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RESEARCH ARTICLE

Association between microRNA-21, microRNA-150, and micro-RNA-451 expression and clinical outcome of patients with acute lymphoblastic leukemia

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BACKGROUND: Acute lymphoblastic leukemia (ALL) occurs owing to the defective maturation, increased proliferation, and lack of differentiation of lymphoid cells. Evaluation of the expression levels of microRNAs (miRNAs) could help in the prognosis and improve the clinical outcome of ALL patients. Given the role of miR-21, miR-150, and miR-451 as oncogenes and tumor suppressors in lymphocytes, this study explored the relation between the expression levels of these miRNAs and the clinical outcomes of ALL patients.
METHODS: cDNA synthesis and RT-PCR were performed for peripheral blood samples from 41 patients with ALL, as well as for U937 and Jurkat cell lines to examine the expression of miR-451, miR-150, and miR-21 after miRNA purification. We also performed an epidemiological analysis in which Mann–Whitney and Chi-square tests were used to investigate the relationship between the expression of miRNAs and other clinical and laboratory data. Binary logistic regression models were used to estimate the odds ratio in univariate and multivariate analyses for clinical outcomes.
RESULTS: miR-21 and miR-150 expression was found to be decreased, while miR-451 expression showed no difference compared to the control group. There was a significant relationship between miR-451 expression and hemoglobin (Hb) levels, as well as between miR-150 expression and clinical outcomes of ALL patients.
CONCLUSION: Increased expression of miR-451 decreased the Hb levels; reduced expression of miR-150 was associated with increased relapse rate in patients. Age, increased WBC, and decreased Hb levels were associated with increased relapse rates in ALL patients. Therefore, miR-150 could be used as a biomarker to determine the clinical outcome of ALL patients.

Keywords acute lymphoblastic leukemia, miRNA, clinical outcome

Introduction

Acute lymphoid leukemia (ALL) is the most common heterogeneous hematologic malignancy in children, and is characterized by an increased number of blast cells in the bone marrow and peripheral blood. ALL is associated with specific molecular abnormalities and shows different clinical features (Anindo and Yaqinuddin, 2012; Lawrie, 2013). Therefore, there are differences in the prognosis of ALL patients belonging to similar ages or risk groups. Epigenetic factors such as microRNAs (miRNAs) have been reported to contribute to these differences in ALL patients (Anindo and Yaqinuddin, 2012; Duyu et al., 2014). MiRNAs form a family of small (18-25 nucleotide), single stranded, non-coding RNAs that regulate gene expression by targeting and degrading mRNAs, as well as by affecting post-translational mechanisms in proteins (Gordon et al., 2013).

MiRNAs play an important role in the regulation of hematopoiesis, and dysregulation of their expression could result in malignancy and leukemogenesis. MiRNAs also play important biological roles in apoptosis, cell growth and differentiation, and response to stress. MiRNAs have been reported to act as both oncogenes and tumor suppressors (Anindo and Yaqinuddin, 2012; Babashah et al., 2012). Abnormal expression of miRNAs has been reported in several

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hematological malignancies, including lymphoma and leukemia. Alteration of miRNA expression may be caused by genetic and epigenetic changes or by modification of their biogenesis pathways (Anindo and Yaqinuddin, 2012; Zhao et al., 2010). Polymorphisms in miRNA genes or their target sites (miR SNPs) can also affect the function of miRNAs. Several studies have indicated abnormal expression of miRNAs in ALL patients (Fig. 1) (Salzman and Weidhaas, 2013). MiR-21 is an oncomiR overexpressed in a large number of cancers, including breast and prostate cancer, leukemia, lymphoma, and glioblastoma (Yan et al., 2008). Studies have shown that miR-21 is essential for growth and proliferation pathways, as well as for maintenance of leukemic cells, and that its overexpression is associated with poor prognosis in patients (Shi et al., 2016). Some studies have indicated that it plays an important role in the development of drug resistance in solid tumors (Rossi et al., 2010). MiR-150 is a lymphoid-specific tumor suppressor miRNA expressed in T- and B cells, causing increased activity, growth, and proliferation. This miRNA has been reported to have several roles such as inhibition of proliferation and induction of apoptosis in malignant cells. Reduced expression of MiR-150 has been observed in malignant cells in several types of leukemia, indicating its role as a tumor suppressor (Ghisi et al., 2011). MiR-451 is a tumor suppressor miRNA that shows reduced expression during the early stages of tumor activity. Studies have shown that this miRNA also shows reduced expression during the transformation of T cells to malignant cells in ALL (Li et al., 2011). Analyzing the expression levels of miRNAs could help in identifying their different biologic functions and in recognizing their roles in the different stages of ALL, thus improving the treatment of these patients. Based on these results that indicate the role of miR-21 as an oncomiR as well as that of miR-150 and miR-451 as tumor suppressors in T- and B cells during cancer, we examined the expression of miR-21, -150, and -451 in ALL patients and its role in the clinical outcome of patients with this malignancy.

