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Expression of micro RNA-9 in Patients with Acute Myeloid Leukemia and its Impact on Treatment Strategy

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ABSTRACT

Background: Acute myeloid leukemia (AML) is a malignant disorder of hematopoietic tissue. It is one of the most common malignancies in adults. It is characterized by its heterogeneous and complex nature that includes chromosomal, genetic and epigenetic abnormalities. MicroRNAs have been described to be one of the epigenetic regulators in AML.

Objective: The present study focused on measuring expression levels of miR-9 in de novo AML patients. 30 bone marrow samples were collected from patients attending at Hematology and Oncology Unit of Aldemerdash Hospitals.MiR-9 expression levels were measured using real time PCR. These expression levels were compared to 30 adult healthy volunteers.

Patients and Methods: The present study was carried out in medical oncology and Clinical Pathology Departments of Ain Shams University and National Research Centre, Egypt during the period between September 2017 and April 2018.

Results: Statistical analysis of the results showed that miR-9 was significantly down regulated in AML patients in comparison to controls. It is also significantly correlated to platelet count. Regarding diagnostic performance, this study showed that miR-9 has weak diagnostic performance but regarding screening function, it has perfect sensitivity but weak specificity so it can be used as an excluding test.

Conclusion: This study showed reduction of miR-9 in bone marrow of AML patients and opened the horizon for future studies on diagnostic and screening performance of miR-9 to be performed on larger scale and different ethnic groups.

Keywords: Leukemia, myeloid, acute,miR-9,treatment outcome.

INTRODUCTION

Acute myeloid leukemia (AML) is a malignant clonal disorder of immature cells in the hemopoietic hierarchical system. These cells have abnormal function characterized by a failure to progress through the expected differentiation program and / or to die by the process of apoptosis. This leads to the accumulation of a clone of cells which dominates bone marrow activity and leads to marrow failure [(1)].

MicroRNAs are a class of small, noncoding RNAs. They are epigenetic regulators that regulate gene expression by either degradation of mRNA or inhibition of translation. Their physiological roles start from development, differentiation, cell cycle regulation and end by aging and metabolism. Consequently, they are frequently deregulated in human's cancers. The Roles of microRNAs in carcinogenesis are very complex. Numerous microRNAs promote tumorigenesis and cancer progression by enhancing tumor growth, angiogenesis, invasion and immune evasion, while others have tumor suppressive effects. As a result, the expression profile of cancer microRNAs can define tumor type, susceptibility, prognosis and response to treatment [(2)].

Different microRNA expression profiles are seen in various cytogenetic groups of AML. Moreover, AMLs with specific gene mutations also harbor distinct sets of microRNA signatures. This may play a part in the pathogenesis and prognosis in AML [(3)].

Evidence is emerging that micro RNA-9 is an important regulator of normal development and diseases. Its aberrant expression levels have been reported in many types of cancers, having opposing effects on proliferation through regulation of various mRNA targets. It is over expressed in Hodgkin lymphoma, primary brain tumors as Neuroblastoma and Medulloblastoma. In contrast, it is down regulated in ovarian tumor [(4)].

Regarding hematological malignancies, miR-9 is an important player in scope of AML prognosis. It is overexpressed in MLL rearranged AML, causing a significant increase in cell viability and a decrease in apoptosis. On the other hand, miR-9 repress the oncogenic LIN28B/HMGA2 axis, thus, acting as a tumor suppressor-miRNA which acts in a stringent cell context [(5)].

AIM OF THE WORK

Measure miR-9 expression levels in AML patients group and to correlate different expression levels of miR-9 to clinical and laboratory data to evaluate its role on treatment strategy in AML cases.

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PATIENTS AND METHODS

The present study was carried out in Medical Oncology and Clinical Pathology Departments of Ain Shams University and National Research Centre, Egypt during the period between September 2017 and April 2018.

