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The Expression of Different Micrornas in Iraqi Patients with Childhood Acute Leukemia and Their Association to C/EBP-B Serum Level

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Abstract

Leukemia is the most common cancer in children which causes death despite the high survival rate. Therefore, new methods are required to find a suitable therapy. A small RNA called microRNAs (miRNAs) is used as a biomarker for cancer diagnosis and early prognostic evaluation. Expression levels of three miRNAs from the 3' arm (*miR-142-3p*, *miR-223-3p* and *miR-146-3p*) were detected in serum samples from 30 acute leukemic children and from 30 healthy individuals by using qPCR. The m*iR-142-3p* and *miR-146-3p* profiles were significantly downregulated (P=0.0010 and 0.0012, respectively), while *miR-223* was found to be significantly upregulated (P= 0.0044) in the pateints. Serum level of C/EBP-β (CCAAT-enhancer-binding protein) was also determined by enzyme-linked immunosorbent assay (ELISA) and showed a significantly higher level in the patients ($p \leq 0.0001$). However, this higher enhancer level is probably not the reason for the abovementioned differences in miRNAs expression, as indicated by the weak correlation values with the three miRNA molecules (0.5737, 0.6625 and 0.7769, respectively). In conclusion, the fold change of *miR-142-3p*, *miR-223-3p* and *miR-146-3p* in serum could be potentially used as an early indicator of the occurrence of acute leukemia in children.

Keywords: microRNA; C/EBP-β; acute leukemia; Real Time PCR; Reverse Transcription.

التعبير عن الرنا الميكروي المختلفة في المرضى العراقيين المصابين بسرطان الدم الحاد في مرحلة الطفولة وارتباطهم بمستوي C/EBPB في المصل

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الخلاصة

سرطان الدم هو اكثر انواع السرطان شيوعايسبب الوفاة على الرغم من ان معدل البقاء على قيد الحياة عالي، لذلك هناك حاجةلايجادطريقة جديدة للعلاج المناسب. حديثا,الحمض النووي الريبوسومي الصغير يستخدم كعلامة حيوية في تحديد السرطان والتشخيص السرطاني. الحمض النووي (microRNAs (miRNAsالرايبي الصغيرالمسمى استخدم كمؤشر حيوي لتشخيص وتقييم التنبؤ لسرطان.

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.تم تحديد التعبير لثلاث انواع من الرنا الميكروية في الذراع في المصل)miR-142-3p وmiR-223-3p
و (miR-146-3p 3 p لثلاثين طفلاً عراقياً مصابا بسرطان الدم الحاد مقارنةً بثلاثين طفلاً يتمتعون بصحة
باستخدام تقنية تفاعل البلمرة المتسلسل مع تقنية المسبر المشع.
كان التعبير في الرنا الميكروية منخفض لكل من على التوالي miR-146a (P=0.0010 and 0.0012)-
miR-142-3p-3 (P= 0.0044) miR -223-3p بينما كان التعبير عالي جدا في
C/EBP-β لمستوى ل ايضاتم فحص المصل بواسطةالاليزا وكان المستوى عالي جدا في مرضى ابيضاض
الدم الحاد مقارنة بالاصحاء (p≤0.0001)
ولكن كانت علاقتهامع الرنا الميكروية غير كافية لاحداث الهبوط , miR-142-3p miR-146a-3p
بالتعبير لل ولاحداث الزيادة بالتعبير miR-223-3p في ال
على التوالي. (P = 0.5737; 0.6625;0.07769 حيث كانت النسبة)
الملخص انه هنالك تغيير عالي في التعبيرالجيني للرنا الميكروية في الاطفال المصابين بابيضاض الدم الحاد
ويمكن استخدامها كمؤشرحيوي

