | 0.004 | |
| hsa-miR-29a | 2.56 | 0.042 | AML, CLL [81,82] | hsa-miR-27a | −2.22 | 0.006 | ALL, AML [18,36] |
| hsa-miR-502-3p | 2.48 | 0.039 | | hsa-miR-30c | −2.12 | < 0.001 | AML [83] |
| hsa-miR-195 | 2.39 | 0.002 | CLL, AML [82,84] | hsa-miR-374a | −2.10 | < 0.001 | |
| hsa-miR-20b | 2.20 | 0.009 | ALL [85] | hsa-miR-331-3p | −2.06 | 0.001 | |
| hsa-miR-579 | 2.17 | 0.029 | | hsa-miR-28-5p | −2.05 | < 0.001 | |
| hsa-miR-7 | 2.08 | 0.024 | ALL [77] | | | | |
| hsa-miR-19a | 2.05 | 0.01 | CLL, CML [51,86] | | | | |
| hsa-miR-768-3p | 2.04 | 0.043 | | | | | |

ALL: Acute lymphoblastic leukemia. CLL: Chronic lymphoblastic leukemia. AML: Acute Myeloid leukemia. APL: Acute promyelocytic leukemia. CML: Chronic myelocytic leukemia.

Table 3
Evaluation of potential capacity for ALL diagnosis of miRNA selected

| Parameter | miRNA Overexpressed | | | miRNA Underexpressed | | |
|---|---|---|---|---|---|---|
| | miR-511 | miR-34a | miR-222 | miR-26a | miR-221 | miR-223 |
| Area under the ROC curve | 1 | 0.98 | 0.91 | 0.91 | 0.92 | 0.93 |
| Sensitivity | 1 | 0.92 | 0.79 | 0.79 | 0.83 | 0.89 |
| Specificity | 1 | 1 | 1 | 1 | 1 | 1 |
| PPV | 1 | 1 | 1 | 1 | 1 | 1 |
| NPV | 1 | 0.70 | 0.54 | 0.47 | 0.54 | 0.64 |

ROC: Receiver Operating Characteristic; PPV: positive predictive value; NPV: negative predictive value.

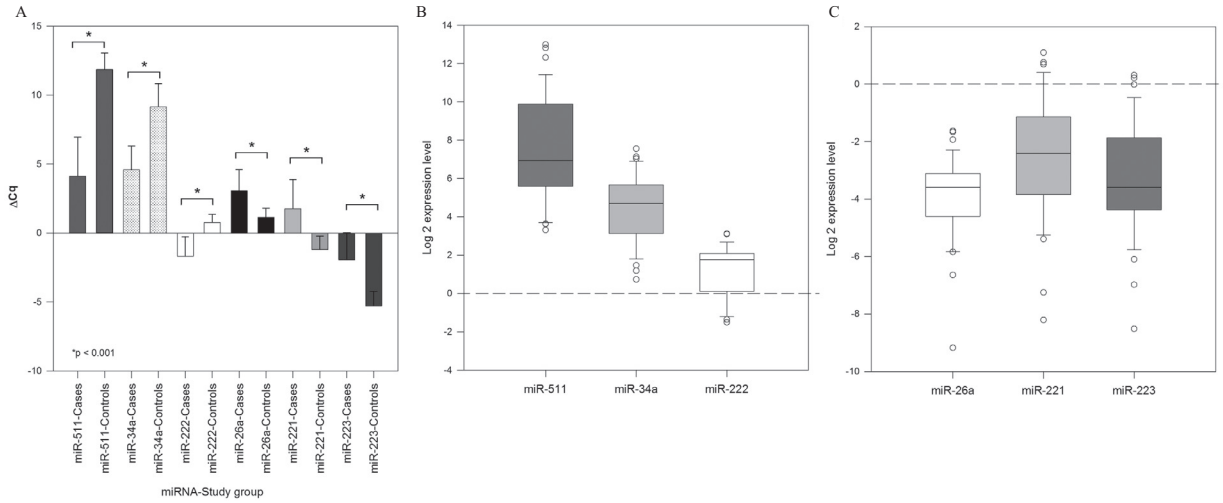


Fig. 1. miRNA validation. Expression levels of six miRNA were validated using 39 samples from ALL patients, and 7 controls by qRT-PCR. Figure 2A shows $\Delta$Cq values ($y$ axis) obtained for 3 miRNA initially overexpressed (hsa-miR-511, hsa-miR-34a, and hsa-miR-222, respectively), and 3 miRNA underexpressed (hsa-miR-26a, hsa-miR-221, and hsa-miR-223 respectively) in the ALL group compared with the controls ($x$ axis). B and C plots illustrate the Log 2 of the ex pression levels ($y$ axis) of the six miRNA included in the validation stage.

