Differential miRNA expression in childhood acute lymphoblastic leukemia and association with clinical and biological features

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A B S T R A C T

The present study aimed to analyze the expression profile of the microRNAs previously described as associated with childhood ALL, miR-92a, miR-100, miR-125a-5p, miR-128a, miR-181b, miR-196b and let-7e, and their association with biological/prognostic features in 128 consecutive samples of childhood acute lymphoblastic leukemia (ALL) by quantitative real-time PCR. A significant association was observed between higher expression levels of miR-196b and T-ALL, miR-100 and patients with low white blood cell count at diagnosis and (12;21) positive ALL. These findings suggest a potential activity of these microRNAs in pediatric ALL biology.

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1. Introduction

Acute lymphoblastic leukemia (ALL) represents the most common type of childhood malignant neoplasia and, despite the significant progress in current treatment, 20–30% of affected children relapse and the causes are still uncertain [1]. ALL is a heterogeneous disease, composed of many subtypes that represent different clinical behaviors and need different therapy schemes [2]; however, the complex mechanism driving to distinct outcomes in each subtype is still poorly understood.

In this context, it is important to study microRNAs (miRNAs), small (17–25 nucleotides) single-stranded noncoding RNAs that function predominantly as sequence-targeted modifiers of gene expression through translational repression. Currently, more than 1000 miRNAs have been described for the human genome and, although most of them do not have established function, the association between the disruption of their expression and tumor progression and aggressiveness is widely accepted [3,4]. The miRNAs play an important role in the regulation of normal hematopoiesis and their disruption could contribute to leukemogenesis [5–8]. Few studies have been conducted until now concerning the role of miRNA in childhood ALL [9–14]. These studies have shown that miRNA expression analysis may help to understand the development of different phenotypes and the biological functions of these miRNAs in childhood ALL.

Based on this information, we aimed to evaluate the expression of miRNAs previously reported to be differentially expressed in childhood ALL [9] (miR-92a, miR-100, miR-125a-5p, miR-128a, miR-181b, miR-196b and let-7e), in consecutively diagnosed bone marrow samples from children with ALL treated according to the GBTLI-ALL 99 protocol and to correlate their expression levels with biological/prognostic features.

2. Materials and Methods

2.1. Patients

Bone marrow (BM) samples were obtained at diagnosis from 128 consecutive patients with childhood ALL classified and treated from January 2002 to May 2005 according to the Brazilian Childhood Leukemia Treatment Group (GBTLI-ALL-99) protocol [15,16] at two different treatment centers. In addition, 11 normal BM samples from children without hematological diseases (aged 1 month to 13 years) were analyzed. The study was approved by the National Research Ethics Committee (CONEP, No. 7329/2009) and was based on the Helsinki convention criteria. Samples were collected after written informed consent was obtained from the persons responsible for the children.

The diagnosis was made by standard morphological analysis and flow cytometry in BM at diagnosis, with all patients presenting more than 70% of blasts cells. Among the 128 ALL patients studied, 108 (84.4%) had B-derived ALL (pro-B ALL in 10 cases...
and common-ALL/pre-B ALL in 98), and 20 (15.6%) had T-ALL; 69 were boys and 59 were girls ranging in age from 13 months to 17 years (median 5.2 years). The white blood cell count (WBC) ranged from 800 to 849,000/mm³ (median: 17,700/mm³). Seven patients presented t(9;22), five t(4;11), twenty-one t(12;21) and one t(1;19) was detected by reverse transcription (RT)-PCR [17]. Conventional G-band karyotyping was performed in 62 patients, with 12 of them presenting a hyperdiploid karyotype.

Patients were characterized as poor responders, regardless of the initial risk group, if they met one or more of the following criteria during the phase of remission induction: WBC > 5000/mm³ on day 7 (D7) as a proxy for peripheral blast count, M3 bone marrow (≥25% blasts) on day 14 (D14), or M2/M3 bone marrow (>5 and 25% blasts, respectively) on day 28 (D28) [16]. Eighty-three patients were classified as high risk (64.8%) and 20 (15.3%) as poor responders.