Introduction

Highly sensitive and specific biomarkers are required for early diagnosis of leukemia in order to enable better decisions for treatment [1]. Functional investigations showed that miRNAs play crucial roles in cell proliferation, apoptosis, immune response, and tumorigenesis [2]. Specific miRNA molecules demonstrated high functional values in different leukemia subtypes, including those of diagnostic management and prognostic assessment [3]. Induction of oncogenes and negative regulation of tumor suppressor genes are the responsible mechanisms for relapse after the change in miRNAs expression. Hence, testing miRNA expression can enhance an efficient early evaluation of leukemic patients [4]. A biomarker that is used in screening the progress of leukemia, e.g. through the usage of miRNA expression, can provide an indication of relapse-free survival or as an overall survival [5]. A previous study demonstrated that the expression level of *miR-142-3p* has about 90% sensitivity and 100% specificity in peripheral blood mononuclear cells in acute myeloid leukemia (AML) when compared to the control, which justified its employment as a biomarker [6]. In another study, this miRNA showed low level of expression in acute lymphoid leukemia (ALL) in comparison with AML [7]. *miR-146a* is highly expressed in children with ALL and AML; therefore, it could be used to significantly differentiate these cases from those of chronic lymphoid leukemia (CLL) and AML in adult. The same study revealed that, despite that ALL patients at the age group of 1–14 year could cure with the current available treatments, one third of them underwent miRNA profiling continue to relapse after one year of follow up. The expression of several miRNAs, such as *miR-7*, *miR-216*, and *miR-100*, *miR-486*, *miR-191*, *miR-150*, *miR-487*, and *miR-342* could distinguish between cases of relapse and complete remission (CR) in the patients [8]. The possible association between serum levels of CEBP-β and fold change in the expression of miRNAs in the patients has been scarcely investigated in the literature. Normally, the expression of m*iR-142* and *miR-223* decreases during hematopoietic cells proliferation. Some studies showed that the *miR-223*, *miR-142* and CEBP-β are involved in a regulatory pathway during hematopoiesis and that C/EBPβ increases at later stages of differentiation. It was also shown that *miR-223* can upregulate the expression of *miR-142* through CEBP-β [9,10].

The aim of this study is to determine the expression of different miRNAs (m*iR-142-3p*, *miR-22-3p3* and *miR-146a-3p)* in Iraqi patients with childhood acute leukemia along with their potential use as diagnostic biomarkers. A second aim is to evaluate the relationship between serum level of C/EBP and the fold change in the expression of these miRNAs in the pateints.

Materials and Methods

Serum samples from leukemia patients

Family history of leukemia, age, gender, and other data were recorded. Whole blood samples were collected in special test tubes for serum separation. A total of sixty serum samples were collected, including thirty from healthy children and thirty from children diagnosed with acute leukemia who attended the acute leukemia unit/ Children's Central Teaching Hospital/ Baghdad (from December 2018 to March 2019). Serum was collected in 2ml tubes by allowing the blood to coagulate at room temperature for 30 minutes followed by centrifugation for 5 minutes at 1600 rpm. Finally, serum was stored at -80℃ for further analysis.

Extraction procedure of miRNAs

MiRNAs were extracted from serum samples by suing the kits purchased from Qiagen/ USA. After thawing, 200μl of serum was mixed with the denaturation buffer. Nuclease-free water was added (50-100 μl) for elution, according to manufacturer's instruction. Finally, miRNA was stored at -80°C in DEPC-treated water after determining the concentration by NanoDrop-5000 spectrophotometer (Nano Drop Technologies, USA).

Reverse Transcription

The TaqMan miRNA reverse transcription kit (Applied Biosystems, USA) was used for the achievement of the reverse transcription reaction. The reaction mixture contains 10 ng of RNA extract, 100 mM of each deoxynucleotide triphosphate, RT buffer, 50 U/L of reverse transcriptase, 20 U/mL of RNase inhibitor, miRNAs (15 ul), nuclease-free water, and specific TaqMan primers (*miR-142-3p*, *miR-223-3p*, *miR-146a-3p* and endogenous control -*RNU48*)] (Applied Biosystems, USA). The reaction mixture was incubated at 42 °C for 30 min then at 85 °C for 5 min and hold at 4 °C by using Eppendorf/Hamburg/Germany RT-PCR.

