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Diagnostic significance of microRNA-100 and -196b as blood markers for acute lymphoblastic leukemia among children

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ABSTRACT

The present study was undertaken to investigate the relative expression levels of miRNA-100 and -196b in childhood acute lymphoblastic leukemia (ALL) and its phenotypes. Peripheral blood mononuclear cells (PMNCs) were isolated from peripheral blood samples of 40 pediatric ALL patients and 10 healthy controls. We assessed the expression levels of miRNA-100 and -196b by quantitative real time polymerase chain reaction (qRT-PCR) assay. A significant upregulation in the expression levels of miRNA-100 and -196b in ALL patients is reported, compared to the normal controls. T-ALL patients manifested a higher expression of miRNA-100 and-196b than those with pre-B-ALL and biphenotypic ALL. MiRNA-100 and -196b distinguished ALL patients from the normal controls at cut-off values 6.54 (92.5 % sensitivity, 100 % specificity) and 5.49 (92.5 % sensitivity, 100 % specificity), respectively. As well, miRNA-100 and -196b discriminated between the different ALL phenotypes with high sensitivity, specificity and accuracy levels. Two correlation coefficients are herein reported including a significant positive correlation between miRNA-100 and -196b expression levels ($r = 0.328$, $p < 0.05$) and a significant negative correlation between miRNA-100 expression level and the platelets count ($r = -0.448$, $p < 0.01$) in ALL patients. Our findings concluded that miRNA-100 and -196b could be considered as good noninvasive blood biomarkers for the diagnosis of ALL, and in distinguishing its phenotypes.

Introduction

Acute lymphoblastic leukemia (ALL) is one of the most common hematologic malignancies observed in childhood [1]. It is characterized by the clonal proliferation of a lymphoid progenitor that has arrested its development at any early stage of B- or T-cell differentiation and results in the accumulation of leukemic lymphoblast in the bone marrow and extramedullary sites [2]. It accounts for approximately 25 % of cancers and 80 % of all leukemias in children who are younger than 15 years [3].

Recurrent chromosomal abnormalities are a hallmark of lymphoblastic leukemias and provide insight into the molecular mechanisms of leukemogenesis. In childhood B-ALL, the most common specific abnormality is the t(12;21) ETV6-RUNX1 translocation, in which the RUNX1 gene, encoding a protein involved in transcriptional control of hemopoiesis, has been translocated and repressed by the ETV6-RUNX1 fusion protein [4]. CDKN2A/2B and NOTCH1 are the most frequently affected genes in T-ALL, with 60 % of T-ALL patients showing activation of the NOTCH1 signaling pathway and up to 80 % harboring deletions and/or mutations inactivating the CDKN2A/B genes at chromosome 9p [5].

The development and activation of B- and T-cells at the periphery are controlled by complex protein signaling pathways, which are regulated by the microRNAs [6]. MicroRNAs represent an interesting class of small (18-25 nucleotides) noncoding RNAs that act as post-transcriptional regulators of gene expression through binding to the 3′ untranslated regions (UTR) of the target mRNAs and promoting mRNA degradation or translational repression [7]. miRNAs expressed differentially at distinct stages of lymphopoiesis, can hybridize with target messenger RNAs, regulating their post-transcriptional expression, and influence the direction

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of lymphoid precursor maturation. As miRNAs have been found to be overexpressed or downregulated in cancer tissues, they can be considered to act functionally as oncogenes (oncomiRNAs) or tumor-suppressors (tumor-suppressor miRNAs) [8]. In malignant lymphopoiesis, there is an aberrant expression of miRNAs that can be used as biomarkers in diagnosis, differential diagnosis, prognosis and therapy of leukemia [9,10].

Among the miRNAs that demonstrated aberrant expression in childhood ALL were miRNA-100 and miRNA-196b [11]. Few studies have demonstrated that the aberrant expression of miRNA-100; located on chromosome 11, is associated with pediatric ALL and considered one of the top 45 most highly significant differentially expressed miRNAs that distinguish ALL patients from controls [12,13]. In addition, miRNA-100 has been reported to function as an oncogenic or a tumor suppressor, depending on the tumor type and the microenvironment [14,15]. On the other hand, miRNA196b is located on chromosome 7 in an evolutionarily conserved region between HOXA9 and HOXA10 genes [16], which have been recognized as major transcription factors involved in the survival and proliferation rates of leukemia cells [17].

The present study aimed at assessing the diagnostic efficacy of peripheral blood miRNA-100 and 196b as diagnostic markers for pediatric ALL since most previous studies include miRNAs taken from the bone marrow. Also, the discriminative role of the expression levels of miRNAs 100 and 196b in the different pediatric ALL phenotypes relative to normal controls was assessed using quantitative real-time PCR (qPCR). Correlations between miRNA-100 and/or miRNA-196b and other hematologic parameters were studied.

