Original Research Article

Study of MicroRNA-326 and MicroRNA-200c Expression in Pediatric Acute Lymphoblastic Leukemia

Abstract

Background: Acute lymphoblastic leukemia (ALL) is cancer of lymphocytes, characterized by over production and accumulation of cancerous immature lymphocytes known as lymphoblasts. The purpose of this research was to estimate micRNA-326 and micRNA-200c expression in children with ALL before and after chemotherapy to explore their potential value in diagnosis and prognosis of pediatric ALL.

Methods: This case control researchwas conducted on 30 children with ALL (group I) and 10 healthy children with matched age and sex as controls (group II). All participants were subjected to history taking, clinical examination and laboratory investigations including routine investigations for cases and controls (CBC on ERMA PCE-210N cell counter, serum LDH, ESR assessment), investigations for cases only (bone marrow aspiration and examination of stained films, cytochemistry, and immunophenotyping) and specific laboratory test for cases and controls: MicRNA-326 and MicRNA-200c relative expression by quantitative-real time PCR at diagnosis and after one year of chemotherapy.

Results: MicRNA-326 and micRNA-200c relative gene expression levels were significantly down regulated in ALL cases than in control group. As regard ALL subtypes micRNA-326 was significantly down regulated in T-ALL cases than in B-ALL cases. While micRNA-200c was significantly down regulated in B-ALL cases than in T-ALL. After a year of chemotherapy for ALL cases, micRNA-326 and micRNA-200c expression levels were still down regulated in relapsed cases while upregulated in cured cases.

Conclusions: The present research showed a statistically significant lower expression of micRNA-326 and micRNA-200c in newly diagnosed ALL children and in relapsed cases after one year of chemotherapy. These data suggests that micRNA-326 and micRNA-200c may play a role in diagnosis, prognosis, and early detection of relapsed cases.

Keywords: Acute Lymphoblastic Leukemia, MicroRNA-326, MicroRNA-200c, Pediatric, Diagnosis, Prognosis.

Introduction:

Acute lymphoblastic leukemia (ALL) is cancer of lymphocytes, characterised by over production and accumulation of cancerous immature lymphocytes known as lymphoblasts. These cells are over produced in bone marrow and multiply causing damage and replace normal cells[1].

ALL is the most prevalent childhood cancer and the leading cause of cancer mortality in cases under the age of 20. Although a small percentage of ALL cases are linked to inherited genetic syndromes, the underlying genetic mecanisms in many other cases remain unexplained [2].

MicroRNAs (micRNAs) are single-stranded noncoding RNAs with a length between 19 and 25 nucleotides that are typically formed from hairpin-shaped precursors [3]. They perform crucial roles in endogenous gene regulation and defending the genome from being intruded by exogenous oncogenes. This occurs through multiple mechanisms, by promoting cell differentiation, maturation, proliferation, apoptosis. Apparently, micRNA dysregulation results in disorder of cell cycle cellular functions. Recently, micRNAs have gained considerable importance for their central role in the occurrence and progression of multiple diseases, particularly malignancies and autoimmune diseases (AD) [4].

Studies on MicRNA-326 have demonstrated that it functions as a tumour suppressor gene by operating upstream of cancer-associated genes in various cancers. Several solid tumours, including gastric cancer (GC), non-small cell lung cancer (NSCLC), glioblastoma (GBM), and osteosarcoma, have been shown to have down-regulated MicRNA-326 and up-regulated its oncogenic targets [5]. Previous investigations demonstrated that overexpression of ABCA2 and ABCA3 (which are predicted target for micRNA-326) genes significantly increases the risk of multidrug resistance and relapse in children with ALL [6].

MicRNA-200c has been identified in a number of cancers, including esophageal, non-small cell lung cancer (NSCLC) and cancer bladder and prostate. MicRNA-200c downregulation may contribute to multidrug resistance, primarily by affecting epithelial-mesenchymal transition (EMT) in malignant cells [7].

The purpose of this research was to estimate micRNA-326 and micRNA-200c expression in children with ALL before and after chemotherapy to explore their potential value in diagnosis and prognosis of pediatric ALL.

Subjects and Methods:

The present research was conducted in Clinical pathology and Pediatric Departments on 30 subjects. They into two groups: Group (I): Thirty newly diagnosed ALL cases, presented to Pediatric Department, Tanta University Hospital and were followed up for one year of chemotherapy. Group (II): Ten apparently healthy children as a control group. Written informed consents was obtained from the parents of cases subjected to the research. The research was approved by the Ethical Committee of Faculty of Medicine, Tanta University.