Materials and methods

Study group: patients and samples

ALL diagnoses were confirmed when the bone marrow (BM) aspirate was found to contain at least 30% blast cells, based on the FAB classification. After morphologic, cytogenetic, and clinical examination of 41 ALL patients, they were enrolled in this study. BM samples (5 mL) were collected from each patient in falcon tubes containing EDTA anticoagulant. The subjects included 24 males (58.53%) and 17 females (41.46%) (ages ranged from 1 to 26 years; median age: 8.05 years). For assessing the quality and efficiency of the real-time polymerase chain reaction (RT-PCR), a T-ALL-lineage Jurkat cell line (suspension, lymphoblast-like,

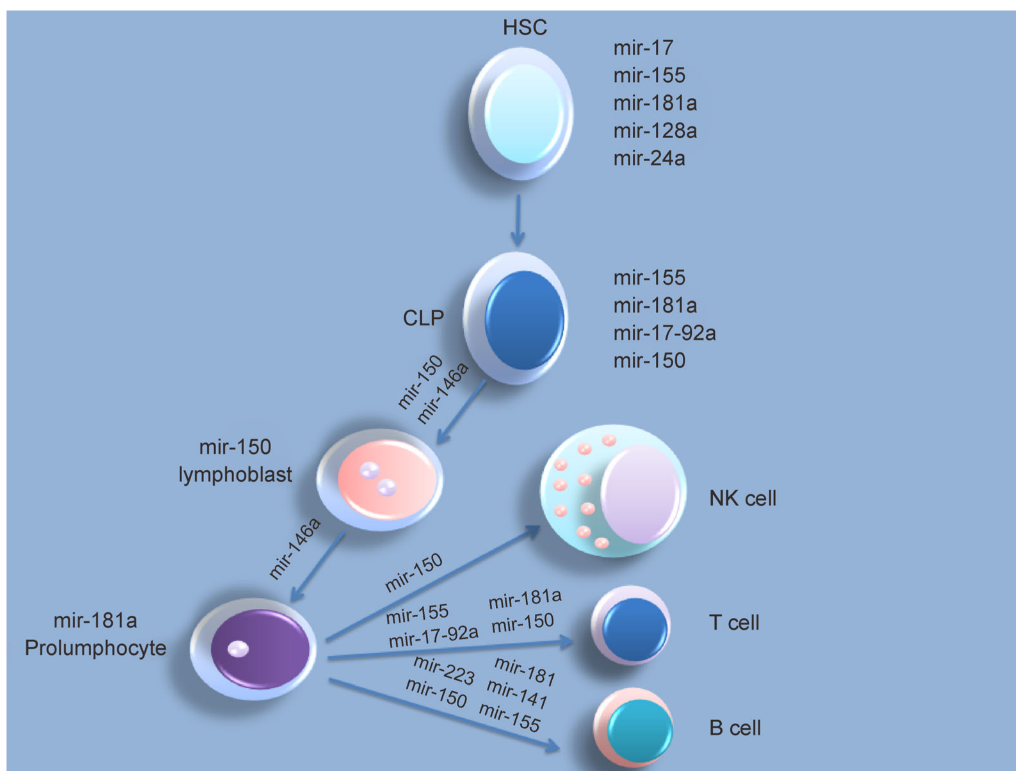

Figure 1 MiRNAs involved in development and differentiation of lymphoid cells. MiRNAs affect different stages of lymphoid cell proliferation and differentiation from HSCs to mature B, T, and NK cells. Abbreviations: HSC, hematopoietic stem cell; CLP, common lymphoid progenitor; NK cell, natural killer cell.