Subjects and Methods

Subjects

In the present study, a total of 40 children with newly diagnosed and untreated ALL (22 males and 18 females aged from 1-15 years old) and 10 normal control children (5 males and 5 females aged from 4 to 12 years) were recruited from Ain Shams Pediatrics Hospital. Children's parents were asked for a written consent before sample collection. ALL patients were categorized into 21 cases with pre-B-ALL (52.5 %), 11 cases with biphenotypic ALL (27.5%) and 8 cases with T-ALL (20 %). Diagnosis of all patients was based on standard clinical criteria, including complete blood count, morphological bone marrow examination, immunophenotyping and cytogenetic analysis. Inclusion criteria were ALL patients with a number of blasts in peripheral blood more than 25 % according to the WHO classification [18]. Exclusion criteria were ALL patients that received any treatment or had suffered from other types of cancer.

Blood sampling and isolation of blood mononuclear cells

Peripheral blood samples (1-3 ml) were collected into EDTA containing vials, mixed well then diluted with an equal volume of phosphate-buffered saline (PBS). Each blood sample was carefully layered over Ficoll-Hypaque solution (Sigma-Aldrich, USA), then centrifuged at 400×g for 40 min at room temperature. The peripheral blood mononuclear cells (PMNCs) were carefully aspirated and suspended in PBS, then spinned at 100×g at room temperature for 10 min. The supernatant was removed, and the leukocytes pellet was washed once with PBS, and then centrifuged. The supernatant was discarded, and the packed leukocytes were suspended in 1 ml of PBS and stored at -80˚C until miRNA extraction [19].

RNA extraction, cDNA synthesis and qPCR analysis of selected miRNAs

Total RNA was extracted from PMNCs using miRNeasy Mini kit (Qiagen, Hilden, Germany). The concentration of extracted RNA was estimated by measuring the absorbance at 260 nm using Q-5000 NanoDrop Spectrophotometer (Quawell Technology, Inc., USA). The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the extracted RNA, where a ratio of ~2.0 is generally accepted as "pure" for RNA. Mature miRNAs were reverse transcribed into cDNA using a miScript HiSpec buffer supplied in miScript II RT Kit (Qiagen, Hilden, Germany) into a thermal cycler (Biometra, Germany). Quantitative real-time PCR (qRT-PCR) was performed using miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For each candidate miRNA, a universal primer (reverse primer) specific for miRNAs and a miScript primer assay (forward primer) specific to the miRNA of interest were used (miRNA-100/Hs–miR-100–2 miScript Primer Assay [MS00031234] [AACCCGUAGAUCCGAACUUGUG] and miRNA 196b/Hs–miR-196b*–2 miScript Primer Assay [MS00031577] [UCGACAGCACGACACUGCCUUC] (Qiagen, Germany). The miRNA levels were analyzed using StepOne Real-Time PCR Analyzer (Applied Biosystems, USA). The amplification program was adjusted as follows; initial activation step for 15 min at 95˚C to activate HotStar Taq DNA polymerase, and 40 cycles (denaturation at 94˚C for 15 sec, annealing at 55˚C for 30 sec and extension at 70˚C for 30 sec). Melting curves were performed by rapid heating to 95˚C for 15 sec to denature the DNA, followed by cooling to 60˚C to assure the purity and specificity of amplified products. The relative quantification of miRNA-100 and miRNA-196b was calculated using the comparative CT method ($2^{-\Delta\Delta Ct}$) where ΔΔCt is the difference of ΔCt value between the patient and the control (ΔΔCt=ΔCt patient miRNA - ΔCt control miRNA), and ΔCt is the difference of CT value between the target (miRNA-100 or miRNA-196b) and endogenous housekeeping miRNA reference (miRNA SNORD68-11) [20].

Statistical analysis

The distributions of quantitative variables were tested for normality using Shapiro-Wilk's test [21]. For parametric data, comparison between two variables was

done using independent t-test. For nonparametric data, comparisons between different categories were done using Kruskal-Wallis test followed by Mann-Whitney U test. Comparison between the different groups regarding the categorical variables was analyzed using Chi-square test. Receiver operating characteristic (ROC) curves were constructed to detect the sensitivity, specificity and the diagnostic efficacy of miRNA -100 and/or miRNA-196b in ALL and its different subtypes. The data were statistically analyzed using Statistical Program for Social Science (SPSS) version 16 **(Chicago, Illinois, USA)**.

Results

Hematological profile of ALL patients

Table 1 demonstrates that ALL patients showed significant decreases in white blood cells count (WBCs), red blood corpuscles count (RBCs), hemoglobin (HGB) concentration, hematocrit value (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), neutrophils and lymphocyte counts ($p < 0.001$), compared to normal controls. By contrast, a significant increase in red cell diameter width (RDW) ($p < 0.001$) was recorded, compared to the control group. Non-significant differences between ALL patients and normal controls were recorded regarding the mean corpuscular volume (MCV) and the counts of platelets, monocytes and eosinophils.