Newly diagnosed pediatric ALL cases were included. Any case with malignant diseases other than ALL was excluded.

All participants were subjected to 1) Detailed history taking and thorough clinical examination. 2) Laboratory investigations including: **1) Routine investigations for cases and contrls:** a) Complete blood picture done on ERMA PCE-210N cell counter with examination of peripheral blood smears stained with Giemsa stain. b) Serum LDH: was measured using Konelab60 iThermo Scientific (Thermo Scientific -Finland) auto analyzer.c) Assessment of ESR.

2) Investigations for cases only: a) Bone marrow aspiration and examination of stained films. b) Cytochemistry (Sudan black and myeloperoxidase). c) Immunophenotyping by BD FACS CaliburFlowcytometer. Using the following T-cell markers (CD2, CD3, CD7, TdT),

B-cell markers (CD10, CD19, TdT), Myeloid markers (CD13, CD33, CD117, MPO), monocytic markers (CD64, CD14), erythroid marker (glycophorin A), megakaryocytic marker (CD61) and other non-lineage specific markers as (HLA-DR, CD34).

3) Specific laboratory test for cases and controls: MicRNA-326 and MicRNA-200c relative expression was measured by quantitative-real time PCR at diagnosis and after one year of chemotherapy.

Sampling: Two millilitres of plasma were placed in an EDTA vacutainer tube for a complete blood count and Giemsa-stained smears. 1.6 millilitre of PB was added to 0.4 ml of Na citrate (32%) for the purpose of measuring ESR. One millilitre of blood was collected into an empty tube, allowed to coagulate, and separated to measure serum LDH. One millilitre of BM aspirate in an EDTA vacutainer tube was utilised for immunophenotyping. Each subject had approximately 3 ml of fresh blood collected in sterile vacutainers with a clot activator and allowed to coagulate for 30 minutes prior to MicRNA -326 and MicRNA-200c analysis. The separated serum was aliquoted and stored at –80°C until further use.

MicRNA extraction protocol: MicRNA was isolated from the serum by using MicRNA extraction kits (miRNeasy Mini Kit, Catalog No 217004).

Real time PCR for MicRNA -326 and MicRNA-200c R-quantiation: miRCURY LNA SYBR® Green PCR Kit Catalog no.339345: 2x miRCURY SYBR® Green PCR Master Blend includes the following components: QuantiNova® DNA Polymerase is composed of Taq DNA Polymerase, QuantiNova Antibody, and QuantiNova Protection. Reference Dye ROXTM. Water without nuclease.

miRCURY LNA micRNA PCR Assay Catalog no. 339306:miRCURY LNA micRNA PCR Primer Mix, dried down.

Procedure: First-Strand cDNA Synthesis: First-strand cDNA synthesis reactions using the miRCURY LNA RT Kit (cat. no. 339340).

Quantitative, Real-Time PCR Using miRCURY LNA miRNome PCR Panels.

Statistical analysis

SPSS v26 was used to perform statistical analysis (IBM Inc., Armonk, NY, USA). The ANOVA (F) test was used to compare the mean and standard deviation (SD) of quantitative variables between the three groups. The Chi-square test was utilised to analyse qualitative variables presented as frequency and percentage (%). In addition, ROC curve analysis was performed. A two-tailed P value less than or equal to 0.05 was deemed statistically significant.

Results:

Demographic and laboratory characteristics of the studied participants aresummarised in Table 1.

Table 1. Clinical and Laboratory Characteristics of 30 Pediatric Acute Lymphoblastic Leukemia

Parameter	Findings
Age	6.83±4.46
< 2 years	6 (20.0)
≥ 2 years	24 (80.0)
Sex	
Male	19 (63.3.0)
Female	11 (36.7)
Hepatomegaly	17 (56.7.0)
Splenomegaly	17 (56.7)
Lymphadenopathy	19 (63.3)
Total leukocytic count x 109/L	34.27±25.65
< 50 X 109/L	22 (73.3)
≥ 50 X 109/L	8 (26.7)
Hemoglobin gm/dl	9.30±2.05
<7 gm/dl	18 (60)
≥7 gm/dl	12 (40)
Platelet x 109/L	93.33±39.53
<100	20 (66.7)
≥100	10 (33.3)
Peripheral blood blasts	56.8 ± 24.44
Bone marrow blasts	70.20 ± 11.67
Bone marrow cellularity	
Normocellular	10 (33.3)
Hypercellular	18 (60)
Hypocellular	2 (6.7)
Cytogenetics and molecular	
Normal karyotype	14 (46.7)
Hyperdiploidy	6 (20)
t (1;19)	2 (6.7)
t (9;22)	5 (16.6)

t (12;21)	3 (10)
Immunophentyping	
B-phenotype	19 (63.3)
T-phenotype	11 (36.7)
CD 34	
Positive	17 (56.7)
Negative	13 (43.3)

