Expression of Micro-RNA 128 and Let-7b in Pediatric Acute Lymphoblastic Leukemia Cases

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Abstract

Background: MicroRNAs (miRNAs) play important roles in the pathogenesis of leukemia and their altered expression is associated with many types of solid and hematological malignancies. Methods: The study was performed on 70 consecutive newly diagnosed pediatric acute lymphoblastic leukemia (ALL) patients, of which 56 were evaluated for both bone marrow miR-128 and let-7b (all 70 for let-7b) by real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR). In addition, seven age and sex matched healthy controls were assessed. Results: miR-128 expression was significantly higher in ALL patients compared with healthy controls (p<0.001). However, the expression levels of let-7b showed no statistical significant difference between the groups. No significant links were noted with clinical details, laboratory data and response to treatment. Conclusion: The results suggest that determination of miR-128 expression level may provide a tool for confirmation of a diagnosis of childhood ALL, follow up for response of treatment and a possible predictor of early relapse. Any role of let-7b in pediatric ALL needs to be further assessed.

Keywords: ALL- RT-qPCR- micro-RNA

Introduction

MicroRNAs (miRNAs) are a family of small (17-25 nucleotides), single stranded and non-coding RNAs. They are involved in the regulation of more than 5300 human genes by affecting post-transcriptional status (Lewis et al., 2005).

Altered expression of miRNAs are associated with development and progress of various solid and hematological tumors through down-regulation of tumor suppressor genes or oncogene over-expression (Wang et al., 2014; Lu et al., 2008).

Recently, specific miRNAs have been discovered to be involved in the pathogenesis of leukemia serving as a biomarker for detection of leukemia at diagnosis and relapse (Tombak et al., 2015).

MicroRNA-128 (miR-128) is a brain enriched microRNA involved in nervous system development. It is encoded by two distinct genes, miR-128a and miR-128b and it plays an important regulatory role in the context of tumor cells (Huang et al., 2015).

Expression of miR-128 is altered in tumor cells through a variety of genetic and epigenetic events. Altered miR-128 has an important effect on oncogenesis through alterations in cellular proliferation, differentiation, metabolism and apoptosis (Huang et al., 2015).

The function of miR-128 in regulating tumors is controversial, as most of studies assumed it as a tumor suppressor (Liu et al., 2014; Adlakha et al., 2013; Donzelli et al., 2012; Shi et al., 2012; Cui et al., 2010). However it was reported to function as a potential oncogene in other studies, suggesting that the functional role of miR-128 depends on the cellular context (Shen et al., 2014, Lin et al., 2013, Zhu et al., 2012 and Mi et al., 2007).

Let-7 family members negatively regulates oncogenes expression as RAS, MYC and HMGA2, that is why it was believed to be a tumor suppressor and its loss is responsible for the poor prognosis in many human cancers as lung cancer (Di Leva et al., 2010).

Let-7b is one out of twenty seven microRNAs that were differentially expressed between ALL and acute myeloid leukemia (AML) and could accurately discriminate ALL from AML (Mi et al., 2007).

In this study, we aimed to evaluate the expression of miR-128 and let-7b in bone marrow of pediatric acute lymphoblastic leukemia (ALL) patients and correlate their expression with clinical, laboratory and prognostic data of patients.

Materials and Methods

Patients and methods

This study was carried on 70 consecutive newly diagnosed pediatric ALL patients who presented to the

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pediatric Medical Oncology Department, National Cancer Institute (NCI) over a period of two years. Seven age and sex matched healthy children taken as control group from donors of bone marrow transplantation.

Diagnosis was established after clinical, morphological, cytochemical, flow cytometric and cytogenetic analysis. All the cases met the ALL diagnosis standards.

A Written informed consent (obtained from persons responsible for the children) was approved by the Institutional Review board (IRB) ethical committee of the NCI which follows the rules of Helsinki IRB.

Sample collection, RNA preparation and cDNA synthesis
Bone marrow samples (1 ml) were collected on EDTA from pediatric ALL patients and controls taken from bone marrow donors. Bone marrow was treated with erythrocytes lysis solution. Leukocytes were collected and stored in QIAzol lysis reagent at -80 °C till use for RNA extraction.

Total RNA was extracted from mononuclear cells using miRNeasy Mini kit (QIAGEN), following the manufacturer’s instructions. The amount of RNA was measured by nanodrop spectrophotometer at 260 and 280 wave length; (a ratio of 1.8-2.1= a ratio of 1.8-2.1) denoted good quality of RNA. Subsequently, 1.0 µg of total RNA was reverse transcribed into cDNA in 20 µL reaction using random hexamer using miScript II RT kit (QIAGEN) according to manufacturer’s instructions and stored at -20 °C till use.