Thirty adult patients with de novo acute myeloid leukemia were recruited from outpatient clinic of Medical Oncology Department of Ain Shams University. All patients were enrolled in the study after establishment of diagnosis of AML before induction of therapy as baseline level of hematological data and our study parameters were assessed prior to initiation of therapy. The study was approved by the Ethics Board of Ain Shams University.

The control group included 30 age and sex matched normal healthy donors for bone marrow transplantation. All patients were subjected to the following: (A) Informed consent and ethical approval were collected. (B) General medical evaluation: 1.Thorough history taking (age, sex, complaint, onset, course, duration of disease, present history, past history and family history). 2. Full clinical examination particularly for pallor, petechiae, lymphadenopathy, hepatomegaly, splenomegaly, skin lesions. (C) Routine laboratory investigation for appropriate diagnosis: 1. Complete blood count: 2 ml blood samples were withdrawn under complete aseptic condition on EDTA tubes for CBC analysis using SysmexXN-1000 SA-01 and peripheral blood smear examination. 2. Bone marrow aspiration (Sternal): 3 ml were withdrawn for morphologic examination of Leishmania stained smear, blast count detection, immunophenotyping and miR-9 expression. Bone marrow samples for miR-9 analysis were preserved using Qiazole reagent (100 µ sample+500 µ Qiazole) then stored in -80 freezer. 3. Immunophenotyping for acute leukemia panel that includes CD13,CD117, MPOX CD33, using Beckman Coulter Navios Flowcytometer. 4- Cytogenetic analysis: 2 ml of samples were collected on heparin tubes and analysed for t(8:21), t(15:17), inv (16) and t(11q22)

Statistical Methods

The collected data were coded, tabulated, and statistically analyzed using IBM SPSS statistics (Statistical Package for Social Sciences) software version 18.0, IBM Corp., Chicago, USA, 2009.

Descriptive statistics were done for quantitative data as minimum& maximum of the range as well as mean±SD (standard deviation) for quantitative normally distributed data, while it was done for qualitative data as number and percentage.

Independent sample t-test in cases of two independent groups with normally distributed data. In qualitative data, inferential analyses for independent variables were done using Chi square test for differences between proportions. While correlations were done using Pearson correlation for numerical normally distributed data. ROC curve was used to evaluate the performance of different tests differentiate between certain groups. The level of significance was taken at P value < 0.050 is significant, otherwise is non-significant.

Diagnostic characteristics were calculated as follows:

Sensitivity = (True positive test / Total positive golden) x 100

Specificity = (True negative test / Total negative golden) x 100

Predictive positive value = (True positive test / Total positive test) x 100

Predictive negative value = (True negative test / Total negative test) x 100

LR+ = (sensitivity/ 1-specificity)

LR- = (1- sensitivity / specificity)

Diagnostic accuracy = ([True positive test + True negative test] / Total cases) x 100

Youden's index = sensitivity + specificity − 1

RESULTS

Table (1): FAB classification of AML patients.

FAB	N	%
M1	3	10.0
M2	17	56.7
M3	6	20.0
M4	3	10.0
M5	1	3.3

Table (2): Cytogenetics of case group.

Variables	N	%
t(8:21)	8	26.8
Inv (16)	3	10
t(15:17)	2	6.6
Trisomy 8	2	6.6
CN	15	50

Table (3): Immunohistochemistry of case group.

Variables	N	%
MPO	30	100.0
CD2	0	0.0
CD10	0	0.0
CD13	29	96.7
CD14	3	10.0
CD19	11	36.7
CD20	4	13.3
CD33	30	100.0
CD34	21	70.0
CD117	29	96.7
HLA-DR	27	90.0

Table (4): Response to treatment in case group.