The time of follow-up of patients in complete clinical remission ranged from 34 to 74 months, with a median observation of 56 months. Unfavorable events (death due to any cause or relapse) occurred in 31 patients (24.2%). Of these, 17 relapsed and 14 died during induction (n = 9) or in clinical remission (n = 5). Among these patients, all casualties were associated with treatment complications: sepsis (n = 12) and hemorrhagic disorders (n = 2).

Minimal residual disease (MRD) was detected by a simplified PCR method with consensus primers for T cell receptor (TCR) and immunoglobulin (Ig) gene rearrangements on days 14 and 28, as previously described [15]. The 5 years event free survival according clinical and biological features are shown in supplemental Table S1.

2.2. RNA extraction, cDNA synthesis and quantitative real-time PCR (RQ-PCR)

BM mononucleated cells were separated by a Ficoll-Hypaque centrifugation gradient and the total cellular RNA was extracted from BM samples using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The RNA of each sample was stored in DEPC-treated water at –80 °C and, before use; the quantity and quality of samples were evaluated with an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies). Total RNA (100 ng) was retrotranscribed with microRNA-specific primers using a TaqMan microRNA reverse transcription kit (Applied Biosystems, Foster City, CA), and qRT-PCR was then performed using Taqman® microRNA assays according to the manufacturer’s protocol.

The microRNA levels of mir-92a, miR-100, miR-125a-5p, miR-128a, miR-181b, miR-196b and let-7e were measured using the ABI 7500 Real Time PCR System (PE Applied Biosystems). The relative expression was calculated using the 2−ΔΔCT method [18] with two internal controls, small nuclear RNU6B and RNU48, used to normalize the cDNA levels. The amplification efficiency of each microRNA and endogenous probes was 92–103%, and the plot of cDNA dilutions versus delta C was close to zero (slope ≤ ±0.1). The mean expression in the 11 normal bone marrow samples was used as calibrator.

Real-time PCR was performed in duplicate and a standard deviation (SD) of <0.5 between duplicates was accepted. A blank control was run in parallel to determine the absence of contamination within each experiment.

2.3. Statistical analysis

The association between the variables analyzed (age, WBC count at diagnosis, immunophenotype, risk group, molecular cytogenetics, hyperdiploid karyotype,

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Minimum, maximum and median microRNA expression levels in normal bone marrow and in ALL samples.</th>
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<tbody>
<tr>
<td>microRNA</td>
<td>Normal BM (n = 11)</td>
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<tr>
<td></td>
<td>Median (range)</td>
</tr>
<tr>
<td>miR-92a</td>
<td>0.97 (0.45–3.91)</td>
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<tr>
<td>miR-100</td>
<td>1.04 (0.34–3.90)</td>
</tr>
<tr>
<td>miR-125a-5p</td>
<td>1.27 (0.48–4.25)</td>
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<tr>
<td>miR-128a</td>
<td>1.02 (0.57–2.23)</td>
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<tr>
<td>miR-181b</td>
<td>1.41 (0.33–3.40)</td>
</tr>
<tr>
<td>miR-196b</td>
<td>1.07 (0.29–3.70)</td>
</tr>
<tr>
<td>let-7e</td>
<td>1.16 (0.54–3.25)</td>
</tr>
</tbody>
</table>

P value calculated by the Mann–Whitney test. Bold value indicates statistically significant (P < 0.05).

BM, bone marrow.

Bone marrow status at day 28, MRD at day 14 and 28 and microRNA expression levels was determined by the Mann–Whitney test.

For survival analysis, patients were stratified into values below and above the median, the quartiles (P25 and P75) and the average of expression levels in normal bone marrow. The Kaplan Meier curves and log rank test were used to estimate event-free survival (5-year EFS), which was calculated from the date of complete continuous remission (CCR) to the last follow-up, or unfavorable event (induction failure, relapse and/or death due to any cause). Patients who did not attain CCR or died during induction were considered as an unfavorable event at time zero.

The correlation between the expression values of the microRNAs was determined by the Spearman correlation coefficient. Data were analyzed statistically using the Statistical Package for the Social Sciences (SPSS) software for Windows, version 15.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Expression of microRNAs in ALL and in normal bone marrow

ALL samples showed a lower expression of miR-100 (P < 0.01), miR-196b (P < 0.01) and let-7e (P < 0.01) and a higher expression of miR-128 (P < 0.01) and miR-181 (P < 0.01) compared to control BM samples. The expression of miR-92a and miR-125a-5p was similar for both groups (Table 1).