### *3.5. Evaluation of B-ALL diagnosis capacity of the six miRNA validated*

To assess the B-ALL diagnosis capacity of the six circulating miRNA validated, their specificity, sensitivity, positive and negative predictive values, were analyzed by ROC curves (Table 3) and the associated area under the curve (AUC). From the miRNA overexpressed, the ROC values (including AUC) obtained for hsa-miR-511 and hsa-miR-34a were better than hsa-miR-222 and/or the three underexpressed genes included in the analyses (hsa-miR-26a, hsa-miR-221 and hsa-miR-223), demonstrating their best B-ALL diagnosis capacity (Table 3). According to ROC analysis (highest values obtained in sensitivity and specificity parameters), the $\Delta$Cq cutoff values for hsa-miR-511, hsa-miR-34a, hsa-miR-222, hsa-miR-26a, hsa-miR-221, and hsa-miR-223 were fixed in 9.458, 7.179, $-0.1325$, 2.073, $-0.1861$, and $-4.309$ respectively. Figure 2 shows a comparison between $\Delta$Cq values obtained for hsa-miR-511, hsa-miR-34a, hsa-miR-222 in the study groups, and their respective cutoff values. Most of $\Delta$Cq values from controls samples were grouped under the cutoffs fixed for each miRNA, confirming the diagnostic power of hsa-miR-511 and hsa-miR-34a for B-ALL.

### *3.6. Pathways analysis and biological targets*

In our study, pathways analysis of 19 miRNA (hsa-miR-511, -19b, -195, -565, -34a, -222, -363, -181a, -181c, -199a-3p, -340, -335, -99b, -221, -744, -223, -26a, -224, and -151-3p) with different over or under expression, showed that mainly Wnt, MAPK, TGF-beta, p53, Jak-STAT, NOTCH, and B and T cell receptor signaling pathways (among others), are actively regulated by these miRNA (Table S2 and Fig. 3 respectively). When grouped by disease, KEGG pathways regulated by these 19 miRNA included CML, AML, and other cancers (see Table S2). Considering hsa-miR-511 only