Variable	N	%
Complete remission(CR)	24	80
No complete remission	6	20

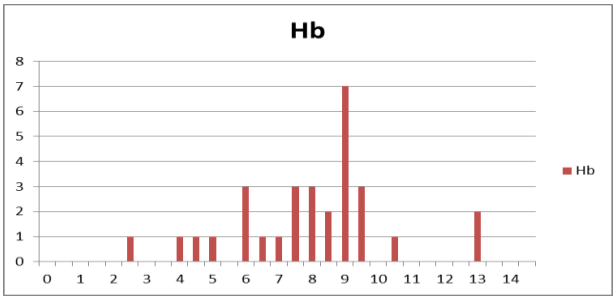
Table (5): Correlation between miR 9 and other factors among cases.

Variables	Case (N=30)		Control (N=30)	
	R	P	R	P
Age	-0.269	0.150	-0.010	0.957
Hb	-0.094	0.622	0.113	0.554
WBCs	-0.298	0.110	0.265	0.158
PLT	0.517	0.003*	0.215	0.254
BM blast	0.187	0.324		

Table (6): Diagnostic characteristics of mRNA≤8.96 and Platelets ≤155.5 in differentiating case group from control group

Characters	Value	95% CI
miR-9≤8.96		
Sensitivity	100.0%	88.4%–100.0
Specificity	20.0%	7.7%–38.6%
Diagnostic accuracy (DA)	60.0%	46.5%–72.4%
Youden's index	20.0%	5.7%–34.3%
Positive Predictive value (PPV)	55.6%	41.4%–100.0%
Negative Predictive value (NPV)	100.0%	54.1%–100.0%
Platelets ≤ 155.5		
Sensitivity	100.0%	39.8%–100.0%
Specificity	100.0%	86.8%–100.0%
Diagnostic accuracy (DA)	100.0%	88.4%–100.0%
Youden's index	100.0%	100.0%–100.0%
Positive Predictive value (PPV)	100.0%	39.8%–100.0%
Negative Predictive value (NPV)	100.0%	86.8%–100.0%

CI: Confidence interval



Figure(1): Hb concentration in AML cases

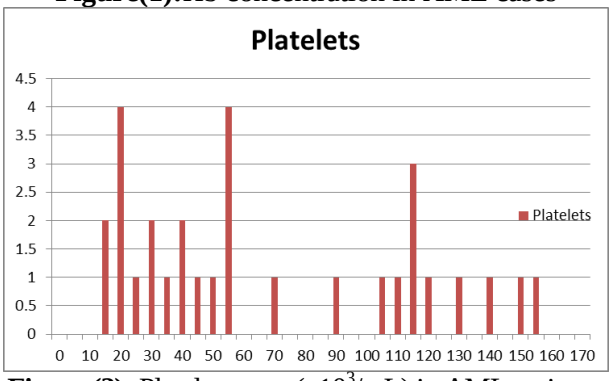
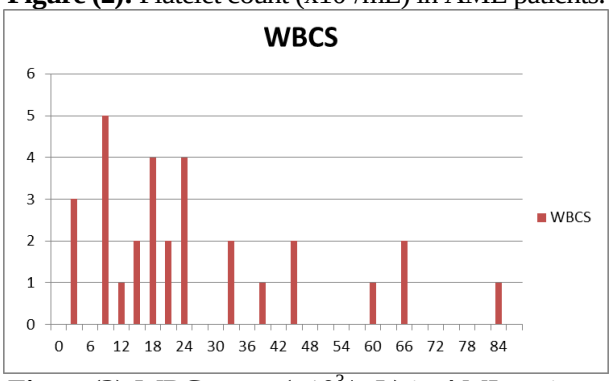
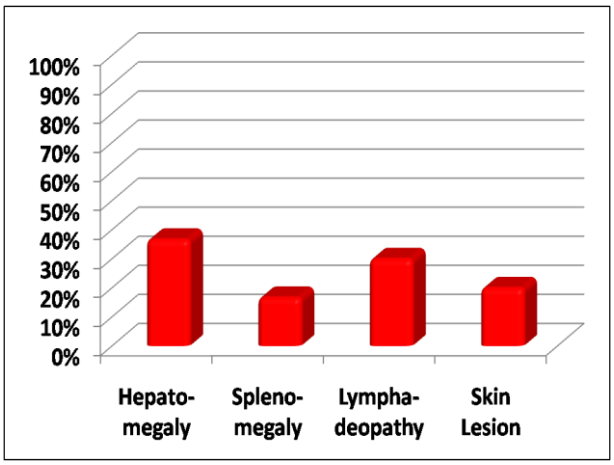