3.2. Relation between microRNA expression and clinical/biological features

The microRNAs studied presented a wide variability of expression in ALL patients (Table 1). A significant positive correlation was observed by the Spearman’s correlation test between miR-92a,
miR-128a, miR-181b and let-7e expression; miR-125a-5p, miR-22a, miR-128a and let-7e and miR-100; and miR-196b and let-7e (Table 2).

A higher MiR-196b expression was found in T-ALL (P=0.01). MiR-100 expression was higher in patients who presented a WBC count at diagnosis <50,000/mm³ (P=0.01). In the group of B-cell ALL patients, higher miR-100 expression was associated with the presence of t(12;21) (P=0.04) and the absence of a hyperdiploid karyotype (P=0.04) (Fig. 1) (Table S2).

The presence of t(4;11) was associated with miR-181b (P=0.02) and miR-128a (P=0.03) (Fig. 2).

3.3. Relation between microRNA expression and treatment response parameters

Despite the low number of patients in each group, the expression of miR-125a-5p (P=0.02) and let-7e (P=0.04) was lower in patients who presented BM status M2/M3 at day 28 of induction therapy (n=7). Additionally, let-7e expression was higher in patients with MRD at D14 (P=0.02) (n=12) (Fig. 3) (Tables S3–S5).

In order to analyze a possible correlation between the expression of the miRNAs analyzed and EFS the patients were divided into two groups using the values below and above the median as cut-off. None of the miRNAs analyzed were associated with 5y EFS (Table S6). The same was true when the quartiles (P25 and P75) and the average of expression levels in control BM were used as a cut-off between low and high expression (data not shown).

4. Discussion

MicroRNAs are associated with the regulation of normal hematopoiesis and their disruption has been related to many types of cancer, including hematological malignancies, but little is known about their effects on childhood ALL. In the present study, pediatric ALL BM samples showed lower expression levels of miR-100, miR-196b and let-7e, while miR-128a and miR-181b were over-expressed in patients when compared to normal pediatric BM samples. Similar to our results, miR-128a and miR-181 have been previously reported to be up-regulated in ALL compared to normal donors [10] and also to normal CD34+ progenitor cells [9]. Additionally, down-regulation of miR-100, miR-196b and let-7e was demonstrated in B-ALL without translocations compared to CD34+ cells [9]. Other studies have also shown that some of the miRNA analyzed here influence hematopoietic differentiation including miR181 [19], miR196a [20,21], and mir125 [11,22,23]. However, since normal BM and CD34+ cells do not reproduce exactly the...
normal counterpart of ALL BM, these results should be viewed with caution.

Besides the described pattern of expression in normal progenitor cells, these miRNAs have also been found to be differentially expressed in different subtypes of ALL [9]. These genes could also have led to inappropriate expression of proteins that act as tumor suppressors or oncogenes. Some of them such as let-7e, miR100 and miR196b have been reported to have potential tumor suppressive function [20,24,25], while miR181 targets oncogenes [24].

Analysis of the correlation of expression levels among the genes under study by the Spearman’s correlation coefficient showed a significant positive correlation between some of them, suggesting a possible co-regulation in childhood ALL.

Regarding the biological features, miR-196b analyses demonstrated higher expression in T-cell ALL when compared to B-cell patients. The association between miR-196b and ALL has been reported in association with t(4;11) [9,26]. In the present study, despite a higher expression in patients with t(4;11), this difference was not significant. High expression of miR-196b is not exclusively induced by MLL chimeras, but has been also correlated with other leukemia types, including T-cell ALL, with increased HOXA gene expression [27].