MicRNA-326 and MicRNA-200c relative expression were significantly lower in cases than controls ($P < 0.05$). Table 2

Table 2: Comparison between cases and control according to MicRNA-326 and MicRNA-200c relative expression:

		Mean ± S. D	t. test	p. value
MicRNA-326	Case	0.69 ± 0.27	6.174	0.001*
	Control	1.24 ± 0.09		
MicRNA-200C	Case	0.61 ± 0.31	6.896	0.001*
	Control	0.94 ± 0.22		

Data were expressed as mean ± standard deviation (SD), *: significant as p value < 0.05.

MicRNA-326 was significantly lowered in T-ALL cases compered to B-ALL case ($p = 0.001$). MicRNA-200c was significantly lowered in B-ALL cases compered to T-ALL cases ($p = 0.004$). Table 3

Table 3: Comparison between B-ALL and T-ALL cases according to MicRNA-326 and MicRNA-200c relative expression:

		Mean ± S. D	t. test	p. value
MicRNA-326	B-ALL	0.84 ± 0.24	5.467	0.001*
	T-ALL	0.44 ± 0.07		
MicRNA-200C	B-ALL	0.49 ± 0.26	3.155	0.004*
	T-ALL	0.81 ± 0.28		

Data were expressed as mean ± standard deviation (SD), *: significant as p value < 0.05.

MicRNA-326 was significantly lowered in relapsed cases compered to cases in remission (p value 0.026). MicRNA-200c was significantly lowered in relapsed cases compered to cases in remission (p value 0.001). Table 4

Table 4: MicRNA-326 and MicRNA-200c relative expression in relapsed cases and cases in remission:

		Mean ± S. D	t. test	p. value
MicRNA-326	Relapsed	0.49 ± 0.13	2.356	0.026*
	Cases in remission	0.75 ± 0.28		

MicRNA-200C	Relapsed	0.30 ± 0.15	3.572	0.001*
	Cases in remission	0.70 ± 0.28		

Data were expressed as mean ± standard deviation (SD), *: significant as p value < 0.05.

There was significant difference between MicRNAN-326 (p value 0.001) and micRNA-200c (p value 0.001) relative expression in ALL cases before and after chemotherapy. Table 5

Table 5: MicRNA-326 and MicRNA-200c relative expressions in ALL cases pre- and post-chemotherapy:

		Mean ± SD	t. test	p. value
MicRNA-326	Pre	0.69 ± 0.27	4.582	0.001*
	Post	1.24 ± 0.59		
MicRNA-200C	Pre	0.61 ± 0.31	6.320	0.001*
	Post	2.27 ± 1.40		

Data were expressed as mean ± standard deviation (SD), *: significant as p value < 0.05.

Regarding the ROC curve analysis of MicRNA-326 relative expression as a diagnostic marker in ALL: 1.1 was set as cutoff value for detection of MicRNA-326 relative gene expression with 72% NPV, 93% PPV, 90% Sensitivity and 80% Specificity. Figure 1

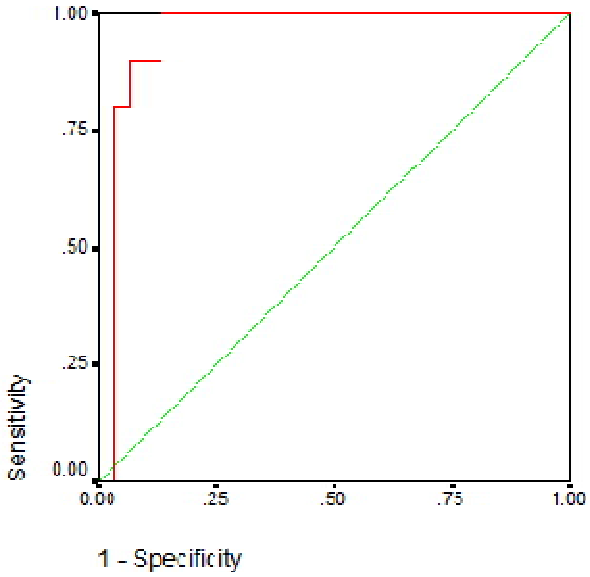

Figure 1: ROC curve analysis of MicRNA-326 relative expression as a diagnostic marker in ALL.