obtained from ALL patients) that expressed miR-21, -150, and -451 in low levels was used as the negative control. A U937 (suspension, monocyte-like, derived from histiocytic lymphoma lymphocyte and myeloid cells) cell line was also used for comparison of the expression levels with the Jurkat cell line. The cell lines were purchased from the Pasteur Institute of Iran and cultured in a RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 2 mmol/L glutamine, 25 mmol/L HEPES, 1.5 g/L sodium bicarbonate, 10% fetal calf serum, 50 U/ml penicillin, and 50 g/ml streptomycin at 37°C in 5% CO_2. Among the subjects, 38 cases (92.68%) were B-lineage ALL, while 3 (7.31%) were T-lineage ALL. The 41 healthy control subjects, matched for age (1-26 years old; median age: 8.05 years) and sex (including 24 males [58.53%] and 17 females [41.46%]), with no morphologic, cytogenetic, and clinical disorders were also enrolled in the study. Patients were treated based on the ALL protocol used in the Shafa Hospital in Ahvaz. They received induction therapy with a combination of drugs, including vincristine, prednisone, cyclophosphamide, doxorubicin, and L-asparaginase, which were administered over 4-6 weeks. All demographic, clinical, and laboratory features of the patients and the cell lines used, including the WBC and platelet count, hemoglobin (Hb) count, age, sex, clinical outcome, and organomegaly, are presented in Table 2. All peripheral blood samples were collected within four months after obtaining written informed consent from the subjects. The protocols used in this study were approved by the local ethics committee of the Ahvaz Jundishapur University of Medical Sciences (AJUMS.REC.1393.310), and was conducted within six months.

MiRNA extraction, cDNA synthesis, and real-time PCR assay

After drawing blood samples and isolating the peripheral blood mononuclear cells using a Ficoll-Hypaque centrifugation gradient, the miRNA was extracted according to the isolation protocol for the RIBO-Prep kit (Toronto, Canada). The quantification of the extracted miRNAs was performed by measuring the absorbance at 260 nm. cDNA synthesis was performed as described below. First, 1.5 μL of the specific primer, 3 μL RNA, and 15.5 μL distilled water were mixed. This mixture was placed in an ABI Step One Plus PCR instrument for 5 min in 95°C and for 10 min in 70°C. After completing this step, 3 μL RT buffer, 3 μL dNTP, 1 μL RT enzyme, and 3 μL distilled water were added to this mixture and incubated for 15 min at 25°C, 15 min at 37°C, 60 min at 42°C, and 10 min at 75°C in the instrument, respectively. The miRNA was quantified by real-time PCR with 4 μL Fermentas SYBR Green Mastermix (Fermentas Life Sciences, St Leon-Rot, Germany) in a Rotor-Gene 6000 system (Corbett, Concorde, NSW, Australia) according to manufacturer's instructions. After quantification, 2 μL template, 2 μL target specific stem loop primers, and 12 μL distilled water were added. The initial polymerase activation was done at 95°C for 15 min. For miRNA quantification, 40 amplification cycles were performed at 95°C for 10 s, 58°C for 20 s, and 72°C for 20 s, and the mRNA was then detected with fluorescence detection. The test was performed in duplicate for each sample. In addition, we followed the guidelines for the minimum information for publication of quantitative RT-PCR experiments (MIQE) to ensure more reliable results (Bustin et al., 2009). The relative expression levels of the target miRNAs were quantified using the comparative cycle threshold method. The raw data were presented as the relative amount of the target miRNAs, normalized for small nucleolar RNA and C/D box (snord) as the endogenous control. Primers for snord 47 were prepared based on the sequences derived from the study by Naderi et al. (Table 1)(Naderi et al., 2015). The primer sequences for miR-21, -150, and -451 were used for synthesizing cDNAs from the miRNAs, as shown in Table 1.