Real Time PCR

TaqMan miRNA probes (*miR-142-3p*, *miR-223-3p*, *miR-146a-3p* and endogenous control -*RNU48*) were used according to manufacturer's protocol (TaqMan fast advanced master mix kit) to quantify miRNA in 20 μl reaction volume. Gene expression relative and quantitative levels were evaluated by using the "ΔΔCt "comparative Ct method. Fold inductions were calculated using the formula" 2^ (ΔΔCt) " whereΔΔCt = ΔCt (patient's group) − ΔCt (control's group)".

Statistical analysis

ANOVA (one-way analysis of variance) was performed to test whether the differences between groups were significant or not. Statistical significance was defined as $p \leq 0.05$. Data were expressed as mean ± standard deviation and statistical analysis was carried out using Graph Pad Prism version 6 (Graph Pad Software Inc., La Jolla, Ca, USA). Significant variation between acute leukemic and normal individuals was determined by student's T -test. R value was used to determine significant correlation between gene expression and CEBP-B concentration.

Results and Discussion

The patients group consisted of thirty patients diagnosed with acute leukemia, including ALL and AML. The number of Children with ALL was 20 and that for patients with AML was 10 (Figure- 1), including 16 males with a mean age of 5.25 ± 2. 8 and 14 females with a mean age of 6.53 ± 3.09. These data are in agreement with those of Ruiz-Delgado and colleagues who showed that ALL represents the most common malignancy in pediatrics, the age range of patients with ALL was 2-9 years, and there was a slight predominance in males [11]. Also, Obaid *et al.*, (2010) found that AML incidence was higher in adults than children [12], while Mohammed and colleagues showed that ALL incidence was higher in children (72%), with 51.6% in males and 49% in females [13]. The healthy group (control) in the present study consisted of 17 males with a mean age of 7.73 ± 2.76 years and 13 females with a mean age of 6.19 ± 1.95 years.

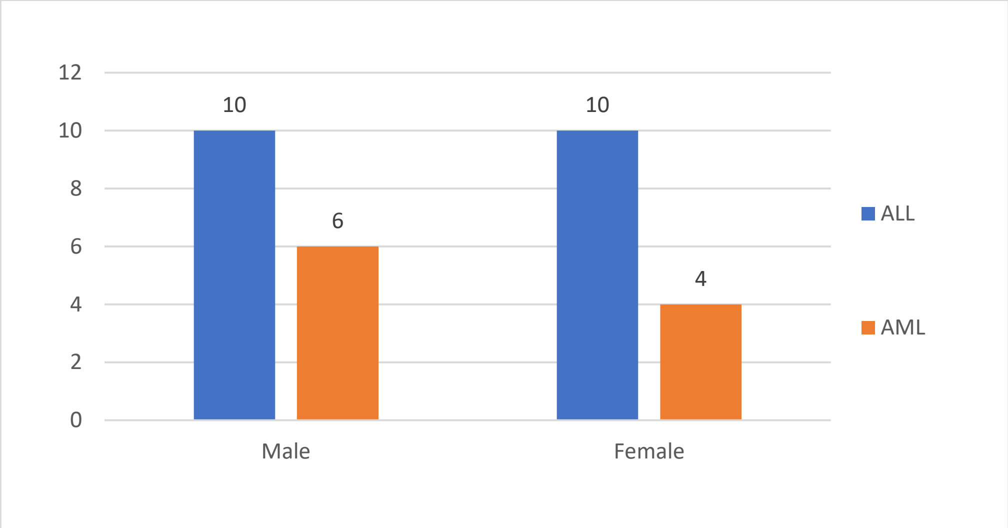

Figure 1-Distribution of the gender among the 30 patients with acute lymphoid leukemia and acute myeloid leukemia.

The distribution of the age and gender groups among children with acute leukemia is presented in Figure-2, which reveals that the frequency of the age group of 1-5 years in male patients with leukemia was higher than that in females, a similar result to that shown by Ismael and Hassan [14].