ROC curve analysis of MicRNA-200c relative expression as a diagnostic marker in ALL: 1.2 was set as cutoff value for detection of MicRNA-200c relative gene expression with 64% NPV, 90% PPV, 87% sensitivity and 70% specificity. Figure 2

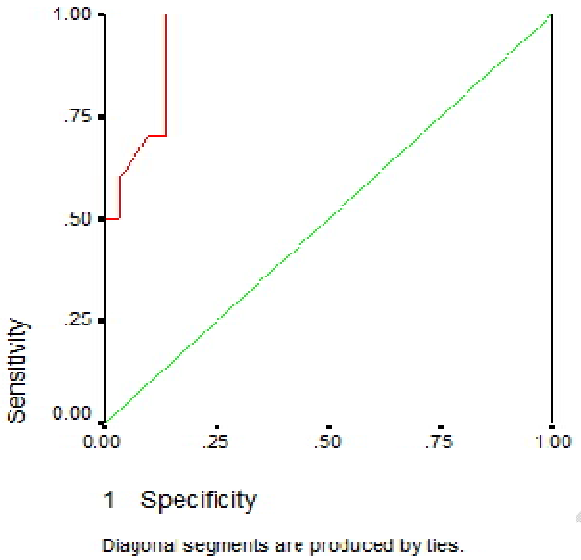

Figure 2: ROC curve analysis of MicRNA-326 relative expression as a diagnostic marker in ALL.

ROC curve analysis of MicRNA-326 relative expression as a prognostic marker in ALL: 0.60 was set as cutoff value for detection of MicRNA-326 relative gene expression with 38% NPV, 88% PPV, 65% sensitivity and 71% specificity. Figure 3

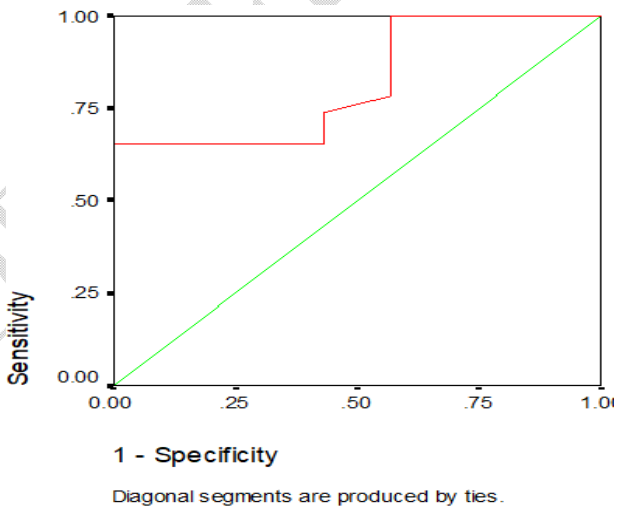

Figure 3: ROC curve analysis of MicRNA-326 relative expression as a prognostic marker in ALL

ROC curve analysis of MicRNA-200c relative expression as a prognostic marker in ALL: 0.45 was set as cutoff value for detection of MicRNA-200c relative gene expression with 56% NPV, 91% PPV, 83% sensitivity and 71% specificity. Figure 4

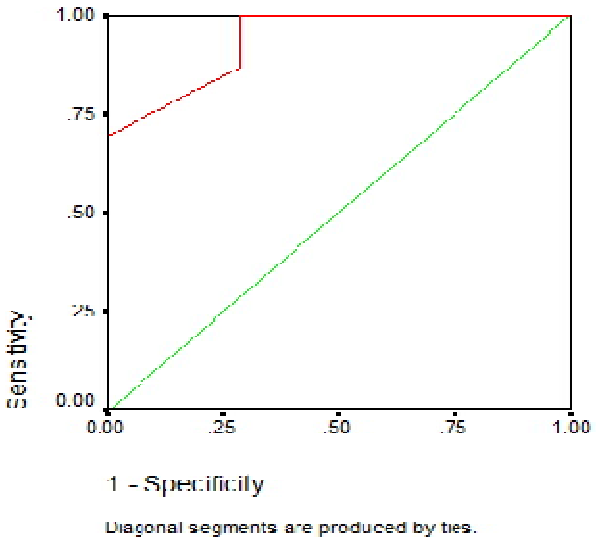

Figure 4: ROC curve analysis of MicRNA-200c relative expression as a prognostic marker in ALL.