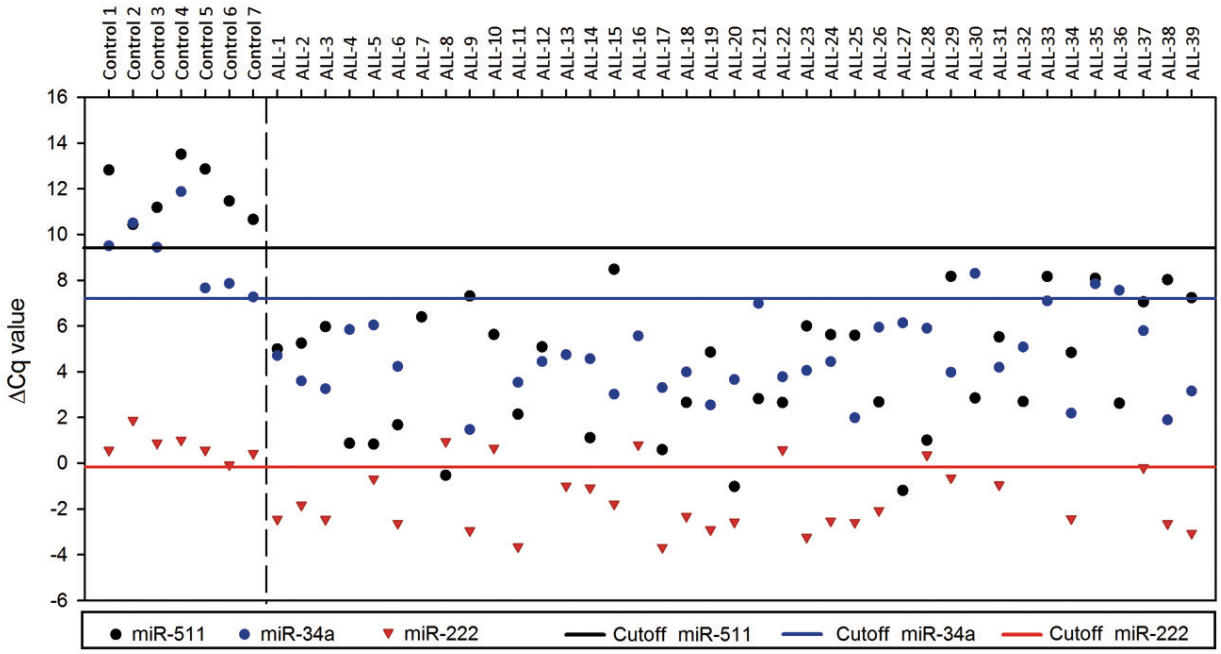


Fig. 2. Evaluation of ALL diagnosis capacity of hsa-miR-511, hsa-miR-34a, and hsa-miR-222. ALL diagnosis capacity of the miRNA with high values of area under the ROC curve is shown in this figure. ΔCq cutoff values for hsa-miR-511, hsa-miR-34a, and hsa-miR-222 were fixed at 9.458, 7.179, and −0.1325, respectively, according to their highest values obtained in the sensitivity and specificity parameters. The vertical dotted line shows that the ΔCq values obtained from controls samples are grouped within the cutoffs fixed for each miRNA (horizontal lines). Study subjects are represented in the $x$ axis, while $y$ axis displays the expression level of hsa-miR-222, and hsa-miR-511, and hsa-miR-34a, respectively.

(most overexpressed) and hsa-miR-199a-3p (most underexpressed), about one hundred biological targets were identified (100 for hsa-miR-511 and 98 for hsa-miR-199a-3p, respectively). Common targets identified by the two miRNA included *PPP3CC, AKT2, RUNX1,* and other genes belonging to the pathways of Wnt signaling, MAPK signaling, the hematopoietic cell lineage, and the chronic/acute myeloid leukemia pathways, among others (Fig. 3).

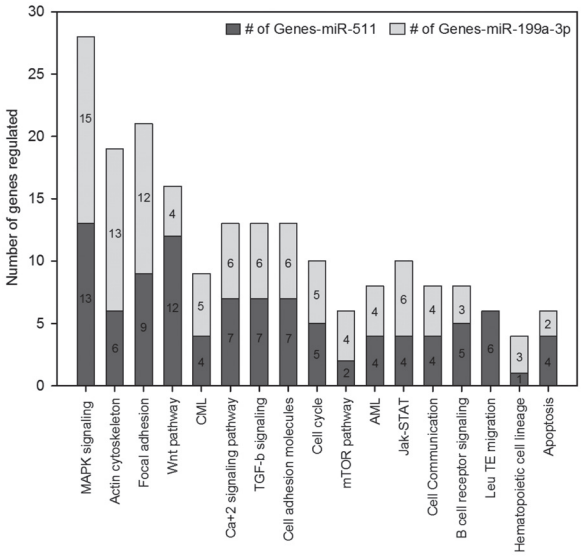


Fig. 3. hsa-miR-511 and hsa-miR-199a-3p pathways analysis.

## 4. Discussion

Previous studies have suggested improvements in detecting malignancies by using specific extracellular miRNA that perform better than other markers for disease classification [14]. The presence of altered miRNA profiles in plasma has been reported for several types of tumors, including CLL [15,16]. Since plasma-derived biomarkers are more accessible, simple to analyze, and correlate to malignant processes [17], we investigated the expression profile of miRNA in plasma of B-ALL patients. Compared to healthy controls, 77 miRNA were B-ALL differentially expressed. Thirty-nine of them were previously associated with leukemia and 38 corresponded to unpublished miRNA markers. Among these novel biomarkers, over-expression of hsa-miR-511, hsa-miR-565,

hsa-miR-34a/a*, and hsa-miR-222, was more strongly associated with B-ALL. A remarkable finding of this study was the under-expression of hsa-miR-199a-3p, hsa-miR-340*, hsa-miR-26a, hsa-miR-223, and hsa-miR-151-3p. There is a discrepancy regarding the expression of hsa-miR-708, a marker associated with relapse and favorable response to glucocorticoid therapy. This molecule is reported as overexpressed in bone-marrow samples of pediatric-ALL [18], in our study this miRNA was over-expressed 94.2 times respect to controls; however the difference observed only showed a statistical trend ($p = 0.058$); the reason for these differences is unclear to us, but this may reflect differences in miRNA concentrations between the blood and the bone marrow milieus due to *in situ* differences in production, transportation, and stability, among other factors. With the exception of hsa-miR-151-3p (no current association with disease), deregulated miRNA observed in our study, have been associated with other pathologies different than B-ALL. For example, hsa-miR-511 is differentially expressed between non-alcoholic steatohepatitis (NASH) patients and non-NASH subjects [19]; the over-expression of hsa-miR-34a (a modulator of p53 negative regulator MDM4) is associated with hypermethylation in non Hodkin Lymphoma, and is also up-regulated in the squamous cell lung carcinoma tissues [20–23], and hsa-miR-708 has been associated with bladder, prostate, and lung cancer [24–27]. Finally, breast lobular neoplasia progression is related to up-regulation of hsa-miR-565 [28] and deregulation of hsa-miR-199a-3p has been associated with Epstein-Barr virus-infection in diffuse large B-cell lymphoma [29,30].