Figure (2): Platelet count (x10^3/mL) in AML patients.



Figure(3):WBC count (x10^3/mL) in AML patients



Figure(4): Clinical data in AML patients.

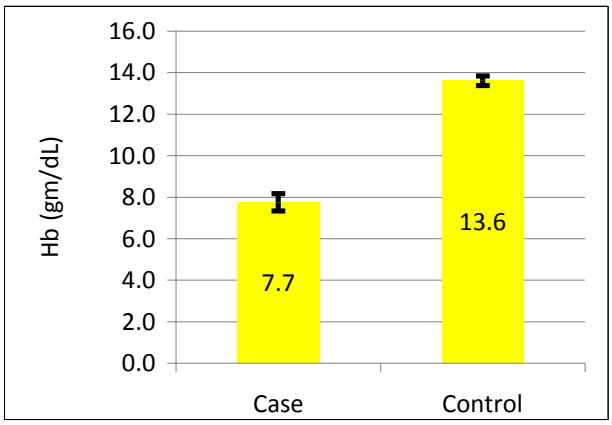


Figure (5): Comparison between case and control groups regarding Hb concentration

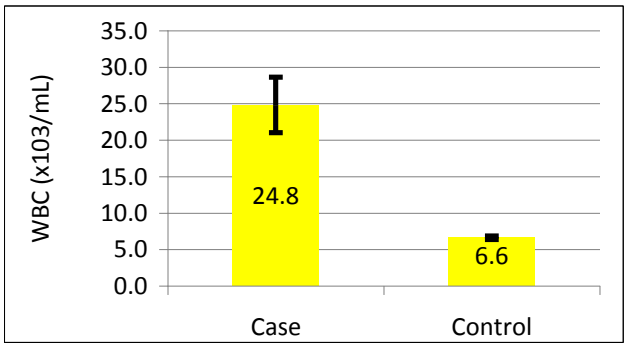


Figure (6): Comparison between case and control groups regarding WBCs.

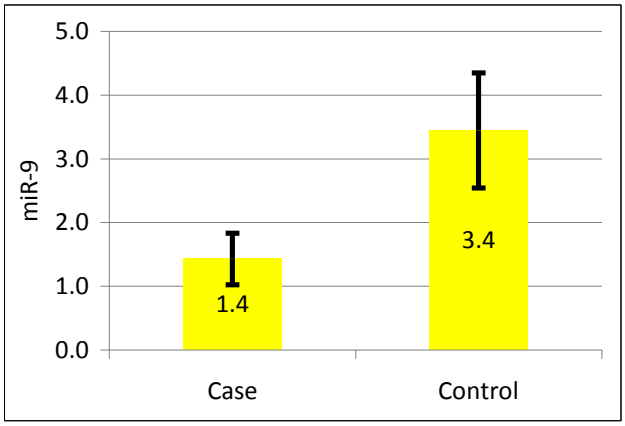


Figure (7): Comparison between case and control groups regarding miR-9.