Discussion

ALL is an acute form of leukemia of lymphocytes, characterised by overproduction of cancerous, immature lymphocytes known as lymphoblast. Lymphoblasts are overproduced in the bone marrow and continuously multiply causing inhibition of normal marrow elements [8]. MicRNA-326 and micRNA-200c may be involved specifically in leukaemogenesis and serve as potential reliable non-invasive diagnostic biomarkers of paediatric ALL [9].

The positive data of clinical evaluation of cases group was 60% cases had hepatosplenomegaly, 66.7% had lymphadenopathy, 46.6% presented with fever, 36.7% presented with bleeding, This was consistent with the findings of Banihashem et al. [10], who reported that children with ALL typically exhibit symptoms of bone marrow infiltration and extra-medullary infiltration. Among the clinical manifestations are fatigue, pallor, petechiae, bleeding, and fever. Additionally, lymphadenopathy and hepatosplenomegaly may be signs of leukemic spread. Other symptoms and signs include loss of weight, bony pain, breathlessness, and infection.

In the present research, there were statistically significant differences regarding to laboratory parameters (LDH, ESR, hemoglobin, white blood cells count, platelets and marrow blast) among the studied groups.

The current research demonstrated that serum LDH level was elevated in ALL cases in comparison with the control group. This was in agreement with Bishop[11], who reported that prior to treatment, mild to moderate increase in serum LDH are usual due to cell destruction and tumor cells turnover.

ESR value in ALL cases was higher than that in control group. A significant difference existed between the newly diagnosed ALL cases and controls. Brochmanet al. [12] reported that ESR is elevated in many malignancies including ALL due to serum monoclonal antibody.

As regard hemoglobin levels the mean value in cases' group was significantly lower than in controls, this indicates that anemia is a direct consequence of lymphoblasts' heavy and diffuse bone marrow infiltration (Whitlock and Gaynon, 2004).

Considering the white blood cells count, it was high in most leukemic cases while in controls total white blood count was in normal range. Significant differences existed between the two groups. Kjeldsberg explained this phenomenon as the result of the uncontrolled proliferation of blast cells in the peripheral blood and bone marrow.

Almost all ALL cases in the present research had moderate to severe thrombocytopenia (thrombocytopenia was manifested with bleeding manifestations). Significant differences existed between the two groups. Tkachuk and Hirschmann reported that the reduced platelet count in ALL is typically the consequence of marrow infiltration or chemotherapy, which was supported by our findings.

In this research also, blast cells percentage in the bone marrow of ALL cases at diagnosis ranged from 49 to 92%. Usmani et al.[8] reported that immature lymphocytes known as

lymphoblast are continuously overproduced in the bone marrow and multiply causing inhibition of normal marrow elements.

In the current research there was down regulation of MicRNA-326 and MicRNA-200c relative gene expression in newly diagnosed ALL cases compared to the controls.

As regard micRNA-326 relative expression, it was downregulated in ALL cases when compared to controls. There was significant difference between the 2 groups.

Also, micRNA-200c showed downregulation in ALL cases when compared with the controls.

Expression of micRNA-326 and micRNA-200c in this research was consistent with the findings of Ghodousi and Rahgozar[9], who reported that both MicRNA-326 and MicRNA-200c had relative expression levels. In addition, the ROC analysis between ALL cases and non-cancer controls revealed a larger total AUC value, which indicates the overall performance of the medical test in correctly differentiating between two situations. Consequently, the total area under the ROC curves indicated that MicRNA-326 and MicRNA-200c levels may function as negative biomarkers for discriminating between ALL cases and non-cancerous controls.

In addition, Lv et al.[13] provided evidence that micRNA-326 and micRNA-200c were downregulated in ALL cases and may modulate a variety of transport proteins to affect treatment response.

Considering ALL phenotypes, micRNA-326 expression was reduced in T-ALL cases compared to B-ALL cases. Significant differences existed between the two phenotypes. In contrast to micRNA-326, micRNA-200c was under-expressed in B-ALL more than T-ALL cases.