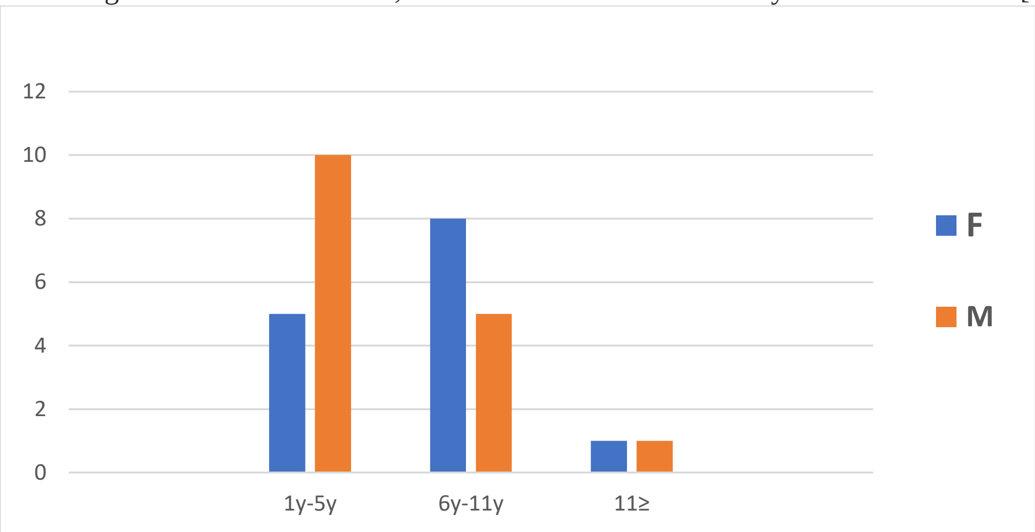

Figure 2-Distribution of the age and gender groups among the 30 children with acute leukemia.

miRNAs expression levels in patients with acute leukemia

The expression of miRNAs found in the circulating blood is either due to death and lyses of tumor cells or the release by tumor cells into blood vessels [15].

MiR-223-3p

The results of the present study revealed that children with acute leukemia had an elevated level of the expression of *miR-223-3p*, as presented in Figure- (3A). This over-expression was associated with a significant difference in comparison with the levels in healthy children (P= 0.0044). However, the difference in the expression level in ALL as compared to AML pateints was not significant ($p \leq 0.05$) (Fig.3B). In hematopoietic tissues, *miR-223-3p* was shown to be highly expressed and its level is increased during WBCs and RBCs development [16]. In addition, the phenotype of this molecule was demonstrated to be possibly changed in hematological and solid cancers [17]. Many studies reported that *miR-223* tends to be upregulated in several cancers, as in ovarian cancer tissues, advanced pathological stage gastric cancer, and non-small cell lung cancer cases [18, 19, 20]. Also, up-regulation of this miRNA can distinguish early stage pancreatic adenocarcinoma from chronic pancreatitis [21], whereas it is highly deregulated in metastasis and advanced clinical stages in the serum of patients with osteosarcoma and colorectal cancer [22, 23]. The high serum level of this miRNA in lymphoma B cell was shown to correlate with good survival in comparison to low level [24, 25].

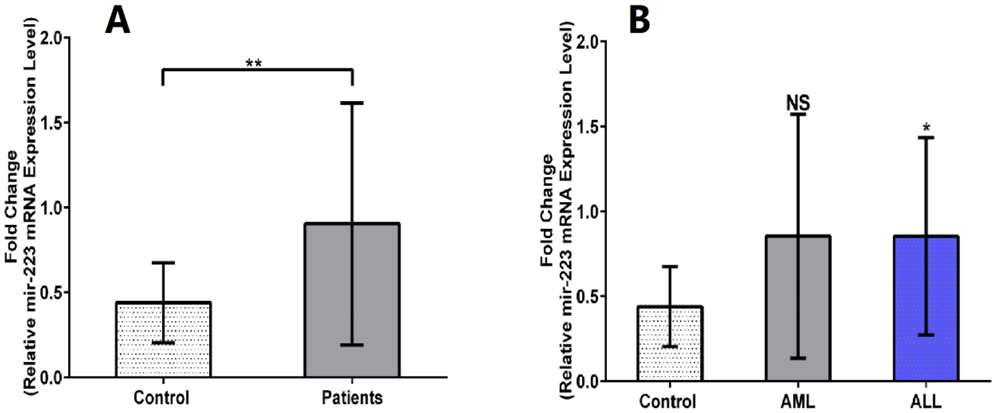

Figure (3A)-Relative *miR-223* expression level in 30 patients with acute leukemia and 30 healthy children, (**):significant difference, (*)non- significant difference. B): Relative *miR-223* expression level in serum samples from 13 children with acute lymphocytic leukemia and 17 children with acute myelocytic leukemia in comparison to 30 healthy children, (**):significant difference, (*)non-significant difference.