Statistical analysis

This study was an epidemiological analysis. The demographic, laboratory, and clinical data of patients lacking the expression of miR-21, -451 and -150 or with low expression of these miRNAs were compared using Mann–Whitney and

Table 1 Primer sequences of miR-21, -150, -451 and Snord 47

Primer	sequence	Primer length (bp)
miR-21	Forward: 5'- ACGTGTTAGCTTATCAGACTG A-3'	21
	Reverse: 5'- GAGCAGGGTCCGAGGT-3'	16
	RT: 5'- GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACTCAACA-3'	51
miR-451	Forward: 5'- CGAGAAACCGTTACCATTAC-3'	20
	Reverse: 5'- GAGCAGGGTCCGAGGT-3'	16
	RT: 5'-GTCGTATGCAGAGCAGGGTCC GAGGTATTCGCACTGCATACGACTCAACA-3'	51
miR-150	Forward: 5'- ACATCTCCCAACCCTTGTAC-3'	18
	Reverse: 5'- GAGCAGGGTCCGAGGT-3'	16
	RT: 5'-GGTCGTATGCAGAGCAGGGTCCGAGGTATCCATCGCACGCATCGCACTCATACGA CCCACTGG-3'	64
Snord 47	Forward: 5'- ATCACTGTAAAACCGTTCA-3'	19
	RT: 5'- GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACCACCTC-3'	51

Chi-square tests. The data for the clinical outcome (relapse time and remission time) were collected, and a follow-up was done for every patient separately after diagnosis. The OR (odds ratio) was calculated based on the relapse or remission, as well as other clinical data from the patients. Binary logistic regression models were used to estimate the OR for univariate and multivariate analyses for clinical outcome. In univariate analyses, the low expression levels of miR-21, -451, and -150 were evaluated in addition to other covariates. The significant variables (with 0.1 level of significance) in univariate analysis were considered as candidates for multivariate analyses. The relative quantification software (REST) (2009, QIAGEN, Valencia, USA) was used to analyze relative RT-PCR data (Pfaffl et al., 2002; Saki et al., 2014). The REST analysis software was used with efficiency correction, and the statistical method used was as follows: $(\mathrm{Etarget})^{\mathrm{DCP}}$ target = (MEAN control – MEAN sample)/(ERef index)$^{\mathrm{DCP}}$; Ref index = (MEAN control – MEAN sample). $p < 0.05$ was considered as statistically significant.

Results

Expression of miRNAs in ALL samples

The expression of miR-21 was found to be decreased in 8 patients (19.5%), as well as in Jurkat and U937 cells; in 33 samples (80.5%), no difference was found in level of miR-21, compared to the control (Fig. 2). The expression of miR-451 was decreased in 3 ALL patients (7.32%), as well as in Jurkat and U937 cells, but there was no difference in its expression compared to the controls in 38 cases (92.7%) (Fig. 2). MiR-150 expression was decreased in 18 cases (43.9%), as well as in Jurkat and U937 cells, while no difference was observed in its expression compared to the control group in 23 cases (56.1%) (Fig. 2 and Table 2). Overall, miR-21 and miR-150 expression was reduced in ALL samples, while miR-451 expression in ALL samples showed no difference compared to the control group (Fig. 3).

Correlation between miRNA expression and demographic, clinical, and laboratory data

In this study, the relationships between the expression levels of miR-21, -150, and -451 and the demographic, laboratory, and clinical data of patients and cell lines, including the WBC count, age, sex, clinical outcome, organomegaly, Hb count, and PLT, was assessed (Table 2). Sex, organomegaly, and clinical outcome were expressed in numbers and percentages, while the age and WBC count were expressed in medians and ranges.

There was no significant correlation between the expression level of miR-21 and the WBC count, age, sex, Hb count, clinical outcome, organomegaly, or PLT in ALL patients (p>0.05). However, there was a significant correlation between miR-451 expression and Hb levels (p = 0.004), but no correlation between miR-451 expression and WBC count, age, sex, organomegaly, clinical outcome, or PLT (p>0.05). In addition, there was a significant correlation between miR-150 expression and the clinical outcome (p = 0.049), but none between miR-150 expression and the WBC count, Hb, age, sex, organomegaly, or PLT (p>0.05). In our study, among the 41 patients, 3 were BCR-ABL-positive, 2 were 11q23-positive, and 1 showed t(1;14), a rare chromosomal abnormality. Our findings showed no significant relationships between the expression levels of miR-21, –150, and –451 and any of the measured parameters in these patients.