The relative expression levels of miRNA-100 and -196b in the PMNCs of normal and ALL cases.

Non-parametric analysis was used to compare between the expression levels of miRNAs-100 and -196b in ALL patients and normal control subjects. Both miRNA-100 and -196b were significantly overexpressed in ALL patients (median = 25.62 vs 1.42; 17.54 vs 0.57, respectively) **Table 2**.

Table 1: Complete blood count of normal controls and ALL patients

Parameters			Normal Control (n = 10)	ALL (n = 40)	$p <$
White blood cells (10^9/L)	**Mann-Whitney U Test**	**Median (25^{th}-75^{th})**	10.15 (7.03 - 11.25)	3.45 (2.05 - 5.1)	0.001*
Red blood corpuscles (10^{12}/L)		**Median (25^{th}-75^{th})**	5.41 (5.05 - 6.12)	3.72 (3.13 - 4.52)	0.001*
Mean corpuscular volume (fl)		**Median (25^{th}-75^{th})**	82.35 (81.22 - 84.47)	81.85 (79.12 - 88.07)	NS*
Red cell diameter width (%)		**Median (25^{th}-75^{th})**	8.2 (5.92 - 9.37)	15 (13.95 - 18)	0.001*
Platelets (10^9/L)		**Median (25^{th}-75^{th})**	265.5 (211 - 320)	252.5 (189 - 311)	NS*
Neutrophils (10^9/L)		**Median (25^{th}-75^{th})**	9.8 (7.42 - 11.37)	1.55 (0.7 - 2.77)	0.001*
Lymphocytes (10^9/L)		**Median (25^{th}-75^{th})**	3.9 (3.52 - 4.97)	1.19 (0.69 - 1.72)	0.001*
Monocytes (10^9/L)		**Median (25^{th}-75^{th})**	0.37 (0.18 - 0.44)	0.43 (0.25 - 0.6)	NS*
Eosinophils (10^9/L)		**Median (25^{th}-75^{th})**	0.1 (0 - 0.2)	0.1 (0 - 0.2)	NS*
Hemoglobin (g/dL)	**Student-t-test**	**Range Mean ± SE**	(11.2 - 13) 12.03 ± 0.17	(6.8 - 13.5) 10.41 ± 0.27	0.001**
Hematocrit (%)		**Range Mean ± SE**	(36 - 38.2) 37.08 ± 0.21	(20.2 - 40.6) 31.74 ± 0.81	0.001**
Mean corpuscular hemoglobin (pg)		**Range Mean ± SE**	(31.89 - 33) 32.53 ± 0.1	(23.5 - 34) 28.26 ± 0.42	0.001**
Mean corpuscular hemoglobin concentration (g/dL)		**Range Mean ± SE**	(35.2 - 36.9) 36.11 ± 0.2	(30.3 - 35.3) 33.06 ± 0.19	0.001**
Lymphoblasts (%)		**Range Mean ± SE**	(0) 0 ± 0	(42 - 89) 70.23 ± 2.31	-
Basophils (10^9/L)		**Range Mean ± SE**	0 0 ± 0	0 ± 0.2 0.01 ± 0	-

SE: Standard error; NS: Non-significant.

* Parametric data were statistically analyzed by Student-t-test

** Non-parametric data were statistically analyzed by Mann-Whitney U test.

Table 2: The relative expression levels of miRNA-100 and -196b in the normal control and ALL cases

Parameter	Control (n=10)	ALL (n=40)	*P* <
MiRNA-100			
Median	1.42	25.62	0.001
Percentiles 25	0.37	17.39	
75	2.51	65.77	
IQR	2.14	48.38	
MiRNA-196b			
Median	0.57	17.54	0.001
Percentiles 25	0.19	10.15	
75	0.71	20.06	
IQR	0.52	9.91	

Statistical analysis was performed using Mann-Whitney U test for non-parametric data.
IQR: Interquartile range

Statistical significance of miRNA-100 and -196b expression levels in the PMNCs of ALL phenotypes

Kruskal-Wallis test (nonparametric test) showed significant changes in the relative expression levels of miRNA-100 and 196b in the different ALL phenotypes. Inter-comparison between the different phenotypes of ALL using Mann-Whitney U test demonstrated a non-significant change in miRNA-100 or 196b expression level between pre-B-ALL and biphenotypic patients, whereas a significant elevation in both miRNAs was recorded in T-ALL patients, compared to pre-B-ALL and biphenotypic patients **Table 3**.