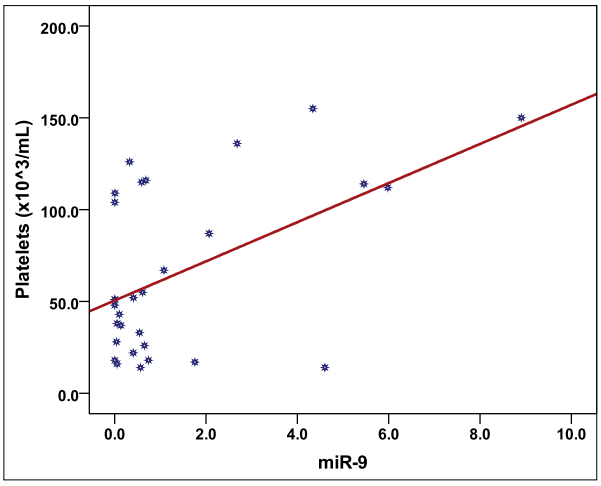
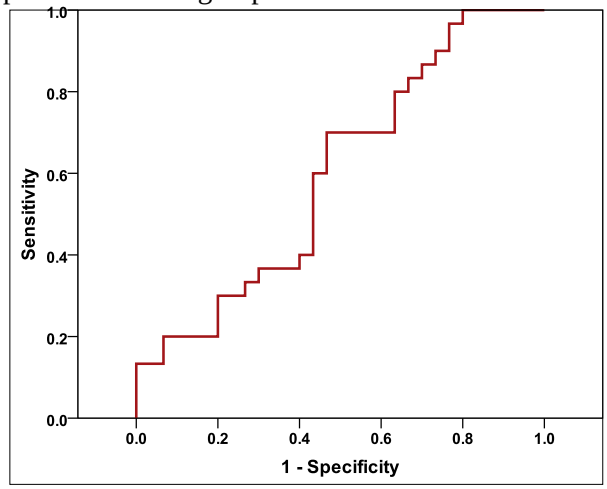


Figure (8): Correlation between miR-9 and platelets in case group.



Figure(9): ROC curve for miR-9 in differentiating case group from control group.

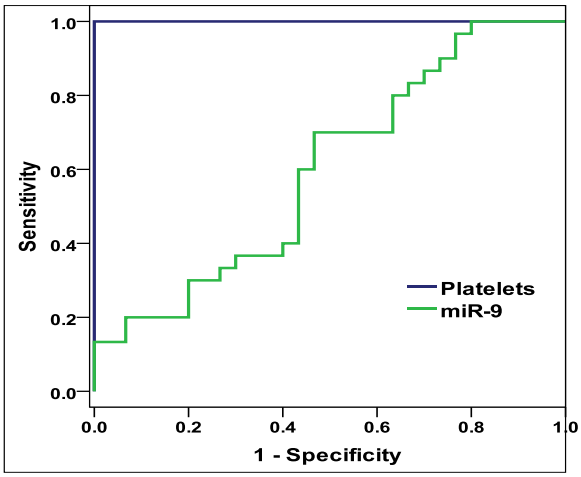


Figure (10): ROC curve for platelet in differentiating case group from control group

DISCUSSION

Acute myeloid leukemia (AML), is a malignancy which is seen in any age. AML constitutes 45% of all blood malignancies and represents about 33% of adolescent and 50% adult leukemia [6,7].

In Egypt, the hemopoietic and lymphatic cancer incidence in 2001 has increased approximately 11-fold compared with that in 1972 [8].

In 2010, leukemias in Egypt ranked the fourth common cancer together with urinary bladder cancer (7.2%), in all ages and both genders. In females, also together with lymphoma, for all ages, they constituted 6.5% and coming the second after breast cancers. While in males leukemias are the fourth after liver cancer, urinary bladder cancer and lymphomas forming 8.0% of all cancers for all ages [9].

Acute myeloid leukemia (AML) is a form of cancer characterized by infiltration of the bone marrow, blood and other tissues by proliferative, clonal, abnormally differentiated and occasionally poorly differentiated cells of the hematopoietic system [10]. It results from diverse phenotypic, genetic and epigenetic alterations in the differentiation of hematopoietic stem cells [11]. It has already been reported that epigenetic modifications are involved in the regulation of hematopoietic development [12,13].