Relationship between clinical outcome and other covariates in ALL patients

Univariate analysis for clinical outcome as a dependent parameter was performed, along with other covariates in the ALL patients. Age (OR = 0.001, p = 0.001), Hb count (OR = 0.57, p = 0.004), WBC count (OR = 0.61, p = 0.004), and miR-150 expression level (OR = 3.8, p = 0.007) were found to be significant predictors of the clinical outcome (Table 3). According to our results, younger age, low Hb and WBC counts, as well as decreased expression of miR-150 were all associated with increased relapse rates in patients. There was no significant correlation between PLT, sex, and miR-21 and –451 expression levels and clinical outcome (Table 3). In addition, multivariate analysis for clinical outcome as a dependent parameter was performed with other covariates in ALL cases. Only WBC count (OR = 0.69, p = 0.02) was found to be a significant predictor of clinical outcome (relapse-remission) in ALL patients (Table 4).

Discussion

ALL is a hematological malignancy associated with increased proliferation and defective maturation of lymphoid blast cells. MiRNAs have also been reported to be involved in the proliferation and differentiation of blast cells. Therefore, altered expression of miRNAs might indicate malignancy of the cells and leukemogenesis (Zhao et al., 2010; Anindo and Yaqinuddin, 2012; Salzman and Weidhaas, 2013). In this study, we examined the expression levels of miR-21, -150, and -451 and their correlation with clinical and laboratory data from ALL patients. The relationships between the expression levels of miRNAs and other laboratory parameters were assessed to determine the factors that predict the clinical outcome of ALL patients. Our results showed that the expression levels of miR-451 were not different in ALL patients and controls (Figs. 2, 3). In addition, no significant correlation was found between miR-451 expression and the clinical outcome (Table 1, 2) (Figs. 2, 3). In 2011, Li et al. described miR-451 as a new tumor suppressor that inhibited the initiation and progression of leukemogenesis through the