Quantitative Real-time PCR (qPCR) for miRNAs
The expression of miR-128 was determined using real-time quantitative RT-PCR. For real-time RT-PCR, the 25 µl reaction contained 12.5 µl 2X SYBR GREEN PCR Master Mix (QIAGEN), 5pmol (2.5 µl) for miR-128 and/or let-7b, 10X miScript primer assay, 10X miScript universal primer and 2.5 µl of the diluted cDNA (diluted with 25 µl of Nuclease-free water). Reactions were run with the following thermal cycles parameters: 95 °C for 15 minutes, followed by 40 PCR cycles at 94 °C (15 seconds, denaturation), 55 °C (30 seconds, annealing) and 70 °C (30 seconds, extension). Relative expression of miR-128 and let-7b was analyzed by the comparative Ct method (2−ΔΔCt) (Livak and Schmittgen, 2001), using SNORD 68 RNA as the endogenous control. Data were expressed as the fold change in gene expression in the patients normalized to the expression levels of the endogenous control and relative to the healthy controls.

Statistical methods
Statistical analysis was done using IBM© SPSS© version 22. Numerical data were expressed as means and standard deviation or medians and ranges as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher’s exact test was used to examine the relation between qualitative variables. For the non-normally distributed data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Spearman-rho method was used to test correlation between numerical variables. Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. Relation between the expressions of two genes was done using Mc-Nemar test. All tests were two tailed. A p-value < 0.05 was considered significant.

Results
The study was done on 70 consecutive newly diagnosed pediatric acute lymphoblastic leukemia (ALL) patients, out of which, 56 were evaluated for both miR-128 and let-7b and all 70 patients were evaluated for let-7b together with seven age and sex matched healthy controls.

Expression levels of miR-128, let-7b and SNORD 68 RNA were determined by real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR).

Mean value of expression level of miR-128 in control group was 0.035 and this value was taken as a cut off, where patients having values above this cut off were considered as high expressers for miR-128 and patients having values below this cut off were considered as low expressers for miR-128. Similarly, the mean value of expression level of let-7b in the control group was 0.0437 and this value was taken as cut off for classifying patients as high or low let-7b expressers. (Demographic and laboratory characteristics of patients are summarized in Table 1).

The expression of miR-128 was statistically significantly elevated in ALL patients compared with control group (p< 0.001). However, no statistical significant difference was detected between two groups as regard let-7b expression (p=0.126) (Table 2).

No statistically significant differences were encountered between high and low miR-128 and let-7b expressers as regards age, sex, organomegaly or lymphadenopathy. Neither were there significant difference between high and low miR-128 and let-7b expressers with regards to Hb level, TLC, platelet count, peripheral blood, bone marrow blasts or immunophenotyping (Table 3).

High let-7b expressers show positivity toward t(9;22) at presentation than those with low expression but due to small number of patients in one of the cells we can’t do statistical analysis. However, no statistical significant difference was detected between high and low miR-128 expressers regarding cytogenetic abnormalities (Table 3).

No statistically significant differences were detected between high and low expressers of miR-128 and let-7b as regards response to induction therapy (p=0.45 and 0.18 respectively) (Table 4).

In our study, eight patients died, out of which 6 (75%) show low expression and 2 (25%) show high expression for let-7b. As regard miR-128 out of eight died patients only five were evaluated for miR-128 where 4 (80%) were high expressers and one (20%) show low expression.

The median follow up duration was 15.2 months (range 0.03-35.6 months). At the end of the study eight patients died. Median overall survival was not reached. 87.8% of the patients were still alive at 18 months.

Discussion
Acute lymphoblastic leukemia represents 75% of
pediatric leukemia and 30% of childhood malignancies and despite of intensive combination therapy which improve the therapeutic outcome, 15 to 20% of patients fail to achieve response to treatment and relapse is the main reason for therapeutic failure (Kaatsch et al., 2010; Pui et al., 2006).

Altered expression of microRNAs (miR) has been observed in association with different hematological malignancies which could serve as a marker for detection of leukemia, affecting prognosis and also predict for relapse (Tombak et al., 2015).

In this study, we evaluated the expression of miR-128 and let-7b in bone marrow of newly diagnosed pediatric ALL patients and correlated their levels with clinical and laboratory data especially that are known to affect the prognosis.

In our study, we found that miR-128 was significantly higher in pediatric ALL patients compared to control group (P<0.001). Similar results were obtained by Nemes et al., (2015) who reported higher expression of miR-128b in ALL cells obtained from pediatric patients. Similarly, Duyu et al., (2014) observed that miR-128 was significantly up regulated in ALL patients at diagnosis compared to control group and the most discriminatory miRNAs involving ALL and AML being significantly highly expressed in ALL than in AML.

Also, De Oliveria et al., (2012); Zhu et al., (2012), wang et al., (2010); Zhang et al., (2009) and Mi et al., (2007) all reported that miR-128 is significantly expressed in pediatric ALL patients at diagnosis compared to control group and the most discriminatory miRNA between ALL and AML.