Correlations between miRNA-100 or -196b expression and the hematological parameters in ALL patients

A novel significant positive correlation was observed between miRNA-100 and -196b expression levels in ALL patients ($r = 0.328$, $p < 0.05$) **Table 4**. No significant correlation was found between the hematological parameters and either miRNA-100 or -196b expression in ALL patients, except for miRNA-100, which was negatively correlated with the platelets count ($r = -0.448$, $p < 0.01$) **Table 4**.

Diagnostic efficacy of miRNAs among ALL phenotypes

The diagnostic significance of miR-100 or -196b and their combination as markers for ALL were evaluated by receiver operator characteristics (ROC) analysis. The area under the curve (AUC) of miRNA -100 and -196b were calculated to be 0.996 ($p < 0.001$) and 0.985 ($p < 0.001$), respectively **Table 5**. The cut-off point of miRNA-100 was 6.54 with 92.5 % sensitivity, absolute specificity and 94 % accuracy. On the other hand, the cut-off point of miRNA-196b was 5.49 with 92.5 % sensitivity, absolute specificity and 94 % accuracy. The AUC for the combined miRNA-100 and -196b was 1 with absolute sensitivity, specificity and accuracy (100 %) **Table 5**.

The ROC curve was also applied to assess the diagnostic performance of miRNA-100 and -196b in distinguishing the ALL phenotypes. As for pre-B-ALL, miRNA-100 expression level recorded an AUC value of 0.993 ($p < 0.001$) and a minimum cut-off value of 2.99 with absolute sensitivity and 90 % specificity, while T-ALL patients have shown AUC equal to 1 ($p < 0.001$) and a maximum cut-off value of 21.5 with absolute sensitivity and specificity. In biphenotypic patients, AUC was equal to 1 ($p < 0.001$) and showed a cut-off value of 7.45 with absolute sensitivity and specificity. On the other hand, the relative expression level of miRNA-196b in pre-B-ALL patients showed AUC equal to 0.995 ($p < 0.001$) and recorded a cut-off value of 5.49 with 90.5 % sensitivity and absolute specificity, while T-ALL patients recorded AUC equal to 1 ($p < 0.001$) and a cut-off value of 5.51 with absolute sensitivity and specificity. In case of biphenotypic patients, the AUC was 1 ($p < 0.001$), and the cut-off value was 5.51, with 90.9 % sensitivity and absolute specificity **Table 5 & Fig. 1-3**.

The distinguishing efficacy of the cut-off values of miRNA-100 and -196b in ALL immunophenotypes

All normal control cases (100 %) showed miRNA-100 and -196b expression levels below their corresponding cut-off values (≤ 6.54 and ≤ 5.49, respectively). However, the relative miRNAs -100 and -196b expression levels were higher than their corresponding cut-off values in 37/40 ALL patients (92.5 %), and by contrast lower in 3/40 ALL cases (7.5 %) **Table 6**. This indicates that these cut-off values significantly distinguish ($p < 0.001$) ALL patients and normal controls.

Evaluation of the discriminative efficacy of the cut-off values of miRNA-100 and -196b in ALL immunophenotypes revealed that all pre-B-ALL patients (21/21; 100 %) and only 1 out of 10 (10 %) normal cases showed higher miRNA-100 expression level than the calculated cut-off value (2.99). On the other hand, 90.5 % (19 out of 21) of pre-B-ALL patients showed miRNA-196b expression level above the cut-off value (5.49), whereas the remaining 2 pre-B-ALL patients (9.5 %) and all normal control cases (100 %) showed miRNA-196b expression level lower than the calculated cut-off value (5.49) **Table 6**.

Similarly, all T-ALL patients (100 %) showed miRNA -100 and -196b expression levels above the corresponding cut-off values (21.51 and 5.51, respectively), whereas all normal control cases had miRNA-100 and -196b expression levels below the corresponding cut-off values **Table 6**. In case of biphenotypic patients, the expression level of miRNA-100 in all patients (100 %) was above the calculated cut-off value (7.45), whereas all normal control cases had miRNA-100 below the cut-off value. On the other hand, the expression level of miRNA-196b was above the cut-off value (5.51) in 10 out of 11 biphenotypic patients (90.9 %), while the remainder (9.1 %) and all normal control cases had miRNA-196b expression level lower than the calculated cut-off value **Table 6**.