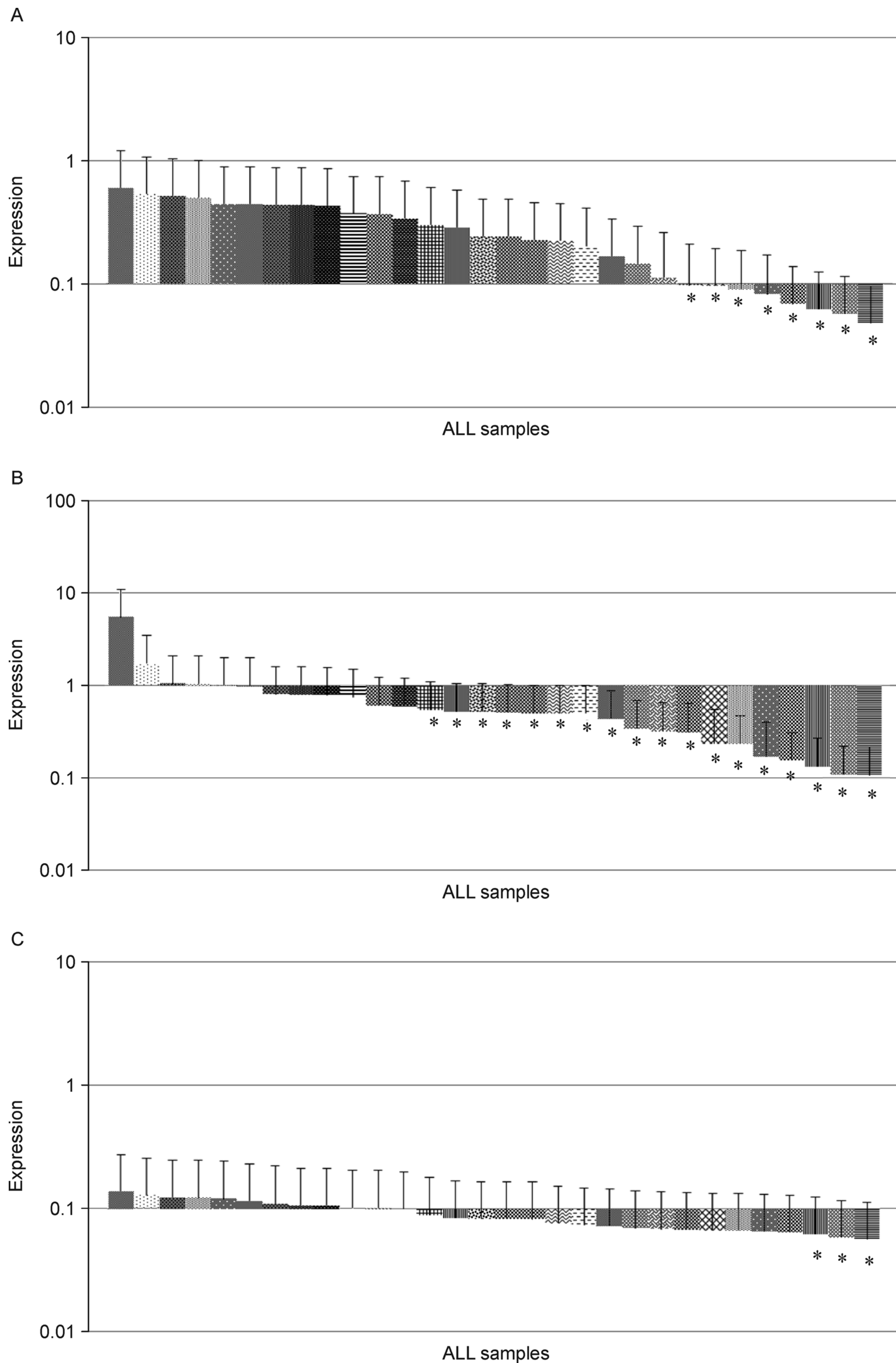

Figure 2 Expression analysis of miR-21 (A), miR-150 (B), and miR-451(C) in 41 ALL samples using real-time PCR.

Notch-1 pathway. Myc is a Notch-1 target essential for the formation and proliferation of T-ALL cells. They showed that ICN1 caused the degradation of E2a and the decreased expression of miR-451 in T-ALL cells, which lead to the increased expression of several oncogenes such as Myc, Akt, and Ras-GRF1 (Li et al., 2011; Saki et al., 2015). Some studies have also shown that the expression levels of miR-451 decreased in AML patients who showed normal cytogenetics,

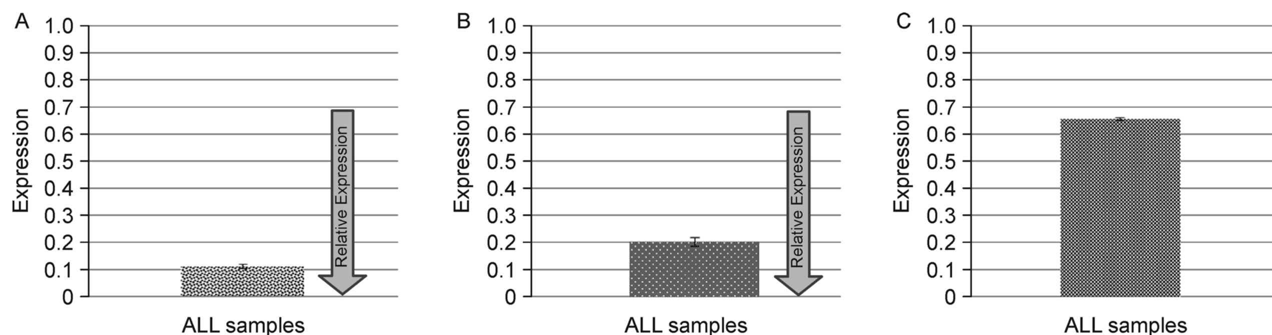

Figure 3 Relative expression levels of miRNAs in ALL samples showing downregulation of miR-21(A) and miR-150 (B) expression levels, and no difference in miR-451 (C) expression level, compared to the control group.