Epigenetics include all molecular pathways that modulate the expression of a genotype to a specific phenotype. It is the study of changes in gene function that do not include a change in DNA sequence. These modulations have a crucial role in the silencing and expression of noncoding sequences [14].

MicroRNAs are essential epigenetic modifiers, they are directly related to genetic regulation which may occur at different levels by different mechanisms [15]. They act mainly at post transcriptional level. Occasionally, genetic regulation by miRNA may occur by histone modification or DNA methylation of promoter sites [16]. All these mechanisms of genetic regulation result in either gene silencing or overexpression.

There is an increasing number of studies trying to correlate miRNA expression in normal hematopoiesis to that in hematological malignancies. Through gene silencing, miRNAs play important roles in tumorigenesis. Either as oncogenes by suppressing tumor suppressor mRNA or as tumor suppressor by suppressing oncogenic mRNA [17].

In AML patients, early screening and intervention can increase survival chances. Consequently, there is a need to find new biomarkers for the diagnosis and prognosis of AML. MiRNAs expression profiling has been shown to classify tissue and tumor type accurately in different types of cancers [18].

MiR- 9 has been proven to have an essential regulatory role in organogenesis, neuronal function and evolution of different solid tumors. Different levels of expression have been described in different phases of hematopoietic differentiation [19].

In this study, we aimed at comparing miR-9 expression levels in between AML patients and control group and correlate these expression levels to the clinical and laboratory data of the patients to observe the impact on the treatment outcome.

To achieve this aim, we applied a quantitative Real Time PCR (qRT-PCR) assay to relatively quantify miR-9 in bone marrow samples from 30 de novo AML patients attended at Hematology and Oncology Unit of Ain Sham University and control group of 30 age and sex matched healthy volunteers.

Normalization is a key step for the accurate quantification of miRNA levels with qRT-PCR. In our study, RNU-48 was used as an internal control for miR-9 quantification.

This study included AML patients with the FAB classification subtypes of M1, M2, M3, M4, M5, while AML patients with M0, M6 and M7 and M8 subtypes were not represented in the study.

Regarding demographic data, there was no significant difference between cases and controls regarding age and sex which is a good indicator of the strong similarity between patients and controls regarding these criteria.

Regarding comparison of laboratory data between cases and controls; our results showed significantly lower hemoglobin ($p<0.001$), platelets ($p<0.001$) and significantly higher WBCs ($p<0.001$). These results are consistent with the

anticipated effect from infiltration of bone marrow with immaturely differentiated blast cells.

Our study showed that miR-9 was significantly under expressed in AML cases in comparison to controls ($p<0.049$). Although our study was performed on de novo AML cases with, it was in agreement with some previous studies, on the other hand the results were in contrast to others.

Our results are in agreement to study done by ***Cammarata et al.*** [2] that measured the expression profiles of miR-9 in AML with recurrent genetic abnormalities and demonstrated that it was significantly under expressed.

The results are in accordance with study performed by ***Emmrich et al.*** [20] that showed down regulation of miR-9 in t(8;21) AML and explained that this low expression has oncogenic effect through its impact on specific genes (HMGA2, LIN28B) causing increased proliferation and decreased monocytic differentiation.

This study was also consistent with study performed by ***Senyuk et al.*** [21] that showed that low expression of miR-9 has leukemogenic effect in ectopic viral integration -1(EV-1) mutated AML. The study showed that miR-9 binds to specific sites causing under expression of genes called FOXO1 and FOXO3 that regulate the cell cycle and in case of under expression of miR-9,these genes are up regulated causing AML through increased rate of cell cycle consequently, increasing proliferation of myeloid precursors.

Maki et al. [22] described an over expression of miR-9 in AML patients. The explanation proposed by the study was that over expression of miR-9 caused down-regulation of specific target genes including Catenin Alpha 1(CTNNA1), suppressor of cytokine signaling (SOCS4) and (SOCS5) could cause abnormal regulation of cell cycle progression, cytokine signals and cell adhesion.