Table 3: Statistical significance of miRNA-100 and -196b relative expression levels in the PMNCs in the different ALL phenotypes

Parameter		Phenotypes			*p* <
		Pre-B-ALL (N=21)	**T-ALL (N=8)**	**Biphenotypic (N=11)**	
MiRNA-100	Median	20.08*	100.71#	21.52*	0.001
	Percentiles 25	10.26	64.83	16.4	
	75	30.68	139.25	63.07	
	IQR	20.42	74.42	46.67	
MiRNA-196b	Median	19.61*	23.14#	12.15*	0.001
	Percentiles 25	10.11	12.61	10.07	
	75	20	30.4	15.03	
	IQR	9.9	17.79	4.96	

Statistical analysis was done using Kruskal-Wallis test for non-parametric data. Values with dissimilar symbols are considered significantly different, IQR: Interquartile range

Table 4: Spearman's correlation coefficients between the relative expressions of miRNA-100 and -196b and the hematological parameters in ALL patients

	miRNA-100	**miRNA-196b**
RBCs (10^{12}/L)		
r-value	0.051	0.004
***p* <**	NS	NS
WBCs (10^9/L)		
r-value	-0.126	0.207
***p* <**	NS	NS
HGB (g/dl)		
r-value	-0.064	-0.018
***p* <**	NS	NS
PLT (10^9/L)		
r-value	-0.448	-0.208
***p* <**	0.01	NS
LYM (10^9/L)		
r-value	-0.135	0.039
***p* <**	NS	NS
Lymphoblasts (%)		
r-value	0.089	-0.031
***p* <**	NS	NS
miRNA-196b		
r-value	0.328	-----
***p* <**	0.05	

Table 5: Cut-off values, sensitivity, specificity and accuracy of miRNA-100 and miRNA-196b in ALL patients and the different ALL phenotypes.

	Marker	AUC	95 %CI(±SE)	Cut-off	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)
ALL (n = 40)	MiRNA-100	0.996*	0.985-1 (± 0.006)	6.54	92.5	100	94	100	76.9
	MiRNA-196b	0.985*	0.957-1 (± 0.014)	5.49	92.5	100	94	100	76.9
Pre-B-ALL (n = 21)	MiRNA-100	0.993*	0.972-1 (± 0.011)	2.99	100	90	96.8	95.5	100
	MiRNA-196b	0.995*	0.980-1 (± 0.008)	5.49	90.5	100	93.5	100	83.3
T-ALL (n = 8)	MiRNA-100	1*	1-1(± 0)	21.51	100	100	100	100	100
	MiRNA-196b	1*	1-1(± 0)	5.51	100	100	100	100	100
Biphenotypic (n = 11)	MiRNA-100	1*	1-1(± 0)	7.45	100	100	100	100	100
	MiRNA-196b	0.995*	0.861-1 (± 0.048)	5.51	90.9	100	95.2	100	90.9

AUC= Area under the curve, CI= Confidence interval, SE= Standard error
* statistically significant at $p < 0.001$