Table 2 Demographic, laboratory and clinical data in ALL cases according to miR-21, miR-451 and miR-150 expression

Clinical characteristics		miR-21 (Down regulation) ($n = 8$)	miR-21 (ND) ($n = 33$)	*p* value	miR-451 (Down regulation) ($n = 3$)	miR-451 (ND) ($n = 38$)	*p* value	miR-150 (Down regulation) ($n = 18$)	miR-150 (ND) ($n = 23$)	*p* value
Sex	Male	7	17	0.11	3	21	0.25	10	14	0.73
	Female	1	16		0	17		8	9	
Organomegaly	yes	1	10	0.41	1	10	0.8	5	6	0.91
	no	7	23		2	28		13	17	
Clinical outcome	Remission	3	9	0.67	2	10	0.20	8	4	0.049
	Relapse	5	24		1	28		10	19	
Age		6.5 (3-14)	5 (1-18)	0.62	14 (6-15)	5 (1-18)	0.11	5.75 (1-18)	5 (2.5-18)	0.42
WBC		8.15 (2.5-20)	6.7(1.4-50)	0.84	15 (10-20)	5.9 (1.4-50)	0.18	10 (1.4-37)	5.2 (2.5-50)	0.23
Hb		6.45 (2.2-12)	7.8(4.1-14)	0.29	12(10.9-13)	7.05(2.2-14)	0.004	7.85(4.1-12.5)	7(2.2-14)	0.77
PLT		50.5(30-279)	52(10-400)	0.80	123(102-125)	47(10-400)	0.08	45(10-279)	52(20-400)	0.71

Abbreviations: WBC, white blood cell; Hb, hemoglobin; PLT, platelet; ND, No difference.

Table 3 Univariate analysis for clinical outcome (relapse-remission) as dependent parameter with other covariates in ALL cases

	p value	OR	95% CI
Age	0.001	0.74	(0.61 , 0.89)
Hb	0.004	0.57	(0.38 , 0.84)
PLT	0.12	0.99	(0.98 , 1.01)
WBC	0.004	0.61	(0.43 , 0.86)
Sex (Male vs. Female)	0.48	1.64	(0.42 , 6.36)
21 expression (vs. down regulation)-MiR	0.57	1.6	(0.32 , 8.11)
451 expression (vs. down regulation)-MiR	0.18	0.18	(0.02 , 2.20)
150 expression (vs. down regulation)-MiR	0.007	3.8	(0.92 , 15.78)

Abbreviation: WBC, white blood cell; Hb, hemoglobin; PLT, platelet; OR, odds ratio; CI, confidence interval.

Table 4 Multivariate analysis for clinical outcome (relapse-remission) as dependent parameter with other covariates in ALL cases

	p value	OR	95% CI
Age	0.13	0.72	(0.47 , 1.10)
WBC	0.02	0.69	(0.51 , 0.95)
Hb	0.37	0.70	(0.32 , 1.53)
150 expression (vs. down regulation)-MiR	0.42	3.94	(0.15 , 106.7)

Abbreviation: WBC, white blood cell; Hb, hemoglobin; OR, odds ratio; CI, confidence interval.