In our study, no statistical significant difference was found between miR-128 expression level and age, gender, clinical parameters, hematological parameters (white blood cell count, hemoglobin, platelets, peripheral blood and bone marrow blasts), immunophenotyping and karyotyping. Similar results were reported by Nemes et al., (2015).

Also, no statistical significant difference was found regarding response to treatment. In contrary to our results, Nemes et al., 2015 showed a significant correlation between lower miR-128 expression and the poor prognosis, as well as the poor response to prednisolone on day eight.

As regard let-7b, no statistical significant difference

| Parameter                      | Findings  | Age | 7.3 ± 5.1* |
|--------------------------------|-----------|-----|------------|
| Age                            |           |     |            |
| < 2 years                      | 11 (15.7)** |
| ≥ 2 years                      | 59 (84.3)** |
| Sex                            |           | Male | 37 (52.9)** |
|                               |           | Female | 33 (47.1)** |
| Hepatomegaly                   | 42 (60)** |
| Splenomegaly                   | 41 (58.6)** |
| Lymphadenopathy                | 37 (52.9)** |
| Total leukocytic count x 10^9/L | 86.49 ± 154.41* |
| < 50 X 10^9/L                  | 49 (70)** |
| ≥ 50 X 10^9/L                  | 21 (30)** |
| Hemoglobin gm/dl               | 8.01 ± 2.1* |
| < 7 gm/dl                      | 23 (32.9)** |
| ≥ 7 gm/dl                      | 47 (67.1)** |
| Platelet x 10^9/L              | 81.0 ± 118.14* |
| < 100                          | 57 (81.4)** |
| ≥ 100                          | 13 (18.6)** |
| Peripheral blood blasts        | 54.3 ± 33.4* |
| < 10                           | 13 (18.6)** |
| ≥ 10                           | 57 (81.4)** |
| Bone marrow blasts             | 87.3 ± 12.40* |
| < 90                           | 23 (32.9)** |
| ≥ 90                           | 47 (67.1)** |
| Bone marrow cellularity        |           | Normocellular | 14 (20)** |
|                               |           | Hypercellular | 53 (75.7)** |
|                               |           | Hypocellular | 3 (1.4)** |
| Cytogenetics and molecular     |           | Normal karyotype | 56 (80)** |
|                               |           | Hyperdiploidy | 5 (7.1)** |
|                               |           | t (1;19) | 1 (1.4)** |
|                               |           | t (9;22) | 5 (7.1)** |
|                               |           | t (12;21) | 3 (4.3)** |
| Immunophenotyping              |           | B-phenotype | 57(81.4)** |
|                               |           | Pre-B | 43 (61.4)** |
|                               |           | Common-ALL | 14 (20)** |
| T-phenotype                    |           | Early-T | 8 (11.4)** |
|                               |           | Intermediate-T | 3 (4.3)** |
|                               |           | Late-T | 2 (2.9)** |
| CD 34                          |           | Positive | 39 (55.7)** |
|                               |           | Negative | 31 (44.3)** |

Table 1. Clinical and Laboratory Characteristics of 70 Pediatric Acute Lymphoblastic Leukemia

Table 2. Expression of miRNA-128 And Let-7b In Pediatric Acute Lymphoblastic Leukemia Patients Versus Control Group

| Gene expression | ALL group | Control group | P-value |
|-----------------|-----------|---------------|---------|
| miRNA-128       | (n=56)    | (n=7)         | <0.001  |
| Over expression | 47 (83.9)** | 1 (14.3)**    |         |
| Under expression| 9 (16.1)** | 6 (85.7)**    |         |
| Let-7b          | (n=70)    | (n=7)         | 0.126   |
| Over expression | 33 (47.1)** | 1 (14.3)**    |         |
| Under expression| 37 (52.9)** | 6 (85.7)**    |         |