These was in harmony with Ghodousi and Rahgozar[9] who discovered a significant variation in micRNA expression between the two ALL phenotypes; T-ALL cases had a lower

expression of micRNA-326 than B-ALL cases. In contrast, B-ALL cases had lower micRNA-200c expression levels than T ALL cases.

After one year of chemotherapy micRNA-326 relative expression was still downregulated when comparing relapsed cases to cases in remission. Significant differences existed between the two groups.

Also, micRNA-200c relative expression was significantly downregulated in relapsed case when compared to cases in remission.

These agreed with Ghodousi and Rahgozar[9] who sated that regarding micRNA expression profiles micRNA-326 relative expression in relapsed cases and ALL cases who are in remission, showed a significantly decreased expression of micRNA-326 in relapsed cases compared with cases in remission. A significant decrease in micRNA-200c relative gene expression level was observed between cases in remission and relapsed ALLs.

Also, Mardani et al.[14] found that deregulation of many micRNAS (e.g., micRNA-326 and micRNA-200c) correlated with the development and progression of leukaemia. Consequently, these molecular studies could be utilised as diagnostic, prognostic, and therapeutic markers at various disease stages.

Conclusions:

The present research showed a statistically significant lower expression of micRNA-326 and micRNA-200c in newly diagnosed ALL children and in relapsed cases after one year of chemotherapy. These data suggests that micRNA-326 and micRNA-200c may play a role in diagnosis, prognosis, and early detection of relapsed cases.

References:

1. Terwilliger T, Abdul-Hay M. Acute lymphoblastic leukemia: a comprehensive review and 2017 update. Blood Cancer J. 2017;7:e577.

2. Pinto MT, Cárcano FM, Vieira AGS, Cabral ERM, Lopes LF. Molecular Biology of Pediatric and Adult Male Germ Cell Tumors. Cancers. 2021;13:2349.

3. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. Front Endocrinol (Lausanne). 2018;9:402.

4. Bushati N, Cohen SM. microRNA functions. Annu Rev Cell Dev Biol. 2007;23:175-205.

5. Kim Y, Kim H, Park H, Park D, Lee H, Lee YS, et al. miR-326-histone deacetylase-3 feedback loop regulates the invasion and tumorigenic and angiogenic response to anti-cancer drugs. J Biol Chem. 2014;289:28019-39.

6. Rahgozar S, Moafi A, Abedi M, Entezar EGM, Moshtaghian J, Ghaedi K, et al. mRNA expression profile of multidrug-resistant genes in acute lymphoblastic leukemia of children, a prognostic value for ABCA3 and ABCA2. Cancer Biol Ther. 2014;15:35-41.

7. Mutlu M, Raza U, Saatci Ö, Eyüpoğlu E, Yurdusev E, Şahin Ö. miR-200c: a versatile watchdog in cancer progression, EMT, and drug resistance. J Mol Med (Berl). 2016;94:629-44.

8. Usmani S, Sivagnanalingam U, Tkachenko O, Nunez L, Shand JC, Mullen CA. Support of acute lymphoblastic leukemia cells by nonmalignant bone marrow stromal cells. Oncol Lett. 2019;17:5039-49.

9. Ghodousi ES, Rahgozar S. MicroRNA-326 and microRNA-200c: Two novel biomarkers for diagnosis and prognosis of pediatric acute lymphoblastic leukemia. J Cell Biochem. 2018;119:6024-32.

10. Banihashem A, Ghasemi A, Tavasolian L. Association of cytogenetics and immunophenotype in prognosis of children with acute lymphoblastic leukemia: Literature Review. Rev Clin Med 2014;12:134-9.

11. Bishop JF. Does it matter how remission is achieved in acute leukemia? Leukemia. 1996;10 Suppl 1:S7-9.

12. Borchmann P, Eichenauer DA, Engert A. State of the art in the treatment of Hodgkin lymphoma. Nat Rev Clin Oncol. 2012;9:450-9.

13. Lv M, Zhu S, Peng H, Cheng Z, Zhang G, Wang Z. B-cell acute lymphoblastic leukemia-related microRNAs: uncovering their diverse and special roles. Am J Cancer Res. 2021;11:1104-20.

14. Mardani R, Jafari Najaf Abadi MH, Motieian M, Taghizadeh-Boroujeni S, Bayat A, Farsinezhad A, et al. MicroRNA in leukemia: Tumor suppressors and oncogenes with prognostic potential. J Cell Physiol. 2019;234:8465-86.