Mir-142-3p

mir-142-3p expression was examined in 30 serum samples in children with acute leukemia compared to 30 normal serum samples in healthy children by qPCR using TaqMan probes. *mir-142-3p* showed low expression in acute leukemia patients in comparison to healthy children, as presented in Figure- (4A.) This downregulation was significant (p= 0.0010). More investigation showed no differences in *mir-142-3p* expression in serum of patients with AML compared with ALL ($p \leq 0.01$) (Fig. 4B). *mir-142-3p* has a role in cancer progression related to cell cycle and invasion, regulating cell migration and apoptosis [2, 26, 27]. The downregulation was reported in both solid cancer and leukemia [28, 29]. Low expression was reported in solid cancers, such as hepatic cancer, with poor prognosis [30, 31]. It also has a role in growth inhibition in lung cancer and acute lymphoblastic leukemia through inhibiting TGFβ-1 receptor and MLL-AF4 oncogene, respectively [32, 33]. This miRNA was also reported to be downregulated in AML **[**6].

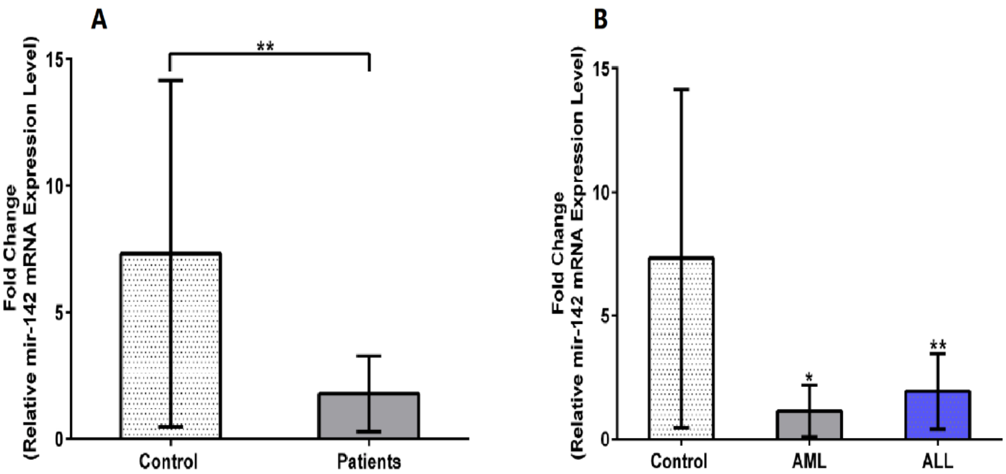

Figure (**4A**)-Relative *miR-142* expression level in 30 Iraqi children with acute leukemia in comparison to 30 healthy volunteers, (**):significant. B) Relative *miR-142* expression level in serum of 13 children with acute lymphocytic leukemia and 17 with acute myelocytic leukemia in comparison with healthy children, (**):significant.

MiR-146a-3p

As shown Figure-(5A), the expression of *miR-146a-3p* was significantly downregulated (*P*=0.0012)in children with acute leukemia in comparison to healthy donors. . Swellam and El-Khazragy showed similar results; they found that ALL children had a lower expression level in WBCs in comparison to the healthy group [34]. *miR-146a* was reported to be highly expressed in bone marrow monocytes in cases of AML [35]. Our results also showed no significant difference in the expression of *miR-146a-3p* between AML and ALL patients (*P*≤ 0.05) (Figure-5B). The expression level of *miR-146a* was reported to be positively correlated to the clinical outcomes [36]. Low expression was also found in prostate, gastric and glioma malignancies [37, 38, 39].