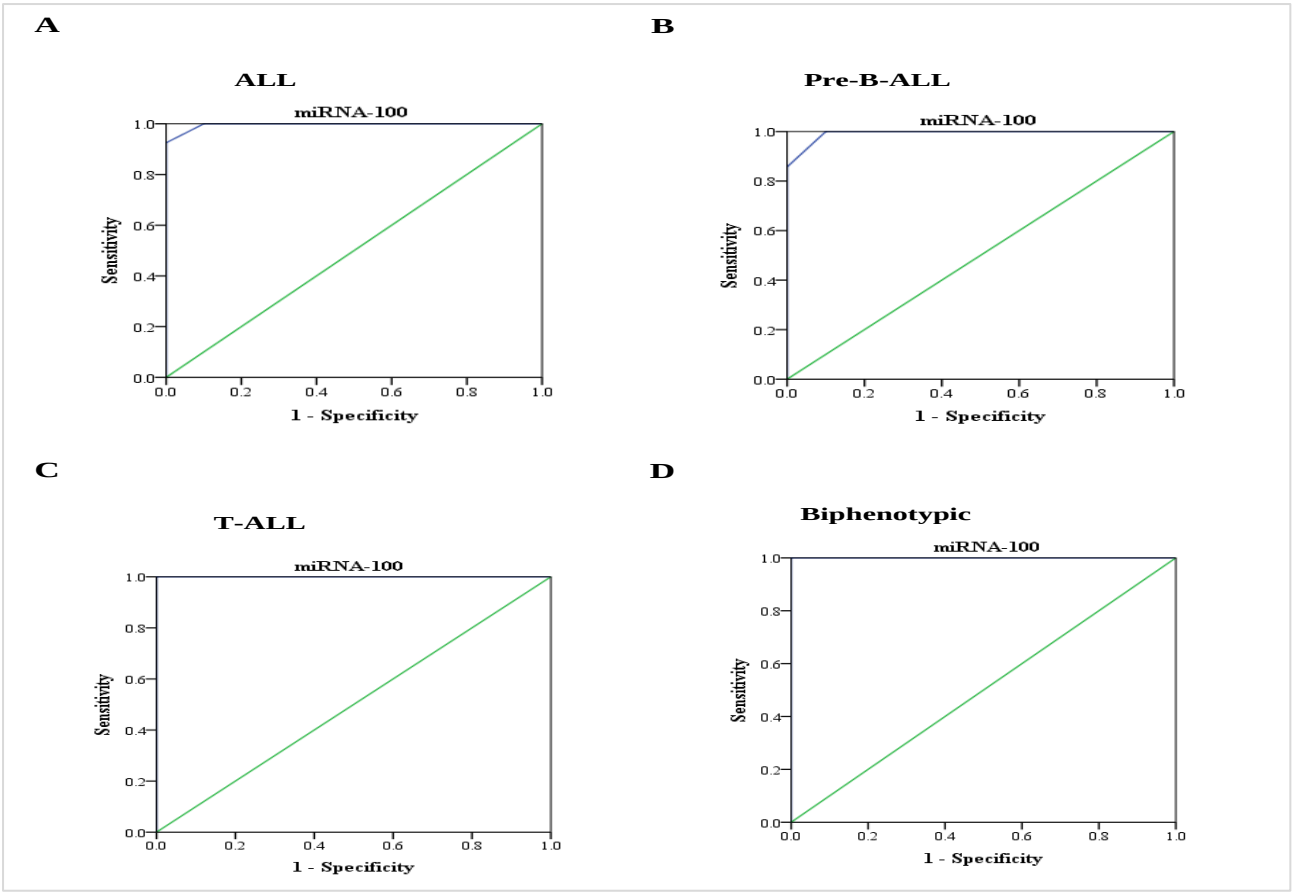

Fig. 1: ROC curves of miRNA-100 to distinguish the normal controls from ALL and its immunophenotypes.

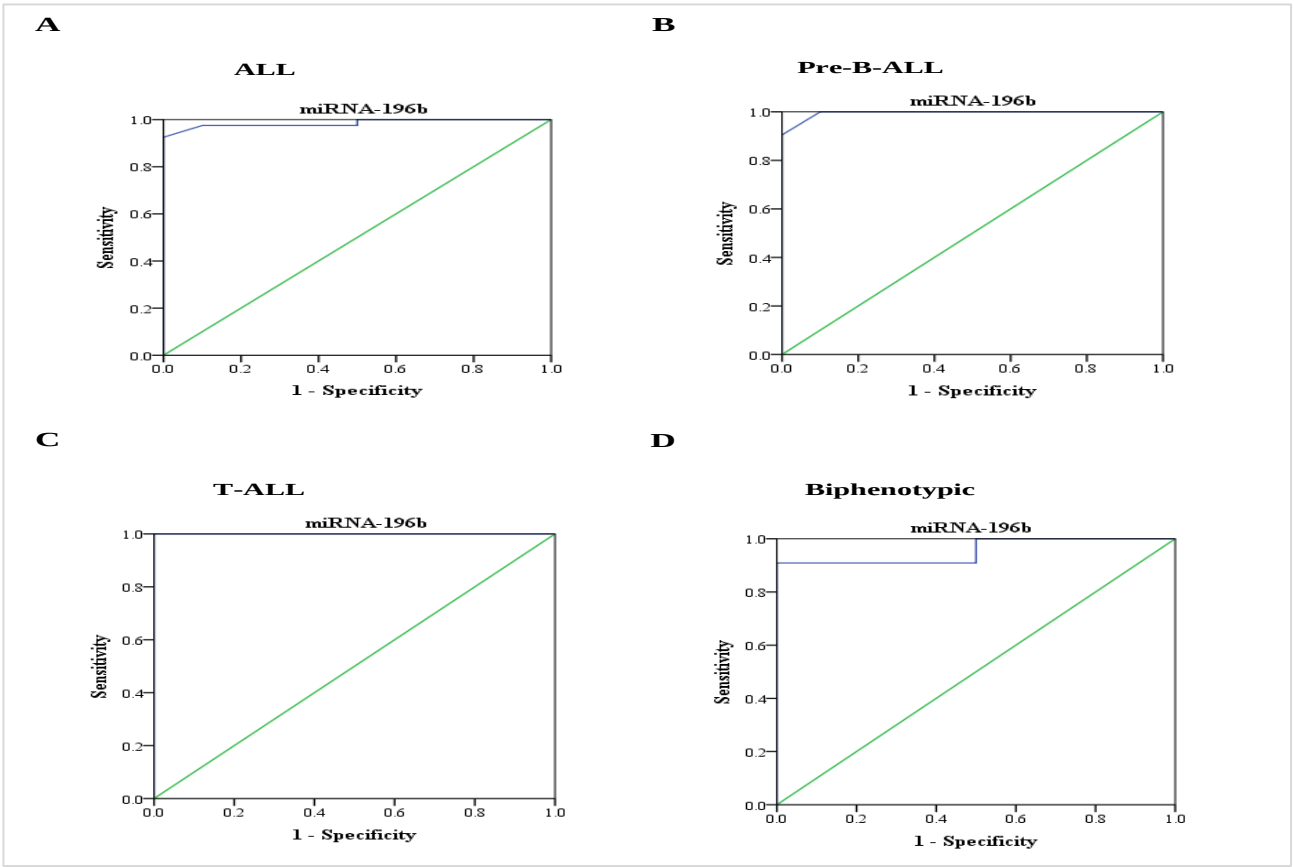

Fig. 2: ROC curves of miRNA-196b to distinguish the normal controls from ALL and its immunophenotypes.