MiR-196b is encoded in the HOXA-cluster, mapped between the HOX9 and HOX10 genes and is most likely co-regulated in ALL [27]. Although the involvement of HOXA genes in the survival and proliferation rates of leukemia cells is generally recognized [28], the overexpression of mir-196b in bone marrow progenitor cells also leads to increased proliferative capacity and survival [26]. In spite of this, its role in leukemia is still controversial, since there are descriptions of its involvement in the regulation of oncogenes such as ERG and c-myc, suggesting a tumor suppressor activity [20,21]. Noteworthy, miR-196b has been associated with the down-regulation of c-myc in B-cell ALL [20], an event not occurring in T-cell ALL as a consequence of mutations in the 3′-untranslated region (3′-UTR) of this gene as reported for some cell lines and patient samples [29]. It is interesting to point that these subtypes related to higher miR196b expression are associated with a poor clinical outcome.

We found lower levels of miR-100 expression in ALL patients as a whole and even significantly lower levels in patients with a WBC count higher than 50,000/mm3. Additionally, increased miR-100 expression was associated with the presence of t(12;21), biological features classically associated with a good outcome. The association between miR-100 expression and t(12;21) patients agrees with previous studies [30]. Furthermore, we found an association between low miR-100 expression and hyperdiploid karyotypes, that are infrequent in t(12;21) ALL (~1%) [31]. This fact may reinforce a t(12;21) specific regulation of miR-100. Likewise, previous reports on oral cancer have demonstrated that forced expression of miR-100 results in decreased proliferation [32]. Furthermore,
miR-100 is known to regulate central elements in signaling pathways involved in cell growth control and proliferation such as PLK1, IGFR2 and FRAP1/miTOR [33–35]. In contrast, a positive correlation between miR-100 and cell growth has been demonstrated in vitro for AML, where this miRNA is required for cell growth through the regulation of the tumor suppressor RBSP3 [36]. Of note, when compared to ALL and normal cells, miR-100 levels are exceedingly higher in AML [10], suggesting a different responsibility for each lineage.

In the present study, some miRNAs were associated with other clinical and biological features. Among them, lower expression of miR-125a-5p was associated with M2/M3 bone marrow status on D28 (n = 7).

MiR-125a expression seems to be conflicting in different tumors, with hyperexpression occurring in lung squamous cell carcinoma and hypoxpression in glioblastomas, medulloblastomas and oral cancer [37–39]. In hematopoietic malignancies, lower expression has been related to HTLV-1–associated acute adult T–cell leukemia [40]. Specifically, miR-125a-5p is an independent prognostic factor in gastric cancer and inhibits proliferation in vitro [41]. Furthermore, this miRNA induces apoptosis by activating p53 in lung cancer cells [42].

The results of miRNA let-7e expression were inconsistent when clinical outcome was considered. While this expression was associated with a good morphological response in bone marrow at D28 (n = 7), it was also associated with the presence of higher levels of DRM on D14 (n = 12). These discrepancies could be related to the small number of cases analyzed and further studies are necessary to determine the relationship between this miRNA and ALL outcome.

Literature data also seem to be controversial about the tumor suppressor role of let-7e. Lower expression levels have been described for malignant mesotheliomas [43], as opposed to overexpression in retinoblastomas [44]. Nevertheless, there is compelling evidence of down-regulation of let-7e involvement in resistance to Cetuximab in colorectal cancer and to Gemcitabine in pancreatic and ovarian cancer [45–47], with let-7e being a regulator of the proto-oncogene MYCN [48].

Our study also points to an association between miR-181b and miR-128a expression and t(4;11) ALL. Due to the small number of cases carrying this translocation, additional studies on a higher number of cases are necessary to corroborate these data.

In conclusion, miR-196b expression was found to be higher in T-ALL and miR-100 expression was higher in patients with a WBC count <50,000/mm³ at diagnosis and in t(12;21)-positive patients. These findings suggest a potential role of these microRNAs in specific subtypes of pediatric ALL and in the development of different phenotypes. Further studies are needed to corroborate and extend our results.

Conflict of interest
None.

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Contributions: JCO, MSB, CAS and LGT designed the study and supervised the project; JCO, AGM, JAP, RPQ performed the molecular genetic analyses; JCO and CAS analyzed the data and performed the statistical analyses; JCO, MSB, CAS and LGT wrote the article with the contribution of JAY and SRB. CAS, LJT, JAY and SRB were the principal investigators and recruited patients. All authors read and approved the final version of the manuscript.

Appendix A. Supplementary data
Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.leukres.2011.10.005.

References