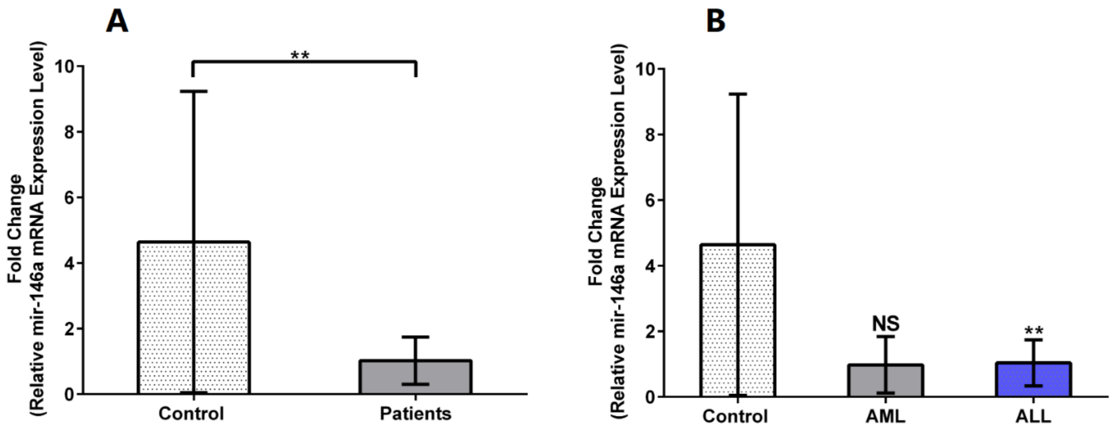

Figure (5A)-Relative *miR-146a* expression level in serum of 30 Iraqi children with acute leukemia and 30 healthy volunteers, (**):significant, (*)non- significant. B) Relative *miR-146a* expression level in 13 children with acute lymphoblastic leukemia and 17 acute myelocytic leukemia in comparison with 30 healthy children, (**):significant.

Detection of C/EBP-β level

There are many targets for each miRNA [40]. CEBP-β is an important transcriptional regulator which causes changes in gene expression and affects hematopoiesis in a regulatory network targeting other miRNAs [41]. The level of C/EBP-β in pediatric acute leukemia and the control group was detected by ELISA. Our results showed a highly significant difference in the level of C/EBP-β ($P < 0.0001$) in the patients in comparison to healthy children, as presented in Figure- (6A). Also, the difference in the level of C/EBP-β in serum of children with AML and ALL was significant ($P \leq 0.0001$) (Figure-6B).

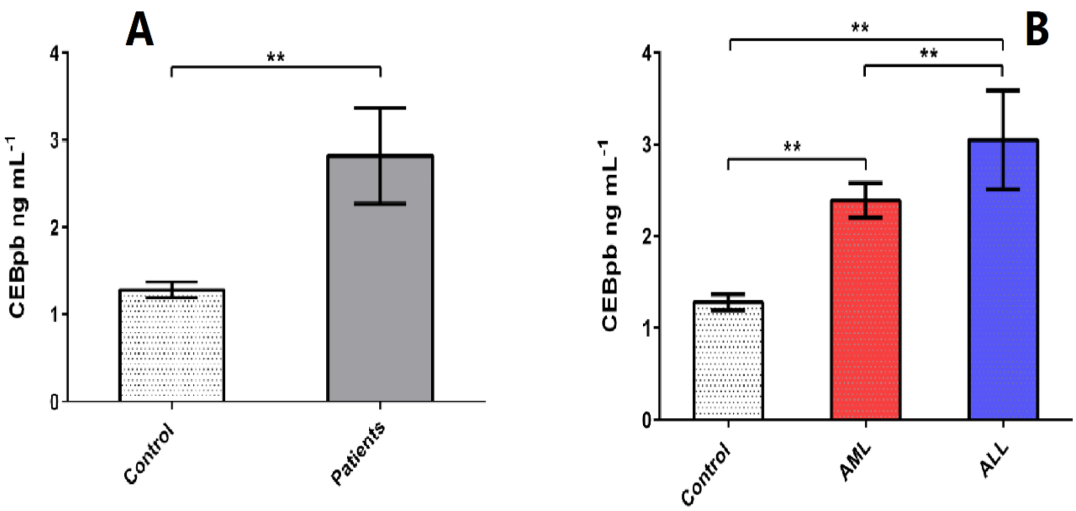

Figure (6A)-Serum level of C/EBP-β in Iraqi children with acute leukemia and healthy children, (**):significant, (*)non-significant. B) Serum level of C/EBP-β in Iraqi children with acute leukemia and healthy volunteers, (**)significant, (*)non- significant.