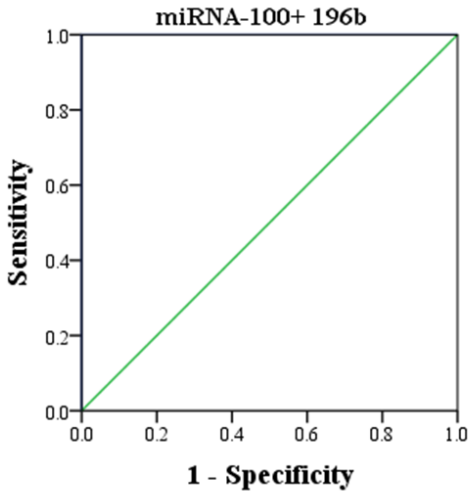

Fig. 3: ROC curve of combined miRNA-100 and -196b to distinguish ALL patients from the normal controls.

Table 6: The distinguishing efficacy of the cut-off values of miRNA-100 and -196b between the normal controls and ALL patients and their immunophenotypes

Parameter	Cut-off value	Group		χ^2
		Control (n=10)	ALL (n=40)	
MiRNA-100	(> 6.54)	0 (0 %)	37 (92.5 %)	35.577 ($p < 0.001$)
	(≤ 6.54)	10 (100 %)	3 (7.5 %)	
MiRNA-196b	(> 5.49)	0 (0 %)	37 (92.5 %)	35.577 ($p < 0.001$)
	(≤ 5.49)	10 (100 %)	3 (7.5 %)	
		Control (n=10)	Pre-B-ALL (n=21)	
MiRNA-100	(> 2.99)	1 (10 %)	21 (100 %)	26.63 ($p < 0.001$)
	(≤ 2.99)	9 (90 %)	0 (0 %)	
MiRNA-196b	(> 5.49)	0 (0 %)	19 (90.5 %)	23.37 ($p < 0.001$)
	(≤ 5.49)	10 (100 %)	2 (9.5 %)	
		Control (n=10)	T-ALL (n=8)	
MiRNA-100	(> 21.51)	0 (0 %)	8 (100 %)	18 ($p < 0.001$)
	(≤ 21.51)	10 (100 %)	0 (0 %)	
MiRNA-196b	(> 5.51)	0 (0 %)	8 (100 %)	18 ($p < 0.001$)
	(≤ 5.51)	10 (100 %)	0 (0 %)	
		Control (n=10)	Biphenotypic ALL (n=11)	
MiRNA-100	(> 7.45)	0 (0 %)	11 (100 %)	18 ($p < 0.001$)
	(≤ 7.45)	10 (100 %)	0 (0 %)	
MiRNA-196b	(> 5.51)	0 (0 %)	10 (90.9 %)	17.35 ($p < 0.001$)
	(≤ 5.51)	10 (100 %)	1 (9.1 %)	

Discussion

In the present study, the relative expression levels of miRNA-100 and -196b in the PMNCs were determined as potential blood markers for the diagnosis and differentiation of pediatric ALL immunophenotypes. A dramatic upregulation in the relative expression levels of miRNA-100 and -196b genes were recorded in ALL patients **(Table 2)**. We previously reported on a significant upregulation of miRNA-100 expression level in children with ALL (aged from 13 months to 14 years old) [22]. Also, our findings are in harmony with the previous study of Schotte *et al.* [23], who stated that pediatric ALL patients with TEL/AML1 translocation t (12;21) are distinguished from those with other genetic phenotypes through the differential de-regulation of various miRNAs, including the 30-fold increase in the expression of miRNA-100. As well, Popovic *et al.* [24] emphasized the overexpression of miRNA-196b in 29 patients with MLL-associated leukemias (10 ALL and 19 AML), compared to non-MLL patients. Schotte *et al.* [25] have demonstrated that the aberrant overexpression of miRNA-196b is not restricted to mixed lineage leukemia (MLL)-rearranged ALL cases (T-ALL or precursor B-ALL), but also occurs in T-ALL patients with other genetic abnormalities that activate the HOXA gene cluster.