which plays an important role in pathogenesis (Whitman et al., 2010). Lopotova et al. showed that the increased expression of miR-451 in CML patients caused the inhibition of BCR/ABL, which is a major target of miR-451 and plays a key role in reducing the expression of miR-451 as well as in leukemogenesis (Lopotová et al., 2011). In this study, we found a significant correlation between miR-451 expression and the Hb levels in ALL patients (Table 2; Figs. 2, 3). The level of Hb in our patients may indicate a relationship between the expression levels of miR-451 and erythroid series. Studies have shown that the increased expression of miR-451 improves the proliferation of cells in the erythroid lineage. Our results showed that the expression level of miR-451 was not different in ALL patients and the control group, and that there was a correlation between its expression and Hb levels (Table 2, 3; Figs. 2, 3). MiR-150 regulates Myb, a transcription factor that binds to DNA and regulates hematopoiesis. The transcriptional activity of Myb is stimulated by P27 in erythroid progenitors. Myb also causes C-kit expression, which is a critical mediator of erythropoiesis (Hussein et al., 2010; Shahrabi et al., 2014). Our results showed that the overall expression of miR-150 was decreased in ALL patients compared with that of the control group (Figs. 2, 3). In 2011, Ghisi et al. showed that miR-150 was a lymphoid-specific miRNA that showed increased expression in the maturation stages of B and T lymphocytes. Notch-3 receptors play an important role in the cell cycle progression and apoptosis of lymphoid cells, and are also primary regulators of differentiation of lymphoid progenitors. They showed that the increased expression of miR-150 might suppress Notch-3 and thus regulate the proliferation and differentiation of lymphoid cells (Ghisi et al., 2011). In another study in 2008, Wang et al. found that the increased expression of miR-150 played an effective role in CLL pathogenesis. This miRNA also targeted C-myb, controlled B cell differentiation, and played an important role in the regulation of immune response (Wang et al., 2008). Given the role of miR-150 in the proliferation of B and T lymphocytes, its decreased expression in our patients was probably not associated with Notch-3 suppression, which would have caused apoptosis and ALL pathogenesis. However, a significant correlation was found between miR-150 expression and the clinical outcome of patients. Our results also showed that the overall expression of miR-21 was reduced in ALL patients compared to the control group (Figs. 2, 3). However, no significant correlation was found between miR-21 expression and clinical outcome in patients (Table 2, 3) (Figs. 2, 3). Rossi et al. in 2010 found that the increased expression of miR-21 was associated with poor prognosis in CLL patients with impaired 17pDEL. MiR-21 decreased the overall survival rates in these patients by targeting CCND2 and controlling the cell cycle. They demonstrated that miR-21 could be used as a useful diagnostic marker for this disease and for other malignancies (Rossi et al., 2010). Bai et al. in 2011 also showed that the increased expression of miR-21 (as an oncomiR) increased the survival and drug resistance of K562 cells in CML. MiR-21 also targeted PTEN and altered the PI3K/Akt pathway in these patients (BaiLopotová, 2011). Based on the expression levels of miR-21 obtained from our study, the majority of our patients were not in the early stages of disease; in addition, the decreased expression of this miRNA was associated with increased PTEN as well as increased drug sensitivity of the cells. We also found that younger age, lower Hb, and lower WBC counts were associated with increased relapse rates in ALL patients. Among these factors, WBC count was found to be the most important factor associated with the clinical outcome (Table 4).

In our pervious study, we had shown that the function of miRNAs depends on the expression and repression of other genes, transcriptional factors, and other miRNAs (Tavakoli et al., 2016). The expression levels of miR-451 and its correlation with Hb levels in ALL patients, as well as the decreased expression levels of miR-150, suggested that the expression of miR-150 leads to decreased erythroid proliferation, and therefore does not affect Myb expression or erythropoiesis. In addition, the lack of difference in the expression of miR-451 in the ALL patients and the controls probably led to the decreased levels of Hb. Additionally, we found a relationship between low Hb levels and increased relapse rates in our patients, which indicated that the decreased expression of miR-150 (as a tumor suppressor) was associated with increased relapse rates in patients with decreased Hb.

By examining the expression levels of miR-451, -150, and -21 in ALL patients, it could be stated that miR-150 would be a useful biomarker to predict the clinical outcome of ALL patients. In addition, younger age, increased WBC count, and decreased Hb levels in ALL patients were found to be associated with increased relapse rates. Furthermore, the expression levels of miR-150 and the Hb levels in ALL patients could be simultaneously used for the prediction of the clinical outcome. In conclusion, our study, involving the evaluation of clinical and laboratory data and the expression levels of miRNAs, showed that miRNAs could be important factors for predicting the clinical outcome of ALL patients.

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Compliance with ethical guidelines

Authors declare that they have no conflict of interest. All the procedures performed in the studies involving human participants were in accordance with the ethical standards of the local ethics committee of the Ahvaz Jundishapur University of Medical Sciences (AJUMS. REC.1393.310), as well as the 1964 Helsinki declaration. Written informed consent was obtained from all patients and control subjects.

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