The correlation between C/EBPβ and *miRNAs* expression levels in serum samples from Iraqi children diagnosed with acute leukemia and healthy children

For further examination, we tested whether we could find an association between C/EBPβ and *miR-142-3p*, *miR-146a-3p* and *miR-223-3p* expression in the serum of pediatrics with acute leukemia. The test was conducted on 20 serum samples from children with acute leukemia and 10 normal serum samples from healthy children. Our results indicated that the high levels of C/EBPβ were not sufficient to induce the down-regulation of the expression of *miR-142-3p*, *miR-146a-3p* and the upregulation of *miR-223-3p* in serum samples, as indicated by the weak correlation values (R= 0.5737, 06625 and 0.7769, respectively), as shown in Figure- 7 (A, B,C). To reach a final conclusions about the relation between C\EBPβ and miRNAs, the sample size needs to be increased. Also, there is probably a need to find the effects of other factors like LMO2-L/-S isoforms (LIM domain only 2) and the relation of other C/EBP family members, such as alpha, gama,…etc. with miRNAs in cancers.

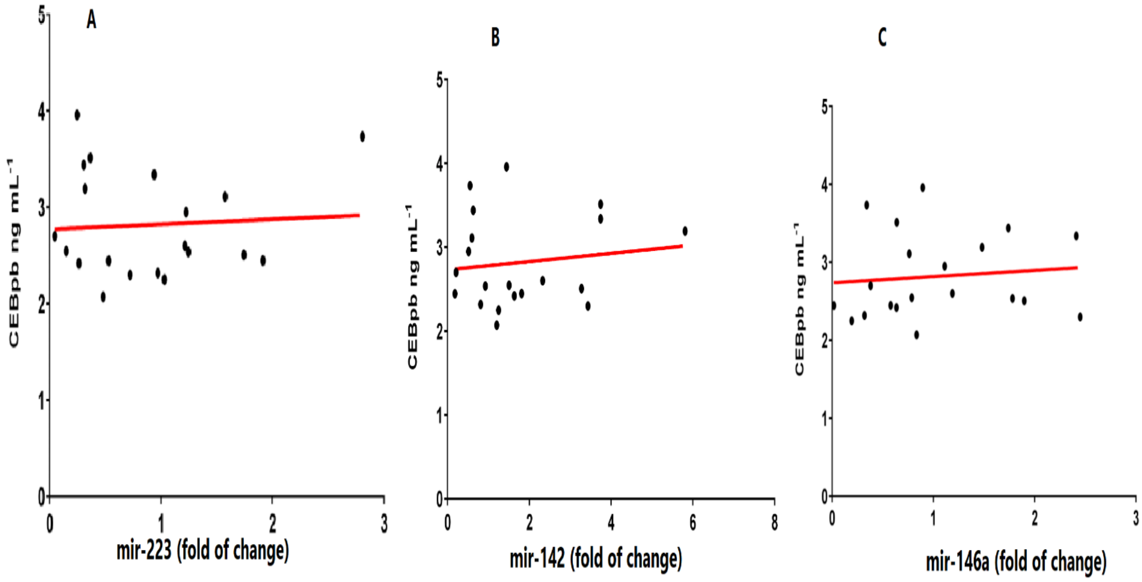

Figure 7-Correlations between serum level of C/EBP-β and A) *miR-223*; B) *miR-142* and C) *mir-146a*.

Conclusions

The serum expression levels of *miR-142-3p*, *miR-223-3p* and *miR- 146-3p* can act as useful indicators for early detection of pediatric acute leukemia. Advanced research is needed to find the association between these miRNAs and the C/EBP-β level in acute leukemia.

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