However, our results contrast some previous studies. Bhatia *et al.* [26] found that the expression of miRNA-196b was significantly decreased in lymphoid leukemic B-cell line (EB-3) and in B-cell ALL patients with respect to normal B-cells, and this downregulation might lead to the overexpression of c-myc gene, suggesting the possible involvement of miRNA-196b in the pathogenesis of human ALL. In another study [27], bone marrow samples were obtained from 128 ALL children that were further classified into 108 patients with B-derived ALL (10 pro-B ALL cases and 98 pre-B ALL cases) and 20 patients with T-ALL. The authors reported a lower expression of miR-100 ($p < 0.01$) and miR-196b ($p < 0.01$) in ALL patients, compared to the control samples. Also, Li *et al.* [28] reported a lower expression level of miR-100 in ALL patients carrying the mixed lineage leukemia (MLL)-rearrangement and BCR-ABL fusion genes, compared to patients without these two fusion genes, which indicates that the expression of miR-100 is associated with specific risk groups within childhood ALL.

In the current study, the expression levels of the candidate miRNAs in the different ALL phenotypes are statistically studied. MiRNA-100 and 196b are significantly upregulated in T-ALL ($p < 0.001$), compared to preB-ALL and biphenotypic phenotypes, while a non-significant change in miRNA-100 and 196b genes expression was aberrant between preB-ALL and biphenotypic patients (**Table 3)**. In agreement with our findings, de Oliveira *et al.* [27] affirmed a significantly higher expression of miRNA-196b in T-ALL patients than in B-ALL subjects. The authors also reported a higher miRNA-100 expression in B-ALL patients associated with the presence of t(12p13;21q11) (ETV6/RUNX1) translocation than in non t (12;21) translocation. Our results agree with the previous studies, which identified the upregulation of miRNA-196b and its ability to significantly distinguish T-ALL from other ALL subtypes [29,30]. These data confirm the usefulness of the candidate miRNAs as discriminative markers for the different ALL phenotypes. In contrast to our findings, a significant downregulation in the expression level of miRNA-100 ($p < 0.01$) in the bone marrow of T-ALL patients was recorded, as compared to B-ALL patients [28]. The discrepancy might be due to the different type of sample.

Two novel correlation coefficients are herein reported including a significant negative correlation ($r = -0.448$, $p < 0.01$) between miRNA-100 and the platelets count, and a significant positive correlation ($r = 0.328$, $p < 0.05$) between miRNA-100 and -196b in ALL patients **(Table 4)**, suggesting the regulatory role of the overexpression of miRNA-100 and -196b in the pathogenesis of ALL.

To evaluate the diagnostic efficacy of the studied miRNAs in ALL and its phenotypes, ROC curves and AUC analyses were performed. ROC curve analysis and the cut-off values of miRNA-100 and -196b **(Tables 5&6, Fig. 1&2)** have distinguished healthy subjects from ALL patients on one hand, and between the different ALL phenotypes on the other hand, which indicates that miRNA-100 and -196b have high diagnostic accuracy for ALL and its immunophenotypes. Both cut-off values are accurate in distinguishing ALL patients from the normal controls, with a sensitivity of 92.5 % and absolute specificity. In addition, combining both markers gave rise to a higher AUC value and absolute sensitivity and specificity **(Fig. 3)**, suggesting that combination of miRNA-100 and -196b might be more reliable in the diagnosis of ALL patients. The literature contains a few studies on the diagnostic potential of miRNA-100 or -196b using ROC curve analysis in various types of cancer. Torres *et al.* [31] affirmed that miRNA-100 has a good diagnostic efficacy for endometrioid endometrial cancer (EEC) patients with AUC of 0.652 for tissue samples and a cut-off value of 6.5 (with 86 % sensitivity and 50 % specificity), and AUC value of 0.74 for plasma samples and a cut-off value of 1.5 (with 64 % sensitivity and 79 % specificity). Also, Wang *et al.* [14] have demonstrated that miRNA-100 can distinguish gastric cancer patients from healthy controls with a cut-off value of 3.33, 71 % sensitivity and 58 % specificity. Lu *et al.* [32] reported the ability of plasma miRNA-196b to discriminate between oral cancer patients and normal subjects (AUC=0.96), with 97.8 % sensitivity and 81.1 % specificity.

Conclusion

miRNA-100 and -196b are overexpressed in the PMNCs of Egyptian ALL children with high sensitivity, specificity and accuracy, which nominates them as good noninvasive blood biomarkers for the diagnosis of pediatric ALL, and further in distinguishing its phenotypes.

In addition, peripheral blood miRNA-100 and-196b distinguished T-ALL from pre B-ALL and biphenotypic subtypes, while miRNA-100 alone showed a better diagnostic efficacy for pre-B-ALL and biphenotypic ALL patients. Further investigations are recommended to assess the target oncogenes/tumor suppressor genes expression of miRNA-100 and-196b at both the transcriptional and translational levels to detect their regulatory roles in leukemogenesis. In view of the fact that genes coding miRNAs have been found to be localized in chromosomal regions susceptible to genetic translocations, further studies are needed to identify and characterize other miRNAs that are present near/within the regions involved in genetic translocations characteristic of B-cell acute lymphoblastic leukemia.

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