**, Number (%)
Table 3. Characteristics of Pediatric ALL Patients According to miRNA-128 and Let-7b Expression

| miRNA-128          | P-value | Let-7b          | P-value |
|--------------------|---------|-----------------|---------|
|                    | Over expression (n=45) | Under expression (n=8) |          | Over expression (n=31) | Under expression (n=33) |
| Age                |          |                 |          |                  |                   |
| < 2 years          | 0.658   | 0.9             |          |                  |                   |
| ≥ 2 years          | 1       |                 |          |                  |                   |
| Sex                |          |                 |          |                  |                   |
| Male               | 0.658   | 0.9             |          | 0.241            | 0.832            |
| Female             | 0.9     |                 |          |                  |                   |
| Hepatomegaly       | 0.529   | 0.282           |          |                  |                   |
| Splenomegaly       | 0.481   | 0.518           |          |                  |                   |
| Lymphadenopathy    | 0.716   | 0.832           |          |                  |                   |
| Total leukocytic count x 10^9/L | 0.073   | 0.638 |          |                  |                   |
| < 50 X 10^9/L      | 35 (74.5)** | 4 (44.4)**     | 0.246   | 0.348            |                   |
| ≥ 50 X 10^9/L      | 12 (25.5)** | 5 (55.6)**     | 0.482   | 0.937            |                   |
| Hemoglobin gm/dl   |          |                 |          |                  |                   |
| <7 gm/dl           | 14 (29.8)** | 1 (6.7)**      | 9 (27.3)** | 14 (37.8)**      |                   |
| ≥7 gm/dl           | 33 (70.2)** | 8 (19.5)**     | 24 (72.7)** | 23 (62.2)**      |                   |
| Platelet x 10^9/L  |          |                 |          |                  |                   |
| <100               | 37 (78.8)** | 8 (17.8)**     | 27 (81.8)** | 30 (81.1)**      |                   |
| ≥100               | 10 (21.3)** | 1 (9.1)**      | 6 (18.2)** | 7 (18.9)**       |                   |
| Peripheral blood blasts | 50.1±32.8* | 69.8±36.4*    | 0.17    | 0.115            |                   |
| Bone marrow blasts | 87.6±10.9* | 90.9±5.6*      | 0.63    | 0.244            |                   |
| Bone marrow cellularity | 0.834   |           |         | 0.12             |                   |
| Normocellular versus hypocellular | 12 (25.5)** | 2 (22.2)**    | 10 (27)** | 4 (12.1)**       |                   |
| Hypercellular      | 35 (74.5)** | 7 (77.8)**     | 27 (73)** | 29 (87.9)**      |                   |
| Immunophenotyping  |          |                 |          | 0.937            |                   |
| B-ALL              | 39 (83)** | 6 (66.7)**     | 27 (81.8)** | 30 (81.0)**      |                   |
| T-ALL              | 8 (17)** | 3 (93.3)**     | 6 (18.2)** | 7 (19.0)**       |                   |
| CD34               | 0.719   | 0.937          |          |                  |                   |
| Positive           | 4 (44.4)** | 26 (55.3)**   | 15 (45.5)** | 16 (43.2)**      |                   |
| Negative           | 5 (55.6)** | 21 (44.7)**   | 18 (54.5)** | 21 (56.8)**      |                   |
| Cytogenetics       |          |                 |          |                  |                   |
| Hyperdiploidy      | 5 (10.5)** | 0 no           | 1 (3)**  | 4 (10.8)**       | 0.36              |
| t(1;19)            | 1 (2.1)** | 0 no           | 0 (0.2)** | 0 (0.5)** no     |                   |
| t(9;22)            | 3 (6.4)** | 2 (22.2** 0.178 | 5 (15.2)** | 0 (0.2)**    | 0.02              |
| t(12;21)           | 3 (6.4)** | 0 no           | 1 (3)**  | 2 (5.4)** no     |                   |

* Mean ± SD, ** Number (%)

Table 4. Response to Induction Therapy According to Mir-128 and Let-7b Expression Levels in Pediatric ALL Patients

| miRNA-128          | P-value | Let-7b          | P-value |
|--------------------|---------|-----------------|---------|
|                    | Over expression (n=45) | Under expression (n=8) |          | Over expression (n=31) | Under expression (n=33) |
| Hematological response | 0.185  | 0.185           |          |                  |                   |
| Complete remission | 41 (91.1)** | 6 (75)**     | 27 (87.1)** | 30 (85.7)**      |                   |
| Not in complete remission | 4 (8.9)** | 2 (25)**     | 4 (12.9)** | 5 (14.3)**       |                   |
| Minimal Residual Disease (MRD) | 0.453  |           |         | 0.135            |                   |
| Complete remission | 25 (55.6)** | 3 (37.5)**   | 19 (61.3)** | 15 (42.9)**      |                   |
| Not in complete remission | 20 (44.4)** | 5 (62.5)** | 12 (38.7)** | 20 (57.1)**      |                   |

** Number (%)
was found in its expression level between pediatric ALL patients and control group \( p=0.126 \). In contrary to our results, Zhu et al., (2012) and Mi et al., (2007) reported significant down regulation in let-7b level in pediatric ALL patients compared to pediatric AML patients and control group. Also, Wang et al., (2010) observed that let-7b was expressed at a significantly higher level in AML compared to ALL indicating the role of let-7b mainly as a discriminatory miRNA marker between ALL and AML rather than a diagnostic marker for ALL.

In conclusion, overexpression of miR-128 is characteristic of childhood ALL which may help to provide new insights in the diagnosis of childhood ALL. The role of the let-7b in pediatric ALL has to be verified by further large scale sample and further studies.

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