The results were in contrast to study done by ***Chen et al.*** [5], which concluded that miR-9 is the most specifically and consistently upregulated miRNA in MLL-rearranged AML but this difference could be explained by earlier studies that demonstrated that MLL fusion proteins function predominantly as transcriptional activators, rather than inhibitors.

Overall, these discrepancies in results between different studies might be due to differences in sample size, age group, clinical characteristics or ethnic origin of the studied patients, where the association between cancer and a particular gene mutation in one population might be of limited value as a biomarker for cancer compared to another different population.

After applying statistical correlation of miR-9 to different demographic and laboratory data; there was no significant correlation of miR-9 expression levels to age, sex, Hb, WBCs, cytogenetic analysis and immunephenotyping.

This study showed that there was a significant positive correlation ($p<0.003$) between miR-9 expression levels and platelets. Although this result is in alignment with a study done in 2009 by ***Landry et al.*** [23] which showed that mRNA translation in blood platelets is regulated by miRNA and that different miRNAs was described to affect biosynthesis of platelets like miR-15 a, miR-339-3 p and miR-365 [24]. There were no previous studies that specifically correlated miR-9 to platelets or explained the exact mechanism by which miR-9 affect megakaryocytopoiesis.

We employed the ROC curve to analyze the diagnostic value of miR-9 expression levels in AML patients subsequently; ROC demonstrated that miR9 ≤ 8.96 non-significant weak diagnostic performance in differentiating case group from control group. It has perfect sensitivity and NPV and low specificity and PPV in differentiating case group from control group. Although these results show the weak screening performance of miR-9, they put miR-9 in the prospective to be used as a good excluding test for AML.

Depending on the significant correlation between miR-9 and platelet count; we applied ROC curve to assess the diagnostic performance of platelet count in AML and the results demonstrated that platelet count has a strong diagnostic performance and screening performance. In this context, our Study showed that platelet count can be used either alone as a screening test for AML after excluding other causes of thrombocytopenia or in combination with miR-9 in a two step screening method where the first step is screening via platelet count as an including test and miR-9 as an excluding test.

This study tried to correlate miR-9 expression levels to the treatment outcome in the form of complete remission or no complete remission. Statistical analysis showed that miR-9 was significantly lowered in CR patients in relation to no CR patients.

Although-to the best of our knowledge- no previous study correlated AML to the treatment outcome, our study is in alignment with previous study performed by ***Maki et al.*** [(22)] that concluded that an overexpression of miR-9 in AML patients is significantly correlated to bad prognosis in the form of overall survival (OS) and relative free survival (RFS). The explanation proposed by the study was that overexpression of miR-9 caused down-regulation of specific target genes including Catenin alpha-1 (CTNNA1), suppressor of cytokine signaling (SOCS4) and (SOCS5) could cause abnormal regulation of cell cycle progression, cytokine signals and cell adhesion which eventually result in bad prognosis.

Although our results are promising regarding the authentication of miR-9 as a reliable excluding biomarker, this study has several limitations. First, the sample size was still small, further validations in large cohorts or in different ethnic groups are recommended. Second, the exact mechanism by which under expression of miR-9 affects leukemogenesis is still uncertain and further studies are recommended for specifying targets of miR-9 to be aim of future therapies.

This study shows a reduction in miR-9 in bone marrow of AML patients in comparison to controls. miR-9 has weak diagnostic and screening performance but can be used as an excluding test for AML as it has perfect sensitivity in diagnosis. miR-9 is significantly correlated to platelet count. Platelet count has strong diagnostic and screening performance.

CONCLUSION

This study showed reduction of miR-9 in bone marrow of AML patients and opened the horizon for future studies on diagnostic and screening performance of miR-9 to be performed on larger scale and different ethnic groups.

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