Regarding the validation of results, the accuracy of miRNA expression data analysis depends on the proper normalization to reach correct conclusions [31, 32]. TLDA platform includes 3 small nucleolar RNAs (snoRNA) stably expressed in several tissues that are useful as reference genes during data analyses; however, the expression of these genes was not a constant in our samples. Some explanation for this result is that the expression of these genes was evaluated in tissues samples, but not in plasma. miRNA expression may differ among biological fluids. Accordingly, selection of endogenous controls is crucial for these studies. In this sense, hsa-miR-106a, hsa-miR-26b (from plate A), hsa-miR-30a and hsa-miR-30d (from plate B) showed to be the most appropriate endogenous control for miRNA plasma normalization in B-ALL patients, due to their stability (smaller M value). Although the expression stability of these miRNA has been individually reported in some human tissues, cell lines [31], prostate and breast cancer models [33], human plasma and serum samples [34], this is the first report that evaluates their utility in plasma of B-ALL patients and we postulated them as reference miRNA for this disease.

The B-ALL aberrant expression patterns identified by TLDA for six of the most deregulated miRNA were successfully confirmed using RT-qPCR in a greater sample number and their diagnostic properties were also assessed by ROC analysis. With the exception of hsa-miR-511 and hsa-miR-34a, the circulating miRNA validated in this study, have been previously associated with ALL [35–38] and we provide an additional support for their role in the pathogenesis of this disease. ROC analysis showed that in plasma at $\Delta$Ct cutoff of 9.458, hsa-miR-511 was the most valuable biomarker for distinguishing B-ALL from normal controls, with an AUC value of 1, and 100% of sensitivity and specificity respectively; these values indicate the maximum performance that can be reached in a marker for B-ALL diagnosis. Remarkably, B-ALL cases were not stratified considering any other variable (age, sex, translocations, etc.) therefore we suggest that the miRNA biomarkers reported in this study are valid for all the subjects with a diagnosis of B-ALL.

Pathway classifications related to 19 miRNA differentially expressed revealed that the most significant biological targets were genes involved in cell adhesion (pathway KEGG ID: hsa04514, and hsa04510), cell proliferation, differentiation, apoptosis (WNT and MAPK signaling: KEGG IDs hsa04310 and hsa04010, respectively), hematopoietic cell lineage (hsa04640), and regulation of transcription (hsa03022). Target prediction of hsa-miRNA-511 and hsa-miR-199a-3p (the most overexpressed and underexpressed, respectively) revealed that they may be involved in the KEGG pathways of CML (hsa05220) and AML (hsa05221). Notably, we found that the *AKT2* and *RUNX1* genes are the shared targets for the two miRNA in myeloid leukemia. *AKT2* is a putative oncogene that plays a key in regulating cell survival, insulin signaling, angiogenesis and tumor formation [39]. It has been previously reported overexpressed in ovarian, pancreatic, breast, prostate, renal, colon, lung, and hepatocellular cancer, as well as in myeloid leukemia [40–42]. *RUNX1* is thought to be involved in the development of normal hematopoiesis. Chromosomal translocations involving this gene are well-documented and have been associated with several types of leukemia [43,44]. Therefore, *AKT2* and *RUNX1* are activated in human cancers and consequently may be considered in the de-

sign of molecular therapies. These results showed that miRNA-511 and hsa-miR-199a-3p might be associated with the pathogenesis of B-ALL, and can be used as circulating biomarkers in the detection of the disease, and potentially as ALL development and progression markers.

## 5. Conclusion

Measuring circulating levels of specific miRNA implicated in cell differentiation and/or cell proliferation such as hsa-miRNA-511, offers high sensitivity and specificity in B-ALL detection and may be potentially used for the identification of progression markers and in the assessment of biological and/or therapeutic targets for patients with B-ALL.

## Abbreviations

| | |
|---|---|
| ALL: | Acute lymphoblastic leukemia. |
| CLL: | Chronic lymphoblastic leukemia. |
| AML: | Acute Myeloid leukemia. |
| APL: | Acute promyelocytic leukemia. |
| CML: | Chronic myelocytic leukemia. |
| qRT-PCR: | Quantitative real time polymerase chain reaction. |
| AUC: | Area under curve. |
| PPV: | Positive predictive value. |
| NPV: | Negative predictive value. |

## Supplementary data

The supplementary files are available to download from http://dx.doi.org/10.3233/CBM-150465.

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## Conflict of interest

Authors do not have any financial and/or personal relationships with other people or organizations that could inappropriately influence (bias) this work.

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